# Triggerlike Stimulation of Cholesterol Accumulation and DNA and Extracellular Matrix Synthesis Induced by Atherogenic Serum or Low Density Lipoprotein in Cultured Cells

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A 72-hour incubation of cultured cells with blood sera or plasma of patients suffering from coronary heart disease (CHD) with angiographically assessed coronary atherosclerosis caused a threefold to fourfold elevation of intracellular cholesterol. An elevated cholesterol level in the cells precultured with patients' sera was retained several days after the removal of the examined serum from culture. The accumulation of intracellular cholesterol was accompanied by enhanced synthesis of DNA, total protein, collagen, sulfated glycosaminoglycans, and hyaluronic acid. Enhanced DNA and total protein synthesis was retained for at least 9 days after the serum had been removed from culture. The obtained results suggest that the sera of CHD patients possess an atherogenic potential that manifests itself at the arterial cell level in the stable stimulation of atherosclerotic cellular processes: proliferation, lipidosis, and fibrosis. The examined sera of healthy donors were devoid of such an atherogenic potential. The low density lipoprotein (LDL) fraction (density, 1.030-1.050 g/cm<sup>3</sup>) obtained from an atherogenic serum had the same atherogenic potential as a whole serum. Atherosclerotic alterations in cultured intimal cells caused by atherogenic LDL were retained for at least 3 days after the removal of the lipoprotein from culture. Preincubation of intimal cells with LDL obtained from healthy donors had no effect on the intracellular cholesterol level or the synthesis of DNA and extracellular matrix. One may assume that the atherogenic potential of CHD patients' sera is related to the presence of LDLs that are qualitatively different from the LDL of healthy subjects. (Circulation Research 1990;66:311-320)

e have recently found that the blood sera of most (>90%) patients with angiographically assessed coronary atherosclerosis are able to induce the accumulation of lipids in cultured smooth muscle cells obtained from grossly normal intima of human aorta.<sup>1-4</sup> This property of patients' sera was termed "atherogenicity." Atherogenic sera caused a significant elevation of free cholesterol, triglycerides, and, especially, cholesteryl esters in cultured cells. The degree of intracellular lipid accumulation depended on the concentration of serum added to culture: the total cholesterol level reached the maximum at 20-40% atherogenic serum. The sera of most healthy donors had no atherogenicity.

Subsequently, it was demonstrated that low density lipoprotein (LDL) isolated from an atherogenic serum possesses the capacity to induce the accumulation of intracellular cholesterol in intimal cell cultures.<sup>2–4</sup> Most preparations of other lipoprotein classes as well as LDL obtained from healthy donors were nonatherogenic.

In addition to the accumulation of intracellular lipids, atherosclerotic lesions also are characterized by cell proliferation activity and connective tissue accumulation. We investigated whether the sera and LDLs obtained from the blood of patients with coronary heart disease (CHD) also possessed the ability to stimulate the other two essential atherogenic processes: cell proliferation and extracellular

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matrix formation. For this purpose, we compared the effects of the atherogenic sera obtained from CHD patients with the effects of nonatherogenic sera taken from healthy donors.

#### **Materials and Methods**

# Reagents

The reagents were purchased from Sigma Chemical, St. Louis, Missouri, if not stated otherwise.

# Donors' Sera and Plasma

For this study, we selected the sera of six CHD patients with angiographically documented coronary atherosclerosis and the sera of six healthy donors, each selected on the basis of a single criterion: the ability or inability of the serum to induce the accumulation of cholesterol in cultured cells (atherogenic and nonatherogenic sera, respectively). All the selected sera of CHD patients were atherogenic, and all the sera of healthy subjects were nonatherogenic. During 24 hours of incubation, the sera of CHD patients raised the cholesterol level in the cells by 75-262% (average, 150%). The atherogenicity of corresponding plasma was lower (49-162%: average, 98%). Ability of patients' sera to induce the accumulation of cholesterol was within the variation range characteristic of atherogenic sera.<sup>1</sup> Neither sera nor plasma of healthy donors' blood caused a statistically significant accumulation of intracellular cholesterol. The characteristics of CHD patients and healthy donors were described in detail elsewhere.<sup>1,2</sup> The group of patients and healthy donors was comparable with respect to such parameters as sex and age, and none of the 12 participants had diabetes mellitus. The mean cholesterol level in both types of the sera was the same:  $217 \pm 6 \text{ mg/dl}$  for patients and  $219 \pm 7$ mg/dl for healthy subjects.

In the morning, before meals, blood was drawn from the cubital vein into plastic tubes with caps. For serum preparation, the blood was incubated for 1 hour at 37° C and centrifuged for 20 minutes at 3,000 rpm. Blood sera were used immediately after preparation. For preparation of plasma, the blood was collected into 1 mg/ml Na<sub>2</sub>EDTA and then centrifuged. The lipoprotein-deficient fraction was obtained by centrifuging the serum at 300,000g (density, 1.250 g/cm<sup>3</sup>) for 48 hours at +4° C according to Lindgren<sup>5</sup> and dialyzing as described below. The obtained sera and plasma were sterilized by filtration (pore size, 0.22  $\mu$ m).

# Lipoproteins

LDL (density, 1.030–1.050 g/cm<sup>3</sup>) was isolated from the plasma obtained from patients and healthy donors according to the conventional method, described elsewhere,<sup>2,4</sup> of ultracentrifugation in a stepwise gradient of NaBr<sup>3</sup>. Protein content in the lipoprotein preparations was determined according to Lowry et al.<sup>6</sup> Lipid composition of LDL preparation was estimated as described earlier.<sup>7</sup> The percent composition by weight of the 1.030–1.050 g/cm<sup>3</sup> fraction was 22%protein, 52% cholesterol, 6% triglycerides, and 20% phospholipid for healthy donors' LDL. The corresponding ratio for CHD patients' LDL was 22%, 50%, 8%, and 20%, respectively (not statistically different from the values for the LDL of healthy donors). LDLs obtained from healthy donors were modified by malondialdehyde according to Haberland et al.8 Insoluble LDL complexes with heparin, fibronectin, and gelatin were prepared according to Falcone et al<sup>9</sup> as described elsewhere.<sup>10</sup> Lipoprotein preparations and the lipoprotein-deficient serum were dialyzed for 24 hours against 2,000 vol phosphate-buffered saline (PBS), sterilized by filtration, and stored for not more than 2 weeks at  $+4^{\circ}$  C. Lipoproteins were used within 1-4 days of preparation.

### Cell Cultures

Cells were obtained from thoracic aorta of 40- to 60-year-old men and women within 1.5-3 hours of sudden death caused mainly by myocardial infarction. Subendothelial smooth muscle cells were isolated from a grossly normal intima by dispersion of aortic tissue with collagenase and were cultured as described elsewhere.<sup>7,11</sup> A suspension of the isolated cells was resuspended in the growth medium containing Medium 199, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2.5  $\mu$ g/ml fungizone (all reagents from GIBCO, Grand Island, New York), and seeded into Linbro® 24-well tissue culture plates (Flow Laboratories, UK) with a density of  $2-4 \times 10^4$  cells/cm<sup>2</sup> of the growth area. The cells were cultured at 37° C in an atmosphere of 95% air-5%  $CO_2$  in a humidified  $CO_2$ -incubator. The primary cultures contained a mixed cell population composed primarily of typical and modified smooth muscle cells.7 The medium was changed every day. All additions were diluted to the final concentration with Medium 199. During the experiments, the medium in all cultures was changed every day with standard growth medium or replaced with growth medium containing 40% serum under investigation or 10%lipoprotein-deficient human serum, combined with 100  $\mu$ g LDL protein/ml. Twenty-four hours before the end of the experiment radioactively labeled precursors were added to culture to determine the synthesis of DNA and extracellular matrix.

#### Cholesterol

Before cholesterol determination the cells were rinsed three times with PBS, treated with 0.025% Trypsin-EDTA for 5 minutes, and washed with PBS five times (all reagents were from GIBCO). The cells were removed from the substrate with 0.25% Tryspin-EDTA and washed twice by centrifugation (200g for 10 minutes). Lipids were extracted from cells with a chloroform-methanol mixture (1:2 vol/ vol) according to Bligh and Dyer.<sup>12</sup> The total cholesterol content in the lipid extracts was determined using Boehringer-Mannheim Monotest®, Cholesterol CHOD-PAP Method (catalogue No. 236691,

Serum	Cholesterol content (µg/mg*)	Proliferation (dpm/µg*)	Total protein synthesis (dpm/µg*)	Collagen synthesis (dpm/µg*)	Sulfated glycosaminoglycan synthesis (dpm/µg*)	Hyaluronic acid synthesis (dpm/µg*)				
Version 1	Studied serum was added on days 6, 7, 8, and 9; cellular indexes were measured on day 10									
Nonatherogenic	64±13	185±13	$1,412 \pm 98$	$306 \pm 21$	$2,093 \pm 115$	$1,027 \pm 115$				
Atherogenic	208±15†	452±42†	$1,422\pm94$	297±23	2,239±139	1,275±87†				
Version 2	Studied serum was added on days 6, 7, and 8; studied serum was removed on day 9; cellular indexes were measured on day 10									
Nonatherogenic	55±7	$142 \pm 11$	$423 \pm 28$	22±2	994±72	721±53				
Atherogenic	172±13†	234±19†	606±52†	37±3†	$1,524 \pm 106 \dagger$	1,327±97†				
Version 3	Studied serum was added on days 3, 4, and 5; studied serum was removed on day 6; cellular indexes were measured on day 10									
Nonatherogenic	65±6	107±9	$258 \pm 30$	8±1	606±32	$402 \pm 43$				
Atherogenic	161±14†	$240 \pm 18^{+}$	405±22†	23±2†	$590 \pm 60$	711±50†				
Version 4 (control)	Cultivation under standard conditions from days 1 to 10; cellular indexes were measured on day 10									
Fetal calf serum	55±6	96±11	363±33	11±1	$565 \pm 58$	337±11				

 TABLE 1. Effects of Patients' Sera on Atherosclerotic Parameters of Subendothelial Cells Cultured From Grossly Normal Intima of

 Human Aorta

Atherogenic sera of patients and nonatherogenic sera of healthy donors were used. For each examined serum, atherosclerotic cellular indexes were determined in three separate wells. Version 1, studied serum was added on days 6, 7, 8, and 9; cellular indexes were measured on day 10. Version 2, studied serum was added on days 6, 7, and 8; removed on day 9; and cellular indexes were measured on day 10. Version 3, studied serum was added on days 3, 4, and 5; removed on day 6; and cellular indexes were measured on day 10. Version 4 (control), cultivation under standard conditions from days 1 to 10; cellular indexes were measured on day 10.

Values listed are means of the respective index for cells cultured with the sera of patients and healthy donors±SEM.

\*Micrograms (milligrams) of cell protein.

+Significant difference between the sera of patients and healthy subjects (p < 0.05).

Boehringer-Mannheim GmbH, Mannheim, FRG). Cholesterol stock standard (stock No. 965-25, Sigma Chemical) was used for standard preparation.

#### DNA Synthesis

DNA synthesis was evaluated by the incorporation of [<sup>3</sup>H]thymidine into the acid-insoluble cell fraction as described elsewhere.<sup>13</sup>

#### Total Protein Synthesis

After incubation with 1  $\mu$ Ci/ml [4,5-<sup>3</sup>H]leucine (135 Ci/mmol, Amersham International, Amersham, UK), the incubation medium and the cells, suspended with a rubber policeman, were heated for 10 minutes at 80° C, and proteins were precipitated by 10% trichloroacetic acid (TCA). The precipitate was washed twice with 5% TCA and twice with diethyl ether, dissolved with 0.5N KOH, and precipitated again by 10% TCA. The precipitate was resuspended in 5% TCA, and its radioactivity was measured.

# Collagen Synthesis

The synthesis of secreted collagen was determined according to Peterkofsky and Diegelmann<sup>14</sup> on [<sup>3</sup>H]proline incorporation in the collagenase-sensitive fraction.

## Glycosaminoglycan Synthesis

The synthesis of sulfated glycosaminoglycans and hyaluronic acid was determined according to Saarni

and Tammi<sup>15</sup> on incorporation of [<sup>3</sup>H]glucosamine hydrochloride.

#### Statistical Analysis

The significance of differences was evaluated by dispersion analysis methods using a statistical program package.<sup>16</sup>

#### Results

We compared the atherosclerosis-related effects of the atherogenic sera taken from CHD patients with the effects of nonatherogenic sera taken from healthy donors. Our experiments were threefold. First, atherosclerotic indexes were measured in the presence of the serum (Table 1, version 1). Second, the same indexes were determined on the next day after serum removal (Table 1, version 2). Third, the determination of atherosclerotic indexes was performed 3 days after serum removal (Table 1, version 3). As a control, we used cells cultured under standard conditions, that is, in the presence of fetal calf serum (Table 1, version 4).

Cultivation of intimal subendothelial cells in the presence of atherogenic sera for 96 hours (from day 6 to day 9 in primary culture) led to a nearly fourfold rise in the total intracellular cholesterol by day 10 (Table 1, compare versions 1 and 4). Similar exposure of nonatherogenic sera did not significantly alter the cholesterol level in cultured cells.

Cultivation of intimal cells in the presence of human sera stimulated the DNA synthesis. Incorporation of [<sup>3</sup>H]thymidine into the DNA of cells cultured with nonatherogenic sera of healthy subjects was nearly twofold higher compared with the [<sup>3</sup>H]thymidine incorporation into cells cultured under standard conditions, that is, with fetal calf serum (Table 1, compare versions 1 and 4). Atherogenic sera had an even stronger stimulative effect on the DNA synthesis of cultured cells. The incorporation of [<sup>3</sup>H]thymidine in the presence of atherogenic sera was substantially higher than in the presence of nonatherogenic sera.

Cultivation of intimal cells with both atherogenic and nonatherogenic sera sharply increased the synthesis of total protein, collagen, sulfated glycosaminoglycans, and hyaluronic acid. As compared with the cells cultured in the presence of fetal calf serum, the above-mentioned indexes in the cells cultured with human sera were several times higher (Table 1, compare versions 1 and 4). Atherogenic and nonatherogenic sera were no different with respect to their ability to increase the synthesis of total protein, collagen, and sulfated glycosaminoglycans. However, atherogenic sera were much more powerful in the stimulation of hyaluronic acid synthesis as compared with nonatherogenic sera.

To find out how the presence of serum affects the parameters in question, we modified the experiment so that cells were cultured with atherogenic or nonatherogenic sera for 72 hours, from day 6 to day 8. On day 9, the examined sera were replaced with a standard fetal calf serum and cells were cultured for another 24 hours. Then, on day 10, the cholesterol level and the synthesis of DNA, total protein, collagen, sulfated glycosaminoglycans, and hyaluronic acid were measured (Table 1, version 2). Under these conditions, we revealed a nearly threefold elevation of cholesterol in the cells cultured with atherogenic sera (Table 1, compare versions 2 and 4). Preexposure to nonatherogenic sera had no effect on intracellular cholesterol (Table 1, version 2).

A 3-day preexposure of intimal cells to atherogenic sera had a significantly higher stimulative effect on the synthesis of DNA, total protein, collagen, sulfated glycosaminoglycans, and hyaluronic acid compared with the preexposure to nonatherogenic sera (Table 1, version 2). Thus, preexposure of intimal cells to atherogenic sera of CHD patients led to the accumulation of intracellular cholesterol, enhanced synthesis of DNA, and extracellular matrix, that is, all the major manifestations of atherosclerosis at the cellular level. On the other hand, preexposure to nonatherogenic sera of healthy donors had little or no effect on atherosclerotic parameters.

We investigated whether the activation of atherosclerotic cellular manifestations induced by an atherogenic serum is retained for a certain period of time. A new modification of the experiment was as follows (Table 1, version 3). On days 3, 4, and 5, the culture medium was replaced with a fresh one containing atherogenic or nonatherogenic sera. On day 6, (i.e., after 72 hours of incubation with the examined sera), the medium was again replaced with a standard one containing fetal calf serum, and the cells were cultured under standard conditions for another 72 hours (i.e., until day 10). Then, all the atherosclerotic indexes were measured.

On day 10, none of the indexes in the cells preincubated with nonatherogenic sera of healthy donors from day 3 to day 6 significantly differed (p>0.05) from the respective characteristics of the cells cultured under standard conditions in the presence of fetal calf serum for the whole experimental period, that is, from days 1 to 10 (Table 1, compare versions 3 and 4). Thus, a 72-hour preexposure of intimal cells to nonatherogenic sera does not affect atherosclerotic cellular indexes even 3 days after the removal of nonatherogenic sera from culture.

A 3-day preexposure of cells to atherogenic sera of CHD patients led to stable alterations in atherosclerotic cellular indexes. Even 3 days after the transfer of culture into standard conditions, the cells preexposed to atherogenic sera had a much higher cholesterol level and a higher synthesis of DNA, total protein, collagen, and hyaluronic acid than the control cells (Table 1, compare versions 3 and 4). Of all the atherosclerotic parameters, only the synthesis of sulfated glycosaminoglycans showed nonsignificant differences from the control (standard) culture.

In connection with the ability of atherogenic sera to cause stable atherosclerotic alterations in intimal cells, it was essential to determine the durability of these alterations. Intimal cells were cultured with atherogenic or nonatherogenic sera from day 3 to day 6. On day 6, the medium was replaced with a standard one containing fetal calf serum. From day 7 to day 14, the three major parameters were measured: total intracellular cholesterol and the synthesis of DNA and total protein.

From day 7 to day 10, the cholesterol level in the cells precultured with atherogenic sera from day 3 to day 6 was 2.2-fold higher than in the cells precultured with nonatherogenic sera (Figure 1). After day 10, the intracellular cholesterol level decreased, although by day 14 it significantly exceeded the cholesterol content in the cells preexposed to nonatherogenic sera. The cholesterol content in the cells preexposed to nonatherogenic sera showed no changes from day 7 to day 14.

The [<sup>3</sup>H]thymidine incorporation into intimal cells increased with a period of cultivation (Figure 1). This reflects the process of gradual involvement of cultured human aortic cells into proliferation. As was shown earlier,<sup>11,13</sup> this process is especially active during the second week in primary culture. From day 8 to day 14, the [<sup>3</sup>H]thymidine incorporation into the DNA of the cells preexposed to nonatherogenic sera increased from  $188\pm15$  dpm/µg cell protein to  $379\pm19$  dpm/µg cell protein while in the cells preexposed to atherogenic sera it increased from  $291\pm15$ 



a medium containing 40% serum of coronary heart disease patients or 40% serum of healthy donors. On days 4 and 5, the culture medium was replaced with a fresh one containing the tested serum. Starting at day 6, the culture medium was replaced daily with fresh standard medium containing 10% fetal calf serum. Twenty-four hours before the determination of atherosclerotic parameters, radioactively labeled precursors  $[^{3}H]$ thymidine or  $[^{3}H]$ proline were added to the respective culture. The total cholesterol level and precursor incorporation were determined on days 7, 8, 10, 12, and 14. The respective indexes for each tested serum were determined in three separate wells. Values listed are mean  $\pm$  SEM of the corresponding index for the cells preexposed to patients' sera (solid line) or healthy donors' sera (broken line). Asterisks mark significant differences between the indexes of cells preexposed to these sera (p<0.05).

FIGURE 1. Total intracellular cholesterol content, prolifera-

tive activity, and total protein synthesis in cultures of intimal cells preexposed to the sera of patients with coronary heart

disease or healthy donors. In a 3-day primary culture of

subendothelial cells isolated from grossly normal intima of human aorta, the standard culture medium was replaced with

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dpm/ $\mu$ g cell protein to 495±38 dpm/ $\mu$ g cell protein. Any time the measurements were taken (up to day 14), the DNA synthesis in the cells preexposed to atherogenic sera was significantly higher than in the cells preexposed to nonatherogenic sera (Figure 1).

Atherogenic sera caused a stable stimulation of total protein synthesis. At least until day 14 in primary culture, the intimal cells preexposed to atherogenic sera from day 3 to day 6 synthesized protein at a considerably higher rate as compared with the cells preexposed to nonatherogenic sera (Figure 1).

Thus, the interaction of atherogenic CHD patients' sera with subendothelial cells of human aortic intima for 3 days led to a long-term activation of lipidosis, proliferation, and fibrosis, that is, exactly

Addition	Cholesterol content (µg/mg*)	Proliferation (dpm/µg*)	Total protein synthesis (dpm/µg*)	Collagen synthesis (dpm/µg*)	Sulfated glycosaminoglycan synthesis (dpm/µg*)	Hyaluronic acid synthesis (dpm/µg*)
None	43±3	35±7	$207 \pm 21$	15±1	$408 \pm 27$	235±29
Nonatherogenic LDL	45±4	92±9	$215 \pm 20$	14±1	$452 \pm 52$	$279 \pm 18$
Atherogenic LDL	82±5†	$138 \pm 14^{+}$	$305 \pm 14^{+}$	43±4†	$1027 \pm 89 \ddagger$	632±32†
MDA-LDL	112±9†	148±12†	$310 \pm 21 \dagger$	29±2†	832±96†	607±54†
LDL-Fn-Hp-G	$162 \pm 14^{+}$	$145 \pm 11^{+}$	387±15†	37±5†	$1043 \pm 115 \dagger$	$709 \pm 71 \ddagger$

TABLE 2. Effects of LDL Isolated From Atherogenic Plasma on Atherosclerotic Parameters of Subendothelial Cells Cultured From Grossly Normal Intima of Human Aorta

MDA, malondialdehyde. Atherogenic low density lipoprotein (LDL) was isolated from atherogenic plasma of patients, and nonatherogenic LDL was isolated from nonatherogenic plasma of healthy donors. Modified and insolubilized LDL were prepared from nonatherogenic lipoprotein of healthy donors. Until day 3 in primary cluture, intimal cells were cultured in the standard medium containing 10% fetal calf serum. On days 3, 4, and 5, the culture medium was replaced with a fresh one containing 10% lipoprotein-deficient serum of a healthy donor and 100 µg protein/ml of respective LDL. From day 6, the culture medium was replaced with a fresh standard medium every day. On day 9, labeled precursors were added to culture. Atherosclerotic cellular indexes were determined for each type of LDL on day 10 in three separate wells.

Values listed are means of the respective index for the cells preexposed to LDL or 10% fetal calf serum (control)±SEM.

\*Micrograms (milligrams) of cell protein.

†Significant difference from the control (p < 0.05).

those cellular processes that determine atherogenesis at the cellular level. It should be pointed out that the intracellular cholesterol level, which increased during cultivation with atherogenic sera, showed a tendency toward gradual decline 5 to 6 days after the removal of these sera from culture (Figure 1). The other two indexes, DNA and protein synthesis, remained elevated for at least 14 days in primary culture (Figure 1), which apparently points to the absence of a direct interrelation between the intimal cells' lipid level and their DNA and protein synthesis.

We have recently shown that LDL is the component of atherogenic CHD patients' sera that is responsible for its ability to induce the accumulation of intracellular lipids.<sup>2,4</sup> We decided to elucidate whether LDL obtained from atherogenic plasma of CHD patients can induce, similarly to their whole plasma, not only the accumulation of lipids but other atherosclerotic alterations as well. In other words, we attempted to find out whether the LDL fraction is atherogenic in a broad sense of the word, that is, not only lipidogenic but mitogenic and fibrogenic, too.

The LDL fraction (100  $\mu$ g protein/ml) obtained from atherogenic or nonatherogenic plasma (atherogenic and nonatherogenic LDL, respectively) was added to the culture medium containing 10% lipoprotein-deficient serum of a healthy donor. The experiments were carried out according to the protocol developed for version 3 of the experiments with whole serum (see Table 1). Cultivation with LDL continued for 72 hours, after which (on day 6) the cells were returned to the standard culture conditions. Atherosclerotic characteristics of cells were determined on day 10 in culture. The intracellular cholesterol level, synthesis of DNA, total protein, collagen, sulfated glycosaminoglycans, and hyaluronic acid in the cells preexposed to nonatherogenic LDL showed no statistically significant differences from the respective indexes of the cells cultured only under the standard conditions (Table 2). At the same time, preexposure of cells to atherogenic LDL led to a considerable increase in all atherosclerotic indexes.

Besides LDL isolated from atherogenic and nonatherogenic plasma, intimal cell cultures were supplemented with LDL chemically modified with malondialdehyde (MDA-LDL) as well as LDL within an insoluble complex containing, in addition to lipoprotein, fibronectin, heparin, and gelatin. Preexposure to these LDL also facilitated the accumulation of cholesterol in cultured intimal cells and increased the other parameters (Table 2). It should be pointed out that MDA-LDL and, especially, insoluble LDL-containing complexes caused a significantly greater accumulation of intracellular cholesterol as compared with atherogenic LDL. However, the other indexes (synthesis of DNA, total protein, collagen, glycosaminoglycans, and hyaluronic acid) increased approximately to the same extent as in the case of naturally occurring atherogenic LDL. This finding corraborates our previous assumption that there is no direct interrelation between the intimal cell lipid levels and the degree of other atherosclerotic parameters' increase.

### Discussion

Previous investigation of populations of CHD patients and healthy subjects (no signs of CHD) has shown that a small number of patients' sera were devoid of atherogenic properties (i.e., failed to stimulate the accumulation of cholesterol in aortic cells). At the same time, some sera of healthy subjects proved to be atherogenic.<sup>1</sup> In the present study, we compared the properties of atherogenic and nonatherogenic sera. For detailed investigation we selected the atherogenic sera of CHD patients since atherogenic sera are found more frequently in subjects suffering from heart disease. Nonatherogenic sera were taken from healthy donors since this type of serum is most often seen in healthy subjects.

Earlier, we used the term "atherogenicity" to describe the lipidogenic properties of CHD patients'

sera manifested in the elevation of intracellular lipids.<sup>1-4</sup> The use of this term proved to be quite justified. We have shown in this study that CHD patients' sera are not only lipidogenic but are mitogenic and fibrogenic, too. In other words, they possess a universal atherogenicity manifested under the conditions of culture. Thus, blood serum of most CHD patients contains all the elements necessary for the onset of atherogenesis in the vessel wall.

Sera of healthy donors (subjects with no signs of CHD) were nonlipidogenic, but had mitogenic and fibrogenic properties (i.e., stimulated the synthesis of DNA and extracellular matrix components). However, the mitogenic and fibrogenic effects of healthy donors' sera proved to be substantially lower after the serum was removed from culture, and completely disappeared 3 days later. Consequently, to manifest these properties, the sera of healthy donors require a constant contact with the cells. At the same time, the effects exerted on the intimal cells by CHD patients' sera were noticeable for at least 8 days after the serum was removed from culture. This means that the CHD patients' sera may act as a trigger, inducing cellular atherogenesis. Subsequently, the atherogenic process can develop even in the absence of the trigger.

The cellular indexes associated with atherosclerosis were investigated in many studies performed on different cultures. These studies focused on the effects of hyperlipidemic and diabetic sera upon the major atherosclerotic cellular indexes: accumulation of lipids, proliferative activity, and extracellular matrix synthesis. A summary of the obtained results follows.

Hyperlipidemic sera from cholesterol-fed animals induced lipid accumulation in various tissue culture cells and organ cultures.<sup>17-29</sup> The results of studies on the effect of hyperlipidemic serum on the growth of culture cells are conflicting. Many authors observed that hyperlipidemic sera from patients and cholesterolfed animals enhanced proliferation of cultured cells of various origins<sup>30–37</sup> and promoted labeling of cells in organ arterial culture with [<sup>3</sup>H]thymidine.<sup>27,38-40</sup> At the same time, other workers showed that during incubation, human hyperlipidemic serum either did not stimulate or slightly inhibited human arterial smooth muscle cell proliferation.<sup>41,42</sup> Sera of type 2 diabetics and sera of diabetic rabbits stimulated the growth of homologous smooth muscle cells more than normal sera.<sup>33,43</sup> Hyperlipidemic serum did not affect or slightly inhibited the synthesis of collagen by cultured arterial smooth muscle cells and isolated aortas.<sup>32,42,44-48</sup> However, it was found that hyperlipidemic serum stimulated the synthesis of collagen by freshly isolated cells.<sup>32,49</sup> Sera from type 1 diabetic patients significantly increased the production of collagen by cultured cells,<sup>50</sup> but in the case of sera from type 2 diabetic patients any stimulatory effect was absent.51 On the other hand, a clearcut increase in the synthesis of hyaluronic acid was found in the presence of type 2 diabetic sera.51 The synthesis of hyaluronic acid by cultured smooth muscle cells was reduced in the presence of hyperlipidemic sera.<sup>41</sup>

It can be concluded from these findings that hyperlipidemia and diabetes mellitus, important risk factors for atherosclerosis and CHD, are characterized by the presence of blood components that facilitate the development of certain atherogenic manifestations at the vascular cell level. However, very often atherosclerosis and CHD are not accompanied by diabetes mellitus and/or hyperlipidemia. Recently, we have reported that the blood sera of patients with CHD demonstrate lipidogenic properties irrespective of the presence or absence of concomitant diabetes mellitus.<sup>52</sup> The sera used in present study were not markedly hyperlipidemic. Consequently, diabetes mellitus and/or hyperlipidemia cannot be regarded as prerequisites for the realization of CHD patients' serum atherogenic potential at the arterial cell level.

Certain differences between the data obtained in the present study using the sera of CHD patients with angiographically assessed atherosclerosis from results obtained using hyperlipidemic or diabetic serum may be explained by the two factors. First, we used a homologous system including human serum and primary culture of human aortic subendothelial cells, that is, exactly those cells in which primary atherosclerotic alterations occur. The cited studies of other researchers were performed on medial smooth muscle cells or a heterologous combination of the sera and cells. Second, the difference of our results from other published studies may reflect specific properties of CHD patients' sera that cause it to differ from hyperlipidemic and diabetic sera. Recently, Laughton et al<sup>29</sup> confirmed our earlier observations.1 They found that sera from patients with angiographically documented coronary atherosclerosis cause marked lipid accumulation in cultured human arterial smooth muscle cells.

It was demonstrated that certain atherogenic properties of the serum are mostly associated with lipoproteins contained in these sera. Avogaro et al<sup>53</sup> have recently found a subfraction of LDL in normal subjects which causes the deposition of free cholesterol in cultured macrophages. This atherogenic modified LDL differed from native LDL by a higher negative charge and the somewhat altered lipid composition. Many studies have demonstrated that LDL obtained from normolipemic, hyperlipemic, or diabetic serum are mitogenic for arterial cells.<sup>30,34,54-63</sup> Others have shown little or no mitogenic effect of normal LDL<sup>34,64</sup> and an inhibitory effect of diabetic LDL.65 It was suggested that LDL are not themselves growth factors but rather may supply lipids required for maximal growth of mitogen-stimulated cells.<sup>61</sup>

Earlier, we demonstrated that LDL, unlike other classes of lipoproteins isolated from atherogenic sera, possesses lipidogenicity.<sup>2,4</sup> In the present study, we have found that this LDL possesses mitogenicity and fibrogenicity as well. Consequently, the LDL obtained from the blood of CHD patients with coronary atherosclerosis has the same atherogenic potential as whole serum. At the same time, preincubation of cells with LDL obtained from nonatherogenic plasma did not induce the accumulation of intracellular lipids or increase the rate of DNA and extracellular matrix synthesis. The presence of atherogenic potential in LDL obtained from the blood of CHD patients allows us to assume that this lipoprotein fraction is responsible for the atherogenicity of their whole blood serum.

The basic differences in the properties of LDL obtained from CHD patients and healthy donors that manifest themselves during the interaction with intimal cells led to the assumption that these types of LDL are qualitatively different. It is known that in culture, normal LDL fails to induce the accumulation of intracellular lipids,66-69 whereas chemically modified LDL or LDL insolubilized by forming a complex with extracellular matrix components is able to cause massive deposition of lipids within the cells.7,8,66,69-73 In present work, we have tested two types of modified lipoproteins: MDA-LDL and LDL insolubilized by fibronectin, heparin, and gelatin. In both cases, we observed the accumulation of cholesterol in cultured intimal cells accompanied with increased proliferation and production of connective tissue matrix. Thus, not only LDL obtained from CHD patients' blood but initially normal LDL modified by different methods, too, possesses an atherogenic potential manifested in cell culture.

Recently, we have demonstrated that the difference of CHD patients' LDL from native LDL of healthy donors is explained by low sialic acid content.<sup>74</sup> Atherogenic patients' LDL had a twofold to fivefold lower level of sialic acid as compared with native LDL. On the other hand, initially native LDL modified by partial desialylation with neuraminidase caused lipid accumulation in cultured cells. A detailed study of naturally occurring modified (desialylated) LDL is the subject of our current investigations.

In our view, the fact that the blood of CHD patients contains LDL that possesses atherogenic properties constitutes a very important finding. As is known, LDL can penetrate through the endothelial lining and accumulate within the intima.<sup>75</sup> One may assume that once within the intima atherogenic LDL, through interaction with subendothelial cells, can cause a whole range of atherosclerotic processes occurring at the cellular level: lipidosis, enhanced proliferation, and fibrosis.

We assume that the major event inducing atherogenesis is primary cellular lipidosis, that is, the accumulation of intracellular lipids induced by atherogenic LDL. In our view, stimulation of cell proliferation and enhanced connective tissue synthesis accompanying primary lipidosis are the consequences of intracellular lipid deposition. The mitogenic and fibrogenic effects of cell preincubation with atherogenic LDL were unrelated to the presence of lipoprotein in culture, since these effects were manifested 3 days after the removal of LDL from the culture medium. This finding brought us to the conclusion that the stimulation of proliferation and enhanced production of the extracellular matrix components arise from the deposition of intracellular cholesterol caused by atherogenic LDL. We think that the absence of a direct relation between mitogenicity, fibrogenicity, and the degree of primary cellular lipidosis means that the decisive factor is the deposition of excess lipid and not the amount.

Furthermore, our data allow the assumption that the development of cellular atherogenesis does not require constant transport of atherogenic LDL into the intima, since atherosclerotic alterations caused by this lipoprotein proved to be rather stable. This suggests two important conclusions. First, a brief local disturbance in the permeability of the endothelial lining, leading to massive penetration of atherogenic LDL within the subendothelial intima, may initiate atherogenesis even if normal permeability is subsequently restored. Second, constant presence of atherogenic LDL in the circulation is not necessary for the development of atherogenesis in the vessel wall. The emergence of atherogenicity in the blood even for a short time can be sufficient for the onset of atherosclerotic alterations in the vessel, after which atherogenesis would develop even if atherogenic factors disappear. This assumption may account for the fact that in a broad population of patients with coronary atherosclerosis, we found about 10% of patients whose blood had no atherogenic properties at the moment of study.<sup>1</sup>

## Acknowledgments

The authors thank Dr. S.N. Pokrovsky and his coworkers for their assistance with LDL isolation and Miss I. Beskhlebnova for illustrative work.

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KEY WORDS • blood serum • coronary atherosclerosis • intimal cells • fibrogenicity • lipidogenicity • mitogenicity • primary cell culture • human aorta • atherogenicity • low density lipoprotein