

# PLATELET FACTORS STIMULATE FIBROBLASTS AND SMOOTH MUSCLE CELLS QUIESCENT IN PLASMA SERUM TO PROLIFERATE

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## ABSTRACT

Whole blood serum is widely recognized as essential for the growth of diploid cells in culture. Dermal fibroblasts and arterial smooth muscle cells fail to proliferate in culture in the presence of serum derived from platelet-poor plasma. Platelet-poor plasma serum is capable of maintaining monkey arterial smooth muscle cells quiescent in culture at either low ( $1.5 \times 10^3$ ) or high ( $2.0 \times 10^4$ ) population densities. The proportion of cells traversing the cell cycle under these conditions was approximately 3%. Equal numbers of quiescent smooth muscle cells initiated DNA synthesis and cell division when treated with whole blood serum or with an equivalent quantity of platelet-poor plasma serum supplemented with a factor(s) derived from a supernate obtained after exposure of human platelets to purified thrombin *in vitro*.

Exposure of quiescent cells to 5% whole blood serum or 5% platelet-poor plasma serum plus 5  $\mu\text{g/ml}$  of crude platelet factor(s) preparations for 1 h stimulated as many cells to initiate DNA synthesis as did exposure for 24 hr, and was independent of the cell densities used in these experiments. Thus, one of the principal mitogens for fibroblasts and for arterial smooth muscle cells in culture present in all blood sera thus far examined appears to be derived from platelets. The system of diploid fibroblasts or smooth muscle cells grown in serum lacking platelet factor(s) permits one to use cells quiescent in the presence of optimal levels of serum and of the low molecular weight components of the defined medium for the study of growth control in culture. This system also permits the study of smooth muscle proliferation as it may relate to the proliferative response observed in atherogenesis *in vivo*.

Recently, attention has been focused on the role of arterial smooth muscle cells in the pathogenesis of arteriosclerosis (11, 14). Focal proliferation of smooth muscle cells in the intima of the affected arteries is the principal early feature of the development of the lesions of atherosclerosis, however,

the events preceding this proliferation and the factors that are important in the initiation of this cellular accumulation are not well understood. Ross and Glomset (14) have suggested that endothelial "injury" leads to exposure of subendothelial connective tissue with resultant platelet aggrega-

tion and focal release of material from the platelets; subsequently, smooth muscle cells migrate into and proliferate within the intima at the site of injury (14, 16). In addition, the release of platelet material as a result of bloodclotting in a skin wound could play a role in the migration and proliferation of dermal fibroblasts that occur in healing. We are presently examining these hypotheses with the use of an in vitro model.

In vitro primate (*Macaca nemestrina*) arterial smooth muscle cells (SMC) reach significantly greater cell population densities when treated with medium supplemented with serum derived from homologous whole blood (WBS) than when treated with serum derived from homologous platelet-poor plasma (PPPS). Smooth muscle cell cultures treated with medium supplemented with platelet-poor plasma serum (PPPS) to which purified platelets were added before clotting, or with PPPS enriched with the supernate from a thrombin-induced in vitro platelet release reaction, reach densities equal to those obtained in whole blood serum (WBS)-treated cultures (16). These observations suggested that platelets release material that enhances the proliferation of SMC in culture. Other investigators have published data describing the diminished capacity of plasma to support the growth of chicken fibroblasts (1) and the presence in platelets of "growth-promoting activity" for 3T3 cells (9).

Here we report the responses of dermal fibroblasts and arterial smooth muscle cells from the same animal (*M. nemestrina*) to the growth-promoting substances present in different preparations of homologous sera. The principal interest in our laboratory is directed toward understanding the biology of the smooth muscle cell, the nature of the response of these cells to material released from platelets, and the factors important in inducing smooth muscle proliferation in atherogenesis. Therefore, the majority of the experiments in this report were designed to answer specific questions concerning the nature of the response of quiescent cultured smooth muscle cells to treatment with the supernate from an in vitro platelet release reaction (16), or fractions of this material which have been shown to stimulate proliferation of SMC in cultures (unpublished observations). These experiments were carried out to determine: (a) the growth fraction (fraction of total cell population traversing the cell cycle) of cells previously rendered quiescent in culture in medium containing platelet-poor plasma serum (PPPS); (b) the ki-

netics of the onset of DNA synthesis and cell division in quiescent cultures of varying densities treated with PPPS supplemented with platelet release material (platelet factors, PF) or whole blood serum (WBS); and (c) the minimum time of exposure of cells to WBS or PPPS+PF-containing medium that will result in a maximal increase in the number of cells engaged in DNA synthesis.

## MATERIALS AND METHODS

### Cell Cultures

SMC were subcultured from explants of *M. nemestrina* thoracic aortas according to previously published methods (12). The dermal fibroblasts were subcultured from skin biopsies of the same animals (13). The cells were grown in Dulbecco-Vogt's modification of Eagle's basal medium (MDV) supplemented with varying concentrations of homologous whole blood serum (WBS) or with equivalent (on a protein basis) concentrations of platelet-poor plasma serum (PPPS) derived from the same donor pool of blood. Equivalence calculations were based upon the protein concentrations of PPPS after dialysis. These values ranged from 58 to 65 mg/ml (see next section). The cells were kept at 37°C in a moist 5% CO<sub>2</sub>-95% air atmosphere. Assays of cell accumulation were performed as described (15, 16).

### Preparation of Sera from Whole Blood and Platelet-Poor Plasma

Blood was periodically drawn into 3.5% sodium citrate (0.35% or 13 μmol/ml final concentration) from each of four adult male *M. nemestrina*, pooled, and divided into two fractions for preparation of WBS and PPPS. Blood specimens from four donors were pooled to avoid the commonly observed but poorly understood toxicity often seen in serum from single donors. The fraction for the preparation of WBS was kept at room temperature until small aliquots were removed for platelet counts and hematocrits. Calcium was then added to the remaining blood to 14 μmol/ml Ca<sup>++</sup> and the blood was incubated at 37°C for 2 h. Higher calcium levels had no effect on the growth of the cells in culture. After clot formation the blood was centrifuged in an SS-34 rotor (Dupont Instruments, Sorvall Operations, Newtown, Conn.) at 3,000 rpm for 15 min at 4°C. The supernate was then centrifuged at 13,500 rpm for 30 min at 4°C, pooled, and dialyzed against Ringer's solution at 4°C for 24 h (three changes). The serum was incubated at 56°C for 30 min, filtered, and frozen at -70°C. Aliquots were taken before and after dialysis for determination of protein concentration (10). The fraction to be prepared as PPPS was placed at 4°C immediately after drawing and the formed elements were sedimented at 4°C at 3,000 rpm for 30 min. The supernates were decanted and pooled. The plasma was incubated at 4°C for 3 h, calcium was

added (14  $\mu\text{mol/ml}$   $\text{Ca}^{++}$ ), and the plasma was incubated at 37°C for 2 h. The clot was sedimented at 13,500 rpm for 30 min at 4°C. The serum was decanted and then handled exactly as that prepared from whole blood. Before clotting, the blood was handled in siliconized glass or plastic vessels. The dialysis tubing (Dialya-Por, National Scientific, Cleveland, Ohio) had an exclusion limit of 6,000–8,000 mol wt.

### Preparation of Platelet Release Material

Human blood-bank platelets were purified (18) and released in vitro by treatment with thrombin as previously reported (16). This material, termed the whole release fraction, was utilized in some experiments on a platelet per milliliter concentration equal to twice that found in the blood from which the PPPS was prepared (16). The whole release fraction was further purified by chromatography on carboxymethyl-Sephadex (G-50-Pharmacia). The column was developed by a step-wise addition of 0.1 M Tris (pH 7.4), 0.1 M NaCl + 0.1 M Tris, and 1.0 M NaCl + 0.1 M Tris. The material eluting at 1.0 M NaCl still contained several proteins as seen on acrylamide and SDS gels and brought about an increase in the number of SMC in vitro (Glomset and Ross, unpublished observations). It was utilized at a concentration of 5  $\mu\text{g}$  protein per milliliter of medium and is referred to in the text as platelet factor(s) (PF).

### Steady-State Cultures at Different Densities

SMC were plated on round, 25-mm diam plastic cover slips (Lux Scientific) and the cover slips were placed in 35-mm plastic culture dishes (Falcon Plastics, Oxnard, Calif., or Corning Glass Works, Science Products Div., Corning, N.Y.) at densities which varied initially by a factor of five. Actual cell densities varied between experiments and are presented in the appropriate figure legends. 16–18 h after plating, an equal number of cover slips with cells from each density group were placed into 100-mm plastic dishes containing 10 ml of MDV containing 5% PPPS. These cultures were then refed every 48 h and cell accumulation was monitored by daily counts of the cells in one 100-mm dish (two or three cover slips per density group). For counting, the cover slips with cells were washed in isotonic Tris-Versene and then placed singly in 35-mm dishes containing 1 ml of 0.05% trypsin (Difco Laboratories, Detroit, Mich.) in Tris-Versene and incubated for 10 min at 37°C. The cover slips were then rinsed five times with the 1 ml of trypsin cell suspension which was pipetted into an equal volume of 20% Formalin in a 50–50 mixture of PBS and methanol. The cells were counted in a Cytograf (Bio/Physics Systems, Inc., Mahopac, N.Y.). Cultures were considered to be in a steady state as regards cell accumulation when there was no net change in cell density over a 48-h period. Cultures typically reached this state 4–7 days after transfer to PPPS medium.

The effect of WBS and platelet factors was tested on steady-state SMC on cover slips. These cover slips were

removed from the PPPS medium, washed twice in Tris-Versene, and equal numbers of cover slips from each density group were placed in the appropriate test medium in new 100-mm plastic dishes.

### Labeling with [ $^3\text{H}$ ]Tdr and [ $^3\text{H}$ ]Tdr Autoradiography

Two different labeling protocols were utilized. In experiments designed to determine the growth fraction (fraction of total cell population traversing the cell cycle), steady-state cells were exposed to 0.1  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]Tdr (sp act 6.7 Ci/mmol) for varying time-periods up to 48 h. For other experiments, 0.2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]Tdr was added for 1 h at different times between the treatment of steady-state cells with WBS, PPPS+PF, or PPPS, and the onset of a measurable increase in cell number. At the end of the labeling period, the cover slips were removed, washed by sequential immersions in PBS, fixed with Formalin (7), air dried, and mounted on microscope slides with Permout. The slides were dipped into Kodak NTB-2 emulsion (40°C), air dried for 1 h, and then stored at 4°C for 10–14 days. The autoradiographs were developed in Dektol, rinsed in running water, stained with hematoxylin and eosin, and dehydrated in successive ethanol baths. The fraction of labeled nuclei (labeling index, [4]) was determined at a magnification of 250 in a Zeiss photomicroscope. The total number of nuclei counted for each determination is presented in the respective figure legends.

The limited number of cells obtained from each group of explants prevented the use of more than three cover slips per density group at each time-point in any of these experiments. This restriction was particularly stringent when cells subcultured from the explants of a single donor were utilized for different experiments. Therefore, each experiment was repeated on cells from three or four different donors, and large numbers of nuclei were counted for each determination. One representative experiment of each type is included in this report. The growth fraction of quiescent smooth muscle cells varied from 3% to 5% among different donor animals (Fig. 2). The maximum labeling index after a 1-h pulse with [ $^3\text{H}$ ]thymidine 24 h after introduction of the test agent varied from 10% to 20% among different donor animals. However, the kinetics of initiation of DNA synthesis and cell division and the duration of treatment required to initiate these responses were highly reproducible among several experiments in which cells from different donors were utilized (Figs. 3 and 4).

## RESULTS

### Proliferative Response of Fibroblasts and Smooth Muscle Cells to Platelet-Poor Plasma Serum

The growth curves in Fig. 1 compare the responses of skin fibroblasts and arterial smooth

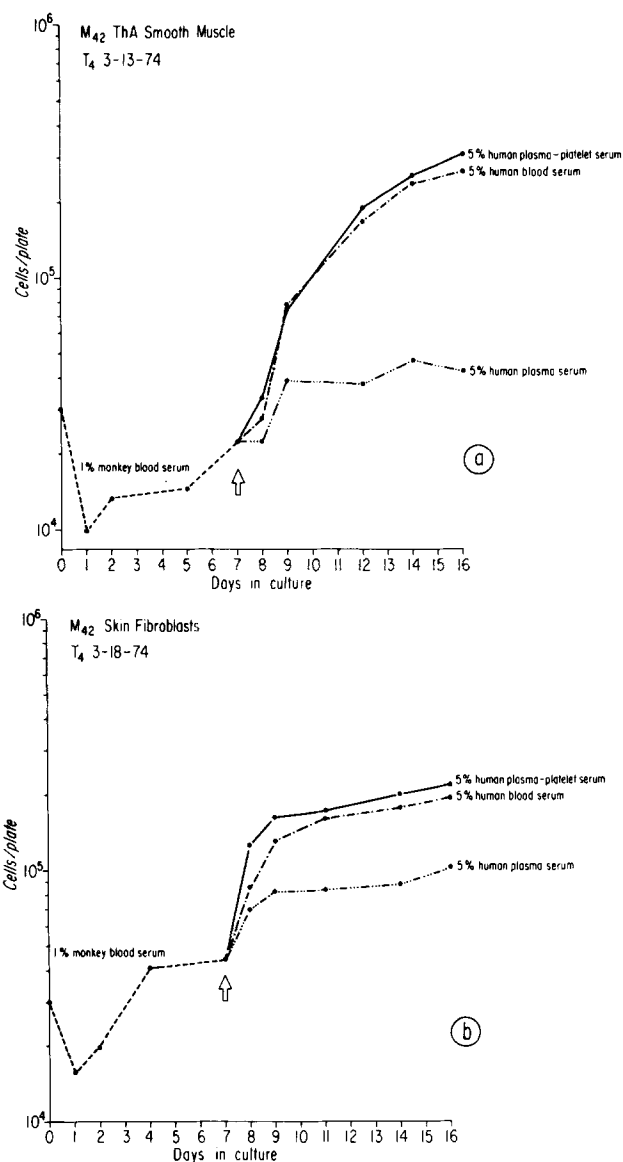


FIGURE 1 The accumulation of dermal fibroblasts (*b*) and arterial smooth muscle cells (*a*) in the presence of whole blood serum, platelet-poor plasma serum, or platelet-poor plasma serum plus platelet factor is plotted as a function of time. Initially,  $3 \times 10^4$  cells were plated in 35-mm dishes in 1% whole blood serum medium and refed every 48 h. At day 7, the appropriate test media were added and daily counts were made from triplicate dishes. Variation among dishes averaged less than 10%.

muscle cells from the same animal to whole blood serum and platelet-poor plasma serum (PPPS) prepared from the same blood pool. Fibroblasts as well as smooth muscle cells cultured in PPPS medium fail to accumulate to the same levels as cells cultured in whole blood serum (WBS)-containing medium. While there were variations among final population densities achieved and the slopes of the curves, the data clearly demonstrate

that the material released from platelets restores the mitogenic capacity present in WBS and lacking in PPPS for both homologous fibroblasts and arterial smooth muscle cells.

#### Definition of Quiescent Cells

Smooth muscle cells cultured for 4–7 days in 5% PPPS medium that was replaced every 48 h showed no net change in cell number (steady state).

The percentage of the total cell population traversing the cell cycle under these conditions was approximately 3% in the experiment depicted here and was not significantly different for cells cultured at the two different population densities (Fig. 2). We defined steady-state cells as quiescent when the growth fraction as determined by continuous labeling with [<sup>3</sup>H]Tdr (2) was 5% or less. The sharp increase in the labeling index between 15 and 20 h observed in Fig. 2 probably represents partial synchrony of the cell population. The reason for this is unknown.

### *Kinetics of Onset of DNA Synthesis and Cell Division in Quiescent Cells*

Quiescent smooth muscle cells maintained in replenished 5% platelet-poor plasma serum (PPPS) at different culture densities had a labeling index (2) of less than 1% (Fig. 3). Treatment of these cells with whole blood serum (WBS) or PPPS plus platelet factor (PF) resulted in an increase in the fraction of S-phase cells (labeling index) followed by a net increase in the number of cells. In no experiments did the peak labeling index exceed 20%, and it usually ranged between 10% and 15% at 24 h after treatment. The cell population densities doubled within 48–60 h after a single treatment with either WBS or PPPS+PF. Control

cultures fed with medium containing only PPPS demonstrated minimal change in the labeling index or culture density.

Quiescent smooth muscle cells maintained at several different initial culture densities varying by three- to five-fold demonstrated no differences in their response to WBS or PPPS+PF. In these experiments, direct comparison of the response of cells at different population densities could be made since an equal number of cover slips from each density group was placed in the test medium. The variation in cell number among cover slips was 5% for the high density and 10% for the low density groups.

### *Effect of Varying Exposure Times of Quiescent Cells to Platelet Factors*

The cultures utilized to study the kinetics of the initiation of DNA synthesis and cell division were maintained in the appropriate test medium for the duration of the experiment. To estimate the minimum time of exposure to the test agents that was required to initiate cell cycle traverse, quiescent cells were treated with WBS or PPPS+PF for varying periods of time. Cells treated for 1 h manifested the same labeling index 24 h after treatment as did cells treated for 24 h (Fig. 4). This result was also obtained in experiments in which

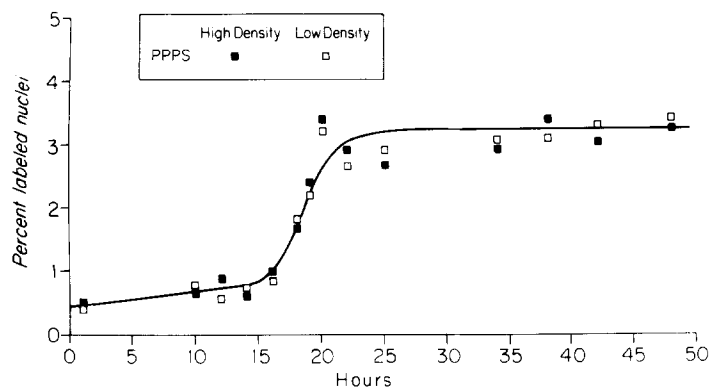


FIGURE 2 This experiment demonstrates the accumulation of labeled nuclei in continuously labeled cultures of steady-state SMC cells treated with 0.1  $\mu$ Ci/ml [<sup>3</sup>H]Tdr (sp act 6.7 Ci/mmol) in medium supplemented with homologous plasma-derived serum lacking platelet factor(s) (PPPS) to a final concentration of 5%. [<sup>3</sup>H]Tdr was added 24 h after a medium change in cultures which were at a steady state for 4 days before the start of the experiment. At each specified interval, three cover slips from each density group were removed and processed for autoradiography. 1,000–1,200 nuclei were counted per cover slip and the average labeling index (percent labeled cells) was calculated for the high and low density cover slips. The variation among cover slips for each time-period was less than 10%. At time zero, cell population densities for this experiment were  $3.3 \times 10^5$  cells/cm<sup>2</sup> and  $1.2 \times 10^4$  cells/cm<sup>2</sup>. Low density cultures, □; high density cultures, ■.

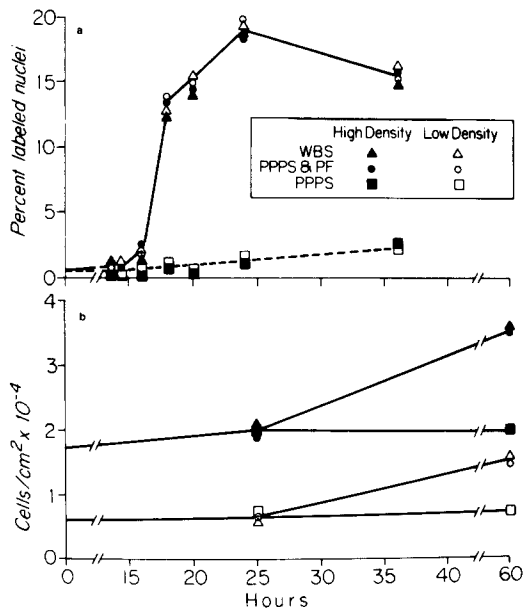


FIGURE 3 The kinetics of onset of DNA synthesis (a) and cell division (b) in SMC maintained in 5% plasma-derived serum lacking platelet factor(s) (PPPS) medium and treated at time zero with 5% whole blood serum (WBS), 5% PPPS + 5  $\mu\text{g/ml}$  platelet factor(s) (PF), or 5% PPPS alone. At intervals after treatment, cells from each treatment group were labeled with 0.2  $\mu\text{Ci/ml}$  (final concentration diluted in the particular test medium) [ $^3\text{H}$ ]Tdr (sp act 6.7 Ci/mmol) for 1 h and then processed for autoradiography. The initial cell densities were  $6 \times 10^8$  cells/cm $^2$  and  $1.8 \times 10^4$  cells/cm $^2$ . Each point in Fig. a represents the average labeling index of three cover slips per density group, with 800–1,000 nuclei counted per cover slip. WBS,  $\Delta$ ; PPPS,  $\square$ ; PPPS + PF,  $\circ$ ; high density, solid; low density, open.

the whole release material was utilized. Again, there were no apparent differences in response among cultures of different densities. The cell number for each group, except those treated with PPPS, doubled by 60 h after treatment (data not shown).

## DISCUSSION

These experiments were designed to provide a system for studying the events that are important in the initiation of in vitro proliferation of smooth muscle cells and fibroblasts. Our approach was (a) to optimize conditions necessary to maintain cells quiescent in vitro; (b) to stimulate quiescent cells to initiate DNA synthesis and cell division; and (c) to study the effects of cell population density and

duration of stimulatory treatment on the transition from quiescence to growth.

## Quiescent Smooth Muscle Cells

Cells in culture have usually been rendered quiescent by relative nutrient or serum starvation at very low (1% or less) serum concentrations (5–8), or by being grown to a stationary phase at a particular serum concentration (22), which may represent another form of serum deprivation. The fact that smooth muscle cells in culture failed to proliferate in 5% PPPS (16) suggested that these cells could be rendered quiescent in this medium. Our results clearly confirm this idea (Figs. 1–3). Cells cultured in defined medium or in very low concentrations of serum tend, with time, to lose their capacity to respond to mitogenic stimuli (3). The present studies demonstrate that after 7 days in platelet-poor plasma serum (PPPS) medium, quiescent cells responded in a quantitatively equivalent manner to the mitogenic effects of either WBS or platelet factor(s), and that, within the limits of population sizes and the serum and platelet factor concentrations used in these studies, this response was independent of cell population density (Figs. 3 and 4). These data and those from Fig. 1 suggest that platelet-poor plasma serum provides the necessary factors for the maintenance of cells in culture and that the principal mitogen in whole blood serum for both cultured arterial smooth muscle cells and dermal fibroblasts is derived from platelets.

Factors derived from platelets may also be important in response to injury. Dermal fibroblasts would only be in contact with serum, or with material released from thrombocytes by clotting during injury such as in wound repair. Similarly, in vivo smooth muscle cells would not come into contact with these agents except during platelet aggregation and release. This could occur at foci of endothelial cell damage where platelets could be exposed to collagen or other constituents that would stimulate the release reaction.

Thomas (21) has obtained data from swine that suggest that in vivo the growth fraction of arterial medial smooth muscle is small. Therefore, smooth muscle cells quiescent in platelet-poor plasma serum may be more analogous to intact tissue when cells would normally contact plasma or a filtrate of plasma but not serum; therefore, this may represent a more physiological model for the study of the effects of platelet factor(s) and other

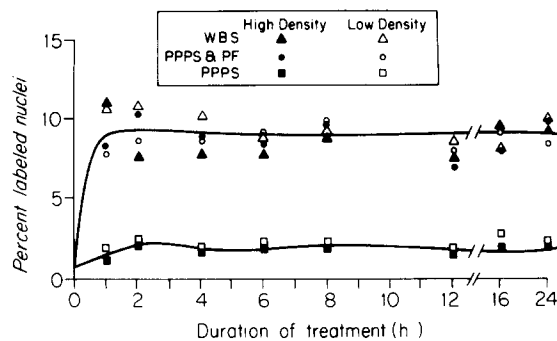


FIGURE 4 The fraction of SMC in S after treatment of quiescent cells for varying periods of time with 5% homologous whole blood serum (WBS) or 5% plasma-derived serum lacking platelet factor(s) (PPPS) + 5  $\mu\text{g/ml}$  platelet factor (PF). At 24 h after replenishing the PPPS medium, equal numbers of cover slips from each density group were placed into the different test media and, after the indicated periods of time, the cover slips were washed three times with Tris-Versene and then replaced into the same PPPS medium from which they were removed. Incubation was continued so that each group was incubated for 24 h after initiation of treatment. All cells were then labeled with 0.2  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]Tdr (sp act 6.7 Ci/mmol) for 1 h (at 24–25 h after start of treatment) and processed for autoradiography. The labeling index represents the percentage of cells in S between 24 and 25 h after initiation of treatment. 800–1,000 nuclei per cover slip were counted, and the average labeling index was calculated from three cover slips per treatment group per point, WBS,  $\Delta$ ; PPS + PF,  $\blacktriangle$ ; PPPS,  $\circ$ ;  $\square$ ; high density, solid; low density, open.

“growth-promoting” agents on the initiation of proliferation of these cells.

#### Initiation of DNA Synthesis and Cell

##### Division in Quiescent Smooth Muscle Cells

Quiescent SMC respond to treatment with whole blood serum (WBS) or platelet-poor plasma serum and platelet factor(s) (PPPS+PF) by an increase in the proportion of cells that synthesize DNA and subsequently by a doubling of the culture density (Fig. 3). The sequence of events after stimulation suggests that the population of SMC, quiescent in PPPS and responsive to treatment with WBS or PPPS+PF, resides in the  $G_1(G_0)$  stage of the cell cycle. Other cells in culture rendered quiescent by nutrient limitation, serum starvation, or growth to high culture densities also respond to release from the particular restrictive condition by initiating DNA synthesis and cell division although the kinetics of the response differs among cell types (reviewed in reference 20).

One need expose SMC to medium containing platelet factors for only 60 min or less (Fig. 4) to stimulate as many cells to initiate DNA synthesis as are stimulated by a 24-h exposure. We cannot be sure that simply removing the cells from PPPS+PF or WBS medium and washing them in serum-free buffer disengages the active agent(s) from the cells. However, these results support the

notion of a “serum-sensitive” phase followed by a “serum-insensitive” phase (19, 23) for the initiation of DNA synthesis in cultured cells. In this system the minimum time as estimated was shorter than that obtained in other systems (17, 19, 23). This result may be at least partially explained by the different conditions utilized to render cells quiescent in our studies. Other investigators decreased the serum concentration (23) or omitted it entirely (17, 19).

The variation in the maximal labeling index observed in Figs. 3 and 4 is probably due to the fact that cells from different donor animals were utilized in these experiments and that there appears to be a difference in the growth fraction of SMC cultured from different animals (18) (Nist et al., manuscript in preparation).

#### Mode of Action of Platelet Factor(s)

These observations support a hypothesis which proposes that platelet factor(s) functions as a regulatory agent to stimulate cell proliferation (16). The fact that the proliferative responses of widely varying numbers of cells to low (5  $\mu\text{g/ml}$ ) concentrations of this material are quantitatively similar, and that cells need be in the presence of the platelet factor(s) for only a relatively short period of time supports this idea. These and other experiments with primate dermal fibroblasts (13) demon-

strate a proliferative response to platelet factor(s) similar to that seen for smooth muscle (16), and suggest a ubiquitous role for platelet factors in inducing proliferation of at least these types of cells and of 3T3 cells (9). Presently, no data are available regarding the site or mechanism of action of the platelet factor(s). Experiments involving the purification, characterization, and further tests of the biological activity of platelet factor(s) are currently in progress.

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