ROLE OF SERUM COMPONENTS IN DENSITY-DEPENDENT INHIBITION OF GROWTH OF CELLS IN CULTURE

Platelet-derived Growth Factor Is the Major Serum Determinant of Saturation Density

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ABSTRACT

The effects of the platelet-derived growth factor and plasma components on saturation density in cultures of 3T3 cells were investigated. Both of these components of whole blood serum affect saturation density; however, when 3T3 cells become quiescent at high density in medium containing whole blood serum, only platelet-derived growth factor and fresh whole blood serum are capable of stimulating proliferation. Addition of fresh plasma-derived serum has little effect on cell growth. These results suggest that the platelet factor is the major determinant of saturation density in cultures of 3T3 cells maintained in medium supplemented with whole blood serum.

Experiments were performed to investigate the mechanism by which platelet-derived growth factor regulates saturation density. We investigated the possibilities of inactivation of growth factors by proliferating cells, and the effects of cell density on the response of 3T3 cells to platelet-derived growth factor. The amount of platelet-derived growth factor required to initiate DNA synthesis increases with increasing cell density. Some inactivation of growth factors by growing cells was detected, but this depletion was only evident at high cell density. We propose that density-dependent inhibition in cultured 3T3 cells is the result both of an increased requirement for the platelet-derived growth factor as the cultures become more crowded and of inactivation of growth factor activity by growing cells.

The phenomenon of density-dependent inhibition of proliferation in cell culture has been extensively studied, but the mechanism involved in this process remains unclear. Two factors have been implicated in the regulatory mechanism: the amount and availability of growth factors in the culture medium, and the effects of cell-cell contact. Many data have been presented to support the hypothesis that cessation of growth at high density occurs because of limiting amounts of growth factors in the culture medium (2, 4, 10, 24, 25). In cultures of cells such as 3T3 cells, the final saturation density is dependent upon the concentration of serum in the growth medium, suggesting that quiescence at high density results from restricting levels of growth factors (8, 10, 25, 26). Holley (8)

has suggested that this limitation of growth factors may result from inactivation by growing cells.

The principal data suggesting that cell-cell contact is important come from "wound" experiments in which a small area of cells is removed from a confluent monolayer (3, 5, 25). In such experiments, cells bordering the wound start to divide, whereas distant cells in the monolayer remain quiescent. The explanation for these results has been that cell-cell contact inhibits growth in the monolayer, whereas the loss of contact in the wound permits cell proliferation.

In this investigation, we studied the effects of components of blood serum on saturation density in 3T3 cells. Whole blood serum is composed of plasma-derived molecules and a platelet-derived growth factor that is released from platelets during the process of serum formation (1, 7, 12, 19, 20, 30). Both of these components of whole blood serum are required for proliferation of 3T3 cells. Restricting the amount of either platelet-derived growth factor or plasma components limits proliferation in sparse cultures of 3T3 cells, fibroblasts, and smooth muscle cells (15, 16, 22, 27). We therefore wanted to determine the effects of both plasma-derived serum (PDS) and the platelet-derived growth factor (PDGF) on saturation density in confluent cultures of 3T3 cells.

We report here that both plasma components and PDGF can affect saturation density; however, when 3T3 cells are cultured in medium containing whole blood serum, the level of PDGF, and not the level of plasma, determines the saturation density. The amount of the PDGF required to stimulate cell growth increases as the cell density increases. In addition, some inactivation of growth factor activity is evident at high cell density. Density-dependent inhibition of growth appears to be the result of these two phenomena.

MATERIALS AND METHODS

Cells, Medium, and Preparation of PDGF and PDS

Swiss 3T3 cells were used in all experiments. The cells were passaged as previously described (27).

The methods of preparation of PDGF, whole blood serum (WBS), and PDS are those described by Ross et al. (21, 22, 27). Both human and monkey blood were sources of serum components.

PDGF was partially purified from outdated human platelets by carboxy-methyl Sephadex chromatography, followed by gel filtration on Bio-Gel P-100. Material purified by CM-Sephadex chromatography is designated CMS-III, and Bio-Gel P-100 purified material is designated BG-2.

PDS is made from cell-free plasma from which the platelet and other cellular components of blood have been removed by centrifugation before coagulation. It therefore contains little PDGF activity. WBS contains PDGF because it is prepared from blood in which the platelets are present during the process of coagulation.

Measurements of Cell Growth and DNA Synthesis

Cell numbers were determined by trypsinization and counting with a Coulter Counter. Two plates from each experimental group were counted daily. DNA synthesis was measured by autoradiography of [^{3}H]Tdr incorporation. Cells were plated in 35-mm dishes and processed for autoradiography by fixation in acetic acid: ethanol (1:2). The dishes were overlayed with melted Kodak NTB-2 emulsion and developed 2–4 d later (17).

Conditioning of Growth Medium

Cells were allowed to grow to confluent cell densities for 3 d in medium supplemented with 5% calf WBS. The medium was removed from the dish, centrifuged at 10,000 rpm for 20 min at 4°C in a Sorvall RC-2, and sterilized by filtration with Nalgene 0.22 μ filters. This medium was then used for further studies in cell growth. Mock conditioning of growth medium was accomplished by incubating calf serum-supplemented medium in plastic dishes without cells for 3 d. This mock conditioning medium was harvested and filtered.

RESULTS

Effects of PDS and PDGF on Saturation Density

Figs. 1*A* and 1*B* show the final saturation density of 3T3 cells grown in varying concentrations of PDS and platelet factor. For a constant amount of PDS, the more platelet factor present, the higher the saturation density (Fig. 1*A*). Conversely, at constant concentrations of platelet factor, the saturation density increases with increasing amounts of PDS. (Fig. 1*B*). Therefore, both PDGF and plasma components regulate final cell density. This result is consistent with previous reports showing that both of these components of whole blood serum are required for optimal growth of 3T3 cells (15, 21, 22, 27).

Addition of PDS and PDGF to Density Inhibited Cultures

The previous experiment demonstrates that both PDS and PDGF affect saturation density. Since cells grown in medium with WBS are exposed to both of these entities, either one, or both, may be the principal determinant of final cell number. To examine this, 3T3 cells were grown to

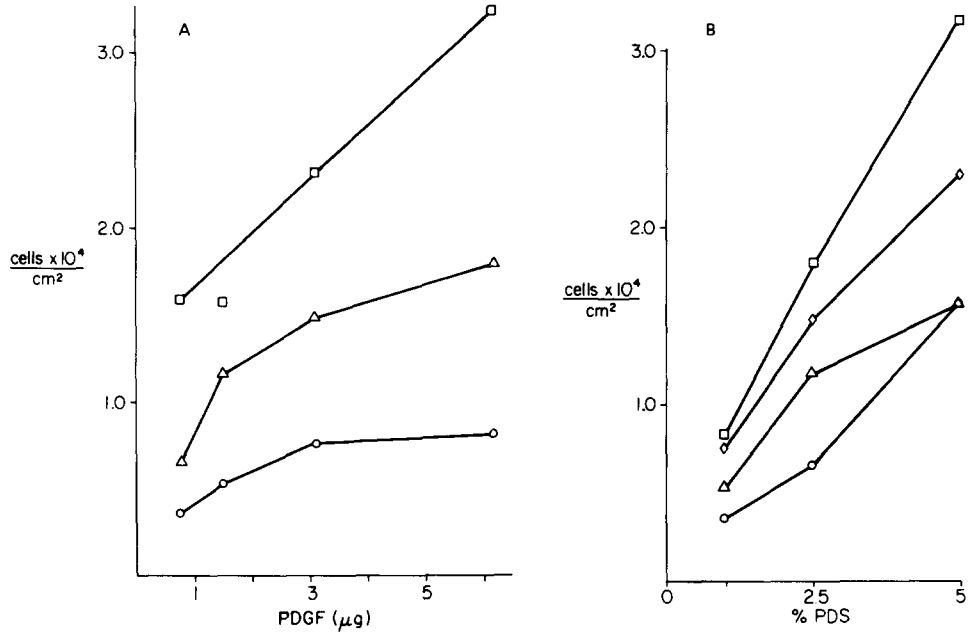

FIGURE 1 (*A*) Saturation density in constant PDS and increasing PDGF. (*B*) Saturation density in constant PDGF and increasing PDS. 3T3 cells were plated at a density of 4×10^4 cells/plate containing 1.5 ml of growth medium supplemented with 1% monkey PDS. 24 h later, 1.5 ml of growth medium containing various concentrations of PDS and platelet factor (partially purified by carboxymethyl-Sephadex chromatography) was added. Cells were counted daily. The medium was changed every 3 d during growth determinations. The cells became quiescent 2–6 d after addition of the various combinations of PDGF and PDS. (*A*) ○, 1.0% PDS; △, 2.5% PDS; □, 5% PDS. (*B*) ○, 0.77 μg PDGF; △, 1.5 μg PDGF; ◇, 3.1 μg PDGF; □, 6.2 μg PDGF.

confluence in 5% calf WBS until they became quiescent and, then, either PDGF, PDS, or additional fresh WBS was added to the quiescent cells. Addition of up to 600 μl of PDS caused only a slight increase in DNA synthesis and cell division, while partially purified PDGF was almost as effective as WBS in stimulating cell proliferation (Figs. 2*A* and *B*). This experiment suggests that PDGF is the growth-limiting molecule in dense cultures maintained in WBS. In addition, stimulation of proliferation by PDGF in these conditions does not require addition of more PDS. This regulation of final saturation density by platelet-derived growth factor could occur by a variety of mechanisms. First, the amount of PDGF *per cell* required to stimulate cell proliferation may increase as the number of cells per dish increases. In other words, cells at high density may require more PDGF than sparse cells. This increased requirement may reflect the effects of increased cell density and/or cell number.

Second, cells may inactivate PDGF as they grow, resulting in depletion of the growth-promoting activity in the medium.

Effects of Cell Density and Cell Number on the Response of 3T3 Cells to PDGF

The dose response of 3T3 cells at different densities to PDGF is shown in Fig. 3. The data are plotted as fraction labeled nuclei versus the amount of PDGF *per cell* added to the medium. If the amount of factor per cell required to initiate DNA synthesis remains constant with increasing cell density, then plots of fraction labeled nuclei versus factor per cell should be identical for cells at different densities. Fig. 3 shows that cells at higher density require more factor per cell than do cells at lower density. Thus, the amount of PDGF per cell required to stimulate proliferation appears to increase as the cell density increases.

In this experiment, increased numbers of cells were added to the same sized dishes; therefore, both the cell number and cell density varied. To

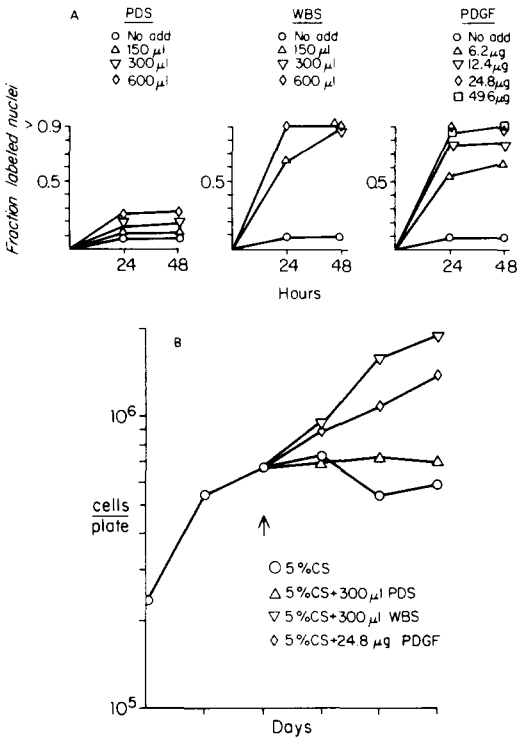

FIGURE 2 (*A*) Stimulation of DNA synthesis in confluent 3T3 by human PDS, WBS, and PDGF. (*B*) Stimulation of confluent 3T3 by human PDS, WBS, and PDGF. 3T3 cells were plated at 2×10^5 cells/plate in 35-mm dishes containing 1.5 ml of 5% calf serum-supplemented medium. 3 d later when the cells had become quiescent, PDGF, human PDS, or WBS was added. [^{3}H]Tdr (2.5 μCi/ml, 6.17 Ci/mm) was added at the time of addition of growth factors. Plates were processed for autoradiography and determination of cell number at the indicated times. Platelet factor was partially purified by carboxymethyl-Sephadex chromatography.

determine whether we were observing the effects of increased cell number or increased cell density, the same number of 3T3 cells was plated in different sized dishes containing equal volumes of growth medium, and the response to varying doses of PDGF was measured (Fig. 4). In this experiment, only cell density varied, so that any difference in dose response should be the result of the difference in cell density. Fig. 4 demonstrates that cells at lower density require less PDGF per cell than do cells at higher density. This difference manifests itself predominantly as an increased ability of the sparse cells to synthesize DNA in the absence of additional platelet factor. This phenomenon is presumably caused by the ability of the sparse cells to respond to the small amounts of PDGF present in the PDS. (Since we do not yet have a radioimmunoassay to measure levels of PDGF, we cannot test this hypothesis.) This experiment demonstrates that a 2.5-fold difference in cell density results in a marked alteration in dose response to PDGF. We therefore conclude that the cellular requirement for the factor increases with increasing cell density.

Effects of Medium Conditioning on Growth-Promoting Activity

To examine the possibility of inactivation of growth-promoting activity by growing cells, 3T3 cells were grown to confluence in 60-mm dishes containing 4 ml of 5% calf serum-containing medium. The cells reached confluence after 3 d at which time the medium was harvested and the cells were trypsinized and plated at different cell numbers in 100-mm dishes containing either 4 ml or 10 ml of the previously conditioned medium. Fig. 5 demonstrates the design of these experiments. Fig. 6 shows that cells plated at low density in the conditioned medium underwent two rounds of division (Fig. 6*C*). Therefore, the initial proliferation in the 60-mm dishes did not remove all the growth-promoting activity from the medium. However, there is some loss of activity because the final density of the cells in 100-mm dishes in conditioned medium is somewhat less than the density of cells grown in 60-mm dishes.

Effects of depletion are also seen if the confluent cells in 60-mm dishes are trypsinized and plated at the same cell number in 100-mm dishes containing 4 ml of medium (Fig. 6*A*). No growth was observed in this experiment even though the cell density was decreased by a factor of 2.5.

Loss of growth stimulation is also demonstrated when the cells are plated in 100-mm dishes containing 10 ml of conditioned medium or 10 ml of fresh culture medium (Fig. 6*B*). In conditioned medium, there was no growth. However, in fresh medium the cells underwent one round of division (Fig. 6*B*).

In parts *A*, *B*, and *C* of this experiment (see Fig. 5), the cells eventually ceased proliferating after transfer to 100-mm dishes (Fig. 6). To determine whether this cessation of growth was due to restricting amounts of the PDGF or other components of the culture medium, we added fresh serum-free medium, PDS, PDGF, or WBS to the quiescent cells in 100-mm dishes containing 10 ml

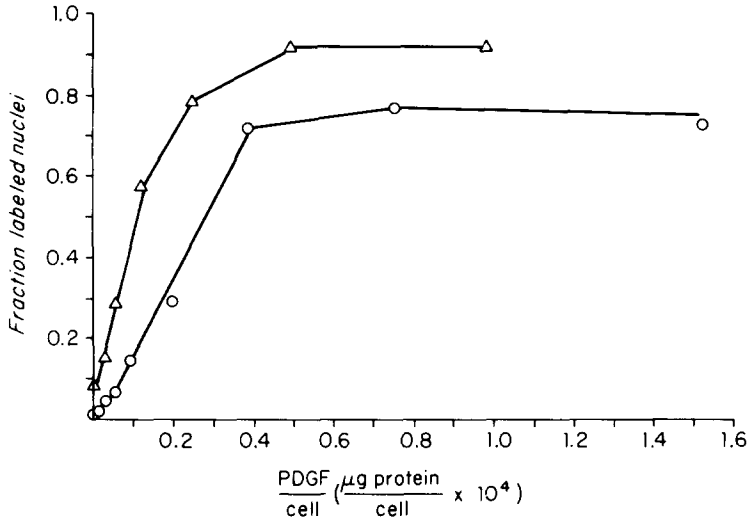

FIGURE 3 DNA synthesis in 3T3 cells at different densities in 5% MPDS. 3T3 cells were plated at 4 × 10^4 and 2 × 10^5 cells/plate into 35-mm dishes containing 1.5 ml of growth medium supplemented with 5% monkey PDS. 2 d later, partially purified platelet factor was added along with 2.5 μCi/ml [^{3}H]Tdr. Cells were processed for autoradiography 28 h later. The platelet factor was partially purified by CM Sephadex chromatography followed by chromatography on Bio-Gel P-100, (BG-2). ○, 1.5 × 10^5 cells/plate; △, 5.9 × 10^4 cells/plate.

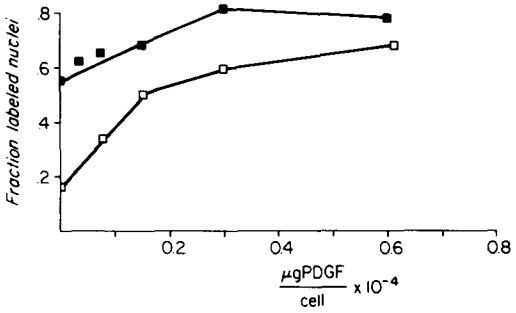

FIGURE 4 Dose response of 3T3 to PDGF; constant cell number (8.2 × 10^4) different density. 3T3 cells were plated at 6 × 10^4 cells in 35-mm or 60-mm dishes containing 1.5 ml of growth medium supplemented with 5% monkey PDS. 2 d later, partially purified platelet factor (BG-2) and [^{3}H]Tdr were added, and the plates were processed for autoradiography 24 h later. □, 35-mm dish, 1 × 10^4 cells/cm^2; ■, 60-mm dish, 0.39 × 10^4 cells/cm^2.

of medium (Fig. 6, B; arrow, day 5) and determined the cell number 3 d later (Table I). Only PDGF and WBS stimulated cell multiplication. PDS and serum-free medium alone had little effect on proliferation of these quiescent cells (Table I). Therefore, PDGF appears to be the growth-limiting molecule in these conditions.

To further evaluate possible depletion of growth factors, we determined whether sparse cells made quiescent in PDS could be stimulated to synthesize DNA in conditioned medium in the presence of a confluent monolayer of cells. To do this, 3T3 cells were plated at sparse density on glass coverslips in PDS and allowed to become quiescent. 48 h later, the coverslips containing the sparse, quiescent cells were transferred to dishes containing confluent monolayers of 3T3 cells in 5% calf serum-containing medium (Table II, *b*), or 5% calf serum-containing medium without cells (Table II, *c*)). The same stimulation of DNA synthesis was observed in conditioned medium with the confluent monolayer as in medium without the confluent cells. Therefore, medium in which 3T3 cells have grown to confluence contains sufficient growth-promoting activity to stimulate the growth of sparse cells. This observation is presumably the result of the ability of the sparse cells to utilize maximally the available PDGF present in the medium.

The results of these experiments suggest that growing cells do not deplete all of the growth factor activity from the medium. However, we did detect some reduction in final cell density in cultures grown in conditioned medium and we assume that this phenomenon may be the result of

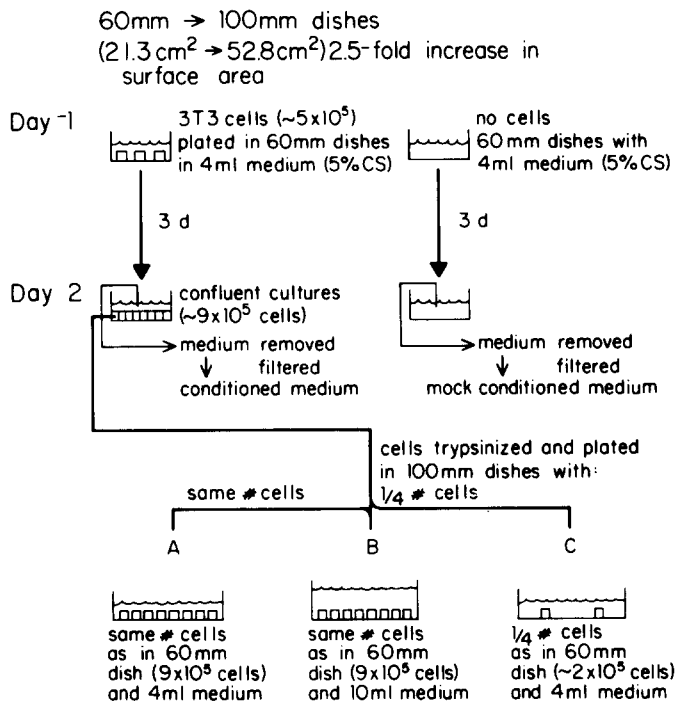

FIGURE 5 Experimental protocol of Fig. 6.

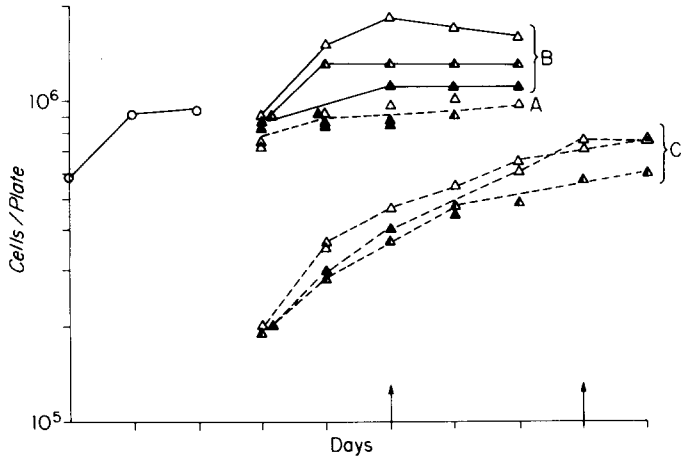

FIGURE 6 3T3 cell growth in 60-mm dishes and 100-mm dishes in 5% calf serum. 3T3 cells were plated in 60-mm dishes containing 4 ml of 5% calf serum containing medium at approximately 5×10^4 cells/plate. 3 d later, when the cells had reached confluent cell densities (○), the medium was harvested, and the cells were trypsinized and plated at different densities in 100-mm dishes containing 4 ml (---) or 10 ml (—) of growth medium. Cell number was determined daily. ○, 60-mm dish; △, 100-mm dish, fresh medium; ◭, 100-mm dish, mock conditioned medium; ▲, 100-mm dish, conditioned medium.

partial depletion of growth factor activity which is only evident at high cell density. These data are consistent with previous reports that suggest that it is easier to deplete medium of the ability to stimulate a confluent monolayer of 3T3 cells than it is to deplete medium of the ability to support growth of sparse cells (11, 26).

The data in Figs. 3–6 suggest that density-de-

TABLE I

Growth Response of 3T3 Cells at Confluence to Addition of Fresh Medium, PDS, PDGF, or WBS

	Saturation density in 10 ml of medium	Plus medium	Plus PDS	Plus PDGF	Plus WBS
			cells/plate		
Fresh	1.9×10^6	1.9×10^6	2.5×10^6	3.6×10^6	4.4×10^6
Mock	1.6×10^6	1.5×10^6	1.9×10^6	3.5×10^6	3.9×10^6
Depleted	1.1×10^6	1.2×10^6	1.5×10^6	3.0×10^6	3.3×10^6

3T3 cells in 100-mm dishes with 10 ml of fresh, mock-conditioned or conditioned medium (Fig. 5 *B*) received the various test materials on day 5 (arrow). 2 ml of monkey PDS, 2 ml of monkey WBS, 0.82 mg of partially purified platelet factor (CMS III), or 5 ml of fresh serum-free medium were added to separate plates, and cell number was determined 3 d later (day 8, arrow).

TABLE II

DNA Synthesis of Sparse 3T3 Cells on Coverslips in the Presence of Confluent 3T3 Cells

	Fraction labeled nuclei
(*a*) 5% plasma-derived serum	0.21
(*b*) 5% calf serum, confluent cells	0.58
(*c*) 5% calf serum, no cells	0.59

3T3 cells were plated in 5% monkey PDS-supplemented medium at 5×10^4 cells into 35-mm dishes containing 16-mm round glass coverslips. After 48 h, the coverslips were transferred to 35-mm dishes containing (*a*) 5% PDS medium and no cells, (*b*) 5% calf serum medium with confluent 3T3 cells, and (*c*) 5% calf serum medium with no cells. [^{3}H]Tdr (2.5 μCi/ml) was added at the time of transfer, and coverslips were processed for autoradiography 30 h later. In (*b*) the cells were grown to confluence in 5% calf serum and were confluent for 2 d before the transfer of the coverslips. In (*a*) and (*c*), the medium was incubated in 35-mm dishes for the same time as in (*b*) but without cells.

pendent inhibition of growth is the results of both an increased requirement for PDGF and inactivation or loss of growth factor activity by growing cells. The relative importance of each of these processes in density-dependent inhibition of growth is unclear. One could argue that the increased cellular requirement for PDGF is solely the result of increased cellular inactivation of the molecule. Two facts argue against this hypothesis: first, if this were true, one would expect the increased cellular requirement for platelet factor to be dependent on cell number rather than cell density. However, Fig. 4 shows that cell density is the determinant of the increased platelet factor requirement. In the second instance, Fig. 6 C and Table II demonstrate that, in conditioned medium, cells at low density are able to proliferate even in the presence of large numbers of confluent cells. Therefore, conditioning of growth medium does not remove all of the growth factor activity. Until we are able to accuately determine the amount of PDGF in the culture medium, we will not be able to determine the relative roles of depletion and increased requirement for the PDGF in density-dependent inhibition of growth.

Effects of Amount and Concentration of PDGF

During the course of these experiments, we were confronted with the question of whether the *amount* of PDGF or the *concentration* of platelet factor in the culture medium was more important in the determination of final cell density. To examine this question, 3T3 cells were plated in 35-mm dishes containing 1, 2, or 3 ml of medium supplemented with a constant volume of PDS, and constant amounts of PDGF were then added to each of these dishes. Cell number was determined daily until the cells reached confluence. Because the amount of platelet factor is constant and the volume of medium increases, the concentration of platelet factor is decreased in cultures containing 3 ml of medium vs. cultures containing 1 ml of medium. If the amount of PDGF is critical, then adding the same amount of factor to 1, 2, or 3 ml of medium should result in identical final cell densities. If the platelet factor concentration is important, then the cell density should decrease as the concentration of the factor decreases. Table III shows that the saturation density reflects the amount rather than the concentration of PDGF. The saturation densities of cultures receiving the same amount of platelet factor in dishes containing 1, 2, or 3 ml of medium were identical. This phenomenon occurs over the threefold range in concentration shown in Table III. However, drastic reduction in platelet factor concentration will

TABLE III

Effects of Amount and Concentration of PDGF on Saturation Density

	Saturation density		
Dose of PDGF	1 ml	2 ml	3 ml
μg	*cells/plate × 10⁵*		
0	3.1	3.0	2.9
4.3	5.0*	5.1△	4.8
6.5	5.6○	5.8	5.7△
12.9	7.5	7.6○	7.0*

3T3 cells were plated at 1.5×10^5 cells per 35-mm dish in 1.5 ml of medium supplemented with 5% PDS. 48 h later, the medium was removed and replaced with 1, 2, or 3 ml of medium containing 150 μl of fresh PDS and different amounts of PDGF. The amount of PDS in each dish was constant (150 μl), but the concentration of plasma varied because of the difference in volume. The amount of plasma was kept constant because preliminary experiments had shown that the amount and not concentration of PDS influenced cell density.

The concentration of PDGF is twofold less in plates containing 2 ml of medium compared with plates containing 1 ml of medium. Similarly, there is a threefold reduction in platelet factor concentration in plates containing 3 ml of medium. Columns with identical symbols (○, *, △) denote plates that contain the same concentration of PDGF, but different amounts of factor.

eventually reduce cell density (unpublished data). For example, the same amount of PDGF added to 10 ml of medium in a 100-mm dish results in a lower saturation density than that seen in Table III.

DISCUSSION

The data presented here suggest that the amount of PDGF in the growth medium determines the final saturation density in cultures of 3T3 cells maintained in medium supplemented with WBS. The occurrence of a round of division in 3T3 cells in medium containing WBS, which contains adequate amounts of plasma components, depends mainly upon the amount of PDGF and the cell density. Cell density affects the amount of platelet factor required to initiate division, with greater amounts of factor needed as the cell density increases (Figs. 3 and 4). Cell proliferation ceases at high density because of insufficient quantities of platelet factor in the growth medium. This "lack" of factor is the result of the increased cellular requirement for platelet factor and possibly of partial inactivation or removal of the factor.

This phenomenon of increased requirement per cell for PDGF at higher cell density provides an explanation of "wound" experiments in which a confluent monolayer of cells in conditioned medium has a small strip of cells removed from the center of the monolayer (3, 5, 25). In such experiments, the cells bordering the wound commence proliferation, but cells in the monolayer distant from the wound remain quiescent. We believe that these results are best explained on the basis of varying requirement for platelet factor by cells at different densities. In these cultures, there is not sufficient factor to stimulate growth of cells at high density in the middle of the monolayer, because each of these cells requires more factor than is present in the growth medium, while cells at the wound edge require less factor and are therefore able to divide.

The data presented here are consistent with data published on density-dependent inhibition of proliferation in 3T3 cells. Many reports present evidence that saturation density is regulated by the concentration of growth factors in the medium (2, 4, 10, 11, 24–26). In a review in 1975, Holley (8) stated that: "density-dependent regulation is due to a quantitative increase in the requirements for macromolecular growth factors as cell density increases." He postulated that this increased requirement is the result of destruction of factors by growing cells and cell contact reducing surface area which can interact with the factors. Our data are consistent with this hypothesis.

The way in which cell crowding increases the requirement for platelet factor is unknown, but various possibilities exist. Increased cell density may affect cell spreading and exposed surface area which in turn may affect the response to growth factors (6, 14). Assuming that platelet factor interacts with a specific receptor on the cell surface, then cell-cell interactions may prevent access of the platelet factor to the receptor by covering the receptors, reducing the surface area containing receptors available to interact with the factor, or creating a diffusion boundary layer that prevents factor-receptor interaction (23, 31). Alternatively, increased cell density may inhibit certain functions that occur after the factor has bound to the receptor. For example, movement of macromolecules in the plane of the membrane or into the cell may be reduced at high cell density, thus preventing stimulation of growth. Each of these hypotheses can be tested further with purified, labeled platelet factor.

This phenomenon of density-dependent re-

sponse to a growth factor has been previously described for epidermal growth factor (EGF). Cultures of human glial cells and mouse 3T6 cells show a decreased response to EGF with increasing cell number (13, 28). For glial cells, this decreased response is not the result of alterations in the number of EGF receptors with increasing cell density (29). However, reports of both increased (18) and decreased (9) EGF binding with increasing cell density are present in the literature.

3T3 cells are similar to diploid fibroblasts, smooth muscle cells, glial cells, and many others in that they are exquisitely sensitive to density-dependent growth regulation. Consequently, the phenomenon of increased requirement for a specific growth factor is not unique to 3T3 cells (28, 29) and may represent a generalized phenomenon that affects the requirements of cells for many growth regulatory molecules.

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