Original Article



Myofibroblast transdifferentiation of mesothelial cells is mediated by RAGE and contributes to peritoneal fibrosis in uraemia

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Abstract

Background. Uraemia is associated with fibrosis of the peritoneal membrane, even prior to the start of peritoneal dialysis. Increased carbonyl stress and the resultant formation of advanced glycation endproducts (AGEs) are potentially involved. The interaction of AGEs with their cell surface receptor for AGE (RAGE) induces sustained cellular activation, including the production of the fibrogenic growth factor- β (TGF- β). TGF- β is pivotal in the process of epithelial-to-mesenchymal transition with the acquisition of myofibroblast characteristics. We investigated whether antagonism of RAGE prevents uraemiainduced peritoneal fibrosis. In addition, we examined whether myofibroblast transdifferentiation of mesothelial cells contributes to peritoneal fibrosis in uraemia.

Methods. Uraemia was induced in rats by subtotal nephrectomy. Uraemic and age-matched shamoperated rats were treated for 6 weeks with neutralizing monoclonal anti-RAGE antibodies or placebo. Expression of AGE, RAGE, cytokeratin and α -smooth muscle actin was evaluated using immunohistochemistry. TGF- β expression was examined with immunostaining and western blotting, and Snail expression with western blotting. Fibrosis was quantified with a picro-sirius red staining and measurement of the hydroxyproline content of the tissue.

Results. Uraemia resulted in the accumulation of AGE, up-regulation of RAGE and TGF- β and the development of interstitial fibrosis and vascular sclerosis in the peritoneal membrane. Prominent myofibroblast transdifferentiation of mesothelial cells was identified by colocalization of cytokeratin and α -smooth muscle actin in submesothelial and interstitial fibrotic tissue. The antagonism of RAGE prevented the up-regulation of TGF- β , epithelial-to-mesenchymal transition of mesothelial cells and fibrosis in uraemia.

Conclusion. The ligand engagement of RAGE and the subsequent up-regulation of TGF- β induces peritoneal fibrosis in chronic uraemia. The process may be mediated by the conversion of mesothelial cells into myofibroblasts.

Keywords: AGE; myofibroblast; peritoneal membrane; RAGE; uraemia; TGF-β

Introduction

Data from the Peritoneal Biopsy Registry have indicated that peritoneal fibrosis appears in predialysis and haemodialysis patients and thus precedes the start of peritoneal dialysis (PD) [1,2]. The thickness of the submesothelial compact zone was larger in PD patients with clinical problems than in those in whom biopsies were taken at random, suggesting that peritoneal fibrosis is clinically relevant [2].

Carbonyl stress is actively involved in the development of uraemic complications [3]. Reactive carbonyl compounds derived from either carbohydrates or lipids accumulate in uraemic plasma and contribute to the formation of advanced glycation end-products (AGEs). AGE accumulation has been demonstrated in the peritoneum of PD patients [4-6], more specifically in the mesothelium, submesothelial stroma and blood vessels, and correlated with the extent of vascular sclerosis and interstitial fibrosis [5]. AGEs exert their biological effects by receptor-independent and dependent pathways. Several cell surface receptors, such as 80 K-H, OST-48, galactin-3, macrophage scavenger receptor and receptor for AGE (RAGE) have been identified, the latter of which is the best characterized [7]. RAGE is a member of the immunoglobulin superfamily of cell surface molecules and interacts with diverse ligands, including AGEs, S100/ calgranulins, amphoterin and amyloid-B-peptid [8]. This ligand-receptor interaction activates multiple cellular signal transduction pathways, leading to the secretion of inflammatory cytokines, up-regulation of

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adhesion molecules and the production of growth factors, such as VEGF and TGF- β [8].

Several in vivo studies support the importance of AGE-RAGE interaction in the development of diabetic complications [8-10]. The interaction of AGE with RAGE induced a TGF-β-dependent epithelial-myofibroblast transdifferentiation in a proximal tubule cell line [11], providing a novel mechanism for tubulointerstitial fibrosis in diabetic nephropathy. Exposure of cultured human peritoneal mesothelial cells to TGF- β resulted in the conversion of these cells into myofibroblasts [12], suggesting that a similar mechanism could be operative in the peritoneal membrane. Overexpression of TGF- β in the peritoneal membrane of experimental animals induced expression of genes and cellular changes characteristic for epithelial-to-mesenchymal transition [13]. In peritoneal biopsies of PD patients, fibroblast-like cells in the stroma were found to express mesothelial markers, supporting the contention that epithelial-tomesenchymal transition of mesothelial cells could be a source of myofibroblasts in the peritoneum [14,15].

The present study evaluates the contributory role of AGE–RAGE interaction to peritoneal fibrosis in uraemia and examines whether epithelial-tomesenchymal transition of mesothelial cells is involved in the process of uraemia-associated peritoneal fibrosis. We used a model of subtotal nephrectomy for the induction of chronic renal failure and antagonized RAGE with a neutralizing monoclonal anti-RAGE antibody (Ab).

Materials and methods

Laboratory animals

The studies were performed in 73 female Wistar rats (Iffacredo, Brussels, Belgium) with an initial mean body weight of 217 ± 1 g, receiving care in accordance with the national guidelines for care and use of laboratory animals. The rats were randomly assigned to uraemic (n=45) or sham-operated groups (n=28). Uraemia was induced using a standard procedure of subtotal nephrectomy as described earlier [16]. Rats were anaesthetized with halothane (Fluothane, Astra-Zeneca, Destelbergen, Belgium) and a flank incision was made to expose the left kidney. Subsequently, the upper and lower poles as well as the anterior and posterior lateral sides of the kidney were cryoablated. One week later, a right nephrectomy was performed. Animals undergoing subtotal nephrectomy received buprenorphine (0.1 mg/kg 1 m/12 h, Temgesic, Schering-Plough, Brussels, Belgium) post-surgically for 48 h and erythropoietin (100 IU/kg s.c., Neo-Recormon) twice weekly to correct anaemia due to uraemia. In sham-operated rats. flank incisions were made and the left and right kidneys were manipulated without tissue destruction, respectively separated by 1 week. After the induction of uraemia, rats were treated for 6 weeks with neutralizing monoclonal anti-RAGE Ab (n = 15) or with isotype-matched irrelevant murine IgG/EBSS (n = 30). Similarly, sham-operated rats were treated with anti-RAGE Ab (n=10) or murine IgG/EBSS (n = 18). One milligram of Ab was injected intraperitoneally thrice a week. After 6 weeks, the animals were anaesthetized with thiobutabarbital (Inactin, RBI, Natick, MA; 100 mg/kg s.c.) and the abdomen was opened. A sample of the visceral peritoneum of the most distal loop of the ileum was fixed in 4% neutral buffered formalin and embedded in paraffin. The visceral peritoneum of the small and large bowel was entirely resected, snap frozen in liquid nitrogen and maintained at -80° C until analysis.

Neutralizing monoclonal anti-RAGE Ab

The preparation and characterization of the neutralizing monoclonal anti-RAGE Ab followed procedures described previously [9,10,17]. The human RAGE extracellular domain encompassing residues 23-340 (sRAGE) was expressed and purified from Escherichia coli using the pET thioredoxin system (Novagen, Madison, WI, USA). Female 8-week-old BALB/c mice were immunized, then boosted three times, 21 days apart, by intraperitoneal and subcutaneous injections of 100 µg of sRAGE protein in Complete Freund's adjuvant for the primary immunization and an additional 50 µg of sRAGE in Incomplete Freund's adjuvant was injected for secondary immunizations. The mouse with the highest serum titre to sRAGE as measured by enzyme-linked immunosorbent assay was injected intravenously with an additional 30 µg immunogen in phosphate-buffered saline (PBS), 21 days after the last immunization. Three days later, spleen cells were harvested for production of hybridomas to sRAGE using previously described techniques [17]. The hybridoma cell line with the highest Ab titre and neutralizing Ab activity was selected after cloning 3-4 times by limiting dilution in 96-well microtitre plates, then grown in a Cellmax Bioreactor (Spectrum; Rancho Dominguez, CA, USA) using DMEM culture media. Purified IgG was prepared by Protein A chromatography. The determination of the isotype (IgG3) and light chain composition (κ) of the Ab [17] and the characterization of the RAGE neutralizing activity of the Ab [10] were performed as described previously.

Histology and immunohistochemistry

The degree of fibrosis was evaluated using a picro-sirius red staining F3B (Klinipath, Geel, Belgium). Sections were deparaffinized, rehydrated and stained briefly with Giemsa. Subsequently, sections were washed and stained with the sirius red solution, resulting in a brick red staining of all fibrillary collagen.

Immunostainings for AGE, RAGE, TGF- β , α -smooth muscle actin (α -SMA) and cytokeratin, as well as a double staining for α -SMA and cytokeratin were performed. Sections were deparaffinized, rehydrated, incubated in 3% H₂O₂ in PBS to block endogenous peroxidase and washed in 10% normal horse serum (Sigma, St Louis, MO, USA) in PBS to block non-specific binding. Subsequently, they were incubated with the primary Ab: a mouse antihuman AGE (6D12, Cosmo Bio Ltd, Tokyo, Japan), a goat anti-human RAGE Ab (Research Diagnostics, Flanders, NJ), a rabbit anti-human TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-human α -SMA (clone 1A4, DAKO, Glostrup, Denmark) and a polyclonal rabbit anti-cytokeratin (wide spectrum screening, DAKO). For the AGE, RAGE and TGF- β staining,

a biotinylated IgG (Vector Laboratories, Burlingame, CA, USA) and streptavidine-peroxidase were applied and 3.3'-diaminobenzidine (DAB) was used as the chromogenic substrate, resulting in a brown precipitate. For the α -SMA staining, a peroxidase-labelled IgG (rabbit anti-mouse IgG/HRP, DAKO) was applied and immunolabelling was visualized with DAB. For the cytokeratin staining, an alkaline phosphatase-labelled IgG (goat anti-rabbit IgG/AP, DAKO) was applied and Fast Red (DAKO) was used as chromogenic substrate, resulting in a red precipitate. For the double α -SMA/cytokeratin immunostaining, tissues were pre-treated with a Tris/EDTA epitope retrieval solution (pH 9.0, DAKO) and incubated with the polyclonal rabbit anti-cytokeratin and the goat anti-rabbit alkaline phosphatase-conjugated IgG. After colour development with Fast Red, the peritoneum was treated with 3% H₂O₂ to inactivate the endogenous peroxidase, incubated with the mouse anti-human anti-α-SMA and the rabbit anti-mouse peroxidase-conjugated IgG and developed with DAB.

Morphometric measurements of the AGE, RAGE, TGF- β and picro-sirius red staining were made by a blinded operator with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) at magnification 200×. For each sample of peritoneum, two tissue sections were analysed quantitatively with a computerized image analysis system (Zeiss, Oberkochen, Germany). A camera sampled six images of each tissue section and generated an electronic signal proportional to the intensity of illumination, which was then digitized into picture elements or pixels. The digital representation of the tissue was analysed with KS400 Software (Zeiss, Oberkochen, Germany). Each pixel in a colour image was divided into three colour components (hue, saturation and intensity). The threshold for each colour component of the staining was defined and kept constant throughout the analysis. In a predefined area, RAGE, AGE, TGF-B and picro-sirius Red staining were measured and expressed as a percentage.

A semi-quantitative assessment of the double α -SMA/ cytokeratin staining was performed independently by two blinded operators with a light microscope at magnification $100 \times$. The whole tissue section was screened to estimate the extent and distribution of colocalization of α -SMA and cytokeratin in each slide. Staining results were classified from 0 to 3: 0 = no, 1 = mild, 2 = moderate and 3 = pronounced colocalization. The results were calculated as the mean of the individual scores of the two operators.

Immunoblotting for $TGF-\beta$ and Snail

For total protein extraction, ~100 mg of peritoneum tissue was homogenized on ice using 1 ml Dounce homogenizers at ice-cold temperature in a 10-fold excess (wt/vol.) lysis buffer containing 25 mM HEPES, pH 7.4, 1% Triton X-100, 140 mM NaCl, 1 mM PMSF, 5 mM DTT, protease ($10 \mu g/$ ml pepstatin A, $10 \mu g/$ ml leupeptin, $10 \mu g/$ ml STI, $10 \mu g/$ ml aprotinin) and phosphatase (1 mM sodium orthovanadate and 30 mM sodium fluoride) inhibitors. Cellular debris was pelleted by centrifugation at 13 200 r.p.m. at 4°C for 20 min. A supernatant protein concentration was determined (Bio-Rad Protein Assay) and 200 μg of protein were pooled from each experimental animal.

Fifty micrograms of pooled tissue lysate from each experimental group was electrophoresed on a 7.5%

SDS-polyacrylamide gel and analysed by immunoblotting after transfer to nitrocellulose membranes. Immunoblotting was performed using a goat anti-human TGF-B1 antibody (Santa Cruz) or a rabbit anti-human Snail antibody (Abgent 2054a; 1:500 dilution), followed by the appropriately diluted, corresponding anti-IgG-fluorescent-conjugated Ab. Molecular weights of the bands were determined by simultaneous electrophoresis of molecular weight markers (Amersham). Densitometric quantitation of the appropriate band was performed using the LI-COR Bioscience OdysseyTM Imaging System with infared fluorescence detection. Specificity of the detected band was assayed using a 50-fold molar excess of the peptide immunogen used to generate the Ab added to the primary Ab. Individual band intensities were normalized to β-actin, which was detected with mouse anti-human β -actin antibody (Sigma A1978).

Hydroxyproline assay

Peritoneal tissue (30–60 mg) was lyophylized for 8 h, hydrolysed in 6 N HCl at 85°C for 14–18 h and lyophylized again. Norleucine was added as internal standard and pH was set at 2.2 by adding lithium citrate buffer (Biochrom, Cambridge, UK). The hydroxyproline content of the samples was quantified by HPLC using a Biochrom 20 Plus Amino Acid Analyser (Ansynth Service Roosendaal, The Netherlands). After reaction with ninhydrine, photometric detection was performed at a wavelength of 440 nm and the hydroproline content was expressed as micromole per milligram peritoneal tissue.

Statistical analysis

The results are expressed as mean \pm SEM. Statistical analysis was performed using ANOVA, and the Tukey test was used as multiple-comparison *t*-test. The significance level was set at $P \le 0.05$.

Results

Characteristics of laboratory animals

The cumulative mortality, including the nephrectomy/ sham procedure, was 0% in sham-operated rats vs 18% (8/45) in uraemic rats. About 75% of the observed mortality (6/8) occurred within 10 days, most likely due to surgical complications. The other animals (2/8) died in the last 2 weeks before sacrifice, as a consequence of their uraemic state. The body weight of the uraemic rats was significantly lower than that of the sham-operated rats. Anti-RAGE Ab treatment did not affect body weight (Table 1). Erythropoietin treatment prevented anaemia in uraemic animals. Exposure to anti-RAGE Ab did not alter haematocrit levels (Table 1). Uraemia was documented by significantly increased plasma ureum and creatinine levels in uraemic rats compared with sham-operated animals (Table 1).

Table 1. Characteristics of experimental animals	
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	Sham + placebo $(n = 18)$	Sham + RAGE Ab $(n = 10)$	Uraemia + placebo $(n=25)$	Uraemia + RAGE Ab $(n = 12)$
Body weight (g) Haematocrit level (%) Plasma creatinine (µmol/l) Plasma ureum (mmol/l)	277 ± 2 44.9 ± 0.5 28.6 ± 1.3 5.0 ± 0.2	$265 \pm 544.4 \pm 0.632.1 \pm 0.74.0 \pm 0.4$	$\begin{array}{c} 251 \pm 4^{\rm b} \\ 46.5 \pm 0.6 \\ 63.9 \pm 3.1^{\rm b} \\ 14.3 \pm 0.6^{\rm b} \end{array}$	$\begin{array}{c} 239 \pm 11^{a} \\ 44.4 \pm 1.2 \\ 75.1 \pm 6.8^{b} \\ 16.7 \pm 1.2^{b} \end{array}$

 $^{a}P < 0.05 vs$ sham, $^{b}P < 0.0001 vs$ sham.

Table 2.	Histological	and	immunohistochemical	analyses	of the	e peritoneum
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	Sham + placebo $(n = 10)$	Sham + RAGE Ab $(n = 10)$	Uraemia + placebo $(n = 7)$	Uraemia + RAGE Ab $(n=9)$
AGE staining (%) RAGE staining (%) Picro Sirius Red staining (%) TGF-β staining (%) Double ctaining (%)	$2.3 \pm 0.2 \\ 3.4 \pm 0.3 \\ 0.7 \pm 0.1 \\ 1.8 \pm 0.2 \\ 1.0 \pm 0.2$	- 0.9 ± 0.1 2.0 ± 0.3	$3.7 \pm 0.6^{a} \\ 6.9 \pm 0.4^{b} \\ 3.3 \pm 0.5^{c} \\ 6.1 \pm 0.6^{c} \\ 2.6 \pm 0.2^{c}$	- 2.0 ± 0.3 ^{a,d} 3.8 ± 0.4 ^{a,d} 1.8 ± 0.2 ^e

 $^{a}P < 0.05$ vs sham, $^{b}P < 0.001$ vs sham, $^{c}P < 0.001$ vs sham, $^{d}P < 0.05$ vs uraemia + placebo, $^{c}P < 0.01$ vs uraemia + placebo.

Peritoneal morphology

The staining for AGE and RAGE was greatly enhanced in the peritoneal tissue of placebo-treated uraemic rats compared with placebo-treated sham rats (Table 2, Figures 1 and 2). AGE accumulation and RAGE expression was prominent in mesothelial and endothelial cells, but also in the submesothelial, perivascular and interstitial fibrotic tissue. Picro-sirius red staining of collagen was more pronounced in the uraemic rats than in the sham-operated rats, as evident by an increased staining of the submesothelial compact zone, interstitial tissue and perivascular fibrotic tissue (Table 2, Figure 3). Exposure to anti-RAGE Ab significantly reduced submesothelial, perivascular and interstitial fibrosis in the uraemic animals (Table 2, Figure 3). TGF-B expression was strongly up-regulated in uraemic rats compared with the sham-operated groups (Table 2, Figure 4). Antagonism of RAGE partially prevented the up-regulation of TGF- β expression in uraemic animals (Table 2, Figure 4).

Staining for the epithelial marker cytokeratin was confined to the mesothelial cell layer in sham-operated animals. In uraemic animals, an extensive additional staining was observed in the submesothelial and interstitial fibrotic tissue (Figure 5). Staining for α -SMA was limited to the muscularis of the blood vessels in control rats, but was also found in the fibrotic areas in uraemic animals (Figure 5). Double α -SMA/cytokeratin staining was virtually absent in controls, but was prominent in uraemic animals (Figure 5). In uraemic animals treated with anti-RAGE Abs, colocalization of α -SMA and cytokeratin was significantly lower than in those that received control Abs (Table 2).

Immunoblotting for TGF- β and Snail

Peritoneal TGF- β expression (expressed as band density of TGF- β normalized to the band intensity of the β -actin band ×100), was increased in placebotreated uraemic animals (49.7) when compared with sham-operated rats (19.1). Treatment with anti-RAGE Ab prevented the up-regulation of TGF- β in uraemic animals (25.8) (Figure 6). Snail was down-regulated in uraemic animals treated with RAGE (7.81) as compared with uraemic animals treated with placebo (11.8) (Figure 7).

Hydroxyproline content

Peritoneal hydroxyproline content was significantly increased in placebo-treated uraemic animals $(5.82 \pm 0.17 \,\mu\text{mol/mg}$ peritoneal tissue, n = 18) when compared with sham-operated animals $(3.94 \pm 0.23 \,\mu\text{mol/mg}$ peritoneal tissue, n = 8) (P < 0.0001). The administration of anti-RAGE Ab reduced the hydroxyproline content in uraemic animals $(4.93 \pm 0.80 \,\mu\text{mol/mg})$ peritoneal tissue, n = 3) when compared with the treatment with placebo (P = 0.05).

Discussion

Chronic uraemia results in fibrosis of the peritoneal membrane, as documented by the picro-sirius red staining and the measurement of hydroxyproline content. The results are in accordance with the findings in human biopsies [1,2]. A pronounced deposition of AGEs was observed in uraemic peritonea. The accumulation of AGEs is known to enhance the surface expression of RAGE [8]. A prominent



Fig. 1. AGE immunostaining of the visceral peritoneum (magnification: $\times 200$). AGEs strongly accumulate in the peritoneal tissue of placebo-treated uraemic rats (A), compared with placebo-treated sham-operated rats (B). The staining is prominent in mesothelial and endothelial cells, but also in the submesothelial, interstitial and perivascular fibrotic tissue.



Fig. 2. RAGE immunostaining of the visceral peritoneum (magnification: $\times 200$). RAGE expression was strongly up-regulated in placebo-treated uraemic rats (A), compared with placebo-treated sham-operated rats (B). The staining pattern is similar to that of AGE with pronounced staining in mesothelial and endothelial cells and in the submesothelial, interstitial and perivascular fibrotic tissue.

up-regulation of RAGE was indeed evident in the peritoneal membrane of uraemic animals, while its expression was low-grade in sham-operated animals. The salient observation in the present study is that inhibition of the AGE-RAGE interaction with a neutralizing monoclonal anti-RAGE antibody prevented uraemia-induced fibrosis and vascular sclerosis. Several lines of evidence support that the fibrogenic properties of AGEs are mediated by the up-regulation of TGF-ß [18,19]. Uraemia resulted in an increased expression of TGF-B, documented by both immunostaining and western blotting. The up-regulation of TGF- β in uraemia was prevented by the antagonism of RAGE. The results support the contention that ligand engagement of RAGE induces expression of TGF-B and results in peritoneal fibrosis. RAGE antagonism did not completely prevent fibrosis, indicating that other mechanisms that are independent of RAGE must contribute to peritoneal fibrosis in uraemia.

Exposure of a proximal tubular cell line to AGE-BSA induced tubular-to-myofibroblast transdifferentiation through interaction with RAGE and the resultant generation of TGF- β [11]. Similarly to smooth muscle cells, myofibroblasts are characterized



Fig. 3. Picro-sirius red staining of the visceral peritoneum (magnification: $\times 200$). Prominent submesothelial and interstitial fibrosis and vascular sclerosis was observed in uraemic animals treated with placebo (A) or anti-RAGE-Ab (B), compared with the shamoperated animals, treated with placebo (C) or anti-RAGE-Ab (D). RAGE-Ab partially prevented the development of submesothelial and interstitial fibrosis and vascular sclerosis in uraemic animals.



Fig. 4. TGF- β immunostaining of the visceral peritoneum (magnification: ×200). TGF- β expression was increased in the peritoneum of placebo-treated uraemic rats (A). Antagonism of RAGE partially prevented the up-regulation of TGF- β in uraemic rats (B). TGF- β expression was virtually absent in the sham-operated groups treated with placebo (C) or anti-RAGE-Ab (D), except for a light staining of mesothelial cells.

by the presence of contractile fibres and stain positively for α -SMA [20]. They secrete cytokines and growth factors and are major producers of extracellular matrix molecules, such as collagen, fibronectin



Fig. 5. Serial sections of the visceral peritoneum from placebo-treated sham-operated animals (A–C) and placebo-treated uraemic animals (D–I) were stained for α -SMA (A,D,G), cytokeratin (B,E,H) and double stained for α -SMA and cytokeratin (C,F,I) (magnification: ×200). In sham-operated animals, only vascular smooth muscle cells stain for α -SMA (A), only mesothelial cells stain for cytokeratin (B) and virtually no α -SMA/cytokeratin colocalization (C) occurs. In the peritoneal membrane of uraemic animals, α -SMA staining (D,G) is found not only in the vascular smooth muscle layer of blood vessels, but also in the submesothelial fibrotic tissue. Cytokeratin staining (E,H) is visible in the mesothelial cells, but is additionally found in submesothelial and interstitial fibrotic tissue. Colocalization of α -SMA and cytokeratin (F,I) is pronounced in the submesothelial, perivascular and interstitial fibrotic tissue. Large arrow = vascular smooth muscle cells, small arrow = mesothelial cells.



Fig. 6. Western blot of peritoneal TGF-β expression in uraemic rats treated with anti-RAGE-Ab (UR), uraemic rats treated with placebo (UP) and sham-operated animals (CP). Each lane represents 50 μg of pooled peritoneal tissue lysate. Immunoblotting was performed using a goat anti-human TGF-β1 Ab. Densitometric quantitation of the appropriate band was performed using the LI-COR Bioscience OdysseyTM Imaging System with infrared fluorescence detection. Individual band intensities were normalized to the corresponding β-actin.

and glycosaminoglycans. The activation and proliferation of myofibroblasts, when unchecked, invariably results in tissue fibrosis. The origin of myofibroblasts is not certain, but they have been postulated to arise from resident fibroblasts, perivascular cells or circulating precursor cells. Recently, mesothelial cells and the process of epithelial-to-mesenchymal transition has been implicated as a potential source of myofibroblasts in peritoneal tissues [12–15]. The administration of TGF- β to cultured human peritoneal mesothelial cells was associated with ultrastructural changes, the appearance of α-SMA myofilaments and the deposition of extracellular matrix typical for a myofibroblast phenotype [12]. Adenovirus-mediated gene transfer of TGF- β in the peritoneal cavity of experimental animals resulted in the up-regulation of genes associated with epithelial-to-mesenchymal transition, the disruption of the submesothelial basement membrane and the appearance of cells with both epithelial and myofibroblast phenotype [13]. Mesothelial cells isolated from the effluent of PD patients showed phenotypical changes characteristic of an epithelial-tomesenchymal transition process [14]. In peritoneal biopsies of PD patients, fibroblast-like cells in the stroma stained positive for epithelial cell markers, thus revealing their mesothelial origin [14,15]. In the present study, we performed a double staining for cytokeratin and α -SMA to examine potential conversion of mesothelial cells into myofibroblasts. epithelial Cytokeratin is а typical marker and thus allows the identification of mesothelial cells. As expected, the cytokeratin staining was confined to the mesothelial cell layer in the peritoneal RAGE-mediated myofibroblast transdifferentiation of mesothelial cells



Fig. 7. Western blot of peritoneal Snail expression in uraemic rats treated with anti-RAGE-Ab (UR) and uraemic rats treated with placebo (UP). Each lane represents 50 µg of pooled peritoneal tissue lysate. Immunoblotting was performed using a rabbit anti-human Snail Ab. Densitometric quantitation of the appropriate band was performed using the LI-COR Bioscience OdysseyTM Imaging System with infrared fluorescence detection. Individual band intensities were normalized to the corresponding β -actin.

membrane of the control animals. α-SMA stains smooth muscle cells and is thus found in the vasculature of a normal peritoneal membrane. It is also characteristic for myofibroblasts and is often used as a marker for these cells. We detected a pronounced colocalization of cytokeratin and α -SMA in the submesothelial and interstitial fibrotic tissue of uraemic peritonea. These results suggest that mesothelial cells migrate towards the interstitium and acquire a myofibroblastic phenotype. Importantly, the inhibition of RAGE was not only associated with a lower TGF- β expression and less fibrosis, but also with less α -SMA/cytokeratin colocalization. In addition, the expression of Snail, a regulatory protein involved in epithelial-to-mesenchymal transition, decreased by the inhibition of RAGE in uraemic animals.

In conclusion, chronic uraemia results in the development of submesothelial and interstitial fibrosis of the peritoneal membrane. We identified the interaction of AGE with RAGE and the subsequent up-regulation of TGF- β and myofibroblast transdifferentiation of mesothelial cells as a potential pathogenic pathway for peritoneal fibrosis.

Acknowledgements. The authors thank Tommy Dheuvaert, Julien Dupont, Nele Nica, Mieke Van Landschoot and Marie-Anne Waterloos for their expert technical assistance. A.S.D.V. is supported by a grant from the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (B/02476/05) and S.M. by a grant from the Bijzonder Onderzoeksfonds (BOF/056).

Conflict of interest statement. None declared.

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Received for publication: 21.1.06 Accepted in revised form: 18.4.06