Increase in Glycosaminoglycan Synthesis in Familial Hypercholesterolemic Fibroblasts

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The enzymes of biosynthesis are usually bound to membranes and require an undisturbed lipid environment for regulated activity. In familial hypercholesterolemia, this lipid environment is disturbed and there is a low cholesterol ester level in the cellular membranes that results from impaired processing of low density lipoprotein (LDL). Thus, altered activities of various synthesizing enzymes could be expected. In this study, we found that microsomal glucuronyltransferase activity was 3.4-fold higher in homozygous familial hypercholesteremic skin fibroblasts (n = 5) than in normal fibroblasts (n = 8). The secretion rates of glycosaminoglycans in familial hypercholesterolemia were also higher and the incorporation of radiolabeled precursor into glycosaminoglycans was enhanced. In addition to the differences in total synthesis and secretion, we found an altered pattern of individual glycosaminoglycan species with respect to the carbohydrate backbone and a different degree of sulfation. In familial hypercholesterolemia, heparan sulfate with a high degree of sulfation was secreted at a particularly high rate by hypercholesterolemic fibroblasts. Although the affinity of this glycosaminoglycan for LDL is lower than that of dermatan sulfate, it might cause formation of enhanced LDL-glycosaminoglycan complex and induce the xanthomas and atheromas of familial hypercholesterolemia.

(Arteriosclerosis 5:434-439, September/October 1985)

The main feature of skin fibroblasts from patients with homozygous familial hypercholesterolemia is the absence of specific receptors for low density lipoprotein (LDL). LDL thus cannot enter the receptor pathway or initiate cholesterol acylation, and this results in a low cholesterol ester content of the cells. Low cholesterol ester content is linked to increased membrane fluidity, and membrane fluidity is one of the parameters that control the activity of membrane-bound enzymes. Hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol synthesis, has been shown to depend on the cholesterol ester content of the microsomes. Our present study was conducted to determine if other membrane-bound enzymes are influenced by the low cholesterol ester content of familial hypercholesterolemic cells.

We chose to use microsomal glucuronyltransferase for two reasons. 1) This enzyme is involved in glycosaminoglycan (GAG) synthesis, and in familial hypercholesterolemia, LDL is deposited in tissues rich in glycosaminoglycans like the arterial walls and tendons. This specific targeting is characteristic in this disease, causing death by coronary heart disease when LDL is preferentially deposited in the upper aorta and the coronary arteries. An altered glycosaminoglycan pattern could provide an explanation for this particular targeting. 2) Some types of GAG exhibit a high affinity for LDL. The complex formation between both molecular species might initiate the characteristic macrophage degradation of LDL.

Methods

Chemicals

$^{14}$C-uridine diphosphate glucuronic acid (specific activity, 180 mCi/mmol), $^{14}$C-acetyl-D-glucosamine (specific activity, 55 mCi/mmol), L-1-$^{14}$C-leucine (specific activity, 55 mCi/mmol), $^{3}$H-thymidine (specific activity, 90 mCi/mmol), the scintillation cocktail Aquasol and $^{14}$C-HMG-CoA (specific activity, 45 mCi/mmol) were purchased from New England Nuclear Chemicals (Boston, Massachusetts). Chondroitin sulfates A, B, and C, hyaluronic acid and...
chondroitinase ABC and AC were from Sigma Chemical Company (St. Louis, Missouri). Heparitinase (EC 4.2.2.8) was prepared from fresh human platelets by affinity chromatography on immobilized heparin. Minimal essential medium and fetal calf serum were from Gibco Chemical Company (Grand Island, New York). LDL from human blood and low-protein-deficient medium were prepared as described elsewhere.

**Skin Fibroblasts**

Human skin fibroblasts were established and maintained as previously described. The controls were eight cell lines from healthy donors (five male, three female) 15 to 32 years of age. Informed consent was given in every case. Four cell lines (GM0486, GM0488, GM0701, GM2000) from patients homozygous for receptor-negative familial hypercholesterolemia and three cell lines (GM0283, GM0483, GM0700) from heterozygotes were obtained from the Mammalian Genetic Mutant Cell Repository (Camden, New Jersey). We also examined one cell line from a 14-year-old boy homozygous for receptor-negative familial hypercholesterolemia and one cell line from his heterozygous mother.

We examined all cell lines between the eighth and 12th passage. The cells were tested for 14C-leucine and 3H-thymidine uptake as described below.

Three days after the cells were split, confluent monolayers of each fibroblast line in 25 cm2 Falcon flasks were washed with phosphate-buffered saline. Then 5 ml of fresh medium containing either 5 μCi 3H-thymidine or 2 μCi 14C-leucine was added. After 3 hours at 37° C, the cells were washed three times with phosphate-buffered saline, scraped off, and suspended in 2 ml of saline to which 2 ml of 10% trichloroacetic acid was added. The precipitating material was pelleted by centrifugation and washed twice with 5% trichloroacetic acid dissolved in 200 μl 0.05 N NaOH. This was used for scintillation counting and protein determination. The incorporation rates (cpm/mg cell protein, means ± sD) for 14C-leucine were as follows: controls 588 ± 31 (n = 8); heterozygotes 602 ± 44 (n = 4); homozygotes 563 ± 26 (n = 5). The rates for 3H thymidine (cpm/mg cell protein, means ± sD) were: controls 290 ± 15 (n = 8); heterozygotes 274 ± 21 (n = 4); homozygotes 295 ± 18 (n = 5). Thus, the cells of each group were similar in protein synthesis and proliferation rates. The latter was lower according to the confluent state.

**Preparation of the Microsomes**

Three days after the cells were split, confluent monolayers of fibroblasts were washed twice with phosphate-buffered saline and were incubated for 24 hours with lipoprotein-deficient medium containing 200 μg/ml LDL protein. Then 15 ml of medium was added to each 75 cm2 Falcon flask, and the medium was aspirated.

The cell layers were washed twice with phosphate-buffered saline and were scraped off with a rubber policeman. The cells were suspended in ice-cold isotonic KCl (2 ml/mg cell protein) and were homogenized with a Potter-Elvehjem Teflon pestle homogenizer. Nuclei were removed by centrifugation at 900 g for 20 minutes; the resulting supernatant was centrifuged at 15,000 g for 20 minutes; and this 15,000 g supernatant was centrifuged at 105,000 g for 60 minutes in a Beckman Ti50 rotor with polycarbonate tubes. The resulting microsomal pellet was resuspended in 0.150 M KCl. After protein determination, the protein concentration was adjusted to 1 mg/ml. Glucose-6-phosphatase was assayed as described previously. Its specific activity in the final microsomal suspension was increased 14- to 16-fold as compared to the 900 g supernatant.

**Glucuronyltransferase Assay**

To determine the optimum conditions for the assay, a prestudy was performed with one normal and one homozygous cell line (GM2000). Each sample contained 25 μg of microsomal protein, 5 mM MnCl2, and 50 mM Tris buffer to a final volume of 100 μl. The substrate concentration was varied from 2 to 20 μM. The pH was 6.0, 6.5, 7.0, 7.5, and 8.0. The incubation time was 10, 20, 30, 40, and 60 minutes. The temperature was varied from 10° to 45° C. The following assay conditions were chosen for all cell lines: 10 μM 14C-glucuronic acid (UDP) pH 7.0 (specific activity 180 mCi/mmol) for 15 minutes at 37° C. The reactions were performed in the presence or absence of 1 mM UDP-acetyl glucosamine. After incubation, 400 μl 12% trichloroacetic acid were added. Precipitates were filtered on glass fiber discs (Whatman GF/B) and washed with 10 ml of methanol/methanol (4:1, vol/vol). Dried precipitates were added to 10 ml Aquasol, and radioactivity was determined in a Packard scintillation spectrometer.

**Secretion Rates of Glycosaminoglycans**

Three days after the cells were split, confluent monolayers of fibroblasts were equilibrated for 24 hours with lipoprotein-deficient medium containing 200 μg/ml LDL protein (15 ml/75 cm2 flask). Then the medium was replaced by an equal volume of the same medium to which was added 10 μCi 14C-acetyl-D-glucosamine (specific activity 55 mCi/mmol). The medium was aspirated after either 6, 12, or 24 hours. The cell layers were scraped off and sonicated, and aliquots were taken for protein determination. Then 1% pronase (Sigma Chemical Company) was added to the media followed by enough Tris to obtain a pH of 8.0. The incubation was carried out at 50° C for 24 hours; then the samples were cooled in ice water. Trichloroacetic acid was added to a final concentration of 10% (wt/vol).

The precipitating material was removed by centrifugation at 5000 g for 20 minutes. The supernatants were dialyzed extensively against distilled
water with benzoyl cellulose bags and were concentrated a final time with Amicon P2 filters. The samples were subjected to electrophoresis on cellulose acetate strips according to the method of Wessler. This step separated the GAG on the basis of their degree of sulfation. After elution with water, the uronic acid content of the electrophoretic fractions was measured by the carbazole method. Radioactivity was determined by scintillation spectrometry with Aquasol used at a counting efficiency of 80%. In all experiments, medium samples that had not been conditioned by cells were run as blanks.

**Enzyme Digestion Subtraction**

Aliquots of the electrophoretic fractions (1/6) were lyophilized. Two aliquots were dissolved in 50 μl Tris buffer (100 mM, pH 8.0) containing bovine serum albumin and either 0.1 unit of chondroitinase ABC (EC 4.2.2.4) or AC (EC 4.2.2.5) according to the method of Yamagata et al. One aliquot was dissolved in 50 μl 0.2M acetate buffer (pH 7.0) containing 50 μg of the heparitinase preparation. After 4 hours at 37°C, both reactions were stopped by heating at 100°C for 1 minute. Unreacted GAG were precipitated by adding 200 μl ethanol containing 1% potassium phosphate. After 1 hour at 4°C, the precipitates were collected by centrifugation at 5000 g for 20 minutes and were dissolved in distilled water for uronic acid determination and scintillation counting. GAG digested by heparitinase were regarded as heparan sulfate because this hydrolase is highly specific for this fraction. Material digested only by chondroitinase AC was termed chondroitin sulfate (sum of A and C). The amount of dermatan sulfate was calculated by subtracting the GAG digested by chondroitinase AC from the GAG digested by chondroitinase ABC. The significance was calculated by Student's t test.

In parallel experiments, GAG fractions obtained by electrophoresis were submitted to nitrous acid degradation at pH 1.5. The reaction was terminated by adding NaOg, and the reaction mixture was applied on a small column (tuberculin syringe) filled with Sephadex G-50. Elution was performed with distilled water. Fractions (0.3 ml) were collected, evaporated, and used in uronic acid determination. The retained fraction, regarded as a degradation product of heparan sulfate, was compared to the amount of material degraded by heparitinase.

To determine if the material not migrating in 0.1 N HCl electrophoresis was hyaluronic acid or heteroglycan chains of glycoproteins, we performed hyaluronidase degradation using leech hyaluronidase (100 μg/ml, McIlvaine's buffer, pH 5.6) (Biotrics, Boston, Massachusetts). The reaction mixture was fractionated on Sephadex G-50, as already described.

**Cholesterol Ester Content of the Microsomes**

Aliquots (20 μg) of the microsomal fractions were lyophilized and extracted with chloroform/methanol 2:1 (vol/vol) for 2 hours. Cholesterol esters were purified by thin-layer chromatography, hydrolyzed, and measured by gas chromatography according to published procedures.

**HMG-CoA Reductase Assay**

HMG-CoA reductase activity of the microsomal fractions was determined by using [14C-HMG-CoA (specific activity 45 mCi/mmol) (NEN Chemicals, Boston, Massachusetts). First, 5 pmol was added to 20 μg microsomal protein in 200 μl potassium phosphate buffer (50 mM, pH 7.4) containing 20 mM glucose-6-phosphate, 2 mM TPN, 4 mM dithiothreitol, and 1 unit glucose-6-phosphate dehydrogenase. After 30 minutes at 37°C, 20 μl of 3.6 N HCl was added. Mevalonolactone was isolated and counted as described.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Correlation of glucuronyltransferase activity with temperature. The enzymatic activities of isolated microsomes were plotted against incubation temperature; 25 μg of microsomal protein was used with a substrate concentration of 10 μmol/liter. One normal cell line was compared to one homozygous hypercholesterolemic cell line (GM2000). The insert depicts the corresponding Arrhenius plot in which the logarithms of enzyme activity were plotted against the reciprocal absolute temperatures.
GLYCOSAMINOGLYCANS IN HYPERCHOLESTEROLEMIA

Table 1. Comparison of Cholesterol Ester Content, HMG-CoA Reductase Activity, and Glucuronyltransferase Activity of Microsomes from Normal, Heterozygous, and Homozygous Familial Hypercholesterolemic Skin Fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>No.</th>
<th>Cholesterol ester content (mg/mg protein)</th>
<th>HMG-CoA reductase (pmol/mg min)</th>
<th>Glucuronyltransferase (pmol/mg min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8</td>
<td>4.2 ± 0.32</td>
<td>16.7 ± 1.1</td>
<td>1.3 ± 0.12 (0.07–0.1)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>4</td>
<td>2.5 ± 0.40</td>
<td>48.5 ± 4.6</td>
<td>2.8 ± 0.24 (0.05–0.09)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>5</td>
<td>0.8 ± 0.11</td>
<td>112.2 ± 4.9</td>
<td>4.4 ± 0.27 (0.06–0.09)</td>
</tr>
</tbody>
</table>

The values are expressed as means plus standard deviations of each group. The data on glucuronyltransferase activity that are in parentheses express the ranges of the activities in the absence of added UDP-acetyl glucosamine of three cell lines in each group.

Results

Glucuronyltransferase Activities

The activities of microsomal glucuronitransferase were determined by incorporating radiolabeled glucuronic acid into endogeneous microsomal acceptors. This was expressed as picomoles transferred in 1 minute by 1 mg of microsomal protein. The means and standard deviations of eight normal cell lines (1.3 ± 0.12 pmol/mg/min), four heterozygous lines (2.8 ± 0.24), and five homozygous cell lines (4.4 ± 0.27 pmol/mg/min) are compared in Table 1. The differences between each group were significant (p < 0.001). These data were obtained at 37°C. When UDP-acetyl glucosamine was absent, the activities were considerably lower, indicating that the enzyme is involved in glycosaminoglycan synthesis. The glucuronyltransferase activities were correlated to the HMG-CoA activities and inversely correlated to the cholesterol ester contents of the microsomes (Table 1).

The glucuronyltransferase activity was linear with time up to 30 minutes in every case. The optimum pH was 7.0 in normal and hypercholesterolemic cells, and the optimum temperature was 30°C (Figure 1). The sharp transition between rising and falling activity at 30°C is probably due to the phase transition temperatures of the fatty acid moieties (mainly palmitic acid). The Arrhenius plot (Figure 1, insert) illustrates that the logarithmic decline toward lower temperatures is less pronounced in familial hypercholesterolemia.

Secretion Rates of Glycosaminoglycans

The accumulation of glycosaminoglycan-bound uronic acid was linear with time up to 12 hours (Figure 2). Familial hypercholesterolemic skin fibroblasts secreted higher amounts of GAG-bound uronic acid into the culture medium than did normal fibroblasts; the highest values were for homozygotes. After incubation of the cells with 14C-acetyl-D-glucosamine, the highest radioactivity was found in the nonsulfated fraction of both normal and hypercholesterolemic cells (Figure 3). Digestion of this fraction with leech hyaluronidase yielded products with low molecular weight that were retained on Sephadex G-50. Apparently, this fraction consists of hyaluronic acid. Both hyaluronic acid and the sulfated GAG incorporated 14C-acetyl-D-glucosamine at a higher rate in familial hypercholesterolemia (Figure 3). In familial hypercholesterolemia, the pattern of secreted GAG fractions with respect to carbohydrate backbone and degree of sulfation was altered (Figure 4). Whereas dermatan sulfate was secreted at a slightly lower rate, heparan sulfate was secreted at a considerably higher rate after addition of the chondroitin sulfates.
14 C-acetyl glucosamine label in secreted glycosaminoglycans with different degrees of sulfation. Confluent fibroblasts were incubated with 14-acetyl glucosamine (10 μCi/75 cm² flask) for 6 hours. GAG isolated from the culture media were submitted to electrophoresis in 0.1 N HCl. Radioactivity (dpm/mg cell protein) was plotted against relative mobility (heparin = 1.0) which reflects the degree of sulfation. The bars represent the means of the controls (n = 8) and of the homozygotes (n = 5). Standard deviations are shown as vertical lines.

Figure 4. Pattern of GAG species secreted by fibroblasts. GAG species determined by enzyme digestion-subtraction were termed heparan sulfate (HS), chondroitin sulfate (CS) and dermatan sulfate (HS). Further differentiation was achieved by electrophoresis in 0.1 N HCl. The degrees of sulfation were: low (relative mobility 0.1–0.25), medium (0.25–0.4) and high (0.4–0.6) (heparin = 1.0). The bars represent the means (nanomoles of GAG-bound uronic acid secreted by 1 mg cell protein in 6 hours) of eight normal and five homozygous cell lines. The standard deviations did not exceed 6%.

Discussion

We designed this study to determine if the low cholesterol ester content of familial hypercholesterolemic cells correlates with changes in the activity of membrane-bound enzymes other than HMG-CoA reductase. We found that microsomal glucuronyltransferase, which is involved in glycosaminoglycan synthesis, exhibited higher activities and there was a reciprocal correlation to the cholesterol ester content of the microsomes (Table 1). We used the skin fibroblast system because skin fibroblasts are usually examined in research dealing with familial hypercholesterolemia, and because the typical symptoms of this disease, like xanthomas, occur in the skin.

The primary defect in familial hypercholesterolemia is the lack of defective LDL binding. Thus, the enhanced glucuronyltransferase activity must be due to secondary changes. Our working hypothesis in this study was that glucuronyltransferase, a membrane-bound enzyme, might be affected by the low cholesterol ester content of the cell membranes because of increased membrane fluidity. The changes in enzyme activity with temperature changes (Figure 1) support this idea because the differences between normal and homozygous cell activity are not affected by the phase transition temperature, which is mainly governed by the type of fatty acid moieties. Never-
theless, the enzyme activity falls less rapidly at lower temperatures in familial hypercholesterolemia, which indicates that, at lower temperatures, the membranes remain in a more fluid state than do normal membranes which contain more cholesterol esters.

The higher activities of glucuronyltransferase are correlated with enhanced secretion of glycosaminoglycans into the culture medium. This was our control parameter. In addition, we found that individual glycosaminoglycans are secreted at a higher rate, with different degrees of increase in the secretion rates.

The resulting changes in the glycosaminoglycan pattern in familial hypercholesterolemia show that heparan sulfate, in particular, highly sulfated heparan sulfate, predominate (Figure 4). Iverius7 reported that heparan sulfate exhibits an intermediate affinity for LDL whereas dermatan sulfate binds LDL more efficiently than does dermalan sulfate.21 Furthermore, heparan sulfate, with its simple carbohydrate backbone, forms complexes with LDL that initiate the degradation of LDL by macrophages.8 Iverius7 data do not exclude the possibility that the degree of sulfation, which can vary in the same glycosaminoglycan type,18 is an important factor in the formation of the LDL-GAG complex. It is unclear whether the affinity of heparan sulfate is intermediate7 or high; nevertheless, if it is secreted at a higher rate in familial hypercholesterolemia, this GAG probably enhances formation of the LDL-GAG complex. This may initiate degradation of LDL by macrophages,8 resulting in the characteristic targeting of LDL to the tendons, the skin, and the arteries with consequent xanthomatosis and atheromatosis.

Authors who have examined glycosaminoglycans in atheromas have obtained diverse results. For instance, one group22 found mainly dermatan sulfate; this would be consistent with Iverius' data.7 Others23 found mainly chondroitin sulfate and heparan sulfate. This inconsistency is probably due to an insufficient understanding of the underlying diseases, particularly hyperlipemias. When efficient methods of diagnosing different types of hypercholesterolemia become available,24 the glycosaminoglycans in the xanthomas and atheromas could be examined and better understood. We hope that our study will encourage such investigations.

References


Index Terms: glycosaminoglycans • familial hypercholesterolemia • skin fibroblasts • glucuronyltransferase
Increase in glycosaminoglycan synthesis in familial hypercholesterolemic fibroblasts.
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doi: 10.1161/01.ATV.5.5.434

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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