Endothelial cell adhesion molecule and PMNL response to inflammatory stimuli and AGE-modified fibronectin

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Endothelial cell adhesion molecule and PMNL response to inflammatory stimuli and AGE-modified fibronectin.

Background. Atherosclerotic vascular disease is the leading cause of death in patients with diabetes mellitus and end-stage renal disease. Advanced glycation end products (AGEs) are strongly suggested to be involved in the pathogenesis of atherosclerosis in these patients who also frequently experience infectious complications. We hypothesized that the interaction of AGEs and inflammatory mediators contributes to the up-regulation of endothelial cell activation.

Methods. We investigated the effect of advanced glycated fibronectin in the presence or absence of inflammatory stimuli on the endothelial cell surface and mRNA expression of cell adhesion molecules. Furthermore, the influence of advanced glycated fibronectin on the transendothelial migration pattern of polymorphonuclear cells was analyzed.

Results. Exposure to advanced glycated fibronectin together with inflammatory stimuli such as interleukin (IL)-1α, tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS) led to a significant increase in the surface expression of the cell adhesion molecules E-selectin, ICAM-1, VCAM-1 and PECAM-1 on endothelial cells. Soluble AGEs in combination with advanced glycated fibronectin significantly enhanced the endothelial cell surface expression of ICAM-1, VCAM-1 and PECAM-1, whereas this was not the case for E-selectin. At the transcriptional level short-time exposure of endothelial cells to advanced glycated fibronectin and inflammatory mediators resulted in an increased expression of E-selectin, ICAM-1 and VCAM-1 mRNA levels, whereas PECAM-1 repeatedly showed a significant decrease of gene transcript levels. An increase of mRNA levels was also observed for E-selectin, ICAM-1, VCAM-1 and PECAM-1 following incubation with a combination of advanced glycated fibronectin and soluble advanced glycation end-products. Furthermore, polymorphonuclear cells responded with a sevenfold increase in transendothelial migration following exposure of endothelial cells to advanced glycated fibronectin and inflammatory mediators.

Conclusions. These results suggest that the combination of matrix glycation and inflammation up-regulates the activation of the endothelial cell adhesion cascade, a mechanism that might contribute to the increased burden of atherosclerotic morbidity and mortality in patients suffering from diabetes mellitus or chronic renal failure.

The incidence of vascular disease morbidity and mortality is manifold increased in patients with diabetes mellitus and in patients with end-stage renal failure [1–3]. One key event in the development of atherosclerosis is the adhesion of leukocytes to the vascular endothelium [4]. Cytokine inducible cellular adhesion molecules (CAM) expressed on the surface of endothelial cells including E-selectin, intercellular CAM-1 (ICAM-1), vascular CAM-1 (VCAM-1), and platelet-endothelial CAM (PECAM) have been shown to participate in the atherosclerotic plaque formation [5–13]. Additionally, advanced glycation end products (AGEs) have been suggested to be involved in the pathogenesis of atherosclerosis, since both circulating and connective tissue AGEs have been identified in patients suffering from diabetes mellitus or end-stage renal failure [14, 15].

AGEs represent a heterogeneous group of metabolically altered substrates that accumulate during long-term hyperglycemia. Advanced glycation starts with a non-enzymatic condensation reaction between circulating blood glucose and amino groups, forming slowly reversible Amadori products. Upon prolonged exposure to elevated plasma glucose levels, these early glycated products are transformed into AGEs that are now irreversibly bound to proteins, lipids and nucleic acids [16]. Thus, AGE-formation alters long-lived molecules in particular, such as extracellular matrix components, and increases with age.

One candidate extracellular matrix protein that might be altered by advanced glycation is fibronectin, a component of the basal membrane of the blood vessel wall that is covered by endothelial cells. Endothelial cells are able to interact with AGEs via specific AGE receptors resulting in
Table 1. Relative surface and mRNA expression of E-selectin, ICAM-1, VCAM-1 and PECAM cultured on native fibronectin (N-FN) or AGE-fibronectin (AGE-FN) and stimulated with IL-1α, TNF-α, LPS or AGE-albumin (sAGE) following different times of incubation (in comparison to culture of cells on native fibronectin in medium; n.d., not done).

<table>
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<tr>
<th>Expression of</th>
<th>Location</th>
<th>hours</th>
<th>N-FN+IL-1α</th>
<th>N-FN+TNFα</th>
<th>N-FN+LPS</th>
<th>N-FN+sAGE</th>
<th>AGE-FN-Co</th>
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<tr>
<td>E-selectin</td>
<td>surface, 4 hr</td>
<td>380 ± 27</td>
<td>229 ± 18</td>
<td>366 ± 29</td>
<td>112 ± 6</td>
<td>130 ± 15</td>
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<td>surface, 18 hr</td>
<td>184 ± 30</td>
<td>124 ± 17</td>
<td>123 ± 25</td>
<td>80 ± 5</td>
<td>91 ± 10</td>
<td>302 ± 30</td>
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<td>mRNA, 2.5 hr</td>
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<td>n.d.</td>
<td>317 ± 69</td>
<td>237 ± 62</td>
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<td>mRNA, 10 hr</td>
<td>483 ± 42</td>
<td>n.d.</td>
<td>106 ± 25</td>
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<td>ICAM-1</td>
<td>surface, 4 hr</td>
<td>185 ± 14</td>
<td>162 ± 14</td>
<td>187 ± 17</td>
<td>104 ± 2</td>
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<td>surface, 18 hr</td>
<td>368 ± 23</td>
<td>288 ± 19</td>
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<td>536 ± 41</td>
<td>611 ± 46</td>
<td>124 ± 8</td>
<td>167 ± 14</td>
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<td>963 ± 106</td>
<td>757 ± 74</td>
<td>776 ± 84</td>
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<td>n.d.</td>
<td>171 ± 22</td>
<td>137 ± 21</td>
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<td>surface, 18 hr</td>
<td>107 ± 5</td>
<td>106 ± 5</td>
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<td>91 ± 5</td>
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Abbreviations are in the Appendix.

an increased vascular permeability and the up-regulation of endothelial cell surface procoagulants [17]. Furthermore, AGEs quench nitric oxide and thus interfere with endothelium-dependent vascular relaxation [18].

Since AGEs are strongly suggested to be involved in the pathogenesis of atherosclerosis in patients with diabetes mellitus and renal failure who also frequently experience infectious complications [19, 20], the hypothesis was raised that the interaction of AGEs and inflammatory mediators enhances the up-regulation of endothelial cell adhesion molecules and the transendothelial migration of leukocytes. This interaction could contribute to the multifactorial acceleration of atherosclerosis in these patients who frequently experience episodes of infections.

METHODS

Reagents and antibodies

Fibronectin and endothelial cell growth supplement were purchased from Collaborative Biomedical Products (Bedford, MA, USA). Fetal calf serum, collagenase type I, Hank’s solution, Dulbecco’s phosphate-buffered saline (PBS) and RPMI 1640 medium were obtained from Gibco Laboratories (Grand Island, NY, USA). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). Bovine serum albumin, β2-microglobulin, complete Freund’s adjuvant, fluorescein isothiocyanate (FITC), RNAse and endotoxin of E. coli serotype 026:B6 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human IgG was from Biocheime GmbH (Vienna, Austria). Glucose-6-phosphate and 2,2'-Azinobis-3-ethylbenzthiazoline-sulfonic acid (ABTS) were from Boehringer Mannheim (Mannheim, Germany). RNazol-B was obtained from Biotech Lab. Inc. (Houston, TX, USA). Calcein AM was purchased from Molecular Probes (Eugene, OR, USA). The oligonucleotide primers were purchased from Pharmacia (Vienna, Austria) and are summarized in Table 1. Ampli-Taq DNA-Polymerase was from Perkin Elmer Cetus (Norwalk, CT, USA). The 6% polyacrylamide mini gels were from Novex (San Diego, CA, USA). The limulus assay (Coatest Endotoxin) was obtained from Boehringer Ingelheim (Ingelheim, Germany).

The antibodies 5.6E (PECAM), 84H10 (ICAM-1), 1.2B6 (E-selectin), 1G2 (endoglin), 1G11 (VCAM-1) and 4F9 (von Willebrand factor) were obtained from Immunotech (Marseille Cedex, France). Interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and blocking antibodies BBIG-I (ICAM-1), BBIG-E4 (E-selectin) and BBIG-V1 (VCAM-1) were supplied by R&D Systems (Minneapolis, MN, USA). FITC labeled goat F(ab)2 anti-mouse IgG+IgM and non-specific anti-IgG1+IgG2a antibodies were from An der Grub (Vienna, Austria).

Preparation of advanced glycated fibronectin

Advanced glycated fibronectin (AGE-fibronectin) was prepared using fibronectin (1 mg/ml) in a reaction mixture containing 5 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM glucose-6-phosphate (G-6-P) and proteinase inhibitors (1 mM phenyl-methyl-sulfonil-fluoride, 5 mM EDTA (pH 8.0), trypsin inhibitor 50 μg/ml, aprotinin 2 μg/ml, 1 mM aminocaproic acid and 0.02% sodium azide). Native fibronectin, which served as a control, was incubated under identical conditions without G-6-P. Using Limulus assay, the polysaccharide (LPS) level in the AGE-fibronectin stock solution was determined to be < 0.6 ng/ml, which was further diluted (1:40). Only AGE-fibronectin without detectable LPS was used in all experiments. Quality controls to assess formation of AGE-fibronectin included the measurement of the fluorescence intensity (at 370 nm and 440 nm, respectively) using a spectrofluorometer (Shimadzu RF-551; the specific fluorescence intensity of AGE-fibronectin used in all experiments was 15000 RFUs, native fibronectin showed no fluorescence) and the analysis of the
inhibition properties of AGE antibody binding in the solid phase immunoassay (as described below).

Generation of anti-AGE antibodies using AGE-RNAse

AGE-RNAse was prepared by incubating RNAse (25 mg/ml) in PBS (pH 7.4) containing 0.5 m G-6-P, 0.1% sodium-azide, 100 U/ml penicillin and 100 µg/ml streptomycin for five weeks at 37°C under sterile conditions. Prior to incubation the reaction mixture was passed through a Detoxigel column (Pierce, Rockford, IL, USA) to eliminate contaminating LPS. At the end of the incubation, unbound G-6-P was removed by dialysis against PBS.

For the preparation of anti-AGE antibodies, white female New Zealand rabbits were immunized with 10 mg/ml or 25 mg/ml of AGE-RNAse emulsified in an equal volume of complete Freund’s adjuvant. Two hundred and fifty microliters were injected subcutaneously every two weeks at two different sites of the back of the animals. A total of six injections were given. After the second injection blood was collected for the determination of antibody titers prior to each injection. High titers were observed ten weeks after the immunization. AGE-antibody titers were determined by a solid-phase assay as described below.

Advanced glycedated end product immunoassay

Advanced glycedated albumin (AGE-albumin) was prepared using the same conditions as described above for AGE-RNAse, but with a bovine serum albumin concentration of 10% (RFU 2300). Ninety-six-well microtiter plates were coated with AGE-albumin (50 µg/ml) for one hour at 37°C followed by an incubation period of three days at 4°C. After washing with PBS, the plates were incubated with 0.4% BSA in PBS for two hours at room temperature to prevent nonspecific binding. The AGE containing samples were diluted in 0.4% BSA/PBS (1:10) and were incubated in glass vials with the rabbit anti-AGE-RNAse antisera (1:1000) for one hour at 37°C and 16 hours at 4°C. These mixtures (0.2 ml) were added to the AGE-albumin coated wells and incubated for one hour at 37°C followed by a final incubation step of 30 minutes at 4°C. After thorough washing with PBS peroxidase labeled anti-rabbit antibody (1:2000) was added for one hour at 37°C and 30 minutes at 4°C. Binding of the second antibody was determined by a color reaction induced by the addition of ABTS (0.1% solution in diluent buffer) and the measurement of the optical density at 405 nm. The degree of inhibition of anti-AGE antibody binding to the solid phase was proportional to the AGE concentration of the sample. AGE-modified β2-microglobulin (AGE-β2m, 25 mg/dl), which was prepared under the same conditions as described for AGE-RNAse, was used as a reference. Results were expressed in molar equivalents of AGE-β2m in relative florescence units (RFU). The molar equivalent of AGE-fibronectin was 25 to 34 µg/ml AGE-β2m, and 17 µg/ml for AGE-albumin. Neither native albumin nor native fibronectin showed any inhibition properties of AGE antibody binding in the solid phase immunoassay.

Endothelial cell culture

Human umbilical cord vein endothelial cells (HUVEC) were cultured according to Jaffe et al [21]. Briefly, cells were pooled after collagenase treatment and seeded on six-well cell culture plates coated with native fibronectin (2.5 µg/cm²) or AGE-fibronectin (2.5 µg/cm²). Cells were grown in RPMI 1640 medium supplemented with 20% FCS, 1% L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin and 1 µg/ml fungizone and after the first day with 5% FCS under standard cell culture conditions (humidified atmosphere, 5% CO₂, 37°C). Reaching confluence within three to four days, primary endothelial cell cultures were used for all flow cytometric analyses and preparation of total RNA. All cells showed cobblestone morphology that did not differ between cells grown on native fibronectin or AGE-fibronectin and showed uptake of acetylated LDL and expression of von Willebrand factor and angiotensin converting enzyme. The presence of contaminating CD14 positive cells (monocytes) was excluded by flow cytometry.

Immunofluorescence analysis of cellular adhesion molecule expression

Endothelial cells were incubated in native fibronectin or AGE-fibronectin coated six-well plates in RPMI 1640 medium containing either IL-1α (100 U/ml), TNF-α (500 U/ml), LPS (1 µg/ml), or AGE-albumin (500 µg/ml) or medium alone (control) for four hours or 18 hours. After incubation confluent HUVEC-monolayers were detached by scraping with a rubber policeman in ice-cold PBS. Cells were then washed twice and centrifuged at 300 g for 10 minutes. Fifty microliters of the cell suspension (5 × 10⁶)
were incubated with 50 μl of the respective monoclonal antibody (mAb; 5 μg/ml) for 30 minutes at 4°C. After washing, cells were stained with FITC-conjugated goat F(ab')2 anti-mouse IgG+IgM antibodies for 30 minutes at 4°C. Following two additional washing steps endothelial cells were analyzed using a FACSScan (Becton Dickinson, Mountain View, CA, USA) and the LYSIS II software package (Becton Dickinson). The cell viability of HUVEC prior to staining procedures was typically > 95% as determined by trypan blue exclusion. Cytosfluorometric analysis of cellular adhesion molecule cell surface expression was performed in a total of 10 independent cell culture experiments. Data are given as mean fluorescence intensity in percent of control expriments.

Fig. 1. Expression of E-selectin. (A) Surface expression (mean fluorescence intensity, mfi) of E-selectin on HUVEC cultured on native fibronectin or on AGE-fibronectin after four hours or 18 hours of incubation with IL-1α, TNF-α, LPS, AGE-albumin (sAGE), or medium as determined by flow cytometric analysis. The results are given as percentages of expression on non-stimulated HUVEC grown on native fibronectin ± SEM. P values (**P < 0.01) refer to comparison of equally treated cells grown on native fibronectin or AGE-fibronectin. (B) Semiquantitative RT-PCR analysis of E-selectin transcript levels in endothelial cells. Cells were cultured on native fibronectin or AGE-fibronectin for 2.5 hours or 10 hours with IL-1α, AGE-albumin (sAGE) or medium alone. Results were corrected by the ABL values of the respective cDNA. The results are given as percentages of expression in non-stimulated HUVEC grown on native fibronectin ± SEM. P values (*P < 0.05) refer to comparison of equally treated cells grown on native fibronectin or AGE-fibronectin.

Human umbilical cord vein endothelial cells were cultured on 175 cm² cell culture flasks coated with native fibronectin (2.5 μg/cm²) or AGE-fibronectin (2.5 μg/cm²) in the presence of IL-1α (100 U/ml), AGE-albumin (500 μg/ml) or medium alone for 2.5 hours or 10 hours. After incubation, cells were washed twice in diethylpyrocarbonate-treated PBS and 1 × 10⁷ cell aliquots were lysed by addition of 1.8 ml RNeasy-B. Total RNA was extracted as described [22]. The quality of isolated RNA was controlled by electrophoresis through formaldehyde agarose gels. High quality RNA was quantitated by measuring absorption at
260 nm and 1 μg of total RNA was subjected to cDNA synthesis as described elsewhere [23].

For semiquantitative analysis of PECAM, ICAM-1, E-selectin and VCAM-1 mRNA, a RT-PCR protocol, which allows measurement of relative transcript levels was applied [24]. The oligonucleotide primer sequences used for PCR amplification of the respective mRNAs have been previously described [25, 26]. Polymerase chain reaction amplification of ABL transcripts was used as a reference to assess variations of total RNA or cDNA between samples as described previously [27]. The linear ranges of PCR amplifications were established as a function of cycle number and cDNA dilutions for each primer pair [24].

Reaction conditions included 3 μl cDNA, 10 to 50 pmol of each primer, 2.5 to 3.0 mM MgCl₂, 200 mM of each dNTP, 1.25 U Ampli-Taq DNA polymerase and α (³²P)-dCTP (150,000 cpm) in a 50 μl reaction volume. The thermal cycling conditions were denaturation at 94°C (1 min), annealing at 57°C − 65°C (1 min) and extension at 72°C (1 min), preceded by an initial denaturation step at 94°C for five minutes and followed by a terminal extension of 10 minutes at 72°C. The PCR amplification products were subjected to 6% polyacrylamide gels and dried gels were exposed to Kodak XAR-5 films at −70°C for 12 hours.

For quantification of PCR products incorporated α(³²P)-dCTP was measured on autoradiograms using an imaging densitometer (model 670; BioRad Laboratories, Hercules, CA, USA) and the system’s volume integration program (BioRad Gel DOC 1000 system, Molecular Analyst/PC software). Potential differences of total cellular RNA/cDNA in
PCR analyses were corrected by dividing PECAM, ICAM-1, E-selectin and VCAM-1 values by the ABL value obtained from the respective cDNA. Each relative transcript level was measured in a total of 12 PCR analyses using six cDNAs that were synthesized in duplicates from RNAs of three independent cell culture experiments.

Transendothelial polymorphonuclear leukocyte migration

Isolation of polymorphonuclear leukocyte (PMNL) was performed as described previously [28]. Briefly, heparinized peripheral blood obtained from healthy volunteers was layered over Ficoll-Paque (1.077 g/ml). After an initial incubation step of 45 minutes at room temperature, the supernatants were layered on 63% Percoll underlayed with 72% Percoll. Following centrifugation at 500 g for 25 minutes at room temperature, cells were washed twice in Ca²⁺- and Mg²⁺- free Hank's solution. The cell pellets were resuspended in RPMI 1640 medium at a final concentration of 2.5 × 10⁶ cells/ml. The cell viability in all experiments was >95% as determined by trypan blue exclusion.

Human umbilical vein endothelial cells were grown on native fibronectin or AGE-fibronectin coated permeable membrane inserts (diameter of 9 mm, pore size of 3.0 μm) of a 24 multiwell double-chamber system and were subsequently incubated with IL-1α (100 U/ml) or medium (control) for four hours and 18 hours. Reaching confluence, HUVEC monolayers of passages 2 to 4 were incubated with PMNL (5 × 10⁵) in the upper chambers of the permeable inserts for two hours. At the end of the incubation period,
the membrane inserts containing the non-migrated leukocytes were discarded. Without further washing or transfer steps, migrated leukocytes in the lower chamber were immediately exposed to 2 mM of the fluorescent dye calcein-acetoxymethylester (calcein-AM). Following incubation in the dark at room temperature for 30 minutes (mild shaking), the relative fluorescence intensity was measured using the cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA, USA). Absolute cell counts were determined by comparison with dilution series of calcein-AM labeled PMNLs cultured in RPMI 1640 medium as described [29].

For blocking experiments, HUVEC were pre-incubated for one hour with 300 μl of a mixture containing blocking antibodies against PECAM (50 μg/ml), E-selectin (50 μg/ml), ICAM-1 (50 μg/ml) and VCAM-1 (60 μg/ml).
Polymorphonuclear leukocytes were pre-treated with human IgG (10 mg/ml) at 37°C for one hour, to saturate Fc-gamma receptors and to prevent nonspecific binding to antibodies. Following preincubation, PMNL were coincubated with HUVEC on the membrane inserts for two hours. The number of transmigrated PMNL in individual wells was determined as described above. Each experiment was performed in triplicates for at least three times.

**Statistical analyses**

All data are presented as means ± SEM. Statistical analysis included ANOVA and Student’s t-test, which were applied when appropriate.

**RESULTS**

**AGE-fibronectin up-regulates cytokine induced expression of endothelial cell adhesion molecules**

The detailed results of flow cytometric analysis of cell surface expression and of mRNA expression of endothelial cell adhesion molecules are indicated in Table 1. Cell surface expression of E-selectin, ICAM-1, and VCAM-1 was remarkably higher on HUVEC grown on AGE-fibronectin after four hours of stimulation with IL-1α, TNF-α or LPS, compared to HUVEC cultured on native fibronectin as determined by flow cytometric analysis (Figs. 1, 2, and 3). Additionally, the presence of IL-1α led to a

![Graph showing expression of VCAM-1](image-url)
prolonged elevated E-selectin expression on HUVEC grown on AGE-fibronectin compared to standard culture conditions (Fig. 1). IL-1α, TNFα and LPS enhanced ICAM-1 and VCAM-1 expression on HUVEC after 18 hours of culture on AGE-fibronectin (Figs. 2, 3). AGE-fibronectin significantly up-regulated the IL-1α induced expression of E-selectin mRNA at both timepoints, whereas ICAM-1, and VCAM-1 transcripts were increased after 2.5 hours (Figs. 1 to 3).

Neither AGE-fibronectin nor AGE-albumin nor a combination of both enhanced the cell surface expression of E-selectin in the absence of inflammatory stimuli (Fig. 1). On the other hand, AGE-fibronectin alone increased the surface expression of ICAM-1 or VCAM-1 on HUVEC after four hours of culture. An additive effect of AGE-albumin on AGE-fibronectin-induced ICAM-1 and VCAM-1 surface expression and transcript levels was observed after long-term incubation (Figs. 2 and 3), whereas
the combination of AGE-albumin and AGE-fibronectin and even AGE-fibronectin alone increased E-selectin transcript levels already after 2.5 hours. No significant additional effect of AGE-albumin on the expression of E-selectin mRNA in HUVEC was observed at the later timepoint (Fig. 1).

PECAM expression on HUVEC was slightly, but significantly enhanced by culture on AGE-fibronectin for four hours or 18 hours with or without stimulating agents (Fig. 4). AGE-albumin showed a weak, but significant stimulatory effect on the PECAM transcript levels of endothelial cells grown on AGE-fibronectin at either timepoint (Fig. 4). Most strikingly, IL-1α significantly reduced PECAM mRNA expression independent from the presence of AGE-fibronectin (Fig. 4).

**Effect of AGE-fibronectin on transendothelial migration of PMNLs**

Confluent HUVEC monolayers grown on native fibronectin or AGE-fibronectin were incubated for four hours or 18 hours with IL-1α or with medium (control) prior to co-incubation with PMNL. The spontaneous migration of unstimulated PMNL through HUVEC-monolayers was increased about threefold if endothelial cells were grown on AGE-fibronectin for four hours and 18 hours compared to the migration pattern obtained on native fibronectin.
fibronectin (Fig. 5A). There was a fivefold increase of PMNL migration through IL-1α activated endothelium cultured on native fibronectin that was more than sevenfold increased if endothelial cells were grown on AGE-fibronectin (Fig. 5A). To demonstrate that the enhanced migration of PMNLs is due to the AGE-fibronectin induced expression of cellular adhesion molecules, we performed additional migration experiments in the presence of a cocktail containing blocking antibodies against E-selectin, ICAM-1, VCAM-1, and PECAM. Blocking antibodies suppressed PMNL migration through IL-1α stimulated endothelial cells cultured on AGE-fibronectin to the levels of migration through resting endothelial cells on AGE-fibronectin (Fig. 5B), whereas antibodies against endoglin (antibody 1G2) and von Willebrand factor had no effect on PMNL migration (data not shown).
DISCUSSION

The present study provides evidence that the interaction of inflammatory mediators and glycated matrix proteins results in a significant up-regulation of endothelial cell adhesion structures and in a tremendous increase of the transendothelial migration of PMNL.

It is well established that expression of cellular adhesion molecules on endothelial cells is enhanced by various cytokines during inflammation [9]. Furthermore, it has been described that cellular adhesion molecules are present on endothelial cells in atherosclerotic lesions [5, 7, 12]. Based on the observation that AGES can be found in diabetics and patients with uremia [15], the hypothesis was raised that AGES (matrix bound and soluble AGES) could modulate the cell surface expression of cellular adhesion molecules in these patients. In particular, we were interested in the effect of AGES on endothelial cell surface and mRNA expression of cellular adhesion molecules in the presence of inflammatory stimuli, since patients with diabetes mellitus and uremia frequently experience inflammatory episodes.

In the present study, we observed that AGE-matrix together with inflammatory stimuli significantly increased the cell surface expression of E-selectin, ICAM-1 and VCAM-1. Furthermore, a combination of AGE-albumin and matrix bound AGES enhanced the cell surface expression of ICAM-1 and VCAM-1. This effect of AGE-albumin was not observed for E-selectin. If endothelial cells were exposed to AGE-fibronectin alone, a significant up-regulation of ICAM-1 and VCAM-1 was observed (although to a much lesser extent), but not of E-selectin. At the molecular level, we could demonstrate that mRNA levels of E-selectin, ICAM-1 and VCAM-1 significantly increased following short-time stimulation of endothelial cells with matrix bound AGES and inflammatory mediators. Furthermore, the combination of AGE-albumin and

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**Fig. 5. Effect of AGE-fibronectin on transendothelial migration of PMNLs.** Confluent HUVEC monolayers grown on native fibronectin (□) or AGE-fibronectin (■) were incubated for four hours or 18 hours with IL-1α or with medium (Control) prior to co-incubation with $5 \times 10^5$ PMNL in a double chamber system. Results are shown as number of migrated PMNL ± SEM. (A) Migration of PMNL through HUVEC monolayers. *P < 0.05 refers to comparison between the numbers of migrated PMNL through equally treated HUVEC grown on native fibronectin or AGE-fibronectin. (B) Blocking experiments. Incubation of HUVEC monolayers with a cocktail of mAbs against PECAM, ICAM-1, E-selectin and VCAM-1 for one hour after incubation with medium (Control) or IL-1α and prior to co-incubation with PMNL. #P < 0.05 refers to comparison between the numbers of migrated PMNL through equally treated HUVEC grown on the same type of fibronectin with blocking mAbs (B) versus incubation without blocking mAbs (A).
AGE-fibronectin also resulted in an increase of ICAM-1, VCAM-1 and E-selectin transcript levels.

Several studies demonstrated that AGE-albumin enhances cell surface expression of endothelial ICAM-1 and VCAM-1 [30–32]. In our study, AGE-albumin enhanced the expression of ICAM-1 and VCAM-1 on endothelial cells grown on AGE-fibronectin, but not on endothelial cells cultured on native fibronectin. This discrepancy might be due to the different degrees of long-term glycation used in these studies. The concentration of albumin (500 μg/ml) in our study was higher than that used by Schmidt et al [32]. Though our albumin concentration was high, fluorescence intensity was low compared to that of AGE-fibronectin (2300 RFU vs. 15000 RFU, respectively). The fluorescence intensity of the AGE-albumin used in the present study, however, resembles the in vivo situation, where proteins with longer half life (such as, fibronectin) may be prone to more intense glycation compared to proteins with shorter half life (for example, serum albumin). Furthermore, we used G-6-P instead of glucose for in vitro glycation. Since proteins in diabetic patients are chronically exposed to hyperglycemic conditions for many years, we took highly concentrated (0.5 m) G-6-P as used by others [33] to achieve comparable in vitro glycation in a relatively short time.

In addition to E-selectin, ICAM-1 and VCAM-1, we show that matrix bound AGEs also enhanced the endothelial cell surface expression of PECAM in the presence of inflammatory mediators. This up-regulatory effect was also observed on endothelial cells cultured on AGE-fibronectin in the presence of AGE-albumin. At the gene transcript level we demonstrate that an inflammatory stimulus, such as IL-1α, led to a significant reduction of PECAM mRNA levels, probably due to enhanced translational and cell surface expression activities at the time points investigated in this study.

An interesting finding of the present study is the time dependent modulatory effect of AGE-fibronectin on the surface expression of cellular adhesion molecules. Cell adhesion molecules are responsible for different sequential steps in the leukocyte diapedesis process. E-selectin is involved in “rolling,” ICAM-1 and VCAM-1 in “firm adhesion” and PECAM, ICAM-1 and VCAM-1 in “trans-endothelial migration” of leukocytes [34, 35]. Our results indicate that the cooperation of inflammatory cytokines and AGE-fibronectin leads to a prolonged expression of “early” endothelial adhesion molecules and to a premature expression of “late” adhesion molecules. The co-stimulation via AGE-fibronectin and IL-1α, which is supposed to frequently occur in diabetic or renal failure patients, suggests a mechanism that enhances leukocyte recruitment to the blood vessel wall and finally may be involved in the acceleration of atherogenesis [20, 21].

Migration of leukocytes across the endothelial barrier is one of the key events in atherosclerotic plaque formation [4, 36]. Therefore, we studied transendothelial migration of PMNLs following activation of endothelial cells with IL-1α and observed a more than sevenfold increase in PMNL migration if endothelial cells were grown on AGE-fibronectin. These results demonstrate that the exposure of endothelial cells to AGE-fibronectin enhances the cytokine-induced transendothelial migration of PMNL.

From previous studies with mononuclear phagocytes and AGE-albumin it seems likely that monocytes are able to adhere to and migrate through AGE modified endothelium [37]. They are retained in the subendothelial space due to a chemotactic effect of AGEs and the expression of receptors for AGE and CD49d/29. In the present study, we have used PMNL since they lack AGE-receptors and fibronectin-receptors (CD49d/29, VLA-4). Therefore, they could not be captured by fibronectin or AGE in our migration assay. Since AGE-compounds were described to have chemotactic effects on leukocytes [37], we performed blocking experiments, which clearly confirmed the involvement of IL-1α and AGE-fibronectin induced up-regulation of cellular adhesion molecule expression on HUVEC in transendothelial migration. Furthermore, in our study, growing endothelial cells on AGE-fibronectin did not result in enhanced production of IL-8 (data not shown), which is a potent chemoattractant for PMNL [38].

Studies from other groups demonstrated that advanced glycation of fibronectin does not alter the adhesion properties of endothelial cells on this growth surface and it does not modify spreading of HUVEC in cell culture experiments [33]. Therefore, the augmented transendothelial migration of PMNL cannot be explained by increased porosity of the endothelial cell monolayers. We therefore conclude that the increased transendothelial migration of PMNL through AGE-fibronectin versus native fibronectin in our experiments is due to cellular adhesion molecule-related interactions and was not due to chemotactic factors or structural alterations of the endothelial cell monolayer.

Adhesion of PMNL to endothelial cells and their matrix proteins as well as transendothelial migration of PMNL has recently received considerable interest in the pathogenesis of atherosclerotic lesions [4, 39, 40]. PMNL can release mediators including oxygen-derived free radicals, resulting in an impairment of endothelial cell function [41]. The damaged endothelium then exposes receptors for immunoglobulins and complement, which further promote adherence of PMNL to endothelial cells [42]. The terminal C5b-9 complement complex has been localized in atherosclerotic plaques, indicating that complement activation had occurred. This process was mediated by activation of PMNL [42]. Furthermore, it has been shown that PMNL cause endothelial dysfunction during ischemia and are involved in reperfusion injury [4]. Thus, our data support the concept that the interaction of leukocytes with endothelial cells exposed to glycated matrix proteins and inflammatory mediators is putatively involved in the development of
vascular pathology. These findings suggest some clinical importance of AGEs and inflammatory mediators, especially in patients with recurrent acute infectious episodes.

It was not the purpose of the present study to clarify the regulatory mechanisms of AGE modulated mRNA and protein expression of endothelial cellular adhesion molecules. According to Stern and colleagues [43], however, the NF-κB/IκBα system has been suggested to participate in the regulation of the expression of these molecules. It has been shown that IκBα is capable of displacing NF-κB p50/p65 from E-selectin- and VCAM-1 NF-κB binding elements, thereby negatively regulating the expression of these molecules. It might be possible that signaling via AGE proteins/AGE receptors influences this negative regulatory process that is supposed to prevent the inappropriate expression of adhesion molecules. By offsetting the physiologic and dynamic balance of the NF-κB/IκBα system, AGEs might promote the onset and the perpetuation of vascular disease. Another potential candidate for mediating AGE effects is the cytokine-induced enhancer complex, which provides an attractive model for superinduction of endothelial adhesion structures by the complex interaction of multiple transcription regulatory elements [44].

In conclusion, this is the first study to our knowledge demonstrating that the effect of an AGE-modified matrix protein, AGE-fibronectin, in combination with inflammatory stimuli, enhances the endothelial cellular adhesion molecule expression and augments transmigration of PMNL through cytokine activated endothelial cells. We provide evidence that irreversibly glycated matrix protein fibronectin modulates the endothelial expression of E-selectin, ICAM-1, VCAM-1 and PECAM both on the cell surface expressed protein and the mRNA-level, an effect that is enhanced by the presence of inflammatory stimuli. The irreversibly glycated matrix compound fibronectin caused a dramatic increase of transendothelial migration of PMNL. These findings represent a potentially important link between glycated matrix proteins, inflammation, and activation of the endothelial adhesion cascade that may be involved in vascular pathology contributing to the development of the excess atherosclerotic burden in patients with diabetes mellitus or chronic renal failure.

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APPENDIX

Abbreviations used in this article are: ABTS, 2,2’-Azinobis-3-ethylbenzthiazolene-sulfonic acid; AGE, advanced glycation end products; AGE-albumin, advanced glycated albumin; AGE-fibronectin, advanced glycated fibronectin; Bm, B2-microglobulin; BSA, bovine serum albumin; calcine-AM, calcine-acetoxyethylster; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; G-6-P, glucose-6-phosphate; HUVEC, human umbilical cord vein endothelial cells; ICAM, intercellular cellular adhesion molecule; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibodies; NF-κB, nuclear factor-kB; PBS, phosphate buffered saline; PECA1, platelet-endothelial cellular adhesion molecule; PMNL, polymorphonuclear leukocyte; RFU, relative fluorescence units; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; VCAM, vascular cellular adhesion molecule.

REFERENCES


30. SCHMIDT AM, ZHANG JH, CRANDALL J, CAO R, YAN SD, BRETTE J, STERN D: Interaction of advanced glycation endproducts with their endothelial cell receptor leads to enhanced expression of VCAM-1: A mechanism for augmented monocyte vessel wall interactions in diabetes. (abstract) *FASEB J* 8(Par t II):662, 1994


