EXPERIMENTAL STUDY

The effect of human growth hormone on the carbohydrate units in arterial basement membrane-like material

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Abstract

Objective: The present study focuses on the pathogenesis of the large vessel disease in diabetes. The arterial wall from diabetic individuals displays characteristic alterations of the extracellular matrix. Other observations show that the metabolism is changed with increased levels of growth hormone in diabetes.

Design: The effects of growth hormone on the carbohydrate composition in the basement membrane around the arterial smooth muscle cells were investigated.

Methods: Basement membrane material was obtained from cultures of smooth muscle cells by sonication and differential centrifugation after labeling with either [³H]glucose or [³H]glucosamine. The proportions of galactose, glucose, mannose, xylose, fucose and glucosamine were evaluated after addition of 45.45 pmol/l human growth hormone. Also, the proportion of glycopeptides generated from the basement membrane was analyzed after fractionation on a combination of a Concanavalin A and a Pea Sepharose column.

Results: The proportion of galactose and glucose was changed, and the incorporation of [³H]glucosamine was reduced. The proportion of glycopeptides containing high mannose moieties was increased as well as that of triantennary glycopeptides with internal fucose residues.

Conclusion: The current *in vitro* data indicates that growth hormone may change the carbohydrate composition of the arterial basement membrane.

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Introduction

It is well established that growth hormone is one important regulator of the metabolism of glucose and lipid *in vivo* (1). This may be of particular interest in relation to the late manifestations of the diabetic angiopathy in which accumulation and changes in the basement membrane are characteristic phenomena (2). The carbohydrates in the arterial basement membrane are known to be important for the degradation (3). It is therefore of relevance to evaluate the influence of growth hormone on the carbohydrate composition of the arterial basement membrane. This seems reasonable, keeping in mind that, in diabetic patients, the serum growth hormone concentration is increased (4).

The effect of growth hormone is complex and varies greatly according to the conditions under which experiments are performed *in vitro*. The direct action of growth hormone on protein metabolism may be a stimulation of the local synthesis as seen in the human forearm (5). It has been shown to decrease the insulin-

stimulated glucose utilization and metabolism in cultured human fibroblast (6). Prolonged elevation of the concentration of growth hormone in plasma decreases the sensitivity to the insulin-induced glucose transport in skeletal muscles (1). These various observations suggest the presence of a stimulated non-oxidative glucose utilization, indicated by the reduced oxidation and uptake in a muscle despite an unchanged endogenous glucose production (7). However, it appears from the literature that the influence of growth hormone on the carbohydrate composition in glycoproteins has not been studied previously.

It is characteristic that the arterial smooth muscle cells are covered by basement membrane-like material (BM-like material), which is a pericellular structure containing important constituents such as laminin, type IV collagen, fibronectin and heparan sulfate proteoglycan (8–10). All these components contain carbohydrate units in various amounts and the susceptibility for degradation of the basement membrane by protease has been shown also to be dependent

on the above-mentioned sugar units (3). Previous studies have demonstrated that growth hormone is able to increase the accumulation of BM-like material in cultures of arterial smooth muscle cells (11).

In the present study, we have attempted to elucidate the effect of growth hormone on the composition of glycopeptides. These were obtained from the pericellular BM-like material covering the arterial smooth muscle cells. We have also focused on the distribution of monosaccharides and amino sugars after administration of growth hormone to the cell culture. It was found that growth hormone had a significant effect on the carbohydrate composition in the BM-like material.

Materials and methods

Cell cultures

Non-trypsinized primary cultures of arterial smooth muscle cells were grown out from rabbit aorta as described earlier (8). The experiments were performed on cultures after five weeks of growth when 'the stationary growth phase' was reached.

Incubation conditions

Cell cultures were preincubated for 24 h in the usual growth medium, supplemented with 45.45 pmol/l recombinant human growth hormone (hGH). A previous study has shown that the maximum effect of growth hormone was observed at 45.45 pmol/l (11, 12). The growth medium consisted of basal medium eagle, Hank's balanced salt solution with 10% serum (rabbit), ascorbic acid (50 µg/ml), antibiotic (neomycin sulfate) and a concentration of unlabeled glucose of 5.5 mmol/l. Using radioactive glucose and glucosamine it is possible to label glycosaminoglycans as well as asparagine and serine bound carbohydrate chains. After termination of the 24 h preincubation period with hGH, new medium was applied containing 45.45 pmol/l hGH and 5 µCi/ml [6-³H]glucose. The whole medium with growth hormone and labeled glucose was changed after an incubation period of 24 h in order to get sufficient labeling of glycopeptides. There was no difference in the glucose concentration between the cultures. Parallel incubation of controls was performed and the growth period was extended for an additional 24 h. Some of the cell cultures were also labeled with 0.1 µCi/ml [6-³H]glucosamine using a preincubation period as described above, labeling for 24 h and a growth hormone concentration of 45.45 pmol/l in the medium.

Preparation of BM-like material

After metabolic labeling, the BM-like material was isolated from the cell layer by sonication and differential centrifugation using the modified technique of Spiro (13) as previously described (8). In the presence of

proteinase inhibitors (0.5 mmol/l N-ethylmaleimide, 0.5 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l benzamidine-HCl) the cells were sonicated for 3 × 30 s in 1 mol/l NaCl buffered with 10 mmol/l Tris-HCl at 4 °C. The BM-like material was sedimented by centrifugation at 2100 *g* for 10 min at 4 °C. The pellet was sequentially washed three times in 1 mol/l NaCl, two times in 0.15 mol/l NaCl ± 2, and finally once in distilled H₂O. The material obtained (200–500 µg per culture flask) was freeze-dried and stored at –18 °C until use.

Fractionation of glycopeptides

Isolated BM-like material labeled with [³H]glucose was treated with proteinase (enzyme/substrate ratio 1:10 w/w) (14). The digest was desalted on a column of Sephadex G-15 (1 × 60 cm) in 7% propanol in H₂O. The void fractions were pooled and freeze-dried. The glycopeptides were fractionated on a ConA Sepharose 4B column (0.6 × 10 cm) in 0.1 mol/l Tris-HCl, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, pH 8.0, followed by 10 mmol/l α-methylglucoside at room temperature at a flow rate of approximately 1 ml/min. Finally, 0.1 mol/l α-methylmannoside was applied at a temperature of 60 °C (15).

In another series of experiments, the non-retained peak from the ConA Sepharose 4B column was desalted as described above, and further fractionated on a Pea Sepharose 4B column in the buffer system, also described above, using 10 mmol/l glycopyranoside (15, 16).

The average recovery was 90% for the gel filtration and between 80–92% for the fractionation of the glycopeptides.

Measurements of neutral and amino sugars

The effect of hGH upon the content of neutral and amino sugars within the extracellular BM-like material was also investigated on cultures labeled with [³H]glucose. Neutral sugars were liberated by hydrolysis in 1 mol/l HCl for 5 h at 100 °C under N₂ in sealed tubes. Neutral and amino sugars were obtained by passage of the hydrolysate through coupled columns of AG 50W-X4 (Bio-rad Labs, CA, USA) (H⁺ form: 200–400 mesh) and AG 1-X4 (format form) (17). The monosaccharides were then separated as borate complexes on an anion-exchange chromatography using DA-X4–11 Resin (Dionex Corporation, USA) as earlier described (18). In order to evaluate the incorporation into hexosamine, the BM-like material was hydrolyzed in 4 mol/l HCl for 5 h at 100 °C, also in sealed tubes. The amino sugars were eluted from the cationic resin (AG 50W-X4) and measured using liquid scintillation counting. The average recovery was 77% when standard mixture of hexoses were subjected to the whole procedure (hydrolysis included) and 88% when standards of hexosamine were analyzed by this procedure.

Figure 1 The effect of 45.45 pmol/l human growth hormone on the proportion of monosaccharides in the arterial basement membrane isolated from smooth muscle cells in culture after 48 h incubation with [^3H]glucose. * = $2P < 0.01$, ** = $2P < 0.03$. C = control. Means are shown as horizontal dashed lines.

Statistical methods

The results were based on cultures from at least two aortas. Student's *t*-test was used and a $2P$ value of less than 0.05 was considered as the limit of significance. The data are presented as $\bar{X} \pm \text{s.d.}$

Results

The effect of hGH on the distribution of neutral monosaccharides in the arterial extracellular BM-like material was evaluated using [^3H]glucose as a precursor. A significant shift could be seen in the galactose:

Figure 2 The incorporation of radioactive [$6\text{-}^3\text{H}$]glucosamine into the arterial basement membrane isolated from smooth muscle cells grown in culture with 45.45 pmol/l human growth hormone for 24 h. The difference between the groups was significant, $2P < 0.01$. C = control. Means are shown as horizontal dashed lines.

Figure 3 The effect of 45.45 pmol/l growth hormone on the proportion of glycopeptides released from ConA Sepharose 4B column with 0.1 mol/l mannopyranoside. The glycopeptides isolated from the basement membrane from arterial smooth muscle cells in cultures were radiolabeled with [^3H]glucose for 48 h. The difference between the groups was significant, $2P < 0.02$. C = control. Means are shown as horizontal dashed lines.

45.52% $\pm$ 4.81 ~ 50.71% $\pm$ 2.89 ($2P < 0.01$, Fig. 1) as well as in the glucose: 19.57% $\pm$ 5.47 ~ 13.97% $\pm$ 5.10 in fractions from the BM-like material after treatment with hGH ($2P < 0.03$, Fig. 1). The incorporation into mannose was unchanged. Only insignificant proportions of labeled fucose, xylose and amino sugars were released after labeling with radioactive glucose. Using radioactive glucosamine as a precursor, it was shown clearly that incorporation into the BM-like material was reduced significantly by growth hormone (Fig. 2, $2P < 0.01$, $n = 21$).

The composition of glycopeptides was also affected by hGH, as it appeared after the lectin-agarose affinity chromatography. The major part of the labeled glycopeptides did not bind to the ConA Sepharose 4B column and no difference between hGH-treated and control cultures was found. However, it was observed that 45.45 pmol/l hGH enhanced (by approximately 30%) the proportions of radioactive glycopeptides released from the column with 0.1 mol/l mannopyranoside (3.06% $\pm$ 0.48 ~ 4.53% $\pm$ 0.97, $2P < 0.02$) (Fig. 3). The quantity of glycopeptides leaving the column after the addition of 1 mmol/l glycopyranoside was not influenced by growth hormone.

When the unbound glycopeptides from the ConA Sepharose 4B column were further fractionated on a Pea Sepharose 4B column, only a minor part of the glycopeptides was retained. However, the quantity of radioactivity released by 10 mmol/l glycopyranoside from the Pea Sepharose 4B column was significantly increased when hGH-treated cultures were compared with control cultures (2.8% $\pm$ 2.3 ~ 7.9% $\pm$ 5.2, $2P < 0.01$) (Fig. 4).

Figure 4 The effect of 45.45 pmol/l human growth hormone on the proportion of glycopeptides which were not bound by a ConA Sepharose 4B column, but retarded in a Pea Sepharose 4B column and released with 10 mmol/l glycopyranoside. The glycopeptides isolated from the arterial basement membrane surrounding the smooth muscle cells in cultures were radiolabeled with [^3H]glucose for 48 h. The difference between the groups was significant, $2P < 0.01$. C = control. Means are shown as horizontal dashed lines.

Discussion

The present study demonstrates that growth hormone, at a physiological level, has a significant effect on the carbohydrate composition in the extracellular located BM-like material which surrounds the arterial smooth muscle cells.

It is important to note that the observed changes in the incorporation into galactose and glucose refer only to the relative values. A key question is whether the perceived alterations of the monosaccharides are results of changes in the proportion of peptides or of changes in the carbohydrates *per se*. In a previous study, we have not been able to identify any alterations in the peptide pattern after SDS-PAGE of material isolated from hGH-treated cultures (11). These observations indicate that the enlisted deviations are not a consequence of the presence of new sugar-rich peptides or major extensions of carbohydrate chains located to a few peptides, which in both situations may have altered the mobility of the peptides. Therefore, it seems reasonable to consider the results obtained to be a consequence of carbohydrate changes. The present data also suggest that collagen types IV and V may be involved, since these types of proteins belong to the very few in which glucose is seen. This glucose is bound to galactose in the glucose-galactose-hydroxylysine unit (19). A previous analysis has demonstrated that collagen types I and III are not present in our basement membrane preparation (8). It is noteworthy, that the alterations of the monosaccharides are the opposite of those seen after addition of physiological amounts of insulin (20).

It was not possible to reveal any effect of growth hormone on the incorporation of amino sugars using radioactive glucose as a precursor. However, when tritiated glucosamine was utilized growth hormone reduced the incorporation significantly. This observation is in agreement with our previous data showing a reduced incorporation of radioactive sulfate into the BM-like material after having added growth hormone to the arterial smooth muscle cells in culture (11). The findings are also compatible with the results demonstrating a reduced content of heparan sulfate proteoglycan in the basement membrane from kidneys obtained from individuals with diabetes mellitus (21).

From our analysis, it was observed that mannopyranoside increased the release of radioactive labeled glycopeptides obtained from growth hormone cultures and fractionated on ConA Sepharose columns. It is well known that ConA binds with affinity to the core trimannoside moiety 3,6-di- O -(α -D-mannopyranosyl)- α -D-mannopyranoside which is present in all asparagine-linked carbohydrates (16, 22). α -mannosidase treatment of the glycopeptides eluted in peak 3 from the ConA Sepharose column prevented the binding, as demonstrated by rechromatography (14). It cannot be ruled out that the reduced incorporation of glucosamine resulted in few β -1,4-linked N-acetylglucosamine residues on the α -1,6-linked mannose. This will increase the association constant to the ConA Sepharose and therefore retain more glycopeptides in the column (23).

It came to light that the proportions of glycopeptides released from the Pea lectin column was increased after growth hormone treatment. This type of lectin binds certain triantennary glycopeptides, which contain internal fucose residues. However, interaction can also be seen between the lectin and branched carbohydrate structures, which contain an α -mannose residue substituted at positions C-2 and C-6 (16). Therefore, the present data suggests a rise in the proportion of triantennary glycopeptides containing either mannosyl groups substituted at positions C-2 and C-6 or fucose residues.

The physiological role of the carbohydrate components in the arterial BM-like material or in other glycoproteins is partly unclear. However, it has been shown that sugar is important for the degradation (24). It appears that removal of either glucose or mannose gives a statistically significant increase in the degradation rate of BM-like material (8). Moreover, in a previous study the degradation rate of the basement membrane from the arterial smooth muscle was inhibited after incubation of the cells with growth hormone (11). Therefore, this reduced degradation is compatible with the observed changes in the carbohydrate unit in the BM-like material. The previous data and the present results suggest that growth hormone is of importance for the metabolism of extracellular matrix components

through changes in the carbohydrate units as seen in the BM-like material in the blood vessels.

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