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Increased biosynthesis and processing of fibronectin in fibroblasts from diabetic mice

(diabetes/extracellular matrix/skin)

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ABSTRACT **Diabetic connective tissues exhibit a deranged regulation of extracellular matrix biosynthesis. Fibronectin is shown to be increased in human dermal connective tissue by immunofluorescence, mainly at the dermoepidermal and capillary basement membranes. The rate of fibronectin biosynthesis, excretion, and incorporation in a pericellular polymeric form was investigated using genetically diabetic KK mouse skin and fibroblasts as compared to Swiss and C57BL mouse skin and fibroblasts. The rate of incorporation of [^{35}S]methionine into proteins recovered in the culture medium or in deoxycholate and NaDodSO$_4$ or urea extracts was investigated. The rate of incorporation in the medium and deoxycholate extracts was comparable. However, the relative rate of incorporation of the tracer in the NaDodSO$_4$-extractable, pericellular polymeric form was increased in the diabetic KK fibroblasts both for total proteins and for fibronectin. In pulse–chase experiments, the deoxycholate-soluble and NaDodSO$_4$-soluble fractions exhibited a precursor–product relationship. The rate of passage of fibronectin from the deoxycholate-soluble (cellular compartment) form to the NaDodSO$_4$-soluble (pericellular polymeric) form was strongly accelerated in the diabetic fibroblast cultures. These results confirm the increased rate of synthesis of fibronectin in diabetic fibroblasts as well as its processing from the cellular compartment to the polymeric pericellular form. The increase of fibronectin in diabetic connective tissues, in the matrix as well as in the basement membranes, may play a role in the mechanism of micro- and macroangiopathies and in the perturbed permeability characteristics of the diabetic capillaries, and as a glycoprotein it may contribute to the increased periodic acid/Schiff reagent staining of diabetic capillary basement membranes.**

The diabetic state is accompanied by deranged regulation of the biosynthesis of extracellular matrix macromolecules (1–5). The increased thickness of capillary basement membranes is one of the histologically and electron-microscopically detectable manifestations of this perturbed matrix biosynthesis (6–8). It has been proposed that the deranged regulation of extracellular matrix biosynthesis concerns most if not all mesenchymal cells, not only those involved in basement membrane deposition (1–9). Biosynthetic experiments performed with biopsy specimens of diabetic human conjunctiva and skin, with biopsy specimens from diabetic (KK strain) mouse conjunctiva, and with diabetic (streptozotocin-induced) rat conjunctiva showed an increased rate of incorporation of [^{14}C]proline into polymeric (insoluble) collagen (ref. 1, pp. 314–323; refs. 4–9). In these same experiments, an increased rate of incorporation of glucosamine into connective tissue glycoproteins was also seen (ref. 1, pp. 314–323; refs. 3–9). Experiments performed on genetically diabetic (KK strain) mouse skin explants showed an increased rate of incorporation of [^{14}C]proline into type III collagen, with no increase of total collagen biosynthesis (5). More recently, it was found that skin biopsy samples from human diabetic patients showed a strongly increased immunofluorescence with antibodies to human plasma fibronectin (10, 11). This increase was conspicuous on capillary basement membranes, at the level of the papillary dermis, and also at the dermo-epidermal basement membrane (Fig. 1).

To further explore the mechanism of this increase of dermal fibronectin in diabetic skin, we performed experiments on genetically diabetic (KK) mouse skin explants and on fibroblast cultures derived from KK mouse skin as compared to control (C57BL and Swiss) mouse skin and derived fibroblast cultures. The results confirm and extend previous observations showing that the diabetic skin explants and derived fibroblasts exhibit an increased rate of biosynthesis and processing of fibronectin from the cellular compartment to the pericellular polymeric form.

MATERIALS AND METHODS

Genetically diabetic (KK strain) mice and control (CB57BL and Swiss) mice were used at the age of 3 weeks. The dorsal skin was shaved, depleted of the adipose layer, and minced into pieces about 1 mm on a side. Fresh minced skin of each animal (about 0.5 g) was incubated at 37°C for 24 hr in 5 ml of Dulbecco's modified Eagle's medium containing methionine at 10% of its normal level and 100 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (Amersham) and supplemented with 10% fetal bovine serum (Flobio, Paris) previously depleted of fibronectin by passage through a column of gelatin-Sepharose. After incubation, the medium was collected and exhaustively dialyzed for 48 hr against several changes of 10 mM Tris·HCl buffer (pH 7.4) containing the protease inhibitors phenylmethylsulfonyl fluoride, sodium iodoacetate, *N*-ethylmaleimide, EDTA, and benzamidine at 2 mM.

The tissues were washed three times with Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}, pH 7.4; Seromed, Biochrom KG, Berlin) and homogenized in 5 ml of boiling 1% NaDodSO$_4$. The aqueous phase was removed after centrifugation at 10,000 × *g* for 15 min. Solid ammonium sulfate was then added to 40% of saturation and the mixture was allowed to stand at 4°C for at least 1 hr. The precipitate obtained by centrifugation was redissolved in 2 ml of 0.2 M 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) at pH 11, which dissolved fibronectin. The insoluble proteins were removed by centrifugation. The supernatant was dialyzed overnight against 0.15 M NaCl/1 mM $CaCl_2$/10 mM Caps, pH 11, containing the protease inhibitors as above.

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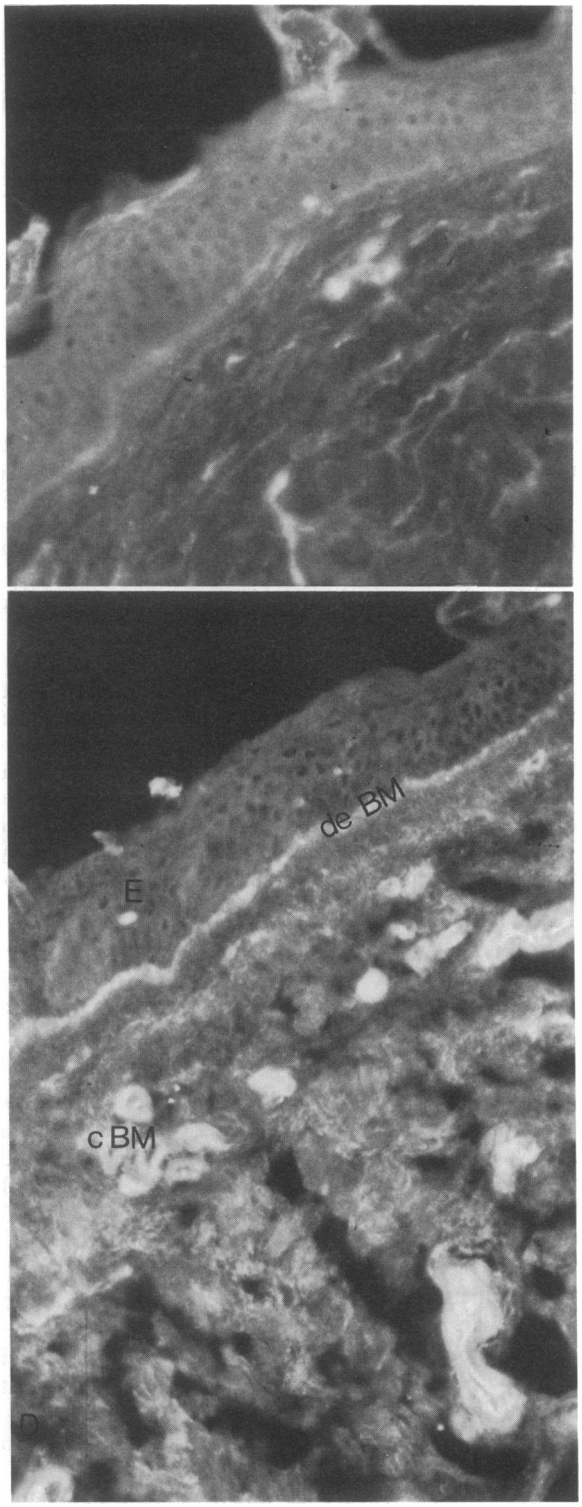

FIG. 1. Fibronectin in the skin biopsy samples of a diabetic patient (*Lower*) and a nondiabetic patient (*Upper*), as shown by indirect immunofluorescence. E, epidermis; de BM, dermo-epidermal basement membrane; cBM, capillary basement membrane; D, dermis. (*Upper*, ×325; *Lower*, ×240.)

Fibronectin Biosynthesis in Fibroblast Cultures. Fibroblast cultures were obtained from the minced dorsal skin of diabetic KK and C57BL mice in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, and further passaged in the same medium with 10% fetal bovine serum depleted of fibronectin. Confluent cultures of the first or second passage were incubated in 3 ml of medium containing [^{35}S]methionine (20 μCi/ml; Amersham) at 37°C for various periods of time. After incubation, the medium was dialyzed exhaustively against 10 mM Tris·HCl (pH 7.4) containing protease inhibitors and kept at −80°C for further processing. The cells were washed three times with phosphate-buffered saline and resuspended, using a rubber policeman, in 2.5 ml of a 2% sodium deoxycholate solution in 20 mM Tris·HCl (pH 8.8) containing protease inhibitors. The cell lysate was centrifuged at 10,000 × *g* for 15 min, and the insoluble residue was homogenized in 2.5 ml of 1% $NaDodSO_4$ containing protease inhibitors and then centrifuged. Fibronectin was immunoprecipitated from the deoxycholate and $NaDodSO_4$ extracts as described by Choi and Hynes (12).

The precipitate was submitted to polyacrylamide gel electrophoresis, radioactivity determination, and fluorography, which confirmed the purity of the fibronectin immunoprecipitate (13). In some experiments, the extraction with $NaDodSO_4$ was replaced by an extraction with 4 M urea according to Hedman *et al.* (14). All experiments were carried out in duplicate or triplicate and were repeated two to ten times. Proteins were determined according to Lowry *et al.* (15) using bovine serum albumin as standard.

Rabbit antiserum to human plasma fibronectin was prepared and rendered monospecific as described (11). It cross-reacted with mouse tissue fibronectin.

$NaDodSO_4$/polyacrylamide gel electrophoresis was carried out in vertical slab gels, with 5% polyacrylamide in the separating gel and 3% in the stacking gel, according to Laemmli (16).

Pulse–Chase Experiments. The pulse–chase experiments were performed essentially as the direct incorporation experiments, except that the culture medium containing [^{35}S]methionine was removed after 30 min of incubation and replaced by fresh medium after three rapid washings with 5 ml of Eagle's minimal essential medium. Parallel culture dishes were harvested after 0.5, 1, 2, 3, 4, and 24 hr for the determination (after exhaustive dialysis) of total radioactivity incorporated in the medium, in the deoxycholate extract, and in the $NaDodSO_4$ or urea extract. Radioactivity in the extracts was expressed as dpm per 10^6 cells (Fig. 2) or as percentage of total incorporation (in all extracts) (Fig. 4 *A* and *B*). Radioactivity found in the specific immunoprecipitate obtained by antifibronectin antibodies and protein A was expressed as radioactivity (dpm) recovered as percentage of total radioactivity obtained in the extract from which the immunoprecipitate was obtained (Fig. 3 and Fig. 4*C*).

RESULTS

Table 1 shows the results obtained with the skin explant cultures: total radioactivity incorporated, as well as the radioactivity in the fibronectin immunoprecipitate as a percentage of total radioactivity incorporated and recovered in the $NaDodSO_4$ extracts. Total incorporation was somewhat

Table 1. Incorporation of [^{35}S]methionine in total proteins and immunoprecipitable fibronectin in mouse skin explants

Mouse strain	Total incorporation, dpm/mg of protein	Radioactivity in fibronectin, % of total	
		Medium	$NaDodSO_4$ extract
Swiss	918 ± 158	2.62 ± 0.12	2.26 ± 0.12
C57BL	1357 ± 264	2.69 ± 0.29	1.71 ± 0.16
KK	749 ± 147	2.66 ± 0.13	3.57 ± 0.36

Explants were incubated for 24 hr at 37°C (expressed as % of total radioactivity). Values are the mean ± SEM of 10 independent determinations. For $NaDodSO_4$ extract: KK vs. Swiss, $P < 0.01$; KK vs. C57BL, $P < 0.001$.

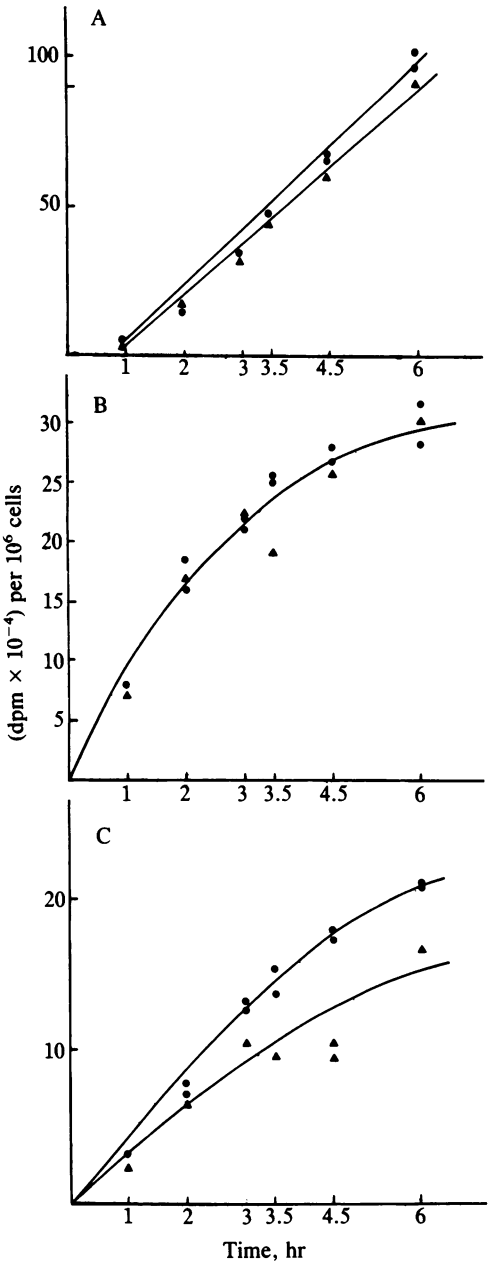

FIG. 2. Kinetics of incorporation of [^{35}S]methionine in the dialyzed culture medium (*A*), the deoxycholate extract (*B*), and the $NaDodSO_4$ extract (*C*) of fibroblasts derived from diabetic KK (▲) and normal C57BL (●) mouse skin.

lower in the KK mouse skin explants than in the nondiabetic controls; but the difference was not significant. Approximately 2.6% of the total radioactivity in the culture medium was immunoprecipitable. There was no significant difference in total radioactivity incorporated between the three mouse strains investigated. In the $NaDodSO_4$ extract of the skin explants, however, significantly higher values were found in the fibronectin immunoprecipitate of the KK mouse skin as compared to the two other strains. This increase is about 100% as compared to the C57BL strain from which the KK strain mutated and about 50% as compared to the Swiss strain.

Cell culture experiments were then performed in order to compare the rate of incorporation of [^{35}S]methionine in the total proteins and in the fibronectin immunoprecipitate. Fig. 2 shows that protein synthesis, expressed as dpm per 10^6 cells, increased during the 6 hr of incubation for both strains. Incorporation of methionine in the medium and in the deoxycholate extracts was comparable for the diabetic KK fibroblasts and the nondiabetic C57BL fibroblasts. Incorporation in the $NaDodSO_4$ extract, which contains mainly the pericellular polymeric form of the glycoprotein (12), was somewhat slower in the diabetic KK fibroblasts than in the nondiabetic C57BL fibroblasts.

Pulse–chase experiments were then carried out, using a 30-min pulse period followed by a 6-hr chase period. Fibronectin was immunoprecipitated from the dialyzed culture medium and its radioactivity was determined. After 1 hr of incubation (i.e., 30-min pulse plus 30-min chase) 5.3% of total radioactivity was recovered in the fibronectin immunoprecipitate of the KK mouse fibroblast culture medium, whereas only 2.7% was recovered in the immunoprecipitate of the control C57BL fibroblast culture medium (data not shown). It appears, therefore, that the secretion of fibronectin during the first hour of incubation of the diabetic fibroblasts is about twice that for the nondiabetic fibroblasts.

Fig. 3*A* shows the incorporation of [^{35}S]methionine in the deoxycholate and $NaDodSO_4$ extracts of the Swiss mouse fibroblasts, and Fig. 3*B* shows incorporation in the same extracts of the KK mouse fibroblasts during a pulse–chase experiment. Both pairs of curves suggest a precursor–product relationship between the immunoprecipitable fibronectin in these two extracts. Radioactivity in fibronectin decreased in the deoxycholate extracts and increased simultaneously in the $NaDodSO_4$ extracts of both control and diabetic cultures. There is, however, an important difference between these two strains. In the Swiss fibroblast cultures, the two curves intersect between 12 hr and 24 hr of chase. In the KK fibroblast cultures, the two curves intersect after only 4 hr of chase.

The radioactivity recovered in the deoxycholate extract, expressed as percentage of total incorporation (sum of incorporation in all extracts) decreased linearly in the nondiabetic fibroblasts as well as in the diabetic fibroblasts between the first hour and the fourth hour of the chase period (Fig. 4*A*). However, the decrease was faster (13.3% per hour) in the diabetic fibroblast extract than in the control fibroblast

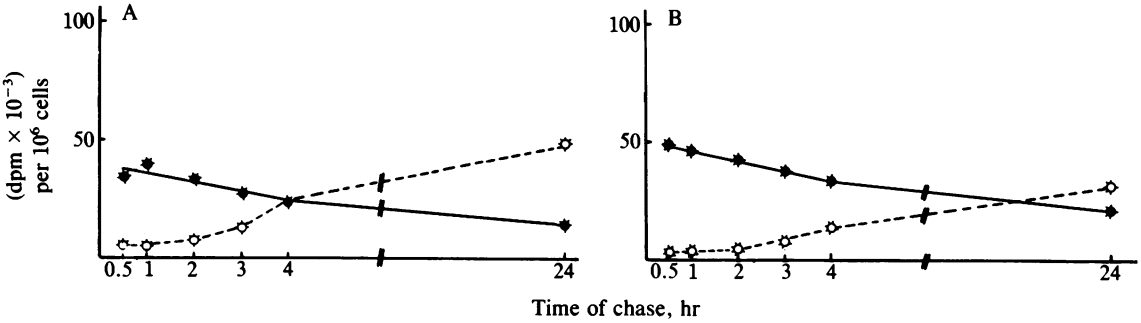

FIG. 3. [^{35}S]Methionine recovered in immunoprecipitable fibronectin in the deoxycholate (solid symbols) and $NaDodSO_4$ (open symbols) extracts of diabetic KK (*A*) and normal Swiss (*B*) mouse skin fibroblasts during chase period of pulse–chase experiment.

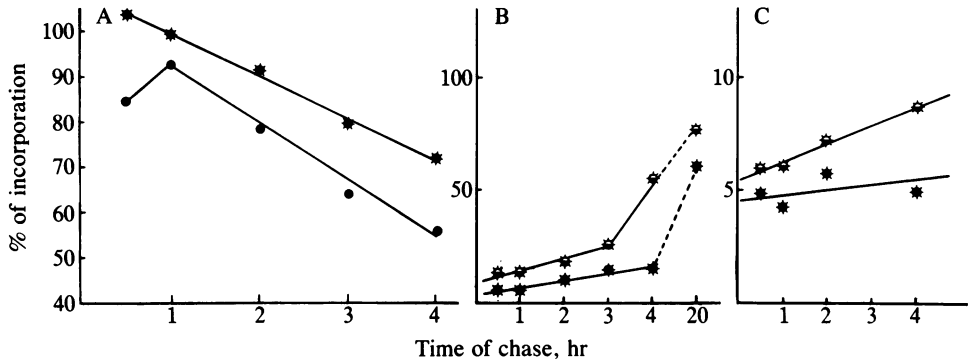

FIG. 4. [^{35}S]Methionine recovered in the deoxycholate (*A*) and NaDodSO$_4$ (*B*) extracts and in the fibronectin immunoprecipitate of the NaDodSO$_4$ extract (*C*) of diabetic KK (smooth filled circles in *A* and spiked half-filled circles in *B* and *C*) and normal Swiss (spiked filled circles) mouse skin fibroblasts during chase period of pulse–chase experiment. Values are expressed as a percentage of the total (medium plus deoxycholate plus NaDodSO$_4$ extracts) incorporation (*A* and *B*) or as the percentage of the incorporation in the NaDodSO$_4$ extract that was immunoprecipitable with antibodies to fibronectin (*C*).

extract (9.6% per hour). Four separate experiments carried out in triplicate gave similar results. This confirms that the deoxycholate-extractable, presumably cellular, compartment of fibronectin (12) is faster processed and incorporated into the NaDodSO$_4$-extractable pericellular polymeric form in the diabetic cells than in the nondiabetic cells.

Fig. 4*B* shows the relative rate of incorporation of the tracer in the NaDodSO$_4$ extract, expressed as percentage of total incorporation. The curve of the diabetic fibroblasts increases faster than the controls. This can be interpreted as a faster rate of incorporation of excreted proteins into the polymeric pericellular form in the diabetic cells. The same is true for immunoprecipitable fibronectin (Fig. 4*C*), which increases faster in the KK fibroblast NaDodSO$_4$ extract than in the control.

It appears, therefore, that the excretion and processing of fibronectin from the cellular compartment to the polymeric pericellular form proceeds faster in the diabetic fibroblasts than in the nondiabetic fibroblasts.

DISCUSSION

The above experiments confirm and extend the immunohistochemical observations made on diabetic human skin biopsy samples (Fig. 1), which show a strong increase of fibronectin immunofluorescence in the diabetic skin at the level of the capillary and dermo-epidermal basement membranes and in the papillary dermis (10, 11). The results obtained on the skin explants showed a stronger increase of radioactivity in the fibronectin immunoprecipitate in the NaDodSO$_4$ extract of the KK mouse skin than in the control mouse skin. A similar increase in the incorporation of radioactive methionine in immunoprecipitable fibronectin was observed in the fibroblast cultures derived from KK mouse skin as compared to C57BL or Swiss mouse skin fibroblasts.

If we compare incorporation in total proteins synthesized by these fibroblasts, no increase in the KK mouse skin was found as compared to the two other strains investigated (Fig. 2). The pulse–chase experiments suggest a precursor–product relationship between the deoxycholate extract, which contains the freshly synthesized cellular compartment, and the NaDodSO$_4$ extract, which contains the pericellular fibronectin present in a polymeric (presumably S-S bound) form, as suggested by Choi and Hynes (12). Thus, it appears that fibronectin biosynthesis, and especially its processing from the cellular to the polymeric form, is selectively increased in the diabetic connective tissue. These results were further confirmed by using a different extraction method, with 4 M urea (data not shown), as suggested by Hedman *et al.* (14).

Both the NaDodSO$_4$ and urea extracts are supposed to contain the pericellular, polymeric form of fibronectin. If this interpretation is correct, the processing and exportation of fibronectin in the pericellular matrix is strongly accelerated in the KK fibroblasts as compared to the nondiabetic fibroblasts. This conclusion is reached also when the results are expressed as radioactivity in the fibronectin immunoprecipitate as a percentage of total radioactivity in the deoxycholate and NaDodSO$_4$ extracts. A faster decrease of the radioactivity was seen in the deoxycholate extract of the KK mouse fibroblasts as compared to the Swiss mouse fibroblasts. Correspondingly, there was a steeper increase of radioactivity in the NaDodSO$_4$ extract of the KK mouse fibroblasts as compared to the Swiss mouse fibroblasts.

These results show that there is an increase in the relative rate of synthesis and processing of fibronectin as compared to total protein synthesis in the KK mouse fibroblasts. This is confirmed by the increased rate of processing and exportation of the pericellular (NaDodSO$_4$- or urea-soluble) matrix-bound fibronectin. The culture medium of the diabetic fibroblasts contained also about twice as much immunoprecipitable fibronectin as the control fibroblast medium.

It appears, therefore, that in the diabetic state, both in humans and in the KK mouse strain, the modification of extracellular matrix biosynthesis concerns not only collagen (1–5) but also fibronectin. This increase in the rate of biosynthesis and processing of fibronectin and the increase of collagen type III (5) appears to be accompanied by a decreased rate of synthesis and/or increased rate of removal of heparan sulfate proteoglycans in diabetic basal lamina (ref. 1, pp. 271–280; refs. 17 and 18).

These perturbations of the biosynthesis of extracellular matrix macromolecules result in the increased thickness and permeability of diabetic basement membranes (1–11). The increased capillary permeability may also enable plasma fibronectin to diffuse in increased amounts to the extracellular matrix (19). The increased frequency and severity of micro- and macroangiopathy in diabetics may well be related to abnormal matrix biosynthesis, as suggested also by the increase of fibronectin deposition in human atherosclerotic lesions (20).

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