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TRANSICIÓN MESOTELIO-MESENQUIMAL INDUCIDA DURANTE LA DIÁLISIS PERITONEAL: EFECTO DE LOS LÍQUIDOS BIOCOMPATIBLES E IMPORTANCIA FUNCIONAL DE VEGF Y SUS RECEPTORES.

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CERTIFICAN

Que Doña María Luisa Pérez Lozano, licenciada en Ciencias Biológicas y Bioquímica por la Universidad de Sevilla, ha realizado bajo su dirección el trabajo titulado ***"Transición mesotelio-mesenquimal inducida durante la diálisis peritoneal: efectos de los líquidos bio-inccompatibles e importancia funcional de VEGF y sus receptores"***, que presenta como Tesis Doctoral para optar al grado de Doctor por la Universidad Autónoma de Madrid.

Fdo: Dr. Rafael Selgas Gutiérrez

Fdo: Dr. Manuel López Cabrera

A mis padres

Después de tantos años se me hace extraño sentarme a escribir esta parte, sobre todo porque significa el fin de una etapa muy importante que, para ser sinceros, ¡ya es hora de cerrarla! Esta tesis ha sido una de las mayores experiencias de mi vida, durante la cual mi personalidad ha ido cambiando y forjando, pasando por buenos y malos momentos, por alegrías y desilusiones; así es una tesis. La ciencia te aporta mucho a nivel personal, la persona que se dedica a este trabajo le ha de gustar (mucho), más aún si tras defender la tesis continúa trabajando en el mundillo (yo espero ser una de esos loc@s); sin embargo, hay que reconocer que es un trabajo que también te desespera y te frustra. Por ello son muy importantes todas aquellas personas que han pasado por mi vida durante los años de tesis, tanto para bien o para mal, ya que de todo y de todos se aprende. Algunas aún formáis parte de ella y otras no y, aunque crea que todas son dignas de mención, seguramente me deje a alguien atrás. Si eso ocurre, disculpadme por ello porque no lo habré hecho de forma consciente. Ya me conocéis, soy un desastre despistado con patas, eso no lo puedo cambiar y además es parte de mi encanto, no? Jajaja. Intentaré (no prometo mucho) expresar todo lo que quiero decir, porque en estado de estrés "pretésico" por los terminar de escribir, plazos de entrega (siempre con el tiempo pegado al culo, para qué cambiar, ¿verdad?), papeleo y más papeleo, mis neuronas no dan para más.

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Ningún descubrimiento se haría ya si nos contentásemos con lo que sabemos.

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ABREVIATURAS

ABREVIATURAS

		CASTELLANO	INGLÉS
•	**AGEs**	Productos de glicosilación avanzada	Advanced glication end products
•	**AngII**	Angiotensina II	Angiotensin-2
•	**CM**	Célula mesotelial	Mesothelial cell
•	**CUB**	Complemento C1r/C1s, Uegf, Bmp1	Complement C1r/C1s, Uegf, Bmp1
•	**DP**	Diálisis peritoneal	Peritoneal dialysis
•	**EGF**	Factor de crecimiento epidérmico	Epidermal growth factor
•	**EMT**	Transición epitelio-mesenquimal	Epithelial-to-mesenchymal transition
•	**ERK-1/2**	Proteína regulada por señal extracelular-1/2	Extracellular signal-regulated kinase-1/2
•	**FGF**	Factor de crecimiento de fibroblastos	Fibroblast growth factor
•	**FGFR-1**	Receptor de FGF-1	FGF receptor-1
•	**FSP-1**	Proteína específica de fibroblastos-1	Fibroblast specific protein-1
•	**GDPs**	Productos de la degradación de la glucosa	Glucose degradation products
•	**GSK-3β**	Quinasa glicógeno sintasa-3beta	Glycogen synthase kinase-3β
•	**GTPasas**	Enzimas de unión a guanosín trifosfato	Guanosin triphosphate binding enzyme
•	**HGF**	Factor de crecimiento de hepatocitos	Hepatocyte growth factor
•	**ICAM-1**	Molécula de adhesión intercelular-1	Intercelular adhesion molecule-1
•	**IFN-γ**	Interferón-γ	Interferon-γ
•	**IGF-1**	Factor de crecimiento similar a insulina-1	Insulin-like growth factor-1
•	**IL**	Interleuquina	Interleukin
•	**ILK**	Quinasa unida a integrina	Integrin linked kinase
•	**JNK**	Quinasa c-Jun	c-Jun kinase
•	**LEF1**	Factor potenciador de unión linfoide-1	Lymphoid enhancer-binding factor-1
•	**MAM**	Meprina/proteína A5/proteína PTPmu	Mephin/A5-protein/PTPμ
•	**MAPK**	Proteína quinasa activada por mitógeno	Mitogen-activated protein kinase
•	**MCP-1**	Proteína quimioatrayente de monocitos-1	Monocyte chemotactic protein-1
•	**MET**	Transición mesénquimo-epitelial	Mesenchymal-to-epithelial transition
•	**MMP**	Metaloproteinasa de matriz	Matrix metalloproteinase
•	**MMT**	Transición mesotelio-mesenquimal	Mesothelial-to-mesenchymal transition
•	**MP**	Membrana peritoneal	Peritoneal membrane

- **Nrp** Neuropilina Neuropilin
- **PAI-1** Inhibidor del activador de plasminógeno-1 Plasminogen activator inhibitor-1
- **PDGF** Factor de crecimiento derivado de plaquetas Platelet derived growth factor
- **PI3-K** Quinasa fosfatidilinositol-3 Phosphatidylinositol-3 kinase
- **PlGF** Factor de crecimiento placentario Placenta growth factor
- **RAGE** Receptor de AGE Receptor for AGE
- **ROCK** Quinasa asociada a Rho Rho-associated coiled-coil kinase
- **ROS** Especies reactivas de oxígeno Reactive oxygen species
- **Sema** Semaforina Semaphorin
- **TCF** Factor de célula T T-cell factor
- **TGF-** Factor de crecimiento transformante beta Transforming growth factor β
- **TNF-α** Factor de necrosis tumoral-alfa Tumor necrosis factor-α
- **tPA** Activador tisular de plasminógeno Tissular plasminogen activator
- **UF** Ultrafiltración Ultrafiltration
- **VCAM-1** Molécula de adhesión celular vascular-1 Vascular cell adhesion molecule-1
- **VEGF** Factor de crecimiento del endotelio vascular Vascular endothelial growth factor
- **VEGFR** Receptor de VEGF VEGF receptor
- **α-SMA** Alfa actina de músculo liso α-Smooth muscle actin

RESUMEN

RESUMEN

La diálisis peritoneal es una terapia alternativa a la hemodiálisis en el tratamiento de la insuficiencia renal en estadíos avanzados o terminales. Sin embargo, este tipo de diálisis implica la continua exposición de la membrana peritoneal a líquidos de diálisis bioincompatibles que, en último término, pueden llevar a un fallo de ultrafiltración. La disfunción peritoneal se caracteriza por la aparición secuencial de cambios estructurales en la membrana peritoneal, tales como la denudación de las células mesoteliales, inflamación, fibrosis tisular y angiogénesis. Estas lesiones parecen ser la causa principal del fracaso de ultrafiltración, que afecta aproximadamente al 20% de los pacientes en diálisis peritoneal.

Los líquidos clásicos empleados en diálisis peritoneal se caracterizan por ser hiperosmóticos, con alto contenido en glucosa, con tampón lactato y con pH ácido. Las altas concentraciones de glucosa y la esterilización de la misma dan lugar a la formación de productos de la degradación de la glucosa, que resultan tóxicos para las células mesoteliales. Asimismo, un pH ácido constituye otro agente dañino, junto con las glicosilaciones no enzimáticas de proteínas tisulares, que dan lugar a la acumulación de productos de glicosilación avanzada. Nosotros hemos estudiado el efecto, a largo plazo, que produce la utilización de líquidos biocompatibles en comparación con aquellos líquidos considerados como bioincompatibles. De este modo y estudiando las células mesoteliales, las citoquinas y los factores secretados, así como la función peritoneal, hemos podido sugerir que los líquidos biocompatibles son capaces de preservar mejor la membrana peritoneal, tanto desde el punto de vista estructural como funcional.

Uno de los mecanismos responsables de la fibrosis asociada a la diálisis peritoneal es la transición mesotelio-mesenquimal que sufren las células cuando son expuestas a los líquidos de diálisis. Paralelamente a la fibrosis peritoneal, se producen vasculopatías y angiogénesis. Esto ha dado lugar a proponer que el factor proangiogénico VEGF (factor de crecimiento de endotelio vascular) debe jugar un papel central en los procesos que conducen a la angiogénesis y al deterioro funcional de la membrana peritoneal. Aunque se sabía que las células mesoteliales secretaban VEGF en respuesta a diversos estímulos, no quedaban claros cuales eran los mecanismos implicados en su sobreexpresión en pacientes en terapia de diálisis peritoneal. Nosotros hemos descrito la correlación entre la conversión mesenquimal de las células mesoteliales, los niveles de VEGF y la funcionalidad peritoneal, observando una clara implicación de la transición mesotelio-mesenquimal en el aumento de la angiogénesis y la permeabilidad vascular.

Adicionalmente, hemos observado que la transición mesotelio-mesenquimal también produce alteraciones en el patrón de expresión de los receptores y co-receptores de VEGF y en la función de las células mesoteliales, dentro del contexto de la diálisis peritoneal.

SUMMARY

Peritoneal dialysis is an alternative therapy to hemodialysis in the treatment of renal failure in advanced or terminal stages. However, this type of dialysis involves the continuous membrane exposure to bioincompatible peritoneal dialysis fluids that ultimately can lead to ultrafiltration failure. Peritoneal dysfunction is characterized by the sequential appearance of structural changes in the peritoneal membrane, such as mesothelial cells denudation, inflammation, tissue fibrosis and angiogenesis. These lesions appear to be the main cause of ultrafiltration failure, which affects approximately 20% of patients on peritoneal dialysis.

The classical fluids used in peritoneal dialysis are characterized by being hyperosmotic, with high glucose content, lactate buffer and acidic pH. Glucose is the most widely osmotic agent used, however, the high concentrations of glucose and its sterilization result in the formation of glucose degradation products, which are toxic to mesothelial cells. Furthermore, acidic pH is other damaging agent together with the non-enzymatic glycosylation of tissue proteins, which lead to the accumulation of advanced glycosylation end products. We have studied the effect of long-term use of biocompatible fluids compared with those considered as bioincompatible. Thus, by studying mesothelial cells, cytokines and secreted factors, and the peritoneal function, we have able to suggest that biocompatible fluids are better to preserve the peritoneal membrane from the structural and functional poitn of view.

One of the responsible mechanisms of fibrosis peritoneal dialysis-associated is the mesothelial-to-mesenchymal transition that cells suffer when are exposed to dialysis fluids. Parallelaly to peritoneal fibrosis, vasculopathy and angiogenesis occur. This led us to propose that the pro-angiogenic factor VEGF (vascular endothelial growth factor) should play a key role in the processes that lead to angiogenesis and the functional deterioration of the peritoneal membrane. Although it was known that mesothelial cells secrete VEGF in response to various stimuli, there were not clear what were the mechanisms involved its overexpression in peritoneal dialysis patients. We have described a correlation between the mesenchymal conversion of mesothelial cells, VEGF levels and peritoneal functionality, showing a clear implication of mesothelial-to-mesenchymal transition in the increase of angiogenesis and vascular permeability.

Furthermore, we have observed that the mesothelial-mesenchymal transition also produces changes in the pattern expression of receptors and co-receptors of VEGF and function of mesothelial cells in the context of peritoneal dialysis.

INTRODUCCIÓN

I. LA INSUFICIENCIA RENAL

El riñón tiene como objetivo mantener la homeostasia del medio interno, cumpliendo funciones complejas como la formación de la orina, el mantenimiento del equilibrio electrolítico y el equilibrio ácido-base, funciones hormonales y metabólicas, desintoxicación sanguínea, así como el mantenimiento del volumen sanguíneo y la presión arterial (Fuentes X et al, 1998; Botella J, 2002). Los continuos cambios en los solutos iónicos de la sangre (sodio, potasio, cloro, magnesio, calcio, fosfatos) generan cambios en el medio interno, que pueden llevar a la muerte si no son regulados a tiempo. Estos cambios se regulan mediante mecanismos compensatorios, uno de los cuales es el mecanismo renal. El mecanismo renal está encargado de filtrar y limpiar la sangre del organismo, extrayendo los productos tóxicos y de desecho (potasio, urea), variando las concentraciones de solutos en sangre y eliminando el exceso de líquido de forma rápida, generando en última instancia la orina. La formación de orina tiene lugar en las nefronas (unidades funcionales del riñón), a través de procesos de filtración, resorción y secreción que suceden en las distintas regiones de su anatomía.

Una alteración en la función de los riñones, por la cual no son capaces de extraer las sustancias tóxicas del organismo, produce una insuficiencia renal. Las causas de la insuficiencia renal son diversas, las cuales conducen a una disminución de la función renal de manera gradual (insuficiencia renal crónica) o rápida (insuficiencia renal aguda) (Mendoza SA, 1977). Cuando se produce un fallo renal, los pacientes sufren *uremia*, término que literalmente significa “orina dentro de la sangre” (Seligman AM et al., 1946). La uremia se define como un conjunto de síntomas generados por la acumulación y retención de tóxicos en la sangre, debido al trastorno del funcionamiento renal. Cuando se produce una insuficiencia renal se ha de buscar una alternativa, como el trasplante de riñón o la diálisis. La diálisis constituye una terapia de reemplazo artificial de la función renal, cuyo principio se basa en la difusión de solutos a lo largo de un gradiente de concentración a través de una membrana semipermeable (**Figura 1**) (Abel JJ. et al, 1914; Kolff WJ et al., 1944; Seligman AM et al., 1946; Evans DB, 1977; Knapp MS, 1982). Hay dos métodos de diálisis: la hemodiálisis y la diálisis peritoneal.

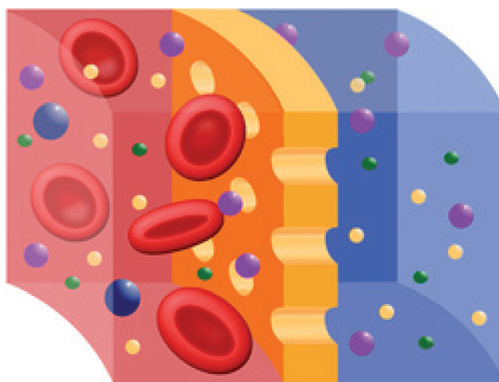

Figura 1. Principio de diálisis. A través de una membrana semipermeable, los productos de desecho y el exceso de agua del organismo pasan desde la sangre a un lado de la membrana, limpiándose la sangre, mientras que un líquido de diálisis pasa hacia el lado contrario.

I. 1. Hemodiálisis

La hemodiálisis es uno de los tratamientos para la insuficiencia renal mediante la cual la sangre del paciente se filtra empleando una máquina (dializador) que utiliza el flujo de contracorriente, donde el fluido de diálisis (dializado) fluye en dirección opuesta al flujo sanguíneo (**Figura 2**) (Abel JJ et al., 1914; Kolff WJ et al., 1944; Evans DB, 1977). De esta forma, la sangre se limpia eliminando las sustancias tóxicas y exceso de líquido, siendo después devuelta al organismo. Este proceso de eliminación de sustancias tóxicas y líquido se denomina *ultrafiltración*. La eficacia de la ultrafiltración de la hemodiálisis es muy alta, los tratamientos no son muy continuos y pueden ser realizados intermitentemente. Las máquinas de diálisis monitorizan continuamente un conjunto de parámetros de seguridad críticos, incluyendo las tasas de flujo de la sangre y del fluido de diálisis, la presión sanguínea, el ritmo cardíaco, la conductividad, el pH, etc.

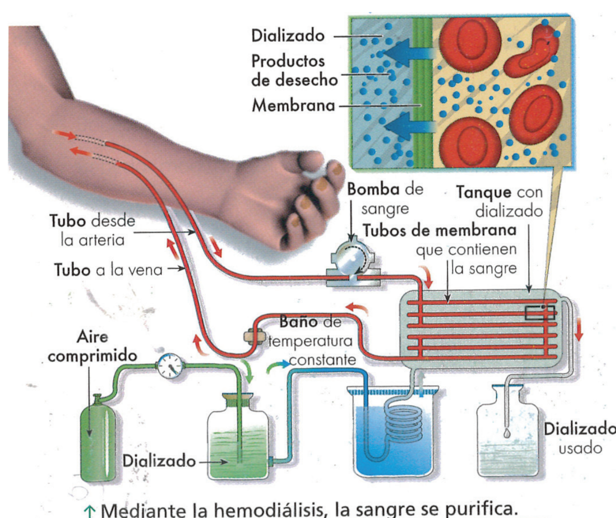

Figura 2. Esquema de un circuito de hemodiálisis.

Las complicaciones más comunes que podemos encontrar en el tratamiento de la hemodiálisis son debidos al acceso vascular, ya sea por infección, bloqueo por coagulación o por mala circulación sanguínea. Estos problemas pueden impedir que el tratamiento dé resultado, sometiéndose el paciente a repetidas cirugías para lograr un acceso que funcione adecuadamente. Otras problemas pueden ser debidos a la aparición de cambios rápidos en el volumen de agua y en el equilibrio químico del cuerpo del paciente durante el tratamiento.

I. 2. Diálisis peritoneal

La diálisis peritoneal surge como una terapia alternativa a la hemodiálisis en el tratamiento de aquellas enfermedades renales que cursan hasta estadíos avanzados o terminales (Pyper R. A., 1948; Chaimovitz C, 1994). Está basada en el hecho fisiológico de que el peritoneo es una membrana

natural, vascularizada y semipermeable que, mediante mecanismos de transporte osmótico y difusivo, permite pasar agua y distintos solutos desde los capilares sanguíneos al líquido dializante que se introduce en la cavidad peritoneal del paciente. Las sustancias que difunden a través de la membrana peritoneal son las de pequeño peso molecular, tales como urea, potasio, cloro, calcio, creatinina, ácido úrico, etc.

Este tipo de diálisis requiere la implantación de un catéter en el abdomen del paciente de forma permanente. El catéter se conecta a la bolsa de diálisis desde la cual se infunde un líquido dializante hiperosmótico. El líquido dializante permanecerá dentro de la cavidad peritoneal durante un periodo de tiempo establecido, para que se realice lentamente la diálisis. Siguiendo el gradiente osmótico se producirá la difusión y ósmosis de tóxicos y electrolitos, de forma que el exceso de líquido y los productos de desecho pasan desde la sangre, a través de la membrana peritoneal, al fluido de diálisis (**Figura 3**).

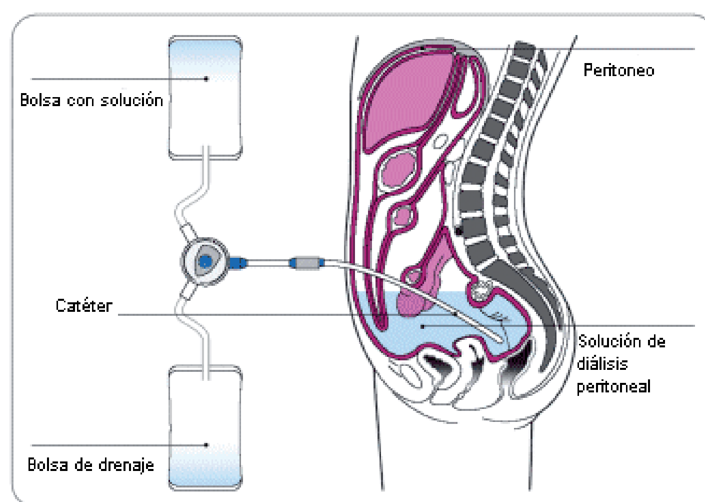

Figura 3. Esquema del tratamiento de Diálisis Peritoneal. Se instila, a través del catéter, una solución de diálisis a la cavidad peritoneal. El exceso de líquido y sustancias de desecho pasan desde la sangre, a través del peritoneo, al líquido de diálisis. Tras un periodo de permanencia, el líquido con las sustancias de desecho es drenado al exterior.

El dializado será drenado a una segunda bolsa de deshecho, administrando seguidamente una nueva solución de diálisis. A este cambio periódico se le llama *intercambio*. La correcta difusión dependerá de tres factores: el gradiente de concentración a ambos lados de la membrana, la superficie de la membrana y la permeabilidad de ésta. La urea difunde con gran rapidez y la creatinina algo menos, por lo que hacia las dos horas de permanencia del líquido dializante en el peritoneo casi se habrá completado la difusión, y poco después se alcanzará el equilibrio entre el líquido dializante y el plasma. Existen dos modalidades de diálisis peritoneal: ambulatoria continua y automatizada.

I. 2. 1. Tipos de diálisis peritoneal

- **a)** La **diálisis peritoneal ambulatoria continua**. Los pacientes en esta modalidad necesirealizar de 3 a 4 intercambios diarios. El tratamiento se puede realizar sin salir de casa, es flexible y puede ajustarse a distintas necesidades y horarios.
- **b)** La **diálisis peritoneal automatizada**. Se realiza por la noche, mientras se duerme. Una máquina controla el tiempo y efectúa los intercambios durante 8 ó 9 horas.

I. 2. 2. Ventajas e inconvenientes de la diálisis peritoneal

a) **Ventajas**

- Es relativamente fácil de aprender y se puede hacer en casa.
- Es mas adecuada en el comienzo de la diálisis, debido a que la salida nativa de la orina se mantiene por más tiempo que en hemodiálisis.
- El proceso de ultrafiltración es más lento y suave que en la hemodiálisis.
- Es más económica que la hemodiálisis.

b) **Inconvenientes**

- Requiere motivación y atención a la limpieza mientras se realizan los intercambios.
- Necesidad de un espacio amplio para almacenar las bolsas de los líquidos de diálisis.
- Resulta algo menos eficiente que la hemodiálisis.

La diálisis peritoneal requiere un acceso al peritoneo y, dado que las personas con fallo renal tienen el sistema inmune levemente suprimido, las infecciones suelen ser comunes. Además, el líquido de diálisis contiene azúcares que pueden favorecer el crecimiento de gérmenes. Si la infección se agrava alcanzando el peritoneo se produce una peritonitis (inflamación del peritoneo), la cual puede requerir antibióticos o, si es severa, un retiro o sustitución del catéter y un cambio de la modalidad de terapia de reemplazo renal a hemodiálisis. Uno de los efectos secundarios más importantes que se producen durante el tratamiento de la diálisis peritoneal es el daño del peritoneo, produciendo modificaciones que conducen a una disminución de la función de la membrana peritoneal como membrana de diálisis. Esta pérdida de función puede manifestarse como una pérdida de capacidad para la diálisis o un intercambio de fluido más pobre, también conocido como *fallo de ultrafiltración*. La pérdida de función de la membrana peritoneal puede implicar un cambio en la técnica de diálisis elegida.

II. EL MESOTELIO Y LA DIÁLISIS PERITONEAL

II.1. Estructura y funciones del mesotelio

El mesotelio está compuesto por un extensa monocapa de células mononucleares y de aspecto poligonal similares a las epiteliales, denominadas células mesoteliales (CMs). Este mesotelio deriva del mesodermo embrionario que recubre el celoma del embrión, y recubre las cavidades serosas

(pleura, pericardio, peritoneo y los órganos internos de nuestro organismo) (Mutsaers SE, 2002). En el peritoneo, el mesotelio da lugar a la membrana peritoneal, formada por tejido conjuntivo y tapizado por una monocapa de CMs. La membrana peritoneal recubre la cara interna de la pared abdominal dando lugar al peritoneo parietal, y los órganos del interior de la cavidad abdominal formando el peritoneo visceral (mesenterio, intestinos, hígado, bazo, etc.). Las CMs se disponen adaptadas unas a otras ofreciendo el aspecto de un empedrado o enlosado, con un diámetro aproximado de 25 µm. Estas células poseen microvellosidades y múltiples vesículas, cuyo número varía dependiendo de la localización; por ejemplo, en el mesotelio visceral las CMs tienen mayor número de vesículas que en el mesotelio parietal, las cuales están implicadas en el transporte de fluidos y sustancias a través del mesotelio. Además, la monocapa de CMs descansa sobre una delgada lámina basal, sostenida por tejido conjuntivo submesotelial que contiene vasos sanguíneos, vasos linfáticos, células y fibras. Es lo que denominamos *intersticio*. El tipo de fibra más abundante que encontramos en el intersticio es el colágeno y la célula principal es el fibroblasto, aunque también podemos encontrar otro tipos celulares tales como mastocitos, monocitos y macrófagos.

Antiguamente, se pensaba que la función del mesotelio consistía en proveer una superficie protectora y no adherente para facilitar los movimientos de órganos y tejidos. Sin embargo, en la actualidad se identifica como una membrana celular dinámica con varias funciones fisiológicas entre las que destacan (**Figura 4**) (Mutsaers SE, 2002; Mutsaers SE, 2004):

1. <u>Barrera de protección y superficie no adherente</u>. Las CMs están estrechamente unidas mediante uniones intercelulares, en cuya superficie secretan glicosaminoglicanos, predominantemente ácido hialurónico, que protege a las células de posibles agentes abrasivos y de agentes infecciosos. Además, las CMs secretan fosfatidilcolina, el componente principal de las lamelas y surfactantes, actuando como lubricante para reducir la fricción entre superficies serosas.

2. <u>Transporte de fluidos y solutos</u> a través de las cavidades serosas, mediante la formación de vesículas pinocíticas, uniones intracelulares y estomas. Los estomas son cavidades de unos 3-12 µm de diámetro que se forman entre la unión de dos o más células. Estas aberturas proveen un acceso directo al sistema linfático submesotelial, permitiendo un rápido movimiento de fluidos y de células, hacia y desde las cavidades serosas.

3. <u>Presentación de antígenos</u> mediante la síntesis y secreción de una gran diversidad de mediadores en respuesta a señales externas, reclutando células hacia las cavidades serosas y presentando antígenos a las células T. En consecuencia, participan en la protección frente a microorganismos. Una de las moléculas que las CMs expresan en su superficie es ICAM-1 (molécula de adhesión intercelular-1), siendo la principal molécula accesoria implicada en la presentación antigénica de las células T $CD4^{+}$. Además, las CMs estimuladas por IFN-γ (interferón gamma), promueven la activación de las células T mediante la secreción de IL-15 (Haussmann MJ et al., 2000).

4. Adhesión y diseminación tumoral. La producción de ácido hialurónico, así como la producción de glicosaminoglicanos y surfactantes, interviene en la prevención de la adhesión, diseminación y crecimiento de tumores. Sin embargo, la superficie de un mesotelio dañado es un lugar ideal para la adhesión tumoral, ya que se produce una unión de las moléculas CD44 de las células tumorales al ácido hialurónico que recubre a las CMs (Cunliffe WJ and Sugarbaker PH, 1989) o bien por la adhesión entre ambos tipos celulares a través de integrinas (Lessan K et al., 1999; Lin MT et al., 2007; Wagner BJ et al., 2011).

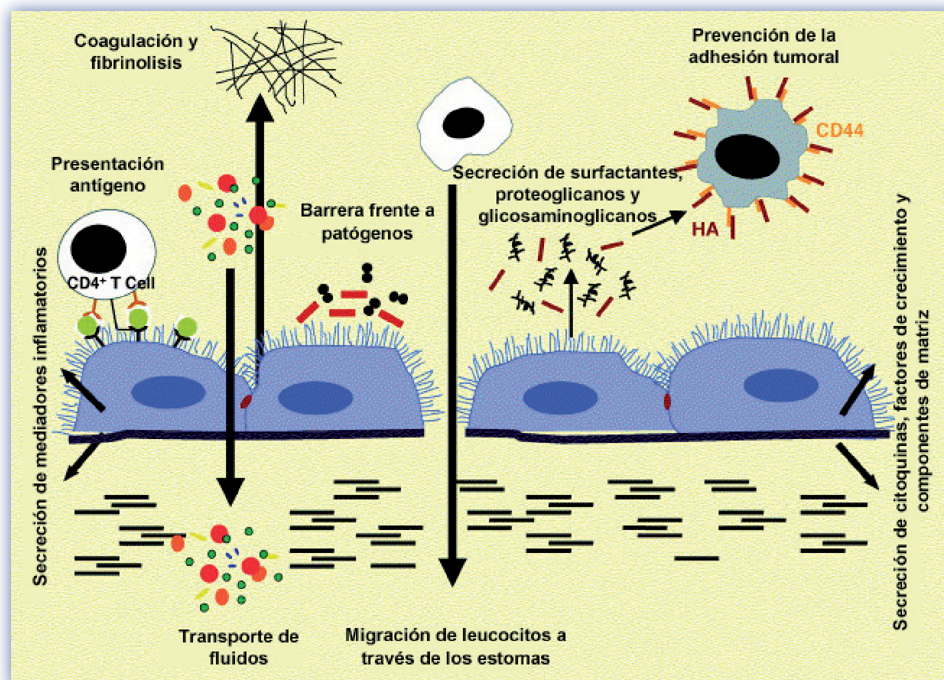

Figura 4. Funciones de las células mesoteliales peritoneales. El mesotelio es una membrana dinámica que realiza diversas funciones: barrera de protección y superficie no adherente mediante la secreción de glicosaminoglicanos, transporte de fluidos y solutos, presentación antigénica, prevención de la adhesión y diseminación tumoral, y reparación de tejidos e inflamación. *Adaptado de Mutsaers SE, 2004.*

5. Reparación de tejidos e inflamación. Las CMs juegan un papel importante en la inflamación y reparación de tejidos, mediante la secreción de factores de crecimiento, citoquinas, prostaglandinas, especies reactivas de oxígeno (ROS) y de moléculas de la matriz extracelular; así como activando sus propiedades proteasas y fibrinolíticas, previniendo la fibrosis y la formación de adhesiones postquirúrgicas. La secreción de quimioquinas promueve la migración transmesotelial de los neutrófilos y monocitos desde el compartimento vascular al espacio seroso (Lanfrancone L et al., 1992), proceso que está facilitado por la expresión de moléculas de adhesión, como ICAM-1. Algunos de los factores de crecimiento que se liberan al medio en respuesta a un daño son: TGF-β (factor de crecimiento transformante-beta), PDGF (factor de crecimiento derivado de plaquetas), FGF (factor de crecimiento de fibroblastos),

HGF (factor de crecimiento de hepatocitos), VEGF (factor de crecimiento de endotelio vascular), EGF (factor de crecimiento epidérmico, etc. (Mutsaers SE et al., 1997; Zweers MM et al., 1999; Zweers MM et al., 2001). Las CMs también sintetizan componentes de la matriz para reparar el tejido dañado, como son colágeno tipo I, III y IV, fibronectina y laminina. Asimismo, y con el fin de regular la síntesis y degradación de estos componentes, las CMs secretan metaloproteasas capaces de degradar la matriz extracelular.

II.2. Alteraciones del mesotelio durante el tratamiento de diálisis peritoneal.

El tratamiento de diálisis peritoneal provoca cambios ó modificaciones en la membrana peritoneal a largo plazo (**Figura 5**). Las modificaciones observadas más comunes son fibrosis (Dobbie, 1992; Williams JD et al., 2002; Margetts PJ and Bonniaud P, 2003; Kaneko K et al., 2007), inflamación y denudación de la monocapa de CMs (Di Paolo N et al., 1986; Chaimovitz, 1994; Baroni G et al., 2012), angiogénesis y vasodilatación (Mateijsen MAM et al., 1999; Krediet RT et al., 2000; Jiménez-Heffernan JA et al., 2005; Aguilera A et al., 2005; Nessim SJ et al., 2010), y cambios morfológicos y estructurales de las CMs pasando de un fenotipo epitelial a un fenotipo fibroblástico mediante un proceso de transición epitelio-mesenquimal (EMT, del inglés "epithelial-to-mesenchymal transition") (Yáñez-Mo, M et al., 2003; Aroeira L et al., 2007). Todos estos procesos culminan con la disminución o pérdida de función de la membrana peritoneal como membrana de diálisis, produciendo un fallo de ultrafiltración (Krediet RT, 1999) y obligando al paciente a cambiar de método de diálisis.

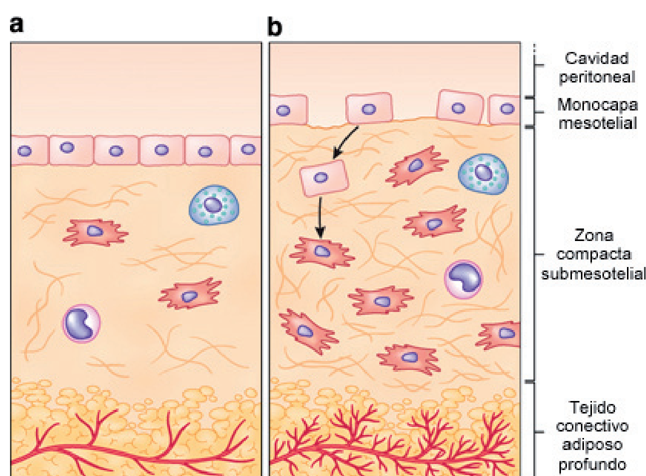

Figura 5. Dibujo representativo de la modificación de la membrana peritoneal: a) Corte transversal de un peritoneo sano. **b)** Alteraciones de la membrana debido a la diálisis peritoneal: denudación celular y conversión mesenquimal de las CMs adquiriendo un fenotipo fibroblástico. La zona submesotelial sufre fibrosis, aumentando el grosor mediante deposición de matriz extracelular. En el tejido conectivo adiposo profundo se encuentra la red vascular a partir de la cual se originan angiogénesis y vasculopatías. *Adaptado de Nessim SJ et al., 2010.*

La causa de este conjunto de alteraciones no está aún bien definida. Pueden estar implicados varios factores, tales como la bioincompatibilidad de los líquidos de diálisis y daños mecánicos producidos por la administración de los mismos, infecciones en el peritoneo (peritonitis), etc. Estos factores desencadenan una serie de procesos que conducen en último término al deterioro de la membrana peritoneal y a una disminución de la capacidad de ultrafiltración.

III. BIOINCOMPATIBILIDAD DE LOS LÍQUIDOS DE DIÁLISIS

La biocompatibilidad de un líquido de diálisis puede definirse como la capacidad de la formulación de una solución para permitir un tratamiento de diálisis a largo plazo sin ningún cambio clínicamente significativo en las características funcionales del peritoneo y, por tanto, es de primordial importancia no sólo en el mantenimiento de la salud de la membrana, sino también en permitir que la diálisis sea un éxito como terapia a largo plazo Los líquidos que no cumplen estas características son considerados como bioincompatibles.

La membrana peritoneal de los pacientes en terapia de diálisis peritoneal está expuesta a líquidos no fisiológicos, caracterizados por ser hiperosmóticos, con alto contenido en glucosa, con tampón lactato y con pH ácido. La continua exposición del peritoneo a estos líquidos bioincompatibles produce lesiones en la membrana, conduciendo, por tanto, a una disfunción peritoneal y fallo de membrana, además de alteraciones sistémicas como diabetes, hiperlipidemias, alteraciones cardíacas, etc. (**Figura 6**).

III.1. La Glucosa

Durante la diálisis peritoneal continuada, un paciente está expuesto a 2200-7000 litros de líquido de diálisis anualmente. La mayoría de las soluciones dializantes utilizan glucosa como agente osmótico, por su bajo coste, por su seguridad y porque se metaboliza fácilmente proporcionando una fuente de energía (Holmes CJ and Shockley TR, 2000). Para generar un gradiente osmótico adecuado, la concentración de glucosa en el líquido de diálisis debe ser superior a 15-40 veces la concentración fisiológica, manteniéndose varias veces por encima de ésta (6-16 veces) una vez alcanzado el equilibrio peritoneal (Chan TM and Yung S, 2007). Sin embargo, las altas concentraciones de glucosa inducen en las CMs la síntesis de factores y proteínas implicados en fibrosis, tales como TGF-β, fibronectina y MCP-1 (Proteína quimoatrayente de monocito-1) (Kang DH et al., 1999; Ha H et al., 2001; Ha H et al., 2002; Wong T et al., 2003) (**Tabla 1**).

III.2. Los productos de la degradación de la glucosa y el pH

Otros componentes no fisiológicos de los líquidos de diálisis son los productos de la degradación de la glucosa (GDPs, del inglés "glucose degradation products") y el pH ácido (Shostak A et al., 1996; Martis L and Henderson LW, 1997). Los GDPs, tales como el glioxal, metilglioxal y 3-deoxiglucosona, se originan en el proceso de esterilización convencional por calor de los

líquidos, dando lugar a la "caramelización" de la glucosa (Witowsky J, et al., 2000). Para reducir la caramelización de la glucosa, se suelen emplear pH ácidos y una solución tampón de lactato (Zareie M et al., 2003, Erixon M et al., 2006); sin embargo, la glucosa, los GDPs, el lactato y el pH ácido son tóxicos para las CMs, generando un proceso inflamatorio que puede verse exacerbado por un tratamiento continuo, desencadenando alteraciones en las funciones de las CMs, fibrosis, angiogénesis, aumento de la permeabilidad vascular y, finalmente, fallo de membrana (Shostak A et al., 1996; Shao JC, 1999; Witowsky J, 2000; Davies SJ et al., 2001).

Agente	Células afectadas	Efectos sobre las células peritoneales residentes o infiltrantes	Efectos sobre la membrana peritoneal
Glucosa	Células mesoteliales	Aumento de la síntesis de MCP-1, TGF-β, laminina y fibronectina.	Inflamación y fibrosis
		Disminución de la expresión de proteínas de uniones estrechas y marcadores epiteliales	Desintegración mesotelial y permeabilidad de la membrana
		Síntesis de perlecan reducida	Aumento de la permeabilidad de la membrana
		Aumento de daño mitocondrial	
		Inhibición de la proliferación celular	Deterioro de la remesotelización
		Aumento de la liberación de lactato deshidrogenasa	
	Fibroblastos	Aumento de la síntesis de proteínas de la matriz extracelular e hialuronano	Fibrosis peritoneal
		Estimulación de la proliferación	Fibrosis peritoneal
		Aumento de los receptores de insulina e IGF-1	Fibrosis peritoneal
	Leucocitos	Inducción de quimioquinas y citoquinas	Inflamación
		Disminución de la fagocitosis	Deterioro de la defensa peritoneal
		Secreción de citoquinas alterada	Deterioro de la defensa peritoneal
GDPs	Células mesoteliales	Disminución de la proliferación y viabilidad celular reducido; formación de ROS	Crecimiento celular alterado, toxicidad de GDPs
		Inhibición de la remesotelización	Denudación mesotelial
		Disminución de la síntesis de proteínas de la matriz y de unión celular	Funciones celulares deterioradas
		Aumento de expresión de citoquinas pro-inflamatorias	Inflamación
	Fibroblastos	Inhibición del crecimiento celular	Cicatrización de las heridas
	Leucocitos	Aumento del número de leucocitos	Inflamación
AGEs	Células mesoteliales	Aumento de la síntesis de VEGF y TGF-β1	Fibrosis peritoneal
		Aumento de expresión de citoquinas pro-inflamatorias	Inflamación
		Acumulación en células	Fibrosis

Tabla 1. Efectos de la glucosa, productos de la degradación de la glucosa, y productos de glicosilación avanzadas en el peritoneo. AGEs, productos de glicosilación avanzada; GDPs, productos de la degradación de la glucosa; IGF-1, factor de crecimiento similar a insulina-1; MCP-1, proteína quimioatrayente de monocitos; ROS, especies reactivas de oxígeno; TGF-β1, factor de crecimiento transformante β1; VEGF, factor de crecimiento endotelio vascular. *Adaptado de Chan TM and Yung S, PDI, 2007.*

Los GDPs producen alteraciones como: aumento de la secreción de factores de crecimiento (VEGF y TGF-β1), aumento de la producción de citoquinas proinflamatorias (IL-6 , IL-8), aumento de la expresión de VCAM-1 (molécula de adhesión celular vascular), inhibición del crecimiento, denudación celular e inducción de apoptosis (**Tabla 1**) (Inagi R et al., 1999; Seo MJ et al., 2001; Leung JC et al., 2005; Witowski J et al., 2001, Welten AG et al., 2003; Chan TM and Yung S, 2007).

Hoy en día se emplean diferentes aproximaciones para evitar estos daños en la cavidad peritoneal. Algunas consisten en el uso de nuevas soluciones tampón, como bicarbonato (Topley N et al., 1996; Ogata S et al., 2004), y el uso de un pH más cercano al fisiológico (**Tabla 2**). Asimismo, actualmente se emplean sistemas multi-bolsa para disminuir el daño peritoneal (Cooker LA et al., 1997; Sundaram S et al., 1997; Rippe B et al., 2001). Este sistema consiste en la utilización de bolsas de diálisis con dos compartimentos separados: uno con glucosa a bajo pH, lo que disminuye la producción de GDPs durante la esterilización, y otro compartimento con la solución tampón, que puede ser lactato, mezcla de bicarbonato/lactato o bicarbonato solo. Estos líquidos son mezclados en el momento de su utilización, siendo el pH final más cercano al fisiológico, de aproximadamente 6.2-7.4.

Marca	Solución	Bolsa	A.Osmótico	Tampón	Calcio	GDPs	pH
Baxter	*Dianeal PD1*	Simple	Glucosa 1.36 2.27 - 3.86 %	Lactato 35 mmol/L	1.75 mmol/L	Si	5.2
	Dianeal PD4	Simple	Glucosa 1.36 2.27 - 3.86 %	Lactato 40 mmol/L	1.25 mmol/L	Si	5.2
	Extraneal	Simple	Icodextrina 7.5 %	Lactato 40 mmol/L	1.75 mmol/L	No	5.6
	Nutrineal	Simple	Aminoácidos 1.1 %	Lactato 40 mmol/L	1.25 mmol/L	No	5.5
	Physioneal 35	Doble	Glucosa 1.36 2.27 - 3.86 %	Lactato 10 mmol/L + Bicarbonato 25 mmol/L	1.75 mmol/L	No	7.4
	Physioneal 40	Doble	Glucosa 1.36 2.27 - 3.86 %	Lactato 15 mmol/L + Bicarbonato 25 mmol/L	1.25 mmol/L	No	7.4
Fresenius	Stay Safe 2 - 3 - 4	Simple	Glucosa 1.5 - 4.25 - 2.3 %	Lactato 35 mmol/L	1.75 mmol/L	Si	5.5
	Stay Safe 17 - 18 - 19	Simple	Glucosa 1.5 - 4.25 - 2.3 %	Lactato 35 mmol/L	1.25 mmol/L	Si	5.5
	Balance 2 - 3 - 4	Doble	Glucosa 1.5 - 4.25 - 2.3 %	Lactato 35 mmol/L	1.75 mmol/L	No	7 -7.4
	Balance 17 - 18 - 19	Doble	Glucosa 1.5 - 4.25 - 2.3 %	Lactato 35 mmol/L	1.25 mmol/L	No	7 -7.4
	BicaVera 2 - 3 - 4	Doble	Glucosa 1.5 - 4.25 - 2.3 %	Bicarbonato 34 mmol/L	1.75 mmol/L	No	7.4
Gambro	Gambrosol Trio 10 A - B - A+B	Triple	Glucosa 1.5 - 2.5 - 3.9 %	Lactato 35 mmol/L	1.79 - 1.75 1.70 mol/L	No	5.5 -6.5
	Gambrosol Trio 40 A - B - A+B	Triple	Glucosa 1.5 - 2.5 - 3.9 %	Lactato 41 - 40 - 39 mmol/L	1.38 - 1.35 1.31 mol/L	No	5.5 -6.5

Tabla 2. Soluciones de líquidos de diálisis.

En los últimos años se ha observado que el uso de nuevos líquidos que contienen menos GDPs, en comparación con aquellos convencionales que tienen altas cantidades de GDPs, conducen a una mejor preservación de la estructura peritoneal y de las CMs que drenan a la cavidad peritoneal (Witowski J et al., 2004; Mortier S et al., 2004; Do YJ et al., 2005; Lee, HY et al., 2005) (**Tabla 2**). También el uso de polímero de glucosa (icodextrina) como agente osmótico, en lugar de glucosa, favorece un aumento de la ultrafiltración y reduce el deterioro de la función de la membrana peritoneal (Cooker LA et al, 2002; Cho Y et al., 2013). Además, hay líquidos basados en aminoácidos, usados normalmente en pacientes que tienen problemas nutricionales (Chang J et al., 2003).

III. 3. Productos de glicosilación avanzada y su receptor

Existen otros factores en los líquidos de diálisis que los hace bioincompatibles induciendo daños en el peritoneo, como son los productos finales de glicosilación avanzada (AGEs, del inglés "advanced glycation end products"). Los AGEs provienen de glicosilaciones no enzimáticas que sufren determinadas proteínas bajo condiciones de alta concentración de glucosa. En condiciones fisiológicas, la aparición de estos compuestos está determinada por la ausencia de enzimas catalíticas, por la alta concentración de azúcares reductores y por el tiempo de exposición de las proteínas tisulares (vida media de la proteína) (Lamb EJ et al., 1995; Friedlander MA et al., 1996). Esta reacción se denomina reacción de Maillard. En las etapas iniciales del proceso, se forman productos tempranos y reversibles denominados Amadori. Sin embargo, si el proceso continúa hasta la formación de AGEs se convierte en irreversible. En proteínas de recambio rápido, la glicosilación no enzimática no suele superar las etapas iniciales del proceso, mientras que las proteínas de vida larga llegan a formar los AGEs.

La glicosilación de proteínas solubles y estructurales en el peritoneo y su posterior acumulación, puede ocurrir como resultado de las altas concentraciones de glucosa que se encuentran en el líquido de diálisis, y por tanto, estar implicada en el fracaso de la ultrafiltración en la diálisis peritoneal (Lamb EJ et al, 1995; Friedlander MA et al., 1996). Interesantemente, los GDPs son los mayores inductores de la formación de AGEs, acusando el daño peritoneal (Lai KN et al., 2004; Williams JD et al., 2004; Schwenger V et al. 2006). Esta acumulación de AGEs en el peritoneo es mayor que en plasma, y correlaciona con el desarrollo de fibrosis, esclerosis microvascular, aumento de la permeabilidad y disminución de la ultrafiltración (Yamada K et al., 1994; Nakayama M et al., 1997; Shaw S et al., 1998; Honda K et al., 1999; Park MS et al., 2000).

Los AGEs ejercen su función tanto por la formación de enlaces cruzados con proteínas como por la interacción de receptores específicos de superficie. El receptor más caracterizado para los AGEs es el denominado RAGE (receptor de AGEs). Este receptor se expresa en una amplia variedad de células tales como macrófagos, células endoteliales, células de músculo liso, CMs del peritoneo y células renales tubulares (Schmidt et al., 1999; Oldfield MD et al., 2001; Boulanger E et al., 2002). La unión de los AGEs a estos receptores desencadena varios procesos, entre los cuales se encuentran la generación de radicales libres de oxígeno, la inducción de la expresión de TGF-β y VEGF, la expresión de citoquinas implicadas en procesos inflamatorios y en la conversión mesenquimal de la CM, el aumento de la permeabilidad vascular, la inducción de fibrosis, etc. (Nakayama M et al., 1997;

Oldfield MD et al., 2001; Boulanger E et al., 2002, Schwenger V et al. 2006; De Vriese AS et al., 2006; Boulanger E et al., 2007; Chan TM and Yung S, 2007; Sandoval P et al., 2010) Todos estos procesos conducen en último término al fallo de la ultrafiltración (**Tabla 1**) .

En conclusión, la composición de los líquidos de diálisis y los procesos mecánicos derivados de la diálisis peritoneal hacen de ésta un proceso que produce un daño a la cavidad peritoneal (Perl J et al., 2011; García-López E et al., 2012). Actualmente existen nuevas soluciones de diálisis que tratan de solucionar estos problemas (**Tabla 2**). Sin embargo, no se ha encontrado aún una solución definitiva que evite los daños en el peritoneo que conducen a la fibrosis y al fracaso de ultrafiltración (**Figura 6**)

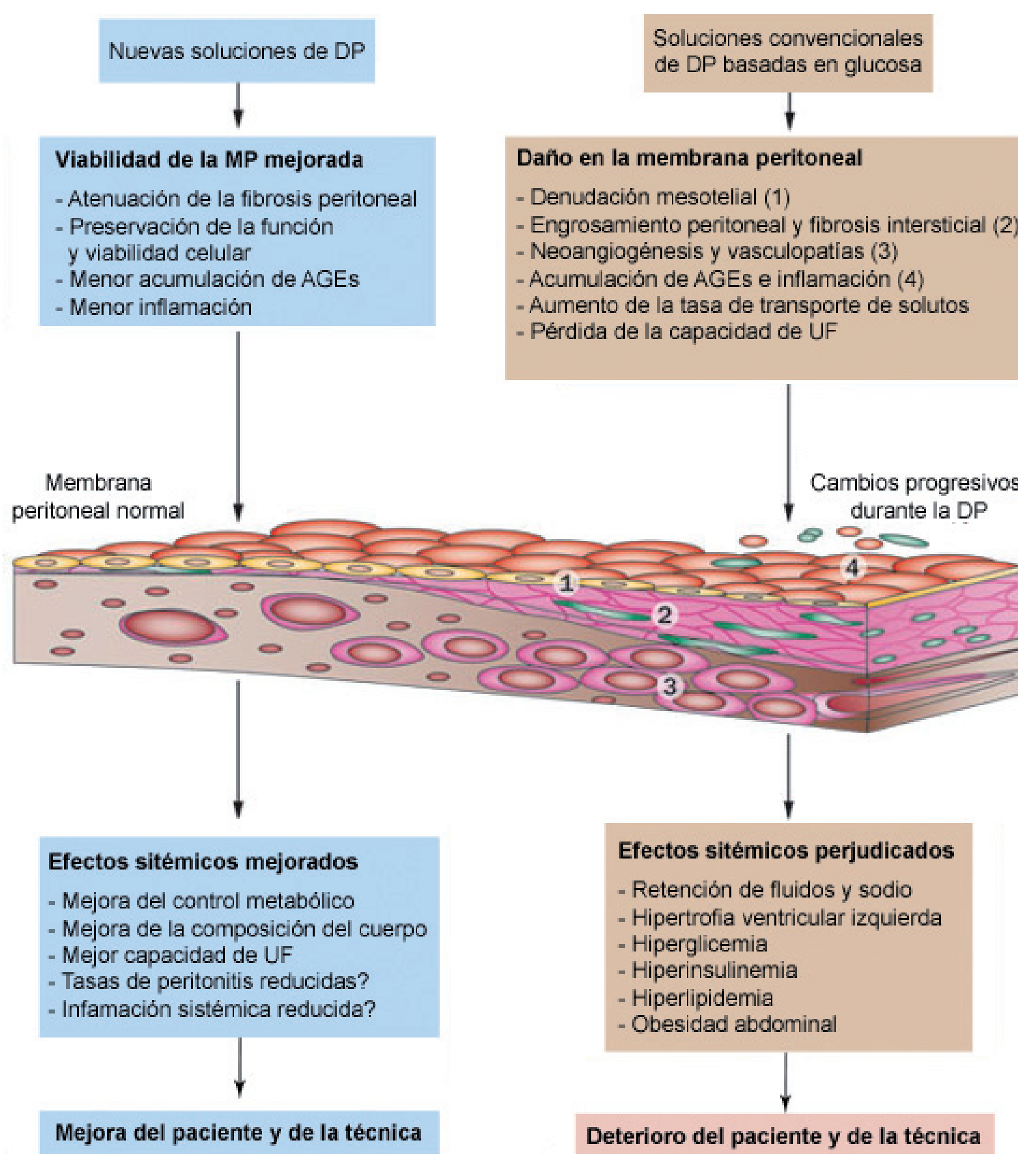

Figura 6. Representación esquemática de los efectos de los líquidos de diálisis convencionales y los nuevos líquidos de diálisis. AGEs, productos de glicosilación avanzada; DP, diálisis peritoneal; MP, membrana peritoneal; UF, ultrafiltración. *Adaptado de, García-López E et al., 2012.*

IV. LA TRANSICIÓN MESOTELIO-MESENQUIMAL INDUCIDA POR DIÁLISIS PERITONEAL

Durante mucho tiempo no estuvo bien definida la patofisiología del peritoneo en la terapia de diálisis peritoneal (Dobbie JW, 1992). Antiguamente, se consideraba que las células mesenquimales o fibróticas que se encontraban embebidas en el estroma submesotelial eran las primeras implicadas en la fibrosis peritoneal, pero nunca se planteó relacionar estas células con las CMs. Sin embargo, hace unos años se descubrió que las CMs que delimitan el peritoneo acaban sufriendo cambios bioquímicos y morfológicos, mostrando una pérdida progresiva del fenotipo epitelial y adquiriendo características mesenquimales mediante un proceso de EMT (Yáñez-Mo M et al., 2003) (**Figura 7**).

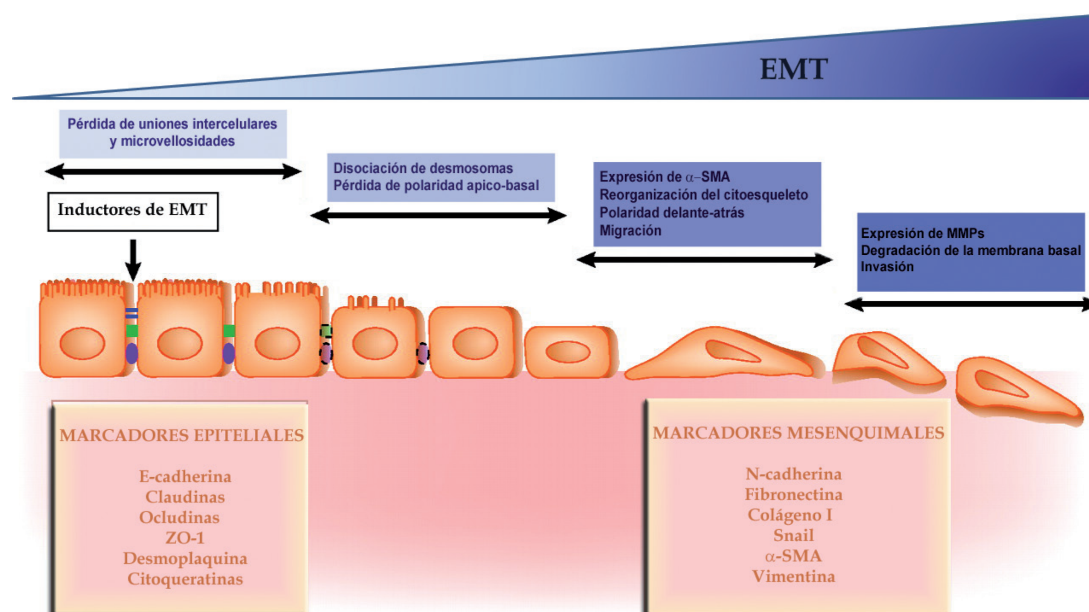

Figura 7. Representación esquemática de una transición epitelio-mesénquima. La figura muestra los 4 pasos esenciales para que se produzca una completa EMT, mostrando los cambios en los marcadores más característicos de las células epiteliales y mesenquimales. α-SMA, alfa actina de músculo liso; ZO-1, zonula occludens-1.*Adaptado del Aroeira L et al., JASN, 2007.*

IV.1. Transición epitelio-mesenquimal

La EMT ocurre cuando una célula epitelial se transforma en una célula mesenquimal (Hay ED, 1995) Las células epiteliales y mesenquimales se diferencian entre sí en características fenotípicas y funcionales. Las células epiteliales se disponen formando una capa, estando estrechamente unidas mediante uniones intercelulares estrechas, uniones adherentes y desmosomas. Además, poseen una polarización apico-basolateral, caracterizada por la distribución de proteínas como cadherinas e integrinas y por la organización del citoesqueleto de actina. Estas células, en circunstancias normales, permanecen apoyadas sobre una lámina basal (Thiery JP and Sleeman JP, 2006). Las células

mesenquimales, a diferencia de las epiteliales, no forman una capa de células organizadas ni poseen polarización apico-basolateral, tampoco disponen de un citoesqueleto de actina organizado y secretan proteínas de la matriz extracelular. Su morfología es de huso alargado y el contacto entre células es solo focal, mediante la emisión de protrusiones pseudopodiales que les permiten una gran movilidad celular.

La EMT se produce de manera natural durante el desarrollo embrionario o en la reparación de heridas, pero también se asocia a enfermedades inflamatorias crónicas y fibrogénicas y a la progresión tumoral; proceso durante el cual se producen cambios morfológicos, bioquímicos y funcionales (Thiery JP, 2002; Thiery JP et al., 2009; Kalluri R and Weinberg RA, 2009). La EMT se activa mediante la interacción de señales extracelulares, incluyendo la interacción con los componentes de la matriz, tales como colágeno y ácido hialurónico; y la liberación de factores de crecimiento solubles, como los miembros de la familia de TGF-β, FGF, EGF y HGF (Massagué J, 1998; Ciruna B and Rossant J, 2001; Kalluri R and Zeisberg M, 2006). La unión de los ligandos a sus receptores desencadena una activación de señales intracelulares, activando moléculas como los miembros de la familia de pequeñas GTPasas, como Ras, Rho y Rac, y miembros de la familia tirosín-quinasa Src. Estas moléculas organizan el desensamblaje de los complejos de unión celular y los cambios del citoesqueleto que ocurren durante la EMT, induciendo la pérdida de la polaridad apico-basolateral y adquiriendo las células un fenotipo mesenquimal (Ikenouchi J et al., 2003). La activación de vías de transducción de señales resulta en la activación de represores transcripcionales como snail, que induce una disminución de proteínas tales como de E-cadherina y citoqueratinas (Cano A et al., 2000). Además, se produce un aumento de la expresión de proteínas como actina de músculo liso (α-SMA), vimentina y proteína específica de fibroblastos 1 (FSP-1), y un aumento en la secreción de componentes de la matriz, tales como fibronectina y colágeno I (**Figura 7**). Paralelamente, se produce un aumento de las capacidades migratorias, invasivas y fibrogénicas de las células junto con la inducción de la expresión de metaloproteinasas necesarias para la invasión celular, ya que degradan la lámina basal y matriz extracelular (Thiery JP and Sleeman J, 2006; Thiery JP et al., 2009; Zeisberg M and Neilson EG, 2009; Acloque H et al., 2009, Kovacic JC et al., 2012). Este proceso puede ser reversible en determinadas circunstancias, es lo que se conoce como transición mesénquimo-epitelial (MET, del inglés "mesothelial-to-mesenchymal transition).

Se han clasificado 3 tipos de EMT (Figura 8) (Thiery et al., 2009; Kalluri R and Weinberg RA, 2009; Kovacic JC et al., 2012) en función del proceso en el que participan:

- Tipo I: Se da durante la implantación, embriogénesis y desarrollo de órganos, donde un epitelio primario se transdiferencia a mesenquimal para dar lugar un epitelio secundario mediante una posterior MET. En este tipo de EMT, no se produce fibrosis, ni se induce un fenotipo invasivo.
- Tipo II: Asociada a la regeneración de tejidos y a la fibrosis de órganos. En este tipo de EMT, un epitelio secundario da lugar a miofibroblastos, estando asociado este proceso con la inflamación y secreción de moléculas de la matriz extracelular.
- Tipo III. Se produce en la metástasis tumoral. Las células epiteliales de un nódulo tumoral primario se transforman en células mesenquimales vía EMT, que migrarán a través del

torrente circulatorio para alojarse en otro órgano y formar un nódulo tumoral secundario vía MET.

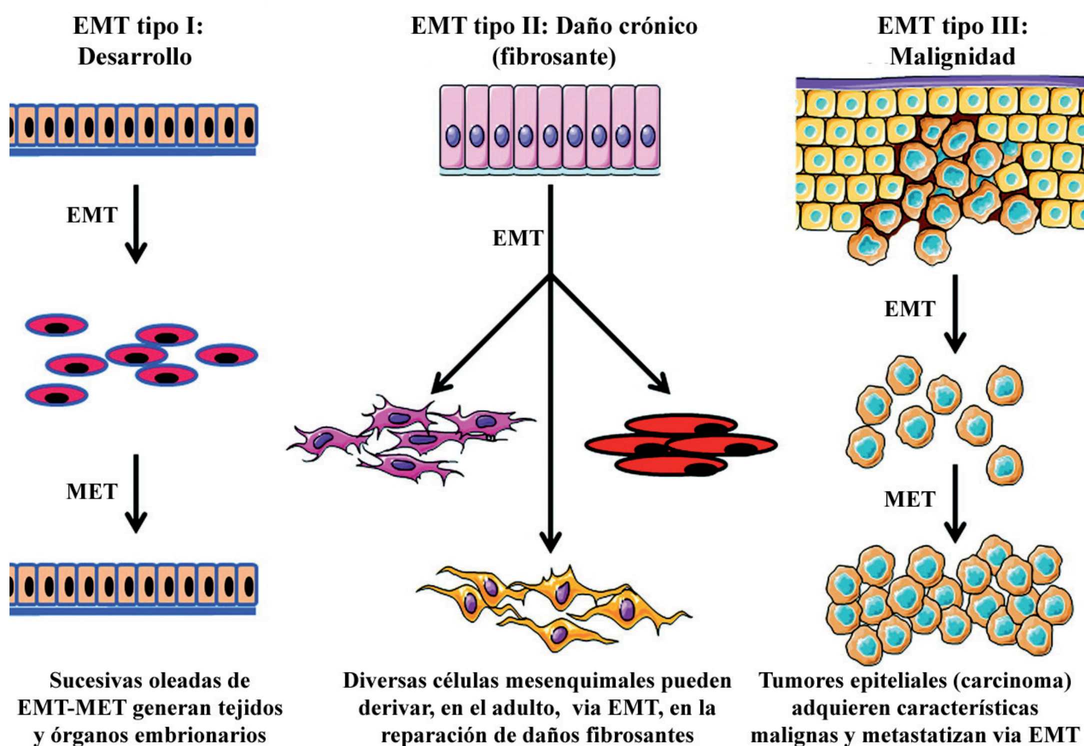

Figura 8. Tipos de transición epitelio-mesénquima. EMT tipo I: ocurre en el desarrollo, donde un epitelio primario, mediante EMT-MET, genera tejidos y órganos. EMT tipo II: se da en la fibrosis de órganos y en la reparación de heridas, dando lugar a células mesenquimales. EMT tipo III: se produce en procesos metastásicos tumorales. EMT, transición epitelio-mesenquimal; MET, transición mesénquimo-epitelial. *Adaptado de Kovacic J C et al., 2012.*

IV.2. Transición mesotelio-mesenquimal

Las CMs sufren una conversión mesenquimal como consecuencia de los prolongados tratamientos de diálisis peritoneal (Yáñez-Mo et al., 2003; Aroeira et al., 2007). Las CMs tienen similitudes morfológicas con las células epiteliales, así como en la expresión de determinados marcadores, como son E-cadherina y ezrina. Sin embargo, son consideradas más bien como un tipo celular mixto, entre epitelial y mesenquimal, debido a su origen mesodérmico. Además, también comparten características con las células endoteliales, como la expresión y secreción de moléculas, tales como ICAM-1, VCAM, VEGF, VEGFR-1,2 (receptor de VEGF-1,2) y neuropilinas (Yañez-Mo et al., 2003; Catalano et al., 2004). Dado que las CMs comparten características tanto con células epiteliales como con células endoteliales, la conversión de las CMs no constituye una EMT en sí misma, ni una transición endotelio-mesénquima, sino que sería, más correctamente denominada, una

transición mesotelio-mesénquima (MMT, del inglés " mesothelial- to- mesenchymal transition"). Existen diversos factores que promueven la MMT en general, y la EMT en particular, provocando cambios en la expresión de determinadas proteínas (**Tabla 3**).

Inductores de MMT/EMT	Proteínas sobre-expresadas	Proteínas en el núcleo
TGF-β	N-Cadherina	β-catenina
FGF-2	Snail	LEF1/TCF
EGF	Vimentina	Snail
AngII	TGF-β	Smad-2/3
PDGF	Fibronectin	NF-κB
IL-1	Colágeno I/III	FGF-2/FGFR-1
AGEs	α-SMA	**Reguladores negativos**
MMP-2 y -3	FGF-1 y -2	Co-represores de Smad
Colágeno I	MMP-2 y -9	Smad-5
Proteínas activadas	FSP-1	Smad-7
ILK	PAI-1	**Cambios celulares**
Wnt	**Proteínas menos expresadas**	Polaridad delante-atrás
MAPK	E-cadherina	Migración aumentada
PI3-K	Citoqueratinas	Invasión Aumentada
Src	Claudinas	Expansión aumentada
Ras/Rho GTPases	Ocludinas	Fibrinólisis disminuida
ROCK	Desmoplaquina	Detención del crecimiento
Proteínas inhibidas	ZO-1	Supervivencia
GSK-3β	Mucina-1	
	tPA	

Tabla 3. Patrones funcionales y moleculares de la EMT/MMT. En esta tabla están señaladas diversas proteínas y factores que inducen una conversión mesenquimal, proteínas que se inhiben y/o activan, los factores y proteínas cuya expresión es aumentada o disminuida, reguladores del proceso y los cambios que se producen en la célula durante la conversión. AGEs, Productos finales de glicosilación avanzada; AngII, angiotensina II; EMT, transición mesotelio-mesenquimal; FGF, factor de crecimiento de fibroblastos; FGFR-1, receptor-1 del factor de crecimiento de fibroblastos; FSP-1, proteína específica de fibroblastos-1; GSK-3β, quinasa glicógeno sintasa-3β; ILK, quinasa unida a integrina; LEF1/TCF, factor potenciador linfoide/factor de célula T; MAPK, proteína quinasa activada por mitógeno; MMP, metaloproteinasa de matriz; PAI-1, inhibidor del activador de plasminógeno-1; PI3-K, quinasa fosfatidilinositol-3; ROCK, quinasa asociada a Rho ; α-SMA, α-actina de músculo liso; tPA, activador tisular de plasminógeno; ZO-1, zonula occludens-1. *Adaptado de Aroeira L, 2007.*

Los estudios de MMT en CMs de pacientes sometidos a diálisis peritoneal se realizaron cultivando las CMs procedentes de la denudación mesotelial y drenadas en los efluentes de los pacientes, estudiando así las características morfológicas y moleculares de las mismas. Así se distinguieron 2 morfologías principales: empedrado tipo epitelio y fusiforme tipo fibroblasto. De este modo, se describió por primera vez cómo las CMs sufren una MMT inducida por diálisis peritoneal, mostrando una pérdida de la polaridad apico-basolateral, uniones intercelulares y microvellosidades. También se observó la inducción de snail y la consecuente represión de la expresión de E-cadherina, un aumento de la expresión de fibronectina y de colágeno I y III, que favorecería la fibrosis peritoneal

(Yáñez-Mo et al., 2003). Adicionalmente, se observó que las CMs empedradas, que poseen una morfología similar a la de un mesotelio normal, mostraban una disminución de la expresión de E-cadherina y citoqueratinas, lo que dio lugar a sugerir que la MMT constituye un proceso temprano que empieza poco después de que se inicie el tratamiento de diálisis peritoneal (Yáñez-Mo et al., 2003).

V VEGF y sus receptores

La fibrosis no es la única alteración que sufre la membrana peritoneal durante la diálisis peritoneal. También se producen vasculopatías y angiogénesis que participan en el fallo de la ultrafiltración. Durante la diálisis peritoneal se produce una secreción de VEGF al efluente, y se ha propuesto que esta producción local e intraperitoneal de VEGF juega un papel central en el proceso que conduce al aumento de la permeabilidad vascular, vasodilatación y angiogénesis (Zweers MM et al., 1999; Inagi R et al., 1999; Pecoits-Filho R et al., 2002; van Esch et al, 2004). Aunque no está claro cuál es la fuente principal de VEGF en pacientes en diálisis peritoneal ni los mecanismos implicados en su inducción, varios estudios proveen evidencias sobre la producción local de VEGF en las células endoteliales y mesoteliales del peritoneo (Inagi R et al., 1999; Selgas R et al., 2000).

VEGF es un potente factor proangiogénico implicado en la proliferación de las células endoteliales y la permeabilidad vascular, por lo que está considerado como un regulador clave en la angiogénesis fisiológica durante la embriogénesis y las funciones reproductivas (Ferrara N, 1999; Ferrara N, 2003). VEGF también está implicado en procesos patológicos asociados a cáncer y a otros trastornos vasculares (Carmeliet P, 2005). Este factor pertenece a una familia de genes que incluye VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F y el factor de crecimiento placentario (P1GF) (Ferrara N, 1999; Lohela M et al., 2009). De VEGF-A existen varias isoformas, denominadas según el número de aminoácidos de cada una: VEGF 121, VEGF 145, VEGF 165, VEGF 189 y VEGF 206. La isoforma más abundante es VEGF165, que comúnmente es denominada como VEGF-A, ya que es la más abundante en el organismo. VEGF-A está implicado fundamentalmente en angiogénesis, migración y vasodilatación; VEGF-B participa en angiogénesis embrionaria y regulación de la degradación de la matriz extracelular; VEGF-C y VEGF-D están relacionados con la angiogénesis linfática; VEGF-E se encuentra codificado en un porxvirus de la familia Orf (Lyttle DJ et al., 1994); y VEGF-F se ha encontrado en venas de serpientes (Yamazaki Y et al., 2005) (**Figura 9**). Existen diversos factores que producen un aumento de la expresión de VEGF tales como hipoxia, citoquinas, factores de crecimiento (TGF-β, TNF-α, FGF, PDGF, IL-6, IL-1) y oncogenes (Ras) (Liu Y et al., 1995; Rak J et al., 1995; Ryuto M et al., 1996; Finkenzeller G et al., 1997; Salven P et al., 2002; Wei LH et al., 2003; Ferrari G et al., 2006).

Los efectos biológicos de VEGF están mediados por tres receptores tirosín quinasa: VEGFR-1 (Flt-1), VEGFR-2 (KDR) y VEGFR-3 (Flt-4) (Ferrara et al., 2003; Cross MJ et al., 2003). Estos constituyen los principales receptores de VEGF y cada receptor participa en diferentes vías de señalización y funciones. VEGFR-1 y VEGFR-2 se expresan fundamentalmente en vasos sanguíneos y VEGFR-3 en vasos linfáticos; también se ha visto expresión de VEGFR-2 en vasos linfáticos

en asociación con VEGFR-3 (Ferrara et al, 2003). La estructura general de los tres receptores está constituida por un dominio extracelular, formado por 7 dominios similares a inmunoglobulinas, una región transmembrana sencilla y una cola citoplásmica con dominios quinasa (**Figura 9**). Cuando VEGF se une sus receptores, se produce una dimerización de los mismos y una activación secuencial de su actividad quinasa intrínseca, activando sitios de unión para moléculas de señalización como proteínas adaptadoras (Grb-2, Shc), lo cual promueve la activación de varias moléculas de transducción de señales como PI3K, ERK-1/2, JNK y p38 MAPK (Cross MJ et al, 2003; Ferrara N et al, 2003; Ferrari G et al., 2006).

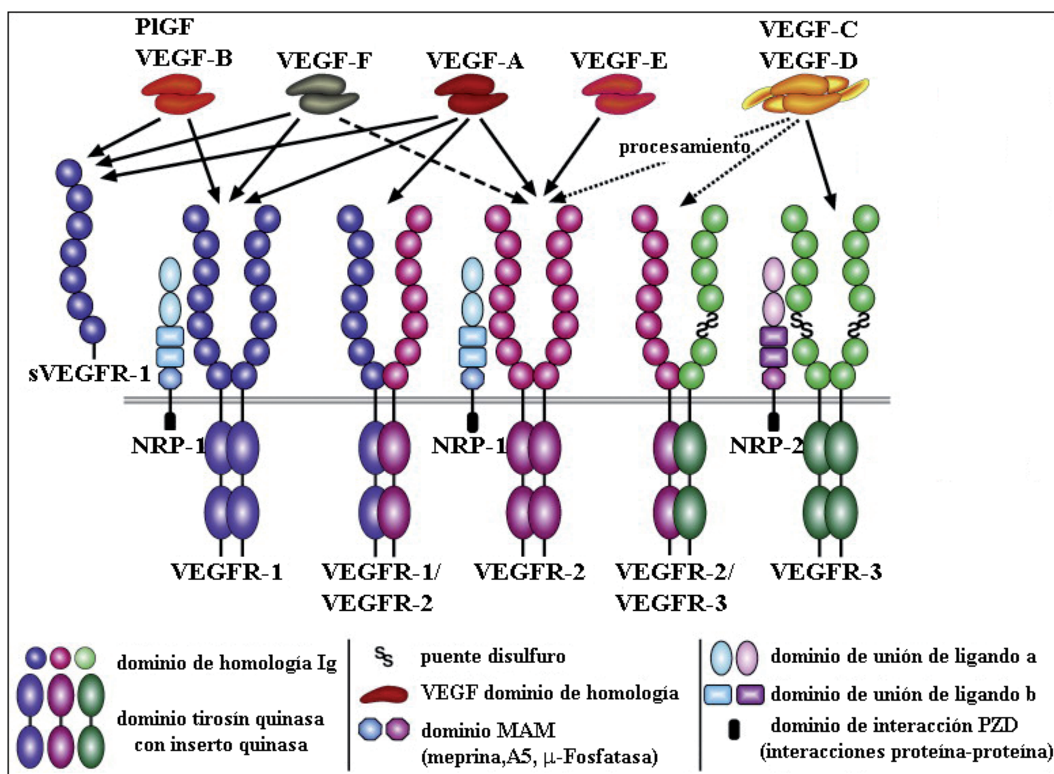

Figura 9. Estructura e interacciones de VEGFs, VEGFRs y co-receptores neuropilinas (NRP). Los factores de crecimiento VEGF se unen a sus receptores con diferentes especificidades (indicado por las flechas). La unión de los ligandos diméricos de VEGF estimula la dimerización y la autofosforilación del receptor, que conduce al reclutamiento de moléculas de señalización. Los receptores de VEGF pueden dimerizar formando homodímeros o heterodímeros. *Adaptado de Lohela M et al., 2009.*

La actividad de VEGF también está regulada por las neuropilinas (Nrps), una familia de glicoproteínas de superficie celular compuesta por dos miembros, neuropilina-1 (Nrp-1) y neuropilina-2 (Nrp-2). Ambas proteínas tienen una estructura primaria bien conservada, manteniendo aproximadamente un 45 % de identidad de aminoácidos. La estructura de estas proteínas está bien caracterizada, formada por un largo dominio extracelular, una región transmembrana sencilla y una corta cola citoplasmática. El domino extracelular está formado por tres motivos estructurales: 2

dominios homólogos a CUB (a1/a2), 2 dominios homólogos a los factores de coagulación V/VIII (b1/b2) y un dominio MAM (meprina/proteína A5/proteína PTPμ) (c) (Gu C et al., 2002) (**Figura 9**). La cola citoplasmática es demasiado corta y no tiene motivo de señalización, por lo que es insuficiente para activar una transducción de señales tras la unión del ligando; sin embargo, se ha visto que puede interactuar con diversas proteínas adaptadoras (Cai H and Reed RR, 1999).

Nrp-1 es la neuropilina más estudiada, caracterizada por primera vez en neuronas como receptor de las semaforinas de clase III (Sema-3) mediando el colapso del cono de crecimiento axonal (He Z and Tessier-Lavigne M, 1997; Kolodkin AL et al., 1997). Posteriormente, fue descrita como co-receptor de VEGF y específico de isoforma en células endoteliales y tumorales, induciendo un aumento de la unión y actividad quimiotáctica de VEGF a VEGFR-2 (Soker S et al., 1998). Además, Nrp-1 ha sido descrita en diversos tipos celulares como células dendríticas y células T (Tordjman R et al., 2002), células hematopoyéticas (Yamada Y et al., 2003), células mesoteliales (Catalano A et al., 2004), células del músculo liso (Liu W et al., 2005) y queratinocitos (Kurschat P et al., 2006).

Los ligandos más estudiados de Nrp-1, Semaforina 3A (Sema3A) y VEGF, son competidores funcionales en su capacidad de unirse a ella (Rossignol M et al., 2000; Gu C et al., 2002) y promueven la internalización de Nrp-1 activada por ligando (Narazaki M and Tosato G, 2006). Sema3A se une a Nrp-1 a través de los dominios a1/a2/b1, mientras que VEGF se une a los dominios b1/b2 (Gu C et al., 2002; Pan Q et al., 2007; Appleton BA et al., 2007). A pesar de la falta de una cola citoplasmática con motivos de señalización, se ha descrito que Nrp-1 puede inducir una señalización independientemente de VEGFR-2, mediando la migración y adhesión tras la unión de VEGF (Bachelder RE et al., 2002; Wang L et al., 2003; Li M et al., 2004; Murga M et al; 2005; Cariboni A et al., 2011). Además de VEGF y Sema-3A, existen otros factores de crecimiento que pueden interaccionar con Nrp-1, entre los que se incluyen FGF (West DC et al., 2005), HGF (Matsushita A et al., 2007) y TGF-β1 (Glinka Y and Prud'homme GJ, 2008; Glinka Y et al., 2011).

Adicionalmente de mediar la angiogénesis (Soker S et al., 1998) y la guía axonal (Kolodkin AL et al., 1997), Nrp-1 participa en otras funciones celulares como en la respuesta inmune primaria (Tordjman R et al., 2002). Sin embargo, también está implicada en procesos patológicos, jugando un papel clave en angiogénesis y progresión maligna de diversos tipos de tumores, tales como cáncer de próstata (Latil A et al., 2000; Pallaoro A et al, 2011), cáncer de mama (Bachelder RE et al., 2002; Barr MP et al., 2005; Glinka Y et al., 2012), cáncer de colon (Parikh AA et al, 2004), carcinoma gastrointestinal (Hansel DE et al., 2004), cáncer de páncreas (Li M et al., 2004; Fukasawa M et al., 2007; Matsushita A, 2007), cáncer de ovario (Baba T et al., 2007) y cáncer de hígado (Berge M et al., 2011).

Hoy en día, las neuropilinas están consideradas como potenciales dianas terapéuticas en el tratamiento del cáncer; no obstante, los complejos mecanismos subyacentes a la interacción de estas proteínas con sus múltiples ligandos no están completamente definidos. En este sentido, se ha descrito recientemente que el bloqueo de la función de Nrp-1 reduce el crecimiento del tumor mediante la inhibición del remodelamiento vascular, haciendo que los vasos sean más susceptibles a la terapia anti-VEGF (Pan Q et al, 2007; Liang WC et al., 2007; Bagri A et al., 2009; Jubb AM et al, 2012).

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS Y OBJETIVOS

Nuestra hipótesis de trabajo es que el incremento de secreción del factor de crecimiento de endotelio vascular (VEGF) durante la diálisis peritoneal es debido a la transición mesotelio-mesenquimal, siendo ésta un mecanismo principal implicado en el fallo de membrana. Además anticipamos que esta transición mesotelio-mesenquimal es en parte inducida por la bioincompatibilidad de las soluciones de diálisis. En base a esta hipótesis planteamos los siguientes objetivos:

1. Estudio de la relación existente entre la transición mesotelio-mesenquimal inducida por diálisis peritoneal, la secreción de VEGF y el fallo de membrana.
2. Estudio del efecto de líquidos de diálisis biocompatibles (con bajo contenido en productos de la degradación de la glucosa) en las células mesoteliales en la relación con la transición mesotelio-mesenquimal.
3. Estudio de los receptores y co-receptores de VEGF durante la conversión mesenquimal de las células mesoteliales. Implicación de VEGF y sus receptores en la capacidad proliferativa e invasiva de las células mesoteliales en distintos estadíos de la transición mesotelio-mesenquimal

MATERIALES, MÉTODOS Y RESULTADOS

1. Estudio de la relación existente entre la transición mesotelio-mesenquimal inducida por diálisis peritoneal, la secreción de VEGF y el fallo de membrana (Am J Kidney Dis 46, 938-948).

Durante la diálisis peritoneal, el peritoneo está expuesto a líquidos de diálisis bioincompatibles que producen modificaciones en la membrana peritoneal. Una de estas modificaciones es el cambio morfológico y estructural de las CMs mediante un proceso de MMT (Yáñez-Mo, M et al., 2003). El proceso de MMT contribuye a que las CMs invadan el estroma submesotelial, favoreciendo la fibrosis del peritoneo mediante la acumulación de componentes de la matriz extracelular. Paralelamente, se producen cambios estructurales que afectan al peritoneo, tales como el aumento del número de vasos sanguíneos y de la permeabilidad vascular, determinantes en el aumento del transporte de solutos a través de la membrana y en consecuencia del fallo de ultrafiltración (Krediet RT et al., 2000). Dichas evidencias dieron lugar a proponer que VEGF (Ferrara N et al., 2003) podría jugar un papel central en los procesos que conducen a la angiogénesis y al fallo funcional de la membrana peritoneal. Aunque se sabía que las CMs eran capaces de secretar VEGF en respuesta a diversos estímulos (Inagi R et al., 1999; Gary Lee YC et al., 2002 Mandl-Weber S et al., 2002), la fuente principal de VEGF en los pacientes sometidos a diálisis peritoneal y los mecanismos implicados en su sobreexpresión no estaban bien definidos. Nosotros analizamos la correlación entre la MMT de las CMs, los niveles de VEGF y el deterioro funcional de la membrana peritoneal dentro del contexto de la diálisis peritoneal.

Para realizar este estudio, aislamos CMs de los efluentes de pacientes en diálisis peritoneal y analizamos el grado de transdiferenciación de las células. Las células fueron clasificadas en 2 grupos atendiendo a su morfologia predominante en: epitelioides, con características morfológicas similares a CMs normales (de omento); y no epitelioides, con fenotipo y características similares a miofibroblastos. Además, aislamos CMs de omento, que fueron estimuladas *in vitro* con TGF-β1 e IL-1β para inducir una MMT que nos sirviese de control. La secreción de VEGF fue medida en los sobrenadantes de los cultivos celulares obtenidos, en los sueros de los pacientes que drenaron las CMs estudiadas, y en biopsias peritoneales de pacientes en diálisis peritoneal y controles sanos. Asimismo, se analizó la función peritoneal de los pacientes mediante el coeficiente de transferencia de masa de creatinina (MTC-Cr). Como resultado, observamos *ex vivo* que las CMs no epitelioides son capaces de producir más componentes de la matriz extracelular (fibronectina y colágeno I) y más VEGF que las CMs epitelioides. Adicionalmente, los pacientes en diálisis peritoneal cuyos efluentes drenaron células no epitelioides, mostraron los niveles séricos de VEGF significativamente aumentados en relación con aquellos pacientes que drenaron células epitelioides. Estos resultados mostraron la existencia de una correlación positiva entre los niveles de VEGF en sobrenadante y en suero. También establecimos correlaciones con los datos clínicos de los pacientes. El MTC-Cr de los pacientes que drenaron CMs no epitelioides fue mayor que aquellos con células epitelioides, correlacionando con los niveles de VEGF.

En conclusión, los resultados mostraron que las CMs que han sufrido una MMT inducida por diálisis peritoneal, son la principal fuente de VEGF en pacientes. Estos datos evidenciaron que la MMT no sólo está implicada en la fibrosis, sino también en la activación y mantenimiento de la angiogénesis peritoneal, así como en el aumento de la permeabilidad vascular.

Mesenchymal Conversion of Mesothelial Cells as a Mechanism Responsible for High Solute Transport Rate in Peritoneal Dialysis: Role of Vascular Endothelial Growth Factor

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• ***Background:*** **During peritoneal dialysis (PD), the peritoneum is exposed to bioincompatible dialysis fluids that cause epithelial-to-mesenchymal transition of mesothelial cells, fibrosis, and angiogenesis. Ultrafiltration failure is associated with high transport rates and increased vascular surface, indicating the implication of vascular endothelial growth factor (VEGF). Sources of VEGF in vivo in PD patients remain unclear. We analyzed the correlation between epithelial-to-mesenchymal transition of mesothelial cells and both VEGF level and peritoneal functional decline.** ***Methods:*** **Effluent mesothelial cells were isolated from 37 PD patients and analyzed for mesenchymal conversion. Mass transfer coefficient for creatinine (Cr-MTC) was used to evaluate peritoneal function. VEGF concentration was measured by using standard procedures. Peritoneal biopsy specimens from 12 PD patients and 6 controls were analyzed immunohistochemically for VEGF and cytokeratin expression.** ***Results:*** **Nonepithelioid mesothelial cells from effluent produced a greater amount of VEGF ex vivo than epithelial-like mesothelial cells ($P < 0.001$). Patients whose drainage contained nonepithelioid mesothelial cells had greater serum VEGF levels than those with epithelial-like mesothelial cells in their effluent ($P < 0.01$). VEGF production ex vivo by effluent mesothelial cells correlated with serum VEGF level ($r = 0.6$; $P < 0.01$). In addition, Cr-MTC correlated with VEGF levels in culture ($r = 0.8$; $P < 0.001$) and serum ($r = 0.35$; $P < 0.05$). Cr-MTC also was associated with mesothelial cell phenotype. VEGF expression in stromal cells, retaining mesothelial markers, was observed in peritoneal biopsy specimens from high-transporter patients.** ***Conclusion:*** **These results suggest that mesothelial cells that have undergone epithelial-to-mesenchymal transition are the main source of VEGF in PD patients and therefore may be responsible for a high peritoneal transport rate.** ***Am J Kidney Dis*** **46:938-948.**

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INDEX WORDS: Peritoneal dialysis (PD); mesothelial cells; peritoneal transport rate; vascular endothelial growth factor.

PERITONEAL DIALYSIS (PD) is an alternative to hemodialysis (HD) for the treatment of patients with end-stage renal disease. In PD patients, the peritoneal membrane acts as a permeability barrier across which ultrafiltration and diffusion take place.[1] Unfortunately, long-term exposure to bioincompatible dialysis solutions and repeated episodes of peritonitis or hemoperitoneum cause injury of the peritoneum, which progressively undergoes fibrosis and angiogenesis and, ultimately, ultrafiltration failure.[2,3] The peritoneal membrane is lined by a monolayer of mesothelial cells (MCs) that have characteristics of epithelial cells and secrete various substances involved in homeostasis of the peritoneum.[4] For a long time, MCs have been considered mere victims of peritoneal injury during PD, whereas resident peritoneal fibroblasts classically have been considered mainly responsible for structural and functional peritoneal alterations.

Recently, we showed that soon after PD therapy is initiated, peritoneal MCs from dialysis effluent show a progressive loss of epithelial phenotype and acquire mesenchymal characteristics.[5] In immunohistochemical studies of peritoneal biopsy specimens from PD patients, we showed the

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expression of mesothelial markers in stromal α-smooth muscle actin–positive myofibroblasts, suggesting that these cells stemmed from local conversion of MCs.[5,6] Recently, myofibroblastic conversion of MCs was confirmed in vivo by injection of an adenovirus vector that transferred active transforming growth factor β1 in rat peritoneum.[7] In addition, we and others showed that this mesenchymal transformation of MCs can be induced in vitro with various stimuli.[2,5,8]

These biochemical and morphological MC changes are reminiscent of those that take place during epithelial-to-mesenchymal transition (EMT).[9] EMT is a complex process that starts with the disruption of intercellular junctions and loss of apical-basolateral polarity typical of epithelial cells, which then are transformed into fibroblast-like cells with pseudopodial protrusions and increased migratory, invasive, and fibrogenic features.[9] Our findings suggest that new fibroblasts may arise from local conversion of MCs by EMT during repair responses of the peritoneal tissue induced by PD.[5,6] A portion of these fibroblast-like MCs are released into PD fluid, and a portion of the remaining cells invade the submesothelium stroma because of their increased migratory/invasive capacity and may contribute to PD-induced fibrosis of the peritoneum.[5] Fibroblast-like MCs may retain a permanent mesenchymal state as long as initiating stimuli persist.

However, fibrosis is not the unique structural alteration in the peritoneal membrane induced by PD. In parallel with this alteration, the peritoneum shows a progressive increase in capillary number (angiogenesis) and vasculopathy.[3,10] There is evidence that angiogenesis and augmented vessel permeability are the main determinants of increased solute transport across the peritoneal membrane and ultrafiltration failure.[2,3] Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor involved, among other molecules, in endothelial cell proliferation and vascular permeability.[11] It was proposed that local VEGF production during PD has a central role, in conjunction with other proangiogenic factors, in processes leading to peritoneal angiogenesis and functional decline.[12-14] Although MCs produce VEGF in vitro in response to various stimuli,[15-18] the main source of VEGF in PD patients and mechanisms implicated in VEGF upregulation during PD remained unclear. Here, we show that nonepithelioid MCs produce much greater amounts of VEGF than epithelial-like MCs, and patients who drain nonepithelioid MCs in their effluent have greater serum VEGF levels than those draining epithelial-like MCs. In addition, we show that peritoneal transport rates correlate with VEGF levels, ex vivo and in vivo, and MC phenotype. A mechanism responsible for high peritoneal solute transport rate based on EMT of MCs is proposed.

METHODS

Patients

We included 37 clinically stable patients on PD therapy; 18 patients on continuous ambulatory PD and 19 patients on automated PD therapy, 27 men and 10 women ranging in age from 25 to 79 years (mean, 61.7 ± 14.5 years). Mean time on PD therapy was 12.6 ± 15.5 months (range, 3 to 62 months). Causes of renal failure were nephrosclerosis in 10 patients, glomerulonephritis in 8 patients, diabetes in 5 patients, chronic pyelonephritis in 5 patients, polycystic kidney disease in 4 patients, unknown cause in 3 patients, and other causes in 2 patients. Twenty-five patients received peritoneal solution based on different glucose concentrations: 1.36%, 75.5 mmol/L; 2.27%, 126 mmol/L; and 3.86%, 214.3 mmol/L and lactate, and 12 of these patients received 1 exchange/d with icodextrin-containing solution. Eight patients were treated with solution containing glucose degradation product–free glucose and lactate; and 4 patients, with solution containing glucose and bicarbonate. Most patients (32 of 37 patients) were administered recombinant human erythropoietin during the study. Duration of active peritoneal inflammation is defined as time (days) from the start of peritonitis (elevation in cell count in PD effluent) and normalizing of cell count. Six patients showed peritonitis, and hemoperitoneum was seen in 4 patients. Mean time of active peritoneal inflammation in these 6 patients was 6.2 ± 5.54 days (range, 3 to 16 days); 2 patients were from the epithelial-like group (range, 4 to 5 days), and 4 patients, from the nonepithelioid group (range, 3 to 16 days).

Peritoneal glucose load was calculated as the sum of glucose contained in each PD fluid bag during the entire time on PD therapy (months). As control groups, 24 patients on HD therapy and 15 young healthy volunteers also were included.

The present study was approved by the Ethics Committee of Hospital Universitario de la Princesa (Madrid, Spain). Written consent was obtained from PD patients included in the study to use serum and effluent samples. Oral informed consent was obtained from omentum donors before elective surgery.

Measurement of Peritoneal Transport Rate

Urea (urea-MTC) and creatinine mass transfer coefficients (Cr-MTC) were measured by using a standard method. Ultrafiltration capacity is defined as a peritoneal exchange of 4 hours using 3.86% (214.3 mmol/L) glucose. Type I perito-

neal membrane failure is defined as a Cr-MTC greater than 11 mL/min and ultrafiltration less than 400 mL/4 hours.[19]

Isolation and Culture of MCs

MCs from PD patients were obtained from PD effluent by using the method described previously.[20] MCs were cultured in Earle M199 medium (Biological Industries, Ashrat, Israel) supplemented with 20% fetal calf serum (Gibco BRL; Life Technologies, Paisley, Scotland), 50 IU/mL of penicillin, 50 μg/mL of streptomycin (ICN Biomedicals, Costa Mesa, CA), and 2% Biogro-2 (Biological Industries).

We previously described that confluent MC cultures from PD effluent showed 3 different phenotypes: epithelial-like, similar to omentum-derived MCs; transitional; and fibroblast-like MCs that remained stable for 2 to 3 cell passages. Frequencies of those MC cultures were approximately 54%, 24%, and 17%, respectively. We also described a less frequent (5%) cell population with mixed phenotypes.[5] Given that transitional and fibroblast-like MCs were similar in terms of molecular expression,[5] in this study, we grouped them into a single category. Therefore, confluent MC cultures were classified according to cellular morphological characteristics and extracellular matrix component expression into 2 groups: epithelial-like (n = 23) and nonepithelioid (n = 14). We did not obtain MC cultures with mixed phenotypes in this instance. The purity of effluent-derived MC cultures was determined by the expression of standard mesothelial markers: intercellular adhesion molecule 1, cytokeratins, and calretinine.[5]

Human peritoneal MCs used as control cells were obtained from omentum samples collected from consenting nonuremic patients undergoing elective abdominal surgery by using the method described by Stylianou et al.[21] To induce transdifferentiation in vitro, human peritoneal MCs were treated with human recombinant transforming growth factor β (0.5 ng/mL) and interleukin 1 (2 ng/mL; R&D System, Minneapolis, MN), as described in our previous study.[5]

The purity of omentum-derived MC cultures was determined by the expression of standard mesothelial markers: intercellular adhesion molecule 1, cytokeratins, and calretinine.[5] These MC cultures were negative for von Willebrand factor, excluding endothelial cell contamination.

Serum Sample Collection

Serum samples were obtained from PD patients at the same times that effluent MCs were collected. Serum samples from HD patients were obtained before the first HD session of the week. All samples were obtained by using vacutainer systems (Becton & Dickson, Frankling Lakes, NJ). After coagulation, samples were centrifuged and sera were collected and stored at –80°C for posterior analysis.

Western Blot Analysis

For Western blotting, first-passage MC cultures were lysed in buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate), and total protein was quantified by using a total-protein assay kit (Pierce, Cambridge, MA). MC proteins (50 μg) were resolved in 8% to 10% sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were blocked with fat-free milk and incubated with specific collagen type I, collagen type IV, and fibronectin monoclonal antibodies (Sigma-Aldrich, San Luis, MO). Membranes were incubated with goat antimouse immunoglobulin G antibody conjugated with peroxidase (Pharmigen, San Diego, CA) and developed with an enhanced chemiluminescence detection kit (Amersham Biosciences, Freiburg, Germany). Blot images were acquired with an LAS-1000 Charge Coupled Device camera (Fujifilm, Cedex, France).

VEGF Measurements

For VEGF concentration analysis, media of confluent MC cultures in the first passage were replaced, and 18 hours later, supernatants were collected and stored at –80% until analysis. Sera and MC supernatant VEGF concentrations were assessed by means of a standard enzyme-linked immunoassay kit (Quantiokine; R&D System). Results of VEGF concentrations in supernatants were normalized with total protein of cell lysate and reported as picograms per microgram. Results of serum concentrations are reported as picograms per milliliter.

Biopsy Processing and Immunohistochemical Analysis

In our nephrology departments, we routinely obtain peritoneal parietal biopsy specimens from PD patients during peritoneal catheter insertion or removal, elective surgeries (inguinal hernia sac, nephrectomy), and renal transplantation. Twelve PD patients included in this study were selected according to their peritoneal transport characteristics and subdivided into 2 groups: low-normal (Cr-MTC range, 4 to 7.2 mL/min; n = 6) and high (range, 12 to 15.2 mL/min; n = 6) transporters. In addition, normal control samples (n = 6) of parietal peritoneum from nonuremic patients who underwent elective surgery also were included in this study. Written consent was obtained from patients before we obtained the peritoneal samples.

To avoid mesothelial artifactual detachment, peritoneal samples were carefully manipulated and immediately fixed with neutral buffered 3.7% formalin (pH 7.3) for 12 to 24 hours. While immersed in formalin, they were attached gently to a flat surface to avoid retraction. Afterward, samples were cut and embedded in paraffin and cut into 3-μm sections. Paraffin sections were mounted on precoated slides, routinely deparaffinized and rehydrated, and incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Antigen retrieval was performed by using a citric acid solution (pH 6) heated with a pressure cooker. Indirect immunohistochemical studies were performed in serial sections from the same peritoneal samples, using anti-VEGF polyclonal antibody (Zymed, San Francisco, CA) and antipancytokeratin monoclonal antibody (Dako, Glostrup, Denmark), as described elsewhere.[6] VEGF and cytokeratin expression was recorded by using a semiquantitative scale described previously.[6]

Statistical Analysis

Results are given as mean $\pm$ SD, whereas median and range were used for such non–normally distributed parame-

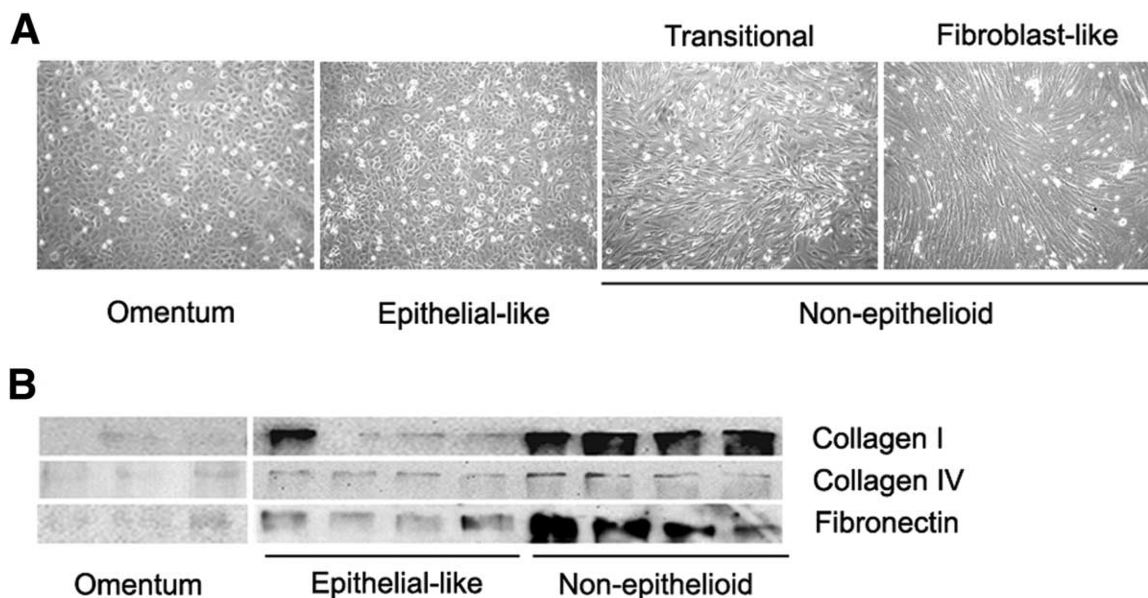

Fig 1. (A) Phase-contrast microscopy shows different morphological characteristics of MCs: omentum, epithelial-like, transitional, and fibroblast-like. Transitional and fibroblast-like MCs were grouped as a single category termed nonepithelioid MCs. (B) Western blot showing expression of extracellular matrix proteins in different MC groups. MCs from PD effluent with the nonepithelioid phenotype show clear upregulation of collagen I and IV and fibronectin.

ters as VEGF levels in sera and supernatants. Comparisons between data groups were performed by using the nonparametric Mann-Whitney rank-sum *U* test, Spearman regression analysis, chi-square, and 2-tail Fisher exact test. *P* less than 0.05 is considered statistically significant. We used the statistical program SPSS, version 11.5 (SSPS Inc, Chicago, IL).

RESULTS

Upregulation of VEGF Expression in Transdifferentiated MCs

In this study, we grouped transitional and fibroblast-like MCs into a single category, hereafter referred to as nonepithelioid MCs (Fig 1A). As shown in Fig 1B, nonepithelioid MCs produced greater amounts of extracellular matrix components than epithelial-like MCs from PD effluent and omentum-derived MCs, reinforcing the concept of EMT of nonepithelioid cells. In addition, effluent MCs showed a progressive increase in VEGF production ex vivo as transdifferentiation proceeded, with VEGF levels maximum in nonepithelioid MC culture supernatants (Fig 2A). Transforming growth factor β plus interleukin 1 treatment of omentum-derived MCs to induce transdifferentiation in vitro resulted in increased VEG production (Fig 2B).

Serum VEGF Concentrations in PD Patients

When PD patients were subdivided into 2 groups according to the phenotype of MCs in their effluent, significantly greater serum VEGF levels were observed in patients who drained nonepithelioid MCs (Fig 3). Furthermore, there was correlation ($r = 0.6$; $P < 0.01$) between VEGF production ex vivo by effluent MCs and serum VEGF levels of patients (data not shown). Circulating VEGF concentrations in PD patients with nonepithelioid MCs in effluent showed a tendency to be greater than in HD patients, although values did not reach statistical significance ($P = 0.078$). Conversely, values were significantly greater than in healthy controls ($P < 0.05$; Fig 3).

Analysis of baseline characteristics of the 37 PD patients included in the study and differences between subgroups according to morphological characteristics of effluent MCs showed important differences in serum and supernatant VEGF levels, Cr-MTC, urea-MTC, and ultrafiltration rate at 3.86% glucose (Table 1).

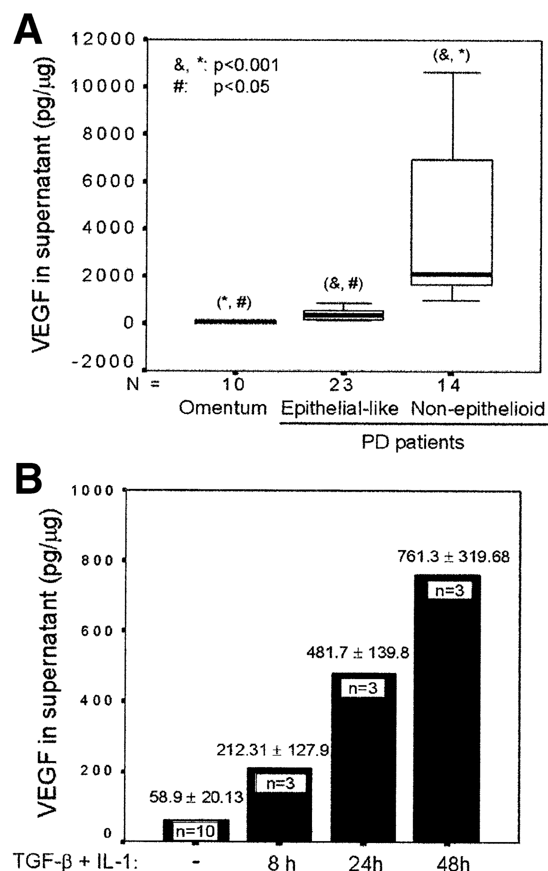

Fig 2. (A) VEGF production in supernatant (picograms per microgram) in MCs from omentum, epithelial-like, and nonepithelioid. Box plots represent 75% percentile, 25% percentile, median, maximum, and minimum values. Symbols show statistical differences between groups: omentum versus epithelial-like (mean, 58.9 ± 20.13 [SD] versus 377 ± 224.5; $P < 0.05$), omentum versus nonepithelioid (58.9 ± 20.13 versus 4,068 ± 3,521.3; $P < 0.001$), and epithelial-like versus nonepithelioid (377 ± 224.5 versus 4,068 ± 3,521.3; $P < 0.001$). (B) VEGF production in supernatant (picograms per microgram) in MCs from omentum treated with transforming growth factor β and interleukin 1 at various times.

Correlation Between Serum and Supernatant VEGF Concentrations and Peritoneal Transport Rate

Because increases in both Cr-MTC and urea-MTC are clinical markers of peritoneal permeability, which is related in turn to augmented blood vessel number, our data suggest that local VEGF production by MCs could have an important role in peritoneal membrane failure. Therefore, we analyzed the correlation between VEGF production in vivo and ex vivo and transport characteristics of PD patients. A significant positive linear correlation ($r = 0.35$; $P < 0.05$) between serum VEGF concentration and Cr-MTC was observed (Fig 4A). Most importantly, when Cr-MTC was related to VEGF production ex vivo by effluent-derived MCs, a strong logarithmic ($r = 0.8$; $P < 0.001$) correlation was obtained (Fig 4B). Thus, Cr-MTC correlated with both serum and supernatant VEGF concentrations (Fig 4C). Urea-MTC also correlated in a logarithmic manner ($r = 0.67$; $P < 0.01$) with supernatant VEGF concentration (data not shown). In addition, both MTCs showed a significant linear correlation ($r = 0.71$; $P < 0.01$; data not shown).

When PD patients were subdivided into 2 groups according to peritoneal transport characteristics (Cr-MTC < 11 mL/min [low and low-average transporters] and Cr-MTC > 11 mL/min [high and high-average transporters]), significantly greater serum ($P < 0.001$) and supernatant ($P < 0.001$) VEGF concentrations were observed in the last group (Fig 5). In addition, Cr-MTC was associated with effluent MC phenotype. No patient with epithelial-like MCs in their effluent showed a Cr-MTC greater than 11 mL/min, whereas 71% of patients (10 of 14 patients) with nonepithelioid MCs showed a Cr-MTC greater than 11 mL/min ($P < 0.001$; Table 2). It is important to note that the remaining 4 patients with nonepithelioid MCs and a Cr-MTC less than 11 mL/min showed a transitional, rather than fibroblast-like, phenotype in their effluent MCs.

Finally, significant positive linear correlations between months on PD therapy and serum VEGF concentrations ($r = 0.39$; $P < 0.05$) and between days of active peritoneal inflammation (peritonitis) and serum VEGF concentrations ($r = 0.41$; $P < 0.05$) were observed, supporting the notion that peritoneal damage contributes to local VEGF production.

VEGF Expression in Peritoneal Biopsy Specimens From PD Patients

To confirm in vivo that local overexpression of VEGF by nonepithelioid MCs was related to peritoneal transport failure, peritoneal biopsy specimens from 6 PD patients considered high transporters, 6 PD patients considered normal or low transporters, and 6 controls (nonrenal patients) were subjected to simple blind (the pa-

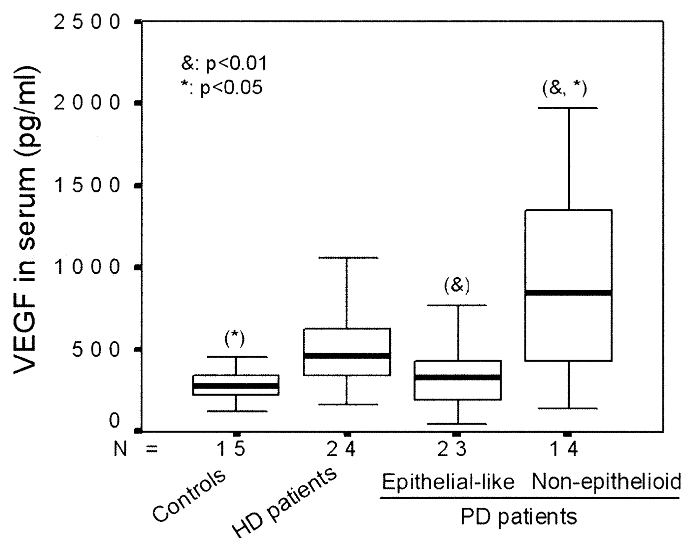

Fig 3. Serum VEGF (picograms per milliliter) levels in PD patients that drained epithelial-like or nonepithelioid MCs in effluent compared with HD patients and healthy controls. Box plots represent 75% percentile, 25% percentile, median, maximum, and minimum values. Symbols show statistical differences between groups: controls versus nonepithelioid (mean, 351 ± 220.6 [SD] versus 894.8 ± 624.3; $P < 0.05$) and epithelioid versus nonepithelioid (331.7 ± 190.4 versus 894.8 ± 624.3; $P < 0.01$). HD patients (456.5 ± 117.2) did not reach statistical differences with the other groups.

thologist) immunohistochemical analysis (Fig 6). Submesothelial fibroblasts from the control group showed no expression of VEGF, with its expression confined to the surface MC monolayer. Despite the low number of cases analyzed, in the high-transporter group, 65% of peritoneal samples (4 of 6 samples) showed clear VEGF expression in spindle-like cells embedded in the fibrotic stroma located mainly in the upper submesothelial area. Conversely, peritoneal biopsy specimens from normal or low transporters showed no or weak VEGF immunostaining in submesothelial fibroblast-like cells, and its expression was confined to deep endothelial cells. Interestingly, serial sections from the same peritoneal samples showed expression of cytokeratins, which overlapped with VEGF expression, in high transporters, whereas normal or low transporters did not show cytokeratin immunostaining in the submesothelial area (Fig 6). Given that the expression of cytokeratins is downregulated gradually during transdifferentiation of MCs, it can be speculated that fibroblast-like cells positive for this marker represent only a portion of the entire population of fibroblastic cells that derive from the mesothelium.[5,6]

DISCUSSION

Peritoneal fibrosis is one of the most common structural changes observed in patients undergoing PD. Degree of fibrosis correlates with time on PD therapy and episodes of peritonitis or hemoperitoneum. For a long time, fibrosis has been considered the main cause of the progressive functional decline in the peritoneum and ultrafiltration failure. In parallel with fibrosis, the peritoneum also shows a progressive increase in capillary number and vasculopathy in response to PD. In this context, recent reports evidenced that enhancement of peritoneal vasculature and vessel permeability is responsible for increased solute transport across the peritoneal membrane and ultrafiltration failure.[3,10,22]

Pathophysiological characteristics of peritoneal functional impairment during long-term PD therapy have remained elusive for a long time. Previous studies showed that MCs from omentum have the capacity to produce VEGF in vitro in response to a variety of stimuli, such as glucose degradation products,[14] advanced glycation end products,[16] transforming growth factor β,[17] and PD fluids.[18] Furthermore, effluent-derived MCs produce spontaneously different levels of VEGF ex vivo, but the reason for these different VEGF production abilities were not established.[23] Results presented in this study clearly show for the first time that the mechanism underlying VEGF upregulation in MCs is EMT of these cells, which is induced by PD. In addition, we also show that patients with nonepithelioid MCs in their effluent show greater circulating VEGF concentrations than those with epithelial-like MCs in effluent. Interestingly, there is a positive correlation between spontaneous VEGF synthesis ex vivo by effluent MCs and serum VEGF concentration, suggesting that MCs

Table 1. Baseline Characteristics and Differences in PD Patients With Different Phenotypes of MCs Cultured From PD Effluent

Parameter	Studied Population (N = 37)	MC Phenotype: Epithelial-Like (n = 23)	MC Phenotype: Nonepithelioid (n = 14)	*P*
Age (y)	62 ± 14.5	60.6 ± 13.8	64.3 ± 16	NS
Time on PD (mo)	12.8 ± 15.4	10.52 ± 15	16.6 ± 15.8	NS
EPO (U/kg/wk)	94.6 ± 94.8	83.9 ± 99.1	112.3 ± 88.1	NS
Peritoneal glucose load (kg)	54.9 ± 34.2	45.2 ± 32.6	64.5 ± 34.8	0.06 (NS)
Urea-MTC (mL/min)	19.8 ± 4.9	17.8 ± 2.8	23.1 ± 5.8	<0.01
Cr-MTC (mL/min)	9.3 ± 2.7	7.94 ± 1.65	11.46 ± 2.6	<0.001
Ultrafiltration 3.86% (mL)*	640 ± 242.3	786.2 ± 117†	508.5 ± 184.1	<0.05
Creatinine clearance (mL/s)	0.08 ± 0.06	0.09 ± 0.06	0.07 ± 0.05	NS
VEGF serum (pg/mL)	375 (42-1,972)	331 (42-876)	847 (139-1,972)	<0.01
VEGF supernatant (pg/μg)	646 (107-10,630)	360 (107-1,100)	2,103 (972-10,630)	<0.001

NOTE. Values expressed as mean ± SD or median (range) for non–normally distributed parameters (serum and supernatant VEGF).

Abbreviations: EPO, recombinant human erythropoietin; NS, not significant.

*Peritoneal exchange with 3.86% glucose (214.3 mmol/L) during 4 hours.

†Ultrafiltration of 1 patient from the epithelial-like group was not available; therefore, in this case, n = 22.

are an important source of VEGF in PD patients and that drained MCs retain their capacity to produce different VEGF levels despite being cultured in homogeneous conditions, at least during the first passage. Our results also indicate that EMT of MCs is involved not only in peritoneal fibrosis, but also in triggering and maintaining peritoneal angiogenesis. Therefore, results obtained with drained MCs in terms of VEGF production ex vivo are keys for understanding the behavior of MCs in the peritoneum.

Peritoneal membrane function is determined by ultrafiltration and small-solute MTC. In agreement with previous results,[12] we found that serum VEGF concentration correlates with Cr-MTC. In this study, we did not measure VEGF levels in dialysate; however, some previous reports,[12-14,24] but not others,[25] also described a correlation between VEGF concentration in PD effluent and peritoneal membrane failure. These discrepancies and the relatively weak associations between serum VEGF level and peritoneal transport rate and between dialysate VEGF level and peritoneal transport rate could be caused by multifactorial influences for VEGF production in vivo; ie, cardiovascular diseases in uremic patients.[26] These multiple factors are no longer present in MC cultures. Therefore, one important finding of this work is that VEGF production ex vivo by effluent MCs shows a stronger correlation with peritoneal transport rate than serum or effluent VEGF concentrations.

Rodrigues et al[27] showed a correlation between MC mass, measured as effluent cancer antigen 125 (CA125), and effluent VEGF level in patients on PD therapy for a few months. In addition, both levels (CA125 and VEGF) correlated with peritoneal transport rate. Conversely, the relationship between CA125 level and both effluent VEGF level and peritoneal transport rate disappeared during long-term PD therapy, probably because of a decline in MC mass. However, these patients maintained the correlation between effluent VEGF level and peritoneal transport rate, and the investigators suggested a non-MC source for VEGF production in late PD. In this study, we clearly show that MCs that have undergone EMT produce a greater quantity of VEGF. These transdifferentiated MCs are located, at least in part, embedded in the submesothelial area because of their increased migratory capacity.[5,6] In this context, our immunohistochemical analysis of peritoneal biopsy specimens from PD patients showed upregulation of VEGF in stromal spindle-like cells in high transporters, but not low transporters, which derived from the mesothelium. Therefore, although MC mass (CA125) declines during long-term PD therapy, MCs that remain in the submesothelial peritoneum, in their mesenchymal stage,

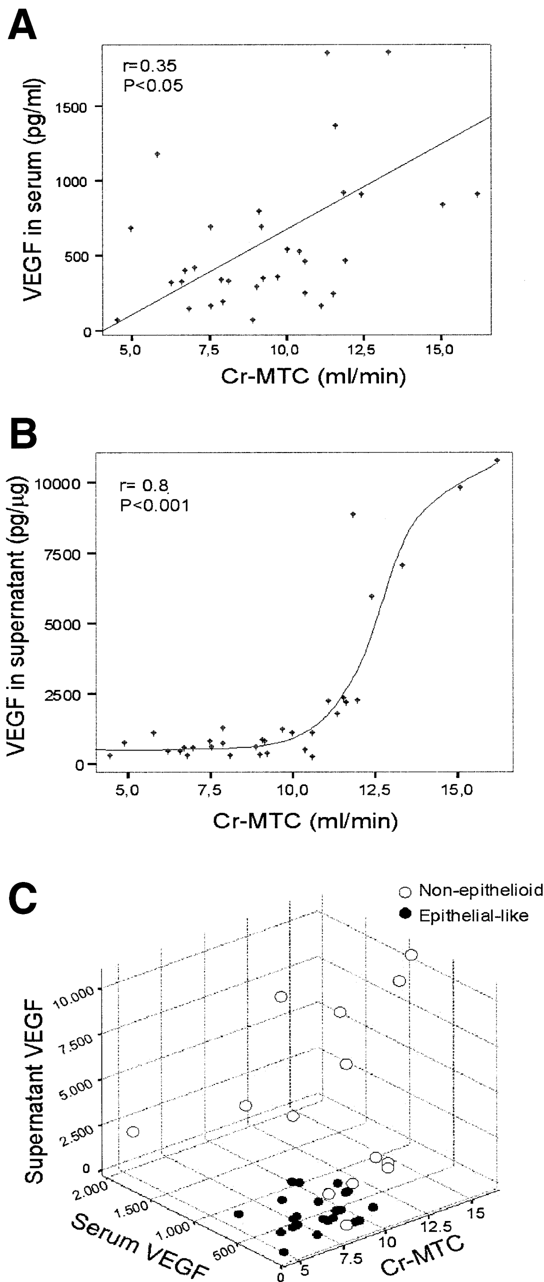

Fig 4. (A) Linear correlation between VEGF levels in serum and Cr-MTC (r = 0.35; P < 0.05) in the PD patient group. (B) Logarithmic correlation between VEGF level in supernatant and Cr-MTC (r = 0.8; P < 0.001). (C) Graphic in 3 dimensions representing the relationship between supernatant, serum VEGF level, and Cr-MTC. Black dots, MCs from PD effluent with epithelial-like phenotype (n = 23); white dots, nonepithelioid phenotype (n = 14).

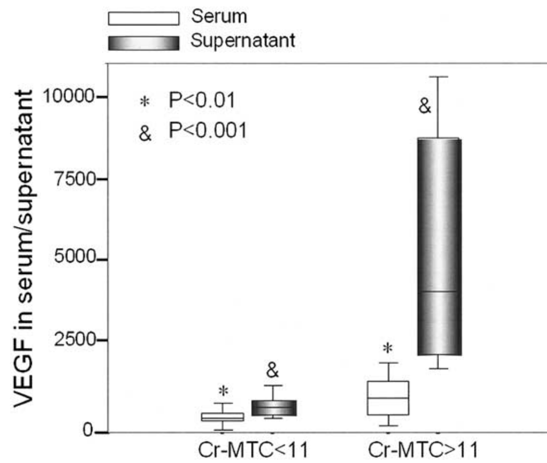

Fig 5. Differences in serum and supernatant VEGF levels in patients with a Cr-MTC less than 11 mL/min (low and low-average transporters) and Cr-MTC greater than 11 mL/min (high and high-average transporters). Serum VEGF values in picograms per milliliter; supernatant VEGF, picograms per microgram. Box plots represent 75% percentile, 25% percentile, median, maximum, and minimum values. Symbols show statistical differences between groups: serum VEGF in patients with a Cr-MTC less than 11 mL/min versus Cr-MTC greater than 11 mL/min (mean, 402 ± 361.5 [SD] versus 928.8 ± 596.7; P < 0.01), supernatant VEGF levels in patients with a Cr-MTC less than 11 mL/min versus Cr-MTC greater than 11 mL/min (506 ± 404 versus 5,196.1 ± 3,591.8; P < 0.001).

also may be responsible for peritoneal transport abnormalities through VEGF overexpression. Although CA125 classically has been used as an index of MC mass in PD patients, recently, the value of CA125 has been questioned because such factors as age and glucose concentration may affect its expression.[28] Thus, use of CA125 as a marker of MC preservation has a limited value. Nowadays, there is no information on

Table 2. Distribution of MC Phenotypes According to Peritoneal Transport Rate

	MC Phenotype		
	Epithelial-Like	Nonepithelioid	Total
Cr-MTC < 11 mL/min	23	4	27
Cr-MTC < 11 mL/min	0	10	10
Total	23	14	37

NOTE. Statistically significant differences in distribution of epithelial-like and nonepithelioid phenotypes in groups of low, low-average (Cr-MTC < 11 mL/min), and high, high-average (Cr-MTC > 11 mL/min) peritoneal transporters (2-tail Fisher exact test, P < 0.001).

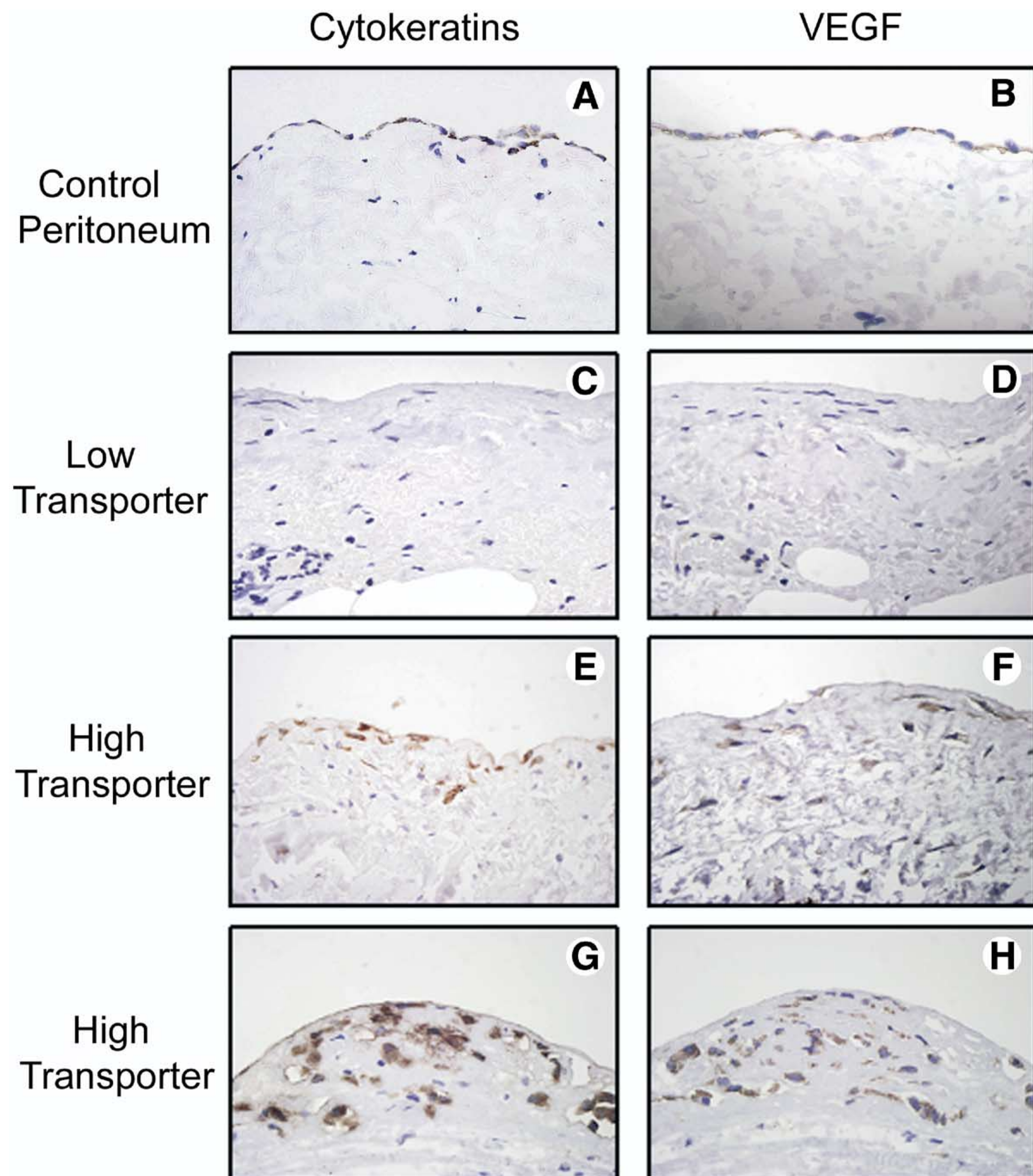

Fig 6. Immunoexpression of (A, C, E, G) cytokeratins and (B, D, F, H) VEGF in representative peritoneal biopsy samples from (A, B) controls and (C, D) low-normal transporter and (E-H) high-transporter PD patients.

CA125 behavior during EMT of MCs; therefore, no conclusion can be inferred from analysis of the association between CA125 and VEGF levels in effluent from long-term PD patients, in which EMT of MCs is massive.

The observation that transdifferentiated MCs invade the submesothelial stroma[5,6] implies that one of the main sources of VEGF in the injured peritoneum is entrapped into the extracellular matrix. Thus, it can be hypothesized that MC-

derived VEGF exerts its effects in a paracrine manner, inducing local angiogenesis and vascular permeability, and only a limited and variable proportion of this VEGF escapes to the effluent and circulation compartments. This could be an additional explanation for the divergent strength of correlations between peritoneal transport rate and VEGF levels produced in vivo (serum and effluent) or ex vivo. Follow-up of VEGF produced ex vivo by effluent MCs may serve as a marker to evaluate the evolution of peritoneal membrane functionality in PD patients.

In this study, we were not able to establish differences between patients using different PD solutions in VEGF production in vivo and ex vivo because of the low number in each group. However, it would be worth comparing the classic and new PD solutions in terms of biocompatibility by using ex vivo VEGF level as a peritoneal function diagnosis marker in longitudinal follow-up.

Data that point to EMT of MCs as a key process in the initiation of peritoneal fibrosis and angiogenesis open new insights for therapeutic intervention. Treatments may be designed toward either direct prevention of EMT of MCs or its deleterious effects, such as extracellular matrix synthesis and/or VEGF production. In this context, it was shown that human growth factor, bone morphogenic protein-7, and inhibitors of the integrin linked-kinase and RhoA-Rho-kinase pathways are able to inhibit and reverse both tissue fibrosis and EMT of tubular epithelial cells in animal models of renal fibrosis.[29] It is conceivable that intervention in either local VEGF synthesis[30] or VEGF activity[31] also might prevent peritoneal neovascularization and ultrafiltration failure. Although VEGF is an important proangiogenic factor, other molecules, such as nitric oxide and angiopoietins, also are involved in endothelial cell proliferation, vascular permeability, and vessel stabilization. Thus, additional studies to analyze the behavior of these molecules during EMT of MCs are required.

In conclusion, our findings suggest that the new fibroblast-like cells that arise from local conversion of MCs by EMT during the repair responses of peritoneal tissue may retain a permanent mesenchymal state as long as initiating stimuli persist and may contribute not only to PD-induced fibrosis, but also to angiogenesis and vascular permeability of the peritoneum through VEGF upregulation.

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2. Estudio del efecto de líquidos de diálisis biocompatibles (con bajo contenido en productos de la degradación de la glucosa) en las células mesoteliales en la relación con la transición mesotelio-mesenquimal (Nephrol Dial Transplant 26, 282-291; Perit Dial Int 32, 292-304).

La exposición crónica del peritoneo a líquidos de diálisis no fisiológicos produce daños en la membrana peritoneal, que conducen a una disfunción peritoneal y a un fallo de membrana (Krediet RT et al, 2000, Williams JD et al., 2002). Este daño de membrana está asociado con la conversión mesenquimal de las CMs, que se cree que es el proceso desencadenante de todos los cambios que se producen y que, en último término, culminarán en un fallo de ultrafiltración. (Yáñez-Mo M et al., 2003; Aroeira L et al., 2005; Aroeira et al., 2007). Se piensa que este proceso de MMT de las CMs se produce principalmente debido a los productos de la degradación de la glucosa (GDPs) presentes en los líquidos de diálisis peritoneal. Estos GDPs son tóxicos para las células y, además, promueven la formación de glicosilaciones no enzimáticas de proteínas, dando lugar a la acumulación de productos finales de glicosilación avanzada (AGEs) (Tauer A et al., 2003; Schwenger V et al. 2006). Estudios previos han descrito que la unión de los AGEs a sus receptores, presentes en las CMs, inducen una transformación miofibroblástica en ellas (De Vriese AS et al., 2006). Asimismo, se ha estudiado el efecto de líquidos con bajo contenido en GDPs a medio plazo, y se ha visto que se produce una menor MMT y una menor secreción de VEGF (Do JY et al., 2005; Do JY et al., 2008). Estas publicaciones nos llevaron a analizar el impacto de los líquidos con bajo contenido en GDPs sobre la MMT de las CMs, *in vitro* y *ex vivo*, en un seguimiento a más largo plazo.

Este estudio se realizó durante un periodo de 4 años, partiendo de un total de 44 pacientes en terapia de diálisis peritoneal. Se analizaron los efectos de 2 líquidos con bajo contenido en GDPs con diferentes soluciones tampón: Balance y Bicavera, ambos de Fresenius Medical Care, en comparación con un líquido con alto contenido en GDPs, pH ácido y lactato como solución tampón (líquido "estándar"). A estos pacientes se les realizó un seguimiento durante 24 meses, recogiendo muestras cada 6 meses. Se aislaron y cultivaron las CMs drenadas en los efluentes de estos pacientes tratados con líquidos con alto y bajo contenido en GDP, para su posterior estudio. Además, se aislaron CMs de omento, que fueron tratadas con los mismos líquidos de diálisis para observar y comprobar si *in vitro* ocurría lo mismo que *ex vivo*. Este trabajo fue dividido en dos partes:

2.1. Efecto del líquido Balance

Balance es un líquido de diálisis con bajo contenido en GDPs y pH fisiológico. Los primeros estudios con este líquido mostraron una mejora en la biocompatibilidad, reduciendo la aparición de un daño de membrana (Williams JD et al., 2004). Observamos que las CMs drenadas en efluentes de pacientes tratados con líquido balance (bajo contenido en GDPs), mostraron menores signos de MMT que aquellos tratados con líquido estándar (con alto contenido en GDPs). *In vitro*, tras tratar durante 72 horas a las CMs obtenidas de omento con el líquido estándar o con TGF-β1, se observó una marcada disminución de los niveles de proteína de E-cadherina, mientras que la exposición al líquido Balance mantuvo los niveles de E-cadherina en comparación con las células no tratadas con líquidos de diálisis. *Ex vivo,* se estudiaron las CMs recogidas de los efluentes, y se observaron resultados

similares. Además, se analizaron otras moléculas asociadas al proceso de MMT, como colágeno I, fibronectina, VEGF, IL-8 y TGF-β. El resultado del análisis mostró una menor inducción de estas moléculas en el grupo tratado con líquido Balance.

Concluyendo, el uso del líquido Balance induce una menor MMT en las CMs, lo que sugiere que este líquido favorece la preservación de la estructura membrana peritoneal en comparación con el uso de líquido estándar.

2.2. Efecto del Líquido BicaVera

BicaVera es un líquido con bicarbonato como solución tampón, con pH fisiológico y con un bajo contenido en GDPs, siendo más biocompatible que los líquidos convencionales (Grossin N et al., 2006). Este estudio se realizó paralelamente al estudio con el líquido Balance, realizando un análisis similar.

In vitro, observamos que el tratamiento con el líquido BicaVera apenas afectó al fenotipo mesotelial y a la expresión de E-cadherina. Sin embargo, la adición del líquido estándar a las CMs de omento dio lugar a la aparición de signos de MMT, como la disminución de los niveles de E-cadherina; observaciones también obtenidas tras el tratamiento con TGF-β para inducir una MMT in vitro. *Ex vivo*, las células no epitelioides mostraron un aumento de los marcadores de MMT (VEGF, fibronectina y colágeno I) y una reducción de E-cadherina. No obstante, en el análisis comparativo de los marcadores entre grupos no dio lugar a la observación de diferencias significativas, a excepción de los niveles secretados de IL-8, que fueron significativamente menores en el grupo tratado con el líquido BicaVera. Pese a estos resultados, el análisis de ambos líquidos a lo largo del tiempo mostró que el uso del líquido BicaVera muestra una progresiva adquisición de un fenotipo epitelioide en las CMs, a diferencia del grupo estándar que tiende a un fenotipo no epitelioide.

Estos resultados sugieren que el uso del líquido BicaVera protege a las CMs a largo plazo, favoreciendo una mejor preservación del peritoneo.

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Original Article

Low-GDP peritoneal dialysis fluid ('balance') has less impact *in vitro* and *ex vivo* on epithelial-to-mesenchymal transition (EMT) of mesothelial cells than a standard fluid

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Abstract

Background. Peritoneal membrane deterioration during peritoneal dialysis (PD) is associated with epithelial-to-mesenchymal transition (EMT) of mesothelial cells (MC), which is believed to be mainly due to glucose degradation products (GDPs) present in PD solutions. Here we investigate the impact of GDPs in PD solutions on the EMT of MC *in vitro* and *ex vivo*.

Methods. For *in vitro* studies, omentum-derived MC were incubated with standard PD fluid or low-GDP solution diluted 1:1 with culture medium. For *ex vivo* studies, 33 patients, who were distributed at random to either the 'standard' or the 'low GDP' groups, were followed over 24 months. Effluents were collected every 6 months to determine EMT markers in effluent MC.

Results. Exposure of MC to standard fluid *in vitro* resulted in morphological change into a non-epitheloid shape, downregulation of E-cadherin, indicative of EMT, and in a strong induction of vascular endothelial growth factor (VEGF) expression. In contrast, *in vitro* exposure of MC to low-GDP solution did not lead to these phenotype changes. This could be confirmed *ex vivo*, as the prevalence of non-epitheloid phenotype of MC in the standard group was significantly higher with increasing PD duration and MC isolated from this group showed significantly higher levels of EMT-associated molecules including fibronectin, collagen I, VEGF, IL-8 and TGF-β levels when compared with the low-GDP group. Over time, the expression of E-cadherin also decreased in the standard but increased in the low-GDP group. In addition, the levels of EMT-associated molecules (fibronectin, VEGF and IL-8) increased in the standard but decreased in the low-GDP group. A similar trend was also observed for collagen I and for TGF-β (for the first year), but did not reach global statistical significance. Accordingly, effluent MC with non-epitheloid morphology showed significantly lower levels of E-cadherin and greater levels of fibronectin, collagen I, VEGF and IL 8 when compared with MC with epitheloid phenotype. The incidence of peritonitis did not significantly influence these results. Drop-out due to technique failure was less in the 'balance' group. The functional, renal and peritoneal evaluation of patients being treated with either standard or 'balance' fluid did not show any significant difference over time.

Conclusions. MC from PD effluent of patients treated with a PD fluid containing low GDP levels show fewer signs of EMT and the respective molecules than MC from patients treated with standard fluid, indicating a better preservation of the peritoneal membrane structure and a favourable outcome in patients using low-GDP fluid. It also confirms the hypothesis that the protection of EMT by GDP-reduced fluids is also present *in vivo*.

Keywords: epithelial-to-mesenchymal transition; low-GDP peritoneal fluid; mesothelial cells; peritoneal dialysis; peritoneal membrane

Introduction

Chronic exposure of the peritoneum to peritoneal dialysis (PD) fluids leads to peritoneal dysfunction and membrane failure [1,2]. Its non-physiological nature is considered to be one of the factors leading to alteration of the peritoneal membrane (PM) [2]. This persistent strain of chronic peritoneal inflammation, exacerbated by acute periodic episodes of peritonitis, contributes to structural abnormalities of the PM. Both processes result in loss of the mesothelial

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cell (MC) monolayer, submesothelial fibrosis, angiogenesis and hyalinizing vasculopathy [3–7]. Such alterations are considered to be the major cause of loss of functional membrane capacity, resulting in ultrafiltration failure. The characterization of this response to PD is based on functional, histological and effluent cytological studies [5–8]. Peritoneal biopsy is the gold standard to investigate PM alterations, but invasiveness precludes its regular use. Based on histological data, we could show that epithelial-to-mesenchymal transition (EMT) of MC is the mechanism that probably initiates the damage of the membrane [9,10]. Transdifferentiated MC acquire a non-epitheloid phenotype with the loss of E-cadherin and cytokeratin expression and an increased production of vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), fibronectin and collagen I [11], which correlates with high peritoneal transport [12]. We have also demonstrated that standard fluids induce EMT of MC *in vitro* [11].

'Balance' is a PD fluid with a low content of glucose degradation products (GDPs) relative to standard solutions, and first clinical studies have suggested an improved biocompatibility [13]. GDPs promote the transformation of precursors of glycosylation (Amadori products) into advanced glycosylation end products (AGE) [14]. MC express AGE receptors (RAGE), and their activation is able to initiate the EMT process [15]. Two different papers published by the same group 3 years apart have shown rapid remesothelization and less EMT using low-GDP solutions in two series of PD patients at medium term [16,17]. In the same sense, we have hypothesized that peritoneal MC of patients exposed to a GDP-reduced fluid ('balance'; Fresenius Medical Care Deutschland GmbH, Bad Homburg, Germany) should be at lower risk of developing mesothelial cell transition, demonstrated both *in vitro* and *ex vivo*, compared with a GDP-rich standard fluid, and they should be at lower risk of deteriorating peritoneal function. The aim of this study, therefore, was to reveal if the expression of EMT markers in MC from effluents of PD patients at medium term is reduced by treatment with the low-GDP solution 'balance'.

Materials and methods

Patients and study design

This prospective study was performed over a 4-year period in two university hospitals using the same PD protocols. Only incident patients were included, and the only inclusion criterion was ability and willingness to perform continuous ambulatory peritoneal dialysis (CAPD) therapy with no expressed indication for automated PD. Patients were alternately assigned to either 'balance' or standard PD fluid depending on the time point of inclusion.

Twenty patients (11 female and 9 male) were allocated to the standard fluid group (Stay Safe) (age 59 ± 15 years; 15% diabetics) and 13 (3 female and 10 male) to the 'balance' group (age 62 ± 11; 38% diabetics) (both solutions from Fresenius Medical Care, Bad Homburg, Germany). Balance, containing 1.5, 2.3 and 4.25% glucose respectively is pH neutral, 'low GDP' solution (Fresenius Medical Care). A follow-up period of 24 months was planned for each patient. The study was performed according to the Declaration of Helsinki and was approved by the ethics committees of both hospitals. Written informed consent was given by the patients. Oral informed consent was obtained from omentum donors submitted to elective surgeries. Small solute and water peritoneal transport was determined during a 4-h dwell peritoneal kinetic study performed with 4.25% glucose peritoneal dialysis fluid, using the allocated fluid type of the patient. Mass transfer area coefficients (MTAC) for creatinine, ultrafiltration capacity for the same period and residual renal function were calculated as previously described [18].

We determined EMT markers in MC released into nocturnal peritoneal effluent every 6 months. If peritonitis or haemoperitoneum occurred, the samples were taken after a 4-week symptom-free period.

Culture of MC and treatments

Human peritoneal MC from PD patients were isolated from PD effluent by using the method previously described [19]. MC were cultured in Earle's M199 medium (Biological Industries, Ashrat, Israel) supplemented with 20% fetal calf serum (Gibco BRL; Life Technologies, Paisley, UK), 50 IU/mL penicillin, 50 μg/mL streptomycin, 2% HEPES 1 M, 10 μg/mL ciprofloxacin (Bristol-Myers Squibb, Columbus, OH, USA) and 2% Biogro-2 (Biological Industries). EMT markers were determined *ex vivo* in cultured MC obtained from effluent of an overnight dwell with PD fluid containing 2.3% glucose (standard or 'balance', depending on group) [19]. For *in vitro* experiments, we used omentum-derived MC that were isolated and cultured from omentum samples as previously described [19]. These cells were used and remained stable for one to two passages. In order to exclude fibroblast contamination, the purity of human MC omentum and effluent-derived cultures was determined by the expression of standard mesothelial markers intercellular adhesion molecule (ICAM-1) [10]. These MC cultures were negative for von Willebrand factor, excluding endothelial cell contamination [19].

Omentum-derived MC were incubated with standard (Stay Safe, 2.3% glucose; Fresenius Medical Care) or low-GDP ('balance', 2.3% glucose; Fresenius Medical Care) solutions diluted 1:1 with culture medium for 48–72 h. MC were also treated with recombinant human TGF-β1 (1 ng/mL) (R&D Systems Inc., Minneapolis, MN, USA) to induce EMT *in vitro* [9,11]. Each experiment was carried out in duplicate, and at least five experiments were performed.

Western blot

MC cultures were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with inhibitor cocktail (Pierce, Cambridge, MA, USA), and total protein was quantified using a total protein assay kit (Pierce). An equal amount of protein (30–50 μg) was fractionated by 8–10% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with non-fat milk and incubated with specific antibodies against E-cadherin (Zymed, San Francisco, CA, USA), Pan-Q Cytokeratin (Sigma-Aldrich, St. Louis, MO, USA) and tubulin (Becton & Dickinson, Franklin Lakes, NJ, USA). Peroxidase-labelled goat anti-mouse antibody (Pharmigen, San Diego, CA, USA) was visualized with an enhanced chemiluminescence detection kit (Pierce), and blot images were acquired with a Kodak Image Station 2000 MM (Kodak, New York, NY, USA).

Quantitative real-time PCR (Q-PCR) analysis

For reverse transcription–PCR analysis, MC were lysed in TRI Reagent (Ambion Inc., Austin, TX, USA), and RNA was extracted as fabricant instructions. cDNA was obtained from 2 μg of total RNA by reverse transcription (RNA PCR Core Kit; Applied Biosystems, Foster City, CA, USA; Roche Systems Inc., Branchburg, NJ, USA). Q-PCR was carried out in a Light Cycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green Kit (Roche Diagnostics GmbH) and specific primer sets for E-cadherin, fibronectin and collagen I. Histone 3 primers were used for PCR reaction control. These studies were performed in MC from patients who reached 18 months of treatment in both groups.

Enzyme-linked immunoassay

For the detection of VEGF, IL-8 or TGF-β in culture supernatants, media of MC cultured under the indicated conditions were replaced and collected 18 h later and stored at −80°C until their analysis. The VEGF concentration in supernatants was determined by enzyme-linked immunoassay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). The levels of fibronectin, procollagen and ICAM-1 levels in cell lysates were assessed by commercially available ELISAs [Biomedical Technologies Inc. (Stoughton, MA, USA), Takara Bio Inc. (Shiga, Japan) and Diaclone (Besancon, France), respect-

ively] according to the manufacturer's protocol and normalized with total protein of cell lysate.

Statistical analysis

Results are given as means ± SEM. Comparisons between data groups were performed using the non-parametric Mann–Whitney rank sum '*U*' test. A P value <0.05 was considered statistically significant. Wilcoxon's test for comparison between periods (intra-group) and Mann–Whitney's test for the comparison between groups were applied.

For studying the complete outcome of each variable over time, we applied the linear mixed model using unstructured covariance matrix for quantitative variables and generalized estimating equations (GEE) for qualitative variables (phenotype) both in the framework of 'Generalized Mixed Models'. These results should be interpreted as follows: (i) significant model means that the interaction 'fluid–time' is $P<0.01$, (ii) significant 'fluid' means that the effect of both fluids is different but the variation over time is not significantly different (maintain parallelism) and (iii) significant 'time' means that the effect of time affects both fluids to a similar degree.

To remove the interference of peritonitis with the studied variables, we followed three different approaches applied on the linear mixed model analysis: (i) isolated analysis of the outcome of patients who never suffered peritonitis vs those with at least one episode, (ii) comparison of samples collected before and after the first episode and introducing it as a co-variable, and (iii) introducing peritonitis (those having one episode vs none, cumulated episodes and days of peritoneal inflammation) as another co-variable in the GEE.

We used SPSS 14.5 (Chicago, IL, USA), which, among others, contains details on the 'Generalized Mixed Models', their method and meaning, and GraphPad Prism 4.0 (La Jolla, CA, USA).

Results

Exposure of MC to low-GDP fluid ('balance') in vitro has less impact on EMT than standard fluid

To analyse the effect of GDPs on EMT *in vitro*, omentum-derived MC were incubated for 48 or 72 h with standard PD fluid composed of 2.3% glucose and buffered with lactate (Stay Safe; Fresenius Medical Care) or low-GDP solutions composed of 2.3% glucose and buffered with lactate ('balance'; Fresenius Medical Care) diluted one-half with culture medium. As a positive control of EMT, omentum-derived MC were treated for 48 or 72 h with human recombinant transforming growth factor (TGF)-β1 (1 ng/mL), which has been proven to be a good model of EMT *in vitro* [10,11]. Exposure of MC to standard PD fluid, with high content of GDPs, resulted in a marked cell death (floating round-shaped cells) and in morphology change at 48 and 72 h, with the acquisition of a spindle-like shape, similar to cells treated with TGF-β1 (Figure 1A). In contrast, exposure of MC to low-GDP fluid had no effect on cellular viability and on cellular morphology. In addition, treatment of MC with standard PD fluid or TGF-β1 induced the down-regulation of E-cadherin, indicative of EMT (Figure 1B). The effect on E-cadherin was more evident at 72 h in cells exposed to standard fluid, indicating that the accumulation of soluble factor(s) was required to repress the expression of this epithelial marker. Interestingly, when incubating MC with the low-GDP fluid, the expression of E-cadherin was preserved (Figure 1B). These data were confirmed in a more quantitative manner by measuring the expression levels of E-cadherin mRNA by real-time RT–PCR. Exposure of omentum MC for 72 h to standard PD fluid or TGF-β1 significantly repressed the expression of E-cadherin mRNA (Figure 1C). In agreement with the results of E-cadherin protein expression shown above, in MC exposed to low-GDP fluid, the expression of E-cadherin mRNA was preserved (Figure 1C).To further explore the effects of PD fluid exposure on EMT, we analysed the expression of VEGF, which has been shown to be up-regulated during the mesenchymal conversion of MC [9,11]. As shown in Figure 1D, exposure of MC to standard PD fluid or treatment with TGF-β1 significantly induced the secretion of VEGF, whereas MC exposed to low-GDP fluid did not show a significant up-regulation of VEGF.

Clinical outcome of patients

Patients could be followed up for 6 (standard/'balance') (20/13), 12 (18/11), 18 (11/11) and 24 months (3/9), respectively. Patients from the 'balance' group showed significantly higher ($P=0.008$) technique survival than those from the standard fluid group, specifically after the first year of treatment. The reasons for drop-out were kidney transplantation in 7/0 patients, transfer to haemodialysis in 5/1, transfer to automated peritoneal dialysis (APD) in 3/2 and death in 1/1 patient (standard/'balance'), respectively. Switch to haemodialysis was more frequent in patients treated with standard fluid ($P=0.008$). The percentage of patients affected by peritonitis was similar in both groups (6 of 20 patients, 30%, for the standard and 5 of 13 patients, 38%, for the 'balance' fluid group). The time to the first episode in each group was also not significantly different (data not shown). The global incidence was slightly higher in the 'balance' fluid group (one episode/16 patient-months vs one episode/27 patient-months in the standard group).

Peritoneal function studies and residual renal function

Creatinine MTAC values were significantly higher in the 'balance' group from the very beginning, but there was no significant change for any group over time. Ultrafiltration capacity was significantly greater in the standard fluid group, but there were no changes for any group over time either. Whereas residual renal function values were slightly higher in the 'balance' group during the first year, the follow-up analysis did not reveal significant differences between groups but a significantly greater 24-h diuresis with 'balance', with no significant modifications for any of the two fluids over time (Table 1).

Ex vivo cell studies

The percentage distribution of MC with epitheloid phenotype was similar in both groups at baseline. However, <25% of patients from the standard fluid group maintained this phenotype in contrast with >75% of 'balance' patients at medium and long term (Figure 2A). The differences reached statistical significance by linear mixed models in the overall observation both for fluids and over time (fluid–time $P=0.00001$). Representative examples of patients of the standard PD fluid group showing an epitheloid to non-epitheloid conversion and of the 'balance' group showing a reversion from non-epitheloid to epitheloid phenotypes, as determined by cellular morphology or cytokeratin expression, are depicted in Figures 2B and C.

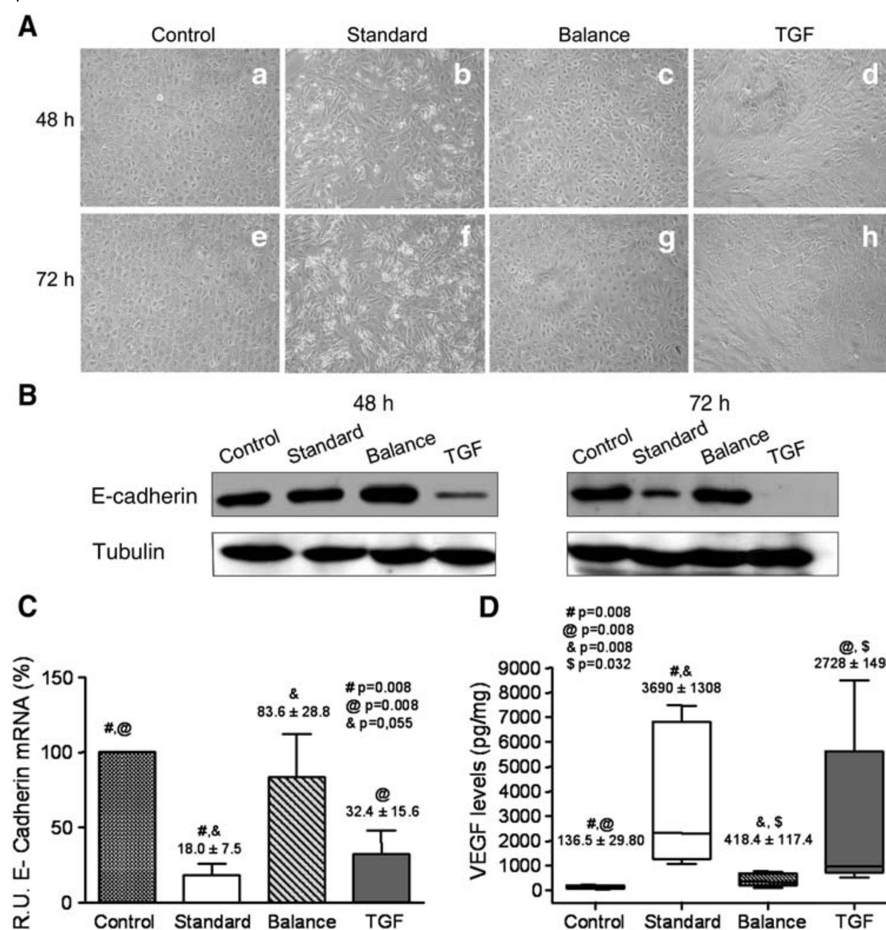


Fig. 1. Effects of PD fluids on MC *in vitro*. (**A**) Effects of PD fluids on MC morphology at 48 and 72 h *in vitro*. Pictures are representative of five independent experiments. (**B**) Western blot showing expression of E-cadherin in MC exposed to PD fluids. Tubulin was used as loading control. Photographs are representative of five independent experiments. (**C**) E-cadherin MRNA levels were analysed by quantitative RT–PCR as described. The results represent the relative expression of E-cadherin mRNA in cells treated with PD fluids or TGF-β1 compared with untreated cells, and data are depicted as mean value±standard error of five experiments. (**D**) VEGF production in supernatant (picograms per milligram of cell protein) in MC from omentum treated with PD fluids or TGF-β1. Box plots represent 75% percentile, 25% percentile, median, maximum and minimum values of five experiments. Symbols show statistical differences between groups.

Figure 3 (A through F) confirms the significant differences between 'balance' and standard groups in terms of EMT-associated molecules in supernatant or cellular extract from effluent-derived MC, along with the observation and with the linear mixed model analysis. E-cadherin expression was progressively lost along the experience in standard fluid patients, whereas it was preserved in 'balance' patients (Figure 3A). On the contrary, and confirming the acquisition of EMT state by MC, VEGF, collagen I (in the limit of significance for fluids, P=0.056), fibronectin, TGF-β and IL-8 levels were significantly (mixed models) higher among standard fluid patients over time (Figure 3B–F).

Table 1. Values of peritoneal transport of creatinine (MTAC), ultrafiltration capacity (UF) and residual renal function in both groups (mean ± SD, control indicates standard fluid group)

	Creatinine MTAC (mL/min)		UF (mL/glucose 4.25%, 4 h)		Residual renal function (mL/min)	
	Standard	Balance	Standard	Balance	Standard	Balance
Baseline	7.6 ± 3.5	12.2 ± 4.6	832 ± 199	615 ± 274	5.8 ± 3.9	7.0 ± 4.3
6 months	6.2 ± 2.04	10.6 ± 2.8	915 ± 247	768 ± 154	3.4 ± 2.5*	6.9 ± 4.2
12 months	6.8 ± 3.1	10.1 ± 2.6	883 ± 273	676 ± 121	4.1 ± 3.1	5.5 ± 3.7
18 months	8.3 ± 2.8	11.6 ± 5.6	833 ± 234	581 ± 296	4.0 ± 2.8	5.0 ± 4.2
24 months	6.1 ± 2.6	9.2 ± 3.4	825 ± 106	720 ± 195	4.2 ± 4.0	4.2 ± 2.6

*P=0.016.

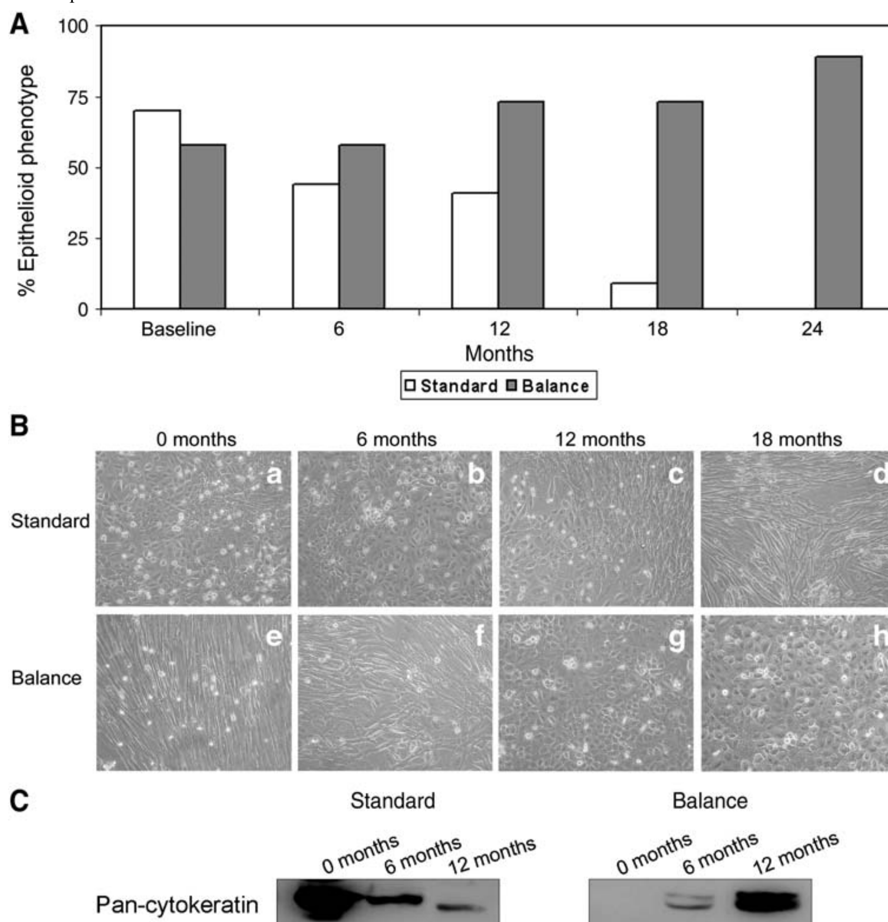


Fig. 2. Epitheloid and non-epitheloid phenotype of MC in standard and 'balance' groups. (**A**) Differences in the percentage of patients showing epitheloid phenotype in MC in each group of fluids over time (mixed models, fluid–time P=0.00001). (**B**) Pictures of MC phenotype from one representative patient from each group of PD fluids (A through D for a standard fluid patient and E through H for a patient treated with 'balance' fluid). (**C**) Western blot of pan-cytokeratin expression in MC from one representative patient of each group of fluids. Red Ponceau staining was used as loading control (data not shown).

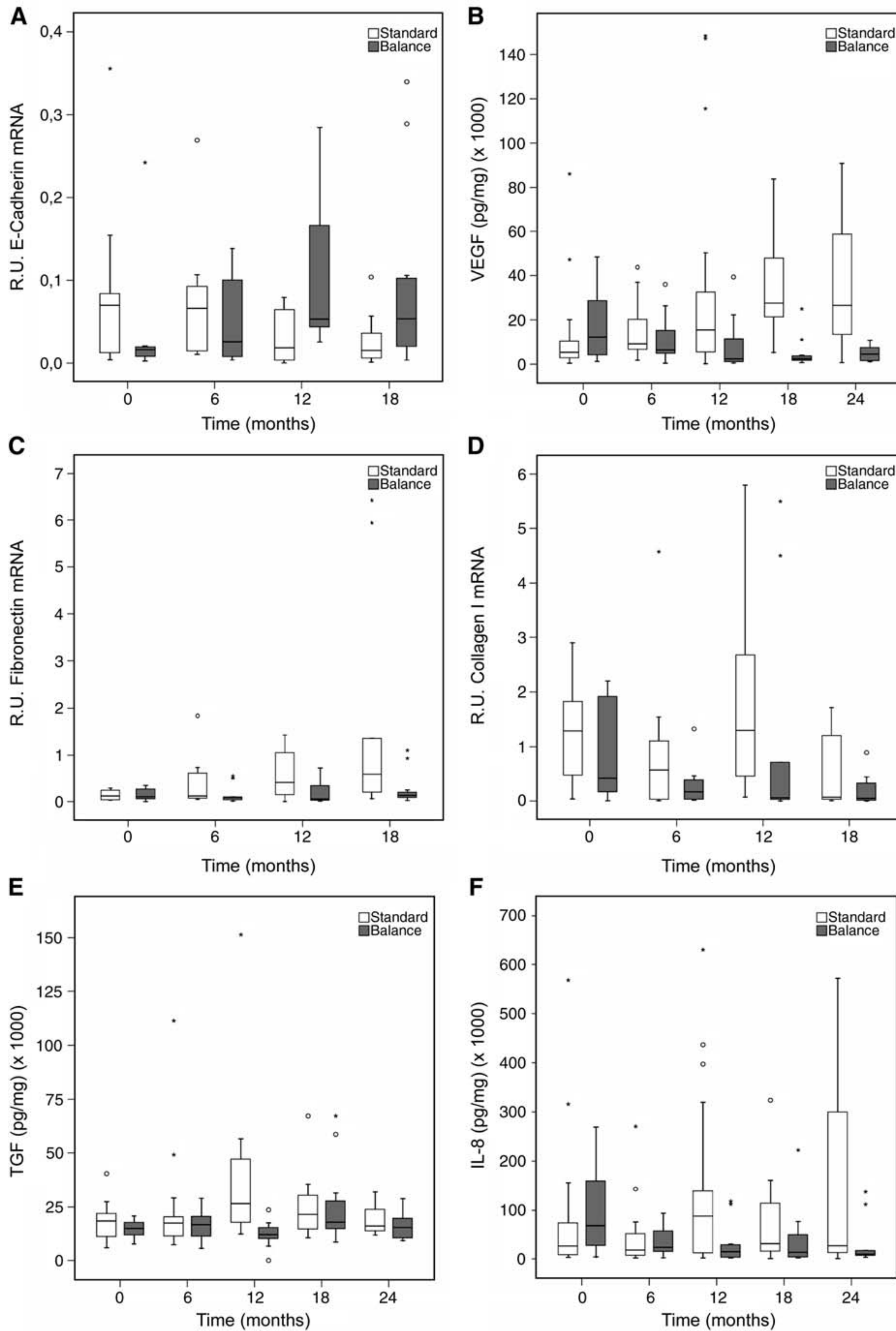
Evaluating all samples by group of fluid, the standard fluid group showed significantly higher values of VEGF, fibronectin mRNA, collagen I mRNA (P=0.056), IL-8 and TGF-β than the 'balance' group; on the contrary, ICAM-1, procollagen and fibronectin proteins showed no significant differences (Figure 4A). E-cadherin mRNA differences did not reach significance (data not shown).

In general, as a confirmation of the hypothesis (agreement between phenotype and EMT products), we found significantly greater levels of VEGF (4-fold), procollagen and collagen I (2- and 1.2-fold, respectively), fibronectin and fibronectin mRNA (5-fold) and IL-8 (2-fold) in non-epitheloid phenotype cells in all samples. However, TGF-β showed similar values for both phenotypes (Figure 4B). In contrast, E-cadherin showed significantly lower values in non-epitheloid cells (0.037 ± 0.04 vs 0.089 ± 0.09, P=0.02).

Effects of peritonitis episodes in variables related to EMT

Peritonitis incidence did not influence these results. In patients with no peritonitis, the MC phenotype outcome was similar to the overall population. However, in patients who suffered peritonitis and were treated with standard fluid, non-epitheloid phenotype was mostly observed over time. In contrast, only few patients in the 'balance' group who suffered peritonitis showed a loss of the usual epitheloid phenotype over time.

In regard to the levels of cytokines and growth factors in the supernatants or MC extracts, patients without peritonitis showed similar values for all markers when compared with the whole group. As these patients were observed after the first episode of peritonitis, in particular the supernatant VEGF levels were markedly increased (6-fold) afterwards for standard fluid (41 555 ± 47 986 pg/mg, n=12 samples vs 7033 ± 6608 pg/mg, n=24 samples in the 'balance' group). Fibronectin and procollagen levels did not show differences for these subgroups. This difference did not reach statistical significance due to intra-group variability. To confirm this trend, we analysed the outcome of supernatant VEGF levels by linear mixed models including as co-variable the cumulated episodes of peritonitis; the significant differences between both groups of fluids per-


A
R.U. E-Cadherin mRNA
Standard
Balance
Time (months)
B
VEGF (pg/mg) (x 1000)
Standard
Balance
Time (months)
C
R.U. Fibronectin mRNA
Standard
Balance
Time (months)
D
R.U. Collagen I mRNA
Standard
Balance
Time (months)
E
TGF (pg/mg) (x 1000)
Standard
Balance
Time (months)
F
IL-8 (pg/mg) (x 1000)
Standard
Balance
Time (months)

sisted, indicating that dialysis fluid influences the VEGF supernatant levels, independent of suffering or no peritonitis. The days of peritoneal inflammation (leukocyte count >100 cells/mm^3) treated as co-variable neutralized the differences between the two dialysis fluids. This indicates that the severity and duration of inflammation determines a loss of the protection by 'balance' fluid in terms of VEGF production.

Discussion

In the present study, we investigated the effect of a low-GDP solution on EMT of MC *in vitro* and *ex vivo* by defining EMT as acquiring a non-epitheloid phenotype associated with the loss of E-cadherin expression and the production of higher amounts of mesenchymal products. Our data obtained from the analysis of EMT *in vitro* and *ex vivo* showed that exposure of MC to a low-GDP solution is consistently associated with the preservation of an epitheloid phenotype. Factors related to mesenchymal conversion were secreted to a much lower extent after treatment with 'balance' compared with standard PD fluid, which strongly induces these molecules.

MC obtained from omentum *in vitro* cultures preserve their physiological phenotype. But if a standard fluid with high content of GDPs is added to cultures, a non-epitheloid phenotype associated to the loss of E-cadherin expression is acquired. This effect on E-cadherin is evident at later time points (72 h) when compared with cells treated with TGF-β indicating that the accumulation of soluble factor(s) was required to repress the expression of this epithelial marker. In this context, we have previously described that the exposure of omentum-derived MC to standard PD fluids induces the expression of TGF-β [20], which in turn may induce the EMT process. In contrast, the addition of low-GDP solution 'balance' to MC cultures barely affects the epitheloid morphology and E-cadherin expression of these cells, revealing the preservation of the physiological MC identity. These results demonstrate that the content of GDPs in PD fluids has an important role in the induction of EMT of MC.

The *ex vivo* studies performed in effluent-derived MC showed similar results. Changes suffered by MC in their morphology and expression of E-cadherin over time have confirmed that a normal epitheloid phenotype was associated with the use of 'balance' fluid. E-cadherin values were similar for both fluids at baseline and at the sixth month. In contrast, values at the 12th and 18th months were significantly higher for the 'balance' group.

The assessment of VEGF levels from cultured MC *in vitro* and *ex vivo* demonstrated a marked increment of this growth factor during the EMT process. Globally, this VEGF production was notably increased by standard fluid compared with 'balance' fluid [9,19,21]. This difference accentuated over time. As VEGF is partially responsible for local vasodilatation, and its levels both *ex vivo* in supernatant and in serum of PD patients correlate with high peritoneal transport [1,2,9], we can suggest VEGF as a key molecule for peritoneal function in this context.

Data obtained with other EMT-associated molecules including collagen I, fibronectin, IL-8 and TGF-β have globally confirmed the differences in MC from peritoneal effluents according to the fluid use, always in the sense that standard fluid induced greater production of these agents.

IL-8 levels were shown to be induced in MC *in vitro* by standard PD fluids [22] and GDPs [23]. Furthermore, there are hints that IL-8 might influence solute transport in PD-related peritonitis and that IL-8 promotes cell migration [24], thereby exacerbating the peritoneal membrane deterioration. Coherently, TGF-β levels did not show any correlation with the phenotype, but comparing MC from the distinctly treated groups, significant differences in TGF-β production for both fluids were only observed when the patients were evaluated by group. The time-dependent course of TGF-β is noteworthy, reaching its maximal levels during the first year but regressing to lower values afterwards. TGF-β has been shown to down-regulate MC proliferation and to accelerate peritoneal fibrosis by inducing the production of extracellular matrix proteins [25–28]. So the down-regulation of several factors associated with membrane deterioration clearly hints towards a benefit of low-GDP solutions in this process.

In agreement with our results, a recent report has shown that different GDPs present in standard PD fluids can induce EMT of MC *in vitro*, in that low-GDP fluid has less impact on peritoneal fibrosis and EMT *in vivo* in a rat model [29].

All these results are in accordance with that obtained in a series of patients (present series and references [16,17]).

Mechanistically, the induction of EMT by standard is related to their high GDP content, which may contribute to AGE accumulation and activation of RAGE, and damage of MC [14,15,30].

The follow-up analysis on EMT markers could be influenced by episodes of peritonitis. Therefore, we compared these markers in MC from patients with and without peritonitis, and we did not find significant influences. However, the variability of the data and the unavoidable reduction of the series precluded a more profound analysis. The most remarkable finding in patients with no peritonitis was that standard

Fig. 3. Box plots representing the cytokine levels in supernatant or extract from mesothelial cells derived from effluents under 'balance' and standard fluid groups. Statistical comparison is based on the mixed model analysis, which determines the significance of the differences between the two fluids and that from each fluid over time. (**A**) E-cadherin (mRNA levels, expressed in relative units) expression shows an increase in the 'balance' group over time, whereas the contrary is observed in the standard fluid group. This difference for time–fluid is significant (P=0.024). (**B**) VEGF production (picograms per milligram) into supernatant demonstrates lower values in the 'balance' group than in the standard fluid group throughout the study. This difference for time–fluid is significant (P=0.017) as well as for fluids (P=0.00001). (**C**) Fibronectin mRNA (relative units) values in MC from the 'balance' group show lower values than that from the standard fluid group over time. This difference is significant just for fluids (P=0.017) but not over time. (**D**) Collagen I mRNA (relative units) values from MC from the 'balance' group show lower values than that from the standard group in the limit of significance (P=0.056) and no differences over time. (**E**) TGF-β cell supernatant levels (picograms per milligram) were globally and significantly higher in the standard fluid group (fluid, P=0.047), specifically during the first year. (**F**) IL-8 supernatant levels (picograms per milligram) (to be correctly read, IL-8 values should be multiplied by 10) showed significantly higher levels in the standard fluid group with a non-significant trend to grow over time (fluid–time, P=0.041).

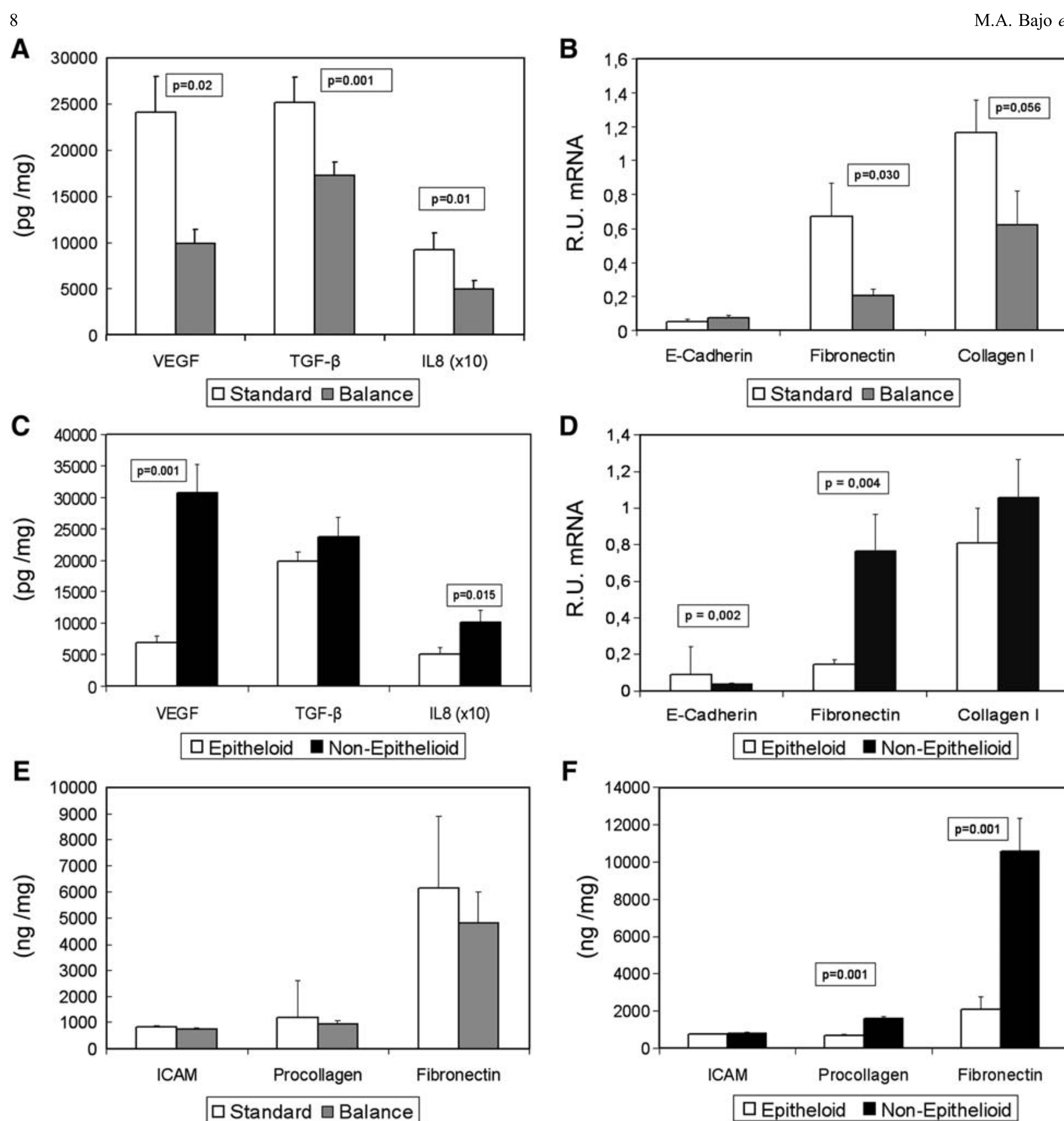


Fig. 4. Mean values of the different mesothelial products studied in culture: evaluation of all samples together. (**A**) Differentiated by group of fluid, the standard fluid group showed significantly higher values of VEGF, TGF-β and IL-8 in supernatant (picograms per milligram). (**B**) Differentiated by group of fluid, the standard fluid group showed mRNA higher values of fibronectin (P=0.03) and collagen I (P=0.05). E-cadherin levels were similar in both groups. (**C**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed higher values of VEGF (P=0.001), TGF-β (NS) and IL-8 (P=0.01) in supernatant (picograms per milligram). (**D**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed lower mRNA levels of E-cadherin (P=0.002) and higher levels of fibronectin (P=0.004) and collagen I (NS). (**E**) Differentiated by group of fluid, the standard fluid group showed no significant differences in cellular lysate (nanograms per milligram) for ICAM-1, procollagen and fibronectin protein levels. (**F**) Differentiated by phenotype of MC, the non-epitheloid phenotype showed higher values of procollagen (P=0.001) and fibronectin (P=0.001) and similar values for ICAM-1 protein levels in cellular lysate (nanograms per milligram).

fluid maintained its association with non-epitheloid phenotype and high VEGF production. In the same sense, patients treated with 'balance' fluid who suffered peritonitis showed the preservation of epitheloid phenotype with lower production of VEGF, which suggests that 'balance' fluid confers additional MC protection against the effects of inflammation.

In summary, we have shown that MC EMT-associated molecules showed favourable differences and outcome for the low-GDP ('balance') fluid relative to standard fluid at medium term. Although a greater series of patients may be required to confirm these results, the consistency with other studies supports our findings. Moreover, our data extend these results beyond the first year on PD. It is noteworthy that we observed that the differences between fluids markedly increased after this first year.

Limitations of our study

The most important limitations of our study are its non-randomized nature and the low number of patients at the final evaluation. Although a random selection cannot be equal to a randomization procedure, the characteristics of patients included in each group were similar, and the important differences we have found probably are not due to these minor differences. It is important to note that our observation is valid for 18–24 months of PD.

In conclusion, non-epitheloid phenotype, lower expression of E-cadherin and higher fibronectin and VEGF production complete the EMT manifestations by mesothelial cells *in vitro* and *ex vivo*. Based on these parameters, we have demonstrated a significantly favourable outcome in patients using 'balance' fluid relative to those using standard fluid at medium term. These findings confirm the hypothesis that GDP-reduced fluid protects mesothelial cells from the development of EMT, and therefore a better peritoneal membrane preservation can be expected in the long term.

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INFLUENCE OF BICARBONATE/LOW-GDP PERITONEAL DIALYSIS FLUID (BICAVERA) ON *IN VITRO* AND *EX VIVO* EPITHELIAL-TO-MESENCHYMAL TRANSITION OF MESOTHELIAL CELLS

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♦ ***Background:*** **Peritoneal membrane damage induced by peritoneal dialysis (PD) is largely associated with epithelial-to-mesenchymal transition (EMT) of mesothelial cells (MCs), which is believed to be a result mainly of the glucose degradation products (GDPs) present in PD solutions.**

♦ ***Objectives:*** **This study investigated the impact of bicarbonate-buffered, low-GDP PD solution (BicaVera: Fresenius Medical Care, Bad Homburg, Germany) on EMT of MCs *in vitro* and *ex vivo*.**

♦ ***Methods: In vitro*** **studies: Omentum-derived MCs were incubated with lactate-buffered standard PD fluid or BicaVera fluid diluted 1:1 with culture medium.**

Ex vivo **studies: From 31 patients randomly distributed to either standard or BicaVera solution and followed for 24 months, effluents were collected every 6 months for determination of EMT markers in effluent MCs.**

♦ ***Results:*** **Culturing of MCs with standard fluid *in vitro* resulted in morphology change to a non-epithelioid shape, with downregulation of E-cadherin (indicative of EMT) and strong induction of vascular endothelial growth factor (VEGF) expression. By contrast, *in vitro* exposure of MCs to bicarbonate/low-GDP solution had less impact on both EMT parameters.**

Ex vivo **studies partially confirmed the foregoing results. The BicaVera group, with a higher prevalence of the non-epithelioid MC phenotype at baseline (for unknown reasons), showed a clear and significant trend to gain and maintain an epithelioid phenotype at medium- and longer-term and to show fewer fibrogenic characteristics. By contrast, the standard solution group demonstrated a progressive and significantly higher presence of the non-epithelioid phenotype. Compared with effluent MCs having an epithelioid phenotype, MCs with non-epithelioid morphology showed significantly lower levels of E-cadherin and greater levels of fibronectin and VEGF. In comparing the BicaVera and standard solution groups, MCs from the standard solution group showed significantly higher secretion of interleukin 8 and lower secretion of collagen I, but no differences in the levels of other EMT-associated molecules, including fibronectin, VEGF, E-cadherin, and transforming growth factor β1.**

Peritonitis incidence was similar in both groups. Functionally, the use of BicaVera fluid was associated with higher transport of small molecules and lower ultrafiltration capacity.

♦ ***Conclusions:*** **Effluent MCs grown *ex vivo* from patients treated with bicarbonate/low-GDP BicaVera fluid showed a trend to acquire an epithelial phenotype, with lower production of proinflammatory cytokines and chemokines (such as interleukin 8) than was seen with MCs from patients treated with a lactate-buffered standard PD solution.**

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KEY WORDS: Biocompatibility; epithelial-to-mesenchymal transition; bicarbonate peritoneal dialysis fluid; glucose degradation products; mesothelial cells.

Peritoneal dialysis (PD) is an established dialysis technique used by approximately 11% of patients

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with end-stage renal disease worldwide (1); however, chronic exposure of the peritoneum to PD fluids causes local inflammation and leads to peritoneal dysfunction and membrane failure (2,3). The loss of the membrane's dialysis capacity is responsible for increased morbidity and mortality.

The non-physiologic nature of PD fluids is considered to be one of the factors leading to alteration of the peritoneal membrane (3). This persistent stress of chronic peritoneal inflammation, exacerbated by periodic acute episodes of peritonitis, contributes to structural abnormalities of the peritoneal membrane, including loss of the mesothelial cell (MC) monolayer, submesothelial fibrosis, angiogenesis, and hyalinizing vasculopathy (4,5). Such alterations are considered to be the major cause of loss of functional membrane capacity, resulting in ultrafiltration (UF) failure.

Characterization of this response to PD is based on functional, histologic, and cytologic effluent studies (6). Peritoneal biopsy is the accepted standard for investigating peritoneal membrane alterations, but its invasiveness precludes regular use. Based on histologic data, we showed that epithelial-to-mesenchymal transition (EMT) of MCs is the mechanism that probably initiates damage to the membrane (7–10). Transdifferentiated MCs acquire a non-epithelioid phenotype, with loss of E-cadherin and increased production of vascular endothelial growth factor (VEGF), fibronectin, and collagen I (7–9), all of which correlate with high peritoneal transport (7). We also demonstrated that standard fluids induce EMT of MCs *in vitro* (7,8).

BicaVera (Fresenius Medical Care, Bad Homburg, Germany) is a bicarbonate-buffered PD fluid with a low content of glucose degradation products (GDPs) relative to standard solutions (11). The first clinical studies have suggested improved biocompatibility for this solution (12,13). Glucose degradation products promote the transformation of precursors of glycosylation (Amadori products) into advanced glycosylation endproducts (AGEs) (14). Mesothelial cells express the AGE receptor (RAGE), and RAGE activation is able to initiate EMT (15). In two series of PD patients, Do and coworkers (16,17) showed rapid remesothelialization and less EMT with the use of low-GDP solutions at the medium term. Based on those data and on our experience with another low-GDP fluid (18), we hypothesized that peritoneal MCs of patients exposed to a GDP-reduced fluid with bicarbonate as buffer (BicaVera) should show an additionally lower risk of EMT development and, by extension, less deteriorated peritoneal function, both *in vitro* and *ex vivo,* than is seen with exposure to lactate/GDP-rich standard fluid. The aim of the present study was therefore to examine whether expression of EMT markers in MCs from effluents of PD patients is reduced by treatment with bicarbonate/low-GDP solution (BicaVera) at the medium term.

METHODS

PATIENTS AND STUDY DESIGN

Two parallel studies to evaluate low-GDP fluids with different buffers—Balance (Fresenius Medical Care) and BicaVera—were simultaneously performed, with both fluids being compared with a standard fluid (Stay•Safe: Fresenius Medical Care). The results obtained with Balance and with the standard fluid have already been published (18).

The present prospective study was performed over a 4-year period in two university hospitals using the same PD protocols (19). Only incident patients were included, and the only inclusion criterion was that patients be able and willing to perform continuous ambulatory PD therapy with no expressed indication for automated PD. Patients were randomly assigned to either BicaVera or the standard PD fluid by the doctors. The standard-fluid (Stay•Safe; 1.5%, 2.3%, and 4.25% glucose) group consisted of 20 patients (11 women, 9 men; mean age: 59 ± 15 years; 15% with diabetes); the BicaVera (1.5%, 2.3%, and 4.25% glucose) group consisted of 11 patients (3 women, 8 men; mean age: 68.22 ± 8.80; 38% with diabetes). All patients were starting PD *de novo,* and every patient in a particular PD group received the same PD solution from the start of PD. The first functional evaluation of the membrane was done before the second month on PD, and that evaluation was considered to be the baseline. The follow-up period for each patient was planned to be 24 months. The study was performed according to the Declaration of Helsinki and was approved by the ethics committees of both hospitals. Written informed consent was given by the patients.

Peritoneal transport of water and small solutes was determined during a 4-hour peritoneal kinetic study performed using the 4.25% glucose version of the fluid to which the patient had been allocated. The patient's mass transfer area coefficient (MTAC) for creatinine, UF capacity for the same period, and residual renal function (RRF) were calculated as previously described (20). Every 6 months, we determined EMT markers in MCs released into nocturnal peritoneal effluent. When a peritonitis or hemoperitoneum occurred, samples were taken after a 4-week symptom-free period.

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MC CULTURES AND TREATMENTS

Human peritoneal MCs were isolated from the effluent of overnight dwells with the 2.3% glucose version of the fluid to which the patient had been allocated, using the previously described method (21). These effluent-derived MCs were cultured until 100% confluence in Earle M199 medium (Biological Industries, Ashraf, Israel) supplemented with 20% fetal calf serum (Gibco–BRL Life Technologies, Paisley, Scotland), 50 IU/mL penicillin, 50 μg/mL streptomycin, 1 mol/L 2% HEPES, 10 μg/mL ciprofloxacin (Bristol–Myers Squibb, Columbus, OH, USA), and 2% Biogro-2 (Biological Industries). Markers of EMT were determined *ex vivo* (21).

The *in vitro* experiments used omentum-derived MCs that were isolated and cultured as previously described (21), remaining stable for 1 – 2 passages. Oral informed consent for the collection of omental MCs was obtained from nonuremic patients undergoing elective abdominal surgery.

To exclude contamination by fibroblasts, the purity of the omentum- and effluent-derived human MC cultures was determined by measuring the expression of standard mesothelial markers (8). These MC cultures were negative for von Willebrand factor, thereby excluding endothelial cell contamination (21). Confluent omentum-derived MCs were incubated for 48 – 72 hours in both standard (Stay•Safe 2.3% glucose) and bicarbonate (BicaVera 2.3% glucose) PD solutions diluted 1:1 with culture medium. As controls, omentum-derived MCs were cultured with M199 0% fetal bovine serum diluted 1:1 with culture medium. Some MCs in control cultures were also treated with recombinant human transforming growth factor β1 (TGF-β1) 1 ng/mL (R&D Systems, Minneapolis, MN, USA) to induce EMT *in vitro* (8). Each experiment was carried out in duplicate, and at least 5 experiments were performed.

The morphology of effluent-derived MCs was assessed at 100% confluence. Each culture of effluent MCs reached confluence at a different time, but always at less than 1 month. Confluent MC cultures from PD effluent were classified into epithelioid and non-epithelioid groups according to their cellular morphology and expression of extracellular matrix components, as previously described (8).

WESTERN BLOT

Cultures of MCs were lysed in radioimmunoprecipitation assay buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with an inhibitors cocktail (Pierce, Cambridge, MA, USA), and total protein was quantified using a total-protein assay kit (Pierce). Equal amounts of protein (30 – 50 μg) were fractionated using 8% – 10% sodium dodecyl sulfate and transferred to nitrocellulose membranes for polyacrylamide gel electrophoresis. Membranes were blocked with nonfat milk and incubated with specific antibodies against E-cadherin (Zymed, San Francisco, CA, USA) and tubulin (Becton Dickinson, Franklin Lakes, NJ, USA). Peroxidase-labeled goat anti-mouse antibody (PharMingen, San Diego, CA, USA) was visualized with an enhanced chemiluminescence detection kit (Pierce), and blot images were acquired using a Kodak Image Station 2000MM (Eastman Kodak, New York, NY, USA).

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

For reverse-transcriptase polymerase chain reaction (PCR) analysis, MCs were lysed in TRI Reagent (Ambion, Austin, TX, USA), and RNA was extracted according to the manufacturer's instructions. Complementary DNA was obtained from 2 μg of total RNA by reverse transcription (RNA PCR Core Kit: Applied Biosystems, by Roche Molecular Systems, Branchburg, NJ, USA). Quantitative PCR was carried out in a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) using a SYBR Green PCR kit (Roche Diagnostics) and specific primers sets for E-cadherin, fibronectin, and collagen I. Histone 3 primers were used as a PCR reaction control (Table 1). These studies were performed using MCs from patients in both groups who had reached at least 18 months of treatment.

TABLE 1
Primer Sequences for Real-Time Polymerase Chain Reaction

Gene	Temperature	Forward primer	Reverse primer
E-Cadherin	62°C	5′TGAAGGTGACAGAGCCTCTG3′	5′TGGGTGAATTCGGGCTTGTT3′
Fibronectin	66°C	5′CCTGAAGCTGAAGAGACTTGC3′	5′CGTTTCTCCGACCACATAGGA3′
Collagen I	64°C	5′GCTATGATGAGAAATCAACCG3′	5′GCTTCCCCATCATCTCCATTC3′
H3	62°C	5′AAAGCCGCTCGCAAGAGTGCG3′	5′ACTTGCCTCCTGCAAAGCAC3′

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ENZYME-LINKED IMMUNOASSAY

For the detection of VEGF, interleukin 8 (IL-8), and active TGF-β1 in culture supernatants, media of MCs cultured under the earlier described conditions were replaced and collected 18 hours later; supernatants were stored at –80°C until analysis. The VEGF, IL-8, and TGF-β1 concentrations in supernatants were determined using ELISA kits (R&D Systems). Levels of fibronectin, procollagen, and intracellular adhesion molecule 1 in cell lysates were also assessed using commercially available ELISA kits (Biomedical Technologies, Stoughton, MA, USA; Takara Bio, Shiga, Japan; and Diaclone, Besaçon, France respectively) according to the manufacturer's protocols. Results were normalized according to the total protein in the cell lysate.

STATISTICAL ANALYSIS

Results are reported as mean ± standard error of the mean. Comparisons between data groups were performed using the nonparametric Mann–Whitney rank sum U-test. Values of $p \leq 0.05$ were considered statistically significant. The Wilcoxon test was used for intragroup comparisons between periods, and the Mann–Whitney test was used for between-group comparisons.

To study the complete outcome of each variable over time, we applied a linear mixed model using an unstructured covariance matrix for quantitative variables and generalized estimating equations for qualitative variables (phenotype), both in the framework of generalized mixed models. The results should be interpreted as follows:

- "Significant model" means that the fluid–time interaction is $p < 0.01$.
- "Significant fluid" means that the effects of the two fluids are different, but that the variation over time is not significantly different (parallelism maintained).
- "Significant time" means that both fluids are affected by time to similar degrees.

To remove the interference of peritonitis from the studied variables, we applied three different approaches to the linear mixed-model analysis:

- Isolated analysis of the outcomes of patients who never experienced peritonitis compared with those who experienced at least 1 episode
- Comparison of samples collected before and after the first peritonitis episode, with the introduction of peritonitis as a covariate
- Introducing peritonitis (1 episode vs 0 episodes, cumulative episodes, and days of peritoneal inflammation) as other covariates in the generalized estimating equations

Analysis of variance was used to establish the distribution of continuous variables between populations.

We used the SPSS software application (version 14.5: SPSS, Chicago, IL, USA), which contains details on generalized mixed models, their method, and their meaning, and GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA) for the statistical analyses.

RESULTS

CHARACTERIZATION OF EFFLUENT-DERIVED MC CULTURES

As previously described (7,8), cultures of effluent MCs can potentially show three different phenotypes: epithelial-like (similar to that of omentum-derived MCs), transitional, and fibroblast-like [Figure 1(A)]. Because of similitude in EMT markers, we group transitional and fibroblast-like MCs into a single category, "non-epithelioid MCs." Using that approach, we have always grouped confluent MC cultures into epithelioid and non-epithelioid MC groups, according not only to their morphologic characteristics, but also to their expression of EMT markers. Furthermore, we use ELISA to measure levels of several markers—VEGF, TGF-β1, IL-8—to better classify confluent MCs.

Compared with MCs from omentum, non-epithelioid MCs showed increased expression of VEGF ($p = 0.0001$) and IL-8. We did not find upregulation of TGF-β1. In addition, in non-epithelioid MCs, quantitative PCR showed significant downregulation of E-cadherin ($p = 0.001$) and important upregulation of fibronectin ($p = 0.005$) and collagen I [$p = 0.01$, Figure 1(B,C)]. In epithelioid MCs compared with MCs from omentum, we also observed upregulation in most EMT markers because, as previously demonstrated, that transition is already ongoing in epithelioid effluent-derived MCs (8).

Exposure of MCs to bicarbonate/low-GDP fluid (BicaVera) *in vitro* had less impact on EMT than did exposure to lactate-buffered standard fluid. The omentum-derived MCs were incubated for 48 or 72 hours with 2.3% glucose standard (Stay•Safe) or bicarbonate/low-GDP (BicaVera) PD fluid diluted 1:1 with culture medium. Positive and negative EMT control MCs were also cultured. Exposure of MCs to standard PD fluid resulted in marked cell death (floating round-shaped cells) and in morphologic change at 48 and 72 hours, with the acquisition of a spindle-like shape, similar to that of TGF-β1–treated cells

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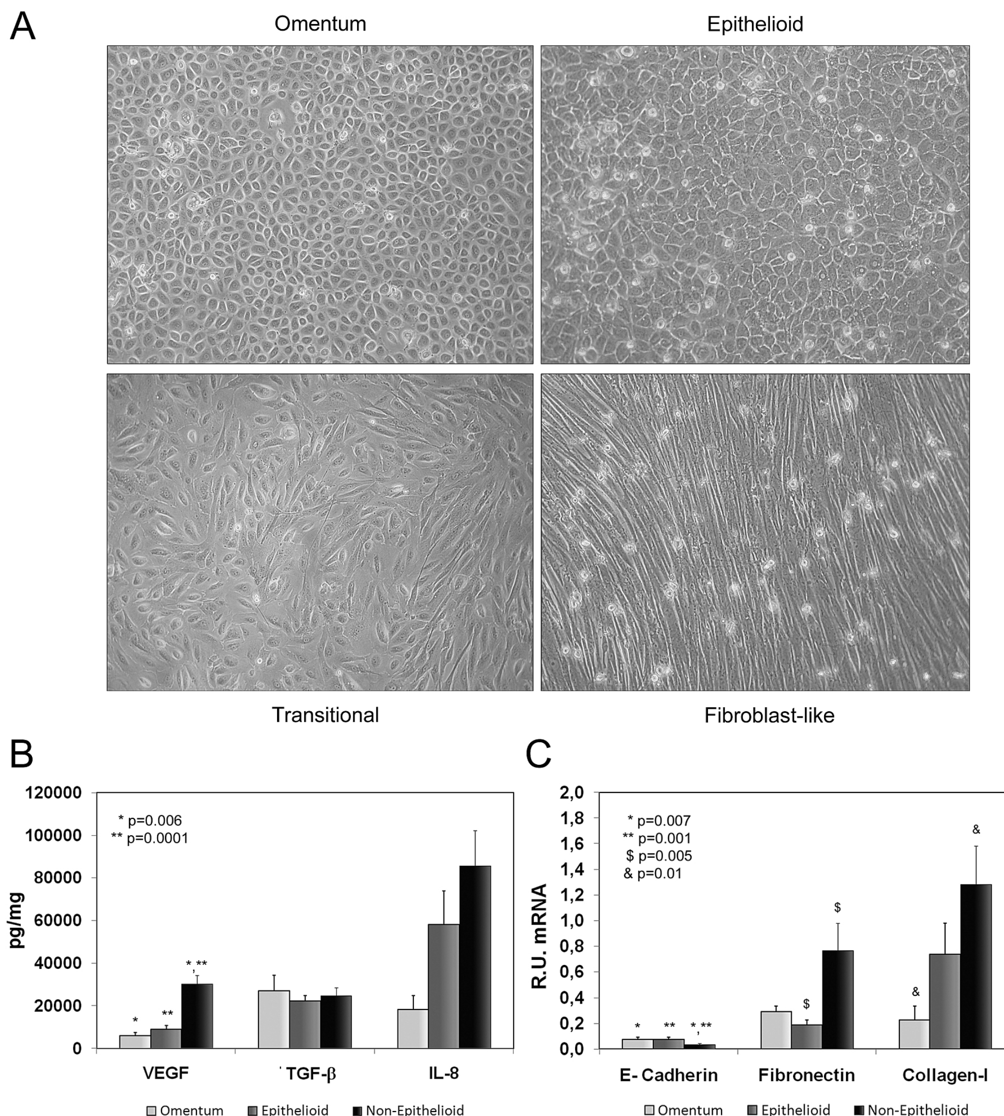

Figure 1 — Characterization of effluent-derived mesothelial cells (MCs). (A) Omentum-derived and the three possible morphologies (epithelial-like, transitional, fibroblast-like) of effluent-derived confluent MCs. Because of similarity in their markers of epithelial–mesenchymal transition (EMT), we grouped transitional and fibroblast-like MCs into a single category: non-epithelioid MCs. (B) Expression levels of EMT markers in supernatants: in non-epithelioid MCs, levels of vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) were seen to be increased [$p = 0.0001$ and 0.089 (not shown) respectively], but levels of transforming growth factor β1 (TGF-β1) were not. (C) In non-epithelioid (compared with omentum-derived) MCs, we found a significant downregulation of E-cadherin ($p = 0.001$) messenger RNA (mRNA) expression and an important upregulation of fibronectin ($p = 0.005$) and collagen I ($p = 0.01$) mRNA expression. R.U. = relative units.

[positive control, Figure 2(A)]. By contrast, no effect on cellular viability or morphology was observed after exposure of MCs to BicaVera. In addition, treatment of MCs with standard PD fluid or with TGF-β1 resulted in downregulation of E-cadherin, which is indicative of EMT. Interestingly, incubation of MCs with BicaVera preserved the expression of E-cadherin [Figure 2(B)]. Those data were confirmed in a more quantitative manner by real-time PCR measurement of expression levels of E-cadherin mRNA. Exposure of omentum-derived MCs to standard PD fluid or to TGF-β1 for 72 hours significantly suppressed the

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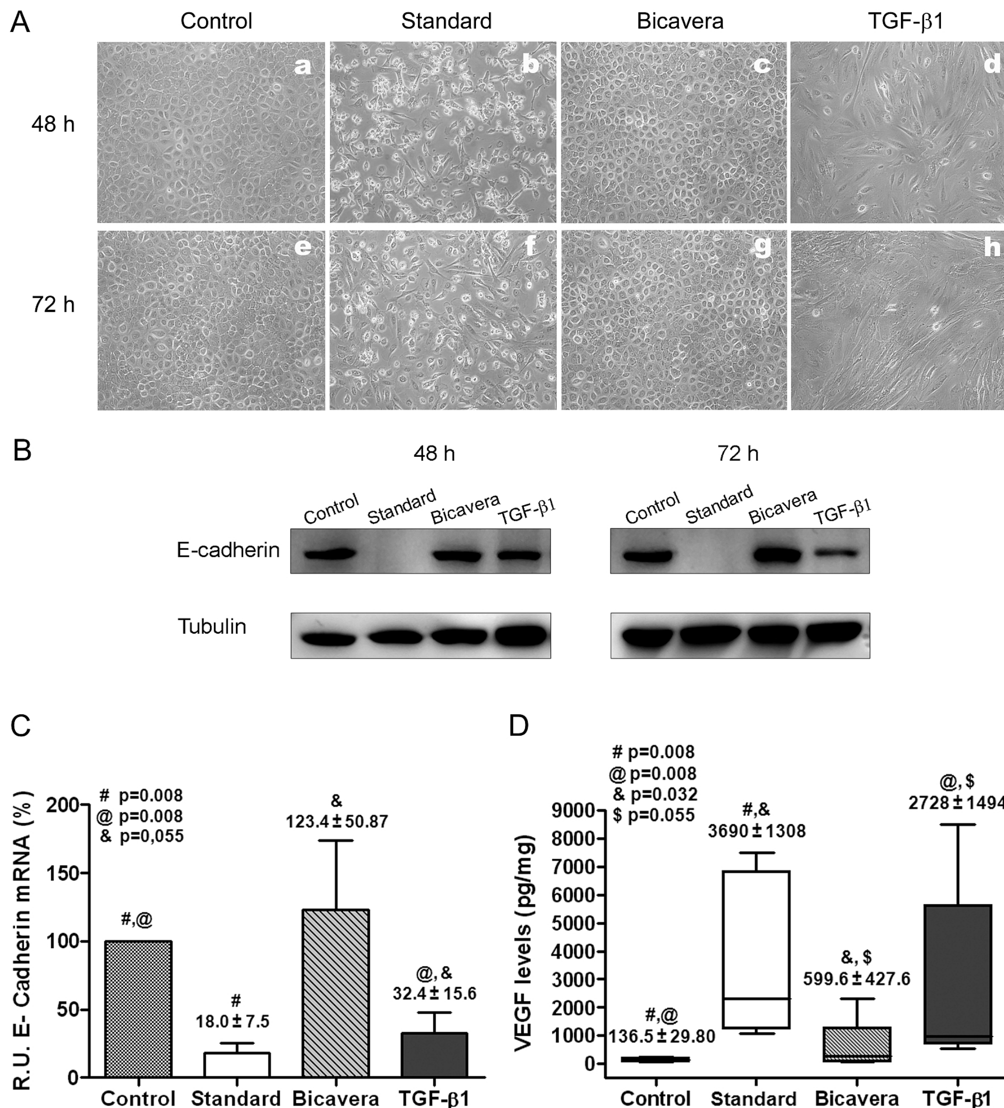

Figure 2 — Effects of peritoneal dialysis (PD) fluids on mesothelial cells (MCs) *in vitro*. (A) Effects on MC morphology at 48 and 72 hours. Images are representative of 5 independent experiments. (B) Western blot results show expression of E-cadherin in exposed MCs. Tubulin was used as a loading control. Images are representative of 5 independent experiments. (C) Levels of E-cadherin messenger RNA (mRNA) analyzed by quantitative reverse transcription polymerase chain reaction [for MCs treated with PD fluids or with transforming growth factor β1 (TGF-β1) relative to untreated cells]. Results are mean ± standard error of 5 experiments. (D) Production of vascular endothelial growth factor (VEGF) in supernatant (picograms per milligram of cell protein) by omentum-derived MCs treated with PD fluids or with TGF-β1. The box plots show 75th percentile, 25th percentile, median, maximum, and minimum values from 5 experiments. BicaVera: solution from Fresenius Medical Care, Bad Homburg, Germany. R.U. = relative units.

expression of E-cadherin mRNA [Figure 2(C)]. In accord with the earlier results of E-cadherin protein expression, MCs exposed to BicaVera showed preserved expression of E-cadherin mRNA [Figure 2(C)].

To further explore the effects of exposure to PD fluid on EMT, we analyzed the expression of VEGF, which has been shown to be upregulated during the mesenchymal conversion of MCs (7,9). As Figure 2(D) shows, exposure of MCs to standard PD fluid or to TGF-β1 significantly induced the secretion of VEGF; MCs exposed to BicaVera did not show significant upregulation of VEGF.

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CLINICAL COURSE OF PATIENTS

In the standard and BicaVera fluid groups, 20 and 11 patients respectively were followed for 6 months, 18 and 11 for 12 months, 11 and 11 for 18 months, and 3 and 5 for 24 months. Technique survival was similar in both fluid groups. The reasons for drop-out were kidney transplantation in 7 (standard-fluid) and 2 (BicaVera) patients, transfer to hemodialysis in 5 and 3 patients, transfer to automated PD in 3 and 0 patients, and death in 1 patient in each group.

The percentage of patients affected by peritonitis was similar in both groups: 9 episodes occurred in the standard group (3 episodes in 1 patient, and 1 episode in each of 6 patients), and 9 episodes in the BicaVera group (3 episodes in 1 patient, 2 episodes in each of 2 patients, and 1 episode in each of 4 patients). The time to first peritonitis episode in each group was not significantly different (data not shown). The global peritonitis incidence was slightly but not significantly higher in the BicaVera group (1 episode in 25 patient–months vs 1 episode in 30 patient–months in the standard group).

In studies of peritoneal function and RRF, the BicaVera group showed higher small-solute transport and lower UF. The linear mixed model using an unstructured covariance matrix for quantitative variables showed, for the fluids alone, higher values for the creatinine MTAC ($p < 0.0001$) and RRF ($p < 0.004$) and lower values for the UF capacity ($p < 0.0001$) in the BicaVera group; however, the fluid–time interaction was not statistically significant (Table 2). Pre-PD RRF values were also higher in the BicaVera group (10.33 mL/min vs 6.06 mL/min in the standard-fluid group). The dialysate-to-plasma ratio of creatinine and the diuresis were similar in both groups (data not shown).

Ex vivo MC cultures from the BicaVera group showed a trend to gain and maintain the epithelial phenotype and a lower level of IL-8 expression. The BicaVera group showed an unexpected higher prevalence of the non-epithelioid phenotype at baseline (72% vs 30% in the standard group). However, the groups behaved differently at the medium- and long-term, showing a trend in patients on standard fluid to gain the non-epithelioid phenotype, compared with a clear and significant loss of the non-epithelioid phenotype in the BicaVera group. At 24 months, all patients in the standard-fluid group had gained the non-epithelial phenotype, but only 20% of patients of the BicaVera group showed that phenotype. The overall differences between the groups were statistically significant by linear mixed-model analysis for both fluid and time [fluid–time intersection, $p = 0.0001$, Figure 3(A)]. Figure 3(B,C) shows representative examples involving 2 patients—1 on standard fluid, and 1 on BicaVera—showing the acquisition of the non-epithelioid morphology and increased expression of fibronectin in the patient on standard fluid, and preservation of the epithelioid morphology and increased expression of E-cadherin in the patient on BicaVera at 18 months' follow-up.

Figure 4(A–F) shows the levels of EMT-associated molecules (E-cadherin, fibronectin, collagen I, VEGF, IL-8, and TGF-β) in supernatant or cellular extract from

TABLE 2
Peritoneal Transport of Creatinine, Ultrafiltration Capacity, and Residual Renal Function Over Time in the Study Groups[a]

Variable	Baseline	6 Months	12 Months	18 Months	24 Months	p Value
Cr MTAC (mL/min)						
Standard	7.6±3.5	6.2±2.04	6.8±3.1	8.4±2.8	6.1±2.6	
BicaVera[b]	10.7±3.4	10.4±1.8	10.3±2.6	11.6±5.6	8.4±2.7	<0.0001
Ultrafiltration (mL)[c]						
Standard	832±199	915±247	883±273	833±234	825±106	
BicaVera[b]	438±282	407±202	573±194	562±190	654±367	<0.0001
RRF (mL/min)						
Standard	5.8±3.9	3.4±2.5	4.1±3.1	4.0±2.8	4.2±4.0	
BicaVera[b]	8.4±4.4	7.2±4.1	5.7±3.1	5.7±3.4	6.0±4.4	<0.004

Cr MTAC = mass transfer area coefficient of creatinine; RRF = residual renal function.
[a] Values from a linear mixed model using unstructured covariance matrix analysis, expressed as mean ± standard deviation.
[b] Fresenius Medical Care, Bad Homburg, Germany.
[c] At 4 hours, using 4.25% glucose.

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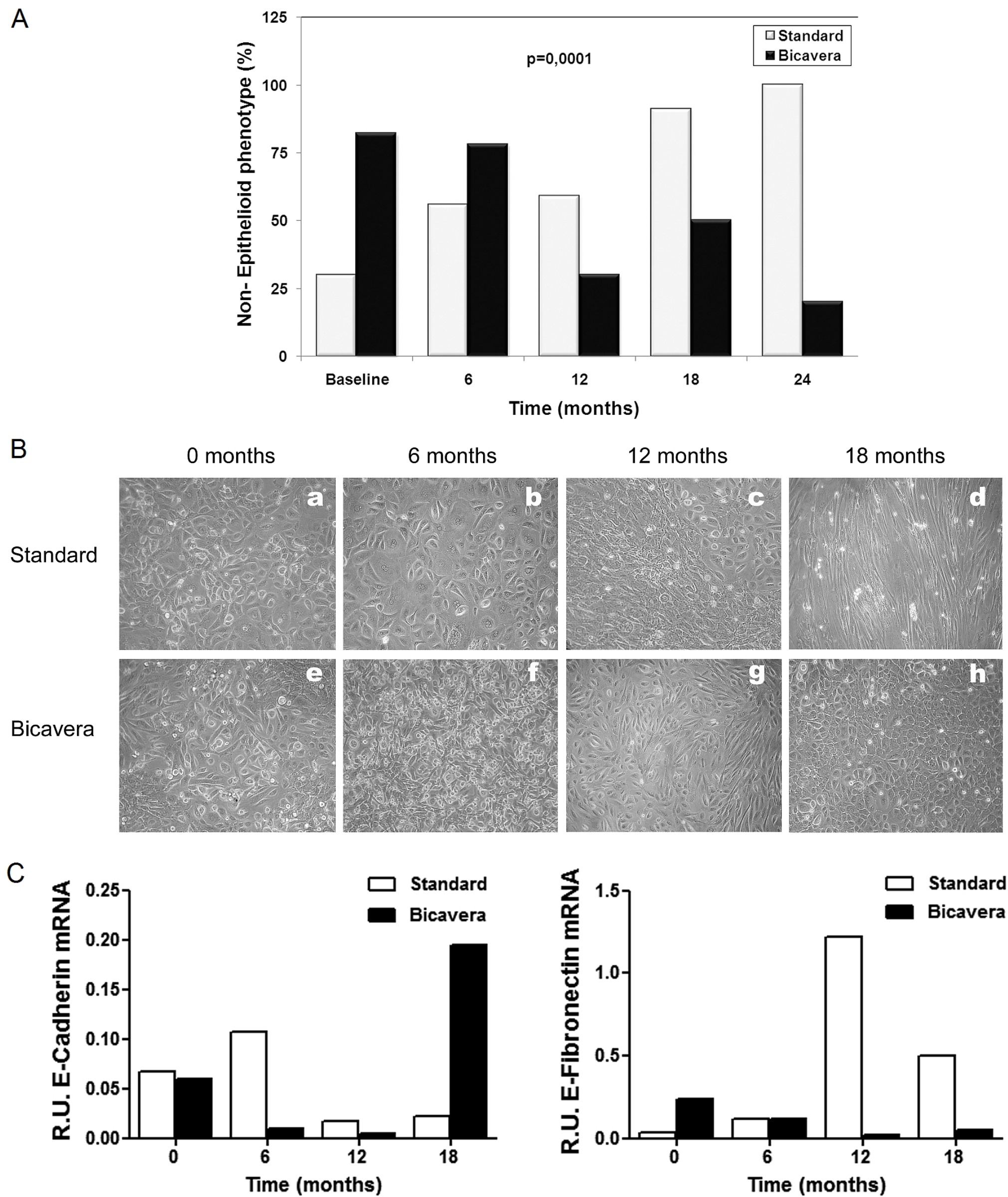

Figure 3 — Epithelioid and non-epithelioid mesothelial cell (MC) phenotypes in the standard fluid and BicaVera (Fresenius Medical Care, Bad Homburg, Germany) study groups. (A) Differences in the percentage of non-epithelioid MC phenotypes in the groups over time (mixed model, fluid–time: $p = 0.0001$). (B,C) Representative images from 2 patients (a – d = standard fluid; e – h = BicaVera) showing acquisition of non-epithelioid MC morphology and increased expression of fibronectin with standard fluid and preservation of epithelioid MC morphology and increased expression of E-cadherin with BicaVera fluid at 18 months of follow-up. R.U. = relative units.

effluent-derived MCs, together with the linear mixed-model analysis. Only the IL-8 level was significant lower in the BicaVera group ($p < 0.004$) than in the standard group. Those data were confirmed when all samples were evaluated according to fluid group only [Figure 5(A,C)].

Studies in cellular lysate [Figure 5(C)] did not show significant differences by fluid group for intracellular adhesion molecule 1, procollagen, and fibronectin. Non-epithelioid MCs showed higher levels of procollagen and fibronectin ($p = 0.0001$, Figure 6).

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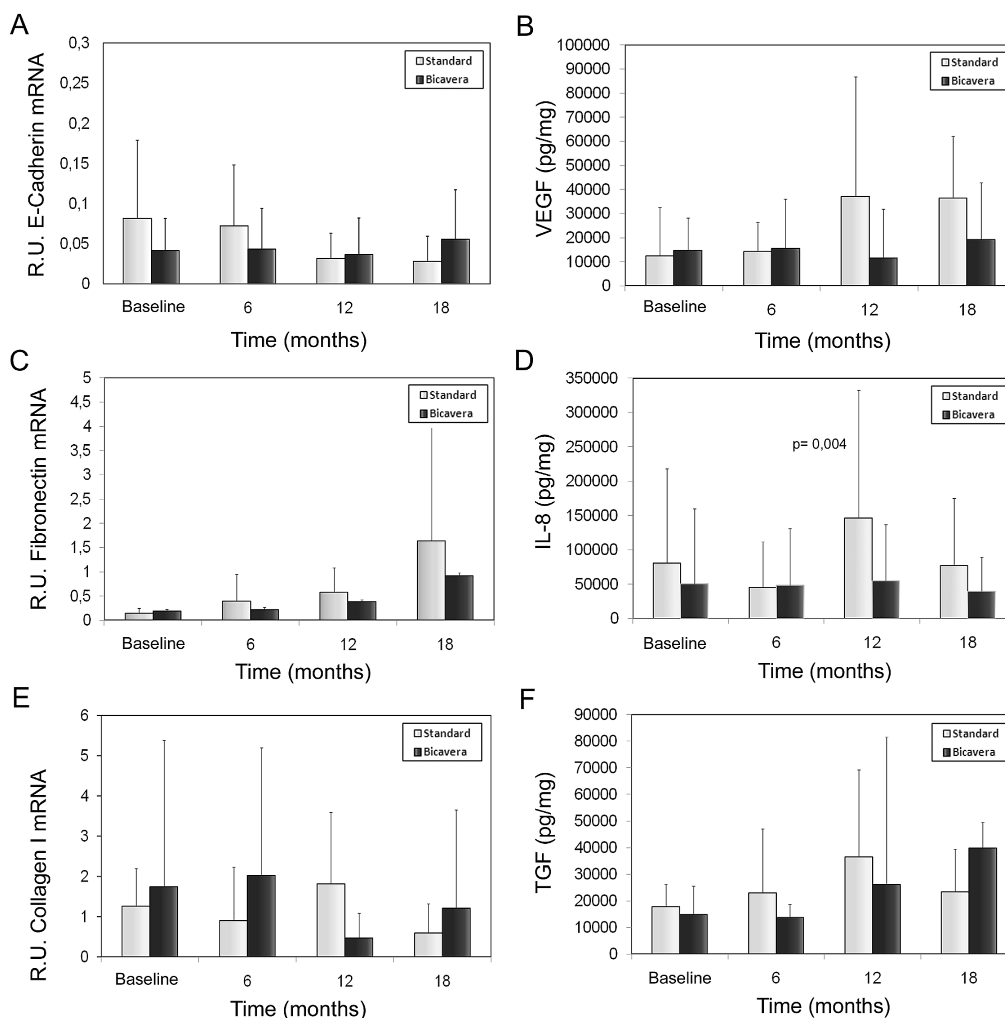

Figure 4 — Bar plots of cytokine levels in supernatant or extract from mesothelial cells (MCs) derived from effluents in the BicaVera (Fresenius Medical Care, Bad Homburg, Germany) and standard fluid (Stay·Safe: Fresenius Medical Care) groups. The statistical comparison uses mixed-model analysis, which determines the significance of differences between the groups and for each group over time. No significant differences were observed for (A) levels of E-cadherin messenger RNA (mRNA) in MCs (relative units); (B) production of vascular endothelial growth factor (VEGF, picograms per milligram) in supernatant; (C) levels of fibronectin mRNA in MCs (relative units); (E) levels of collagen I mRNA in MCs (relative units); and (F) levels of transforming growth factor β1 (TGF-β1, picograms per milligram) in supernatant. Significantly higher levels were observed only for (D) supernatant levels of interleukin 8 (IL-8, picograms per milligram) in the standard fluid group, which showed a significant trend to rise over time (fluid–time: $p < 0.01$). R.U. = relative units.

Because non-epithelioid MCs appeared in effluent in the early and late PD periods in the BicaVera group, we studied molecules associated with EMT from cells at different stages of PD, using analysis of variance to compare results obtained at baseline and after 18 months on PD. The non-epithelioid cells initially observed in the BicaVera group were characterized by lower levels of fibronectin mRNA than were seen with non-epithelioid cells from the BicaVera group at 18 months (0.23 ± 0.2 relative units vs 1.72 ± 2.54 relative units, $p = 0.05$, Figure 7).

INFLUENCE OF PERITONITIS EPISODES ON VARIABLES RELATED TO EMT

The numbers of patients and of peritonitis episodes were small, and so drawing definitive conclusions is impossible. The levels of cytokines and growth factors in

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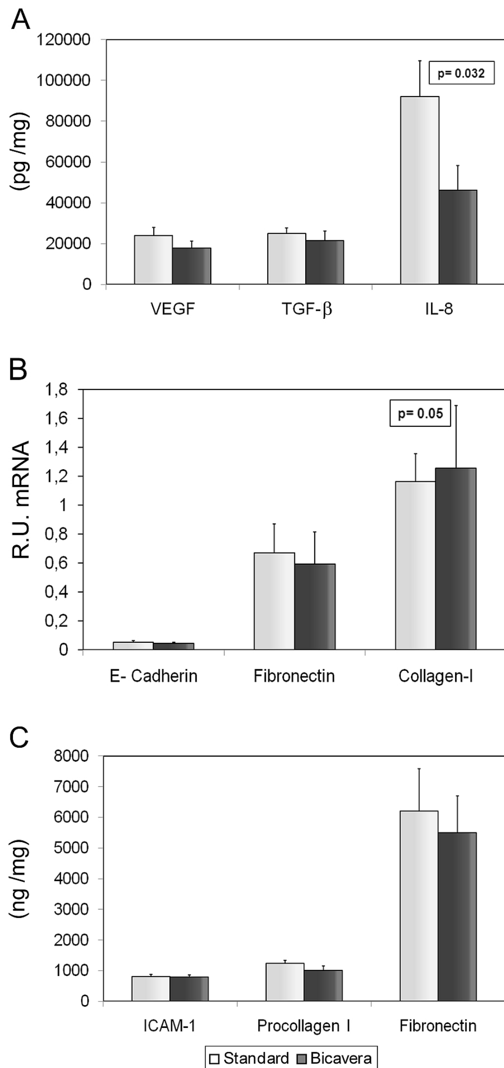

Figure 5 — Mean values of the various mesothelial cell products in culture for the study groups (all samples). We observed (A) significantly higher levels of interleukin 8 (IL-8, picograms per milligram) in supernatant in the standard fluid group; (A,B) similar levels of vascular endothelial growth factor (VEGF), transforming growth factor β1 (TGF-β1), E-cadherin, fibronectin, and collagen I in both groups; and (C) no significant differences in protein levels (nanograms per milligram) of intracellular adhesion molecule 1 (ICAM-1), procollagen I, and fibronectin in MC lysate. BicaVera: solution from Fresenius Medical Care, Bad Homburg, Germany. R.U. = relative units.

supernatants or MC extracts in patients without peritonitis were similar to those in the whole group. The percentage of patients affected by peritonitis was similar in both groups: 9 episodes in the standard group, and 9 episodes in the BicaVera group. The global peritonitis incidence

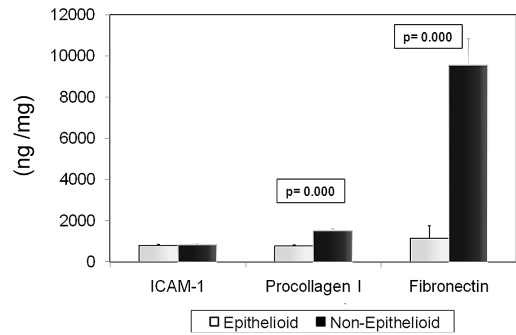

Figure 6 — In a comparison of cellular lysates by mesothelial cell phenotype, protein levels (nanogram per milligram) of procollagen and fibronectin were observed to be higher (p = 0.0001) for the non-epithelioid phenotype; levels of intracellular adhesion molecule 1 (ICAM-1) were observed to be similar for all phenotypes.

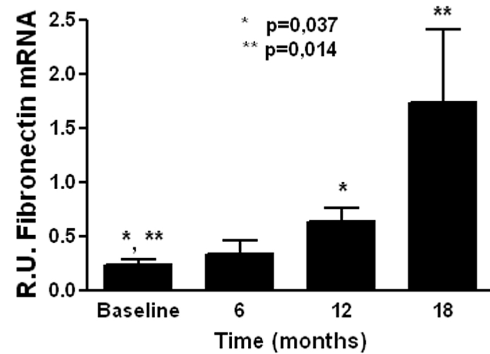

Figure 7 — Expression levels of fibronectin messenger RNA (mRNA) in non-epithelioid mesothelial cells (MCs) from the BicaVera (Fresenius Medical Care, Bad Homburg, Germany) group. Non-epithelioid MCs appear to be associated with levels of fibronectin mRNA that, compared with baseline, are higher at later periods. R.U. = relative units.

was slightly but nonsignificantly higher in the BicaVera group (1 episode in 25 patient–months vs 1 episode in 30 patient–months in the standard group). In the standard group, 4 patients showed non-epithelioid morphology pre-peritonitis; at the end of follow-up, 3 had maintained the same morphology, and 1 had changed to epithelioid morphology. In the BicaVera group, 3 patients showed non-epithelioid morphology, and 1 showed epithelioid morphology pre-peritonitis; all showed the epithelioid phenotype at the end of follow-up.

DISCUSSION

The aim of the present study was to investigate the effect of a bicarbonate/low-GDP PD fluid (BicaVera) on

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EMT of MCs *in vitro* and *ex vivo*. We defined EMT as the acquisition of a non-epithelioid phenotype, associated with loss of E-cadherin expression and production of higher amounts of mesenchymal products. Although specific studies on the correlation between *ex vivo* and *in vivo* methods for accurately diagnosing EMT in the peritoneal membrane are lacking, our data, repeated in multiple studies of PD patients, published and unpublished, continue to demonstrate consistency in findings with both approaches. We hypothesize that, when a patient shows repetitively epithelioid or non-epithelioid phenotype *ex vivo,* the findings from a peritoneal biopsy in the same patient show good correlation. Our data from the analysis of *in vitro* and *ex vivo* exposure of MCs to a bicarbonate/low-GDP fluid are consistently associated with preservation or acquisition of a MC epithelioid phenotype, which contrasts with the trend of MCs to acquire an EMT-associated non-epithelioid status with the use of standard fluid.

In vitro cell cultures of omentum-derived MCs preserve a physiologic phenotype. By contrast, if standard fluid with a high content of GDPs is added to cultures, a non-epithelioid phenotype associated with the loss of E-cadherin expression develops. Compared with cells treated with TGF-β1, cells exposed to standard fluid show this effect on E-cadherin at a later time point (72 hours), indicating that accumulation of a soluble factor or factors is required to repress expression of that epithelial marker. In this context, we previously described induction of TGF-β1 expression with exposure of omentum-derived MCs to standard PD fluids (22), a situation that in turn may induce EMT. By contrast, we showed that the addition of BicaVera (and other low-GDP fluids) to MC cultures barely affects epithelioid morphology and expression of E-cadherin in the cultured cells, revealing preservation of the original physiologic MC identity (18). The foregoing results demonstrate that the GDP content of PD fluids has an important role in the induction of EMT in MCs.

The analysis of EMT markers confirms that non-epithelioid cells show higher levels of VEGF, fibronectin, and collagen I, and reduced levels of E-cadherin, reflecting good consistency between morphology and molecular markers of EMT. However, when we analyzed markers of EMT in *ex vivo* studies in both groups of patients, no conclusive results were observed with respect to EMT-associated molecules, including fibronectin and TGF-β1. Only the level of IL-8 expression was significantly higher in cells from the standard-fluid group. Levels of IL-8 have been shown to be induced in MCs *in vitro* by standard PD fluids (23) and by GDPs (24). Furthermore, there are hints that IL-8 might influence solute transport in PD-related peritonitis and that IL-8 promotes cell migration (25), thereby exacerbating deterioration of the peritoneal membrane. The finding of higher levels of the chemokine IL-8 could point to a pro-inflammatory state in the standard-fluid group.

The foregoing results are tempered by the unexpected appearance at baseline of a higher percentage of patients showing a non-epithelioid phenotype in the BicaVera group. However, despite that initial finding, MCs chronically exposed to BicaVera showed an acquired positive change toward an epithelioid phenotype. By contrast, patients in the standard-fluid group showed a gradual transformation towards a generally non-epithelioid phenotype. Those data suggest that BicaVera fluid protects MCs by encouraging the maintenance of an epithelioid phenotype over the long term. Kalluri and Weinberg (26) described an intermediate state of EMT that is engaged during reparative processes and organ fibrosis. The non-epithelioid MCs initially observed in the BicaVera group might correspond to that intermediate stage of EMT ("type 1") and represent an initial reparative process. The phenomenon of EMT is an adaptive and reparative physiologic phenomenon; it becomes pathological when processes that induce EMT are maintained over time and are not properly regulated. Compared with the non-epithelioid cells from the BicaVera group at 18 months, the non-epithelioid MCs initially observed in the BicaVera group were characterized by lower levels of fibronectin mRNA. That difference over time could represent different functional stages of a common morphology: early reparative non-epithelioid cells compared with late profibrotic non-epithelioid cells.

The development of EMT could be promoted by episodes of peritonitis. We therefore compared EMT markers in MCs from patients with and without peritonitis, finding no significant differences. The most remarkable finding in patients treated with BicaVera who experienced peritonitis was the persistent preservation of the epithelioid phenotype over time. We consider that preservation of the mesothelial phenotype should be beneficial for patients, although we would need tissue samples to confirm that hypothesis.

In agreement with our results, a recent report showed that various GDPs present in standard PD fluids can induce EMT of MCs *in vitro* and that low-GDP fluids have less impact on peritoneal fibrosis and EMT *in vivo* in a rat model (27). All of those results accord with results obtained in several patient series, including the present report (16,17). Mechanistically, the induction of EMT by standard fluid is related to high GDP content in such fluid, which may contribute to AGE accumulation, activation of RAGE, and damage to MCs (14,15,28). The specificity of BicaVera, with its bicarbonate instead of

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the usual lactate buffer, relative to effects on EMT of MCs requires further studies with larger samples than that in the present report.

Our study confirms previous data on the decreased UF capacity and increased transport of small solutes associated with more biocompatible fluids. There is no definitive explanation for those observations, although several authors have speculated that the less biocompatible fluids induce an increase in capillary permeability by increasing the response to molecules such as VEGF.

The most important limitations of our study are its nonrandomized nature and the low number of patients at final evaluation. Although a random selection cannot be equal to a randomization procedure, the characteristics of the patients included in each group were quite similar except for the larger number of patients with diabetes and higher initial RRF in the BicaVera group. In the pre-PD peritoneum, patients with diabetes showed a significant decrease in lumen-to-vessel diameter ratio compared with the ratio in nondiabetic patients. Uremia and diabetes have a significant impact on the pathogenesis of peritoneal sclerosis in the pre-PD peritoneum (29). The higher percentage of patients with diabetes in the group treated with BicaVera might penalize that group. However, the BicaVera group showed progressive phenotypic change and, at follow-up, mostly an epithelioid phenotype, which might suggest a protective role of BicaVera fluid—or at least inhibition of EMT of MCs.

CONCLUSIONS

We showed that, compared with MCs obtained from patients using standard fluid, MCs obtained from effluent of patients treated with the bicarbonate/low-GDP fluid BicaVera can acquire an epithelioid phenotype at medium term, despite the potential deleterious effect of peritonitis. We also observed favorable differences and outcomes for the bicarbonate/low-GDP fluid with respect to other EMT-associated molecules, although with no conclusive results. A larger series of patients might be required to confirm these results, but the consistency of our findings with those from other studies supports the hypothesis of an improvement in biocompatibility.

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DISCLOSURES

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<u>3. Estudio de los receptores y co-receptores de VEGF durante la conversión mesenquimal de las células mesoteliales. Implicación de VEGF y sus receptores en la capacidad proliferativa e invasiva de las células mesoteliales en distintos estadíos de la transición mesotelio-mesenquimal.</u>

El proceso de MMT provoca un aumento de la secreción VEGF sobre las CMs, que se correlaciona con tasas de alto transporte y peor ultrafiltración en pacientes. (Aroeira L et al., 2005). Está descrito que las CMs normales y malignas de pleura expresan receptores (VEGFRs) y co-receptores de VEGF (Nrps) y que VEGF actúa de forma autocrina en mesoteliomas. Nosotros estudiamos la expresión de los receptores y co-receptores de VEGF durante la MMT inducida por diálisis peritoneal, así como las posibles implicaciones de estas expresiones en la funcionalidad celular de las CMs.

Para este estudio se aislaron: 1) CMs de omento, que fueron estimuladas con TGF-β1 e IL-1β con el fin de inducir una MMT *in vitro*; 2) CMs drenadas en los efluentes de 51 pacientes en terapia de diálisis peritoneal. Además, estudió la expresión de los distintos receptores, y medido la secreción de VEGF tanto en sobrenadante como en los efluentes de los pacientes. Los resultados de los experimentos llevados a cabo, tanto *in vitro* como *ex vivo*, demostraron que las CMs que han sufrido una MMT manifiestan una bajada en la expresión de los dos receptores principales de VEGF (VEGFR-1 y VEGFR-2), así como una sobreexpresión del co-receptor neuropilina-1 (Nrp-1). Estos cambios de expresión van acompañados de una disminución de la capacidad proliferativa además de un aumento de la capacidad invasiva de las CMs. Mediante el uso de anticuerpos bloqueantes y proteínas recombinantes se estudió el papel tanto de VEGF como del co-receptor Nrp-1 en la alteración de estas funciones celulares durante proceso de MMT. Se observó que VEGF sólo ejerce un papel clave en la proliferación de las CMs que no han sufrido ningún tipo de conversión mesenquimal, mientras que se observó una importante implicación, junto con Nrp-1, en la capacidad invasiva de las CMs que estuviesen o no en cualquier estadío de MMT.

Los resultados obtenidos sugieren que el cambio de los patrones de expresión de los receptores y co-receptores de VEGF en las CMs durante el proceso de MMT inducido por diálisis peritoneal, determina en parte el comportamiento y funcionalidad de las CMs en respuesta a VEGF.

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Functional Relevance of the Switch of VEGF Receptors/Co-Receptors during Peritoneal Dialysis-Induced Mesothelial to Mesenchymal Transition

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Abstract

Vascular endothelial growth factor (VEGF) is up-regulated during mesothelial to mesenchymal transition (MMT) and has been associated with peritoneal membrane dysfunction in peritoneal dialysis (PD) patients. It has been shown that normal and malignant mesothelial cells (MCs) express VEGF receptors (VEGFRs) and co-receptors and that VEGF is an autocrine growth factor for mesothelioma. Hence, we evaluated the expression patterns and the functional relevance of the VEGF/VEGFRs/co-receptors axis during the mesenchymal conversion of MCs induced by peritoneal dialysis. Omentum-derived MCs treated with TGF-β1 plus IL-1β (*in vitro* MMT) and PD effluent-derived MCs with non-epithelioid phenotype (*ex vivo* MMT) showed down-regulated expression of the two main receptors Flt-1/VEGFR-1 and KDR/VEGFR-2, whereas the co-receptor neuropilin-1 (Nrp-1) was up-regulated. The expression of the Nrp-1 ligand semaphorin-3A (Sema-3A), a functional VEGF competitor, was repressed throughout the MMT process. These expression pattern changes were accompanied by a reduction of the proliferation capacity and by a parallel induction of the invasive capacity of MCs that had undergone an *in vitro* or *ex vivo* MMT. Treatment with neutralizing anti-VEGF or anti-Nrp-1 antibodies showed that these molecules played a relevant role in cellular proliferation only in naïve omentum-derived MCs. Conversely, treatment with these blocking antibodies, as well as with recombinant Sema-3A, indicated that the switched VEGF/VEGFRs/co-receptors axis drove the enhanced invasion capacity of MCs undergoing MMT. In conclusion, the expression patterns of VEGFRs and co-receptors change in MCs during MMT, which in turn would determine their behaviour in terms of proliferation and invasion in response to VEGF.

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Introduction

Peritoneal dialysis (PD) is a therapeutic option for the treatment of end-stage renal disease and is based on the use of the peritoneal membrane (PM) as a permeable barrier across which ultrafiltration and diffusion take place [1], [2]. Continuous exposure of the PM to non-physiologic PD fluids, as well as episodes of peritonitis and hemoperitoneum, may cause inflammation and injury to the PM, which progressively undergoes denudation of the mesothelial cell (MC) monolayer, submesothelial fibrosis and angiogenesis. These structural alterations may lead to the loss of the PM dialytic function [2], [3].

During long-term PD, MCs undergo a progressive loss of epithelial phenotype and acquire myofibroblast-like characteristics by a mesothelial-to-mesenchymal transition (MMT) process [2], [4], [5]. It has been demonstrated that effluent-derived MCs still retaining an epithelioid appearance *ex vivo* already show down-regulated expression of E-cadherin and cytokeratins, suggesting that the MMT of MCs starts soon after PD is initiated [4], [5]. MMT is a complex and stepwise process that is characterized by the disruption of intercellular junctions, loss of apical-basolateral polarity and acquisition of migratory and invasive properties. Cells that have undergone MMT also acquire the capacity to produce extracellular matrix components as well as inflammatory, fibrogenic and angiogenic factors [6], [7], [8], [9]. We have previously shown that effluent-derived MCs produce vascular endothelial growth factor (VEGF) spontaneously and that the MMT process of MCs is associated with strong VEGF up-regulation [10], [11]. Furthermore, we demonstrated that high levels of VEGF production by effluent MCs correlated with high transport rates in PD patients [11], [12].

VEGF is a key regulator of both physiologic and pathologic angiogenesis [13], [14]. The biological effect of this growth factor

is mediated by three VEGF receptors (VEGFRs): VEGFR-1/Flt-1, VEGFR-2/KDR and VEGFR-3/Flt-4, which share similar molecular structure and are composed by seven extracellular immunoglobulin (Ig)-like domains, one transmembrane region, and an intracellular tyrosine kinase domain that is activated via ligand-triggered dimerization, leading to the induction of different signal transduction pathways [15], [16]. The activity of VEGF is also regulated by neuropilins (Nrps), a family of cell surface glycoproteins composed by two members, Nrp-1 and Nrp-2, that have about 45% amino acid identity and show conserved primary structures. These proteins are constituted by, a large extracellular domain, a single transmembrane domain and a short cytoplasmic tail. The extracellular domain contains three structural motifs: two CUB homology domains (a1/a2), two coagulation factor V/VIII homology domains (b1/b2) and a mephrin/A5-protein/protein tyrosine phosphatase μ (MAM) domain (c) [17]. The short cytoplasmic domain has no signalling motif but can interact with several proteins [18].

Nrp-1 was first characterized as a receptor for the class III semaphorins (Sema-3) in neurons mediating axon growth cone collapse [19], [20]. Subsequently, it was also described as an isoform-specific VEGF co-receptor expressed in endothelial and tumour cells, enhancing VEGF binding to VEGFR-2 and its bioactivity [21]. It has been described that Nrp-1 may also signal independently of VEGFR-2 in endothelial cells to mediate VEGF-triggered migration and adhesion [22], [23], [24], [25], [26]. The a1/a2 domains of Nrp-1 are involved in Semaphorin 3A (Sema-3A) binding, whereas VEGF binds to b1/b2 motifs [17], [27], [28]. Sema-3A and VEGF are functional competitors in their ability to bind Nrp-1 [17], [29], and promote ligand-triggered Nrp-1 internalization [30]. More recent studies revealed that Nrp-1 may also interact with other growth factors including hepatocyte growth factor (HGF) [31], fibroblast growth factor (FGF) [32], and transforming growth factor (TGF)-β1 [33], [34]. Besides neurons and endothelial cells, Nrp-1 expression has been described in many other cell types including MCs [35], [36], [37], [38], [39]. Importantly, Nrp-1 is frequently expressed by tumour cells and is involved in their malignant progression [31], [34], [40], [41], [42], [43], [44], [45], [46], [47], [48]. Nowadays, Nrps are considered potential therapeutic targets in cancer but the complex mechanisms underlying the interaction of these molecules with multiple ligands have not been fully elucidated so far. In this context, it has been described that blocking Nrp-1 function reduced tumour growth by inhibition of vascular remodelling, rendering vessels more susceptible to anti-VEGF therapy [27], [48], [49], [50], [51].

It has been demonstrated that normal and malignant MCs express VEGFRs and Nrps and that VEGF is an autocrine growth factor for mesothelioma [37]. However, the role of VEGF/VEGFRs/Nrps axis in peritoneal MCs during PD-induced MMT is still unknown. Herein, we show that MCs change the expression pattern of VEGFRs and co-receptors during MMT, which determines a switch of the VEGF effect on MCs from a proliferation response to an invasive response.

Materials and Methods

Ethics Statements

The protocol and informed consent were reviewed and approved by the Ethics Committee of Clinic Investigation of the 'Hospital Universitario de la Princesa' (Madrid, Spain). All of the patients signed the informed consent prior to the initiation of any study-related activities. This research was carried out in accordance with Good Clinical Practice guidelines, applicable regulations, as well as the ethical principles that have their origin in the Declaration of Helsinki.

Patients

We included 51 clinically stable PD patients in this study (30 men and 21 women), ranging in age from 25 to 78 years. The causes of renal failure were diabetes (n = 16), chronic pyelonephritis (n = 12), glomerulonephritis (n = 10), nephrosclerosis (n = 9), systemic disease (n = 3) and unknown cause (n = 1). Thirty-four patients were treated with standard solution based on glucose and lactate, containing high concentration of glucose degradation products (GDPs) (Dianeal; Baxter Healthcare Corp., Deerfield, IL). Ten of these patients received one dwell per day (generally overnight) with icodextrin-containing solution (Extraneal; Baxter) and 5 received one exchange with amino acid-containing solution (Nutrineal; Baxter). Sixteen patients were treated with low-GDPs solutions buffered with lactate in 11 cases (Balance; Fresenius Medical Care, Bad Homburg, Germany) or bicarbonate in 5 cases (BicaVera; Fresenius). Finally, 1 patient received a combination of different solutions.

MCs from the dialysis effluents of these PD patients were classified according to morphological characteristics and the expression patterns of epithelial or mesenchymal markers (**Figure 1**) into two groups: epithelioid (E) (n = 30) and non-epithelioid (Non-E) (n = 21) [2], [11], [52], [53]. At the moment of effluent-derived mesothelial cells (MCs) sampling, 43 patients were on continuous ambulatory peritoneal dialysis and 8 were on automatic peritoneal dialysis techniques.

The baseline characteristics of the patients and the differences between the subgroups according to the phenotype of effluent-derived MCs are shown in **Table 1**. Most patients (48 of 51) received recombinant human erythropoietin during this study. The mass transfer coefficients for urea and creatinine (Urea-MTC and Cr-MTC) and the creatinine clearance (CCr) were measured using standard methods [54]. Ultrafiltration (UF) capacity was defined as a peritoneal exchange of 4 hours using 3.86% glucose (214.3 mmol/L) [55]. Peritoneal glucose load was calculated by the sum of glucose contained in each PD-fluid bag during the whole time on PD. Eleven patients showed peritonitis, and one experienced hemoperitoneum. MC cultures from effluent were taken at least 3 months after the resolution of peritonitis or hemoperitoneum episodes.

Isolation and Culture of Mesothelial Cells and Treatments

MCs from the dialysis effluents of the PD patients were isolated as previously described [56]. To standardize effluent MC harvesting, the cells were obtained from a long dwell (generally overnight) with a PD fluid containing 2.27% glucose (Dianeal; Baxter). Effluent MCs from patients treated with low-GDP liquids were isolated from a long dwell with biocompatible fluids containing 2.3% glucose and buffered with lactate (Balance, Fresenius) or with bicarbonate (BicaVera, Fresenius). Omentum-derived MCs were obtained from patients that underwent unrelated abdominal surgery as described elsewhere [56], [57]. All cells were cultured in Earle's M199 medium, supplemented with 20% fetal-calf serum (FCS), 2% Hepes 1 M, 50 U/mL penicillin, 50 μg/mL streptomycin and 2% Biogro-2 (Biological Industries, Beit Haemek, Israel). The purity of effluent and omentum-derived MC cultures was determined by the expression of standard mesothelial markers: intercellular adhesion molecule-1, cytokeratins, and calretinine. These MC cultures were negative for von-Willebrand factor, CD31 and CD45, ruling out any contamination by endothelial cells or macrophages [56], [58], [59].

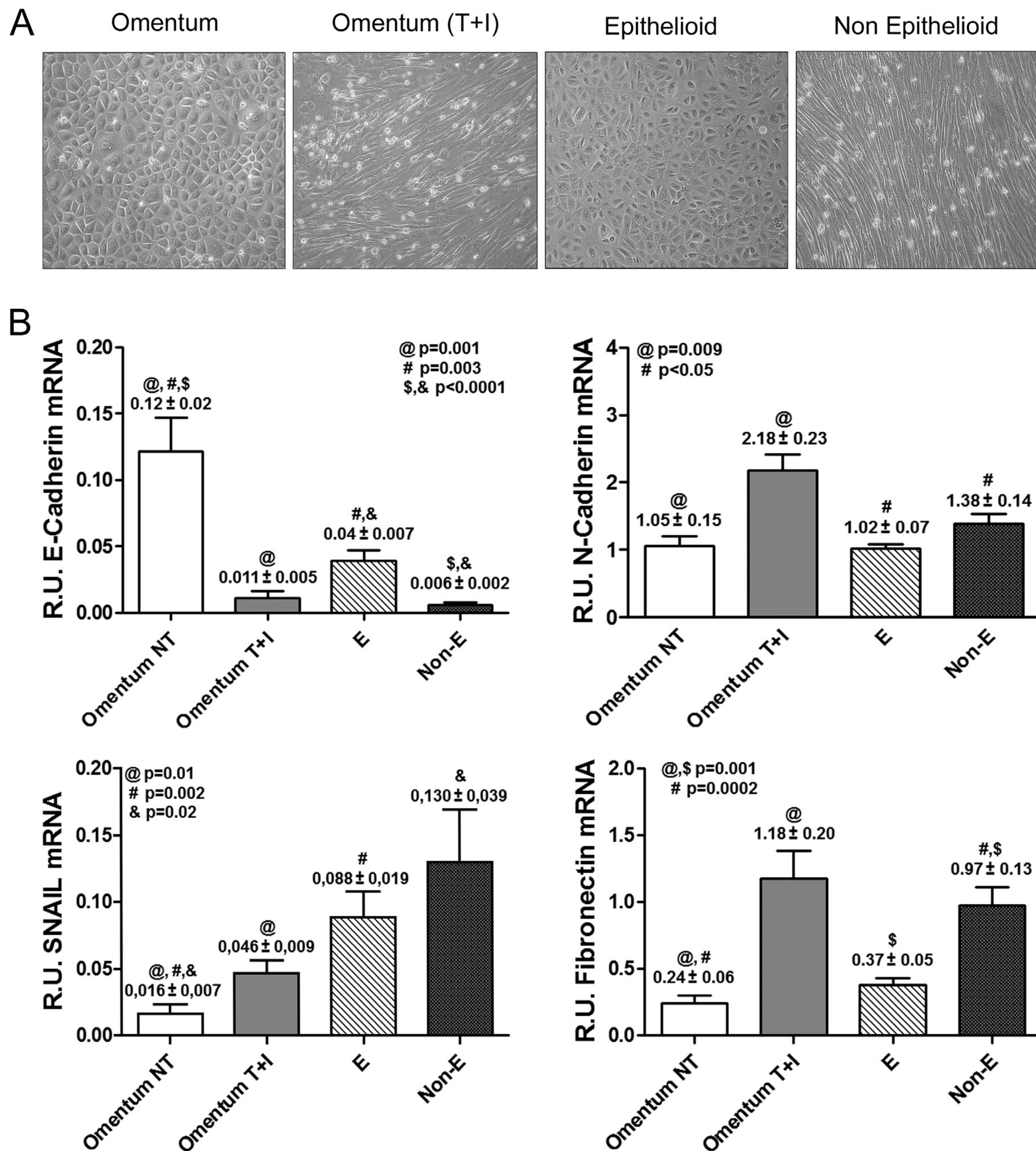


Figure 1. Characterization of MMT *in vitro* and *ex vivo*. (**A**) Representative pictures of omentum-derived MCs, either untreated or treated with TGF-β1 and IL-1β (MMT *in vitro*), and the two morphologies observed in confluent cultures of effluent-derived MCs: epithelioid and non-epithelioid. (**B**) Transcript levels of mesenchymal markers were analyzed by quantitative RT-PCR (n = 11 Omentum, 11 Omentum T+I, 30 E and 21 Non-E). Results show down-regulation of E-cadherin and up-regulation of snail expression during both *in vitro* and *ex vivo* MMT. The histograms also show a significant up-regulation of N-cadherin and fibronectin expression in mesenchymal MCs compared to omentum and epithelioid MCs. Data are depicted as mean value ± SE. Symbols show statistical differences between groups.
doi:10.1371/journal.pone.0060776.g001

To induce MMT *in vitro*, omentum-derived MCs were treated for 72 hours with a combination of human-recombinant TGF-β1 (0.5 ng/mL) and IL-1β (2 ng/mL) (T+I) (R&D Systems, Inc, Minneapolis, MN), which has been proven to be a good *in vitro* model of MMT [4], [8], [11], [58], [59]. The cytokine doses used were in the range of those detected in PD effluents, especially

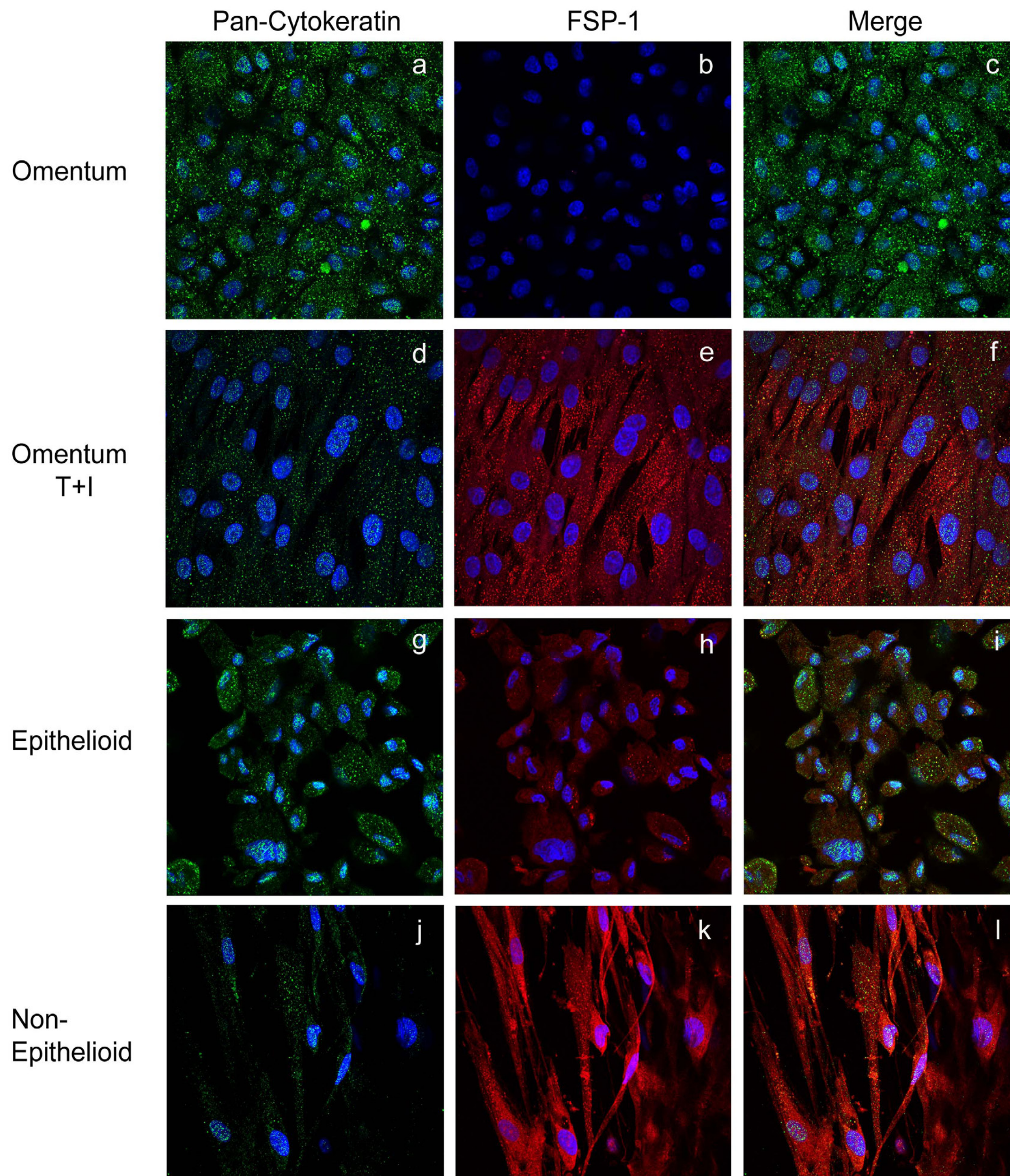


Figure 2. The expression of FSP-1 is up-regulated during *in vitro* and *ex vivo* MMT. The expression of FSP-1 and cytokeratin was analyzed in omentum and effluent-derived MCs by immunofluorescence using confocal microscopy. MCs were double stained for cytokeratin (green) and FSP-1 (red). Nuclei were stained with DAPI. Pictures show *in vitro* a Cyt+++/FSP1- staining for normal MCs (**a–c**), and Cyt+/FSP1++ for MCs treated with TGF-β1 and IL-1β (**d–f**). *Ex vivo*, pictures show Cyt++/FSP1+ staining for epithelioid MCs (**g–i**) and Cyt+/FSP1++ staining for non-epithelioid MCs (**j–l)** proving the mesenchymal conversion of MCs. Data are representative of 5 samples for each condition from PD patients and omentum samples included in the study.

doi:10.1371/journal.pone.0060776.g002

during peritonitis episodes [60], and were similar to those used in previous studies [4], [8], [11], [52], [53], [58], [59], [61]. We included 31 healthy donors of omental tissue for the study, of which 11 donors were used for QT-PCR analysis and 20 for proliferation, invasion and immunofluorescence assays. Furthermore, we included 15 additional clinically stable PD patients randomly selected to obtain effluent MCs for proliferation and invasion assays, whose baseline characteristics where not considered for these experiments.

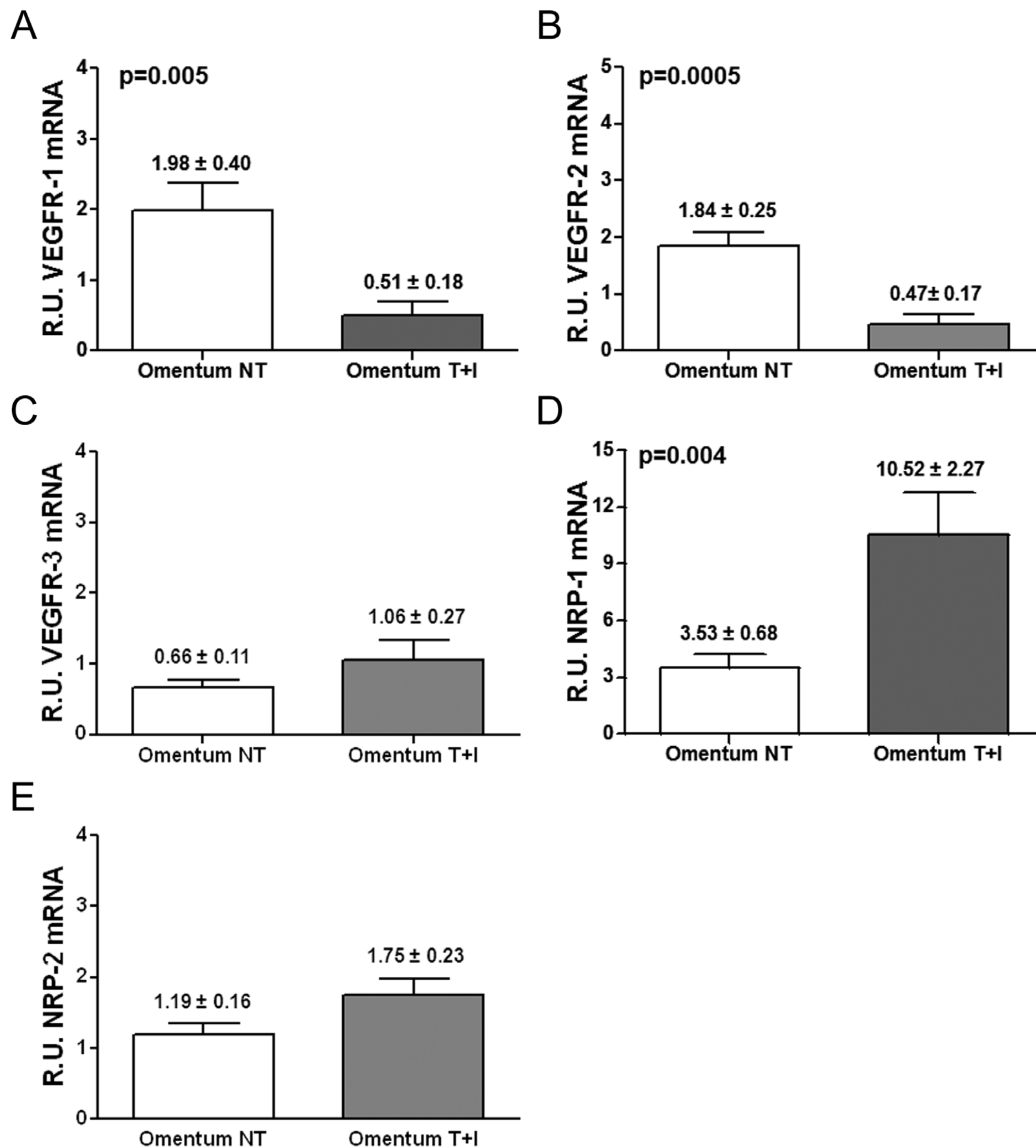


Figure 3. Transcript levels of VEGF receptors/co-receptors during *in vitro* MMT. mRNA levels of VEGF receptors/co-receptors were analyzed by quantitative RT-PCR. The results represent the relative mRNAs expression of VEGF receptors in omentum-derived MCs treated with TGF-β1 plus IL-1 β (Omentum T+I) compared with untreated MCs (Omentum NT). The data are depicted as mean value ± SE of omentum samples from 11 healthy donors. (**A, B and D**) The histograms show a down-regulation in expression of receptors VEGFR-1 (p = 0.005) and VEGFR-2 (p = 0.0005) and up-regulation of co-receptor Nrp-1 (p = 0.004). (**C and E**) The expression of VEGFR-3 and Nrp-2 did not show significant differences.
doi:10.1371/journal.pone.0060776.g003

Reagents and Antibodies

Recombinant human TGF-β1, IL-1β and Sema-3A were purchased from R&D Systems (100-B, 201-LB and 1250-S3, respectively; Minneapolis, MN,). Blocking monoclonal antibody against VEGF (MAB293, R&D Systems), and antibodies against the VEGF-binding motif or the Sema-3A-binding motif of Nrp-1 (Np-1b and Np-1a, respectively; Genentech, San Francisco, USA) [49], were used in the proliferation and invasion assays. Monoclonal antibodies IgG2a and IgG2b (Sigma-Aldrich, St Louis, MO, USA) were employed as isotype controls. Pilot studies were performed to establish optimal antibodies and recombinant protein dosages. For Western blotting anti-Nrp-1 rabbit monoclonal antibody (ab81321, Abcam, Cambridge, UK), anti-VEGFR-2 mouse monoclonal antibody (sc-316, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-tubulin mouse monoclonal antibody (T-5168, Sigma-Aldrich) were used. For immunofluorescence, the same antibodies against Nrp-1 and VEGFR-2 used in Western blot were employed. Cells were also stained with

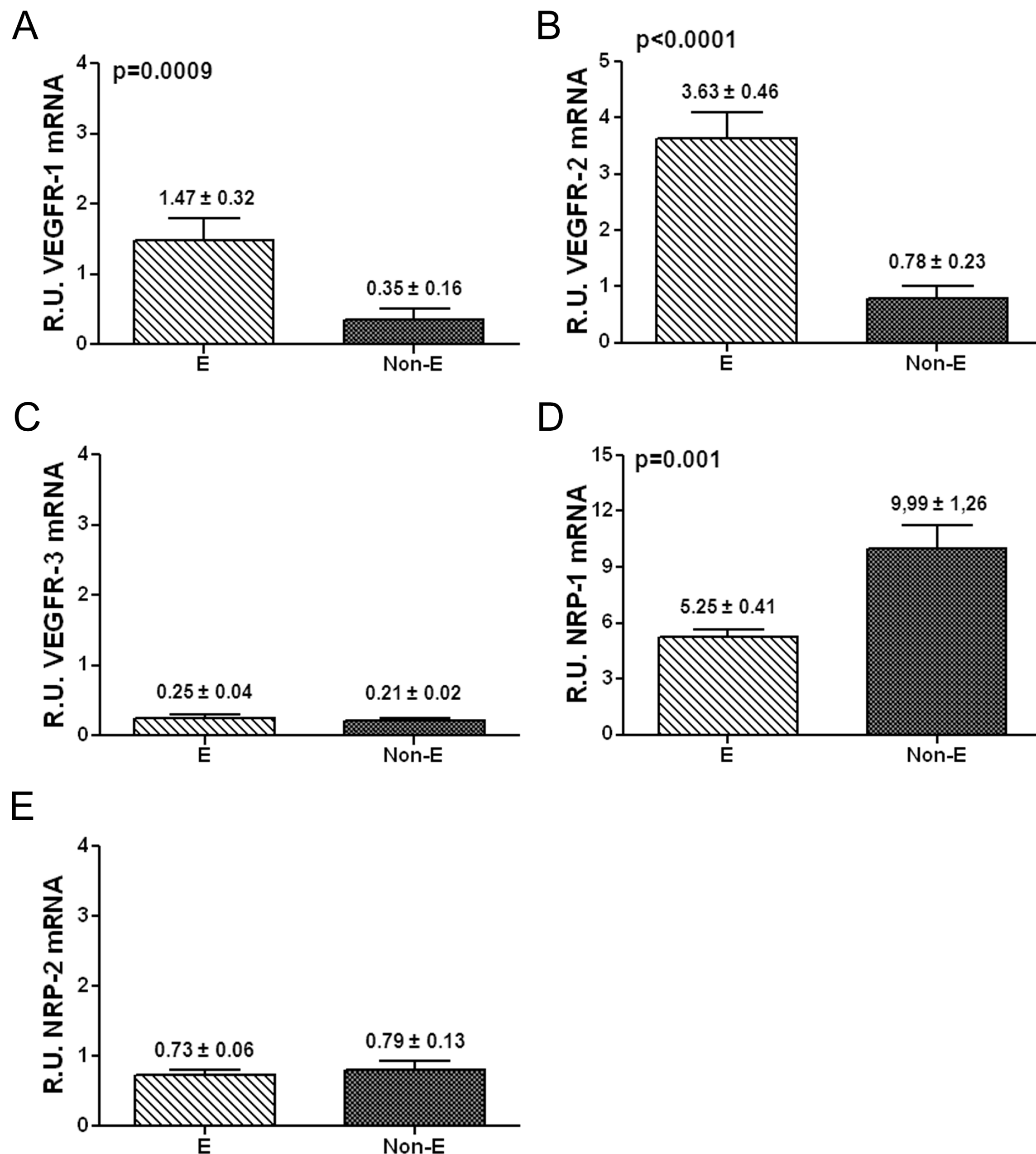
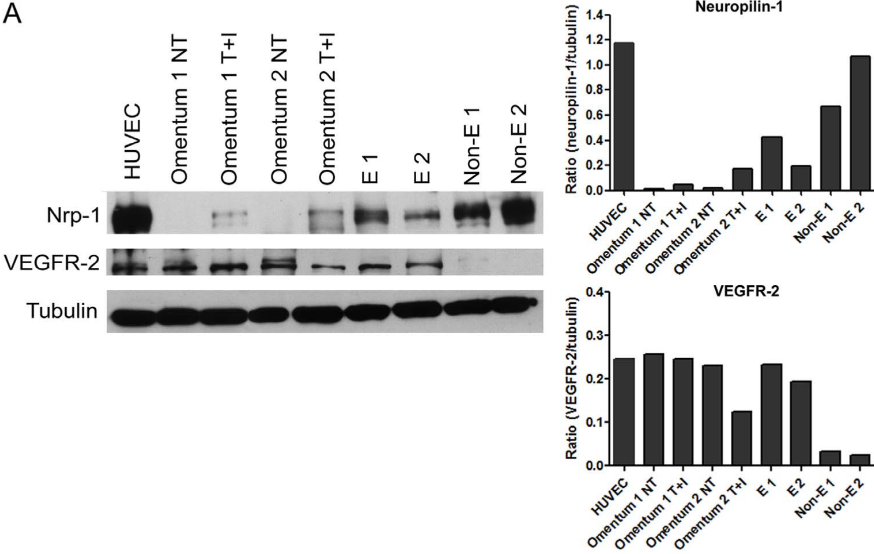


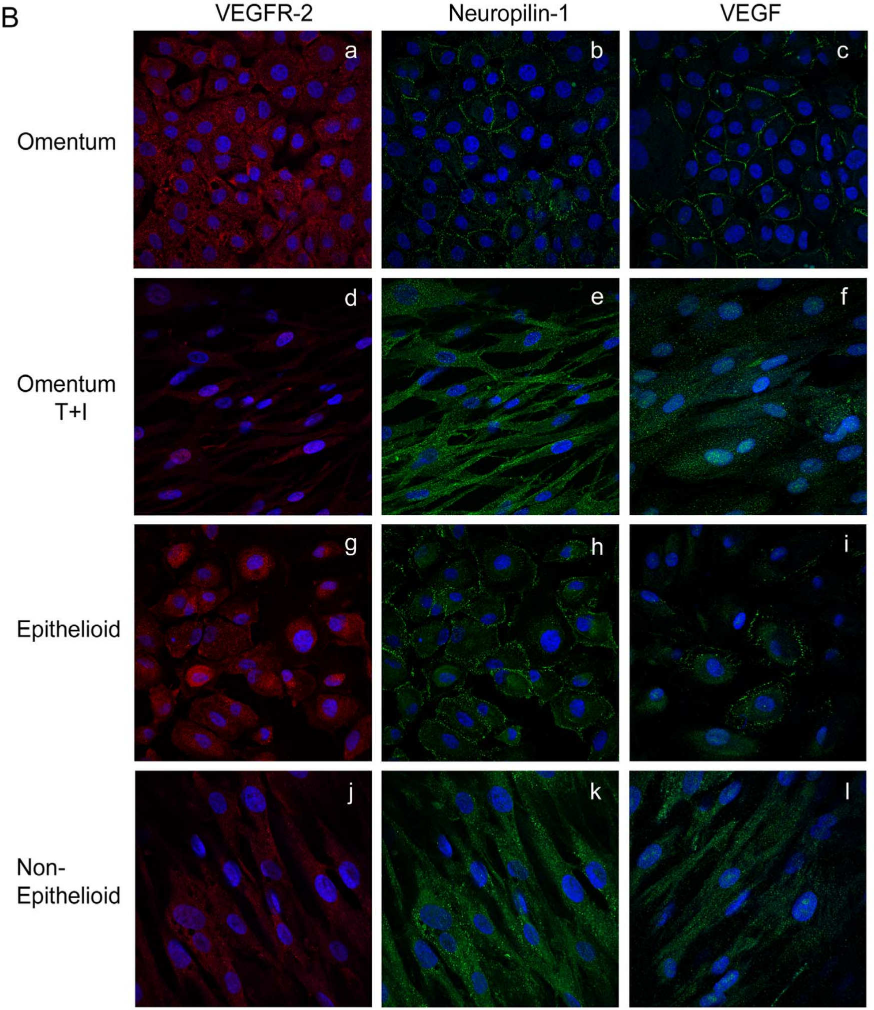
Figure 4. Transcript levels of VEGF receptors/co-receptors during *ex vivo* MMT. The histograms represent the relative mRNA expression of VEGF receptors/co-receptors in non-epithelioid effluent-derived MCs (Non-E, n = 21) compared with epithelioid effluent-derived MCs (E, n = 30). The data are depicted as mean value ± SE of effluent samples from 51 PD patients. (**A, B and D**) Similar results to those of *in vitro* MMT: a significant down-regulated expression of receptors VEGFR-1 (p = 0.0009) and VEGFR-2 (p<0.0001) and up-regulation of co-receptor Nrp-1 (p = 0.001). (**C and E**) The expression of VEGFR-3 and Nrp-2 did not show variation.
doi:10.1371/journal.pone.0060776.g004

anti-VEGF rabbit polyclonal antibody (ab46154, Abcam), anti-Pan cytokeratin mouse monoclonal antibody (c-1801, Sigma-Aldrich) and anti-FSP-1 (A-5114, Dako, Glostrup, Denmark). Secondary antibodies (conjugated to Alexa Fluor 568 and Alexa Fluor 488) were from Molecular Probes (Life Technologies, USA). For immunohistochemistry anti-Nrp-1 mouse monoclonal antibody (sc-5307, Santa Cruz Biotechnology) and mouse monoclonal anti-Pan cytokeratin (c-2931, Sigma-Aldrich) were employed.

Real-time Quantitative PCR Analysis

Total RNA was extracted using TRI Reagent® (Ambion, Inc., Austin, TX) and following manufacturer's recommendations.


A
HUVEC
Omentum 1 NT
Omentum 1 T+I
Omentum 2 NT
Omentum 2 T+I
E 1
E 2
Non-E 1
Non-E 2
Nrp-1
VEGFR-2
Tubulin
Neuropilin-1
Ratio (neuropilin-1/tubulin)
VEGFR-2
Ratio (VEGFR-2/tubulin)


B
VEGFR-2
Neuropilin-1
VEGF
Omentum
Omentum T+I
Epithelioid
Non-Epithelioid
a
b
c
d
e
f
g
h
i
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l

Figure 5. Expression levels and cellular distribution of Nrp-1 and VEGFR2 proteins during *in vitro* and *ex vivo* MMT. (**A**) Western blots show the expression levels of Nrp-1 and VEGFR-2 in total cell lysates during *in vitro* and *ex vivo* MMT. Expression of α-tubulin is employed as a loading control. Human umbilical vein endothelial cells (HUVEC) are used as a positive control. The histograms depict the quantification of Nrp-1 and VEGFR2 levels compared with α-tubulin. Data are representative of 5 samples for each condition from PD patients and omentum samples included in the study. (**B**) The expression of Nrp-1, VEGFR-2, and VEGF was analyzed by immunofluorescence microscopy in omentum and effluent-derived MCs. MCs were double stained for Nrp-1 (green) and VEGFR-2 (red), and single stained for VEGF (green). Nuclei were stained with DAPI. Nrp-1 and VEGF show a membrane distribution in omentum and epithelioid MCs (**b, c, h, i**)**.** During *in vitro* (**e, f**) and *ex vivo* (**k, l**) MMT both proteins change their localization and are internalized. The expression of VEGFR-2 is down-regulated but it does not show differences in localization during *in vitro* (**a, d**) and *ex vivo* (**g, j**) MMT.
doi:10.1371/journal.pone.0060776.g005

Complementary DNA was obtained from 2 μg of total RNA by reverse transcription (RNA PCR Core Kit; Applied Biosystems, Foster City, CA). Quantitative RT-PCR was carried out in a Light Cycler 2.0 using a SYBR Green kit (Roche Diagnostics GmbH, Mannheim, Germany) and specific primers for VEGFR-1/Flt-1, VEGFR-2/KDR and VEGFR-3/Flt-4, Nrp-1, Nrp-2, Sema-3A, E-cadherin, N-cadherin, snail, fibronectin and histone H3 (**Table S1**). Amplification values were normalized with respect to the value obtained for H3.

Enzyme-linked Immunoassay

Albumin, VEGF and Sema-3A concentrations were determined by using standard enzyme-linked immunoassay (ELISA) kits (Abcam; R&D Systems; UscnK Life Science, USA; respectively). For analysis of VEGF levels in supernatants, the media of confluent MC cultures in the first passage was replaced with fresh media and 18 hours later supernatants were collected and stored at –80°C until their analysis. The results were normalized with total protein of cell lysates and depicted as picograms per milligrams (pg/mg). For VEGF concentration in effluents from PD patients, 15 ml of each bag of effluents were collected and stored at –80°C until their analysis. Results were represented as picograms per millilitre (pg/mL).

For Sema-3A concentration in effluents from PD patients, the dialysates were concentrated as previously described [62], using commercial concentrators (Amikon Ultra-15, Millipore). The concentration factor was defined as the albumin concentration in the concentrate divided by the albumin concentration in the original effluent sample. Results were represented as pg/mL.

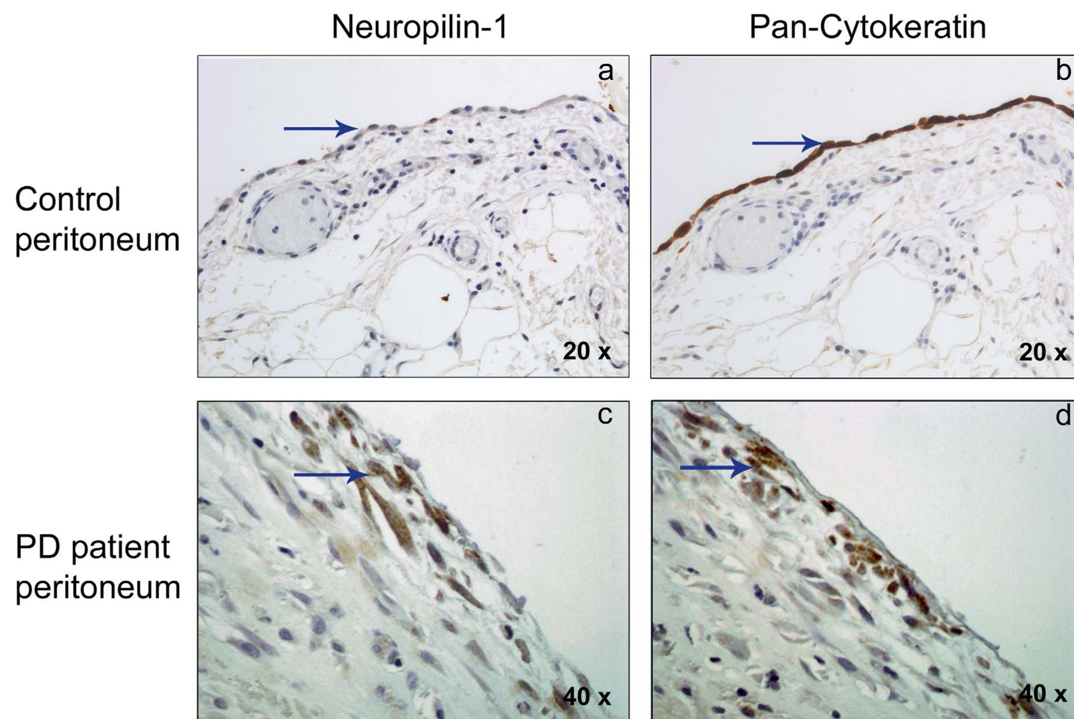


Figure 6. Nrp-1 immunohistochemical analysis in peritoneal human biopsies. The expression of Nrp-1 and the mesothelial marker cytokeratin was analyzed in human peritoneal specimens by immunohistochemistry. Positive cells for antibodies used (Nrp-1 and Cytokeratin) show brown staining. Nuclei are counterstained in blue. (**a, b**) Control peritoneal tissue, with a conserved mesothelial cell monolayer showing an epithelioid morphology (with a 20X objective). These cells show weak expression of Nrp-1 and a marked staining for cytokeratin (arrows). No expression of these proteins was observed in the submesothelial area (region under mesothelial monolayer) (**c, d**) Fibrotic tissue sample from PD patient showing the loss of mesothelial monolayer and invading spindle-like mesothelial cells in submesothelial area (with a 40X objective). These cells present a strong staining for Nrp-1 (**c**), and are also positive for cytokeratin (**d**) (arrows). Pictures are representative of 5 cases of PD patient samples and 4 of control samples.
doi:10.1371/journal.pone.0060776.g006

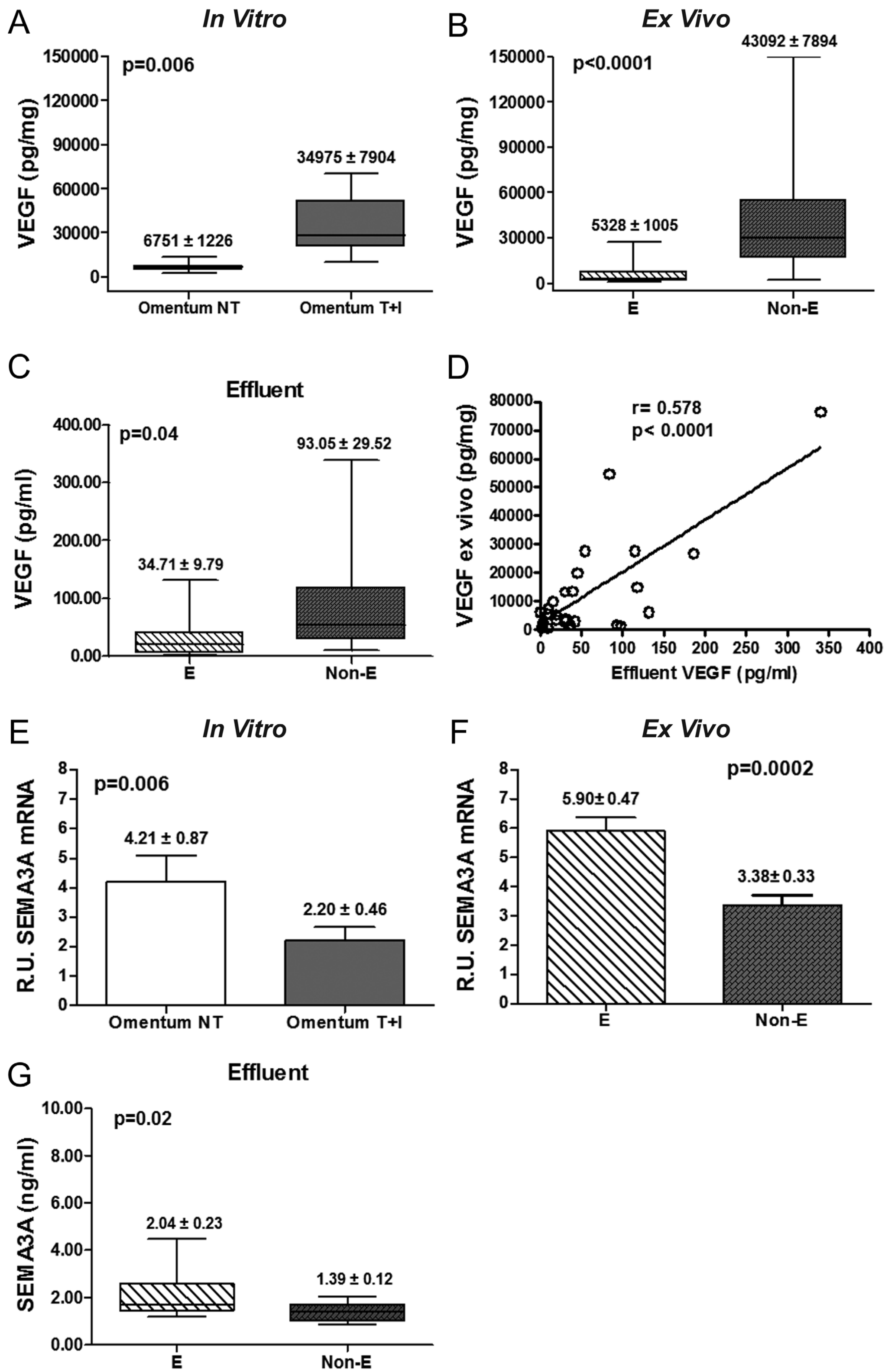

A
In Vitro
p=0.006
VEGF (pg/mg)
150000
120000
90000
60000
30000
0
6751 ± 1226
34975 ± 7904
Omentum NT
Omentum T+I
B
Ex Vivo
p<0.0001
43092 ± 7894
5328 ± 1005
VEGF (pg/mg)
E
Non-E
C
Effluent
p=0.04
93.05 ± 29.52
34.71 ± 9.79
VEGF (pg/ml)
400.00
300.00
200.00
100.00
0.00
E
Non-E
D
r= 0.578
p< 0.0001
VEGF ex vivo (pg/mg)
80000
70000
60000
50000
40000
30000
20000
10000
0
0 50 100 150 200 250 300 350 400
Effluent VEGF (pg/ml)
E
In Vitro
p=0.006
4.21 ± 0.87
2.20 ± 0.46
R.U. SEMA3A mRNA
Omentum NT
Omentum T+I
F
Ex Vivo
p=0.0002
5.90± 0.47
3.38± 0.33
R.U. SEMA3A mRNA
E
Non-E
G
Effluent
p=0.02
2.04 ± 0.23
1.39 ± 0.12
SEMA3A (ng/ml)
10.00
8.00
6.00
4.00
2.00
0.00
E
Non-E

Figure 7. Analysis of the expression levels of VEGF and Sema3A during the MMT process. (**A and B**) The expression of VEGF in MC culture supernatants during *in vitro* (n = 11, p = 0.006) and *ex* vivo (n = 51, p<0.0001) MMT was analyzed by ELISA. (**C**) VEGF secretion in effluents of PD patients draining MCs with different phenotypes was also analyzed by ELISA (E, n = 16 and Non-E, n = 11; p = 0.04). (**D**) Correlation between VEGF levels *ex vivo* and effluents of PD patients (r = 0.578, p<0.0001) (**E and F**) Sema-3A mRNA expression, in both *in vitro* (p = 0.006) and *ex vivo* (p = 0.0002) MMT, was analyzed by quantitative RT-PCR. (**G**) Sema-3A secretion in PD effluents (n = 16 E and 11 Non-E; p = 0.02). Box Plots represent 25% and 75% percentiles, median, minimum and maximum values. Numbers above boxes and histograms depict mean ± SE.
doi:10.1371/journal.pone.0060776.g007

Western Blotting

MC cultures were lysed in RIPA buffer (1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) plus a phosphatase and protease inhibitor cocktail (Pierce) and total protein was quantified by protein assays kit (Pierce). Equal amounts of denatured proteins (30–40 μg) from each sample were resolved by 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred on nitrocellulose membranes, which were then blocked with 5% nonfat milk in TBS-Tween buffer for 1 hour and incubated with specific antibodies against Nrp-1, VERGFR-2 and tubulin in 0.5% milk in TBS-Tween overnight at 4°C. These antibodies were detected with a peroxidase conjugated goat anti-mouse and donkey anti-rabbit antibodies (Pierce, Rockford, IL, USA), and then were visualized with enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA). Blot images were acquired with GS-710 Imaging Densitometer (Bio-Rad, Hercules, CA) and analyzed with Quantify-One software (Bio-Rad).

Confocal Microscopy and Immunofluorescence

Cells were fixed for 10 minutes in 4% paraformaldehyde and permeabilized 10 minutes in 0.1% NP-40. In all cases, 5% goat serum was applied for 20 minutes to block non-specific unions. Cells were stained for Nrp-1 (labeled with Alexa Fluor 488), VEGFR-2 (labeled for Alexa Fluor 568) and VEGF (labeled with Alexa Fluor 488) and mounted with fluorescent mounting media (Dako). Confocal images were acquired with a LSM710 Zeiss Confocal Microscope (with a 40X objective).

Biopsy Processing and Immunohistochemical Analysis

Parietal peritoneum biopsies from PD patients were obtained from the anterior abdominal wall by surgeons during renal transplantation, insertion or removal of the PD catheter, or because of incidental abdominal conditions. Normal control samples of parietal peritoneum from non-uremic patients subjected to elective surgeries were also included in this study. Written consent was obtained from patients prior to obtaining the peritoneal samples. Tissue samples were routinely fixed in neutral-buffered 3.7% formalin (pH 7.3) during 12–24 hours. Afterwards, samples were dehydrated and embedded in paraffin to obtain sections 3–4 μm thick. Deparaffinised sections were stained with Haematoxylin-Eosin and Masson's trichrome to analyze the histological characteristics of each specimen. Samples from 5 PD patients with evident sign of submesothelial fibrosis and 4 controls were included in this study.

For immunohistochemistry, samples were incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Antigen retrieval was performed by heating samples in citrate buffer (pH 6). Monoclonal anti-Nrp-1 (Santa Cruz Biotechnology) and anti-Pan-cytokeratin antibodies (Dako) were applied to detect the antigens by means of a dextran-polymer conjugate technique (EnVision+, Dako). The reactions were visualized by diaminobenzidine chromogen (brown) and tissue sections were finally counterstained with a nuclear haematoxylin staining (blue). Morphological characteristics were studied as previously described [4], [11].

Proliferation Assays

For proliferation assays, 10^4 cells/well of effluent- or omentum-derived MCs, with either epithelioid or non-epithelioid phenotypes, were seeded into 96-well plates and cultured at 37°C and 5% CO2 for 48 hours. Cells were then pulsed with [^{3}H]-thymidine (1 μCi per well) for 16–18 hours and lysed with Filter Mate Cell Harvester (Perkin Elmer, Turku, Finland). Radioactivity was determined for 1 minute in a basic beta liquid scintillation counter (Perkin Elmer). For proliferation-blocking experiments, anti-VEGF (0.5, 1 or 10 μg/mL), anti-Np-1b (10 μg/mL), anti-Np-1a (10 μg/mL), and isotype controls (10 μg/mL) were added in fresh medium eight hours prior to [^{3}H]-thymidine addition. Each experiment was carried out in triplicate, and at least 8 experiments were performed.

Table 1. Baseline characteristics of PD patients with different phenotypes of mesothelial cells.

Parameters	Studied population (n = 51)	Mesothelial Cells Phenotype		P
		Epithelioid (n = 30)	Non-Epithelioid (n = 21)	
Time on PD(months)	7.61±6.52	6.55±6.47	9.41±6.37	0.04
CCr (mL/min)	4.82±3.50	5.74±3.56	3.50±3.00	0.02
Glucose load (Kg)	22.85±28.26	24.55±33.75	20.41±18.27	NS
Urea-MTC (mL/min)	23.20±6.95	23.74±8.06	22.43±5.05	NS
Cr-MTC (mL/min)	10.85±3.13	10.39±2.72	11.50±3.60	NS
UF 3.86% (mL)*	505.90±171.58	508.80±181.64	501.76±160.40	NS
N° Peritonitis	11 from 51	2 from 11	9 from 11	0.03
VEGF (pg/mg)	20878.64±4185.47	5328.63±1005.32	43092.92±7894.40	0.0001

CCr: creatinine clearance. MTC: mass transfer coefficient. UF: ultrafiltration after a 4-hour dwell with glucose 3.86%. VEGF: vascular endothelial growth factor. Values represent mean ± SD for median clinical parameters or mean ± SE for supernatant VEGF.
doi:10.1371/journal.pone.0060776.t001

Table 2. Differences between low and high peritoneal transporters.

	Cr-MTC <11 mL/min (n = 29)	Cr-MTC ≥11 mL/min(n = 22)	P
Urea-MTC (mL/min)	21.23±3.53	25.80±9.30	0.02
Albumin (g/dL)	3.64±0.46	3.46±0.36	0.04
UF rate (3.86%)	574.66±180.61	415.27±107.10	0.0001
Glucose load (Kg)	20.99±33.56	25.29±19.72	0.01
N° peritonitis	4 from 11	7 from 11	NS
VEGF (pg/mg)	14319.43±4242.77	29524.86±7667.62	0.01
VEGFR-1 (R.U)	2.00±0.73	0.93±0.36	NS
VEGFR-2 (R.U)	3.73±0.65	1.52±0.35	0.005
VEGFR-3 (R.U)	0.22±0.02	0.24±0.05	NS
Nrp-1 (R.U)	6.21±0.64	8.29±1.24	0.14 (NS)
Nrp-2 (R.U)	0.74±0.08	0.75±0.10	NS
Sema-3A (R.U)	5.59±0.48	4.06±0.43	0.025

Statistic differences between the groups of low, low-average (Cr-MTC <11 mL/min, range 4.3–10.9) and high, high-average (Cr-MTC ≥11 mL/min, range 11.3–18.3) peritoneal transporters. R.U. mRNA relative units. Values represent mean ± SD for median clinical parameters or mean ± SE for experimental data.
doi:10.1371/journal.pone.0060776.t002

Invasion Assays

Invasion assays were performed in a 24-well insert system (Costar, Cambridge, MA). The filters, 8 µm pore size, were pre-coated with 40 µL of 300 µg/mL solution of collagen type I (PureCol, Inamed, Fremont, Canada) allowing to gel overnight at 37°C. Then, 3×10^4 starved MCs/well were added into the upper chamber. As a chemotactic stimulus, 5% FCS was added into the lower chamber in 600 µL of medium. Cells were allowed to invade for 24 hours at 37°C and 5% CO_2. Transwells were then fixed in 4% formaldehyde. After removing the gel and non-invading cells from the upper face of the membrane with a cotton swab, filters were cut out and nucleus of invading cells were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). Invading cells were counted in ten fields per sample using a fluorescence microscope (with a 40X objective). For invasion-blocking experiments, MCs were pre-incubated during 30 minutes at 4°C with recombinant Sema-3A (150 nM), anti-VEGF (0.5, 1 or 10 µg/mL), anti-Np-1b (10 µg/mL), anti-Np-1a (10 µg/mL), isotype controls (10 µg/mL), before seeding in transwell units. At least 8 experiments were carried out in duplicate.

Statistical Analysis

Experimental data in Figures and Tables are depicted as mean ± standard error (SE). Clinical data of patients included in the study are given as mean ± standard deviation (SD). To assess the changes produced in MCs by *in vitro* MMT, induced by the treatment with TGF-β1+IL-1β, the data was analyzed using a Paired test. Comparison between data groups was performed using the non-parametric Mann Whitney rank-sum U test, Spearman regression analysis, Chi-square and 2-tail Fisher exact test. P values less than 0.05 were considered statistically significant. We used SPSS Inc, version 15 (Chicago, IL) and GraphPad Prism 4.0 (La Jolla, CA).

Results

Characterization of Mesothelial to Mesenchymal Transition (MMT)

For this study, we included omentum MCs from 11 donors that underwent unrelated abdominal surgery and effluent-derived MCs from 51 PD patients. To characterize the MMT process, we first identified the different morphologies of confluent MC cultures (**Figure 1A**). The effluent-derived epithelioid MCs showed cobblestone morphology similar to that of normal MCs (omentum), and effluent-derived non-epithelioid MCs showed a fibroblast-like phenotype similar to that of omentum treated with TGF-β1 and IL-1β (omentum T+I). Then, the cells were lysed for RNA extraction and we performed quantitative RT-PCR in order to study the transcript level expression of the epithelial marker E-cadherin and the mesenchymal markers N-cadherin, fibronectin and snail (**Figure 1B**). We observed a strong down-regulation of E-cadherin and up-regulation of its repressor, snail, during both *in vitro* and *ex vivo* MMT. We also observed a significant up-regulation of N-cadherin and fibronectin expression in mesenchymal MCs compared to omentum and epithelioid MCs. These data demonstrated the mesenchymal conversion of MCs.

To further characterize the MMT process, we studied the expression of fibroblast specific protein-1 (FSP-1), a mesenchymal marker, and the expression of cytokeratin (Cyt), a mesothelial marker, by immunofluorescence and confocal analysis (**Figure 2**). We observed a Cyt+++/FSP1- staining for normal MCs (**Figure 2 a–c**), and Cyt+/FSP1++ for MCs treated with TGF-β1 and IL-1β, as a consequence of *in vitro* mesenchymal conversion (**Figure 2 d–f**). In effluent-derived MCs cultured *ex vivo*, we observed a Cyt++/FSP1+ staining for epithelioid MCs (**Figure 2 g–i**). In this case cytokeratin staining showed less intensity than normal MCs and FSP-1 was also expressed, suggesting that epithelioid MCs were undergoing an early MMT. Non-epithelioid MCs showed Cyt+/FSP1+++ staining, indicating a more advanced mesenchymal transformation (**Figure 2 j–l**).

Switch of VEGF Receptors/co-receptors during in vitro and ex vivo Mesothelial to Mesenchymal Transition

It has been shown that the expression of VEGFR-1/Flt-1, VEGFR-2/KDR, Nrp-1 and Nrp-2 remains unchanged during the malignant transformation of MCs (e.g. mesothelioma) [37]. However, the expression patterns of VEGFRs and co-receptors throughout the mesenchymal conversion of MCs have not been analyzed so far. Thus, we analyzed by quantitative RT-PCR the expression levels of VEGFR-1/Flt-1, VEGFR-2/KDR, VEGFR-3/Flt-4, Nrp-1 and Nrp-2 throughout *in vitro* and *ex vivo* MMT. Treatment of omentum MCs with TGF-β1 plus IL-1β (*in vitro* MMT) significantly down-regulated the expression of VEGFR-1 and VEGFR-2 (**Figures 3A and 3B**) and up-regulated the expression of the co-receptor Nrp-1 (**Figure 3D**). The expression of VEGFR-3 and Nrp-2 did not show statistical differences (**Figures 3C and 3E**). Similarly, comparison of effluent-derived MCs with different phenotypes (*ex vivo* MMT) showed significant down-regulation of VEGFR-1 and VEGFR-2 (**Figures 4A and 4B**) and up-regulation of Nrp-1 (**Figure 4D**) in non-epithelioid cells when compared with epithelioid cells. Again, the expression of VEGFR-3 and Nrp-2 did not show statistical differences (**Figures 4C and 4E**). Thus, these results evidence the switch of the expression patterns of VEGFRs and co-receptors throughout *in vitro* and *ex vivo* MMT.

These alterations are inherent features of the mesenchymal conversion of MCs and not a result of the treatment with TGF-β1 plus IL-1β. We induced a reversible *in vitro* MMT treating MCs

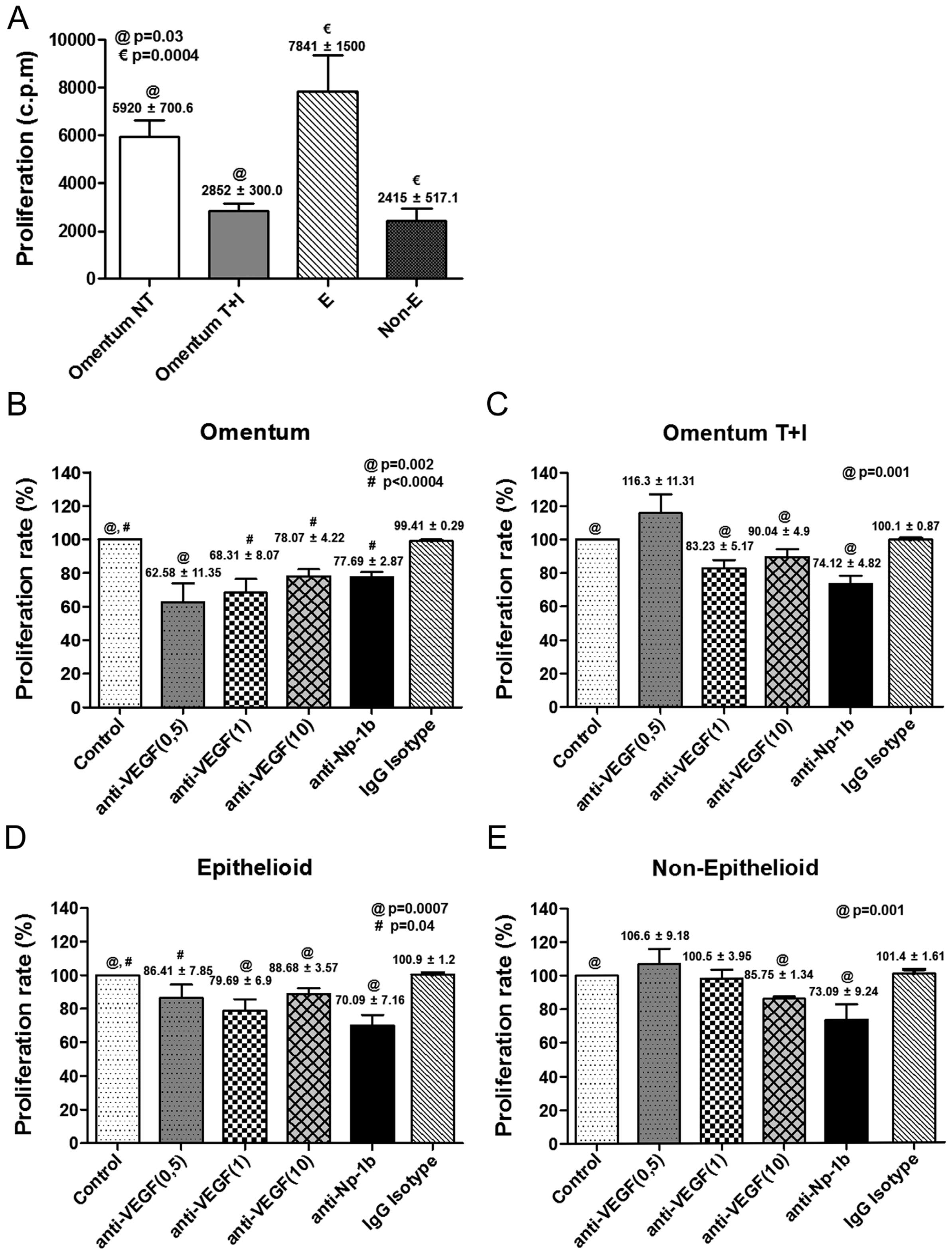

A
Proliferation (c.p.m)
@ p=0.03
€ p=0.0004
@
5920 ± 700.6
@
2852 ± 300.0
€
7841 ± 1500
€
2415 ± 517.1
Omentum NT
Omentum T+I
E
Non-E
B
Omentum
Proliferation rate (%)
@ p=0.002
# p<0.0004
@, #
@
62.58 ± 11.35
#
68.31 ± 8.07
#
78.07 ± 4.22
#
77.69 ± 2.87
99.41 ± 0.29
Control
anti-VEGF(0,5)
anti-VEGF(1)
anti-VEGF(10)
anti-Np-1b
IgG Isotype
C
Omentum T+I
@ p=0.001
@
116.3 ± 11.31
@
83.23 ± 5.17
@
90.04 ± 4.9
@
74.12 ± 4.82
100.1 ± 0.87
D
Epithelioid
@ p=0.0007
# p=0.04
@, #
#
86.41 ± 7.85
@
79.69 ± 6.9
@
88.68 ± 3.57
@
70.09 ± 7.16
100.9 ± 1.2
E
Non-Epithelioid
@ p=0.001
@
106.6 ± 9.18
100.5 ± 3.95
@
85.75 ± 1.34
@
73.09 ± 9.24
101.4 ± 1.61

Figure 8. Treatment with anti-VEGF and anti-Np1b blocking antibodies interferes with the proliferation of mesothelial cells. (**A**) The proliferation capacity of MCs throughout *in vitro* and *ex vivo* MMT was analyzed by the incorporation of [3H]-thymidine (n = 20). Bar graphic depicts the radioactivity emitted (c.p.m.) in each condition. Data are depicted as mean value ± SE. Symbols show statistical differences between groups. (**B and E**) Effect of anti-VEGF (n = 10) and anti-Np1b antibodies (n = 8), or IgG isotype control antibody on the proliferation capacity of omentum-derived MCs, either untreated (**B**) or treated with TGF-β1 plus IL-1β (**C**); and on the proliferation capacity of effluent-derived MCs with epithelioid (**D**) or non-epithelioid phenotype (**E**). Bar graphics represent proliferation percentage of treatments over control cells. Data are depicted as mean value ± SE. Symbols show statistical differences between groups.
doi:10.1371/journal.pone.0060776.g008

with TGF-β1 plus IL-1β for 72 hours, and after that, we replaced the media without the cytokines for another 24 hours. We observed that MCs reverted the mRNA levels of VEGFR and co-receptors after removal of the stimuli (**Figure S1**).

The Expression and Subcellular Localization of VEGFR-2 and Nrp-1 Proteins Change during MMT

We studied the expression of Nrp1 and VEGFR-2 by Western blot to confirm the data obtained by quantitative RT-PCR. Up-regulated expression of Nrp-1 and down-regulated expression of VEGFR-2 proteins were observed during both *in vitro* MMT (omentum vs. omentum T+I) and *ex vivo* MMT (epithelioid vs. non-epithelioid) (**Figure 5A**). We also studied the cellular distribution of both proteins by immunofluorescence and we observed that Nrp-1 showed different localization depending on the cell phenotype. In epithelial-like MCs (omentum and effluent epithelioid cells), Nrp-1 was mainly localized in the membrane **(Figure 5B b, h),** whereas in mesenchymal-like MCs (omentum T+I and effluent non-epithelioid cells) the distribution was cytoplasmic, suggesting its internalization (**Figure 5B e, k**). In contrast, VEGFR-2 had a cytoplasmic distribution in all cell types (**Figure 5B a, d, g, j**). MCs stained with anti-VEGF showed a similar pattern of expression and distribution to cells stained with anti-Nrp-1 (**Figure 5B c, f, i, l**). Moreover, pictures showed different intensity patterns for Nrp-1 and VEGFR-2 during MMT, confirming data previously observed by Western blot.

To confirm *in vivo* the local up-regulation of Nrp-1 by non-epithelioid MCs, peritoneal biopsies from PD patients and controls (non-renal patients) were subjected to immunohistochemical analysis**.** Submesothelial fibroblasts from the control group did not express Nrp-1 (**Figure 6 a**), and a weak expression of this co-receptor was confined to the surface of the MC monolayer. In contrast, peritoneal biopsies from PD patients, with evident signs of peritoneal fibrosis, showed up-regulated expression of Nrp-1 in spindle-like cells embedded in the fibrotic stroma, located mainly in the upper submesothelial area (**Figure 6 c**). Serial sections from the same peritoneal samples showed expression of cytokeratin overlapping with Nrp-1 in the compact zone from PD patients (**Figure 6 d**). The controls showed cytokeratin staining in the mesothelium but not in the submesothelial area (**Figure 6 b**).

Increase of the VEGF/Sema-3A Ratio throughout the MMT Process

A previous study from our group described that MMT is associated with strong up-regulation of VEGF expression [10]. In the present study, we confirmed the induction of VEGF secretion to culture supernatants in omentum MCs treated with TGF-β1 plus IL-1β (*in vitro* MMT) and in effluent MCs with non-epithelioid phenotype (*ex vivo* MMT) (**Figures 7A and 7B**). Furthermore, we determined the levels of VEGF secreted in the dialysates of PD patients and we observed higher levels of VEGF in effluents of patients that drained non-epithelioid MCs than in effluents with epithelioid MCs (**Figure 7C**). Moreover, we found a significant correlation between VEGF levels secreted *ex vivo* and VEGF levels secreted in effluents (**Figure 7D**).

On the other hand, it has been shown that Sema-3A might act as a functional competitor of VEGF-driven cell responses through the competition for Nrp-1 binding [37], [63]. Thus, we measured Sema-3A mRNA expression throughout the MMT process. Significant down-regulation of Sema-3A-encoding transcript took place in both *in vitro* and *ex vivo* MMT (**Figures 7E and 7F**). We also analyzed the concentration of Sema-3A in the effluents. We found significant lower levels of this protein in effluents that drained non-epithelioid MCs. (**Figure 7G**). In addition, the expression of VEGF and Sema-3A showed a significant negative correlation (**Figure S2**). These data demonstrate that the VEGF/Sema-3A ratio increases throughout the MMT process.

The Change of the VEGF/VEGFRs/Nrp Axis is Associated with Peritoneal Functional Decline in PD Patients

We analyzed the baseline characteristics of PD patients included in this study and the differences between subgroups according to the phenotype of effluent-derived MCs (**Table 1**). Significant differences in the time on PD, the CCr and *ex vivo* production of VEGF were found between epithelioid and non-epithelioid subgroups of MCs. Nine of the 11 patients who experienced peritonitis, and the patient that suffered hemoperitoneum, drained non-epithelioid MCs in their effluents ($p = 0.03$). These data suggest that MCs with non-epithelioid phenotype appear at early stages of peritoneal membrane deterioration, even before ultra-filtration failure has been established.

An elevated mass transport coefficient for creatinine (Cr-MTC) is a clinical marker for peritoneal functional decline, which is related to the augmented vessel number and is associated with increased production of VEGF by MCs [10]. Thus, we subdivided the PD patients into 2 groups according to peritoneal transport characteristics: Cr-MTC <11 mL/min (low and low-average transporters) and Cr-MTC ≥ 11 mL/min (high and high-average transporters) [7], [10]. As expected, the high transporter group showed a significant increase of urea-MTC and glucose load and a significant decrease of ultra-filtration (**Table 2**). In addition, Cr-MTC seemed to be associated with effluent MC phenotype; 30% of patients (9 of 30) with epithelioid MCs in their effluent showed Cr-MTC ≥ 11, whereas 62% of patients (13 of 21 patients) with non-epithelioid MCs showed Cr-MTC ≥ 11 (two tails Fischer's exact test; $r = 0.395$, $p = 0.04$) (**Table S2**). In agreement with our previous results [10], effluent-derived MCs from high transporters produced significantly higher *ex vivo* amounts of VEGF than MCs from low transporters (**Table 2**). In addition, we observed a significant correlation between the high peritoneal transport status and the down-regulation of VEGFR-2 and Sema-3A mRNAs *ex vivo*. The high transporter group showed augmented expression of Nrp-1-encoding mRNA, although it did not reach statistical significance (**Table 2**). Of note, the increased expression of Nrp-1 had a significant correlation with the time on PD ($p = 0.001$). These data indicated that the peritoneal functional decline was associated to changes of MC phenotype and to changes of VEGF/VEGFRs/Nrp axis.

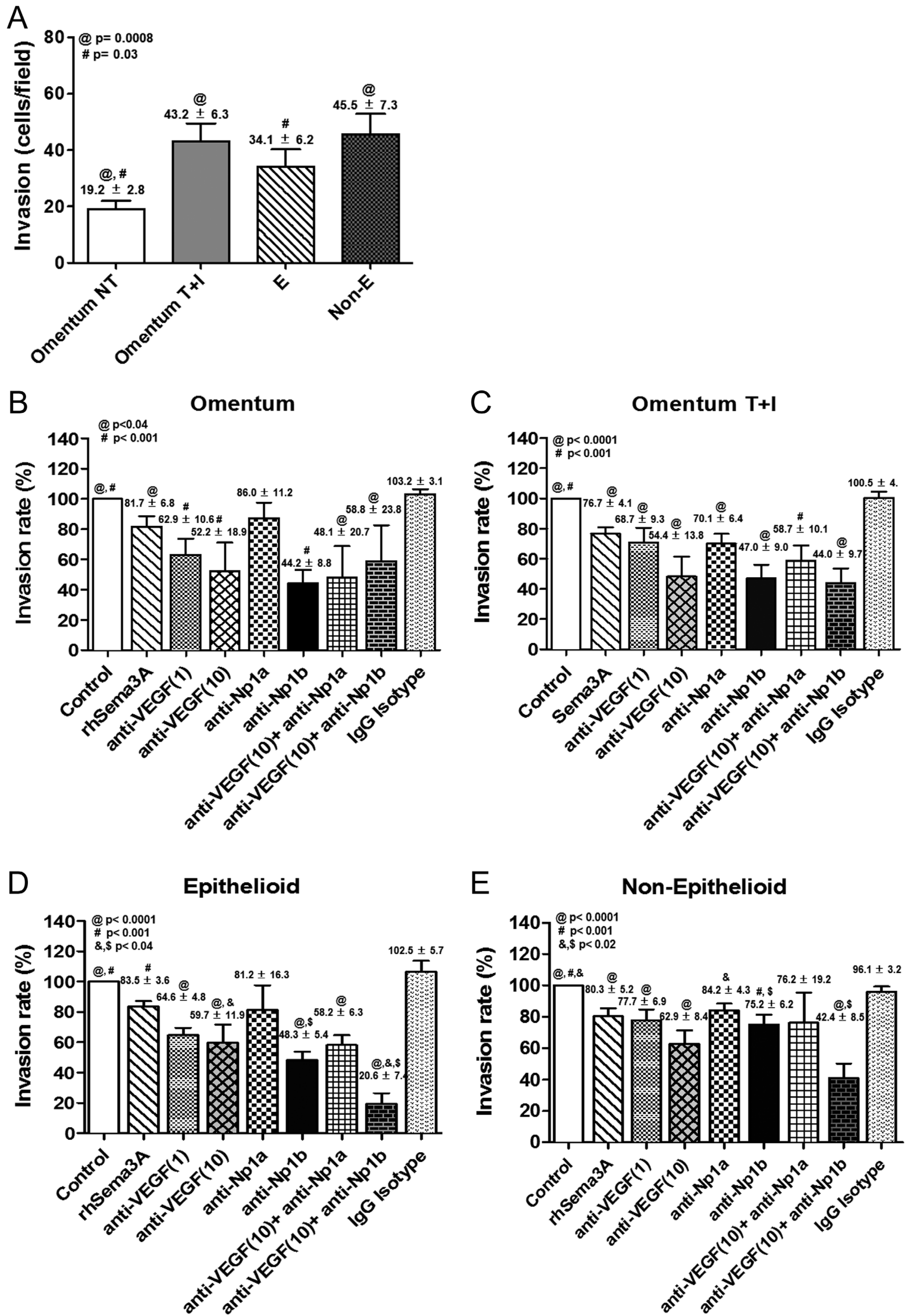

A
Invasion (cells/field)
@ p= 0.0008
# p= 0.03
@, #
19.2 ± 2.8
@
43.2 ± 6.3
#
34.1 ± 6.2
@
45.5 ± 7.3
Omentum NT
Omentum T+I
E
Non-E
B
Omentum
C
Omentum T+I
D
Epithelioid
E
Non-Epithelioid
Invasion rate (%)
Control
rhSema3A
Sema3A
anti-VEGF(1)
anti-VEGF(10)
anti-Np1a
anti-Np1b
anti-VEGF(10)+ anti-Np1a
anti-VEGF(10)+ anti-Np1b
IgG Isotype

Figure 9. Effects of anti-VEGF and anti-Np1b blocking antibodies on the invasion capacity of mesothelial cells. (**A**) The MC invasion capacity throughout *in vitro* and *ex vivo* MMT was analyzed by counting invading cells using a fluorescence microscope (n = 20). Data are depicted as mean value ± SE. Symbols show statistical differences between groups. (**B and E**) Effect of rhSema3A (n = 15), anti-VEGF (n = 10), anti-Np1a (n = 8), anti-Np1b (n = 8) and IgG Isotype antibodies on the invasion capacity of omentum-derived MCs, either untreated (**B**) or treated with TGF-β1 plus IL-1β (**C**); and on the invasion capacity of effluent-derived MCs with epithelioid (**D**) or non-epithelioid (**E**) phenotype. Bar graphics represent invasion percentage of treatments over control cells. Numbers above histograms depict mean ± SE. Symbols show statistical differences between groups.
doi:10.1371/journal.pone.0060776.g009

Effects of VEGF- and Nrp-1-blocking Antibodies on MC Proliferation and Invasion

We hypothesized that VEGF might establish loops in MCs to control key processes, such as proliferation and/or invasion. Our results suggest that the pair VEGF/Nrp-1 appear to gain relevance throughout MMT, given that both are up-regulated during the whole process. Of note, the up-regulated expression of VEGF and Nrp-1 showed a strong correlation (**Figure S2**). Thus, we investigated whether these two molecules played a major role in proliferation and/or invasion of MCs during the MMT process.

Analysis of the proliferation capacity showed that throughout *in vitro* and *ex vivo* MMT there was growth arrest of MCs (**Figure 8A**). Blocking of endogenously produced VEGF with a specific antibody resulted in significant inhibition of the proliferation of omentum MCs (**Figure 8B**). In contrast, the lower proliferation capacity of mesenchymal-like MCs (T+I-treated omentum MCs and Non-E effluent MCs) was not affected, or only marginally affected, by treatment with anti-VEGF (**Figures 8C and 8E**). Surprisingly, the proliferation capacity of effluent MCs with epithelioid phenotype, which was similar to that of omentum MCs, was only slightly affected by the VEGF-blocking antibody (**Figure 8D**). These data suggest that endogenous VEGF only plays a role on cellular proliferation in naïve epithelial-like MCs, but not in MCs at any stage, early or late, of the mesenchymal conversion. Treatment with blocking anti-Np-1b antibody inhibited cellular proliferation to a similar extent (25 to 30%) in MCs with both epithelial and mesenchymal phenotypes **(Figures 8B to 8E**), suggesting that Nrp-1 mediated not only the VEGF-dependent but also the VEGF-independent proliferation response. In this context, anti-Np-1a antibody, which did not interfere in VEGF-Nrp-1 interaction, also partially inhibited the proliferation of mesenchymal-like MCs (data not shown).

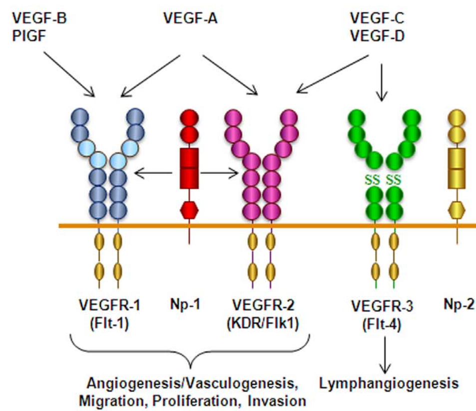
Invasion assays using omentum MCs demonstrated that *in vitro* MMT enhanced the invasion capacity. On the other hand, effluent-derived MCs with both epithelioid and non-epithelioid phenotypes showed increased invasion capacity when compared with omentum-derived MCs (**Figure 9A**). Treatment of omentum MCs with anti-VEGF and anti-Np-1b antibodies significantly inhibited the invasion capacity, and co-treatment with both antibodies had no additive effect (**Figure 9B**). Treatment of MCs that were at any stage of the MMT process including epithelioid MCs from effluents with anti-VEGF and anti-Np-1b antibodies showed also an important significant inhibition of the invasion capacity, and co-treatment with both antibodies had co-operative effects (**Figures 9C to 9E**). Treatment of omentum- and effluent-derived MCs with anti-Np-1a, which did not block the VEGF-Nrp-1 interaction [49], showed partial (**Figures 9B and 9D)** or no statistically significant effects on cellular invasion (**Figures 9C and 9E**). In addition, treatment of MCs with recombinant Sema-3A, which has been shown to compete with VEGF for binding to Nrp-1, resulted in a statistically significant diminished invasion capacity, reinforcing the notion that the interaction of VEGF with Nrp-1 participated, at least partially, in MC invasion (**Figures 9B to 9E**).

Discussion

The presence of MCs that have undergone a MMT in the effluent and in the peritoneal tissue of PD patients was first demonstrated in a landmark paper published in 2003 [4]. MCs with mesenchymal phenotype acquire the capacity to synthesize extracellular matrix components as well as pro-inflammatory and pro-angiogenic factors [2]. During the last few years, emerging evidence has suggested that the mesenchymal conversion of MCs is an important event for structural and functional peritoneal deterioration [7], [10], [64], [65]. In this context, we have previously shown that during the MMT process there is a strong up-regulation of VEGF and that high levels of VEGF production by effluent-derived MCs correlated with high transport rates in PD patients [10]. In this study we show that, not only VEGF expression levels, but also the whole VEGF/VEGFRs/co-receptors axis, is associated with high peritoneal transport status.

Peritoneal fibrosis is the most common structural change observed in PD patients, and it has been considered the main cause for the progressive functional decline of the peritoneum. However, in parallel with fibrosis, the peritoneum may also experiment an increase in capillary number (angiogenesis) in response to PD. Some reports have evidenced that enhancement of peritoneal vasculature and vessel permeability appear to be responsible for an increase in solute transport across the peritoneal membrane [66], [67]. It is generally accepted that local production of VEGF by MCs during PD may exert paracrine effects on endothelial cells to induce peritoneal angiogenesis and functional decline [10], [62], [68], [69]. Furthermore, VEGF has been shown to inhibit endothelial to mesenchymal transition [70], [71]. Therefore, it is conceivable that VEGF secreted by mesothelial cells could exert its function on myofibroblasts with an endothelial origin localized in the compact zone [5], inhibiting the mesenchymal phenotype by a mesenchymal to endothelial transition and, consequently, inducing angiogenesis. However, the possible autocrine effects of VEGF on MCs throughout the MMT process have been overlooked. The expression of VEGFRs and Nrps by MCs has been previously described [37]. Furthermore, it has been shown that the expression pattern of VEGFRs and co-receptors remains unaltered during the development of mesotheliomas [37]. In contrast, herein we show that throughout the mesenchymal conversion of MCs, the expression of the receptors VEGFR-1 and VEGFR-2 is down-regulated, and the expression of the co-receptor Nrp-1 is induced. In addition, we demonstrate that the ratio VEGF/Sema-3A increases sharply during the MMT process. It is intriguing that the main receptor VEGFR2, involved in VEGF-mediated proliferation, is repressed during MMT while the expression of VEGF is strongly induced. It is also noteworthy that the expression of Nrp-1, implicated in multiple cellular functions including invasion, is induced during MMT in parallel with VEGF. In this context, the expression of VEGF shows a significant negative correlation with VEGFR2 and a significant positive correlation with Nrp-1 (**Figure S2**). Interestingly, VEGF and Nrp1 have a very similar distribution in MCs and during MMT, when both proteins seem to internalize. However, VEGFR2 has a different distribution. Thus, it is tempting to speculate that VEGF might have divergent autocrine functions (e.g. proliferation

A



B

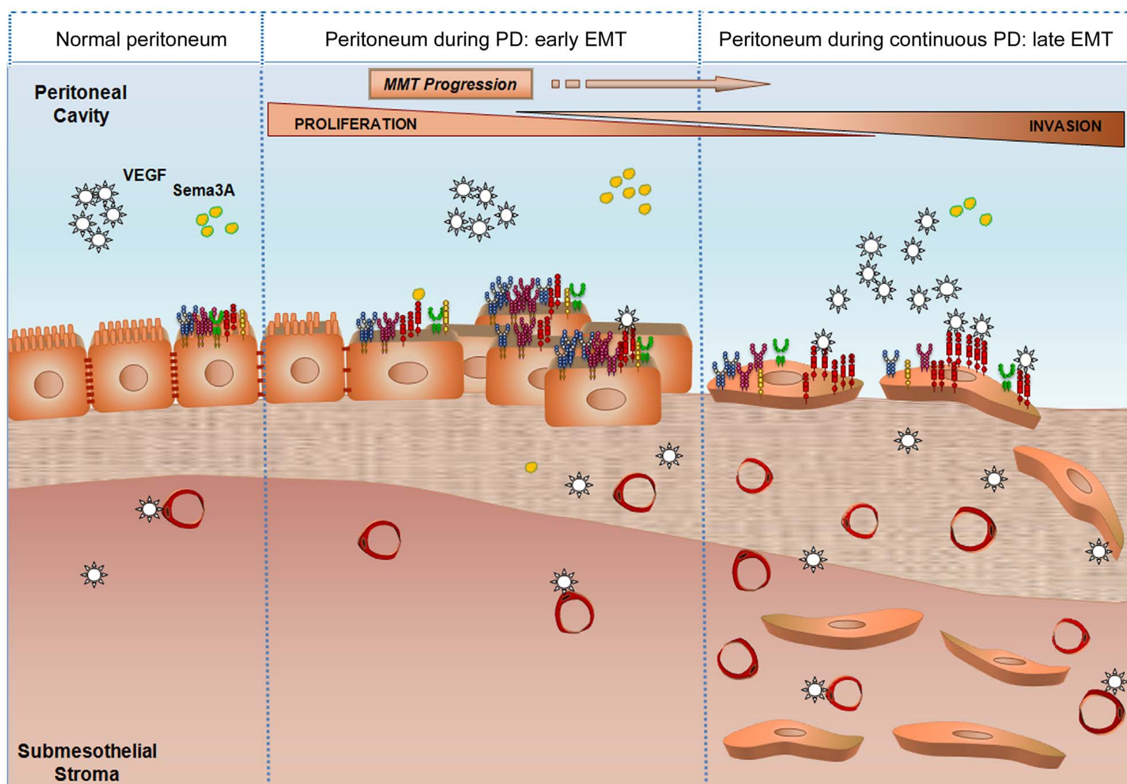


Figure 10. Proposed model of the role of VEGF receptors and co-receptors during MMT induced by peritoneal dialysis. (**A**) General scheme of VEGF receptors and co-receptors and the processes in which they are involved. (**B**) A normal peritoneum shows baseline VEGF receptors and co-receptors expression, and normal VEGF and Sema-3A levels are secreted at the peritoneal cavity. During continuous peritoneal dialysis, MMT takes place in the peritoneum. Denudation of MC monolayer, submesothelial fibrosis, and augmented vessel number begin to appear as visible signs as a result of the MMT. In addition, MCs change the expression pattern of VEGFRs and co-receptors. At an initial stage, a higher proliferation rate of MCs takes place, perhaps in order to repair the damage in the monolayer. However, MCs could fail to repair the peritoneal damage and MMT progress. As a result of MMT progression, MCs increase secreted VEGF levels while its receptors are down-regulated and Nrp-1 co-receptor increases at late stages of the MMT. The binding of VEGF to Nrp-1 would induce MC invasion to the submesothelial stroma. Therefore, MMT would determine MC behaviour in terms of proliferation and invasion in response to VEGF.
doi:10.1371/journal.pone.0060776.g010

vs. invasion) on MCs with either epithelial or mesenchymal phenotype (**Figure 10**).

In this study, we demonstrate that MCs that have undergone an *in vitro* or *ex vivo* MMT proliferate less than epithelial-like MCs. These data are in agreement with previous results that demonstrated that during EMT, there is an arrest of the cell cycle and the cells acquire resistance to death [72]. Furthermore, we show that blockade of endogenous VEGF inhibits the proliferation of omentum MCs. Surprisingly, the proliferation capacity of epithelioid MCs from effluents is only marginally affected by VEGF-blocking antibody, in spite of producing similar amounts of VEGF and expressing even greater levels of VEGFR2 than omentum MCs. It is noteworthy that effluent MCs, still retaining an epithelioid appearance, already show down-regulated expression of E-cadherin and induction of snail, suggesting that these cells are already in early stages of the MMT process [4] (**Figure 1**). Thus, our results indicate that endogenous VEGF plays a key role in cellular proliferation in naïve epithelial-like MCs, but not in MCs undergoing the MMT process. On the contrary, we demonstrate that MCs at any stage of the mesenchymal conversion acquire an increased invasion capacity compared with naïve epithelial-like MCs. In addition, we show that the enhanced invasion can be partially inhibited by treatment with anti-VEGF or anti-Np-1b antibodies, and is almost completely abrogated by a combination of both antibodies. These results strongly suggest that the interaction of VEGF with Nrp-1 may have a role in MC invasion (**Figure 10**).

Perhaps the most important aspect of the identification of the MMT as a key event in peritoneal deterioration is that this process can be modulated *in vivo* with a number of endogenous factors and pharmaceutical agents [2]. In recent works, therapeutic strategies were designed either to prevent or reverse the MMT process itself or to reduce the MMT-inducing stimuli such as inflammation and AGEs [7], [11], [12], [73–75]. It is important to point out that MMT is a physiologic process necessary for wound healing during PD-induced aggression of the peritoneum; therefore, it is plausible that chronic blockade of MMT would result in inefficient tissue repair. Thus, alternative therapeutic approaches should be addressed to treat the consequences of the MMT, instead of the MMT process itself. One of such consequences of MMT is the increased cellular invasion. The identification of VEGF and Nrp-1 as important molecules controlling MC invasion might contribute to the design of therapeutic approaches to avoid the accumulation of MC-derived myofibroblasts in the submesothelial compact zone (**Figure 10**).

Supporting Information

Figure S1 Effect on VEGF receptors and co-receptors after TGF-β1 and IL-1β removal. (A–C) mRNA levels of VEGF receptors/co-receptors were analyzed by quantitative RT-PCR. The results represent the fold induction of mRNA expression of VEGF receptors in omentum-derived MCs treated with TGF-β1 plus IL-1 β (T+I 72 h) and omentum-derived MCs after T+I withdrawal (T+I 72 h/24 h wo) compared with untreated MCs (NT). **(D–F)** mRNA levels of mesenchymal markers were analyzed by quantitative RT-PCR. Histograms represent the expression of E-cadherin, collagen I and fibronectin after the treatment with T+I (T+I 72 h) and after T+I withdrawal (T+I 72 h/24 h wo) compared to non treated omentum-derived MCs (NT).
(TIF)

Figure S2 Correlations between secreted levels of VEGF and mRNA levels in effluent-derived MCs. (A) Negative correlation between VEGF levels and VEGFR-2 mRNA expression levels ($p = 0.004$). **(B)** Negative correlation between VEGF levels and Sema-3A mRNA expression ($p = 0.04$). **(C)** Positive correlation between secreted VEGF and Nrp1 mRNA expression levels ($p < 0.0001$). Data are depicted as mean value ± SE. Symbols show statistical differences between groups.
(TIF)

Table S1 Oligonucleotides Sequences.
(DOC)

Table S2 Distribution of MCs Phenotype According to Peritoneal Transport Rate.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MLPL MLC. Performed the experiments: MLPL PS ARV PA JAJH. Analyzed the data: MLPL AA PLM. Contributed reagents/materials/analysis tools: JAST RS PLM. Wrote the paper: MLPL MLC.

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DISCUSIÓN

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El peritoneo sufre diversas modificaciones estructurales durante el tratamiento de diálisis peritoneal, que conllevan una disfunción peritoneal progresiva y a un fallo de la ultrafiltración. La fibrosis constituye una de las causas principales, y la presencia de CMs en el efluente y en el tejido peritoneal que han sufrido una MMT contribuyen en gran parte a dicha fibrosis, debido a su capacidad de sintetizar componentes de la matriz extracelular (Yañez-Mo M et al., 2003; Aroeira L et al., 2007). Además, el peritoneo también muestra un aumento de capilares sanguíneos y un aumento de la permeabilidad vascular, que son responsables en gran medida de una alteración en el transporte de solutos a través de la membrana y, por tanto, del fallo de la ultrafiltración (Williams JD et al., 2002; Numata M et al., 2003). Se sabe que las CMs secretan VEGF *in vitro* en respuesta a una gran variedad de estímulos, tales como GDPs, AGEs, TGF-β1 y líquidos de diálisis (Zweers MM et al., 2001; Mandl-Weber S et al., 2002; 143. Gary Lee YC et al., 2002; Ha H et al., 2002). Las CMs también secretan VEGF de forma espontánea durante la diálisis peritoneal (Selgas R et al., 2000; Selgas R et al., 2001); sin embargo, los mecanismos subyacentes a esta producción de VEGF no estaban bien esclarecidos.

En este trabajo de investigación hemos demostrado que el mecanismo implicado en el aumento de la secreción de VEGF por parte de las CMs es el proceso de MMT que sufren durante la terapia de diálisis peritoneal. De este modo, se observó que los pacientes en diálisis peritoneal que drenaron CMs no epitelioides en sus efluentes mostraron niveles séricos de VEGF significativamente mayores que aquellos pacientes que drenaron CMs epitelioides. Estos niveles séricos correlacionaron de manera directa con las concentraciones de VEGF detectadas en los cultivos celulares, donde las CMs no epitelioides aisladas secretaron también grandes cantidades de VEGF. Estos primeros resultados obtenidos sugieren que las CMs constituyen una fuente importante de VEGF en pacientes en diálisis peritoneal, que deben participar en el mantenimiento de la angiogénesis peritoneal y, consecuentemente, en el funcionamiento de la membrana peritoneal. El estado funcional de la membrana peritoneal suele determinarse midiendo la capacidad de ultrafiltración y los niveles de MTC-Cr. Considerando estos parámetros clínicos, se establecieron correlaciones entre éstos y los niveles de VEGF secretados por las CMs *ex vivo*. Como resultado observamos una correlación estrecha y directa entre los niveles de VEGF secretado y el coeficiente MTC-Cr. Sin embargo, en estudios previos encontraron una débil asociación entre niveles de VEGF en el efluente de pacientes en diálisis peritoneal y el coeficiente de transporte de solutos, a diferencia de la encontrada en este estudio, que es fuerte. No obstante, tales discrepancias pueden ser debidas a la variabilidad de factores existentes entre pacientes, como por ejemplo la presencia de enfermedades cardiovasculares en pacientes urémicos, que pueden influir en las variaciones séricas de VEGF (Pecoits-Filho R et a., 2002; Nakajima K et al., 2004). En las CMs cultivadas *ex vivo* no hay factores externos o adicionales que puedan alterar la secreción de VEGF, ya que esta secreción se mantiene estable durante el cultivo y no hay factores externos fisiológicos que puedan estar afectando a las células, lo que apoya fuertemente la implicación del VEGF secretado por las CMs en el deterioro peritoneal. Además, los análisis inmunohistoquímicos de biopsias de pacientes en diálisis peritoneal mostraron un cambio de localización de las CMs desde la superficie mesotelial hacia el estroma submesotelial, quizás debido

al aumento de su capacidad migratoria e invasiva. Las células embebidas en el estroma submesotelial mostraron una mayor expresión de VEGF en aquellos pacientes con un índice alto de MTC-Cr (altos transportadores) en comparación con aquellos considerados como bajos transportadores. Este hallazgo implicaría que una de las principales fuentes de VEGF debe encontrarse atrapada en la matriz extracelular, donde podría actuar de manera paracrina sobre las células endoteliales adyacentes provocando una inducción de la angiogénesis y un aumento de la permeabilidad vascular. Sólo una pequeña parte de este VEGF escaparía al efluente o a los compartimentos circundantes. Quizás esto explicaría la fuerte correlación directa encontrada entre la tasa de transporte peritoneal y los niveles de VEGF *in vivo* o *ex vivo*. Estos datos refuerzan la hipótesis de la implicación de la alta secreción de VEGF por parte de las MCs en el daño peritoneal.

El hecho de que la MMT de las CMs suponga un proceso clave en la fibrosis y la angiogénesis peritoneal nos abre nuevas estrategias de investigación. Por consiguiente, un seguimiento de los niveles de VEGF producido por las CMs drenadas en los efluentes de los pacientes en diálisis peritoneal podría servir como marcador de la evolución de la función peritoneal. Sería interesante estudiar si existen diferencias en la producción de VEGF entre pacientes que usan líquidos de diálisis diferente, para lo cual sería necesario realizar un estudio comparativo entre las soluciones de diálisis peritoneal clásicas y nuevas, en términos de biocompatibilidad, empleando los niveles de VEGF como posible marcador de diagnóstico y/o pronóstico de la función peritoneal en un estudio longitudinal.

El siguiente paso a desarrollar consistió en investigar el efecto de nuevos líquidos de diálisis más biocompatibles sobre la MMT de las CMs en un estudio longitudinal a largo plazo. En este estudio investigamos el efecto de dos nuevos líquidos con bajo contenido en GDPs y de un líquido convencional (estándar, de alto contenido en GDPs). Estos dos nuevos líquidos biocompatibles son Balance y Bicavera. El estudio comparativo aportó datos muy interesantes relativos al efecto de los líquidos sobre la MMT y, por consiguiente, sobre el VEGF secretado por las CMs.

En una primera parte de este estudio, se evaluó el grado de MMT hallados *in vitro* y *ex vivo*, comparando las soluciones de diálisis Balance y estándar. Como resultado observamos que la exposición de las CMs al líquido Balance, con bajo contenido en GDPs, se asocia a una mejor preservación de la morfología epitelial. Por el contrario, la exposición mesotelial al líquido estándar, con alto contenido en GDPs, se asoció con un aumento en la secreción de los factores normalmente inducidos en una conversión mesenquimal. *In vitro* se observó una progresiva disminución de E-cadherina tras tratar las CMs de omento con el líquido estándar o con TGF-β1. Esta observación puede ser debida a que la exposición de CMs de omento a líquidos estándar induce la expresión de TGF-β1, el cual está considerado como un importante inductor de la MMT (Loureiro J et al., 2010). Por el contrario, la exposición al líquido Balance mantuvo la expresión de E-cadherina así como el fenotipo epitelial de las células. El estudio *ex vivo* dio lugar a la observación de resultados similares. A corto plazo(al inicio y a los 6 meses) el efecto de ambos líquidos sobre las CMs fue parecido, mostrando similares niveles de E-cadherina. Sin embargo, a medio-largo plazo (12 y 18 meses) los niveles de E-cadherina y la frecuencia del fenotipo epitelial fueron mayores en el grupo Balance. Además, se analizaron otras moléculas asociadas a la MMT, como colágeno I, fibronectina, IL-8 y TGF-β. El resultado del análisis mostró una menor inducción de estas moléculas en el grupo con

líquido Balance en comparación con el grupo estándar. IL-8 es una citoquina proinflamatoria e inductora de la migración celular cuya expresión se induce en respuesta a GDPs y a líquidos de diálisis estándar (Welten AG et al., 2003; Bates RC et al., 2004; Bender TO et al., 2007), lo que favorecería el deterioro de la membrana peritoneal. TGF- β1, que es un factor de crecimiento implicado en la disminución de la proliferación de las CMs y en la fibrosis peritoneal, no mostró ninguna correlación con la morfología celular; sin embargo, sí observamos diferencias en los niveles secretados de TGF-β1 cuando comparamos globalmente los grupos de líquidos (Border EA y Noble NA, 1994; Ha H et al., 2001; Wong TY et al., 2003).

Una de las metas en este estudio incluía el análisis de VEGF como posible marcador de la función peritoneal. En este sentido, el análisis de los niveles de VEGF en sobrenadante de los cultivos de MCs *in vitro* y *ex vivo* dio lugar a la observación de una fuerte secreción de este factor de crecimiento durante el proceso de MMT (Aroeira L et al., 2005; López-Cabrera M et al., 2006). Además, observamos que los niveles de VEGF estaban muy aumentados en el grupo estándar en comparación con el grupo Balance, acentuándose esta diferencia a lo largo del tiempo. Estos datos, sumados al hecho que VEGF es, al menos en parte, responsable de un aumento de angiogénesis y del transporte peritoneal, sugieren que VEGF debe ser un factor clave en la función peritoneal (Krediet RT et al, 2000; Aroeira L et al., 2005). Los resultados obtenidos en este estudio están en concordancia con aquellos publicados recientemente, tanto en humanos como en modelos de rata, los cuales demuestran que diferentes tipos de GDPs contenidos en líquidos de diálisis estándar inducen una MMT y que los líquidos con menos GDPs provocan un menor impacto en la MMT y, en consecuencia, en la fibrosis peritoneal (Do JY et al., 2005; Do JY et al., 2008; Oh EJ et al., 2010).

Tras el estudio de la asociación de la MMT con los líquidos de diálisis peritoneal con alto y bajo contenido en GDPs, nos planteamos la cuestión de si los niveles de estos marcadores podrían estar influenciados por episodios de peritonitis. Realizamos un estudio comparativo de estos marcadores entre pacientes con y sin peritonitis, y no encontramos diferencias significativas. Sin embargo, los pacientes tratados con líquido estándar que no sufrieron peritonitis mantuvieron la morfología no epitelioide y la alta secreción de VEGF. De la misma forma, los pacientes tratados con Balance, y que sí sufrieron episodios de peritonitis, preservaron el fenotipo epitelioide y la baja producción de VEGF. Estas observaciones sugieren que el líquido Balance confiere una protección adicional frente los efectos de una posible inflamación.

En este estudio hemos confirmado que la solución de diálisis Balance con bajo contenido en GDPs, protege y/o no induce el desarrollo de una MMT debido a la diálisis peritoneal a medio y largo plazo, mejorando por tanto la preservación de la membrana peritoneal. No obstante, sería necesaria la realización de un estudio más profundo, con un mayor número de pacientes y a tiempos más prolongados que 24 meses.

Continuando con este trabajo, nos centramos en la segunda parte del estudio de los líquidos, basada en investigar el efecto de la solución de diálisis BicaVera (con solución tampón bicarbonato y con bajo contenido en GDPs). Este estudio dio lugar a resultados consistentes con la preservación de un fenotipo epitelioide *in vitro* y *ex vivo*, al igual que los resultados observados en el estudio anterior con el líquido bajo en contenido de GDPs, Balance. El tratamiento *in vitro* con el líquido BicaVera

apenas afectó al fenotipo mesotelial y a la expresión de E-cadherina. Estos resultados, junto a los ya obtenidos en el estudio del líquido Balance, demuestran que el contenido en GDPs en de los líquidos de diálisis ejercen un papel crucial en la inducción de una MMT en las CMs. *Ex vivo*, el análisis de los marcadores de MMT dieron lugar a la observación del aumento de VEGF, fibronectina y colágeno I, y la reducción de E-cadherina, en aquellas células con morfología no epitelioide. Sin embargo, cuando se analizaron estos marcadores comparando los grupos de líquidos, no se observaron diferencias concluyentes. Sólo se encontraron diferencias en los niveles de IL-8, que fueron significativamente mayores en el grupo tratado con líquido estándar. Este resultado indica que el grupo estándar sufre un estado pro-inflamatorio debido a los GDPs. El hecho de que no encontráramos diferencias significativas entre grupos con los marcadores de MMT, fue debida a la inesperada aparición, en el grupo BicaVera en el inicio de la diálisis, de un mayor porcentaje de CMs con morfología no epitelioide. No obstante, a pesar de ello, la exposición crónica a BicaVera mostró la adquisición de un fenotipo epitelioide a lo largo del tiempo; a diferencia del grupo estándar, que pasó a un fenotipo no epitelioide. Estas observaciones sugieren que el líquido BicaVera protege a largo plazo a las CMs, fomentando un fenotipo epitelioide. No tenemos una explicación para las observaciones sobre el fenotipo no epitelioide al inicio de la diálisis con Bicavera, sin embargo, podría ser que se diese esta situación porque las células se encuentran en un estado intermedio de transdiferenciación, en un estado reparativo, lo que se describe como EMT tipo I (Kalluri R y Weinberg RA, 2009). Nos basamos en esta suposición al comparar las CMs no epitelioides del grupo BicaVera al inicio de la diálisis y a los 18 meses, encontrando que los niveles de fibronectina al inicio de la diálisis son menores que los observados a los 18 meses. Esta diferencia a lo largo del tiempo representa diferentes estados funcionales bajo la misma morfología: CMs no epitelioides en estadío temprano reparativo y CMs no epitelioides en estadío tardío pro-fibrótico .

En cuanto al estudio de función peritoneal, observamos una menor capacidad de ultrafiltración y un mayor transporte de pequeños solutos en grupo de BicaVera, confirmando los datos previos obtenidos con líquidos biocompatibles. Al igual que en el estudio anterior con Balance, los episodios de peritonitis no causaron diferencias significativas en los marcadores de MMT. Sin embargo, los pacientes que fueron tratados con BivaVera y sufrieron peritonitis preservaron el fenotipo epitelioide a lo largo del tiempo. Para obtener respuestas concluyentes sobre los efectos específicos de BicaVera sobre la MMT de las CMs, deben realizarse un estudio con un mayor número de muestras para la obtención de respuestas concluyentes.

Hemos visto que el uso del líquido BicaVera induce la preservación de un fenotipo epitelioide, y por tanto, una menor conversión de las CMs, a largo plazo. Sin embargo, un estudio más amplio sería necesario para obtener una mayor información sobre los datos inesperados. Lo único que pensamos que ha podido afectar en nuestro estudio es la diabetes. En la pre-diálisis peritoneal, el peritoneo de los pacientes con diabetes muestra un aumento del ratio del diámetro del lúmen de los vasos en comparación con pacientes no diabéticos. Está descrito que la uremia y la diabetes tiene un impacto significativo sobre la patogénesis de la esclerosis peritoneal en el peritoneo durante la pre-diálisis peritoneal. Quizás el hecho que en el grupo BicaVera haya un mayor porcentaje de pacientes con diabetes, penalice este grupo en el inicio del tratamiento (Honda K et al., 2008).

Nuestros resultados sugieren que las CMs no epitelioides, provenientes de la conversión mesenquimal de las CMs durante las respuestas de reparación del tejido peritoneal a daños (daños mecánicos, efectos de líquidos bioincompatibles, etc.) contribuyen a la fibrosis, angiogénesis y la permeabilidad vascular, que conllevan al fallo de la ultrafiltración. Por consiguiente, los posibles tratamientos deberían estar diseñados hacia el bloqueo de la MMT y/o disminuir sus inductores como inflamacion y AGEs, o en su defecto, de sus efectos perjudiciales, tales como la síntesis de componentes de la matriz extracelular y/o de la secreción de VEGF. En recientes trabajos se desarrollaron estrategias para bloquear la MMT y sus efectos, como la secreción de COX-2, la inflamación y la producción de AGEs (Strippoli et al., 2008; Yu MA et al., 2009; Aroeira L et al., 2009; Kihm LP et al., 2011; Sandoval P et al., 2010;, Loureiro J et al., 2010; Loueiro et al., 2011, Loureiro et al., 2013; Strippoli et al., 2012). Además, también se usan actualmente líquidos biocompatibles con bajo contenido en GDPs y un pH más neutro, que como hemos visto en nuestros estudios, reduce la MMT de las CMs a largo plazo. Sin embargo, el desarrollo de estrategias frente a la secreción de VEGF no está establecido, y ello requiere un estudio en profundidad del eje VEGF/VEGFRs/CMs.

Está aceptado de forma general que la producción local de VEGF por parte de las CMs durante la diálisis peritoneal puede tener un efecto paracrino sobre las células endoteliales induciendo un aumento de la angiogénesis y de la permeabilidad vascular, conllevando al declive de la función peritoneal (Zweers MM et al., 1999; Selgas R et al., 2000; Pecoits-Filho R et al., 2002; Boulanger E at al., 2007). Sin embargo el efecto autocrino de VEGF sobre las CMs durante la MMT no se había estudiado. Catatalo A et al. describieron que los receptores (VEGFRs) y co-receptores (Nrps) de VEGF en CMs de pleura, se expresaban de forma constitutiva, y que sus expresiones no mostraban alteraciones cuando las células adquirían características de malignidad. Nosotros hemos realizado el estudio de la expresión de estos receptores en las CMs peritoneales durante el proceso de MMT inducido por diálisis peritoneal. Observamos que la conversión mesenquimal de las CMs produce una disminución de la expresión de receptores VEGFR-1 y VEGFR-2, y una inducción de la expresión del co-receptor Nrp-1. Además, observamos que la expresión de Sema-3A, un competidor funcional de VEGF por la unión a Nrp-1, está también disminuida, produciéndose un considerable aumento del ratio VEGF/Sema-3A durante el proceso de MMT, lo cual favorece posible acción de VEGF. Es sorprendente que el principal receptor de VEGF, VEGFR-2, implicado principalmente en proliferación celular, esté reprimido durante la MMT. VEGF es fuertemente inducida; y que la expresión de Nrp-1, co-receptor implicado en funciones como la invasión celular, está inducida durante la MMT. Además, encontramos que los niveles de expresión de VEGF muestran una correlación negativa con los niveles de VEGFR-2 y una correlación positiva con los niveles de Nrp-1. Fue también intrigante encontrar que VEGF y Nrp-1 muestran una distribución similar en las CMs antes y durante la MMT, produciéndose una internalización de las mismas; mientras que VEGFR-2 muestra una localización diferente. Estos datos sugieren que VEGF ejerce funciones autocrinas en las CMs durante el proceso de MMT.

En este estudio, demostramos que las CMs que han sufrido una MMT, *in vitro* o *ex vivo*, muestran una menor tasa de proliferación que aquellas con fenotipo tipo epitelial. Estos datos están en

concordancia con resultados previos, donde se observa una detención del crecimiento y un aumento de la supervivencia celular (Vega S et al., 2004). Cuando bloqueamos el VEGF endógeno se produjo una inhibición de la proliferación de las CMs de omento, sin embargo, la proliferación de las CMs epitelioides de efluentes sólo fue marginalmente aceptada por el bloqueo de VEGF, a pesar de producir cantidades similares de VEGF y expresar mayores niveles de VEGFR-2 que las CMs de omento. Quizás sea debido a que estas células, a pesar de mantener el fenotipo epitelial, muestran ya una disminución de las expresión de E-cadherina y una inducción de snail, lo que sugiere que están en un estado inicial de MMT (Yáñez-Mo M et al., 2003). Por lo tanto, VEGF sólo muestra jugar un papel clave en la proliferación celular en las CMs nativas, sin que hayan sufrido un proceso de MMT. Por el contrario, las CMs que han sufrido una MMT adquieren una mayor capacidad invasiva, la cual es parcialmente inhibida tras el tratamiento con anticuerpos bloqueantes frente a VEGF y Nrp-1; siendo casi totalmente bloqueada con la combinación de ambos anticuerpos. Estos resultados sugieren en gran medida que la interacción de VEGF con Nrp-1 juega un importante papel en la invasión de las CMs. Sin embargo, el bloqueo de Nrp-1 produjo una mayor disminución de la capacidad invasiva que con el bloqueo VEGF, lo que sugiere que, además de VEGF, otras moléculas podrían estar implicadas en esta capacidad invasiva.

En este estudio, hemos demostrado que durante la MMT cambia el patrón de expresión de los receptores y co-receptores de VEGF, lo que conlleva a cambios en las funciones de las CMs en respuesta a VEGF, favoreciendo la fibrosis, angiogénesis, y consecuentemente, fallo de membrana. Es importante señalar que la MMT es un proceso necesario para reparar el daño que sufre el peritoneo durante la diálisis peritoneal; por ello es posible pensar que un bloqueo estable de la MMT provoque una reparación tisular ineficiente. Por tanto, quizás las aproximaciones terapéuticas deberían estar dirigidas al tratamiento de las consecuencias de la MMT. La identificación de VEGF y Nrp-1 como moléculas importantes en la capacidad invasiva de las células, establece nuevas bases para el desarrollo de nuevas estrategias usando estas moléculas como posibles dianas terapéuticas. De este modo, se podría evitar la acumulación de miofibroblastos derivados de CMs en la zona submesotelial, lo que podría reducir tanto la fibrosis, como la neoangiogénesis y el aumento de la permeabilidad vascular, y por tanto, reducir la posibilidad de un fallo de la ultrafiltración.

CONCLUSIONES

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1. Las CMs que han sufrido una MMT secretan altos niveles de VEGF, constituyendo la principal fuente de VEGF en los pacientes en terapia de DP, contribuyendo a la fibrosis, neoangiogénesis y aumento de la permeabilidad vascular.

2. El VEGF secretado por las CMs transdiferenciadas es el responsable del alta tasa de transporte peritoneal; por lo tanto el seguimiento del VEGF producido por las CMs podría servir como marcador para evaluar la funcionalidad de la membrana peritoneal en pacientes en DP.

3. Los líquidos o soluciones de diálisis con bajo contenido en GDPs inducen una menor MMT en pacientes en DP, que los líquidos estándar, y muestran una tendencia a la adquisición de una morfología epitelioide.

4. Las CMs sufren un cambio en el patrón de expresión de los receptores VEGFR-1, VEGFR-2 y coreceptor Nrp-1 durante el proceso de MMT inducido por DP. Además, también se reduce la expresión de Sema-3A, que es un competidor funcional de VEGF.

5. En paralelo a la alteración de la expresión de los receptores y co-receptores de VEGF durante la MMT se produce una disminución de la capacidad proliferativa y un aumento de la capacidad invasiva, a medida que progresa la MMT.

6. VEGF y Nrp-1 muestran una localización similar antes y después de la MMT; y su bloqueo induce una fuerte reducción de la invasividad de las células, sugiriendo un papel importante de ambas moléculas durante la MMT de las CMs.

7. La identificación de VEGF y Nrp-1 como moléculas clave en el control de la invasividad de las CMs, permite el diseño de nuevas estrategias para evitar la acumulación de miofibroblastos en el estroma submesotelial, reduciendo de este modo la fibrosis, neoangiogénesis y permeabilidad vascular que conllevan al fracaso de UF.

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ANEXO

Durante el desarrollo de esta tesis se han producido colaboraciones que han dado lugar a las siguientes publicaciones:

1. Pilar Sandoval, Jose Antonio Jiménez-Hefferman, Ángela Rynne-Vidal, María Luisa Pérez-Lozano, Álvaro Gilsanz, Vicente Ruiz-Carpio, Raquel Reyes, Julio García-Bordas, Konstantinos Stamatakis, Javier Dotor, Manuel Fresno, Carlos Cabañas, Manuel López-Cabrera. "Carcinoma-associated fibroblasts derive from mesothelial cells via mesothelial to mesenchymal transition in peritoneal metastasis". Under review

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