Nonenzymatic Glycation of Fibronectin Impairs Adhesive and Proliferative Properties of Human Vascular Smooth Muscle Cells

F. Cavalot, G. Anfossi, I. Russo, E. Mularoni, P. Massucco, L. Mattiello, S. Burzacca, A.W.A. Hahn, and M. Trovati

Nonenzymatic glycation of proteins is involved in the pathogenesis of diabetes vascular complications. Extracellular matrix proteins are a prominent target for nonenzymatic glycation because of their slow turnover rates. The aim of this study was to investigate the influence of human fibronectin (F) nonenzymatic glycation on adhesion and proliferation of cultured human vascular smooth muscle cells (hVSMC). Incubation of human F with 500 mmol/L D-glucose at 37°C induced a time-dependent increase in fluorescence detectable at 440 nm after excitation at 363 nm. Nonenzymatic glycation did not affect binding of F itself to the plates. Adhesion of hVSMC to F increased with the increase of incubation time of the cells on the protein from 30 minutes up to 120 minutes and remained stable thereafter. Adhesion to glycated fibronectin (GF) was reduced in comparison to control F at all the different adhesion times. Adhesion of hVSMC to GF was reduced when F was exposed to glucose for 4, 9, or 28 days ($P = .0417$ to .0025), but not when F was exposed to glucose for 1 day. Adhesion of hVSMC to GF was reduced compared with adhesion to nonglycated F at all coating concentrations from 0.2 to 10 μg/mL ($P = .05$ to .014). Thus, nonenzymatic glycation of F impairs adhesion of hVSMC in vitro. Proliferation of hVSMC on F increased with increasing concentrations of the protein as coating agent (ANOVA: $P < .0001$ for both nonglycated F and GF). Proliferation with F glycated for 4, 9, and 28 days was reduced at concentrations of 1, 3, and 10 μg/mL as compared with proliferation with nonglycated F ($P = .0253$ to .0001). Proliferation on F glycated for only 1 day was not significantly reduced. When the number of hVSMC plated on control F was reduced by 25% to take into account the reduced adhesion, the number of cells that proliferated on F was still reduced. In conclusion, nonenzymatic glycation of F impairs adhesive and proliferative properties of hVSMC.

NONENZYMATIC glycation is a process involved in the pathogenesis of many late complications of diabetes mellitus.[1] It occurs also in nondiabetic subjects, where it proceeds with age, but it is greatly enhanced in diabetic patients.[2] All cellular proteins are susceptible to nonenzymatic glycation: however, since the process progresses slowly, proteins with a slow turnover rate are the most relevant targets. Among them there are the constituents of the extracellular matrix. Nonenzymatic glycation occurring in this compartment was shown to cause decreased proteolytic degradation of collagen[3] and to promote covalent attachment of circulating proteins, including growth modulators, to basement membrane.[4] Both phenomena may be relevant to the thickening of the basement membrane and the deposition of basement membrane–like material in the vessel wall that are structural changes of diabetes angiopathy.[1] Moreover, advanced glycation of proteins may be a signal for vascular remodeling.[5]

It has been shown that nonenzymatic glycation of fibronectin (F), laminin, type IV collagen, and gelatin (obtained from denatured collagen) dramatically decreases their ability to self-associate and to interact with each other and with heparin.[6-8] However, the effects of nonenzymatic glycation of extracellular matrix components on the vascular cell types embedded therein has not yet been extensively evaluated. In particular, as far as we know, no study has taken into account the influence of F glycation on the vascular smooth muscle cells (VSMC) that are deeply involved in the modulation of vascular tone and in atherogenesis.

F is a high–molecular-weight glycoprotein consisting of two similar subunits joined by disulfide bonds.[9] Its molecule contains functional domains that display different biological activities, eg, interaction with the cell surface or specific interaction with other extracellular matrix components, such as collagen, heparan sulfate, heparin, and hyaluronic acid.[10,11] The interaction of F with the specific cell surface receptors, known as integrins, through the specific sequence RGDS (arginine-glycine-aspartate-serine)[12] is important for cell morphology, adhesion, growth/differentiation, and migration, and cell-to-cell interaction.[13-16] F is present in large amounts in plasma, at the surface of cells, in the extracellular matrix,[17,18] and in basement membrane.[18,19] F is localized in the kidney mesangium and the pericyte-endothelial interstitium.[20] It is not clear whether F present in the subendothelial part of the glomerular basement membrane derives from local production[21,22] or from entrapment of circulating F by the glomerular filtration barrier.[23] F mRNA has been localized in the arterial wall; furthermore, the alternatively spliced form of F containing the EIIIA insert, which is known not to circulate in plasma, is synthesized at this level. These two facts indicate that in the arteries F is locally produced and not only infiltrated from plasma.[24] Regardless of its origin, F plays an important role in modulating the phenotype of vascular cells, both endothelial cells[25] and smooth muscle cells.[26] In vitro studies in which arterial smooth muscle interaction with F and laminin was evaluated have shown that newly synthetized F is incorporated into a network of pericellular and intercellular fibrils, whereas laminin forms a layer covering the cells in a basement membrane–like manner.[26] The same investigators conclude their study by stating that "fibronectin stimulated modulation of the [smooth muscle] cells from

From the Diabetes Unit, Department of Clinical and Biological Sciences, University of Turin, San Luigi Gonzaga Hospital-Orbassano, Turin, Italy; and the Department of Research, Basel University Hospital, Basel, Switzerland.

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Address reprint requests to M. Trovati, MD, Diabetes Unit, Department of Clinical and Biological Sciences of the University of Turin, Ospedale San Luigi Gonzaga, 10043 Orbassano, Torino, Italy.

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a contractile to a synthetic phenotype," and that "in the intact organism, fibronectin and laminin may fulfill important functions during development and growth of the vascular system, as well as in atherogenesis."[26]

A developmentally regulated increase in F synthesis by smooth muscle cells is involved in the intimal proliferation that occurs in ductus arteriosus.[27] F is indeed present in the wall of normal arteries, and is increased in the arteries in different disease states such as diabetes and atherosclerosis[28-31] and in an experimental model of hypertension induced by deoxycorticosterone acetate or angiotensin II administration.[32] F plays a pivotal role in wound healing[33,34] and in postinfarction cardiac remodeling.[35] Furthermore, it has been shown that upregulation of F synthesis induced by interleukin-1β in coronary artery smooth muscle cells is involved in the progressive intimal thickening associated with post–cardiac-transplant coronary arteriopathy in piglets.[36] Further studies concerning diabetes showed that levels of plasma F were elevated in diabetic retinopathy[37] and in incipient[38] and overt[39] diabetic nephropathy. Increased F has been found within diabetic human glomeruli at the level of mesangium in early and moderate stages of expansion[40] and is associated with mesangial or endothelial cell proliferation.[22] F synthesis is stimulated by interaction of advanced glycation end products with mesangial cells (a cell type closely related to smooth muscle cells) in culture, through an increase of platelet-derived growth factor synthesis.[41] Endothelial and mesangial cells in the presence of high glucose synthesize increased amounts of F, together with other extracellular matrix components.[42-44]

Since F is particularly susceptible to nonenzymatic glycation,[6] we aimed at investigating the biological effects of this process on F interactions with human VSMC (hVSMC), to add to the understanding of the pathogenesis of diabetic angiopathy. In particular, we aimed at evaluating (1) if nonenzymatic glycation of F influences hVSMC adhesion; (2) if different adhesion times influence the extent of hVSMC adhesion to either control or glycated F; (3) if different incubation times of F with glucose influence the extent of adhesion of hVSMC to the protein; (4) if nonenzymatic glycation of F influences hVSMC proliferation; and (5) if a putative effect of F nonenzymatic glycation on proliferation is attributable only to impaired adhesion or also to a direct effect on proliferation.

MATERIALS AND METHODS

Preparation of Nonenzymatically Glycated F

Human sterile F (Boehringer Biochemia, Mannheim, Germany) was nonenzymatically glycated at a concentration of 1 mg/mL in sterile phosphate-buffered saline ([PBS] 137 mmol/L sodium chloride, 2.68 mmol/L potassium chloride, 1.46 mmol/L potassium phosphate monobasic, 8 mmol/L sodium phosphate dibasic—tissue culture reagent; Sigma, St Louis, MO) supplemented with 500 mmol/L D-glucose in the presence of 1.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L EDTA, and 0.5 mmol/L EGTA as protease inhibitors, and 40 μg/mL gentamicin, pH 7.4. The solution was incubated at 37°C for 1, 4, 9, and 28 days; solution pH was checked at the beginning and end of the incubation and did not show any variation. Bovine serum albumin ([BSA] Sigma) was dissolved in PBS, filter-sterilized, and nonenzymatically glycated using conditions identical to those for glycation of F. Nonglycated control F (F) and BSA were treated identically except for D-glucose addition to PBS. After nonenzymatic glycation, protein preparations were dialyzed against PBS at 4°C with four changes of dialysis buffer; thereafter, protein concentrations were measured according to the method of Bradford,[45] and the occurrence of browning was assessed by spectrofluorimetry (emission at 440 nm and excitation at 363 nm) in a Perkin Elmer (Norwalk, CT) spectrofluorimeter.[2] Emission intensity was corrected for control values and expressed as arbitrary optical units per milligram protein. Values are expressed as the mean of three independent determinations. Protein solutions were stored as aliquots at −20°C before use. To evaluate physicochemical characteristics of F or glycated F (GF), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted[46] using a 7% separating gel and a 3% stacking gel. F and GF were dissolved in sample buffer (0.0625 mol/L Tris, 2% SDS, and 1.0 mol/L 1,4-dithiothreitol, pH 7.0) and boiled for 5 minutes and 2 to 10 μg were placed in each lane of the gel after addition of bromophenol blue at 1:4 with sample buffer. Neither F nor GF showed any evidence of degradation, and both showed an identical electrophoretic pattern.

Tissue Culture

Tissue for isolation of hVSMC was obtained from patients undergoing surgery, and consisted of microarterioles. Cells for culture were prepared as described and shown to accumulate acetylated low-density lipoprotein, and to stain positively with antibody to smooth muscle α-actin but negatively with antibody to factor VIII–associated antigen.[47] The cells were grown in minimum essential medium (MEM) supplemented with 10 mmol/L L-glutamine, 10 mmol/L TES/HEPES, 100 U/mL penicillin and streptomycin, 1 mmol/L sodium pyruvate, and 10% fetal calf serum, all from Boehringer Biochemia. Confluent cells were passaged by trypsinization and were used at passages 6 to 10 for the experimental procedures.

Assay for Binding of F and GF to 96-Well Microtiter Dishes

The extent of stable binding of F to polystyrene plates (96 wells; Sterilin Limited, Hounslow, UK) was evaluated according to methods previously described.[48] The wells were coated for 2 hours at 37°C with either F or GF dissolved in MEM at 0.2 to 10 μg/mL. Then the wells were blocked with MEM/0.25% BSA for an additional hour at 37°C and rinsed twice with PBS. Each F dilution was tested in quadruplicate, and each assay was repeated three times. One hundred microliters of a 1:200 dilution of goat anti-F antiserum (Boehringer Biochemia) was added to the coated wells, incubated at room temperature for 2 hours, and then washed three times with PBS/0.1% Tween 20 (Sigma). Subsequently, peroxidase-coupled antibodies (Sigma) were added and incubated for 1 hour, and then the wells were washed three times with PBS/0.1% Tween 20. As substrate for the enzyme, 30 mg orthophenylenediamine was diluted in 1 mL methanol and 100 mL distilled water, followed by addition of 10 μL 30% hydrogen peroxide. The reaction was stopped by addition of 50 μL 2.0-mol/L sulfuric acid. The microtiter plates were read at 492 nm in a Titertek Multiskan Plus MKII ELISA reader (Flow, Milan, Italy).

Adhesion Assay With hVSMC

The assay was performed as described by Bourdon and Ruoslathi.[49] Ninety-six–well polystyrene plates were coated as described above. Coating concentrations were between 0.2 and 10 μg/mL. Subconfluent monolayer cultures were harvested by treatment with Trypsin/EDTA/EGTA (0.05%:0.1%:0.01%) and resuspended in MEM/0.25% BSA at a concentration of 2.5×10^5/mL. One

hundred microliters of cell suspension was added to coated wells and incubated for different times ranging from 30 to 240 minutes. Since the number of cells adhering remained stable after 2 hours, this time was chosen for further experiments. In experiments in which the dose-response curve for adhesion was tested, each concentration of both F and GF was tested in four wells per plate, four plates were used for each experiment, and the experiments were repeated three times. After adhesion, the wells were washed three times with 0.2 mL per well PBS, and adherent cells were fixed to the substratum using 3.5% formaldehyde in PBS for 10 minutes and then stained with 0.5% toluidine blue in water. The number of adherent cells was counted in four fields in an inverted microscope at a magnification of 320-fold. As an alternative method to evaluate the number of adherent cells, the cells were lysed with 100 μL per well 1% SDS, and the optical density (OD) was read at 592 nm in the ELISA reader.[49] Since the two methods showed a high degree of correlation ($r = .901$, $P < .0001$), after the initial experiments, the second method was preferred as a faster way to evaluate adhesion.

As a control for unspecific binding, adhesion of hVSMC to either BSA or glycated BSA was evaluated by coating the wells of microtiter plates at 1 and 10 mg/mL. Experiments were then performed as just described.

When small amounts (30%) of GF were added to F, the biological properties of F on hVSMC proliferation were not significantly impaired.

Test of hVSMC Proliferation

For the assays, 96-well polystyrene plates were coated with different concentrations of either F or GF as described above. After coating, the plates were blocked with MEM/0.25% BSA for 1 hour at 37°C to avoid adhesion of cells to the wells through cell-surface F. Afterward, hVSMC were suspended at 5×10^4/mL in complete medium, and 100 μL was added per well. After a 3-day incubation at 37°C, the cells were treated with Trypsin/EDTA/EGTA and the number of cells per well was counted in a hemocytometer. Each concentration was tested in four wells per plate, four plates were used for each experiment, and the experiments were repeated three times.

To evaluate if GF influenced hVMSC proliferation by a pure effect on adhesion or by some direct effect on proliferation, in some experiments the number of VSMC placed on F to proliferate was reduced to mimic the number of cells adhering to GF.

When small amounts (30%) of GF were added to F, the biological properties of F on hVSMC proliferation were not significantly impaired.

Statistical Analysis

Results are expressed as the mean ± SEM. Statistical evaluation has been performed by ANOVA and, when appropriate, by Student's *t* test for unpaired data.

RESULTS

F Glycation

Emission at 440 nm after stimulation at 363 nm showed a progressive increase with the time of protein incubation in the presence of 500 mmol D-glucose versus incubation in the absence of glucose (arbitrary absorbance units, mean of three determinations). Emission for GF was 43 after 1 day, 199 after 4 days, 460 after 9 days, and 1,420 after 28 days; emission for F was 24 after 1 day, 25 after 4 days, 23 after 9 days, and 26 after 28 days.

F Binding to Polystyrene Plates

When the ability of F or GF (28 days) to coat polystyrene plates was evaluated with an ELISA system, they showed comparable behavior (Fig 1): OD increased with increasing protein concentrations (ANOVA, $P = .0001$ for either F or GF). The difference in ODs between F and GF at all coating concentrations used was not significant.

Adhesion of hVSMC to F

When we evaluated the effect of different incubation times on adhesion to 28-day GF or F, we observed that the number of adhering cells increased with time from 30 minutes up to 120 minutes; thereafter, the number of cells remained stable with time (Fig 2). For this reason, we chose 120 minutes as the usual incubation time. The reduction of adhesion to GF compared with F was present at each incubation time tested (Fig 2).

The effect of F glycation on adhesion of hVSMC was not statistically significant with day 1 GF, but reached statistical significance with day 4, 9, and 28 GF ($P = .0025$ to $.0465$; Fig 3). Adhesion to F itself was not different with day 1, day 4, day 9, or day 28 F; adhesion to GF itself was not different with day 4, day 9, or day 28 GF. Adhesion of hVSMC to F or GF (28 days) increased with increasing concentrations from 0.2 to 10 μg/mL (ANOVA, $P = .0001$ for F and GF; Fig 4); hVSMC adhesion to GF versus F was significantly reduced at all concentrations in the range from 0.2 to 10 μg/mL ($P = .05$ to $.014$). hVSMC did not show any adhesion to nonglycated or glycated BSA.

Proliferation of hVSMC on F

The effect of F glycosylation on hVSMC proliferation is shown in Fig 5: (1) cell proliferation showed a dose-

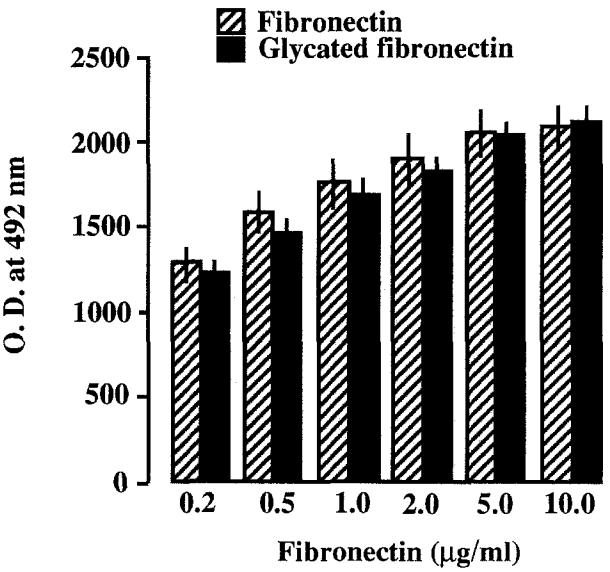

Fig 1. Coating ability of F or GF (28 days) on polystyrene plates, evaluated with an ELISA and expressed as OD at 492 nm. The increment of OD with different F concentrations (ANOVA) is significant ($P = .0001$) for both F and GF; the difference between each concentration of F and GF, evaluated by Student's *t* test, is not significant.

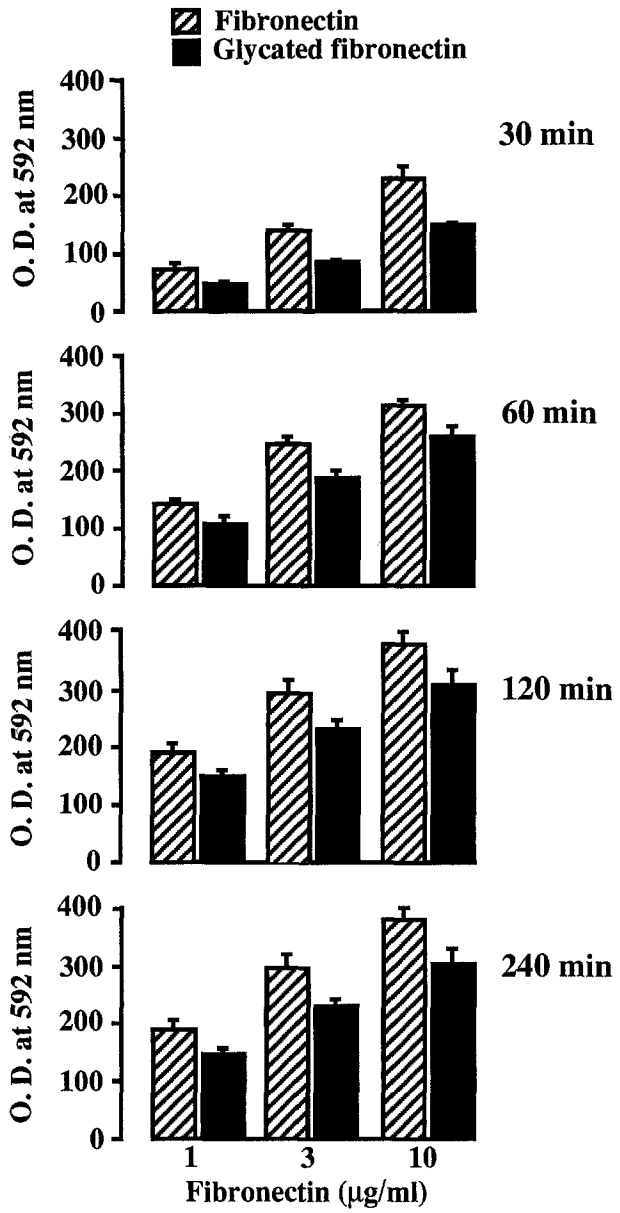

Fig 2. Effect of different adhesion times on adhesion of hVSMC to F incubated for 28 days without or with 500 mmol/L D-glucose. 5 × 10^4 cells per well were seeded and incubated for 30, 60, 120, or 240 minutes as indicated. The number of adhering cells increased with time up to 120 minutes and remained stable thereafter. Adhesion to GF was reduced at each time and concentrataion tested.

response behavior, since the cell number increased with the increased amount of coated F or GF (ANOVA, $P < .0001$); and (2) hVSMC proliferation on GF was not significantly reduced after 1 day of glycation. The reduction of hVSMC proliferation reached statistical significance with day 4, 9, and 28 GF at each concentration tested in comparison to proliferation on F ($P = .0002$ to $.0253$). Proliferation on F itself was not different with day 1, day 4, day 9, or day 28 F. Proliferation on GF itself was not different with day 4, day 9, or day 28 GF.

When the number of hVSMC that were seeded for each well coated with day 28 F was reduced by 25% (approximate average reduction of adhesion to GF *v* F), the number of cells that proliferated on GF versus F was still significantly reduced at each coating concentration tested (1 μg/mL, $P = .043$; 3 μg/mL, $P = .065$; 10 μg/mL, $P = .019$; Fig 6).

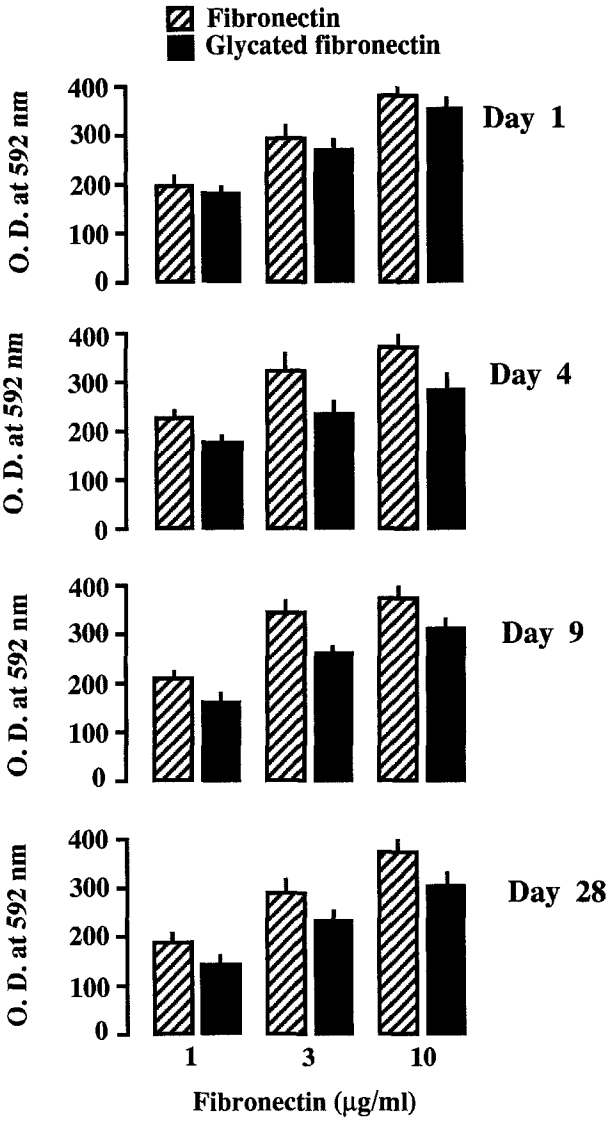

Fig 3. Effect of 1, 4, 9, and 28 days of F incubation with medium without or with 500 mmol/L D-glucose on 120 minutes adhesion of hVSMC to the molecule. Adhesion was evaluated as OD at 592 nm of cells stained with toluidine blue. For each concentration tested, adhesion to GF was lower than adhesion to F. The effect was not statistically significant with day 1 GF, but reached statistical significance thereafter: day 4 (GF *v* F), 1 μg/mL, $P = .0126$, 3 μg/mL, $P = .0335$, and 10 μg/mL, $P = .0147$; day 9 (GF *v* F), 1 μg/mL, $P = .0197$, 3 μg/mL, $P = .0025$, and 10 μg/mL, $P = .0417$; and day 28 (GF *v* F), 1 μg/mL, $P = .0417$, 3 μg/mL, $P = .0465$, and 10 μg/mL, $P = .0446$. Adhesion to F itself was not different with day 1, day 4, day 9, or day 28 F. Adhesion to GF itself was not different with day 4, day 9, or day 28 GF.

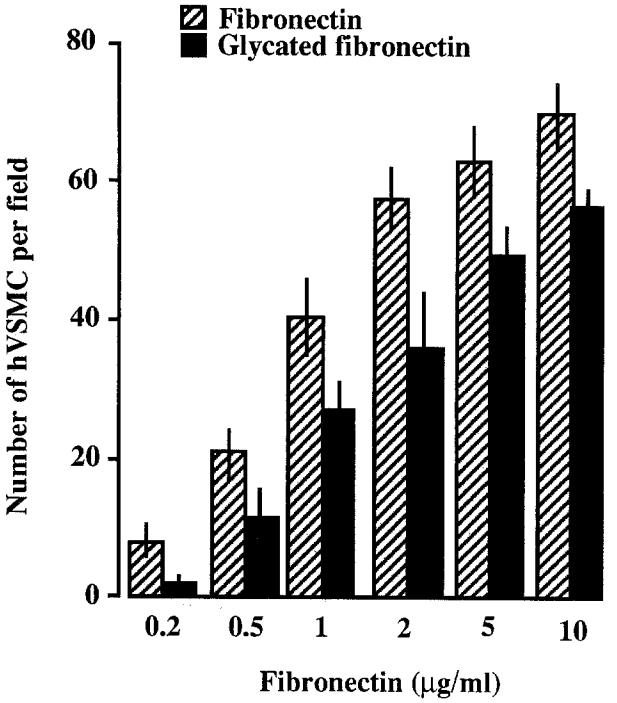

Fig 4. Adhesion of hVSMC to different coating concentrations of F or GF (28 days), evaluated as the number of cells per high-power field. ANOVA for both F and GF, $P = .0001$. The difference between F and GF was significant (Student's t test for unpaired data) from 0.2 to 10 μg/mL: 0.2 μg/mL, $P = .049$, 0.5 μg/mL, $P = .046$, 1.0 μg/mL, $P = .048$, 2 μg/mL, $P = .015$, 5 μg/mL, $P = .027$, and 10 μg/mL, $P = .042$.

DISCUSSION

This study shows that adhesion of hVSMC to F is impaired by nonenzymatic glycation of the protein; moreover, GF is less able to sustain hVSMC proliferation. These observations indicate that the glycation process impairs not only the reciprocal interactions of extracellular matrix proteins,[6,7] but also their ability to interact with cells. The reduction of both adhesion and proliferation was observed after 4, 9, and 28 days of F incubation with glucose; nonenzymatic glycation for 1 day was not able to induce a statistically significant decrease of adhesion and proliferation to F. Our experiments show that the impaired adhesion and proliferation of hVSMC to GF could not be explained by a different coating ability of the glycated protein on the polystyrene plates. Furthermore, the time course experiment of adhesion (30 to 240 minutes) shows that GF impairs and does not simply delay hVSMC adhesion. Proliferation on nonenzymatically glycated F is still impaired even when the cells on control F are reduced by 25% to take into account the reduced adhesion. This fact suggests that the impaired interaction between integrin and GF could have an effect on hVSMC proliferation additive to that on adhesion. Since the effects on adhesion and proliferation are mediated through the same receptors, the reduced adhesion obviously plays a role in but does not completely explain the reduced proliferation, thus making plausible an autonomous effect of nonenzymatic glycation on proliferation.

Tarsio et al[6] showed that the extent of nonenzymatic glycation of F increases with glucose concentration and exposure time of the protein to glucose. It was observed that in diabetic dogs plasma F was nonenzymatically glycated 2.3 times more than in nondiabetic animals; on the other hand, a 1-day exposure to 500 mmol/L D-glucose

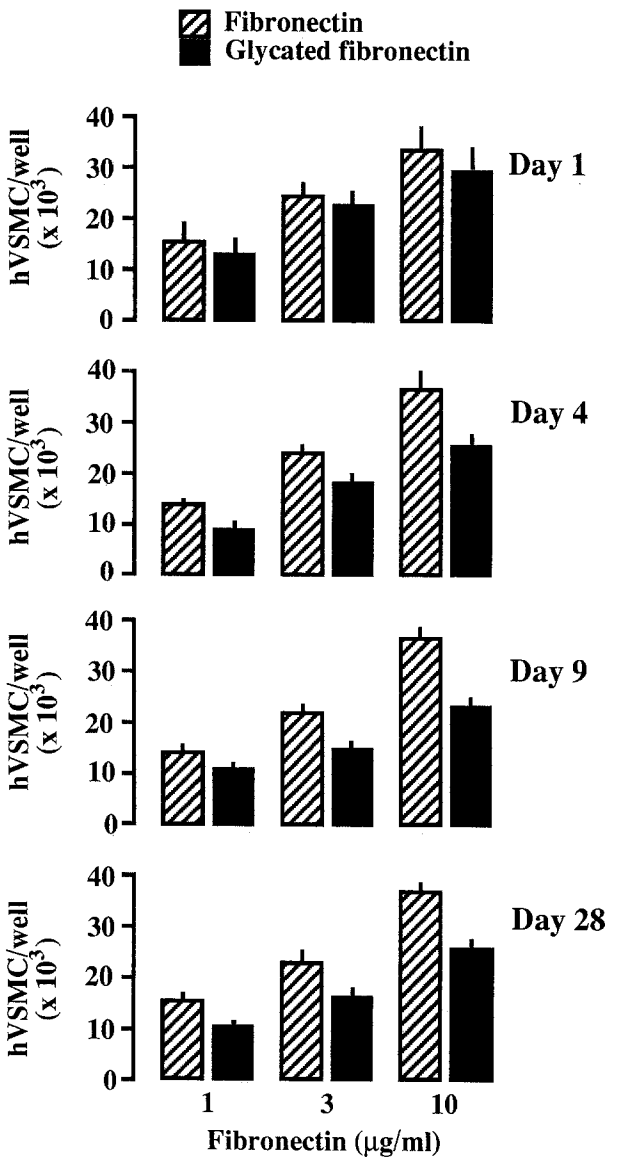

Fig 5. Effect of 1, 4, 9, and 28 days of F incubation with medium without or with 500 mmol/L D-glucose on proliferation of hVSMC. 5 × 10^3 cells were seeded per well. The number of cells per well increased dose-dependently with increasing concentrations of F: $P < .0001$ for both F and GF (ANOVA). The number of cells per well was lower in the presence of GF at all concentrations tested: day 4 (GF v F), 1 μg/mL, $P = .0025$, 3 μg/mL, $P = .022$, and 10 μg/mL, $P = .0091$; day 9 (GF v F), 1 μg/mL, $P = .0253$, 3 μg/mL, $P = .0008$, and 10 μg/mL, $P = .0001$; day 28 (GF v F), 1 μg/mL, $P = .0031$, 3 μg/mL, $P = .0122$, and 10 μg/mL, $P = .0002$. Proliferation on F itself was not different with day 1, day 4, day 9, or day 28 F. Proliferation on GF itself was not different with day 4, day 9, or day 28 GF.

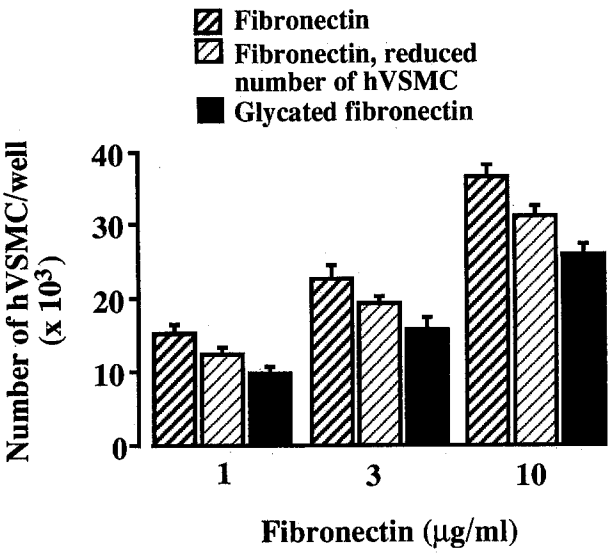

Fig 6. Effect of 28-day GF on hVSMC proliferation adjusted for the effect on adhesion. 5 × 10^3 or 3.75 × 10^3 (ie, 25% less cells than on GF hVSMC were seeded on F incubated without or with 500 mmol/L D-glucose. The cells were allowed to proliferate for 3 days. At the end, the number of cells counted on GF was reduced *v* control F on which a lower number of hVSMC were seeded: 1 μg/mL, *P* = .043, 3 μg/mL, *P* = .065, and 10 μg/mL, *P* = .019.

induced a threefold increase in the nonenzymatic glycation of F, an increment that continued in a time-dependent fashion in the following days. The glucose concentration (500 mmol/L) and times of incubation that we used (1, 4, 9, and 28 days) for the in vitro preparation of GF are comparable to those used for other experiments of in vitro F glycation.[6-8] Moreover, in many published studies, similar concentrations and incubation times have been used with substances (like glucose-6-phosphate) with higher ability than glucose itself to induce glycation and advanced-glycation end-products formation. Recent evidence suggests that nonenzymatic glycation may already start at the intracellular level, where highly reactive carbohydrate moieties are present.[50,51] The observation that the abnormal F behavior was not significant after 1 day but became significant after 4 days of exposure to glucose indicates a time-dependency for nonenzymatic glycation to impair adhesion and proliferation. The detection of an effect after a relatively short time (4 days) suggests that the phenomenon may be relevant in vivo, where, even though glucose concentrations are lower, the incubation time may be much longer, especially at the level of the extracellular matrix.

It has been reported that plasma F of diabetic patients is functionally preserved[52]; however, since the turnover rate of matrix proteins is much slower than that of serum proteins, these results could not be extended to F incorporated in the extracellular matrix.[52] Many studies (eg, Monnier et al[2,53]) have evaluated the extent of collagen advanced-glycation end-product formation; however, the use of arbitrary units of fluorescence and the different amino acid compositions and turnover rates between collagens and fibronectin make comparisons with the actual degree of fluorescence we obtained with F difficult and of dubious meaning. As far as we know, no study has evaluated the extent of F nonenzymatic glycation in tissues of normal or diabetic subjects, also because of the insoluble nature of tissue F.[54] Further studies are needed to extend our knowledge in this field, to give the appropriate physiological significance to experimental data obtained using in vitro procedures of nonenzymatic glycation. On the other hand, both in the present investigation and in the large majority of studies published in the literature, in vitro experiments of glycation are performed with a relatively short exposure of proteins to relatively high concentrations of glucose; thus, the results obtained could not be completely representative of what happens in vivo, where tissues are exposed for years to different degrees of hyperglycemia.

Our data confirm that F, coated on a solid substrate, is able to support hVSMC proliferation. They are in agreement with the observations that insolubilized F stimulates in a dose-dependent way the synthesis of DNA in endothelial cells,[25] and that the spreading of cells on F causes intracellular alkalinization, a phenomenon usually observed in response to growth factors.[25] In this respect, hVSMC behave like other anchorage-dependent cells, since they must first adhere to the substrate to proliferate.[55] The reduced ability of these cells to adhere and proliferate on GF may be explained by considering that extracellular matrix binds to the cells via integrin receptors,[14,15] which are synthesized continuously and recycled to the cell surface. These interactions may be hindered by nonenzymatic glycation of the matrix. The integrin-binding site of F contains the sequence RGDS (arginine-glycine-aspartate-serine)[12]—arginine and lysine both contribute to the formation of glycation products.[56] It has already been described that glycation of collagen type IV and laminin reduces adhesion, cell spreading, and cell perimeter of aortic endothelial cells.[57] Comparable phenomena, possibly explained by the altered association with cellular integrin receptors, may be assumed for glucose-modified F. The present data do not exclude the possibility that smooth muscle cells are able to overcome the reduced proliferative effect observed on GF if they are maintained in culture for a period longer than we tested.

Maintenance of normal microvessel structure and function requires a complex interplay between extracellular matrix and cells, and requires both soluble stimuli, like nitric oxide and endothelin, and a supporting scaffolding; nonenzymatic glycation has been shown to adversely affect both sides.[58] Our results concerning the reduced adhesive and proliferative properties of hVSMC exposed to GF suggest that nonenzymatic glycation of extracellular matrix components may play a role in the pathogenesis of diabetic angiopathy. For instance, they could help to explain why pericytes, which are specialized VSMC, are lost from the capillary wall in diabetic retinopathy.[59]

REFERENCES

1. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101:527-537, 1984
2. Monnier VM, Kohn RR, Cerami A: Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci USA 81:583-587, 1984
3. Lubek G, Pollack A: Reduced susceptibility of nonenzymatically glycosylated glomerular basement membrane to proteases: Is thickening of diabetic glomerular basement membranes due to reduced proteolytic degradation? Renal Physiol 3:4-8, 1980
4. Brownlee M, Pongor S, Cerami A: Covalent attachment of soluble proteins by nonenzymatically glycosylated collagen: Role in the in situ formation of immune complexes. J Exp Med 158:1739-1744, 1983
5. Vlassara H: Advanced nonenzymatic tissue glycosylation: Cell-mediated interactions implicated in the complications associated with diabetes and aging. Blood Purif 8:223-232, 1990
6. Tarsio JF, Wigness B, Rhode TD, et al: Nonenzymatic glycation of fibronectin and alterations in the molecular association of cell matrix and basement membrane components in diabetes mellitus. Diabetes 34:477-484, 1985
7. Tarsio JF, Reger LA, Furcht LT: Decreased interaction of fibronectin, type IV collagen, and heparin due to nonenzymatic glycation. Implications for diabetes mellitus. Biochemistry 26:1014-1020, 1987
8. Tarsio JF, Reger LA, Furcht LT: Molecular mechanisms in basement membrane complications of diabetes. Alterations in heparin, laminin, and type IV collagen association. Diabetes 37:532-539, 1988
9. Hynes RO: Fibronectins. Sci Am 254:42-51, 1986
10. Yamada KM: Cell surface interactions with extracellular materials. Annu Rev Biochem 52:761-799, 1983
11. Hynes RO: Interactions of fibronectins, in Hynes RO (ed): Fibronectins. New York, NY, Springer-Verlag, 1990, pp 84-112
12. Pierschbacher MD, Ruoslathi E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309:30-33, 1984
13. Vartio T: Fibronectin: Multiple interactions assigned to structural domains. Med Biol 61:283-295, 1983
14. Buck CA, Horwitz AF: Cell surface receptors for extracellular matrix molecules. Annu Rev Cell Biol 3:179-205, 1987
15. Juliano RL: Membrane receptors for extracellular matrix macromolecules: Relationship to cell adhesion and tumor metastasis. Biochim Biophys Acta 907:261-278, 1987
16. Nathan C, Sporn M: Cytokines in context. J Cell Biol 113:981-986, 1991
17. Linder E, Vaheri A, Ruoslahti E, et al: Distribution of fibronectin in human tissues and relationships to other connective tissue components. Ann NY Acad Sci 312:151-159, 1978
18. Stenman S, Vaheri A: Distribution of a major connective tissue protein, fibronectin, in normal human tissue. J Exp Med 147:1054-1064, 1978
19. Laurie GW, Leblond CP, Martin GR: Light microscopic immunolocalization of type IV collagen, laminin, heparan sulphate proteoglycan and fibronectin in the basement membrane of a variety of rat organs. Am J Anat 167:71-82, 1983
20. Courtoy PJ, Boyles J: Fibronectin in the microvasculature: Localization in the pericyte-endothelial interstitium. J Ultrastruct Res 83:258-273, 1983
21. Courtoy PJ, Kanwar YS, Hynes RO, et al: Fibronectin localization in the rat glomerulus. J Cell Biol 87:691-696, 1980
22. Dixon AJ, Burns J, Dunnill MS, et al: Distribution of fibronectin in normal and diseased human kidneys. J Clin Pathol 33:1021-1028, 1980
23. Martinez-Hernandez A, Marsh CA, Clark CC, et al: Fibronectin: Its relationship to basement membranes. II. Ultrastructural studies. Collagen Relat Res 5:405-418, 1981
24. Glykhovas MA, Frid MG, Shekhonin BV, et al: Expression of extradomain A fibronectin sequence in vascular smooth muscle cells is phenotype dependent. J Cell Biol 109:357-366, 1989
25. Ingber DE, Prusty D, Frangioni JV, et al: Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J Cell Biol 110:1803-1811, 1990
26. Hedin U, Bottger BA, Forsberg E, et al: Diverse effects of fibronectin and laminin on phenotypic properties of cultured arterial smooth muscle cells. J Cell Biol 107:307-319, 1988
27. Boudreau N, Rabinovitch M: Developmentally regulated changes in extracellular matrix in endothelial and smooth muscle cells in the ductus arteriosus may be related to intimal proliferation. Lab Invest 64:187-199, 1991
28. Jensen BA, Holund B, Clemmensen I: Demonstration of fibronectin in normal and injured aorta by an indirect immunoperoxidase technique. Histochemistry 77:395-403, 1983
29. Rasmussen LH, Garbarsch C, Chemnitz J, et al: Injury and repair of smaller muscular and elastic arteries, immunohistochemical demonstration of fibronectin and fibrinogen/fibrin and their degradation products in rabbit femoral and common carotid arteries following a dilatation injury. Virchows Archiv [A] 415:579-585, 1989
30. Orekhov AN, Andreeva ER, Shekhonin BV, et al: Content and localization of fibronectin in normal intima, atherosclerotic plaque, and underlying media of human aorta. Atherosclerosis 53:213-219, 1984
31. Phan-Than L, Robert J, Derouette JC, et al: Increased biosynthesis and processing of fibronectin in fibroblasts from diabetic mice. Proc Natl Acad Sci USA 84:1911-1915, 1987
32. Saouaf R, Takasaki I, Eastman E, et al: Fibronectin biosynthesis in the rat aorta in vitro—Changes due to experimental hypertension. J Clin Invest 88:1182-1189, 1991
33. Grinnel F: Fibronectin and wound healing. J Cell Biochem 26:107-116, 1984
34. Welch MP, Odland GF, Clark RAF: Temporal relationship of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. J Cell Biol 110:133-145, 1990
35. Knowlton AA, Connelly CM, Romo GM, et al: Rapid expression of fibronectin in the rabbit heart after myocardial infarction with and without reperfusion. J Clin Invest 89:1060-1068, 1992
36. Clausell N, Rabinovitch M: Upregulation of fibronectin synthesis by interleukin 1B in coronary artery smooth muscle cells is associated with the development of the postcardiac transplant coronary arteriopathy in piglets. J Clin Invest 92:1850-1858, 1993
37. De Giorgio LA, Seghieri G, Gironi A, et al: Raised plasma fibronectin concentration is related to the presence of diabetic retinopathy. Acta Diabetol Lat 21:251-256, 1984
38. De Giorgio LA, Bartolomei G, Gironi A, et al: Increased plasma fibronectin concentration in diabetic patients with microalbuminuria. Diabetes Care 11:527-530, 1988
39. Solerte SB, Piovella F, Viola C, et al: Plasma fibronectin, von Willebrand factor antigen and blood rheology: Association with diabetic microvascular disease. Acta Diabetol Lat 22:239-246, 1985

40. Falk RJ, Scheinman JI, Mauer SM, et al: Polyantigenic expansion of basement membrane constituents in diabetic nephropathy. Diabetes 32:34-39, 1983 (suppl 2)

41. Doi T, Vlassara H, Kisrstein M, et al: Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. Proc Natl Acad Sci USA 89:2873-2877, 1992

42. Nahman NS Jr, Leonhart KL, Cosio FG, et al: Effects of high glucose on cellular proliferation and fibronectin production by cultured human mesangial cells. Kidney Int 41:396-402, 1992

43. Cagliero E, Maiello M, Boeri D, et al: Increased expression of basement membrane components in human endothelial cells cultured in high glucose. J Clin Invest 82:735-738, 1988

44. Pugliese G, Pricci F, Pugliese F, et al: Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. Diabetes 43:478-490, 1994

45. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254, 1976

46. Laemmli U: Cleavage of the structural proteins during the assembly of the bacteriophage T4. Nature 227:680-685, 1970

47. Scott-Burden T, Resink TJ, Hahn AWA, et al: Induction of growth-related metabolism in human vascular smooth muscle cells by low density lipoprotein. J Biol Chem 264:12582-12589, 1989

48. Rennard SI, Berg R, Martin GR, et al: Enzyme-linked immunoassay (ELISA) for connective tissue components. Anal Biochem 104:205-214, 1980

49. Bourdon MA, Ruoslathi E: Tenascin mediates cell attachment through an RGD-dependent receptor. J Cell Biol 108:1149-1155, 1989

50. Baynes JW: AGEing growth factors: A role in diabetic vascular disease? J Clin Invest 94:2, 1994

51. Giardino I, Edelstein D, Brownlee M: Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters fibroblast growth factor activity. A model for intracellular glycosylation in diabetes. J Clin Invest 94:110-117, 1994

52. Di Girolamo N, Underwood A, McCluskey PJ, et al: Functional activity of plasma fibronectin in patients with diabetes mellitus. Diabetes 42:1606-1613, 1993

53. Monnier VM, Sell DR, Abdul-Karim FW, et al: Collagen browning and cross-linking are increased in chronic experimental hyperglycemia. Diabetes 37:867-872, 1988

54. Rasmussen LM, Heickendorff L: Quantification of fibronectin in extracts of human aortae by an ELISA. Scand J Clin Lab Invest 49:205-210, 1989

55. Easty FC, Easty DM, Ambrose EJ: Studies of cellular adhesiveness. Exp Cell Res 19:539-548, 1960

56. Lyons TJ, Thorpe SR, Baynes JW: Glycation and autoxidation of proteins in aging and diabetes, in Ruderman N, Williamson J, Brownlee M (eds): Hyperglycemia, Diabetes and Vascular Disease: Clinical Physiology Series, American Physiological Society. New York, NY, Oxford University Press, 1992, pp 197-217

57. Haitoglou CS, Tsilibary EC, Brownlee M, et al: Altered cellular interactions between endothelial cells and nonenzymatically glucosylated laminin/type IV collagen. J Biol Chem 267:12404-12407, 1992

58. Bucala R, Tracey KJ, Cerami A: Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J Clin Invest 87:432-438, 1991

59. Engerman RL: Pathogenesis of diabetic retinopathy. Diabetes 38:1203-1206, 1989