EXPERIMENTAL STUDY

Production of hyaluronan and chondroitin sulphate proteoglycans from human arterial smooth muscle – the effect of glucose, insulin, IGF-I or growth hormone

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Abstract

Background: Although it is recognized that the extracellular matrix is important for cell proliferation, migration and metabolism of growth factors, the regulation of the synthesis of hyaluronan and chondroitin sulphate proteoglycan (CSPG) in the vessel wall is poorly understood.

Objective: To examine the role of glucose, insulin, IGF-I and human growth hormone (hGH) on the accumulation of hyaluronan and CSPG using cultures of human aortic smooth muscle cells.

Methods: The cultures were exposed for 36 h. The CSPG content in the incubation medium was measured by a combination of digestion with testicular hyaluronidase and precipitation of label-[35SO4]2- labelled material with ethanol and trichloroacetic acid. Hyaluronan was estimated using a radiometric assay.

Results: Glucose and insulin reduced the amount of synthesized hyaluronan (P < 0.01), Stimulation of synthesis was seen with hGH (P < 0.01), whereas no effect was observed with IGF-I. The production of CSPG was increased with glucose and hGH (P < 0.01), but showed no change with insulin.

Conclusions: The present data obtained with human arterial smooth muscle cells in vitro showed that glucose, insulin and hGH can influence the accumulation of hyaluronan and CSPG. These observations may be relevant for an understanding of diabetic macroangiopathy.

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Introduction

Proteoglycans occur both in the extracellular matrix and at the cell surface in the arterial wall (1). Matrix proteoglycans can be divided into three groups: those present in the basement membrane; the hyalectans, which contain lectin-like domains and interact with hyaluronan and various carbohydrates; the small leucine-rich proteoglycans (2). However, hyaluronan is not classified as a proteoglycan, but as a glycosaminoglycan without sulphate units.

The function of many of these compounds is poorly understood, but they seem to be important for a number of fundamental cellular processes such as cell migration (3), cell proliferation (4, 5), regulation of growth factors (6, 7), lipid binding (8), etc. Therefore, these vascular wall constituents may be expected to operate during the development of abnormalities in the vessels.

In this respect diabetes mellitus is of particular interest because it leads to development of an arterial disease that is a major threat to the health of the patients. The pathogenesis is unknown, but most studies have focused on the relationship between diabetes mellitus, abnormalities of serum lipids, and atherosclerosis. Little effort has been made to integrate the presence of a diabetic macroangiopathy into the development of atherosclerosis in diabetes mellitus. It is reasonable to take the concept of a diabetic macroangiopathy into consideration, as it is regarded as a part of the generalized diabetic angiopathy (9, 10).

However, diabetic macroangiopathy, which is seen as a set of non-atherosclerotic changes in the vessel wall, is defined on the basis of morphological and biochemical criteria, albeit inchoate, rather than by clinical observations.

Tunica media in the coronary arteries and the aortas obtained from patients with diabetes mellitus contains more periodic acid-Schiff positive material, less glycosaminoglycans (11, 12), and more connective tissue, calcium (11, 13), fibronectin (14), type IV collagen (15) and hyaluronan (16) in areas without visible...
atherosclerosis than in corresponding regions from vessels taken from non-diabetic patients. What initiates the development of these changes is unknown, but the hypothesis of a diabetic macroangiopathy ascribes the vessel alterations to abnormalities of diabetic metabolism. Glucose, insulin, insulin-like growth factor (IGF)-I and human growth hormone (hGH) are obvious trigger candidates to be investigated. Because of the importance of chondroitin sulphate and hyaluronan, we have focused on the accumulation of these two substances using cultures of human arterial smooth muscle cells.

Materials and methods
The study was approved by the local ethics committee.

Cells
Human smooth muscle cells were established from explants (2 × 2 mm) of tunica media from normal aortas of humans aged 35–45 years, and incubated in culture flasks (264 ml Nunc, Roskilde, Denmark) with standard growth medium (minimal essential medium with 5% newborn calf serum and 5% human serum) for 4–6 weeks (17). The cells were detached by trypsination, resuspended in standard growth medium, and incubated in culture flasks until they reached confluency. Third passage cells were used in the experiments.

Experimental conditions
The cell cultures were incubated for 36 h in either control medium (with 2.5% newborn calf serum and 2.5% human serum) or test medium (with 2.5% newborn calf serum and 2.5% human serum) with fresh medium after the first 18–20 h. The various test substances included glucose at concentrations of 5.5, 8.0, 16, 20 and 30 mmol/l; insulin was used in concentrations of 10, 50, 100, 200 and 1000 μU/ml. Recombinant hGH was added at concentrations of 0.5, 1.25, 5.0, 10 and 20 ng/ml, and IGF-I was applied in concentrations of 0.6, 6.0 and 60 mmol/l. As an osmotic control for the glucose experiment, 30 mmol/l mannitol was included.

Measurements of chondroitin sulphate proteoglycan
Sulphur-35-labelled sulphate ($^{35}$SO$_4^{2-}$) was used in a concentration of 10 μCi/ml (NEN, Boston, MA, USA) for estimating the rate of production of chondroitin sulphate proteoglycan (CSPG).

The medium was removed, dialysed and lyophilized. Resuspended proteoglycans were precipitated with 95% ethanol at 4 °C, and then dissolved in 800 μl 50 mmol/l sodium acetate, pH 5.0, and divided into two samples.

CSPG was measured as radiolabelled tetra- and hexasaccharides after depolymerization with testicular hyaluronidase (Sigma type IV). The released chondroitin sulphate fragments were separated from larger molecules by precipitation with trichloroacetic acid. The radiolabelled chondroitin sulphate fragments present in the supernatant were counted in a liquid scintillation counter. The amount of CSPG was calculated as c.p.m. in the enzymatically degraded fraction.

For evaluation of the proteolytic activity of hyaluronidase, radiolabelled ([3H]leucine) cell material was used, and approximately 3% of the radioactivity was found in the enzymatically degraded fraction. In order to visualize the action of the hyaluronidase treatment on the sulphate-labelled medium, a high performance liquid chromatography analysis (HPLC, Perkin Elmer) was carried out on the degraded fraction using a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) and a buffer of 100 mmol/l CH$_3$COONH$_4$ with 7% isopropyl alcohol at pH 5.0 (Fig. 1).

Measurements of hyaluronan
Hyaluronan was measured in medium from cultures that were not labelled, using a radiometric assay (HATest, Pharmacia Diagnostics, Uppsala, Sweden) in accordance with the manufacturer’s instructions. The medium was diluted 1:20 with phosphate-buffered saline (PBS) before analysis. Intra- and interassay coefficients of variation of 5.2% and 6.7% were obtained. Hyaluronan could not be detected in samples.

Figure 1 The pattern of elution of $[^{35}$SO$_4^{2-}$]-labelled CSPG after degradation with testicular hyaluronidase using gel chromatography. CSPG was isolated from the medium of human aortic smooth muscle cultures. Standards were various carbohydrates: 1) maltoheptose (heptasaccharide), mol. wt 1153.0; 2) maltohexose (hexasaccharide), mol. wt 990.66; 3) maltopentose (pentasaccharide), mol. wt 828.72; 4) stachyose hydrate (tetrasaccharide), mol. wt 666.66. $V_0$=void volume.
of test medium unexposed to cell cultures (undiluted samples).

**Measurements of DNA and protein**

The cell layer was rinsed twice in saline PBS. The DNA content was measured by fluorometry using bisbenzimide H33258 (Fluka) and calf thymus DNA as standard (Sigma) (18). The protein concentration was determined using bovine albumin as a standard (19).

**Statistical methods**

Each experiment consisted of groups of 10–28 confluent cell cultures. The results were calculated as c.p.m./μg cell protein or DNA, and the data expressed as percent of control values. Statistical evaluation was performed with either ANOVA or the Mann–Whitney U-test. A 2P value less than 0.05 was accepted as statistically significant.

**Results**

Hyaluronan was measured using a commercially available radiometric assay. The data showed that increased glucose concentrations resulted in a significantly reduced accumulation (ANOVA, 2P < 0.01), as can be seen in Fig. 2. A dose-dependent decrease could also be observed when the cultures were supplemented with various amounts of insulin (ANOVA, 2P < 0.01; Fig. 3). hGH stimulated the accumulation of hyaluronan in low concentrations, whereas greater amounts showed no effect (Fig. 4). It was not possible to demonstrate any effect of IGF-I on the concentration of hyaluronan (control: 100 ± 18.0%; 0.6 nmol/l IGF-I: 83.94 ± 9.2%; 6.0 nmol/l IGF-I: 90.1 ± 9.7%; 60 nmol/l IGF-I: 86.9 ± 6.7% (means ± S.E.M.)).

CSPG was quantified using a classic precipitation technique and enzymatic degradation with hyaluronidase (20). The accumulation of CSPG increased significantly after the addition of a high concentration of glucose (5.5 mmol/l glucose (control): 659.26 ± 44 c.p.m./μg protein, n = 19; 20 mmol/l glucose: 1532.00 ± 85 c.p.m./μg protein, n = 9 (means ± S.E.M.); 2P < 0.01). No effect of 15 mmol/l glucose was seen. The increase was not observed when the glucose was replaced by an equimolar concentration of mannitol (5.5 mmol/l glucose (control): 546.00 ± 33 c.p.m./μg protein, n = 20; 30 mmol/l mannitol: 492.00 ± 22 c.p.m./μg protein, n = 15; means ± S.E.M.). The results obtained with various amounts of insulin revealed no change in the accumulation of CSPG, even with a concentration of 1000 μU/ml, as seen in Fig. 5.

hGH significantly increased the concentration of CSPG (ANOVA, 2P < 0.01). The effect of 10 ng/ml seemed to be the same as that of 5.0 ng/ml (Fig. 6).
Discussion

We found that glucose, insulin and hGH were able to change significantly the accumulation of either CSPG or hyaluronan from human arterial smooth muscle cells in culture. These observations have not been demonstrated previously.

As the experiments were performed in the presence of 5% serum, the production of proteoglycan may have been stimulated, to some degree, before the addition of test substances. However, in the incubation medium the concentrations of hGH, IGF-I, IGF binding proteins and insulin were 80% lower than those observed in undiluted serum. Therefore, the observed effects seem to be mainly due to the analysed substances (hGH, insulin, glucose, IGF-I) added to the cell culture medium.

High concentration of glucose reduced the accumulation of hyaluronan. Data obtained from in vitro studies have demonstrated that pooled serum from individuals with insulin-dependent diabetes mellitus stimulated the accumulation of hyaluronan in cultures of arterial smooth muscle cells (21). This observation may be of interest in relation to previous data from diabetic patients demonstrating increased concentrations of hyaluronan in areas of aorta without atherosclerosis (16). However, it is not clear which factor(s) causes this increase in hyaluronan in the vessel wall of diabetic patients. The present result does not support the idea that high glucose concentrations may be partly responsible for the greater amounts of hyaluronan. A similar conclusion was made in a study of non-treated rats with streptozotocin diabetes, which is a model of the effect of a constant, high concentrations of serum glucose (22). However, in another study a concentration of 25 mmol/l glucose significantly increased the production of hyaluronan from isolated glomerular cores from rats (23).

Insulin seems to reduce the concentration of hyaluronan, although previous in vitro studies have not been able to show any effect (21). However, these investigations were carried out in the presence of 15% serum, which may have blurred the effect of insulin. In vivo studies using insulin-treated rats with streptozotocin diabetes were not able to demonstrate any effect on the amount of hyaluronan in intima-media preparations from the aortas (22). Thus the present findings support the concept of insulin as a significant factor for the development of large-vessel disease in diabetes mellitus (24). However, this is not supported by previous data, although insulin receptor has been demonstrated in vascular smooth muscle cells (25).

It seems as if hGH in a concentration of 1 ng/ml stimulates the accumulation of hyaluronan. The reason for the lack of an effect of hGH in greater concentrations is unclear, as hGH receptors have been demonstrated in blood vessels (26). Consequently, the increased amount of hyaluronan in the vessel wall in diabetes seems not to be due to the increased serum hGH concentration.

The vascular smooth muscle cells express IGF-I receptor (27), but no effect of IGF-I was demonstrated. However, IGF binding proteins produced by the cells may interfere with the action of IGF-I (28, 29).

The present data show that glucose is able to increase the accumulation of CSPG in vitro at very high concentrations. This may be an effect of transforming growth factor (TGF)-β, as glucose can enhance the production of this peptide from the mesangial cells – a cell type related to smooth muscle cells (30). It has previously been demonstrated that TGF-β is able to stimulate the production of CSPG from fibroblasts in vitro (31).

hGH stimulated the accumulation of CSPG in our in vitro system. However, some of this increase may have
been due to the increased accumulation of the basement membrane material that occurs after hGH supplementation to cultures of arterial smooth muscle cells (32). This arterial basement membrane contains CSPG in the form of perlecan (33, 34). Furthermore, the alterations may also be confined to changes in the accumulation of biglycan and versican.

No effect of insulin on the production of CSPG was demonstrated in the present study. However, in vitro studies on pigs have shown an increase in the total amount of sulphated proteoglycans after the addition of insulin (35). It has been proposed that hyperinsulinaemia may have a deleterious effect on the vessel wall (24), but our data cannot support this view.

It is important to remember that these short-term investigations of the concentration of hyaluronan and CSPG were performed in cultures of human aortic smooth muscle cells. At present it would be inappropriate to extrapolate our findings directly to the situation prevailing in the large vessels of patients with long-term diabetes. Nevertheless, our findings, together with others — including, for example, the presence of increased plasma glucose, peripheral insulin and GH — suggest that aberrant diabetic metabolism per se may initiate the development of the diabetic macroangiopathy.

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