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Mesothelial RAGE activation by AGEs enhances VEGF release and potentiates capillary tube formation

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Advanced glycation end-products (AGEs) inhibit ischemia-induced angiogenesis but are potential triggers of neoangiogenesis that occurs in peritoneal dialysis (PD) patients. We investigated whether the effect of glucose and AGEs on human peritoneal mesothelial cells (HPMCs) might alter the release of vascular endothelial growth factor (VEGF) and subsequently the formation of capillary tubes by human umbilical vein endothelial cells (HUVECs). HPMCs were exposed to glucose and the glycated protein *Nε*-(carboxymethyl)lysine-human serum albumin (CML-HSA) and VEGF production was measured by reverse transcription-polymerase chain reaction and enzyme-linked immunosorbent assay. Capillary tube formation by HUVECs in presence of HPMC supernatant or co-cultured with HPMC was investigated. AGE and VEGF levels in PD effluents from 11 patients were measured. CML-HSA stimulated VEGF production by HPMCs, $P<0.001$. Glucose and AGE inhibited capillary tube formation by HUVECs, $P<0.001$. HPMC supernatant potentiated capillary tube formation, $P<0.001$. In co-culture with HPMC capillary tube formation was increased, especially by HPMCs stimulated by CML-HSA, $P<0.001$. Anti-VEGF antibody limited this effect, $P<0.001$. Preincubation of HPMCs with anti-receptor for AGEs (RAGE) antibody reduced capillary tube formation, $P<0.001$. AGE and VEGF levels in PD effluents were increased during long dwell time, $P<0.05$ and $P<0.001$, respectively. In a co-culture system, we showed that VEGF production by HPMC favors capillary tube formation through mesothelial RAGE activation and could explain neoangiogenesis in PD patient.

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The treatment of end-stage renal failure by peritoneal dialysis (PD) is associated with a progressive loss of efficacy owing to increased membrane permeability and ultrafiltration failure.[1,2] Fibrosis of the submesothelial layer and neoangiogenesis are observed.[2,3] Peritoneal exposure to PD fluids (PDFs), especially conventional poorly biocompatible PDFs which combine non-physiological pH, high glucose concentrations, and high levels of glucose degradation products (GDPs), is associated with an elevated incidence of peritoneal alterations.[1,4] Heat sterilization of PDFs induces the formation of GDPs such as glyoxal, methylglyoxal, 3-deoxyglucosone, and 3,4-dideoxyglucosone-3-ene which are involved in the formation of advanced glycation end-products (AGEs).[4–6] Glucose, GDPs, and AGEs have distinct and synergistic toxic effects on human peritoneal mesothelial cells (HPMCs), reducing cell proliferation and inducing apoptosis.[7] The binding of AGEs to the receptor for AGEs (RAGE) activates HPMCs and potentiates vascular cell adhesion molecule expression and leukocyte adhesion.[8]

Reduction in ultrafiltration capacity is related to vascular modifications which occur after repeated peritoneal exposure to PDFs, the consequence of a complex interaction between cytokines and vasoactive factors.[3,9] Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor which is involved in endothelial cell proliferation and vascular permeability.[10] Local VEGF production during PD plays a key role together with other factors such as transforming growth factor-β1 in a process that leads to the development of peritoneal neoangiogenesis and fibrosis.[11,12]

In patients with diabetes mellitus, N^{ε}-(carboxymethyl) lysine-human serum albumin (CML-HSA), a high-affinity ligand for RAGE, is potentially involved in the development of microvascular complications.[13] High AGE concentrations observed in patients with this disease also amplify extracellular matrix degradation by metalloproteinases, and may limit the development of neoangiogenesis secondary to ischemia.[14] The relationship between neovascularisation, glucose, and AGEs is not obvious as high glucose concentrations and AGEs are factors that are known to inhibit angiogenesis. In the present work, we hypothesized that mesothelial cells, which undergo alterations following

exposure to PDFs might deliver a message to endothelial cells, which could result in neoangiogenesis. Capillary tube formation by human umbilical vein endothelial cells (HUVECs) was inhibited by high glucose concentrations and AGEs (CML-HSA) in Matrigel but the supernatant of HPMCs enhanced capillary tube formation. The capillary tube formation was potentiated when HPMCs were stimulated by CML-HSA and co-cultured with HUVECs. We explored whether VEGF secreted by HPMCs after mesothelial RAGE activation could result in capillary tube formation stimulation.

RESULTS

CML-HSA enhances VEGF mRNA and protein production in a time- and concentration-dependent manner

In HPMCs, maximum VEGF messenger RNA (mRNA) expression and protein production were reached with 2.5 and 5 nmol/ml CML-HSA concentration, respectively (Figure 1a). HPMCs produced VEGF protein under basal conditions (171.7 ± 16.5 pg/1 $\times 10^3$ HPMCs). CML-HSA enhanced VEGF mRNA expression and protein production by HPMCs in a concentration but also in a time-dependent manner. With the addition of 5 nmol/ml CML-HSA, optimal protein production response was reached after a 40 h period of stimulation (281.0 ± 12.4 pg/1 $\times 10^3$ HPMCs; $P < 0.001$) (Figure 1b). No significant modification of VEGF protein production was induced by a higher glucose concentration, that is, 15 g/l (151.8 ± 13.5 pg/1 $\times 10^3$ HPMCs). CML-HSA enhanced the production of VEGF when associated with glucose 15 g/l (311.8 ± 66.3 pg/1 $\times 10^3$ HPMCs; $P < 0.01$) (Figure 1c).

As maximum VEGF protein production was achieved with 5 nmol/ml CML–HSA, this concentration was used for the following experiments.

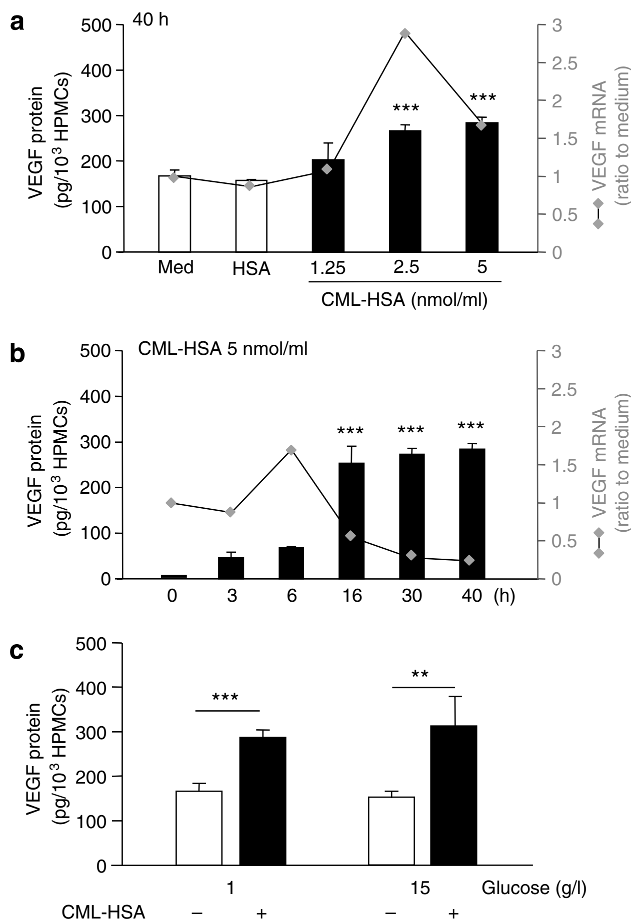

Figure 1 | Mesothelial cell VEGF mRNA expression and protein production are stimulated by CML-HSA in a (a) concentration and **(b)** time-dependent manner. HPMCs were cultured in presence or absence CML-HSA (1.25–5 nmol/ml) during 3–40 h. VEGF mRNA expression in HPMCs was assessed by real-time reverse transcription-polymerase chain reaction (curve). VEGF protein production was assessed by ELISA in culture supernatants (bars). (**c**) CML-HSA in presence of glucose (15 g/l) stimulates VEGF protein production. Results are expressed as the mean $\pm$ s.e.m. of nine determinations (three experiments performed in triplicate) (**$P < 0.01$; ***$P < 0.001$).

Endothelial cell VEGF receptor 1 and 2 regulation by CML-HSA

When HUVECs were incubated (glucose 1 g/l) with CML-HSA, endothelial vascular endothelial growth factor receptor (VEGFR)-1 protein expression was significantly enhanced (2.8 ± 0.1 vs 2.2 ± 0.1 pg/10^3 HUVECs, $P < 0.01$).

Endothelial VEGFR-1 protein expression increase was also observed in presence of 15 g/l glucose ($P < 0.05$) (Figure 2). VEGFR-2 protein was not detectable in HUVECs.

Antiangiogenic activity of glucose and CML-HSA via RAGE

In Matrigel, HUVECs spontaneously formed capillary tubes (12.8 ± 0.3/mm^2; Figure 3 corresponding to the experimental condition A shown in Figure 4). Compared with 1 g/l glucose-containing culture medium, the ability of HUVECs to form capillary tubes in Matrigel was significantly inhibited by 15 g/l glucose (5.6 ± 0.2/mm^2; $P < 0.001$) and CML-HSA had no additional inhibitory effect (5.0 ± 0.3/mm^2). The capillary tube formation was totally inhibited when HUVECs

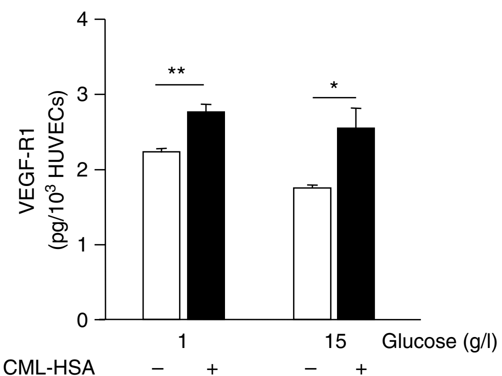

Figure 2 | CML-HSA stimulated VEGF-R1 expression by HUVECs. Confluent HUVECs were incubated 40 h in culture medium (glucose 1 or 15 g/l) with CML-HSA (5 nmol/ml). VEGFR-1 protein expression was measured by ELISA in HUVEC cell lysates. Results are the mean $\pm$ s.e.m. of two experiments performed in triplicate (*$P < 0.05$; **$P < 0.01$).

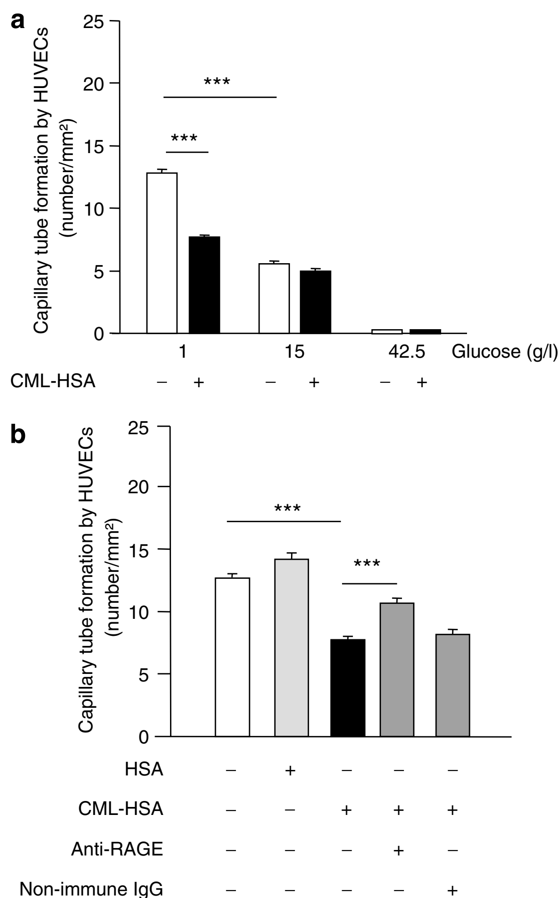

Figure 3 | (a) Glucose inhibits *in vitro* neoangiogenesis. HUVECs were incubated 24 h with culture medium at different glucose concentrations (1, 15, or 42.5 g/l) with or without CML-HSA (5 nmol/ml). (**b**) CML-HSA inhibits via RAGE the capacity of HUVECs to form capillary tubes. HUVECs were cultured 24 h in Matrigel with HSA or CML-HSA (5 nmol/ml) added to the culture medium. To block the AGE-RAGE interaction, HUVECs were preincubated 30 min with anti-RAGE antibody (100 μg/ml). Non-immune IgG was used as control. The results are expressed in number of capillary tubes formed per mm^2, and presented as the mean$\pm$s.e.m. of 36 determinations from six different experiments (***$P<0.001$).

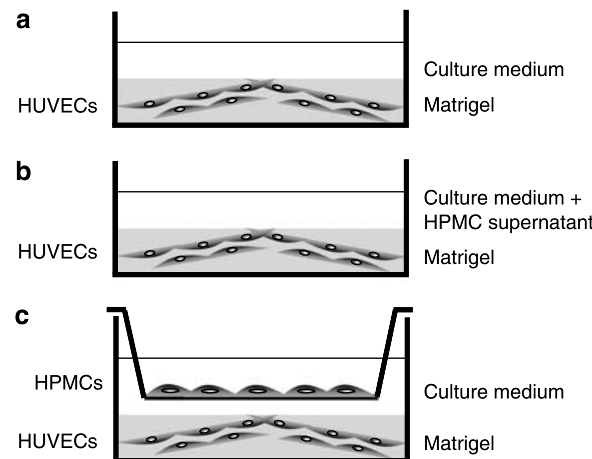

Figure 4 | Experimental conditions for capillary tube formation by HUVECs. (**a**) HUVECs cultured in Matrigel. (**b**) HUVECs cultured in Matrigel exposed to HPMC supernatant. (**c**) Co-culture of HUVECs in Matrigel (lower compartment) with HPMCs on a transwell membrane (upper compartment).

were cultured in high glucose concentration 42.5 g/l (Figure 3a). CML-HSA inhibited the formation of capillary tubes (7.6$\pm$0.3/mm^2; $P<0.001$), and anti-RAGE antibody significantly reduced the antiangiogenic effect of CML-HSA (10.7$\pm$0.4/mm^2; $P<0.001$), whereas non-immune immunoglobulin (Ig) G did not (Figure 3b).

VEGF produced by HPMCs stimulates capillary tube formation

To demonstrate that inhibition of capillary tube formation induced by glucose (15 g/l) can be partially reversed by the addition of HPMC supernatant, HUVECs were exposed to supernatants of HPMCs cultured with glucose 15 g/l. Capillary tube formation was 10.1$\pm$0.7/mm^2 (Figure 5), whereas after the addition of anti-human VEGF antibody to the HPMC supernatant the capillary tube formation was reduced (8.0$\pm$0.4/mm^2). This result suggests that VEGF present in the HPMC supernatant was implicated in the angiogenic mechanism ($P<0.05$).

To investigate the possible presence of a cross-talk between mesothelial and endothelial cells, we developed a co-culture system without direct contact between the two cell types (experimental condition C shown in Figure 4). In this system (using a 24-well plate), the spontaneous generation of capillary tubes by HUVECs (without the addition of cultured HPMCs) was found to be within the same range (11.2$\pm$0.9/mm^2, Figure 6a) as that observed in the 96-well plate experiments (12.8$\pm$0.3/mm^2, Figure 3). VEGF measured by enzyme-linked immunosorbent assay (ELISA) was not detected in the HUVEC supernatant regardless of the presence or absence of CML-HSA, thus ensuring that all the VEGF present in our system was produced by HPMCs. Compared to the spontaneous capacity of HUVECs to form capillary tubes under basal conditions, the co-culture with unstimulated HPMCs enhanced capillary tube formation (15.3$\pm$0.7/mm^2; $P<0.001$, Figure 6b). The stimulation of HPMCs present in the upper compartment by CML-HSA but not by control HSA resulted in increased capillary tube formation by HUVECs in the lower compartment (19.7$\pm$0.6/mm^2; $P<0.001$; Figure 6d). The capillary tube formation promoted by the co-culture of HUVECs with stimulated HPMCs was mediated by mesothelial RAGE, as RAGE blockade by anti-RAGE antibody limited capillary tube development (15.4$\pm$0.6/mm^2; $P<0.001$, Figure 6e). The addition of anti-VEGF antibody to HPMCs significantly reduced capillary tube formation by HUVECs (15.9$\pm$0.7/mm^2; $P<0.001$; Figure 6f). These results indicate that VEGF production is stimulated by CML-HSA through mesothelial RAGE activation, and may well be responsible for a large proportion of the increase in capillary tube formation.

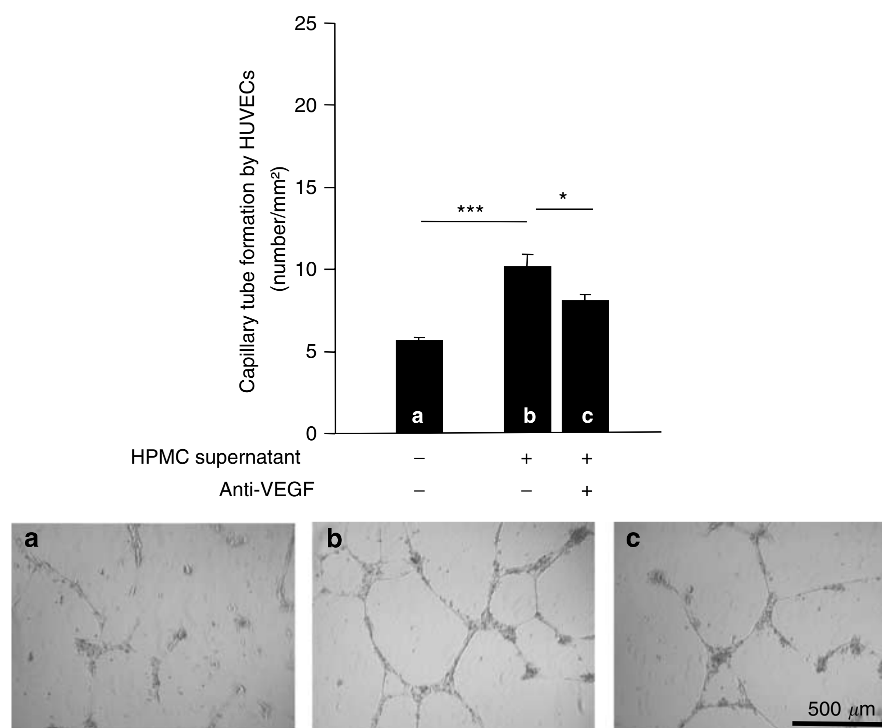

Figure 5 | Capillary tube formation stimulated by HPMC supernatant is reduced by preincubation with anti-VEGF antibody. (**a**) HUVECs were grown for 24 h with glucose (15 g/l). (**b**) Capillary tube formation was stimulated 24 h by HPMC supernatant which could be reduced by preincubation 30 min with anti-VEGF antibody (0.5 μg/ml) (**c**). The results of experiments are expressed in number of capillary tubes formed per mm^2, and presented as the mean $\pm$ s.e.m. of 36 determinations from six different experiments (*$P<0.05$; ***$P<0.001$).

AGE and VEGF concentrations in PD effluents

In a separate study, we found that blood AGE-protein concentration in PD patients was 15.4 $\pm$ 7.8 μg/ml at PD initiation and increased to 20.8 $\pm$ 8.3 μg/ml, 6 months later.[15] In the present work, 6 weeks after PD initiation, AGE-protein concentration in PD effluents was 25.7 $\pm$ 4.1 μg/ml after short dwell and reached 40.3 $\pm$ 4.3 μg/ml after long dwell ($P<0.05$) suggesting local AGE production. VEGF levels were higher in long dwell PD effluents (35.4 $\pm$ 4.3 vs 15.9 $\pm$ 1.8 pg/ml, $P<0.001$; Figure 7a). AGE-protein level correlated with VEGF concentration in PD effluents (Figure 7b).

DISCUSSION

Despite the direct inhibitory effect of AGEs on capillary tube formation by HUVECs, we demonstrated that VEGF produced by mesothelial cells through RAGE activation led to an increase in capillary tube formation by HUVECs. The present study supports the hypothesis of a possible cross-talk between AGE-stimulated HPMCs and endothelial cells, resulting in neoangiogenesis. Higher levels of VEGF in PD effluents after longer dwell time may be a biomarker of HPMC alteration owing to the effect of glucose concentrations and AGEs that may participate in the development of neoangiogenesis. PDFs containing low levels of AGE precursors have the potential to slow down conventional PDF-induced VEGF expression and AGE accumulation, as demonstrated in rat experiments, suggesting a close correlation between AGE concentrations and peritoneal VEGF production.[16] Immunoblotting and immunostaining studies have previously demonstrated that VEGF colocalizes with AGE deposits in the peritoneal membrane and is mainly located in the endothelium lining the peritoneal blood vessels.[3,18] In the present study, AGEs were detected by immunoassay that did not assess the presence of glycation-free adducts which are measured by liquid chromatography-mass spectrometry. Different compounds are classified as being glycation products: early glycation adducts, monolysyl adducts including CML, hydroimidazolones, bis(lysyl)imidazolium crosslinks, or pentosidine. In PD therapy, protein glycation-free adducts that markedly accumulate in the plasma of uremic patients are mainly eliminated in the peritoneal dialysate.[19,20]

AGEs are involved in the development of microvascular complications in patients with diabetes mellitus and contribute to the deleterious effects upon ischemia-induced neoangiogenesis.[13,14] Inhibition of AGE formation could consequently be used to stimulate collateral vessel formation in the context of ischemia, and may constitute an alternative therapeutic strategy to enhance new vessel growth in the setting of diabetes. Neoangiogenesis, which occurs in the peritoneum exposed to glucose and glycoxidation products, appears to be mediated by a paracrine mechanism. In the co-culture experiments, we demonstrated that *in vitro* HPMC exposure to AGEs could result in a release of VEGF, which

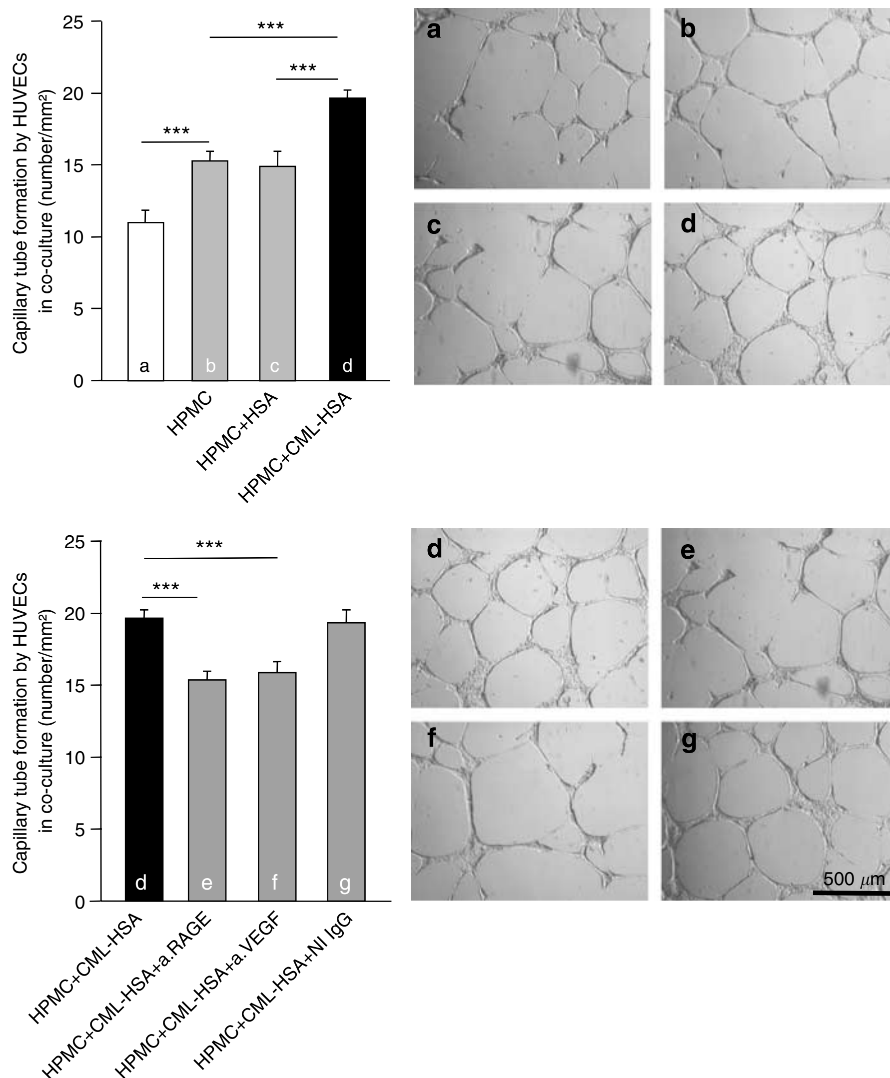

Figure 6 | (Top panel) VEGF produced by HPMCs stimulates capillary tube formation by HUVECs. Formation of capillary tubes by HUVECs in Matrigel was evaluated in a co-culture system with HPMCs. Capillary tube formation by HUVECs was measured (**a**) under basal conditions and (**b**) in a co-culture system with unstimulated HPMCs or (**c**) when stimulated for 24 h by HSA or (**d**) CML-HSA (5 nmol/ml). (Bottom panel) Mesothelial RAGE is involved in increased capillary tube formation. (**e**) For the RAGE blockade experiment, CML-HSA-stimulated HPMCs were preincubated for 30 min with rabbit anti-RAGE antibody (100 μg/ml). In other experiments, (**f**) mouse anti-human VEGF antibody (0.5 μg/ml) or (**g**) non-immune IgG was added to the HPMC and HUVEC culture medium. The results of the experiments are expressed in terms of the number of capillary tubes formed per mm^2, and presented as the mean $\pm$ s.e.m. of 36 determinations from six different experiments (***$P < 0.001$).

enhances capillary tube formation and conceals the anti-angiogenic effect of AGEs. Similarly, in the nervous system, a dysregulation between neuronal and vascular cells contributes to the development of severe disorders such as motor neuron degeneration or Alzheimer's disease.[21] The activity of VEGF-A, the major isoform acting on the endothelial cells investigated in this study, is mediated mainly via two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. VEGFR-1 and VEGFR-2 protein analysis revealed that VEGFR-1 was upregulated by CML-HSA in HUVECs, and may participate in endothelial cell overstimulation. Approaches to disrupt VEGF/VEGF receptor signaling pathways range from small molecular ATP competitive VEGF receptor inhibitors to biological agents such as soluble receptor, anti-VEGF, and anti-VEGF receptor antibodies. Antiangiogenic treatment such as the use of thalidomide and protein kinase-β inhibitors can represent an alternative treatment.[22]

Low-GDP PDFs preserve peritoneal integrity and may thus improve the longevity of the peritoneal membrane. GDPs combined with accelerated AGE formation are the main causative factors in PDF-induced peritoneal damage.[23] RAGE stimulation results in HPMC activation, which may promote local inflammation, and is thus implicated in the peritoneal injury found in long-term PD patients.[8] Glycated serum albumin stimulated VEGF production by HPMCs but not glucose alone.[17] In the present co-culture system that could mimic *in vivo* mesothelial/endothelial VEGF cross-talk, mesothelial cell RAGE activation resulted in increased VEGF

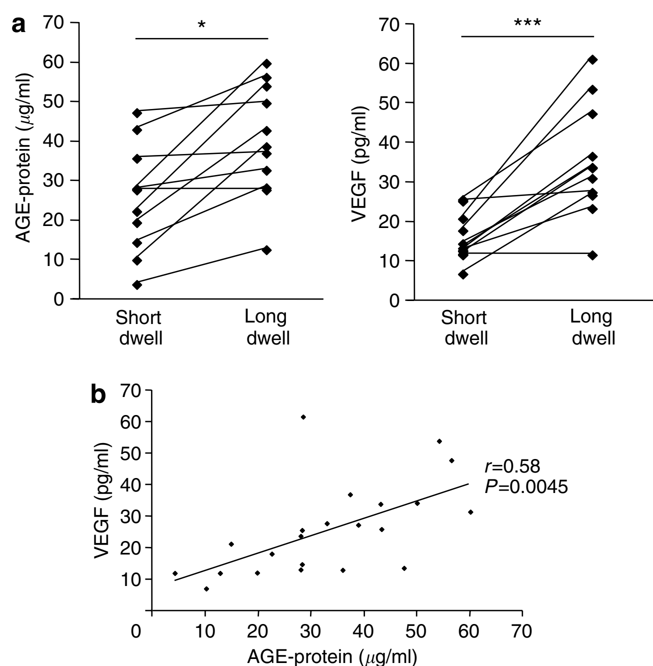

Figure 7 | (a) AGE-protein and VEGF concentrations are increased in PD effluents after long dwell time. AGE-protein and VEGF concentrations assessed by ELISA in PD effluents from 11 PD patients were analyzed after short or long dwell time in the peritoneum for conventional lactate-based PDFs (15 g/l glucose) (*$P<0.05$; ***$P<0.001$ for AGE-protein and VEGF, respectively). **(b)** AGE-protein correlated with VEGF concentration in PD effluents. Correlation between AGE-protein and VEGF concentrations in PD effluents (short and long dwell) was analyzed by the Pearson correlation test.

production leading to capillary tube formation. RAGE-deficient mice failed to develop peritoneal neoangiogenesis and increased VEGF expression after exposure to high GDP-containing PDFs compared to control mice.[24] Animal and *in vitro* studies have evidenced that AGE/RAGE interaction blockade by soluble RAGE or anti-RAGE antibodies could reduce the cellular and organic alterations secondary to RAGE activation, but these molecules have not been used in human therapy.[8,25,26]

The excess of reactive oxygen intermediates, oxidized carbohydrate proteins and lipids observed in hemodialyzed patients shows that this technique also has its limitations. Alternatively, the blood levels of advanced oxidation protein products (opp) can be reduced by PD treatment.[15] Owing to the shortage of kidneys available for transplantation and the increasing number of patients with renal failure the artificial clearance of toxins has to be adopted as the technique of choice, and therefore needs to be improved upon.

MATERIALS AND METHODS

Cell cultures

The study was conducted in accordance with the Declaration of Helsinki Principles, and with the rules of our institution. All the experiments were performed using HPMCs obtained from 15 consenting donors.

HPMCs were isolated from freshly obtained omental tissue and cultured in M199 plus 20% fetal calf serum as described previously.[8] HUVECs were isolated from human umbilical cords by enzymatic digestion with collagenase,[27] grown in M199 plus 20% fetal calf serum, and used for capillary tube formation experiments.

Preparation of CML-HSA

CML is a major lysine-derived AGE. To prepare CML-HSA, HSA (30 mg/ml), and sodium cyanoborohydride (0.45 M) were dissolved in sodium phosphate buffer (0.2 M; pH 7.8).[28] Glyoxylic acid was then added, and the mixture incubated for 24 h at 37°C. Control protein was prepared under the same conditions, except that glyoxylic acid was omitted. The preparation of CML-modified proteins was characterized by modifications in percentage, assessed via 2,4,6-trinitrobenzenesulfonic acid and by gas chromatography-mass spectrometry. CML-HSA and control HSA solutions were extensively dialyzed for 48 h (1 v/100 v). To avoid any residual sodium cyanoborohydride (62.84 Da) or glyoxylic acid (74.04 Da) presence, the membrane cutoff was 15 000 Da. Endotoxin levels evaluated by chromogenic Limulus assay (ACC, Falmouth, MA, USA) amounted to <0.002 EU/mg protein in the experimental preparations.

CML blood concentrations assessed by ELISA in diabetic patients varied between 1.4 and 7 nmol/ml,[13] and evaluated by tandem mass spectrometry in PD patients the mean of CML concentration was 8.01 ± 3.8 nmol/ml.[29] To stimulate the VEGF production by HPMCs, we tested three different CML-HSA concentrations (1.8, 3.75, and 7.5 μg/ml of albumin) corresponding to 1.25, 2.5, and 5 nmol/ml CML-HSA, respectively.

Measurement of VEGF mRNA expression and protein production by HPMCs

Reverse transcription-polymerase chain reaction was performed in a 20 μl reaction system with the use of the RNA Master HybProbe kit in LightCycler technology (Roche Diagnostics, Mannheim, Germany). The content of the amplification mix and the thermal cycling conditions were set to manufacturer's instructions. Following oligonucleotide primers and probes were designed for human VEGF-A (isoform $VEGF_{165}$): sense 5′-CCCTGAGATGCAGTACAT-3′; antisense 5′-AGCAAGGCCCACAGGGATTT-3′ (corresponding to ex5/7); labeled probe (5'FAM, 3'TAMRA) 5′-ATCCTGTGTGCC GATGCGATGCGGT-3′. β2-Microglobulin served as a reference gene: sense 5′-GATGAGTATGCCTGCCGTGTG-3′; antisense 5′-CAATCCAAATGCGGCATCT-3′; labeled probe 5′-CTCCATGA TGCTGCTTACATGTCTCGATCCC-3′. VEGF and β2-microglobulin transcripts were quoted from reference.[30] Negative controls excluded amplification from contaminating genomic DNA. The relative changes in VEGF/β2-microglobulin mRNA ratio between treated and non-treated cells were determined by the $\Delta\Delta C_t$ procedure. Efficiencies of target and reference gene amplification were comparable.

Production of VEGF protein was measured in HPMC supernatants using a Quantikine Human $VEGF_{165}$ Immunoassay system (R&D Systems, Abingdon, Berks, UK). HPMCs (15×10^3/well) were seeded and cultured in a 96-well plate for 24 h. HPMCs were exposed during 40 h to culture medium with or without CML-HSA (1.25, 2.5, or 5 nmol/ml). Effect of CML-HSA was also analyzed in culture medium containing 15 g/l D-glucose (Sigma, St Louis, MO, USA). VEGF protein concentration was also measured in PD effluents.

Anti-VEGF antibodies

Neutralizing mouse anti-human VEGF monoclonal antibody (IgG, Sigma) was used in the capillary tube formation experiments (at 0.1, 0.25, 0.5, and 1 μg/ml). The highest blocking effect was achieved with 0.5 μg/ml. Mouse non-immune IgG was used as control (Sigma).

Anti-RAGE antibody production

Anti-RAGE antibody was obtained using purified recombinant rat RAGE to immunize rabbits.[25] HPMCs or HUVECs were preincubated with anti-RAGE IgG for 30 min at a concentration of 100 μg/ml previously determined as the concentration totally blocking RAGE-ligand interaction.[8] Rabbit non-immune IgG was used as control (Sigma).

Expression of VEGF receptors

VEGF-A activity is mainly mediated via two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. HUVECs were grown until confluence, then stimulated for 40 h with CML-HSA. VEGFR-1 and -2 protein expression was measured by ELISA in HUVEC cell lysates using a Quantikine human soluble VEGFR-1 or VEGFR-2 immunoassay (R&D Systems).

Capillary tube formation in Matrigel

The property of HUVECs to spontaneously form capillary tubes in Matrigel basement membrane matrix (Becton Dickinson, San Diego, CA, USA) was used to assess the effect of glucose and CML-HSA as a model for angiogenesis (Figure 4a). HUVECs (40×10^3/well) were cultured in Matrigel in a 96-well plate (M199 + 10% fetal calf serum) for 24 h with glucose (1 or 15 g/l), with HSA or CML-HSA. To investigate RAGE involvement, a series of experiments were performed in the presence of anti-RAGE antibody or non-immune IgG. Capillary tube formation was quantified by counting the number of capillary tube structures formed per mm^2 using videomicroscopy linked to a computerized analytical system.

To demonstrate that VEGF produced by HPMCs and present in the supernatant could alter capillary tube formation, HPMC supernatant was added to the HUVEC cultures (Figure 4b). HPMCs were cultured for 24 h and then exposed for 40 h to culture medium containing D-glucose (15 g/l). The HPMC supernatants preincubated or not with anti-human VEGF antibody were added to the HUVECs, and their effect on the HUVEC-induced formation of capillary tubes in Matrigel was tested.

Co-culture system

HPMCs (30×10^3/chamber) were initially cultured in M199 + 10% fetal calf serum until confluence on a polyester membrane in an intercup chamber (Becton Dickinson) before the experiments. HUVECs were seeded in a Matrigel matrix in a 24-well plate. After HUVEC culture in Matrigel (3 h), the confluent HPMCs cultured in the membrane of the intercup chamber were added to the wells (Figure 4c). HPMCs were stimulated beforehand for 30 min with CML-HSA. For the RAGE blockade experiment, HPMCs were preincubated with anti-RAGE antibody or non-immune IgG for 30 min before CML-HSA stimulation. In some experiments, anti-VEGF antibody or non-immune IgG was added to the culture medium just before and during the stimulation period. During the co-culture period CML-HSA was still present in the HPMC supernatant, allowing passage into the HUVEC culture medium. After 24 h of co-culture, the number of capillary tubes was counted using a microscope linked to a computerized video system.

Effluents from PD patients

PD effluent from 11 patients (56 ± 14.2 years old) treated by PD for 6 weeks and receiving conventional heat-sterilized lactate-buffered PDFs (pH 5.5, glucose 15 g/l) were collected after short (4.2 ± 0.8 h) or long (9.8 ± 1.9 h) dwell times in the peritoneal cavity. Mean glucose peritoneal equilibration test and daily dextrose exposure (DDE) were measured as described previously.[15] glucose peritoneal equilibration test was 0.4 ± 0.1 and DDE was 114 ± 12.7 g/day.

After collection, the effluents were centrifuged for 7 min at 150 *g*, and the supernatants were collected into sterile tubes then stored at −80°C until the measurements of AGE and VEGF concentrations were performed.

AGE measurement in PD effluents

AGE-protein concentration present in the PD effluents was determined by competitive ELISA.[28] Briefly, glycated proteins (glycated RNase) were used for immunization in hens and IgY were obtained by differential polyethylene glycol precipitation and purified by cryoalcohol treatment (Agrobio, La Ferté St Aubin, France). Antibody specificity has been described previously.[28] The results were expressed in μg/ml AGE-protein.

Statistical analysis

The results of VEGF production by HPMCs and capillary tube formation were presented as the mean ± s.e.m. Statistical significance was determined using one-way analysis of variance followed by the parametric Dunnett's test. Variations in the VEGF and AGE-protein measurements during PDF dwell time were assessed by the Wilcoxon signed rank test for paired comparisons.[31] Correlation between the AGE-protein and VEGF concentration in PD effluents was analyzed by the Pearson correlation test. A *P*-value of under 0.05 was considered as being statistically significant.

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REFERENCES

1. Davies SJ, Phillips L, Griffiths AM *et al*. What really happens to people on long-term peritoneal dialysis? *Kidney Int* 1998; **54**: 2207–2217.
2. Topley N, Kaur D, Petersen MM *et al*. *In vitro* effects of bicarbonate and bicarbonate-lactate buffered peritoneal dialysis solutions on mesothelial and neutrophil function. *J Am Soc Nephrol* 1996; **7**: 218–224.
3. Combet S, Miyata T, Moulin P *et al*. Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long-term peritoneal dialysis. *J Am Soc Nephrol* 2000; **11**: 717–728.
4. Witowski J, Korybalska K, Wisniewska J *et al*. Effect of glucose degradation products on human peritoneal mesothelial cell function. *J Am Soc Nephrol* 2000; **11**: 729–739.
5. Miyata T, Horie K, Ueda Y *et al*. Advanced glycation and lipidoxidation of the peritoneal membrane: respective roles of serum and peritoneal fluid reactive carbonyl compounds. *Kidney Int* 2000; **58**: 425–435.
6. Linden T, Cohen A, Deppisch R *et al*. 3,4-Dideoxyglucosone-3-ene (3,4-DGE): a cytotoxic glucose degradation product in fluids for peritoneal dialysis. *Kidney Int* 2002; **62**: 697–703.
7. Boulanger E, Wautier MP, Gane P *et al*. The triggering of human peritoneal mesothelial cell apoptosis and oncosis by glucose and glycoxydation products. *Nephrol Dial Transplant* 2004; **19**: 2208–2216.

8. Boulanger E, Wautier MP, Wautier JL *et al.* AGEs bind to mesothelial cells via RAGE and stimulate VCAM-1 expression. *Kidney Int* 2002; **61**: 148–156.
9. Devuyst O, Nielsen S, Cosyns J *et al.* Aquaporin-1 and endothelial nitric oxide synthase expression in capillary endothelia of human peritoneum. *Am J Physiol* 1998; **275**: H234–H242.
10. Ferrara N, Gerber H, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**: 669–676.
11. Pecoits Filho R, Araujo M, Lindholm B *et al.* Plasma and dialysate IL-6 and VEGF concentrations are associated with high peritoneal solute transport rate. *Nephrol Dial Transplant* 2002; **17**: 1480–1486.
12. Rougier JP, Guia S, Hagege J *et al.* PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: transcriptional regulation by TGF-beta 1. *Kidney Int* 1998; **54**: 87–98.
13. Wautier MP, Boulanger E, Guillausseau PJ *et al.* AGEs, macrophage colony stimulating factor and vascular adhesion molecule blood levels are increased in patients with diabetic microangiopathy. *Thromb Haemost* 2004; **91**: 879–885.
14. Tamarat R, Silvestre JS, Huijberts M *et al.* Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. *Proc Natl Acad Sci USA* 2003; **100**: 8555–8560.
15. Boulanger E, Moranne O, Wautier MP *et al.* Changes in glycation and oxidation markers in patients starting peritoneal dialysis: a pilot study. *Perit Dial Int* 2006; **26**: 207–212.
16. Mortier S, Faict D, Lameire N *et al.* Benefits of switching from a conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model. *Kidney Int* 2005; **67**: 1559–1565.
17. Mandl-Weber S, Cohen C, Haslinger B *et al.* Vascular endothelial growth factor production and regulation in human peritoneal mesothelial cells. *Kidney Int* 2002; **61**: 570–578.
18. Combet S, Ferrier ML, van Landschoot M *et al.* Chronic uremia induces permeability changes, increased nitric oxide synthase expression, and structural modifications in the peritoneum. *J Am Soc Nephrol* 2001; **12**: 2146–2157.
19. Agalou S, Ahmed N, Babaei-Jadidi R *et al.* Profound mishandling of protein glycation degradation products in uremia and dialysis. *J Am Soc Nephrol* 2005; **16**: 1471–1485.
20. Thornalley PJ. Measurment of protein glycation, glycated peptide, and glycation free adducts. *Perit Dial Int* 2005; **25**: 522–533.
21. Carmeliet P. Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet* 2003; **4**: 710–720.
22. Podar K, Anderson K. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. *Blood* 2005; **105**: 1383–1395.
23. Mortier S, Faict D, Schalkwijk C *et al.* Long-term exposure to new peritoneal dialysis solutions: effects on the peritoneal membrane. *Kidney Int* 2004; **66**: 1257–1265.
24. Schwenger V, Morath C, Salava A *et al.* Damage to the peritoneal membrane by glucose degradation products is mediated by the receptor for advanced glycation end-products. *J Am Soc Nephrol* 2006; **17**: 199–207.
25. Wautier MP, Chappey O, Corda S *et al.* Activation of NADPH oxidase by advanced glycation end-products (AGEs) links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab* 2001; **280**: E685–E694.
26. Schmidt AM, Hori O, Chen JX *et al.* Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 1995; **96**: 1395–1403.
27. Wautier JL, Paton RC, Wautier MP *et al.* Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications. *N Engl J Med* 1981; **305**: 237–242.
28. Wautier MP, Massin P, Guillausseau PJ *et al.* N(carboxymethyl)lysine as a biomarker for microvascular complications in type 2 diabetic patients. *Diabetes Metab* 2003; **29**: 44–52.
29. Teerlink T, Barto R, Ten Brink H *et al.* Measurement of Nepsilon-(carboxymethyl)lysine and Nepsilon-(carboxyethyl)lysine in human plasma protein by stable-isotope-dilution tandem mass spectrometry. *Clin Chem* 2004; **50**: 1116–1117.
30. Wellmann S, Taube T, Paal K *et al.* Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by lightcycler technology. *Clin Chem* 2001; **47**: 654–660.
31. Rosner B, Glynn R, Lee M. The Wilcoxon signed rank test for paired comparisons of clustered data. *Biometrics* 2006; **62**: 185–192.