Alterations in glycosaminoglycans in wounded skin of diabetic rats. A possible role of IGF-I, IGF-binding proteins and proteolytic activity

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In the skin of diabetic animal tissues the amount of extracellular matrix (ECM) components is drastically decreased as a result of a reduced rate of their biosynthesis or increased degradation. In the present study we have investigated the mechanism of poor wound healing in diabetic rats. We have found that wounded skin of diabetic rats shows a significant decrease in glycosaminoglycan (GAG) content compared to that of control animals. This decrease was accompanied by significant depletion of insulin-like growth factor-I (IGF-I), known as a stimulator of GAG biosynthesis, and a distinct decrease in the content of high molecular weight IGF-binding proteins (HMW-BPs) with a simultaneous increase in low molecular weight IGF-binding proteins (LMW-BPs) in the sera of diabetic animals. Basing on determination of proteolytic activities we suggest that insulin shortage in diabetes results in increased proteolytic activity in various tissues. Proteolytic enzymes may cleave the HMW-BPs and convert them to LMW-BPs. The LMW-BPs may inactivate IGF-I and eliminate its stimulatory effects on GAG biosynthesis. The proteolytic enzymes may also digest the protein cores of proteoglycans releasing the GAGs and making them more susceptible to the action of glycosidases. These phenomena may be responsible for the observed marked decrease in GAG content in the skin of diabetic rats and disturb the wound-healing process.

The physiology of wound healing is an integrated process involving wound contraction, inflammation, granulation, reepithelization and remodeling of the connective tissue [1]. In animals with streptozotocin-induced diabetes the inflammatory response after wounding, and fibroblast and epithelial cell proliferation were decreased [2, 3]. Collagen and glycosaminoglycans (GAGs) are the main extracellular matrix (ECM) components of the skin. The amount of these substances may be changed by alterations in the rate of their biosynthesis or degradation. It is well known that both of these processes are regulated by hormones and cytokines. Diabetes impairs collagen biosynthesis by decreasing the amount of procollagen mRNA [4] and the extracellular content of procollagen [5], and decreases the content of GAG in the rat skin [6].

GAGs in most cases are attached to core proteins, forming proteoglycans [7]. They exert a wide range of biological functions. Some of them are involved in cell adhesion [8, 9], migration [10], proliferation [11], protein secretion.

Abbreviations: BPs, binding proteins; BSA, bovine serum albumin; GAG, glycosaminoglycan, IGF-I, insulin-like growth factor; HMW-BP and LMW-BP, high and low molecular weight IGF-binding proteins; ECM, extracellular matrix.
and gene expression [12, 13]. They contribute to general architecture and permeability properties of connective tissue and serve as an anchor for cell-specific growth factors and enzymes in the ECM and at the cell surface [14].

Much attention has been focused on the role of insulin-like growth factors (IGFs) in the regulation of collagen and GAG biosynthesis. IGF-I, previously referred to as somatomedin C or "sulfation factor" [15, 16], is known as a stimulator of both collagen and GAG biosynthesis [17]. It is produced mainly by liver and circulates in blood plasma in a complex with binding proteins (BPs) [18].

At least six separate classes of IGF-BPs have been described (IGF-BP-1, IGF-BP-2, etc.) [18, 19]. During gel chromatography the BPs present in the serum are eluted as two fractions of different molecular weight. The high molecular weight (150 000) binding proteins (HMW-BPs) and low molecular weight (40 000–50 000) binding proteins (LMW-BPs) have been found. Under normal conditions most of the IGF-I circulates in the form bound to HMW-BPs, which contains BP-3 as an active constituent and a so called "acid-labile subunit" of M, about 100 000 [20]. It is known that BPs modify the biological activity of IGF-I. The HMW-BPs keep the IGF-I in an active form, whereas some of the LMW-BPs bind and inactivate this factor [17, 21].

Determination of a possible effects of serum IGF-I and IGF-BP levels and tissue proteolytic activity on GAG content in the wounded skin of diabetic rats is the aim of this study.

MATERIALS AND METHODS

Reagents. Streptozotocin, bovine serum albumin (BSA), papain, cetylpyridinium chloride (CPC), glucuronic acid and carbazole were purchased from Sigma Chemical Co. Cellulose CF-11 and Sephacryl S-200 were from Pharmacia. Bio-gel P-60 was provided by Bio-Rad and Glucostix 2627 by Ames. Cold IGF-I, 125I-IGF-I, antibodies against IGF-I and Amerlex-M-separation reagent were provided as a Somatomedin C reagent pack for RIA by Amersham. All other reagents were commercially available, analytical grade chemicals.

Animals. The experiments were performed on male Wistar rats, body weight of 180–200 g. Animals were kept at room temperature and fed with a standard diet and water ad libitum. The rats were divided into 4 groups of 12 rats each: group I, control intact rats; group II, control wounded rats; group III, diabetic intact rats; group IV, diabetic wounded rats.

The control animals of groups I and II were injected with 0.4 ml of 0.1mM citrate buffer (pH 4.0) into the tail vein. Diabetes was induced by injecting the rats of groups III and IV with a single dose of streptozotocin (65 mg/kg body weight), dissolved in 0.4 ml of the same buffer. The progress of the disease was monitored by the assay of glucose concentration in blood taken from the tail vein with the use of Glukostix 2627. After 2 months the rats of groups II and IV (control and diabetic) were wounded under pentabarbital anaesthesia. Four 2-cm longitudinal incisions (0.5 cm apart) were made through the epidermis, dermis and subcutaneous tissue on the back of animals in sterile conditions. The animals of the remaining two groups: I and III were not wounded. All animals were killed by decapitation, 120 h after wounding. Blood was collected for the assay of IGF-I and IGF-binding proteins. Samples of intact and wounded skin were taken from the back and assayed for GAGs and proteolytic activity.

Analytical procedures

Isolation, fractionation and determination of GAGs. GAGs were isolated as described by Wesicki [22], fractionated according to Svejcar & van Robertson [23] and determined by the method of Bittker & Muir [24].

Separation of IGF-I from IGF-BPs. At acidic pH the IGF-I-BP complexes dissociates releasing free IGF-I which may be determined by radioimmunoassay. This procedure has been described in detail in a previous paper [21]. Briefly, 1 ml of acidified rat serum was applied to 1 cm x 40 cm column with Bio-Gel P-60 (100–200 mesh) and eluted with 1 M acetic acid. Fractions of 2.2 ml were collected and supplemented with 0.4 mg of BSA. Acetic acid was removed by evaporation to dryness under vacuum, and the residues were dissolved in 0.4 ml of water.

To determine the position of IGF-I in the eluate 125I-labeled IGF-I was added to 1 ml of acidified rat serum, and the sera were chromatographed as described above. Samples of eluate taken from each fraction were counted in a
Mini-gamma 1275 counter (LKB Wallac). Fractions (containing IGF-I) were pooled, evaporated to dryness, redissolved in 0.5 ml of assay buffer and subjected to radioimmunoassay for IGF-I, as described below.

**Radioimmunoassay for IGF-I** was performed according to the protocol provided by Amer sham with the somatomedin C reagent pack for radioimmunoassay (code IM 1721). The assay buffer consisted of 0.03 M sodium phosphate, 0.2 mg/ml protamine sulphate, 0.02% sodium azide, 0.01 M EDTA and 0.25% BSA. The reaction mixture contained 100 µl of assay buffer, supplemented with either unlabeled IGF-I (0.05-3.2 ng) or the pooled low molecular weight fraction from the Bio-gel P-60 column (20 µl or 40 µl diluted with assay buffer 1:10) and 100 µl of antibody diluted 1:4000 with assay buffer. Samples were incubated for 0.5 h, at room temperature and then 100 µl of 125I-IGF-I (about 15000 c.p.m., specific activity about 74 TBq/mmol = 2000 Ci/mmol) was added. Incubation was continued at 4°C for 48 h, then 500 µl of Amerlex-M second antibody reagent was added. The mixture was incubated for 10 min, at room temperature with occasional stirring. Then the samples were centrifuged at 25000 × g for 10 min. The supernatant was discarded and radioactivity of the sediment was determined. A standard competition curve prepared using 0.05-3.2 ng of unlabeled IGF-I per tube, was linear in the range of 0.05-0.8 ng/tube. The radioactivity of control samples (containing 100 µg unlabeled IGF-I) was subtracted from the radioactivity of the test samples to correct for nonspecific binding.

**Determination of IGF-BPs in the serum.** The assay was based on the procedure described in [25]. The reaction mixtures, containing 5 µl of serum, 220 µl of 0.1 M Tris/HCl (pH 7.6), supplemented with BSA (2.5 mg/ml) and 25 ml of 125I-IGF-I (about 150000 c.p.m.) in binding buffer were incubated in conical polystyrene tubes, at 30°C, for 3 h. In order to separate free 125I-IGF-I from its protein-bound form, 250 µl of cold 5% charcoal suspension was added. The mixtures were incubated at 0°C for 30 min and then centrifuged at 4000 × g for 15 min at 4°C. Supernatants containing the protein-bound 125I-IGF-I were collected and counted in a Mini-gamma Counter 1275 (LKB Wallac). The sediments (containing free 125I-IGF-I) adsorbed on charcoal were discarded.

**Gel filtration of 125I-IGF-I-BPs.** The supernatants (400 µl samples), containing the 125I-IGF-I bound proteins, were applied to a Sephacryl S-200 column (0.9 cm × 63 cm) equilibrated with 0.1 M ammonium bicarbonate (pH 8.0), at 4°C and eluted with the same solution. Fractions of 2 ml were collected and counted.

**Proteolytic activity assay.** Determination of proteolytic activity in the tissue homogenates was performed according to Worowski & Roszkowska [26]. A4% (w/v) haemoglobin solution (denatured with urea) was used as a substrate. Tissue homogenates, 10% (w/v) were mixed with the substrate at the 1:1 (v/v) ratio, and incubated in 37°C for 2 h. Non-digested proteins were precipitated with 5% trichloroacetic acid and tyrosine content was measured in supernatants according to Folin & Ciocalteau [27]. Proteolytic activity was expressed in nmoles of tyrosine released from haemoglobin per mg of protein contained.

**Statistical analysis.** In all experiments, the mean values for each group ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Student's t-test, accepting P < 0.05, as significant.

**RESULTS**

Injection of rats with a single dose of 65 mg of streptozotocin per 1 kg of body weight resulted in development of chronic diabetes. Such an amount of streptozotocin was found to be optimal for a rapid increase in blood glucose concentration and allows the animals to survive the 2 month long experiment. A significant increase in blood glucose concentration on the 3-rd day after injection and a progressive decrease in body weight were observed. The details were given previously [6].

It was found that the intact skin of control animals contains about 1.7 mg of GAG-bound uronic acids per gram of dry tissue. Wounding of these animals resulted in a significant reduction in GAG content in the skin to about 40% of the initial value (Fig. 1A). Diabetes by itself results in a significant decrease of GAG content and the wounding of skin in diabetic rats results in a further decrease of this value (Fig. 1B).

As can be seen from Fig. 2A the skin of control intact animals contains several GAGs with
The serum of control intact rats contains about 170 ng IGF-I per 1 ml. Wounding of control animals resulted in more than doubling the IGF-I concentration in the serum (Fig. 3 A). Diabetes by itself was associated with a distinct decrease of IGF-I level in the serum to about 35% of the control value. In contrast to control rats, in wounded diabetic animals no changes in IGF-I concentration in the serum were induced (Fig. 3 B).

A comparison of the total IGF-I-binding activity of rat sera of the experimental groups are presented in Fig. 4. Wounding of control animals resulted in a slight, but statistically significant decrease of IGF-binding activity (Fig. 4 B).
A). Diabetes by itself induced slightly this activity. Wounding of diabetic rats evoked its decrease to a value similar to that observed in control wounded animals (Fig. 4 A).

The IGF-BPs of control intact rat serum were eluted during gel chromatography on Sephacryl S-200 in the form of two peaks: high molecular weight binding proteins (HMW-BPs) and low molecular weight binding proteins (LMW-BPs). The HMW-BP-peak, containing about 90% of total $^{125}$I-IGF-I binding activity was the predominant one. It was eluted close to void volume of the column. The second peak, containing about 10% of this activity, was eluted in a volume corresponding to that of the bovine serum albumin (69000). It represents the LMW-BPs. Wounding of healthy rats caused a total disappearance of the latter peak (Fig. 5 A).

The IGF-BPs of diabetic intact rat serum distinctly differed from those observed in the intact control animals. In this case about 85% of total $^{125}$I-IGF-I binding activity was found in LMW-BPs. Furthermore, there was an additional LMW-BP's peak, eluted in the volume corresponding to that of carbonic anhydrase (30000). This fraction (not present in control rat serum) represented about 15% of total $^{125}$I-IGF-I binding activity (Fig. 5 B). The response of diabetic rats to skin wounding was qualitatively similar to that observed in control wounded animals. Although the total IGF-I-binding activity of serum proteins was but slightly de-
creased, significant changes in proportions between HMW-BPs and LMW-BPs were observed. The proportion of HMW-BPs was significantly increased and that of LMW-BPs distinctly decreased, the 30000 fraction completely disappeared (Fig. 5 B).

Table 1 presents the results of proteolytic activity determination in the intact and wounded skin of control and diabetic rats, measured at pH 7.5. As can be seen, diabetes significantly induced proteolytic activity in the skin. In the wounded skin of both control and diabetic animals a further increase of proteolytic activity was observed.

**Table 1**

Proteolytic activities in the skin of control and diabetic rats (intact and wounded), evaluated 72 h after wounding.

The activity was expressed as nmol of tyrosine released from haemoglobin during 2 h incubation with 10% skin homogenates.

<table>
<thead>
<tr>
<th></th>
<th>Proteolytic activity at pH 7.5</th>
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<tbody>
<tr>
<td>Control intact rats</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Control wounded rats</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>Diabetic intact rats</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>Diabetic wounded rats</td>
<td>82 ± 7</td>
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DISCUSSION

Our studies demonstrate a marked decrease of glycosaminoglycan content in the skin of rats with experimentally induced chronic diabetes. This decrease was accompanied by a significant depletion of IGF-I concentration and a distinct qualitative and quantitative changes in the IGF-binding proteins in the sera of these animals: a decrease in high molecular weight binding proteins and an increase in low molecular weight binding proteins. As we have described in this and a previous paper [6] proteolytic activity significantly increased in skin of diabetic rats.

IGF-I is considered a stimulator of GAG biosynthesis [15, 17, 28]. Its activity is modulated by IGF-BPs. It is believed that HMW-BPs bind IGF-I and prolong its half life time [21]. In contrast, some LMW-BPs inactivate IGF-I or IGF-I-dependent functions [17, 21, 29]. Their concentration increases in plasma of scorbatic and fasted guinea pigs. The sera obtained from those animals showed a significant decrease in growth-promoting and collagen biosynthesis-stimulating activities in fibroblast and chondrocyte cultures [17, 21].

Changes in IGF-binding proteins observed in diabetic animals could be due to two co-existing processes: enhanced biosynthesis of LMW-
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BPs and increased degradation of HMW-BPs resulting in their conversion into LMW-BPs.

Oei et al. [29] demonstrated that expression of mRNAs encoding IGF-BP-1 and IGF-BP-2 increases in the liver of rats with streptozotocin-induced diabetes. Similar effects of experimental diabetes on the hepatic level of IGF-BP-mRNAs was described by Boni-Schnetzler et al. [30]. They have found that insulin therapy lowers the levels of those mRNAs in diabetic rats, whereas IGF-I does not exert such an effect. This allows to conclude that insulin is a major regulator of LMW-BP-biosynthesis in rat liver [30]. On the other hand, it may be suggested that some of LMW-BPs are the products of limited proteolysis of HMW-BPs. Such a suggestion can be supported by the previously described co-existence of the increased LMW-BPs level with enhanced proteolytic activity in the liver (where BPs are produced), in the plasma (where they circulate) and in the skin (where they transport IGF-I) [31]. It is possible that the increased proteolytic activity causes the conversion of HMW-BPs into LMW-BPs which may bind and inactivate IGF-I or block its interaction with proper receptors [32]. Furthermore, it is known that insulin contributes to a decrease of proteolytic activity in some tissues [33], therefore it may protect both IGF-I and the HMW-BPs against proteolysis. The shortage of insulin may result in an increase of some proteolytic enzyme activities and enhance the proteolytic degradation of both IGF-I and IGF-BPs. The LMW-BPs may be degraded by some proteinases, producing the LMW-BPs.

It can be concluded from our previous studies that similar mechanism may act also in experimental diabetes. The treatment of control rat serum with a diabetic rat liver extract resulted in partial degradation of HMW-BPs and in increased concentration of LMW-BPs [34].

We suggest that the LMW-BPs released by the proteolysis of HMW-complex are not identical with the normal, well characterized LMW-BPs, BP-1, BP-2, etc. As can be seen from Fig. 5, the molecular weight of these products corresponds to that of bovine serum albumin (69000), which is higher than the molecular weight of any of the known normal LMW-BPs, produced by liver and other tissues.

Clinical studies show that diabetes contributes to impairment of the wound healing process [35]. In the skin of diabetic animals the content of collagen [5, 31] and GAGs [6] is significantly decreased, as compared to controls. This phenomenon could result from binding and inactivation of IGF-I (stimulator of collagen and GAG biosynthesis) by the LMW-BPs.

Wounding of control animals causes almost total disappearance of LMW-BPs and increased IGF-I concentration in the serum, which co-exist with a decrease of GAG content in the skin. The increase of IGF-I concentration in the serum in response to wounding may be a result of increased biosynthesis of this factor in the liver. The decrease in the serum of wounded rats of the content of LMW-BPs (known to be inhibitors of IGF-I dependent functions) allows for enhanced activity of IGF-I in the wound healing process. In diabetic rats such a mechanism seems to be inefficient. The wounding evokes a further increase in proteolytic activity and a decrease of GAG content in the skin. In contrast to control animals, there was no increase in the level of serum IGF-I. The direction of changes in BPs in response to wounding of diabetic animals was similar as in control animals but distinct quantitative differences were found. The increase of HMW-BP-concentration was not as significant as in control animals. Probably, high proteolytic activity in diabetic tissues may convert the newly synthesized HMW-BPs into LMW-BPs. The LMW-BPs may bind and inactivate IGF-I. As a result of increased clearance of such complexes [18] the serum IGF-I level may decline. The availability of IGF-I to the wounded tissue is therefore limited. These phenomena may be responsible for a marked decrease of GAGs content in the skin of diabetic rats and impairment of skin wound healing.

REFERENCES


