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Failure of Hypercholesterolemic Serum to Stimulate Collagen Synthesis in Aortic Smooth Muscle Cells from Two Species of Nonhuman Primates Having Different Rates of Collagen Synthesis[1] (41716)

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Abstract. African green monkeys develop atherosclerotic lesions that are more fibrous than those found in rhesus monkeys. The purpose of this study was to determine if this difference in collagen content could be related to a specific effect of a serum component on collagen synthesis by cells of the arterial wall or to inherent differences in the arterial smooth muscle cells from these two nonhuman primate species. Aortic smooth muscle cells were grown in tissue culture from both rhesus and African green monkeys and incubated for up to 48 hr with different concentrations of serum from normal or hypercholesterolemic animals. Collagen synthesis was determined by measuring the conversion of radiolabeled proline to hydroxyproline. Total protein synthesis was determined from the incorporation of radiolabeled proline into total proteins. Hypercholesterolemic serum from either species did not stimulate collagen or total protein synthesis in either African green or rhesus monkey smooth muscle cells. This was true even though under the same conditions other studies have shown that hypercholesterolemic serum produces an increase in cellular cholesteryl ester content. This suggests that hypercholesterolemia per se is not directly responsible for the stimulation of collagen synthesis typically seen in atherosclerotic lesions. African green monkey smooth muscle cells synthesized from 1.7 to 3.4 times more collagen than did rhesus monkey smooth muscle cells, while total protein synthesis was similar for both cell types. This suggests that the greater amounts of collagen present in the atherosclerotic lesions from African green monkeys may be in part the result of an inherently greater potential of their smooth muscle cells to synthesize collagen.

An increase in the collagen content is a well recognized feature of atherosclerotic lesions (1–3). Differences in the extent of collagen accumulation exist, however, among animal models even when exposed to a similar atherogenic challenge. African green monkeys (*Cercopithecus aethiops*), for example, develop atherosclerotic lesions that are rich in fibrous components, such as collagen, compared with rhesus monkeys (*Macaca mulatta*) which develop a predominantly fatty lesion with a paucity of connective tissue (4). The reasons for these differences are unclear but could be related either to differences in the potential for collagen synthesis by the cells of the arterial wall or to factors in the plasma that either stimulate or inhibit collagen synthesis.

Studies by others have shown that the connective tissue components of the arterial wall are synthesized principally by the arterial smooth muscle cells (5) and that the rate of collagen synthesis is increased in atherosclerotic arteries (6–11). The factors responsible for this stimulation in collagen synthesis appear to be directly related to the severity of atherosclerosis (6, 8, 10) and, in particular, to the extent of fibrosis of the atherosclerotic lesions. Previous studies by others have indicated that hypercholesterolemic rabbit serum when added to whole arterial segments *in vitro* (12) or to arterial smooth muscle cells in culture (13, 14) fails to stimulate the synthesis of collagen. The rabbit, however, develops atherosclerotic lesions that are principally fatty, and increases in collagen synthesis usually are not detected until the lesions are well established. Hypercholesterolemic rat serum also has been reported to have no influence on collagen synthesis of cultured rat smooth muscle cells (15). The rat, however, is resistant to the development of atherosclerosis, making extrapolation to other species uncertain. Thus, a more definitive understanding of the factors responsible for mediating collagen synthesis by arterial smooth muscle cells might be expected with the use of animals that develop

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atherosclerotic lesions with histologic features more typical of the lesions found in human beings.

The present study was based on the observation that atherosclerotic lesions from African green monkeys are more fibrous than those from rhesus monkeys. Thus, we evaluated whether arterial smooth muscle cells from these two species were different in their potential for collagen synthesis. In addition, we determined the ability of normal and hypercholesterolemic serum to stimulate collagen synthesis in arterial smooth muscle cells from these two species. Arterial smooth muscle cells in culture provide an attractive tool by which to study arterial collagen synthesis since they have been shown to actively synthesize both types I and III collagen, the principal collagen types found in the arterial wall (16–20).

Methods. *Cell culture.* Smooth muscle cells were grown from explants of the thoracic aorta of rhesus monkeys and African green monkeys and subcultured as described previously (21). Cells were routinely maintained in 75-cm^2 flasks with growth medium consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum and other components (21). Cells were routinely seeded (3×10^5) into 60-mm tissue culture dishes and grown to confluence prior to initiation of the collagen synthesis studies. Cells were split 1:3 at each passage and were used for these studies from the 5th to 20th passage.

Normocholesterolemic serum (NCS) and hypercholesterolemic serum (HCS) were obtained by venipuncture from rhesus and African green monkeys maintained in our primate colony. Normocholesterolemic rhesus monkeys were fed Purina Monkey Chow 25 (Ralston Purine Co.) and hypercholesterolemic rhesus monkeys were fed the same diet supplemented with 0.73 mg cholesterol/kcal as egg yolk. African green monkeys were fed a semisynthetic diet low in cholesterol (0.16 mg/kcal) or a diet enriched in cholesterol (0.79 mg/kcal) (22). The serum added to cells in culture represented the pooled serum from four animals of each diet group.

Measurement of collagen and total protein synthesis. The rate of collagen synthesis was determined by measuring the radioactive hydroxyproline produced following incubation of the smooth muscle cells with [^{3}H]proline. General protein synthesis was measured by determining the incorporation of the [^{3}H]proline into the total protein fraction. Once cells had reached confluence in 60-mm dishes they were incubated from 8 to 48 hr in 3 ml of Eagle's minimum essential medium containing 1–1.5 μCi/ml of L-[2,3-^{3}H]proline (New England Nuclear, sp act 24.7–35 Ci/mmole), 50 μg/ml sodium ascorbate, 100 μg/ml β-aminopropionitrile fumarate (to inhibit cross-linking), 100 I.U. penicillin/ml, 100 mg streptomycin/ml, 200 m*M* L-glutamine, twice the normal concentration of Eagle's vitamins, 20 m*M* *N'*-2-hydroxyethylpiperazine (Hepes), and the test sera as indicated in the individual experiments.

After incubation, the medium was poured off and the cells rinsed twice with phosphate-buffered saline (23) (pH 7.4) and the rinses combined with the medium. The cells were removed from the dish with 0.05% trypsin–0.02% disodium ethylenediaminetetraacetic acid (EDTA). The cells and the medium were treated separately but identically as follows. The samples were dialyzed exhaustively against distilled water at 4°C using Spectrapor membranes having a molecular weight cut-off of 10,000–14,000, evaporated to dryness under air, then hydrolyzed in 2 ml of 6 *N* HCl at 120°C for 24 hr under N_2. The samples were evaporated to dryness in a water bath at 60–80°C under N_2. The residue was resuspended in 2 ml of 0.1 *N* HCl, 0.1 μCi of L-[U-^{14}C]proline (New England Nuclear) was added as an internal standard and the sample filtered through a 0.45-μm Millipore filter. Proline and hydroxyproline were separated from 1 ml of the hydrolysate with 30×0.7-cm columns of Bio-Rad cation exchange resin, AG 50W-X8, 100–200 mesh. Since the separation of proline and hydroxyproline was essentially complete, we routinely collected hydroxyproline in the first 32 ml following the void volume (approximately 20 ml void volume) and proline in the next 52 ml. Using the [^{14}C]proline internal standard, an average of 4% of the proline fraction was found to contaminate the hydroxyproline fraction. The radioactivity in both the proline and hydroxyproline fractions were corrected for this contamination. Hydroxyproline radioactivity was used as a measure of collagen synthesis and

proline radioactivity was used as a measure of total protein synthesis. The ratio of proline to hydroxyproline radioactivity was calculated in order to determine whether collagen synthesis was altered disproportionately from total protein synthesis. Results were based on milligrams cell protein as determined on an aliquot of the isolated cells. The method of Lowry (24) was used with bovine albumin as the standard. The amount of protein per 60-mm dish ranged from 315 to 605 μg. Under the conditions of cell growth used in this study, the amount of protein per dish closely paralleled the number of cells such that cell protein accurately reflected the relative number of cells per dish.

No attempt was made to selectively extract or precipitate collagen, thus OH-[^{3}H]proline of elastin would also be contained within the protein hydrolysate. The error from this would be small, since the amount of hydroxyproline in elastin is only about 7% of that found in collagen (25).

Results. At all time points studied and with both rhesus and African green cells, between 90 and 95% of the newly synthesized collagen was found in the culture medium. On the other hand, only 60 to 70% of the total, newly synthesized proteins were found in the culture medium. There was no difference in this distribution between cells and culture medium for either the rhesus or African green cells with any of the sera tested. As a result, when calculating the proportion of total protein to collagen synthesis (dpm [^{3}H]proline/dpm OH-[^{3}H]proline) we used the OH-[^{3}H]-proline isolated from the culture media and the sum of the [^{3}H]proline from the media and cells.

In preliminary experiments, we tested the effect of a single addition of 50 μg/ml of Na ascorbate every 8 hr on hydroxyproline production. Similar to the results of Burke and Ross (26), there was no additional stimulation in hydroxyproline production with multiple supplementation, thus only a single initial 50 μg/ml of Na ascorbate was used in all subsequent incubations.

Figure 1 shows the results of an experiment in which collagen synthesis was measured in African green and rhesus monkey smooth muscle cells that were incubated for up to 48 hr in the identical medium containing 10% fetal bovine serum. Collagen synthesis was essentially linear for the entire 48 hr of incubation for rhesus monkey cells, but showed some decline in synthesis rate in the African green cells between 24 and 48 hr. Nevertheless, at all time points, the total amount of collagen synthesized in the African green cells was consistently greater than that of the rhesus monkey cells.

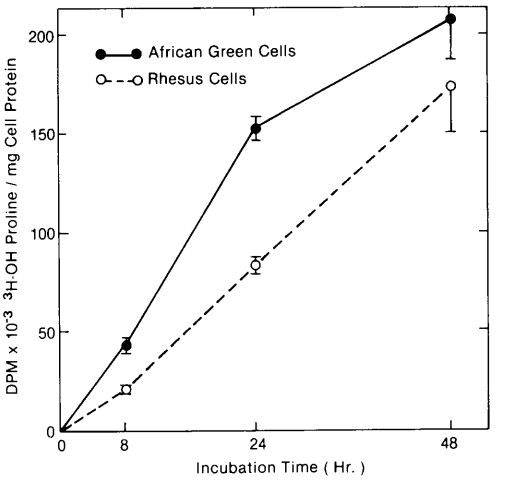

FIG. 1. Effect of incubation time on collagen synthesis in rhesus and African green monkey smooth muscle cells. Cells were grown to confluence in 60-mm dishes with culture medium containing 10% fetal bovine serum. Cells were then incubated for the indicated times in 3 ml of culture medium containing 1.25 μCi/ml of [2,3-^{3}H]proline and 10% fetal bovine serum. Rhesus smooth muscle cell line 423 and African green line 138 were used for this experiment. The African green cells were in the fourth passage and the rhesus cells were in the fifth passage. Results are the mean ± SEM of four replicate cultures except for the 48-hr point where there were two replicate cultures.

In order to confirm that the higher rate of collagen synthesis in African green smooth muscle cells, shown in Fig. 1, was a consistent feature of African green cells and not simply a peculiarity of the particular cells used in that experiment, we compared collagen synthesis in two different smooth muscle cell lines obtained from different animals. As shown in Table I, in three separate experiments in which cells were incubated for either 24 or 48 hr, the African green cells consistently synthesized from 1.7 to 3.4 times more collagen than did the rhesus cells. This was not the result of an overall greater rate of total protein synthesis

TABLE I. COLLAGEN SYNTHESIS IN RHESUS AND AFRICAN GREEN MONKEY SMOOTH MUSCLE CELLS[a]

	Rhesus		African green	
	(dpm OH-[^{3}H]proline)/ (mg cell protein)	(dpm [^{3}H]proline)/ (dpm OH-[^{3}H]proline)	(dpm OH-[^{3}H]proline)/ (mg cell protein)	(dpm [^{3}H]proline)/ (dpm OH-[^{3}H]proline)
Experiment 1 (N = 4)	83,570 ± 3,880[b]	34.4 ± 2.3	152,975 ± 5,373	19.4 ± 1.6
Experiment 2 (N = 4)	206,677 ± 15,014	13.2 ± 0.15	694,781 ± 40,658	8.5 ± 0.12
Experiment 3 (N = 4)	179,067 ± 2,973	15.8 ± 0.97	434,722 ± 24,192	6.7 ± 0.34

[a] Cells were grown to confluency using standard growth medium containing 10% fetal bovine serum and incubated for 24 hr (Expt 1) or 48 hr (Expts 2 and 3) in 3 ml of culture medium with 1.25 μCi/ml of [2,3-^{3}H]proline. These experiments were done at different times using different cells, thus, only comparisons between rhesus and African green cells within each experiment are valid. In Expt 1, rhesus monkey cell line 423 and African green cell line 138 were used. The African green cells were in their 4th passage and the rhesus cells were in their 5th passage. In Expts 2, and 3, rhesus monkey cell line 703 and African green monkey cell line 142 were used. The African green cells were in passage 14 in Expt 2 and passage 9 in Expt 3. The rhesus cells were in passage 20 in Expt 2 and passage 13 in Expt 3. The difference between rhesus and African green cells for both total collagen synthesis and the ratio of dpm [^{3}H]proline/dpm OH-[^{3}H]proline was significant ($P < 0.001$) for all experiments using the Student's t test.
[b] All values $\bar{X}$ ± SEM.

since the [^{3}H]proline/OH-[^{3}H]proline ratio was consistently lower for African green cells, indicating that the African green cells synthesized proportionately more collagen relative to total protein than did the rhesus cells exposed to the same experimental conditions.

Table II shows the effect of normal and hypercholesterolemic serum on collagen synthesis in smooth muscle cells from the two species. This experiment was a crossover design in which African green and rhesus cells were incubated with normal or hypercholes-

TABLE II. INFLUENCE OF NORMO- OR HYPERCHOLESTEROLEMIC SERUM ON COLLAGEN SYNTHESIS IN RHESUS AND AFRICAN GREEN MONKEY SMOOTH MUSCLE CELLS[a]

Treatment	(dpm OH-[^{3}H]proline)/ (mg cell protein)	(dpm [^{3}H]proline)/ (dpm OH-[^{3}H]proline)
Rhesus monkey serum		
Rhesus 703 cell line		
NCS	200,842 ± 7,565[b]	15.4 ± 0.50
HCS	192,459 ± 18,258	17.7 ± 1.5
African green 142 cell line		
NCS	454,839 ± 51,043	10.0 ± 0.45
HCS	378,256 ± 15,749	10.4 ± 0.12
African green monkey serum		
Rhesus 703 cell line		
NCS	185,367 ± 17,330 *	16.2 ± 0.09 *
HCS	129,475 ± 3,062 *	17.6 ± 0.23 *
African green 142 cell line		
NCS	438,327 ± 21,563 *	7.3 ± 0.18
HCS	311,935 ± 5,851 *	7.0 ± 0.07

[a] Cells were grown to confluence, then incubated for 48 hr with 3 ml culture medium containing 1.25 μCi/ml of [2,3-^{3}H]proline and either 10% normocholesterolemic serum (NCS, 112 and 158 mg chol/dl for African green and rhesus monkey serum, respectively) or hypercholesterolemic serum (HCS, 445 and 548 mg chol/dl for African green and rhesus monkey serum, respectively). The (*) indicates that NCS vs HCS are significantly different from one another ($P < 0.05$). The difference between rhesus and African green cells for both total collagen synthesis and the ratio of dpm [^{3}H]proline/dpm OH-[^{3}H]proline was signficant ($P < 0.001$) regardless of the serum used.
[b] All values $\bar{X}$ ± SEM (N = 4).

terolemic serum from either rhesus or African green monkeys. Collagen synthesis was significantly greater in African green cells, regardless of the type of serum added to the culture medium. Consistent with the results of Table I, the ratio of [^{3}H]proline/OH-[^{3}H]proline was lower for the African green cells relative to the rhesus cells. There was somewhat less collagen synthesis with the addition of hypercholesterolemic serum. This difference was statistically significant, however, only for African green serum in which the rate of collagen synthesis was about 30% lower with hypercholesterolemic compared with normal serum. The bulk of this change appeared to be due to a reduction in total protein synthesis as the ratio of [^{3}H]proline/OH-[^{3}H]proline changed only slightly and only for the rhesus cells.

Discussion. The results of this study confirm those of others using rabbit (13, 14) and rat (15) smooth muscle cells in showing that hypercholesterolemic serum does not stimulate collagen synthesis. Thus, even though our previous studies have shown that incubation of smooth muscle cells with whole hypercholesterolemic serum results in a marked accumulation of cellular cholesteryl ester (23, 27), the increase in cellular cholesteryl ester content is not associated with an increase in collagen synthesis.

These findings are consistent with other studies in which the stimulation in collagen synthesis and the resulting accumulation of connective tissue components of the atherosclerotic lesion has been shown to occur only after the lesions have progressed beyond the initial foam cell stage. Previous studies with rabbits (28, 29) and pigeons (30) have indicated that in the early stages of atherosclerosis, when the lesions are predominantly of the foam cell type with little necrosis or extracellular lipid, collagen content and synthesis are not increased substantially. Upon continued exposure to hypercholesterolemia, however, the synthesis of collagen is stimulated and fibrous components, including collagen, become a more prominent feature of the lesions (6, 7). The specific factors that are responsible for this stimulation in collagen synthesis are unknown but probably are associated with cellular necrosis and the release of cellular lipids and other constituents into the extracellular space. Consistent with this conclusion is the observation that cholesteryl esters are themselves sclerogenic if they are introduced into the arterial wall (31).

The present study also demonstrates that African green monkey smooth muscle cells synthesize collagen at a greater rate than do rhesus monkey smooth muscle cells. Both the absolute rate of collagen synthesis as well as the relative rates of collagen to total protein synthesis was greater for African green cells. This was clearly a property of the cells themselves since addition of serum from either species had little effect on collagen or total protein synthesis. In addition, since there was a greater proportion of collagen to total protein synthesis in African green cells, differences in the size of the endogenous amino acid pool probably cannot explain the higher absolute rate of collagen synthesis in the African green cells. As a result, the higher rate of collagen synthesis in African green smooth muscle cells may be one factor contributing to the more fibrous nature of the atherosclerotic plaques in African green monkeys relative to rhesus monkeys.

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