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A Platelet-Dependent Serum Factor That Stimulates the Proliferation of Arterial Smooth Muscle Cells *In Vitro*

(primate/cell culture/atherosclerosis)

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ABSTRACT **Dialyzed serum from clotted monkey blood ("blood serum") promotes the proliferation of monkey arterial smooth muscle cells in culture, but dialyzed serum prepared from recalcified platelet-poor plasma ("plasma serum") is much less effective. Addition of platelets and calcium to platelet-poor plasma increases the activity of plasma serum to the same level achieved with blood serum. Furthermore, addition to plasma serum of a platelet-free supernatant prepared by exposing purified platelets to thrombin also stimulates the proliferation of smooth muscle cells. Thus, much of the growth-promoting activity of dialyzed serum is directly or indirectly derived from platelets. This finding has important implications for the response of arteries to localized injury and provides a key to further understanding of the role of factors derived from blood serum in promoting cell proliferation *in vitro*.**

We have been studying the growth of arterial smooth muscle cells (SMC) in culture as part of an attempt to determine why these cells accumulate focally in atherosclerosis (1). Since our working hypothesis has been that atherosclerosis is an exacerbated arterial response to local endothelial injury, we have been interested in the fact that SMC, like most other diploid, nontransformed cells, do not proliferate in culture except in the presence of blood serum. We have been attempting to identify the serum factors involved, in order ultimately to test the possibility that endothelial injury increases their concentration in the subendothelial space and thereby promotes SMC proliferation in the intima. We have already found (1, 2) that serum lipoproteins are necessary for optimal cell growth in culture, and now wish to report studies of a nondialyzable serum component that is probably not a lipoprotein and that appears to be derived from platelets.

MATERIALS AND METHODS

Procedures described in detail elsewhere (1–3), were used to subculture SMC from explants of the intima and inner media of the thoracic aorta of pigtail monkeys (*Macaca nemestrina*). The explants were grown in a modified Dulbecco–Vogt medium (3) based on that originally described by Eagle, and containing 5% or 10% pooled blood serum from the same species of monkey. SMC from the explants were subcultured in a similar medium that contained 5% blood serum and lacked streptomycin. Cells from the third to sixth subculture were used to test the effect of serum factors upon cell proliferation. The assay procedure used was similar to that of Paul *et al.* (4) (except for the use of monkey serum). A 2-ml Cornwall syringe was used to plate trypsin-treated SMC in equal numbers in 35-mm Falcon plastic petri dishes. The reproducibility of cell plating was confirmed in each experiment. The medium was changed three times per week. For the first 7 days the medium contained 1% pooled monkey blood serum. Thereafter, the 1% serum was replaced with the different serum fractions to be tested. Each fraction was added in an amount that corresponded to that provided by 5% monkey blood serum, and subsequent cell proliferation was compared with that obtained in medium containing 5% blood serum and 0% serum.

RESULTS

In initial experiments nondialyzable components were found to be responsible for most if not all of the effect of serum on SMC proliferation. To increase the supply of these com-

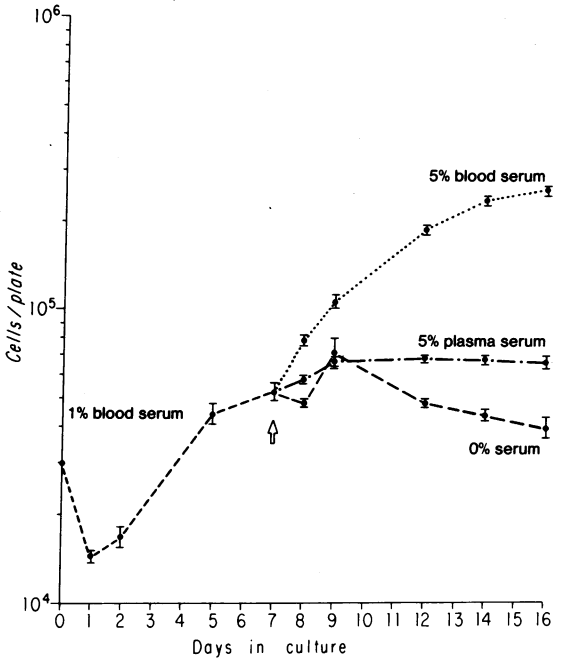

FIG. 1. Response of arterial smooth muscle in cell culture to blood serum versus plasma serum. Equal numbers (3×10^4) SMC were added to a large series of petri dishes and incubated in medium containing 1% pooled serum from several *Macaca nemestrina*. After 7 days (*arrow*) the dishes were separated into three groups. One group was incubated in serum-free medium. The other two groups were incubated in medium containing either 5% dialyzed whole blood serum or 5% dialyzed plasma serum. This experiment demonstrates that 5% dialyzed plasma serum had little to no proliferative effect when compared with dialyzed blood serum.

Abbreviation: SMC, smooth muscle cells.

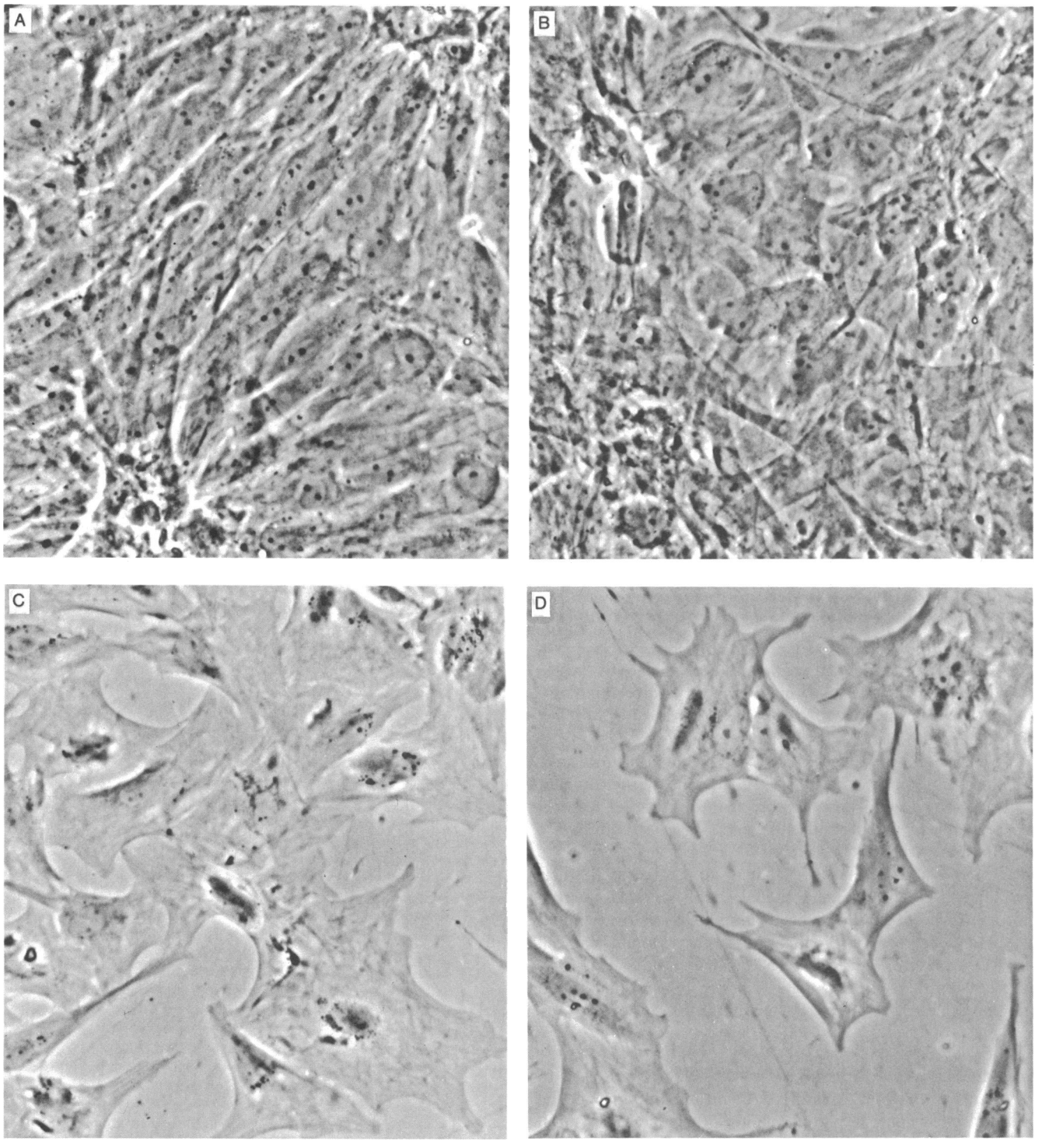

FIG. 2. These phase micrographs demonstrate the appearance of arterial smooth muscle cells (SMC) from *Macaca nemestrina* in culture. The cells were grown in 35-mm plastic Falcon petri dishes. All dishes were inoculated with 3.0×10^4 cells per dish, and incubated for a total of 15 days. The first 7 days the medium contained 1% blood serum. During the last 8 days the medium contained the following test sera or 0% serum. (*A*) Five percent blood serum—This micrograph demonstrates the characteristic appearance of a confluent culture of SMC in 5% blood serum. The cells tend to grow in "hills" (multiple layers of cells) and "valleys" (0-1 cell layer). Two "hills" are present in diagonally opposite corners of the micrograph. Due to the confluency, the cell outlines are somewhat obscured; but in areas containing single cells, they can be seen to have a bipolar, ribbon-like shape with prominent nucleoli. ×465. (*B*) Five percent plasma-platelet serum—The appearance of SMC in 5% plasma platelet serum is quite similar to that seen in *A*. The cells are again confluent and grow in "hills and valleys." ×465. (*C*) Zero percent serum—Smooth muscle cells appear larger than those grown in medium containing 5% blood serum. In addition, they are more irregular and lack a bipolar shape, appear flattened, and have a random distribution of myofilament bundles and more numerous dense cytoplasmic granules. ×465. (*D*) Five percent platelet-poor plasma serum, SMC are similar in appearance to those grown in medium with 0% serum. They often possess finger-like projections at their periphery. ×465.

ponents an attempt was made to utilize plasmaphoresis rather than the withdrawal of whole blood. Acid citrate–dextrose solution (U.S.P. Formula A) was used as anticoagulant, the blood was centrifuged at 100 × *g* for 20 min to remove the bulk of the cells, and the supernatant was centrifuged at 20,000 × *g* for 30 min to obtain essentially platelet-free plasma. The plasma was then dialyzed against Ringer's solution at 4° for 24 hr during which time it clotted. The dialyzed *plasma serum* was heat inactivated at 56° for 30 min and its growth promoting activity was compared with that of dialyzed *blood serum* obtained from the same pool of monkey blood. (Whole blood was allowed to clot at 4° for 24 hr and the resulting serum was dialyzed against Ringer's solution and heat-inactivated as in the case of the plasma serum.) Tests of the two types of sera showed that the dialyzed plasma serum was much less effective than the dialyzed blood serum in promoting cell proliferation (Fig. 1). In addition, phase microscopy of the cells after they had been grown in the test medium for 7 days revealed that SMC grown in the presence of either 0% serum or plasma serum were more irregular in shape and did not have the ribbon-like appearance seen in the presence of 5% blood serum and previously reported (5, 6) (Fig. 2). These findings suggested that much of the growth promoting activity of the dialyzed blood serum was directly or indirectly associated with material released from blood cells during clotting.

Since platelets seemed to be likely candidates responsible for this release (7), the following experiments were performed. Untreated whole blood and blood anticoagulated with sodium citrate (13 μmol/ml) were obtained from a group of monkeys. The whole blood was allowed to clot at 37° for 2 hr after which it was dialyzed and heat-inactivated as above. The citrated blood was divided into three parts. One part was used to prepare platelets by the procedure of Tangen *et al.* (8). The second part was immediately recalcified by adding $CaCl_2$ (14 μmol/ml), allowed to clot at 37° for 2 hr, dialyzed, and heat-inactivated. The third part was centrifuged at room temperature (9) to prepare essentially platelet-free plasma (see above). One aliquot of the plasma was then recalcified (20 μmol of $CaCl_2$ per ml), incubated at 37° for 2 hr, dialyzed, and heat-inactivated to obtain plasma serum. A second aliquot was recalcified after adding purified platelets in numbers equivalent to those present in the original citrated blood. After incubation at 37° for 2 hr the platelet–plasma serum was centrifuged at 20,000 × *g* for 30 min, dialyzed, and heat-inactivated. Thus the groups of smooth muscle cells were exposed to the following constituents added to the culture medium:

(*a*) 5% pooled whole blood serum,
(*b*) 5% pooled platelet-poor plasma serum,
(*c*) 5% pooled serum prepared by re-adding platelets (in amounts equivalent to that present in pooled 5% whole blood) to platelet-poor plasma + calcium.

The growth promoting activity of medium containing each of these dialyzed serum preparations was then compared with that of medium containing 0% serum [group (*d*)] (Fig. 3).

Cells grown in medium containing blood serum prepared from untreated blood or citrated, recalcified blood grew logarithmically for approximately 10 days before becoming stationary. Cells in 0% serum, the additional control, declined slightly in number, while cells grown in plasma serum proliferated minimally. In sharp contrast, cells grown in the presence of plasma serum prepared in the presence of platelets (platelet–plasma serum) grew logarithmically in a fashion identical to that of cells grown in the presence of whole blood serum and were similar in appearance (Fig. 2).

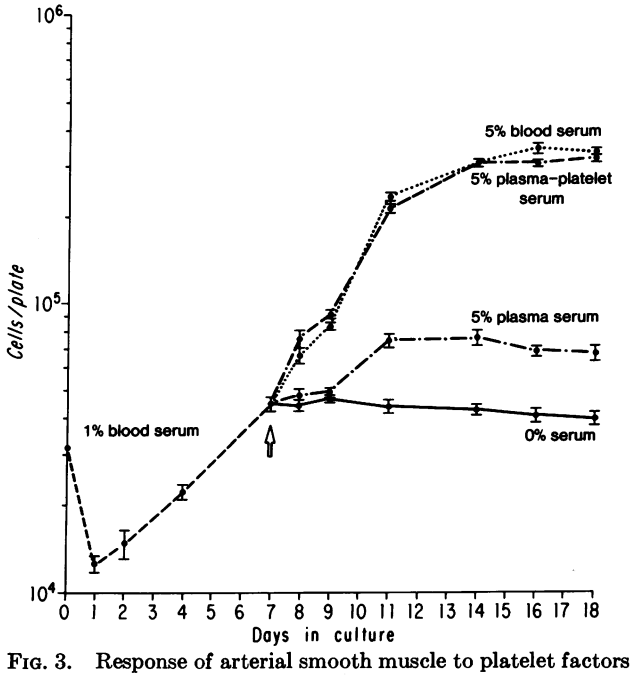

FIG. 3. Response of arterial smooth muscle to platelet factors in plasma serum. Equal numbers (3 × 10^4) of primate arterial smooth muscle cells were added to a large series of 35-mm petri dishes and incubated in medium containing 1% serum pooled from several *Macaca nemestrina*. After 7 days (*arrow*) the dishes were separated into four groups. One group was incubated in serum-free medium. The remaining groups were incubated in medium containing: 5% dialyzed serum from whole blood containing 3.95 × 10^8 platelets per ml; 5% dialyzed plasma serum which had been exposed during the process of recalcification and serum formation to an equivalent number of platelets, derived from the same pool of blood; 5% dialyzed plasma serum in which no platelets were present during the process of serum formation. This experiment demonstrates that 5% dialyzed plasma serum has little or no proliferative effect unless allowed to clot in the presence of platelets.

To determine whether the platelet factor(s) promoting the growth of SMC in culture is secreted during the platelet release reaction (10, 11), intact platelets were incubated for 5 min at 37° with purified thrombin (5 units/ml of platelet suspension), and then centrifuged at 20,000 × *g* for 30 min. The supernatant so obtained was then incubated for 30 min at 37° with recalcified, platelet-poor plasma that had previously been allowed to clot at 37° for 90 min. After dialysis, the mixture of serum and platelet supernatant had about one-half of the activity of whole blood serum when 64 μg of supernatant protein were added per ml of plasma serum. Furthermore, this activity was not diminished when the platelet supernatant and plasma serum were separately heated for 30 min at 56° before being mixed together.

DISCUSSION

These experiments demonstrate that serum prepared by adding calcium to monkey plasma lacks a factor present in monkey blood serum that promotes the proliferation of monkey arterial SMC in culture. These observations confirm those made by Balk (12) and extend them to another species

and another cell type. During studies that compared the effects of chicken plasma serum and blood serum in promoting the proliferation of chicken fibroblasts, he noted that plasma serum was much less effective than blood serum, and that the difference between the two types of serum was only partially related to the calcium levels present. Even with optimal calcium levels, plasma serum did not have the mitogenic effect of blood serum. Although we did not specifically study the role of calcium in promoting the proliferation of SMC in our experiments, the concentrations of calcium in the media containing the plasma serum and blood serum were identical, 2.38 mM, and exceeded the concentration found by Balk (12) to promote the maximal growth of fibroblasts. Thus the difference between the growth-promoting activity of plasma serum and blood serum did not depend upon calcium.

Our observations probably explain the differences Balk (12) noted, by identifying platelets as the source of the factor in blood serum necessary for stimulation of SMC proliferation.

In addition, our experiments demonstrate that the platelet factor(s) is released by the action of thrombin and that its action does not depend on substances, such as the fifth component of complement, that are inactivated by heating for 30 min at 56°. This raises fundamental questions with regard to the source of serum factors found by others (13–15) to modify cell proliferation in culture and with regard to the role of platelets in stimulating the response of arteries and other tissues to injury.

A number of different serum factors have been shown to stimulate either cell proliferation or cell migration *in vitro*. These include lipoproteins (1, 2), insulin (15–17), hormones (18), and fractions purified from whole blood serum by several different procedures (4, 13–15). Clearly, it will be necessary to examine the role of platelets in relation to each of these factors; and we have already performed experiments (unpublished observations) that dissociate the effect of the platelet-derived factor from that of lipoproteins.

Platelets clearly play a complex role in stimulating the response of tissues to injury (19, 20), but their potential role in stimulating the proliferation of subendothelial cells has not previously been recognized. For example, in a recent discussion (1) of the role of subendothelial SMC in atherosclerosis, we suggested that "local injury to the endothelium may increase the concentration of plasma proteins in the vicinity of medial smooth muscle cells and in response to some of these proteins the cells migrate into the intima and proliferate." It is now apparent that this concept must be specifically modified to include concepts developed by others concerning the adherence of platelets to focal areas of injured endothelium (19, 20), the release of specific platelet factors (10, 11) into the subendothelial space, and the proliferative response of SMC to the platelet-derived factor(s) demonstrated in the present investigation.

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