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CHANGES IN THE EXPRESSION OF SMALL INTESTINE EXTRACELLULAR MATRIX PROTEINS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Diabetes mellitus is characterized by anatomical and functional alterations of the intestinal tract. However, the aetiology of these disturbances remains unclear. The aim of the present work was to investigate the effects of diabetes on the expression of laminin-1 and fibronectin in the small intestine of Streptozotocin (STZ)-induced diabetic rats. The Western immunoblotting of the extracts from the small intestine revealed that experimental diabetes resulted in a marked increase in the intensity of the bands corresponding to laminin-1 and fibronectin. Immunohistochemical studies demonstrated a strong labelling to these two extracellular matrix (ECM) proteins in the small intestine of diabetic rats, mainly localized in the smooth muscle layer. These results occur together with a thickening of the basement membrane (BM) of the smooth muscle cells, demonstrated by transmission electron microscopy (TEM). We propose that the accumulation of ECM proteins in the smooth muscle layer may be an effect mediated by hyperglycaemia, since insulin treatment of diabetic rats reversed this accumulation. These results could provide information on the potential role of the ECM in the intestine, an organ which is known to exhibit important alterations in diabetes.

KEYWORDS: extracellular matrix; laminin; fibronectin; diabetes; streptozotocin.

INTRODUCTION

Diabetes mellitus is defined as a chronic disease characterized by metabolic disorders with fasting hyperglycaemia and glycosuria (Renold *et al.*, 1978). The main metabolic complications of diabetes are retinopathy, nephropathy and peripheral vasculopathy. However, symptoms of gastrointestinal dysfunction are not uncommon among the diabetic population, for example, nausea, vomiting, diarrhoea, constipation, early satiety or dysphagia (Feldman *et al.*, 1983). The aetiology of these disturbances remains unclear.

Streptozotocin (STZ), the most potent diabetogenic agent, has been widely used to induce experimental diabetes in rats (Ar'Rajab and Ahrén, 1993) since it causes alterations similar to those found in diabetic humans (Eriksson *et al.*, 1991). STZ-induced diabetes is characterized by morphological alterations such as the proliferation of intestinal epithelial cells, which results in hypertrophy of the mucosa layer (Miller *et al.*, 1977), and also by functional disorders such as the transport of nutrients (Jarvis and Levin, 1996; Thompson, 1982).

It has been well established that the intestine has a high glycolytic activity (Hanson and Parson, 1976; Nicholls *et al.*, 1983) that plays an important role in glucose homeostasis, the highest activity being observed in intestinal epithelial and smooth muscle cells. This glycolytic activity is liable to undergo different alterations, some of which might be attributed to diabetes mellitus.

Even though several biochemical abnormalities have been identified in the diabetic intestinal tract

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(Lacombe *et al.*, 1996), the cellular and molecular mechanisms linking these findings with the diabetic pathology have not yet been completely established.

In the last decade, great advances have been made in understanding the composition of normal extracellular matrix (ECM) components in different tissues and organs (Hay, 1991) including the intestine (Simon-Assmann *et al.*, 1995), but the role of ECM in some diseases has yet to emerge. Abnormalities of the basement membrane (BM), the specialized ECM situated at the boundary between the cells and the underlying connective tissue, are a generalized phenomenon in diabetes mellitus (Østerby, 1990). BM thickening is a prominent failure in diabetes (Reddi, 1995), although its origin is still uncertain. Current consensus favours the idea that this thickening is a consequence of the abnormal carbohydrate metabolism, and findings such as variations in the ECM components have begun to provide a biochemical basis to understand the process.

In recent years, the distribution of some individual ECM components, such as laminin α_2, α_4 and α_5 chains in the developing human and mouse intestine has been studied (Lefebvre *et al.*, 1999; Simon-Assmann *et al.*, 1994). However, to the best of our knowledge, the participation of the ECM in the diabetic intestine has not yet been reported.

The aim of the present immunocytochemical study was to determine the effects of diabetes on the expression and localization of laminin-1 and fibronectin in the small intestine of STZ-induced diabetic rats.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague–Dawley rats (initial weight 250–300 g) were used in this study, STZ being the agent of choice for inducing insulin deficiency in experimental animals. It is known that the effects of the drug are dose-related in rats. Diabetes was induced by an intraperitoneal injection of streptozotocin (50 mg/kg weight, Sigma Chemical Co., St Louis, MO, U.S.A.) dissolved in citrate buffer (pH 4.5); control animals received only buffer. Diabetes was achieved in the majority of animals within 24 h as determined measuring fasting blood glucose and glucosuria with reactive strips (Haemoglukotest and Glucostick, respectively, Boehringer Mannheim, Germany). Only animals showing fasting glucose levels higher than 350 mg/dl 2 days after STZ treatment were included in the study. Immediately after STZ administration, insulin doses (5 UI, Monotard MC-40-Novo) were subcutaneously injected to a group of diabetic rats in order to restore normoglycaemia. This treatment was carried out daily at 9 a.m. and 6 p.m. for 21 days.

The three groups of animals (diabetic, insulin-treated diabetic and control) were pair-fed with standard rat chow and had free access to water.

Tissue preparation

After 21 days of STZ administration the rats were fasted overnight, weighed and sacrificed. Blood samples were taken for determination of fasting blood glucose by the glucose-oxidase enzymatic method. Small intestine was thoroughly washed with cold PBS and a sample of the small intestine was immediately removed. The duodenum was opened longitudinally and subdivided into sections, which were immersed in Bouin's fixative for immunohistochemistry or fixed in 4% glutaraldehyde for electron microscopy. For the SDS-polyacrylamide gel electrophoresis and immunoblotting procedure, extracts from the duodenum were prepared by homogenization at 4°C in extraction buffer (50 mM Tris-HCl pH 7.4, 0.1 M NaCl and 1% Nonidet P-40, containing the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 5 µg/ml pepstatin and 5 µg/ml aprotinin). Then they were centrifuged at $10{,}000 \times \boldsymbol{g}$ for 5 min. Lipids were extracted from the supernatant as described by Heifetz and Snyder (1981) by adjusting the sample to chloroform-methanol-water 10:10:3 (v/v/v). Precipitated proteins were solubilized in sample buffer for SDS-polyacrylamide gel electrophoresis.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedure

Denaturing polyacrylamide slab gel electrophoresis was carried out following Laemmli's procedure (Laemmli, 1970). SDS-denatured proteins were electrophoresed in 5 and 7.5% polyacrylamide gels. Samples were boiled for 3 min in 2% SDS, 2% 2-mercaptoethanol, 20 mM Tris–HCl, pH 7. The molecular weights of the denatured samples were estimated from a calibration curve obtained with the standard proteins in a pre-stained SDS-PAGE standard solution (Sigma). The gels were stained with Coomassie Blue R-250. Protein determination was performed by the method of Lowry *et al.* (1951).

After SDS-PAGE, the immunoblotting analyses were performed according to the method of Towbin (1979). Electrophoresed proteins were

Table 1.
Characteristics of study animals

Rats	*Body weight (g)*	*Blood glucose (mmoll/l)*	*Intestinal weight (g)*	*Intestinal length (cm)*	*Intestinal/body weight (%)*
Control (n=6)	337.5 ± 25.0	7.21 ± 3.94	6.9 ± 0.3	90.6 ± 7.5	2.0 ± 0.9
STZ (n=7)	157.8 ± 39.0	28.9 ± 10.60	10.5 ± 1.3	102.1 ± 3.47	7.0 ± 1.9
STZ+I (n=7)	355.0 ± 31.0	9.15 ± 5.88	10.4 ± 0.8	86.5 ± 8.1	2.8 ± 0.2

Values are means ± SD. Diabetes was induced by an intraperitoneal injection of streptozotocin in adult male Sprague–Dawley rats.

transferred from the gel to a nitrocellulose membrane that was rinsed with distilled water and blocked with 3% BSA-PBS for 1 h at room temperature. A 1:100 dilution of polyclonal antibody to laminin-1 (Sigma) or polyclonal antibody to human plasma fibronectin (Sigma) was added and incubated overnight at 4°C. Then the membrane was rinsed three times for 10 min with PBS with gentle shaking. The antigen–antibody reaction was detected using biotin-conjugated anti-rabbit polyvalent immunoglobulins and ExtrAvidin-Peroxidase conjugate (Sigma), each diluted 1:1000 and incubated for 2 h at room temperature. Between these incubations, and after them, the membrane was rinsed with PBS three times for 10 min each. Peroxidase activity was detected by incubating blots in 3,3′ diaminobenzidine-H_2O_2.

Immunohistochemistry

The localization of ECM proteins in sections of the small intestine of control, diabetic and insulin-treated diabetic rats was determined by an immunoperoxidase method. Specimens fixed in Bouin's solution were dehydrated through a graduated alcohol series and embedded in Paraplast. The sections were cut into 7-μm-thick slices, deparaffinized and rinsed with PBS (pH 7.4). Then they were incubated with 0.3% H_2O_2 in methanol for 30 min to inactivate the endogenous peroxidase and treated with 0.1% Trypsin (Merck kGaA, Darmstadt, Germany) for 10 min at room temperature to unmask antigenic sites of extracellular laminin in the basal lamina. Slides were incubated with 3% BSA-PBS for 1 h at room temperature to avoid non-specific background staining. After blocking, they were treated for 2 h at room temperature with 1:100 dilution of polyclonal antibody to laminin-1 (Sigma) or 1:100 dilution of polyclonal antibody to human plasma fibronectin (Sigma). After rinsing with PBS the sections were treated with 1:500 dilution of biotin-conjugated anti-rabbit polyvalent immunoglobulins for 2 h at room temperature and with ExtrAvidin-Peroxidase conjugate (Sigma) for 2 h at room temperature. Peroxidase activity was detected by incubation with 3,3′ diaminobenzidine-H_2O_2, at room temperature for 10 min. The reaction was stopped by rinsing with distilled water and slides were mounted in Mowiol (Hoechst Verkauf Lackrohstoffe, Frankfurt, Germany) and observed with a Nikon Fluophot microscope. In control experiments, no immunostaining was observed when primary antiserum was omitted.

Transmission electron microscopy (TEM)

For thin sectioning the tissues were fixed for 4 h in 4% glutaraldehyde in 0.1% sodium phosphate (pH 7.4). Afterwards, specimens were washed twice in phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer at 4°C overnight. Samples were dehydrated in an ethanol series and embedded in Spurr resin. Sectioning was carried out with Potter Blum MT1 ultramicrotome. Slices were stained with lead citrate and uranyl acetate. Preparations were examined with a Zeiss EM electron microscope.

RESULTS

Characteristics of study animals

Sublethal doses of STZ (50 mg/kg body weight) elicited a characteristic diabetic response. Twenty-one days after injection of the drug the animals showed hyperglycaemia with fasting serum glucose levels of 521.0 ± 191.0 mg/dl and a significant decrease in body weight (157.8 ± 39.0 g). In contrast, no alteration in these parameters was evident in the control group (130.0 ± 71.0 mg/dl for serum glucose levels and 337.5 ± 25.0 g for body weight).

The weight of the small intestine from diabetic rats was nearly twice that of the control ones (10.5 ± 1.3 g compared with 6.9 ± 0.3 g). With regard to the third group of animals, the one made

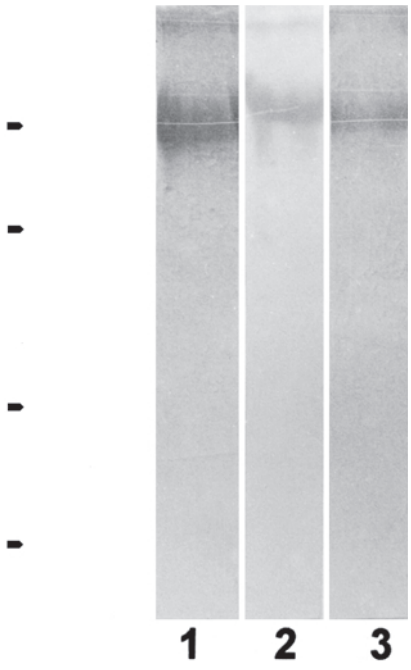

Fig. 1. Western immunoblotting of laminin-1 in tissue extracts of the smooth muscle and serous intestinal layers. Proteins were separated by 5% SDS-PAGE and transferred on to nitrocellulose before immunoblotting was performed, as described in Materials and Methods. Lane 1 corresponds to diabetic rats, lane 2 to insulin-treated diabetic rats, and lane 3 to control rats. On the left are molecular mass markers (from top to bottom, 205, 125, 51, and 35 kDa).

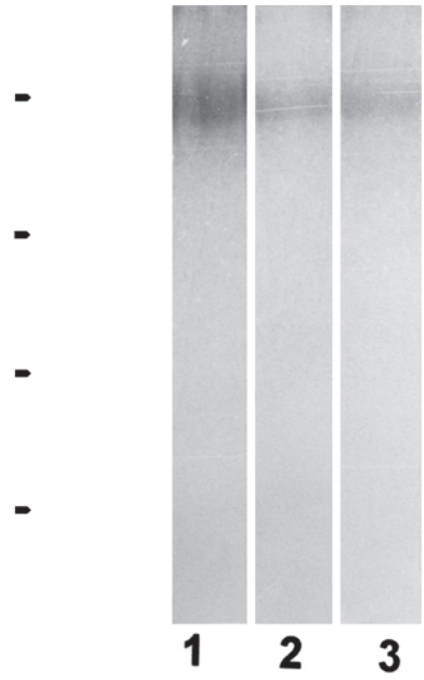

Fig. 2. Western immunoblotting of fibronectin in tissue extracts of the smooth muscle and serous intestinal layers. Proteins were separated by 7.5% SDS-PAGE and transferred on to nitrocellulose before immunoblotting was performed, as described in Materials and Methods. Lane 1 corresponds to diabetic rats, lane 2 to insulin-treated diabetic rats, and lane 3 to control rats. On the left are molecular mass markers (from top to bottom, 205, 125, 51, and 35 kDa).

up of insulin-treated diabetic rats, the body weight and glucose levels were restored to normal values, measured at the end of the 21 day period (355.0 ± 31.0 g and 165.0 ± 106.0 mg/dl respectively). However, the weight of the small intestine was similar to that of the non-treated group of diabetic rats (10.4 ± 0.8 g). The effects of STZ-induced diabetes on these parameters are shown in Table 1. The length of the entire small intestine was significantly increased in diabetic animals compared with control animals (102.1 ± 3.47 cm and 90.6 ± 7.5, respectively). With insulin treatment, diabetic small intestinal length (86.5 ± 8.1 cm) was not significantly different from normal control values.

Expression of laminin-1 in extracts from intestine

In order to determine the expression of laminin-1 in insulin-treated and non-treated diabetic rats, we subjected tissue extracts from the small intestine of the animals to Western immunoblot analysis. The polyclonal antiserum recognized laminin-1 as distinct bands with apparent molecular masses of 400 kDa and 200 kDa (Fig. 1). These results indicate the presence of both laminin α and β-γ chains in rat small intestine tissue, although we cannot exclude the possibility that the 400 kDa band, or a part of it, may represent crosslinked β-γ chains.

The variability in the intensity of these bands could be related to changes in the expression of laminin-1 caused by the diabetic condition of the animals. The relative intensity of the laminin bands was highest in diabetic rat intestine tissue (Fig. 1 lane 1), while a decrease in the intensity of both bands was apparent in tissue extracts from insulin-treated diabetic rats as well as in those from control rats (Fig. 1 lanes 2 and 3, respectively).

Figure 2 shows the Western blot analysis using a polyclonal antibody against human plasma fibronectin. This antibody recognized in the extracts from diabetic rat fibronectin as a broad band with an apparent molecular mass of 200 kDa; the intensity of this band appeared moderately increased as compared to that of extracts from insulin-treated diabetic and control rats.

Immunolocalization of laminin-1 in the intestine of diabetic rats

Immunohistochemical staining with laminin-1 specific antibody was used to determine the exact tissue distribution pattern of laminin-1.

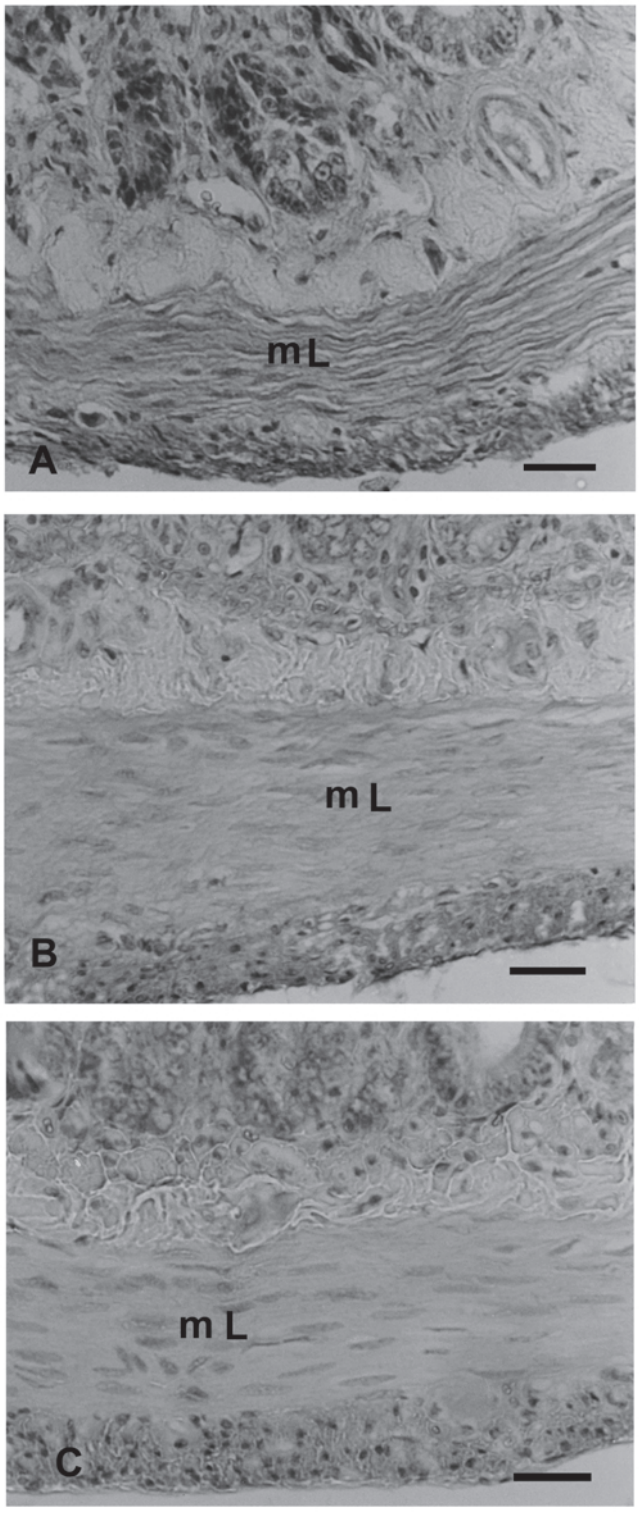

Fig. 3. Immunostaining of laminin-1 in the small intestine of rats. Intestinal samples were analysed in untreated diabetic rats (A), insulin-treated diabetic rats (B) and control rats (C). Immunolabelling in the small intestine was only obvious in the rats with STZ-induced diabetes exhibiting a restricted pattern of expression at the smooth muscle and serous layers. (mL) Muscular layer. Bar 12 μm.

In the small intestine of rats with STZ-induced diabetes, a strong labelling, mainly restricted to the smooth muscle layer, was found in the BM of the smooth muscle cells and in the serous layer. No labelling was determined in the BM of epithelial cells. Figure 3(A) shows a transverse section of the small intestine of diabetic rats stained with anti-laminin-1 antibody. Laminin-1 appears to be distributed more or less evenly along the smooth muscle layer. In contrast, insulin-treated diabetic rats and control rats showed a very weak labelling, which was linearly distributed from crypts to villus tips (Fig. 3B and C, respectively). This suggests that the diabetic state led to important changes in the expression pattern of laminin-1 in the small intestine, whereas insulin treatment restores the expression pattern to normal.

In order to find whether there is any relationship between laminin-1 and other components of the small intestine ECM, we also examined the expression pattern of fibronectin, another important component of the ECM. As with laminin-1, the intensity of fibronectin immunoreactivity was increased in the smooth muscle and serous layer of the small intestine of rats with STZ-induced diabetes (Fig. 4).

Transmission electron microscopy

Data obtained with TEM demonstrated that the BM of the smooth muscle cells that makes up the muscle layer of the small intestine underwent ultrastructural changes as a result of STZ-induced diabetes. As can be seen in Figure 5, the space between smooth muscle cells appears remarkably enlarged in diabetic rats when compared with that of control rats. In addition, many parallel bundles of collagen fibrils were observed in that space.

DISCUSSION

Diabetes mellitus has been shown to be associated with various ultrastructural and biochemical changes in BM, the diffuse thickening of which is the most consistent morphologic feature in this disease (Kreisberg, 1992). However, the involvement of the ECM and especially of BM molecules in diabetic intestine tracts has, as far as we know, not yet been investigated.

The rat small intestine appears to be an interesting model to examine the consequence of STZ-induced diabetes on the expression of laminin and fibronectin. Our results demonstrated that this experimental diabetes resulted in a marked increase in the amount of laminin-1 and fibronectin in the

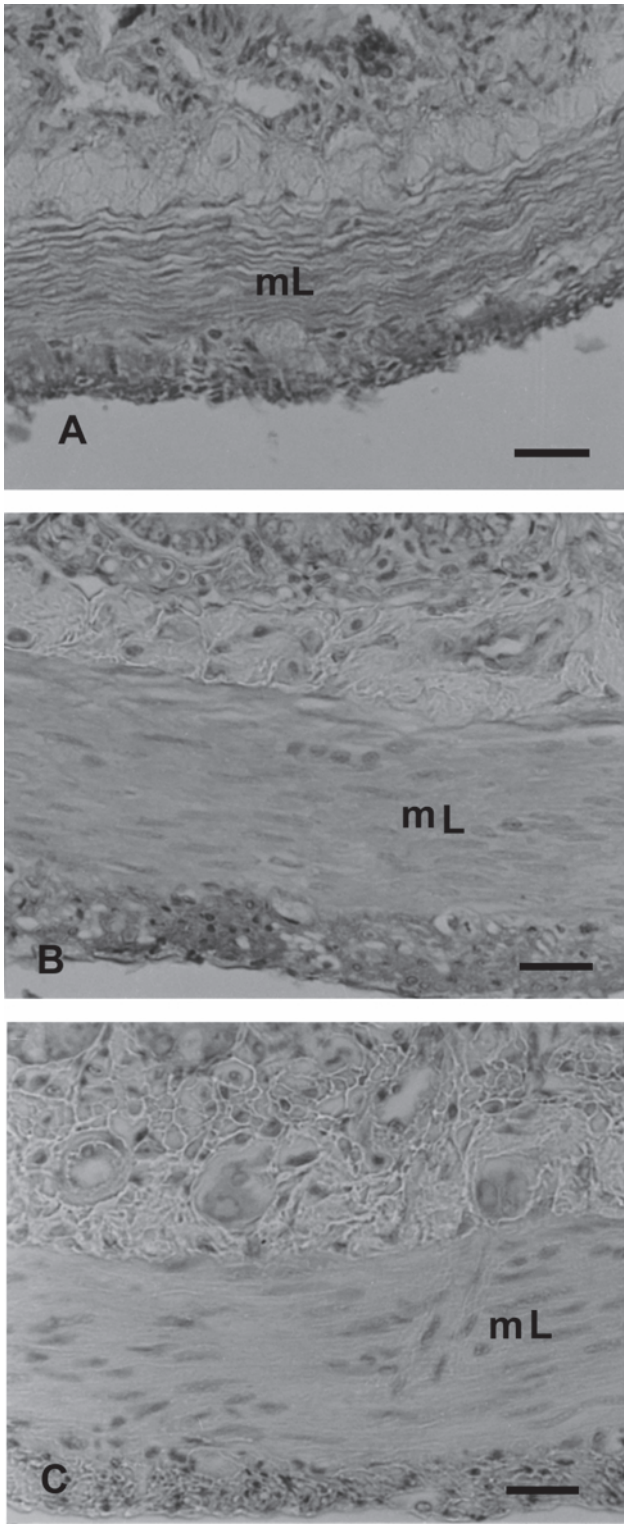

Fig. 4. Immunostaining of fibronectin in the small intestine of rats. Intestinal samples were analysed in untreated diabetic rats (A), insulin-treated diabetic rats (B) and control rats (C). Note the strong immunoreactivity in the smooth muscle and serous layer of the rats with STZ-induced diabetes in contrast with the weak staining in the insulin-treated diabetic and control rats. (mL) Muscular layer. Bar 12 μm.

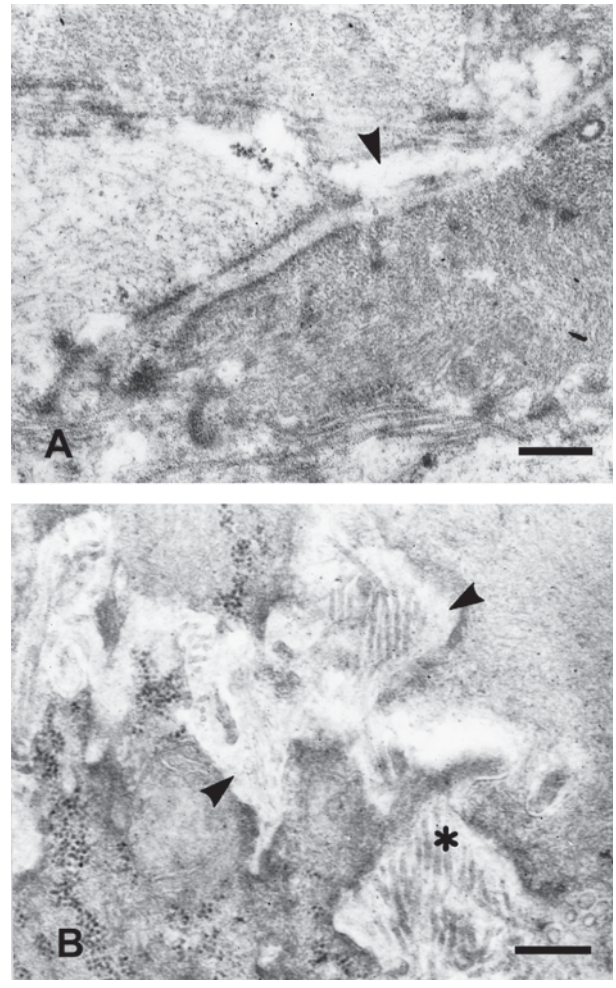

Fig. 5. Electron microscopy of the smooth muscle cells of the small intestine. (A) Control rats: a typical smooth muscle cell. (B) Diabetic rats: the space between smooth muscle cells is larger than in (A), with numerous bundles of collagen fibrils (*). Arrowhead: intercellular space. Bar 0.29 μm.

smooth muscle layers. This evidence is in agreement with the well-established fact that the thickening of the BM is due to an increased accumulation of ECM proteins. This thickening, which is evident in the renal glomerulus, the skin, the skeletal muscle, the nerves and the retina, gives rise to the characteristic microangiopathy of these organs (Cotran *et al*., 1989).

The ability of intestinal cells to interact and adhere to each other and to the BM is important for the maintenance of the intestinal tissue integrity and function (Wilson and Weiser, 1992). Laminin, as a major glycoprotein of BM, is thought to play an important role in various cellular functions including adhesion, proliferation and differentiation (Timpl, 1989). It has been established that the normal intestine subepithelial BM as well as the smooth muscle one express multiple laminin

isoforms and type IV collagen (Simon-Assmann *et al*., 1995; Lefebvre *et al*., 1999). During the development of the small intestine, in particular, laminin is strongly expressed at all embryonic stages (Orian-Rousseau *et al*., 1996). The increased expression of laminin-1 and fibronectin associated with smooth muscle cells provides evidence of alterations in the expression of ECM molecules during diabetes. This fact could be due to a stimulatory effect of high glucose levels on the ECM proteins production, as suggested by Galli *et al*. (1994) to explain the modified pattern of ECM components by cultured bovine bone endothelial cells. These investigators observed that, in the culture medium, hyperglycaemia induced an accumulation of type I and III collagen, fibronectin and glycosaminoglycans. In our experimental conditions, it is likely that hyperglycaemia induced an increased expression of diabetic intestine smooth muscle ECM which, together with the disturbance of the blood-tissue exchange of nutrients and metabolic factors, could lead to tissue damage in diabetes.

Brownlee *et al*. (1988) discovered that one of the major consequences of hyperglycaemia is the excessive non-enzymatic glycation of proteins, while Cohen *et al*. (1981) proposed that this glycation might be the cause of BM thickening. On studying the BM-producing EHS tumour in genetically diabetic mice, Rohrbach *et al*. (1982, 1983) demonstrated the presence of decreased levels of heparan sulfate proteoglycan in the BM and suggested that the loss of this proteoglycan might result in a subsequent compensatory biosynthesis of other BM glycoproteins such as laminin. In the present paper we provide evidence that laminin-1 is strongly expressed in protein extracts of the small intestine tissue of diabetic rats. This finding provides further support for the above hypothesis. We found that the increased laminin-1 expression in the intestine of diabetic rats is partially reduced by insulin treatment. This fact may be due to the effect of insulin on blood glucose levels, since control of blood glucose has been shown to prevent both structural and functional abnormalities in the BM (Nathan, 1994).

The insulin therapy in diabetic animals also restores to normal the serum glucose levels, the body weight and small intestine length. The lack of response in small intestine weight after insulin treatment appears paradoxical. Small intestine growth has been described in rodent models of poorly controlled diabetes (Jarvis and Levin, 1966; Miller *et al*., 1977), the changes being reversible by insulin treatment, but this behaviour appears to vary according to the period of metabolic derangement (Jarvis and Levin, 1966; Zoubi *et al*., 1995) and streptozotocin treatment (Ar'Rajab and Ahrén, 1993). In our experiments in Sprague–Dawley rats we found a higher sensitivity toward streptozotocin to induce a stable diabetes, and a lesser response to insulin to restore euglycaemic levels than those previously reported by several authors. These differences would account for the lack of response in the weight of small intestine of insulin-treated diabetic rats. However, other regulatory mechanisms are probably involved in the relation between insulin and intestinal growth. Recent studies have shown elevated levels of plasma and intestinal trophic factors, such as glucagon-like peptide 2 (GLP-2) in diabetes-associated intestinal growth (Fischer *et al*., 1997) that were proposed as an adaptive response of the small intestine to the STZ-induced diabetes.

The present study also demonstrates that, besides laminin-1, the expression of fibronectin increases in the extracts of the small intestine of diabetic rats. In a similar way, Ayo *et al*. (1990) demonstrated that fibronectin synthesis in cultured mesengial cells is increased by hyperglycaemia. On the basis of the above, we think that the accumulation of fibronectin in the diabetic intestine smooth muscle layer might also be an effect mediated by hyperglycaemia, since insulin treatment of diabetic rats reversed this accumulation, restoring fibronectin to almost normal levels.

Complex interactions between laminin and other components are necessary to form a structure that can be recognized as a BM by TEM. The use of this technique allowed us to show a thickening of the BM of the small intestine smooth muscle cells in diabetic rats. This membrane showed a high amount of materials that seemed to be collagen fibril bundles. Although no complementary studies on the expression of collagen have been carried out, we believe that the increase of laminin-1 expression associated with collagen fibrils may result in the formation of matrices with a very stable structure, highly resistant to proteolysis. In time, the unbalanced accumulation of these components could lead to the deposition of a BM that is thicker than the normal one, but exhibits severe functional alterations.

In conclusion, the results of our experiments using rats with STZ-induced diabetes indicate that the diabetic state is associated with an increase in the expression of laminin-1 and fibronectin, mainly at the smooth muscle layers of the small intestine. This increase, associated with the thickening of the BM, may be the phenomenon that links hyperglycaemia to changes in the intestinal diabetic pathological sequelae.

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