vitro and that this RNA and not contaminating unlabeled RNA is responsible for the translation products obtained. Similarly, while 4-thiouridine labeling partially inhibits reverse transcription of isolated RNA, a series of cDNA's specifically enriched for newly synthesized RNA sequences are generated using affinity-purified thiouridine-containing RNA as substrate (*9*).
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9 August 1984; accepted 8 November 1984

Platelet-Mediated Cholesterol Accumulation in Cultured Aortic Smooth Muscle Cells

Abstract. *Cholesterol accumulates within smooth muscle cells and macrophages in atherosclerotic lesions, thereby contributing to the progressive enlargement of these lesions. The mechanism of this cellular accumulation of cholesterol is not known. The possibility that platelets may have a role in the cellular cholesterol accumulation that occurs during atherogenesis was investigated. Incubation of thrombin-activated washed rat platelets (or platelet-free supernatants prepared from thrombin-activated platelets) with cultured rat aortic smooth muscle cells induced cholesteryl ester lipid droplet accumulation within the smooth muscle cells. No cholesteryl ester lipid droplets accumulated when smooth muscle cells were incubated with unactivated platelets. Smooth muscle cell lipid droplet accumulation occurred in the absence of serum lipoproteins and was not inhibited by mevinolin, a drug that blocks cholesterol synthesis. These findings suggest that activated platelets may release cholesterol, which can be accumulated by cells and stored as lipid droplets.*

Atherosclerosis is characterized by focal thickening of the intima of large- and medium-sized arteries. Thickening is caused in part by the accumulation of cholesterol (*1*) and cells [smooth muscle cells (*2*, *3*) and macrophages (*4*)] within the intima. Much of the cholesterol accumulates within cells. Progressive thickening of atherosclerotic lesions may result in myocardial infarction or stroke. Because the mechanism of vascular cholesterol accumulation is not known, a study was undertaken to examine a possible basis for cholesterol deposition.

Although much of the cholesterol in atherosclerotic lesions accumulates within cells, some accumulates in the extracellular space (*5*, *6*); this was shown with the use of filipin, a fluorescent dye that specifically binds to cholesterol. Cholesterol sometimes also accumulates at the surface of lesions (*6*), apparently within small thrombi. The association of filipin-stained cholesterol with thrombi suggested that platelets may have a role in the origin of vessel cholesterol deposits.

A study was undertaken to investigate whether platelets take part in cholesterol deposition and specifically to determine whether platelets could induce cholesterol accumulation within cultured vascular smooth muscle cells. This possibility was examined by incubating washed rat platelets with cultured smooth muscle cells of rat aorta in the absence of serum. Washed rat platelets were prepared from citrated (3.2 percent) blood removed—with a 19-gauge needle—from the abdominal vein of ether-anesthetized 3-month-old male rats (Sprague-Dawley). Platelet-rich plasma was prepared by centrifugation (3 minutes, 1500*g*, 22°C) of well-mixed blood. The platelet-rich plasma, which was prepared from the blood of each rat (~5 ml), was placed in a 15-ml conical polypropylene tube and centrifuged (30 minutes, 1400*g*, 22°C). The supernatant was removed, and platelet pellets were suspended in 10 ml of Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) containing 1 percent glucose. The platelet suspensions were centrifuged again (20 minutes, 900*g*, 22°C) and suspended in 10 ml of Ca^{2+}- and Mg^{2+}-free DPBS plus glucose. The platelets were counted with an electronic cell counter and centrifuged a final time (20 minutes, 900*g*, 22°C). The washed platelets were suspended in Ca^{2+}- and Mg^{2+}-free DPBS plus glucose at a platelet concentration of 15×10^8 per milliliter of medium. All washed platelet suspensions were pooled and held at room temperature for 2 hours before use.

Cultured rat (Wistar-Kyoto) aortic smooth muscle cells were maintained in polystyrene tissue culture flasks in minimal essential medium (MEM) containing 10 percent fetal calf serum (FCS). Confluent cultures showed the hill-and-valley pattern of growth typical of cultured smooth muscle cells (*7*).

Smooth muscle cell cultures for experiments were initiated by using trypsin to release cells from a confluent culture. Cells were plated at 160,000 cells in 4 ml of MEM and 10 percent FCS in single-well slide chambers and incubated overnight (37°C; 95 percent air, 5 percent CO_2). The medium was then replaced with 4 ml of MEM without FCS. Cell cultures remained in this medium for at least 3 days to eliminate any cholesteryl ester lipid droplets that may have accumulated during cell growth in FCS (*8*, *9*). Cultures seeded at 160,000 cells were confluent at the beginning of experiments. In some experiments, cells were plated at a lower density (40,000 per chamber) to produce nonconfluent cultures.

For experiments, the desired amount of washed platelet suspension (typically 2 ml of platelet suspension containing 30×10^8 platelets) was placed in a 12 by 75 mm polypropylene round-bottom tube and centrifuged (3 minutes, 1000*g*, room temperature). The platelet pellet was then suspended in 0.5 ml of Ca^{2+}- and Mg^{2+}-free DPBS plus glucose. Smooth muscle cultures were prepared for experiments as follows. Culture medium (3.5 ml) was removed and replaced with 3.0 ml of fresh MEM containing 0.35 percent bovine serum albumin (free of fatty acid and globulin). Experimental cultures then received 16 U of human thrombin to activate platelets; control cultures received no thrombin. The concentrated washed platelets (0.5 ml) were added to designated chambers and mixed with the culture medium by repetitive pipetting (three times). Additional control cultures received thrombin without added platelets. Cultures were incubated for specified times, rinsed three times with 4 ml of DPBS, and fixed for 1 hour (room temperature) with 4 ml of 10 percent phosphate-buffered Formalin.

Cell cultures were stained specifically for cholesteryl ester to determine whether cholesteryl ester lipid droplet accumulation had occurred in the cultured smooth muscle cells. Cultures were rinsed three times with 4 ml of DPBS (5 minutes for each rinse). Unesterified cholesterol was extracted from cells with a 5-minute incubation with 70 percent

ethanol (*10*), then rinsed one time in 4 ml of DPBS. Duplicate cultures were incubated (37°C) for 3 hours in 4 ml of 0.1*M* potassium phosphate buffer (*p*H 7.0), with or without 6 U of cholesterol esterase. Enzymatic hydrolysis of cholesteryl esters released unesterified cholesterol, which was stained for 1 hour with 1 ml of filipin solution (2.5 mg of filipin dissolved in 1 ml of dimethylformamide and added to 50 ml of DPBS) (*8, 9*). Cultures were rinsed three times with 4 ml of DPBS (5 minutes each rinse), slide chambers were dismantled, and slides were mounted with glycerol-gelatin containing 1 percent phenol. Slides were examined by phase and fluorescence (excitation filter UG1, barrier filter 520 nm) microscopy. Extraction of cellular unesterified cholesterol was verified by the absence of fluorescence in smooth muscle cells incubated without cholesterol esterase and stained with filipin. In some experiments, cell cultures were stained (60 minutes) with the lipid stain, oil red O.

Platelet activation (indicated by platelet aggregation) occurred when thrombin was present but did not occur when thrombin was absent. Smooth muscle cell cultures incubated with platelets and thrombin contained numerous attached platelet aggregates (Fig. 1, a and c), whereas cultures incubated with platelets without thrombin contained predominantly single attached platelets (Fig. 1e).

Smooth muscle cells incubated with platelets in the absence of thrombin were spread out (Fig. 1e) as were smooth muscle cells incubated without platelets. In contrast, smooth muscle cells incubated with platelets in the presence of thrombin were elongated and less spread out (Fig. 1, a and c). Thrombin alone had no effect on smooth muscle cell morphology.

Smooth muscle cells incubated with thrombin-activated platelets accumulated numerous cholesteryl ester lipid in-

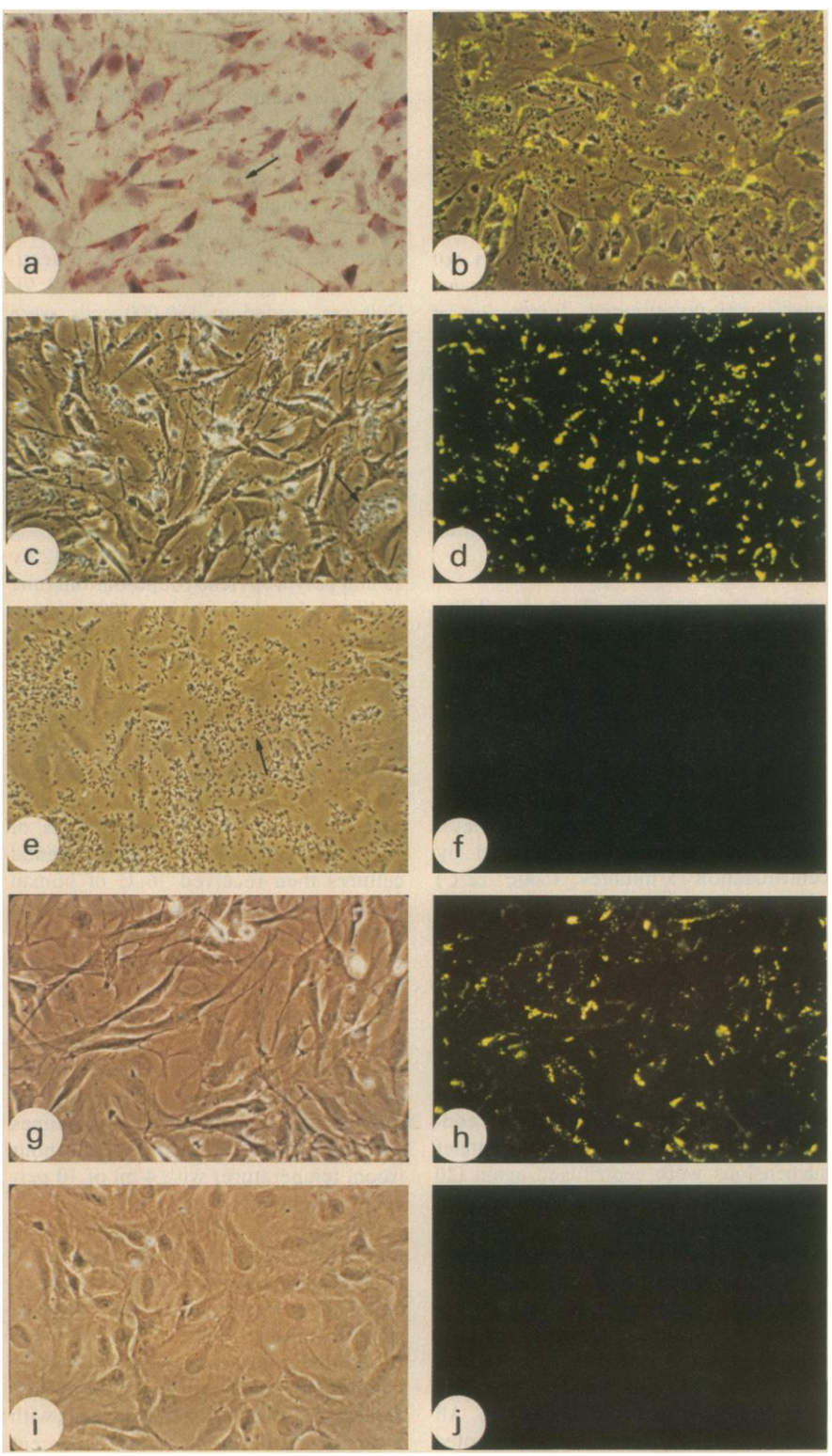

Fig. 1. Photomicrographs of cultured smooth muscle cells of rat aorta. The cells were incubated with (a to d) thrombin-activated platelets (30×10^8), (e and f) unactivated platelets, (g and h) platelet-free supernatants prepared from previously incubated thrombin-activated platelets (30×10^8), or (i and j) platelet-free supernatants prepared from previously incubated unactivated platelets. Magnification of all photomicrographs, ×170. (a) Bright-field photomicrograph of oil red O- and hematoxylin-stained smooth muscle cells shows lipid droplets (stained red) that accumulated within smooth muscle cells incubated with thrombin-activated platelets. No lipid droplets were observed in smooth muscle cells incubated with platelets or thrombin separately (data not shown). Cholesteryl ester (stained green), demonstrated within the lipid droplets by use of the fluorescent cholesterol stain filipin (see text for details of staining), is shown in separate phase (c) and fluorescence (d) photomicrographs of the same microscopic field and in a combined fluorescence and phase photomicrograph (b). Phase (e) and fluorescence (f) photomicrographs of the same microscopic field show that no cholesteryl ester accumulated in smooth muscle cells incubated with platelets in the absence of thrombin (that is, unactivated platelets). The thrombin-activated platelets are aggregated [indicated by arrows in (a) and (c)], whereas the unactivated platelets are not aggregated [indicated by arrow in (e)]. Phase (g and i) and fluorescence (h and j) photomicrographs of the same microscopic fields are shown. Cholesteryl ester accumulated in smooth muscle cells incubated with platelet-free supernatants prepared from platelets previously incubated with thrombin (g and h) (see text for details). No cholesteryl ester accumulated in smooth muscle cells incubated with platelet-free supernatants prepared from platelets previously incubated in the absence of thrombin (i and j).

clusions (Fig. 1, b to d). These cells contained oil red O–stained lipid droplets (Fig. 1a) similar to the oil red O–stained lipid droplets that accumulate in smooth muscle cells within atherosclerotic lesions (*5, 6*). Examination of the cultured smooth muscle cells, prepared for electron microscopic analysis as described (*11*), revealed that non-membrane-bound lipid droplets had accumulated. Addition of increasing numbers of platelets (7.5×10^8, 15×10^8, and 30×10^8 per culture) resulted in increasing amounts of cholesteryl ester within cells. The response of nonconfluent cultures of smooth muscle cells was similar to that of confluent cultures. No lipid inclusions accumulated in smooth muscle cells from cultures incubated with unactivated platelets (that is, in the absence of thrombin) (Fig. 1, e and f) or from cultures incubated with thrombin in the absence of platelets (data not shown).

The possibility that thrombin-activated platelets release a factor that induces cholesteryl ester lipid droplet accumulation in smooth muscle cells was tested. Washed platelets (30×10^8 in 0.5 ml of DPBS plus glucose) were added to 3.5 ml of MEM containing 0.35 percent bovine serum albumin in single-well slide chambers. These platelet cultures were incubated with or without thrombin for 24 hours in the absence of smooth muscle cells. Supernatants from these cultures were collected and then centrifuged at 1000*g* for 3 minutes and filtered (0.4 μm pore) to remove all platelets. These platelet-free supernatants when added to and incubated for 24 hours with smooth muscle cells caused cholesteryl ester lipid droplet accumulation (Fig. 1, g and h). Supernatants centrifuged at 10,000*g* for 10 minutes also induced cholesteryl ester lipid droplet accumulation in smooth muscle cells. Supernatants from platelets incubated without thrombin and then added to and incubated with smooth muscle cell cultures did not promote cholesteryl ester lipid droplet accumulation (Fig. 1, i and j).

Supernatants from platelets incubated with thrombin for increasing lengths of time (0.5, 1, 3, or 24 hours) before being added to and incubated for 24 hours with smooth muscle cell cultures promoted progressively increasing amounts of cholesteryl ester lipid droplet accumulation. However, supernatants from platelets incubated with thrombin for 48 hours before addition to smooth muscle cell cultures did not promote greater lipid droplet accumulation than did supernatants form platelets incubated with thrombin for 24 hours.

Addition of the cholesterol synthesis inhibitor mevinolin (10 μ*M*) (*12*) to smooth muscle cell cultures incubated with thrombin-activated platelets did not diminish cholesteryl ester lipid droplet accumulation. Hence, cholesteryl ester lipid droplet accumulation does not appear to depend on de novo cholesterol synthesis. Also, since platelet-free supernatants could induce cholesteryl ester lipid droplet accumulation, lipid droplet accumulation did not result simply from endocytosis of activated platelets by the smooth muscle cells. It is possible that thrombin-activated platelets release cholesterol in some form that can be taken up by smooth muscle cells and stored as cholesteryl ester lipid droplets. This possibility is supported by previous studies that showed release of cholesterol-containing lipoprotein complexes from thrombin-activated platelets (*13, 14*).

In addition to accumulating in smooth muscle cells, cholesterol accumulates within macrophages in atherosclerotic lesions. Furthermore, thrombin-activated platelets cause cholesteryl ester accumulation within cultured macrophages (*15*). Thus, platelet-mediated cholesteryl ester lipid droplet accumulation in macrophages and vascular smooth muscle cells may be related to cellular cholesterol accumulation in these cells within atherosclerotic lesions.

Induction of vessel-associated thrombi in experimental animals is associated with the development of lipid-containing atherosclerotic lesions (*16–20*). This occurs even when serum cholesterol levels are not high. Lesion development is inhibited in these animals when they are made thrombocytopenic (*20*).

The possible role of platelets in promoting smooth muscle cell proliferation within atherosclerotic lesions was recognized earlier (*21*). Since it has now been shown that platelets can mediate cholesteryl ester lipid droplet accumulation within cultured vascular smooth muscle cells, the possible role of platelets in cholesterol accumulation within atherosclerotic lesions must be considered in evaluating the pathogenesis of atherosclerosis.

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22. I thank G. Johnson, F. Carter, and R. Seabron for technical assistance; C. Kosh for help in preparation of the manuscript; the Upjohn Company for providing filipin; A. W. Alberts (Merck and Company) for providing mevinolin and W. Lovenberg and T. Nabika for providing the smooth muscle cell line.

5 October 1984; accepted 9 November 1984

Auditory Midbrain Responses Parallel Spectral Integration Phenomena

Abstract. *Resolving the frequency components of complex sound spectra including speech is an inherent, important accomplishment of the auditory nervous systems of vertebrates. The critical perceptual unit in the frequency domain, the critical bandwidth, has a simple functional equivalent within the principal midbrain auditory nucleus—the central nucleus of the inferior colliculus.*

The study of the resolution and interaction of components of complex sound spectra has been essential for understanding the basis of the perception of frequency spectra, for example, in speech, in music, and in animal vocalizations (*1*). Frequency resolution in the auditory system has been defined in various psychophysical tests. Common measures obtained are frequency bandwidths crucial for the perception of the quality of the sound stimulus, called "critical bands." Critical bands have been described as "filters" in the auditory sys-