Damage to the Peritoneal Membrane by Glucose Degradation Products Is Mediated by the Receptor for Advanced Glycation End-Products

Vedat Schwenger,* Christian Morath,* Alexander Salava,* Kerstin Amann,† Yuri Seregin,* Reinhold Deppisch,‡ Eberhard Ritz,* Angelika Bierhaus,* Peter P. Nawroth,* and Martin Zeier*

**Department of Medicine, Divisions of Nephrology and Endocrinology, University of Heidelberg, Heidelberg, Germany; †Department of Pathology, University of Erlangen-Nurnberg, Erlangen, Germany; and ‡Gambro Corporate Research, Hechingen, Germany*

Peritoneal dialysis is limited by morphologic changes of the peritoneal membrane. Use of peritoneal dialysis fluids (PDF) that contain glucose degradation products (GDP) generates advanced glycation end-products (AGE) within the peritoneal cavity. It is unknown whether peritoneal damage is causally related to AGE–receptor for AGE (RAGE) interaction. The effects of PDF were compared with different amounts of GDP on morphologic changes of the peritoneal membrane in 48 wild-type (WT) and 48 RAGE-deficient mice. PDF (1 ml) were instilled twice daily over a period of 12 wk. Groups with eight animals each received no manipulation (sham); sham instillation (sham i.p.); or filter-sterilized, glucose-free, conventional low GDP- or high GDP PDF. *In vitro* (generation of AGE fluorescence in PDF) and *in vivo* (immunohistochemistry for carboxymethyllysine), a GDP-dependent increase of AGE formation occurred. Inflammation and neoangiogenesis were augmented in WT mice that were treated with high GDP accompanied by upregulation of $CD3^+$ T cells, increased NF-κB binding activity, increased lectin, and vascular endothelial growth factor expression. Furthermore, pronounced submesothelial fibrosis was found with increased expression of TGF-β1. Exposure to low GDP resulted in only mild inflammation and neoangiogenesis (compared with sham i.p.) and no fibrosis in WT mice. The findings in WT contrasted with those in RAGE-deficient mice, which showed no increased inflammation ($CD3^+$ T cells and NF-κB binding activity), neoangiogenesis (by lectin and vascular endothelial growth factor expression), or fibrosis (expression of TGF-β1) after long-term exposure to GDP-containing PDF. Peritoneal damage by GDP in PDF is dependent at least in part on AGE–RAGE interaction.

J Am Soc Nephrol 17: 199–207, 2006. doi: 10.1681/ASN.2005020155

Peritoneal dialysis (PD) is an accepted alternative to hemodialysis in the treatment of patients with ESRD. Long-term PD is limited by structural and functional changes of the peritoneal membrane resulting in dialysis failure (1). Conventional PD fluids (PDF) are a source of glucose degradation products (GDP; carbonyl compounds) that are generated during heat sterilization under acidic conditions (2). Such GDP are highly reactive substances (2–10) that exhibit considerable direct cytotoxicity. However, GDP lead to the formation of advanced glycation end-products (AGE) (11). The extent to which peritoneal damage from GDP is due either to direct effects, *e.g.*, illustrated by generation of vascular endothelial growth factor (VEGF) by methylglyoxal in peritoneal cells (12), or to indirect effects such as the generation of AGE from precursor GDP is unknown. To investigate mechanisms of AGE-dependent peritoneal damaging, we compared PDF with different GDP contents in wild-type (WT) and receptor for AGE (RAGE)-deficient (−/−) mice. The rationale for this approach was based on the consideration that RAGE, a multiligand member of the Ig superfamily (13,14), is the best characterized signal transduction receptor for AGE. Binding of AGE to RAGE results in an activation of key signal transduction pathways, such as NF-κB and subsequent transcription of mediators for which a role was also claimed in the pathogenesis of uremic complications (15,16). To avoid this confounder, we performed the following study in nonuremic animals.

Human peritoneal mesothelial cells also express RAGE (17). Furthermore, monoclonal anti-RAGE antibodies prevent fibrosis of the peritoneal membrane induced by hyperglycemia in a diabetic animal model (18). However, it is not certain whether anti-RAGE antibodies recognize structures other than the ligand binding domain of RAGE. The purpose of our experimental study was to address the following issues: (*1*) Whether AGE–RAGE interruption, demonstrated in a RAGE −/− mouse model, prevents peritoneal damage after long-term PD; (*2*) whether interruption of AGE–RAGE interaction also pre-

Received February 10, 2005. Accepted October 18, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

V.S. and C.M. contributed equally to this work.

Address correspondence to: Dr. Vedat Schwenger, Department of Medicine/Division of Nephrology, University of Heidelberg, Im Neuenheimer Feld 162, 69120 Heidelberg, Germany. Phone: +49-6221-9112-601; Fax: +49-6221-9112-229; E-mail: vedat.schwenger@med.uni-heidelberg.de

Copyright © 2006 by the American Society of Nephrology

ISSN: 1046-6673/1701-0199

vents peritoneal damage in a nondiabetic model; and (*3*) whether the adverse effects of GDP (as opposed to glucose *per se*) are mediated *via* RAGE.

Materials and Methods

Animal Experiments

The experiments were performed in female mice (48 WT mice expressing RAGE, genetic background C57BL/6 and 48 RAGE −/− mice) that weighed 31.6 ± 0.54 g according to the guidelines of the Institute of Laboratory Animal Science of the University of Heidelberg. The RAGE-targeting construct and the generation of RAGE −/− mice have been described in detail elsewhere (19). The abrogation of RAGE expression at the mRNA level was analyzed by reverse transcription–PCR of lung tissue, in which RAGE is constitutively expressed at high levels. Pair-fed mice were kept in a 12-h day-night rhythm at a constant room temperature of 20°C. WT mice and RAGE −/− mice were randomly allocated to six groups, as shown in Table 1.

Twice daily, the animals received an intraperitoneal (i.p.) injection of 1 ml of the respective PDF solution at 37°C under sterile conditions. The composition of the PDF is given in Table 2. One group of mice was kept completely unmanipulated to obtain baseline histologic and molecular data (sham). In a second group, mice were sham-injected without instilling solution as a control for puncture trauma (sham i.p.). The experiment was terminated for all animals after 12 wk. We also considered the possibility of inflammatory injury to the peritoneum by a reaction to the trauma of twice-daily injection. Indeed, there was some reaction to injection, as evidenced by comparison of sham animals and sham i.p. animals, but the degree of reaction was significantly less than in response to the dialysis fluid. We took particular care to avoid artificial injury by thermal trauma (fluids were injected at body temperature) and contamination by bacteria or lipopolysaccharides.

Histologic and Immunohistochemical Examinations

Visceral peritoneal tissue samples were fixed in 6% phosphate-buffered formalin (pH 7.4) and embedded in paraffin. Four-micrometer-thick tissue sections were stained with hematoxylin & eosin, periodic acid Schiff reagent, and Picro Sirius red staining for detection of fibrous tissue and investigated by light microscopy.

Immunohistochemical stainings were performed in three groups (high GDP, low GDP, and sham i.p.). Tissue sections were deparaffinized, rehydrated, and incubated in PBS that contained 3% H_2O_2 for 15 min to block endogenous peroxidases. For TGF-β1 staining, tissue sections were incubated in PBS that contained 10% normal horse serum for 20 min to block nonspecific binding. Tissue samples were incubated with primary antibodies for 45 min. After incubation with a biotinylated IgG antibody, tissue sections were stained with a peroxidase-labeled streptavidin-biotin staining kit (DAKO GmbH, Hamburg, Germany) according to the manufacturer's recommendations.

Primary antibodies included a tomato anti-lectin (Sigma-Aldrich Co., St. Louis, MO) as an endothelial marker, polyclonal rabbit anti–TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-CD3 (Novocastra Lab Ltd., Newcastle upon Tyne, UK) as a T cell marker, a polyclonal rabbit anti-VEGF (Santa Cruz Biotechnology), a monoclonal mouse anti-methylglyoxal (MGO), and a monoclonal mouse anti-carboxymethyllysine (CML; Biologo, Kronshagen, Germany). Replacement of the primary antibodies with PBS served as control.

Quantification of Histologic and Immunohistochemical Findings

For each visceral peritoneal specimen, >30 cross-sections were evaluated in a blinded manner. Interstitial fibrosis was analyzed using Sirius red–stained sections and a semiquantitative score system: 0, no interstitial fibrosis; 1, mild interstitial fibrosis; 2, moderate interstitial fibrosis with thickening of the peritoneum; and 3, severe interstitial fibrosis with marked thickening of the peritoneum. For determination of inflammatory cell number per area, $CD3^+$ T cells per area and vessel number per area, all cells/vessels on a 121-point grid (Leitz, Wetzlar, Germany) were counted (area 0.028 μm^2). For quantitative analysis of lectin, VEGF, TGF-β1, MGO, and CML, a 121-point grid (Leitz) was used. Points on stained visceral peritoneal tissue and all points on visceral peritoneal tissue were counted. The ratio in percentage was used to determine the degree of staining.

In Vitro *Generation of AGE-Specific Fluorescence in PDF*

The presence of AGE in unused PDF was assessed indirectly by measuring the generation of AGE-specific fluorescence after adding purified human serum albumin with a final concentration of 40 mg/ml (fraction V; Sigma Co., Taufkirchen, Germany). The samples were incubated without any preservatives at 37°C for 3 and 10 d. AGE concentration was assessed in unused and spent PDF recovered after 2 h as fluorescence intensity (excitation 350 nm/emission 430 nm) with a spectrofluorometer (LS 50 B; Perkin Elmer Co., Überlingen, Germany). At the end of experiments, sham i.p. animals received an injection of normal saline (pH 7.4). The analysis of measuring AGE fluorescence has been described in detail elsewhere (20).

Table 1. Treatment groups and characteristics of animals[a]

	WT Mice (n = 8 per Group)			RAGE −/− Mice (n = 8 per Group)		
	Age (Weeks)	Body Weight (g)	Hematocrit (%)	Age (Weeks)	Body Weight (g)	Hematocrit (%)
Sham	14	31.9 ± 1.7	0.53 ± 0.03	14	32.8 ± 1.2	0.48 ± 0.03
Sham i.p.	14	30.8 ± 0.8	0.51 ± 0.02	14	30.7 ± 1.2	0.53 ± 0.02
Filter-sterilized	14	31.5 ± 1.5	0.51 ± 0.04	14	31.0 ± 1.2	0.51 ± 0.09
Glucose-free	14	31.9 ± 1.1	0.52 ± 0.02	14	32.3 ± 2.0	0.52 ± 0.02
Low GDP	14	32.4 ± 1.0	0.52 ± 0.02	14	30.2 ± 1.4	0.52 ± 0.05
High GDP	14	31.6 ± 1.3	0.51 ± 0.02	14	33.3 ± 0.9	0.50 ± 0.04

[a]WT, wild-type; RAGE, receptor for advance glycation end-products; sham i.p., sham injection without instilling solution as a control for puncture trauma; GDP, glucose degradation products.

Table 2. Composition of different dialysis fluids

	Filter-Sterilized	Glucose-Free	Low GDP[a]	High GDP[b]
Sodium (mmol/L)	132	132	132	132
Chloride (mmol/L)	96	96	96	96
Calcium (mmol/L)	1.75	1.75	1.75	1.75
Magnesium (mmol/L)	0.25	0.25	0.25	0.25
Glucose (%)	2.5	0	2.5	2.5
Lactate (mmol/L)	40	40	40	40
GDP concentration	Not detectable	None	Low	High
pH	6.5	6.5	6.5	6.5
Method of sterilization	Filter	Filter	Heat	Heat

[a]Formaldehyde <2 μM, acetaldehyde <1 μM, methylglyoxal <3 μM, 3-deoxyglucosone <13 μM.
[b]Formaldehyde 4.6 μM, acetaldehyde 226 μM, methylglyoxal 22.7 μM, 3-deoxyglucosone 123 μM.

Electrophoretic Mobility-Shift Assay

Visceral peritoneal specimens were quick-frozen in liquid nitrogen, homogenized, transferred into cold buffer A (10 mM Hepes-KOH [pH 7.9, at 4°C], 10 mM KCl, 1.5 mM $MgCl_2$ 0.5 mM DTT, 1 mM EDTA, 0.2 mM PMSF, and 0.6% Nonidet P-40), incubated on ice for 10 min, and centrifuged for 5 min at 8000 rpm at 4°C. The supernatant was discarded, and the nuclear pellet was resuspended in 100 μl of buffer B (25% glycerol, 20 mM Hepes-KOH [pH 7.9 at 4°C], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 0.2 mM PMSF, 2 mM benzamidine, and 5 mg/ml leupeptin) and incubated on ice for 20 min. Cellular debris was removed by 2 min of centrifugation at 4°C, and the supernatant was quick-frozen at −80°C. Protein concentrations were determined by the Bradford assay. Nuclear extract (10 μg) was included in the binding reaction. Binding to an NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCAGGC-3′) was performed in 10 mM Hepes (pH 7.5) that contained 0.5 mM EDTA, 100 mM KCl, 2 mM DTT, 2% glycerol, 4% Ficoll 400, 0.25% Nonidet P40, 1 mg/ml BSA (DNAse free), and 0.3 μg/μl poly(dI/dC) in a total of 20 μl. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides and by characterization with specific polyclonal antibodies (Santa Cruz Biotechnology).

Statistical Analyses

All values are expressed as mean ± SEM. Wilcoxon and Kruskal-Wallis tests were used as appropriate to test statistical significance. Significance level was set at $P < 0.05$. Statistical analysis was performed by PC-Statistik (version 5.0; Hoffmann, Giessen, Germany) and GraphPad Prism (version 3.0.3; San Diego, CA).

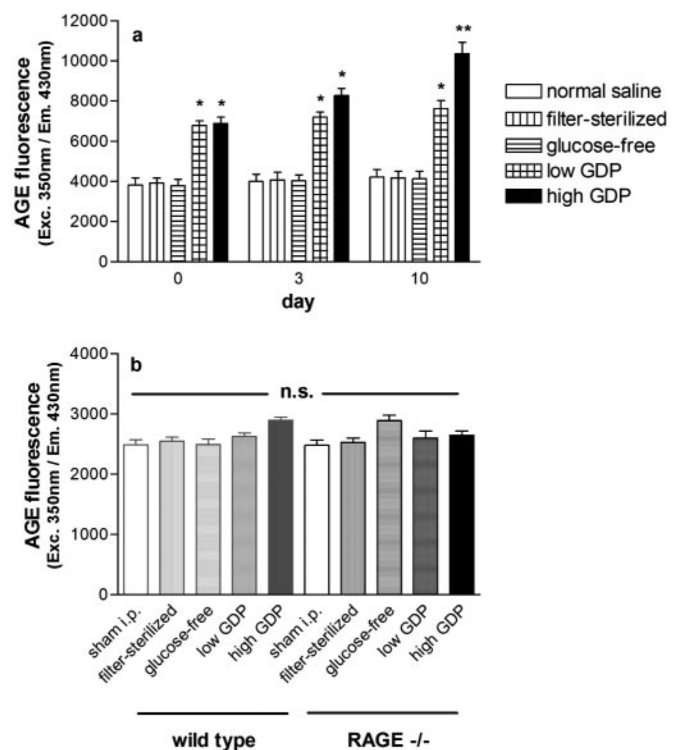

Figure 1. (a) Generation of fluorescence advanced glycation end-products (AGE) during *in vitro* incubation with albumin. Fluorescence intensity (excitation 350 nm/emission 430 nm) was quantified using a spectrofluorometer. *$P < 0.05$ *versus* normal saline on the same day; **$P < 0.01$ *versus* normal saline on the same day. (b) AGE fluorescence quantified with a spectrofluorometer (excitation 350 nm/emission 430 nm) in various spent peritoneal dialysis fluids (PDF).

Results

Animal Data

There were no significant differences with respect to age, body weight, and hematocrit values between RAGE −/− mice and WT mice (Table 1).

AGE Formation

In vitro, a time-dependent increase of AGE formation was demonstrated in low and high GDP PDF ($P < 0.05$ *versus* normal saline; Figure 1a) but not in filter-sterilized and glucose-free PDF. *In vivo*, however, AGE formation was not different in the various spent PDF (Figure 1b).

WT and RAGE −/− mice that were treated with either high or low GDP had an enhanced expression of MGO in peritoneal stainings compared with sham i.p. ($P < 0.01$; Figure 2a). CML was formed at an accelerated rate in peritoneal stainings of WT mice and RAGE −/− mice that were treated with high GDP but not in mice that were treated with low GDP ($P < 0.01$; Figure 2b).

Inflammation

In WT mice that were treated with high GDP, there was a significant increase in inflammatory cell number in visceral

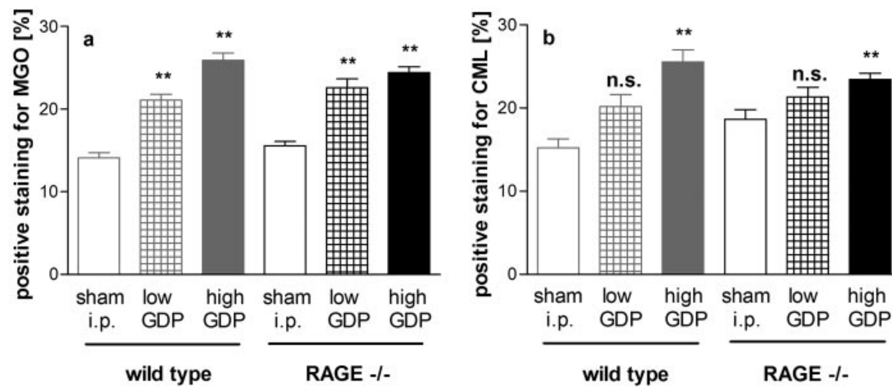

Figure 2. Methylglyoxal (MGO; a) and carboxymethyllysine (CML; b) stainings of visceral peritoneal tissue in wild-type (WT) and receptor for AGE (RAGE)-deficient (RAGE −/−) mice after a 12-wk treatment period with either low or high glucose degradation products (GDP). Mice that were sham-injected without instilling solution as a control for puncture trauma (sham i.p.) received intraperitoneal injections twice daily. $^{**}P < 0.01$ *versus* sham i.p. in the same group.

peritoneal tissue samples ($P < 0.05$ *versus* sham i.p.). This increase was not seen in WT mice that were treated with low GDP, filter-sterilized, and glucose-free PDF. RAGE −/− mice had a higher absolute inflammatory cell number in visceral peritoneal tissue samples at baseline; however, no significant increase was noted after treatment with GDP-containing PDF (Table 3).

These findings were confirmed by stainings for $CD3^+$ T cells and Electrophoretic mobility-shift assay for NF-κB in selected groups (sham i.p., low GDP, and high GDP). There was a significant increase in $CD3^+$ T cells and NF-κB binding activity in visceral peritoneal tissue samples of WT mice that were treated with high GDP ($P < 0.05$ *versus* WT sham i.p.; Figures 3 and 4). At baseline, a mild but statistically NS increase of NF-κB binding activity in peritoneal tissue of RAGE −/− mice was observed (Figure 4). After treatment with high GDP, no further increase could be detected. Furthermore, no increase of $CD3^+$ T cell count was seen in RAGE −/− mice (Figure 3). Consistent with the above results, use of low GDP did not increase $CD3^+$ T cell count and NF-κB binding activity in peritoneum of WT and RAGE −/− mice. Puncture trauma *per se* led to a significant higher inflammatory cell number when sham WT mice were compared with WT sham i.p. treatment ($P < 0.05$; Table 3).

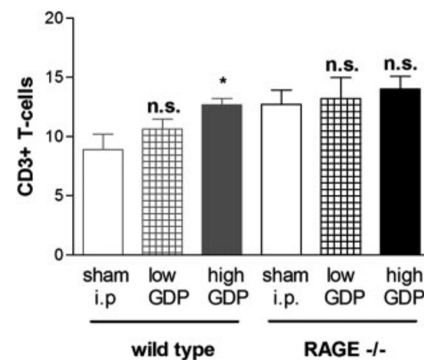

Figure 3. $CD3^+$ stainings in WT and RAGE −/− mice after a 12-wk treatment period with either low or high GDP. Sham i.p. mice received intraperitoneal injections twice daily. $^{*}P < 0.05$ *versus* sham i.p. in the same group.

Neoangiogenesis

WT mice that were treated with high GDP had an increased number of vessels and an enhanced expression of lectin and VEGF compared with sham i.p. (Table 3 and Figure 5, a and b). WT mice that were treated with low GDP had less pronounced

Table 3. Inflammation, neoangiogenesis, and fibrosis in WT and RAGE −/− mice

	WT Mice (n = 8 per Group)			RAGE −/− Mice (n = 8 per Group)		
	Inflammatory Cell Number	No. of Vessels/Area	Fibrosis Score	Inflammatory Cell Number	No. of Vessels/Area	Fibrosis Score
Sham	13.0 ± 2.17[a]	5.69 ± 0.69[a]	0.67 ± 0.41	20.1 ± 2.22	14.0 ± 3.06	0.67 ± 0.82
Sham i.p.	18.0 ± 4.08	8.61 ± 1.38	0.67 ± 0.82	21.5 ± 4.56	13.4 ± 2.16	0.67 ± 0.82
Filter-sterilized	19.2 ± 3.79	10.9 ± 3.25	1.17 ± 0.98	26.8 ± 2.06	14.0 ± 1.44	1.00 ± 0.89
Glucose-free	22.1 ± 4.63	11.1 ± 1.58[a]	1 ± 0.82	24.9 ± 4.6	14.1 ± 2.71	1.00 ± 1.26
Low GDP	20.2 ± 4.19	11.4 ± 1.59[a]	1.00 ± 1.26	24.9 ± 6.82	16.3 ± 3.47	1.17 ± 0.75
High GDP	23.5 ± 2.73[a]	12.4 ± 2.18[a]	2 ± 0.89[a]	25.9 ± 6.01	14.5 ± 2.47	1.5 ± 0.84

[a] $P < 0.05$ *versus* sham i.p.

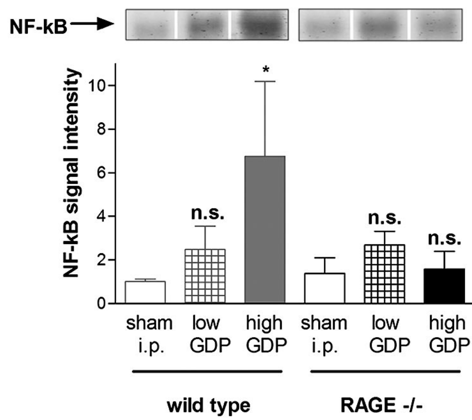

Figure 4. Electrophoretic mobility-shift assay for NF-κB in visceral peritoneal tissue of WT and RAGE −/− mice after a 12-wk treatment period with either low or high GDP. Sham i.p. mice received intraperitoneal injections twice daily. $*P < 0.05$ *versus* sham i.p. in the same group.

neoangiogenesis than WT mice that were treated with high GDP (Figure 5, a and b). RAGE −/− mice showed a higher number of vessels per area at baseline but no further increase in number of vessels and in lectin and VEGF staining after long-term PDF administration (either filter-sterilized, glucose-free, low GDP or high GDP PDF; Table 3 and Figure 5, a and b).

Fibrosis

The fibrosis score was increased only in WT mice that were treated with high GDP but not in WT mice that were treated with low GDP and sham i.p. ($P < 0.05$; Table 3). Sham- and sham i.p.–treated WT mice were not significantly different. Using the Sirius red stain, an increase in fibrous tissue was found in the interstitium as well as in the vascular endothelium of WT mice that were treated with high GDP but not in RAGE −/− mice (Figure 6). Expression of TGF-β1 was more pronounced in WT mice that were treated with both high GDP and low GDP but not in sham i.p. (Figure 5c).

In contrast, RAGE −/− mice had no increase in fibrosis score at baseline and no further increase after long-term PDF treatment (either filter-sterilized, glucose-free, low GDP or high GDP PDF; Table 3). RAGE −/− mice showed higher TGF-β1 expression at baseline but no further increase after 12 wk of treatment with either high or low GDP (Figure 5c).

Discussion

Nondiabetic and nonuremic RAGE −/− mice did not show a progress of inflammation, neoangiogenesis, and fibrosis after prolonged twice-daily instillation of GDP-containing PDF. In contrast, in RAGE-bearing WT mice, a significant increase of inflammation (as indicated by $CD3^+$ T cells and increased NF-κB binding activity), neoangiogenesis (accompanied by enhanced expression of lectin and VEGF), and fibrosis (increased expression of TGF-β1) was noted. This observation was more apparent in high as compared with low GDP, pointing to a dose-response relationship (2,4–8).

Originally, high glucose concentrations and AGE were believed to be the main contributing factors in peritoneal damaging that result in PD failure (1,10). In the meantime, toxic effects of carbonyl compounds (GDP) in PDF have been demonstrated (21). However, GDP lead to formation of AGE and enter the systemic circulation from the peritoneal cavity (20). In addition, AGE have been detected immunohistochemically in the peritoneum of PD patients and likely are responsible for structural and functional peritoneal damage (1,22). One hypothesis postulates that AGE-mediated peritoneal damaging seems to be dependent on the interaction with RAGE (18). RAGE is expressed in a variety of cell types, including endothelial cells, vascular smooth muscle cells, mesangial cells, and neuronal cells (13,14,16,23–25). One study localized RAGE in the peritoneal membrane *in vivo* (17) and confirmed earlier observations of mRNA expression of functional RAGE in human peritoneal mesothelial cells *in vitro* (26). In a diabetic animal model, inhibition of AGE–RAGE interaction with neutralizing monoclonal anti-RAGE antibodies prevents hyperglycemia-induced fibrosis of the peritoneal membrane (18). Although there is no doubt that RAGE is effectively blocked with anti-RAGE antibodies, it cannot be excluded presently that RAGE antibodies also

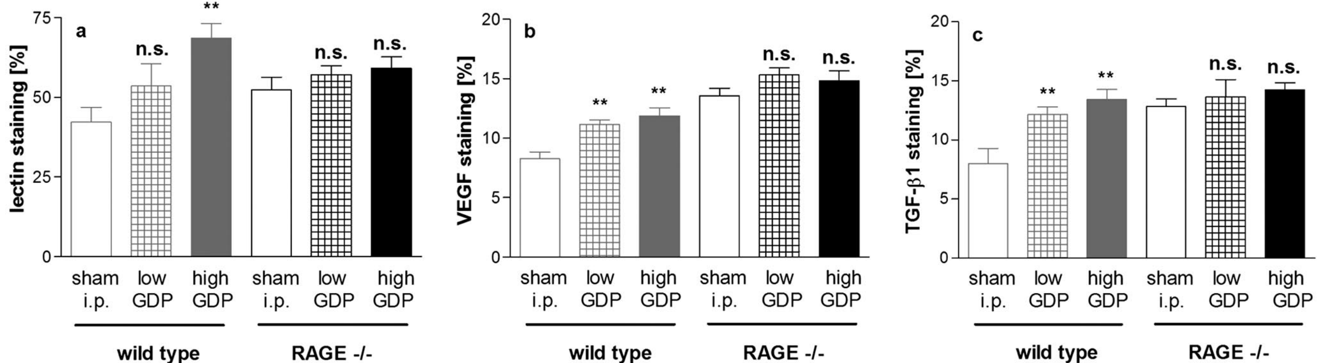

Figure 5. Lectin (a), vascular endothelial growth factor (VEGF; b), and TGF-β1 (c) stainings in WT and RAGE −/− mice after a 12-wk treatment period with either low or high GDP. Sham i.p. mice received intraperitoneal injections twice daily. $**P < 0.01$ *versus* sham i.p. in the same group.

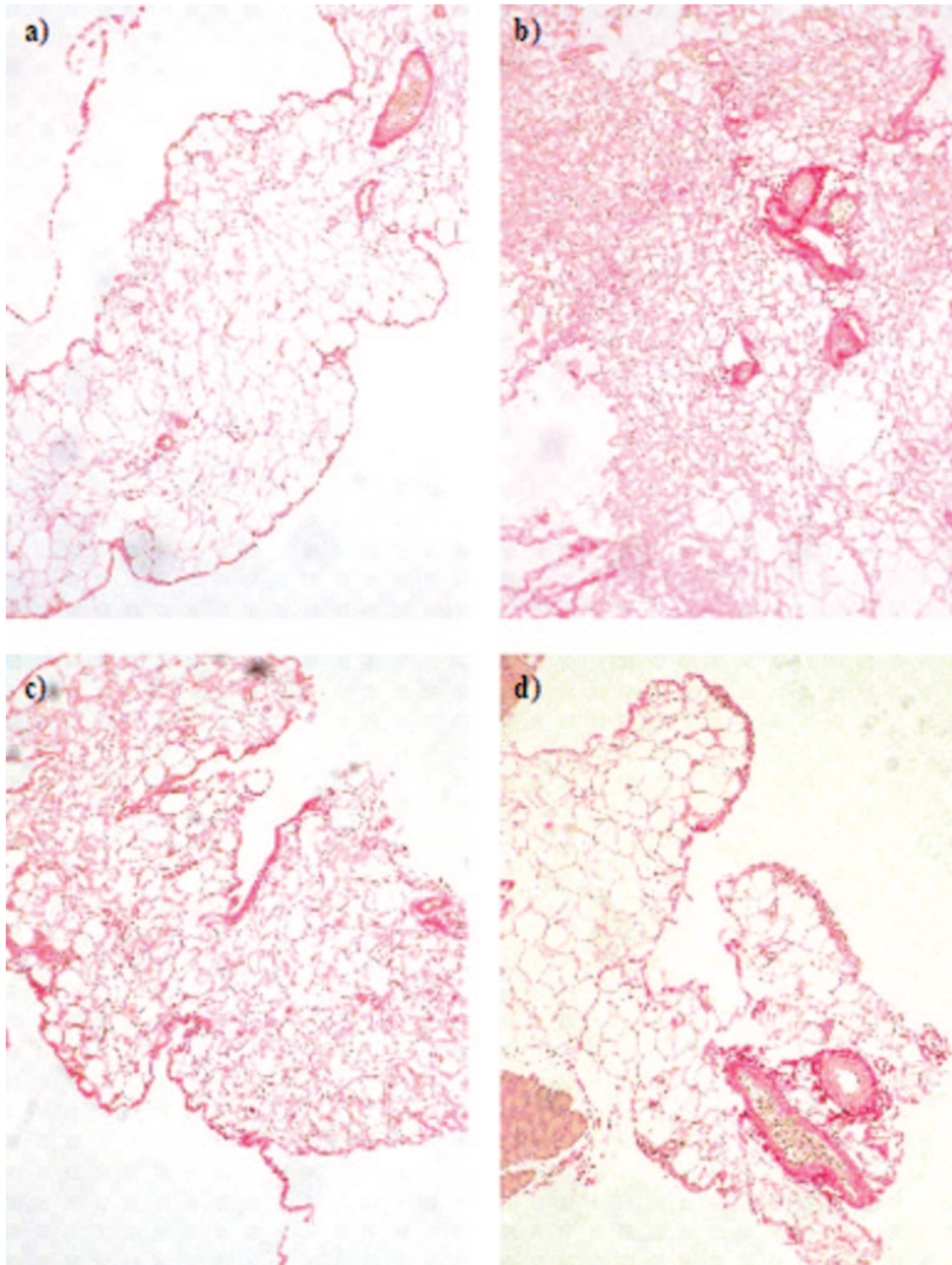

Figure 6. Sirius red staining of visceral peritoneal tissue from WT and RAGE −/− mice. (a) WT sham i.p. (b) WT mice after 12 wk of treatment with high GDP. (c) RAGE −/− sham i.p. (d) RAGE −/− after 12 wk of treatment with high GDP.

affect structures that are different from RAGE. Other groups had demonstrated that application of sRAGE, an extracellular, truncated, soluble form of RAGE, *in vivo* and *in vitro* prevented late complications, *i.e.*, microvascular and macrovascular complications in diabetes models. This raises the question of whether AGE-induced peritoneal damage can also be prevented by sRAGE. Remarkably, in each of these models (diabetic nephropathy, neuropathy, and arterial restenosis), protection from development of pathology was more profound in WT mice that were treated with sRAGE than in RAGE−/− mice (27–29). In diabetic neuropathy, for example, administration of sRAGE to diabetic WT animals completely restored pain perception, whereas diabetic RAGE−/− mice were only partly protected from loss of pain perception (28). These observations suggest that ligands that are sequestered by sRAGE are likely to interact with cellular structures other than RAGE. To avoid these methodologic culprits, we used a RAGE −/− mouse model to study the effects of high GDP PDF and low GDP PDF on the peritoneal membrane. We deliberately used nondiabetic and nonuremic

mice to exclude enhanced AGE production and stimulation of RAGE by the uremic or the diabetic milieu.

AGE Formation

From our study we cannot entirely exclude a direct interaction of GDP products and RAGE, because RAGE is a known pattern recognition receptor that interacts with a variety of ligands. However, the key pathophysiologic step seems to be GDP-dependent AGE formation as shown by *in vitro* experiments (Figure 1a). Although measurement of AGE formation was not different in spent PDF (Figure 1b; most probably because of the short dwell time), there was an increased formation of CML in visceral peritoneal tissue samples of animals that were treated with high GDP (Figure 2b). These data indicate that rapid AGE modifications occur primarily on tissue-bound proteins.

For measurement of AGE formation, we used spectrofluorometry (11,30). Although ELISA could be more sensitive, at least for an individual AGE compound, we prefer, in the sense of chemical identity, the more broad method to detect the sum of a variety of fluorescence AGE as discussed earlier (31).

Inflammation

RAGE was identified as a central signal transcription receptor for NF-κB activation (32,33). In monocytes and in inflammatory lesions in various diseases (*e.g.*, rheumatoid arthritis), increased RAGE expression was observed (25,34). Members of the NF-κB family control the initiation of inflammation by regulating expression of cytokines and also play a central role in terminating inflammation (35). The slight proinflammatory phenotype (also indicated by a slight increase in basal NF-κB binding activity and elevated cytokine levels) supports the hypothesis that RAGE might also be involved in the regulation of anti-inflammatory and antiproliferative processes (36).

It is of note that the otherwise healthy RAGE $-/-$ mice presented a higher baseline of CD3$^+$ T cells, which corresponds with earlier reports describing a slight proinflammatory phenotype in the absence of RAGE (36). This was also demonstrated in the peritoneum of RAGE $-/-$ mice, in which NF-κB binding activity was slightly elevated when compared with WT mice. Despite a minor increased proinflammatory status, NF-κB binding activity did not further increase in RAGE $-/-$ mice (low and high GDP), whereas NF-κB binding activity augmented significantly in the peritoneum of WT mice (Figure 4). In addition, in both RAGE $-/-$ groups that were treated with high and low GDP, no further increase in CD3$^+$ T cells was observed. The difference in the basal proinflammatory phenotype excludes the direct comparison of WT sham i.p. mice *versus* RAGE $-/-$ mice that were treated with low and high GDP PDF. The overall observation that the increase in all parameters (inflammation, neoangiogenesis, and fibrosis) determined between sham i.p.– and GDP-treated animals is significant in WT mice but not in RAGE $-/-$ mice clearly proves the involvement of RAGE. Recently, it was shown that RAGE $-/-$ mice can mount a normal adaptive immune response. This was demonstrated in experimental autoimmune encephalomyelitis as an autoimmune model in which RAGE $-/-$ mice developed the same inflammatory response as WT controls (19), therefore demonstrating that AGE–RAGE interaction must be the main contributing factor in transmitting GDP-dependent peritoneal inflammation. In addition, it should be considered that GDP-mediated toxicity is not only a local peritoneal phenomenon, because GDP enter the systemic circulation from the peritoneal cavity (20). Both findings argue strongly against the use of PDF with a high GDP content. Compared with low GDP, filter-sterilized and glucose-free PDF showed no further benefit in reducing CD3$^+$ T cells.

Neoangiogenesis

WT mice that were treated with either low or conventional high GDP presented an increased number of vessels per peritoneal area, which was accompanied by enhanced expression of VEGF. *In vitro* and *in vivo* exposure of peritoneal cells to high glucose stimulates the expression of VEGF (34,37,38). In our study, exposure of the peritoneum to high GDP resulted in an increased staining for VEGF and increased vascular density. RAGE $-/-$ mice demonstrated no increase of VEGF or vessels per peritoneal area with either low or high GDP. Although a correlation between AGE accumulation and VEGF expression had been demonstrated by de Vriese *et al.* (18), inhibition of AGE–RAGE interaction with anti-RAGE antibodies did not prevent peritoneal neoangiogenesis. In contrast to these observations, our results document a central role for GDP-induced upregulation of peritoneal neoangiogenesis by VEGF *via* AGE–RAGE interaction (12,15,24,32,39–42) in the peritoneal membrane. As mentioned above, the absence of the proposed regulatory function of RAGE might also account for an increase of peritoneal vessels per area at baseline in RAGE-deficient mice.

Fibrosis

Exposure to high glucose concentrations induces collagen, fibronectin, or TGF-β1 production in various cells (18,34,37). The role of high GDP concentrations in "extracellular" fibrosis in the peritoneum is less well understood. After 12 wk of treatment, no peritoneal fibrosis or upregulation of TGF-β1 expression was found in RAGE $-/-$ mice. In contrast, fibrosis score and TGF-β1 expression were increased in WT mice that were treated with high GDP. Our data not only confirm the recent observations of de Vriese *et al.* that AGE–RAGE interaction plays an important role in the induction of extracellular fibrosis in PD in a diabetic mouse model (18) but also extend the hypothesis that AGE–RAGE interaction plays a pivotal role in the induction of peritoneal fibrosis in a nondiabetic (and nonuremic) mouse model.

In conclusion, the study presented here provides evidence that the RAGE is causally involved in the generation of structural and functional damage to the peritoneal membrane induced by GDP-containing PDF. These changes were less pronounced in low GDP PDF, indicating a dose-response relationship.

Acknowledgments

This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (DFG/SFB 405 to P.P.N.).

We thank Monika Klewer and Miriam Ramming (Department of Pathology, University of Erlangen-Nurnberg, Erlangen, Germany), Heike Ziebart (Department of Pathology, University of Heidelberg, Heidelberg, Germany), Ulrike Haug (Gambro Corporation Research, Hechingen, Germany), and Dr. Anders Wieslander (Gambro Corporate Research, Lund, Sweden) for excellent technical assistance.

References

1. Honda K, Nitta K, Horita S, Yumura W, Nihei H, Nagai R, Ikeda K, Horiuchi S: Accumulation of advanced glycation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultrafiltration. *Nephrol Dial Transplant* 14: 1541–1549, 1999
2. Witowski J, Bender TO, Gahl GM, Frei U, Jorres A: Glucose degradation products and peritoneal membrane function. *Perit Dial Int* 21: 201–205, 2001
3. Jorres A: Effect of peritoneal dialysis on peritoneal cell biology: Peritoneal fibroblasts. *Perit Dial Int* 19[Suppl 2]: S348–S352, 1999
4. Wieslander A, Linden T, Musi B, Jarkelid L, Speidel R, Beck W, Henle T, Deppisch R: Exogenous uptake of carbonyl stress compounds promoting AGE formation from peritoneal dialysis fluids. *Contrib Nephrol* 82–89, 2001
5. Jorres A, Bender TO, Witowski J: Glucose degradation products and the peritoneal mesothelium. *Perit Dial Int* 20[Suppl 5]: S19–S22, 2000
6. Witowski J, Jorres A, Korybalska K, Ksiazek K, Wisniewska-Elnur J, Bender TO, Passlick-Deetjen J, Breborowicz A: Glucose degradation products in peritoneal dialysis fluids: Do they harm? *Kidney Int Suppl* 84: S148–S151, 2003
7. Jorres A: Glucose degradation products in peritoneal dialysis: From bench to bedside. *Kidney Blood Press Res* 26: 113–117, 2003
8. Witowski J, Jorres A: Glucose degradation products: Relationship with cell damage. *Perit Dial Int* 20[Suppl 2]: S31–S36, 2000
9. Witowski J, Bender TO, Wisniewska-Elnur J, Ksiazek K, Passlick-Deetjen J, Breborowicz A, Jorres A: Mesothelial toxicity of peritoneal dialysis fluids is related primarily to glucose degradation products, not to glucose per se. *Perit Dial Int* 23: 381–390, 2003
10. Witowski J, Wisniewska J, Korybalska K, Bender TO, Breborowicz A, Gahl GM, Frei U, Passlick-Deetjen J, Jorres A: Prolonged exposure to glucose degradation products impairs viability and function of human peritoneal mesothelial cells. *J Am Soc Nephrol* 12: 2434–2441, 2001
11. Schalkwijk CG, Posthuma N, ten Brink HJ, ter Wee PM, Teerlink T: Induction of 1,2-dicarbonyl compounds, intermediates in the formation of advanced glycation end-products, during heat-sterilization of glucose-based peritoneal dialysis fluids. *Perit Dial Int* 19: 325–333, 1999
12. Inagi R, Miyata T, Yamamoto T, Suzuki D, Urakami K, Saito A, van Ypersele de Strihou C, Kurokawa K: Glucose degradation product methylglyoxal enhances the production of vascular endothelial growth factor in peritoneal cells: Role in the functional and morphological alterations of peritoneal membranes in peritoneal dialysis. *FEBS Lett* 463: 260–264, 1999
13. Schmidt AM, Hori O, Cao R, Yan SD, Brett J, Wautier JL, Ogawa S, Kuwabara K, Matsumoto M, Stern D: RAGE: A novel cellular receptor for advanced glycation end products. *Diabetes* 45[Suppl 3]: S77–S80, 1996
14. Schmidt AM, Yan SD, Yan SF, Stern DM: The biology of the receptor for advanced glycation end products and its ligands. *Biochim Biophys Acta* 1498: 99–111, 2000
15. Wendt T, Bucciarelli L, Qu W, Lu Y, Yan SF, Stern DM, Schmidt AM: Receptor for advanced glycation endproducts (RAGE) and vascular inflammation: Insights into the pathogenesis of macrovascular complications in diabetes. *Curr Atheroscler Rep* 4: 228–237, 2002
16. Greten J, Kreis I, Wiesel K, Stier E, Schmidt AM, Stern DM, Ritz E, Waldherr R, Nawroth PP: Receptors for advance glycation end-products (AGE)—Expression by endothelial cells in non-diabetic uraemic patients. *Nephrol Dial Transplant* 11: 786–790, 1996
17. Boulanger E, Wautier MP, Wautier JL, Boval B, Panis Y, Wernert N, Danze PM, Dequiedt P: AGEs bind to mesothelial cells via RAGE and stimulate VCAM-1 expression. *Kidney Int* 61: 148–156, 2002
18. De Vriese AS, Flyvbjerg A, Mortier S, Tilton RG, Lameire NH: Inhibition of the interaction of AGE-RAGE prevents hyperglycemia-induced fibrosis of the peritoneal membrane. *J Am Soc Nephrol* 14: 2109–2118, 2003
19. Liliensiek B, Weigand MA, Bierhaus A, Nicklas W, Kasper M, Hofer S, Plachky J, Grone HJ, Kurschus FC, Schmidt AM, Yan SD, Martin E, Schleicher E, Stern DM, Hammerling GG, Nawroth PP, Arnold B: Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J Clin Invest* 113: 1641–1650, 2004
20. Zeier M, Schwenger V, Deppisch R, Haug U, Weigel K, Bahner U, Wanner C, Schneider H, Henle T, Ritz E: Glucose degradation products in PD fluids: Do they disappear from the peritoneal cavity and enter the systemic circulation? *Kidney Int* 63: 298–305, 2003
21. Wieslander A, Linden T, Musi B, Carlsson O, Deppisch R: Biological significance of reducing glucose degradation products in peritoneal dialysis fluids. *Perit Dial Int* 20[Suppl 5]: S23–S27, 2000
22. Nakamura S, Tobita K, Tachikawa T, Akamatsu S, Ohno Y, Kan A, Katsuragawa M, Yasumura K, Miyazaki S, Sakai S, Morita T, Hirashawa Y, Niwa T: Immunohistochemical detection of an AGE, a ligand for macrophage receptor, in peritoneum of CAPD patients. *Kidney Int Suppl* 84: S152–S157, 2003
23. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813–820, 2001
24. Schmidt AM, Yan SD, Wautier JL, Stern D: Activation of receptor for advanced glycation end products: A mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. *Circ Res* 84: 489–497, 1999
25. Hofmann MA, Drury S, Hudson BI, Gleason MR, Qu W, Lu Y, Lalla E, Chitnis S, Monteiro J, Stickland MH, Bucciarelli LG, Moser B, Moxley G, Itescu S, Grant PJ, Gregersen PK, Stern DM, Schmidt AM: RAGE and arthritis: The G82S polymorphism amplifies the inflammatory response. *Genes Immun* 3: 123–135, 2002
26. Ogata S, Yorioka N, Nishida Y, Shao JC, Yamakido M: Expression of receptor for advanced glycation end product mRNA by human peritoneal mesothelial cells. *Nephron* 86: 245–246, 2000
27. Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y,

Bucciarelli LG, Rong LL, Moser B, Markowitz GS, Stein G, Bierhaus A, Liliensiek B, Arnold B, Nawroth PP, Stern DM, D'Agati VD, Schmidt AM: RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. *Am J Pathol* 162: 1123–1137, 2003

28. Bierhaus A, Haslbeck KM, Humpert PM, Liliensiek B, Dehmer T, Morcos M, Sayed AA, Andrassy M, Schiekofer S, Schneider JG, Schulz JB, Heuss D, Neundorfer B, Dierl S, Huber J, Tritschler H, Schmidt AM, Schwaninger M, Haering HU, Schleicher E, Kasper M, Stern DM, Arnold B, Nawroth PP: Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J Clin Invest* 114: 1741–1751, 2004
29. Sakaguchi T, Yan SF, Yan SD, Belov D, Rong LL, Sousa M, Andrassy M, Marso SP, Duda S, Arnold B, Liliensiek B, Nawroth PP, Stern DM, Schmidt AM, Naka Y: Central role of RAGE-dependent neointimal expansion in arterial restenosis. *J Clin Invest* 111: 959–972, 2003
30. Tauer A, Knerr T, Niwa T, Schaub TP, Lage C, Passlick-Deetjen J, Pischetsrieder M: In vitro formation of N(epsilon)-(carboxymethyl)lysine and imidazolones under conditions similar to continuous ambulatory peritoneal dialysis. *Biochem Biophys Res Commun* 280: 1408–1414, 2001
31. Henle T, Deppisch R, Beck W, Hergesell O, Hansch GM, Ritz E: Advanced glycated end-products (AGE) during haemodialysis treatment: Discrepant results with different methodologies reflecting the heterogeneity of AGE compounds. *Nephrol Dial Transplant* 14: 1968–1975, 1999
32. Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, Kislinger T, Stern DM, Schmidt AM, De Caterina R: Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: A mechanism for amplification of inflammatory responses. *Circulation* 105: 816–822, 2002
33. Bierhaus A, Schiekofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J, Hong M, Luther T, Henle T, Kloting I, Morcos M, Hofmann M, Tritschler H, Weigle B, Kasper M, Smith M, Perry G, Schmidt AM, Stern DM, Haring HU, Schleicher E, Nawroth PP: Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. *Diabetes* 50: 2792–2808, 2001
34. Ha H, Yu MR, Lee HB: High glucose-induced PKC activation mediates TGF-beta 1 and fibronectin synthesis by peritoneal mesothelial cells. *Kidney Int* 59: 463–470, 2001
35. Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA: Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 7: 1291–1297, 2001
36. Bierhaus A, Humpert PM, Stern DM, Arnold B, Nawroth PP: Advanced glycation end product receptor-mediated cellular dysfunction. *Ann N Y Acad Sci* 1043: 676–680, 2005
37. Kang DH, Hong YS, Lim HJ, Choi JH, Han DS, Yoon KI: High glucose solution and spent dialysate stimulate the synthesis of transforming growth factor-beta1 of human peritoneal mesothelial cells: Effect of cytokine costimulation. *Perit Dial Int* 19: 221–230, 1999
38. Mortier S, Faict D, Schalkwijk CG, Lameire NH, De Vriese AS: Long-term exposure to new peritoneal dialysis solutions: Effects on the peritoneal membrane. *Kidney Int* 66: 1257–1265, 2004
39. Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I, Bucala R, Adamis AP: Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest* 101: 1219–1224, 1998
40. Tamarat R, Silvestre JS, Huijberts M, Benessiano J, Ebrahimian TG, Duriez M, Wautier MP, Wautier JL, Levy BI: Blockade of advanced glycation end-product formation restores ischemia-induced angiogenesis in diabetic mice. *Proc Natl Acad Sci U S A* 100: 8555–8560, 2003
41. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS, Stern D, Schmidt AM: Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat Med* 4: 1025–1031, 1998
42. Mandl-Weber S, Cohen CD, Haslinger B, Kretzler M, Sitter T: Vascular endothelial growth factor production and regulation in human peritoneal mesothelial cells. *Kidney Int* 61: 570–578, 2002