Proc. Natl. Acad. Sci. USA
Vol. 77, No. 5, pp. 2726–2730, May 1980
Cell Biology

Do plasma and serum have different abilities to promote cell growth?

(vascular smooth muscle/fibroblast growth factor/extracellular matrix)

D. GOSPODAROWICZ AND C. R. ILL

Cancer Research Institute and the Departments of Medicine and Ophthalmology, Medical Center, University of California, San Francisco, California 94143

Communicated by Abraham White, January 24, 1980

ABSTRACT **The abilities of plasma and serum to support the growth of vascular smooth muscle cells maintained on uncoated tissue culture dishes or dishes coated with an extracellular matrix (ECM) have been compared. Vascular smooth muscle cells maintained on plastic dishes and exposed to plasma proliferate poorly; when exposed to serum they proliferate actively. Addition of fibroblast growth factor (FGF) increases the growth rate of the cultures in both cases. In contrast, when vascular smooth muscle cells are maintained on an ECM, they proliferate equally well exposed to either plasma or serum. Because the cultures had an average doubling time (15 hr) that was already at a minimum, FGF no longer had an effect on vascular smooth muscle cell proliferation. These results raise the possibility that the lack of response of vascular smooth muscle cells, as well as that of other cell types *in vitro*, to plasma factors is not an intrinsic property of the cells but is rather due to the substrate upon which the cells rest. Because cells maintained on an ECM respond to plasma factors, it is likely that the close contact of the cells with the ECM restores their sensitivity to physiological factors present in plasma.**

Culture of most cells *in vitro* requires the presence of serum (1). Consequently, investigators have spent much effort to identify the various factors in serum that stimulate cell growth *in vitro*. An important step in the search for serum growth factors has been the finding that one of the most potent mitogenic factors present in serum is derived from platelets. Such a possibility, first postulated by Balk (2), was based on studies of the growth of chicken embryo fibroblasts in medium supplemented with plasma or serum. Chicken fibroblasts do not proliferate in plasma-containing medium (2), but when they are exposed to serum they proliferate actively. It was therefore concluded that serum contained growth-promoting activity that was absent from plasma (3). These studies were followed by reports which demonstrated that platelets are the source of a potent mitogen present in serum but not in plasma. Whereas plasma was unable to support the growth of aortic smooth muscle cells (4) or of BALB/c 3T3 cells (5), serum made from the same pool of blood stimulated their proliferation. Addition of a platelet extract to cell-free plasma-derived serum restored the growth-promoting activity (4–6). One could therefore conclude that one of the principal mitogens responsible for the induction of DNA synthesis present in whole blood serum is derived from platelets (4–6). The difference in the proliferative ability of cells exposed to plasma or to serum results from the absence of the platelet factor in the former.

All studies have thus far used cells maintained on plastic rather than on a basal lamina or an extracellular matrix (ECM), which *in vivo* is the natural substate upon which cells migrate, proliferate, and differentiate. This difference in the substrate upon which the cells are maintained could have prevented their response to physiological factors present in plasma, thereby creating the difference in mitogenic activity between plasma and serum. In recent studies the proliferative behaviors of four different cell types maintained on plastic or on an ECM produced by corneal endothelial cells (7) have been compared. Bovine adrenal cortex cells, granulosa cells, and vascular or corneal endothelial cells maintained on plastic and exposed to optimal serum concentration do not proliferate unless they are exposed to fibroblast growth factor (FGF) (8–11). In contrast, when maintained on a corneal ECM these same cell types proliferated actively and FGF was not required in order for the cultures to become confluent (7). One can therefore conclude that the close contact of the cells with the ECM must make them responsive to factors present in serum and that adherence to plastic prevents such a response. That this is likely to be the case was inferred from the observation that the growth rate of cells maintained on an ECM was a direct function of the serum concentration to which they were exposed (7). Therefore, in regard to proliferation, those cells that exhibit a total dependence on FGF but not on serum when maintained on plastic contrariwise exhibit a total dependence on serum and no longer require FGF when maintained on an ECM. The simple change of substrate from plastic to ECM could therefore restore the sensitivity of the cells to mitogens present in serum, which could be the same as those present in plasma.

To explore the possibility that the serum factors to which cells maintained on ECM become sensitive are also present in plasma, in the present study we compared the mitogenic activities of plasma and serum using, as target cells, vascular smooth muscle cells maintained on either plastic or ECM. Our results demonstrate that, when vascular smooth muscle cells are maintained on ECM, they respond and proliferate as well in plasma as in serum. It is therefore likely that the serum factors to which cells respond when maintained on an ECM are the same as those present in plasma.

MATERIALS AND METHODS

Materials. FGF was purified from bovine brains as described (12). Dulbecco's modified Eagle's medium (DME medium, H-16) was obtained from GIBCO. Calf serum and fetal calf serum were obtained from Irvine Scientific. Tissue culture dishes were from Falcon. Dextran (M_r, 40,000) was from Sigma, gentamycin was from Schering, and Fungizone was from Squibb.

Cell Culture Conditions. Cultures of bovine corneal endothelial cells were established from steer eyes as described (11). Stock cultures were maintained on tissue culture dishes in DME

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: ECM, extracellular matrix; FGF, fibroblast growth factor; DME medium, Dulbecco's modified Eagle's medium.

medium supplemented with 10% fetal calf serum, 5% calf serum, and 50 μg of gentamycin and 2.5 μg of Fungizone per ml (13). FGF (100 ng/ml) was added every other day until the cells were nearly confluent.

Primary cultures of bovine vascular smooth muscle cells were prepared from the vascular media of a bovine aortic arch (14). Stock cultures were maintained on tissue cultures dishes in DME medium supplemented with 10% calf serum and 50 μg of gentamycin and 2.5 μg of Fungizone per ml. FGF (100 ng/ml) was added every other day until the cells were nearly confluent.

Preparation of Plates Coated with ECM. Corneal endothelial cells were plated at an initial density of 10^4 cells per 35-mm dish and maintained in the presence of DME medium supplemented with 10% fetal calf serum, 5% calf serum, 5% dextran T-40, and 50 μg of gentamycin and 2.5 μg of Fungizone per ml. FGF (100 ng/ml) was added every other day until the cultures became subconfluent. Once the plates became confluent (ordinarily within 6 days) and signs of basement membrane formation could be observed by phase-contrast microscopy (7), the cultures were washed with phosphate-buffered saline and then exposed for 30 min to buffered saline containing 0.5% Triton X-100. Once the nuclei and the ECM became clearly visible, the cultures were washed three times with phosphate-buffered saline (7). After these washings, only a few cytoskeletons and nuclei could be observed associated with the intact ECM which coated the entire dish (7). To check for the possible adsorption of FGF to the ECM produced by corneal endothelial cell cultures, ^{125}I-labeled FGF (10^5 cpm/ml) was added every other day together with 100 ng of unlabeled FGF per ml. The ECM prepared as described above was then solubilized with 0.5 M NaOH and assayed for radioactivity in a Beckman scintillation gamma counter. Less than 100 cpm of ^{125}I-labeled FGF was found to be associated with the ECM. This is less than 0.1% of the original concentration of FGF to which the cells were exposed and represents an adsorption to the ECM of at most 0.1 ng of FGF. This value is far below the FGF concentration required to induce maximal proliferation of vascular smooth muscle cells (14). Therefore, the proliferation of vascular smooth muscle cells maintained on the ECM and exposed to plasma cannot be attributed to previous adsorption of FGF to the ECM. To test for the possible association of serum proteins with the ECM, the ECM was radiolabeled by using Na^{131}I and lactoperoxidase-catalyzed iodination. The ^{131}I-labeled ECM was subsequently analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. No serum protein associated with the ECM could be detected when such analysis was performed.

Preparation of Bovine Plasma and Serum. Plasma (platelet-poor plasma serum) and serum (whole blood serum) were prepared from bovine blood as described (4, 15). Briefly, blood was drawn into plastic syringes containing 3.8% sodium citrate (1 vol of citrate to 10 vol of blood). The blood was then recalcified with 1 M $CaCl_2$ to a final concentration of 14 μmol/ml and allowed to clot at room temperature for 5 hr. The clotted blood was then centrifuged at 2000 × *g* for 15 min at 4°C. The serum was decanted from the clots and respun (22,000 × *g*) for 30 min at 4°C. The serum was dialyzed (Spectrapor-1, M_r cutoff 6000–8000) against Ringer's solution at 4°C for 24 hr. The serum was then filtered on a 0.2-μm Millipore filter and stored at −70°C.

Plasma was prepared by drawing the blood into chilled (4°C) citrated syringes. All subsequent steps other than the recalcification and clotting were carried out at 4°C. The blood was centrifuged at 1900 × *g* for 15 min and the resulting plasma was pipetted off and respun (22,000 × *g* for 30 min). The platelet-poor plasma was pooled and 1.0 M $CaCl_2$ was added to a final concentration of 20 μmol/ml. The plasma was then incubated at 37°C for 2 hr and then centrifuged at 22,000 × *g* for 30 min to remove the fibrin clot. The supernatant was dialyzed as described with serum. After 24 hr, the plasma was respun (22,000 × *g* for 30 min) and then filtered on a 0.2-μm Millipore filter followed by storage at −70°C.

Cell Growth Measurement. For growth-rate determinations, cultures of vascular smooth muscle cells in either their first or second passage were used. In a few cases, cultures in their 10th passage were used and gave identical results. Vascular smooth muscle cells were plated at an initial cell density of 20,000 cells per 35-mm dish on either plastic or an ECM. The plating efficiency determined 12 hr later was identical (90%) for cells maintained on either plastic or ECM and exposed to either serum or plasma. The cultures were maintained in DME medium supplemented with 2.5 μg of Fungizone and 50 μg of gentamycin per ml and with either 10% serum or 10% plasma. Due to the extremely fast growth rate of cultures maintained on ECM and exposed to either plasma or serum, no medium change was required because cultures became confluent within 4–5 days (final cell density, 7.2 × 10^5 cells per 35-mm plate). Triplicate cultures were trypsinized every other day and cell number was determined with a Coulter Counter.

RESULTS

Comparison of Proliferation of Vascular Smooth Muscle Cells Exposed to Plasma or Serum When Cells Are Maintained on Plastic or ECM. Vascular smooth muscle cells maintained on plastic and exposed to plasma (10%) proliferated poorly. Within 4 days the cells went through one doubling and afterward ceased to proliferate (Fig. 1*A*). When such cultures were observed by phase-contrast microscopy, the cells were considerably enlarged (Fig. 2*A*). When the same cultures were exposed to serum (10%) instead of plasma, the cells proliferated actively over a period of 6–8 days and underwent a 15-fold

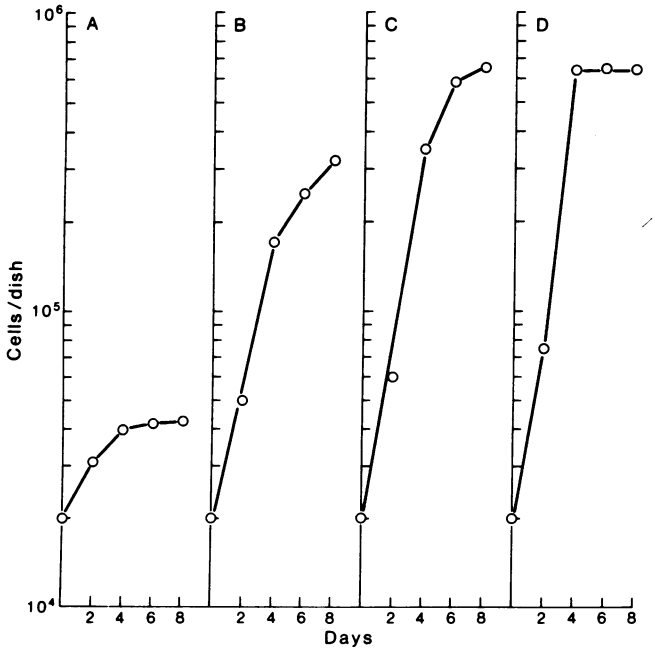

FIG. 1. Proliferation of bovine vascular smooth muscle cells maintained on plastic or ECM and exposed to either plasma or serum. Vascular smooth muscle cells were seeded at 2 × 10^4 cells per 35-mm dish and maintained in the presence of DME medium supplemented with either 10% plasma (*A*, *C*) or 10% serum (*B*, *D*). The cells were grown on either plastic (*A*, *B*) or ECM (*C*, *D*).

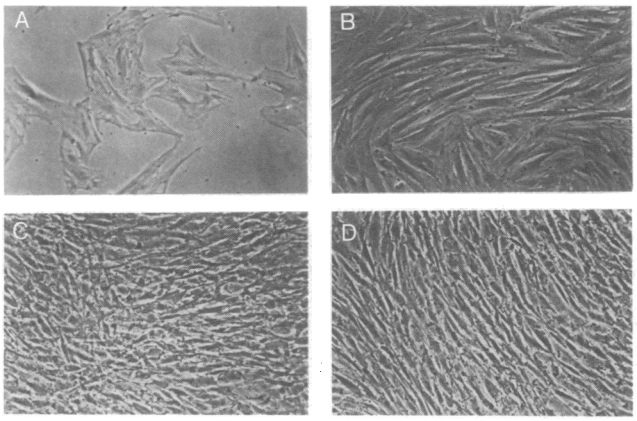

FIG. 2. Morphological appearance of vascular smooth muscle cells maintained on plastic (*A*, *B*) or ECM (*C*, *D*). Cultures were plated and maintained as described in Fig. 1 and exposed to either 10% plasma (*A*, *C*) or 10% serum (*B*, *D*). Pictures were taken on day 8. (Phase-contrast optics; ×100.)

increase in cell number (Fig. 1*B*). During the logarithmic growth phase the mean doubling time of the cultures was 30 hr. These results therefore confirm previous results showing that, when cells are maintained on plastic and exposed to plasma they proliferate poorly or not at all whereas they actively proliferate when exposed to serum (4, 5). In contrast to the above results, when cells were maintained on an ECM and exposed to plasma, they proliferated actively (Fig. 1*C*). Within 6 days the cell number increased by 30-fold and during the logarithmic growth phase the mean doubling time of the cultures was as low as 15 hr (Fig. 1*C*). Plasma was therefore even more mitogenic for cells maintained on an ECM than was serum for cells maintained on plastic. The growth rate and the final cell density of cultures maintained on an ECM and exposed to either plasma (Fig. 1*C*) or serum (Fig. 1*D*) were the same. The differences between plasma and serum in their abilities to support cell growth, which were evident when the cells were maintained on plastic, vanished when the cells were maintained on an ECM.

Morphologically, the difference between a culture maintained on plastic and exposed to serum (Fig. 2*B*) and a culture maintained on an ECM and exposed to either plasma (Fig. 2*C*) or serum (Fig. 2*D*) is apparent in the respective average cell sizes. Cultures maintained on an ECM and exposed to either plasma or serum were composed of small, spindly, overlapping, and tightly packed cells that, on an average, were one-fifth to one-third the mean cell size of cultures maintained on plastic and exposed to serum. Cells maintained on plastic and exposed to plasma (Fig. 2*A*) were, on an average, 10-fold larger than cells maintained on an ECM and exposed to plasma (Fig. 2*C*).

Response to FGF of Vascular Smooth Muscle Cells Exposed to Plasma or Serum When Maintained on Plastic or ECM. Earlier studies have shown that FGF, which has many similarities to the platelet-derived growth factor [same molecular weight and isoelectric point (6)], is a mitogen for vascular smooth muscle cells (14). We therefore compared its effects on cells maintained on plastic or an ECM exposed to either plasma or serum. Cells maintained on plastic and exposed to plasma hardly proliferated (Fig. 3*A*). In contrast, when FGF was added to the cultures, the cells rapidly divided. After 7 days there was a 25-fold increase in cell number. The final cell density of cultures maintained on plastic and exposed to plasma plus FGF was higher than that of cultures maintained on plastic and exposed to serum alone. This demonstrates that the addition of

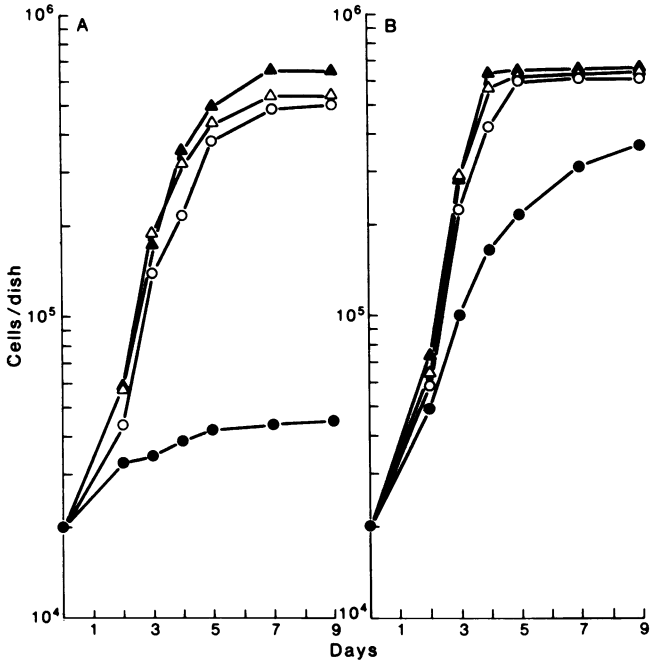

FIG. 3. Effect of FGF on the growth rate of vascular smooth muscle cell cultures. Vascular smooth muscle cell cultures (second passage) were plated at 20,000 cells per 35-mm dish on plastic (●, ○) or ECM (▲, △). The cultures were then maintained with DME medium supplemented with 2.5 μg of Fungizone and 50 μg of gentamycin per ml and with either 10% plasma (*A*) or 10% serum (*B*). FGF (100 ng/ml) was added every other day to half of the cultures (○, △).

FGF to the medium of cells maintained on plastic can make up for the difference in mitogenic activity between plasma and serum. The growth rate of cultures maintained on plastic and exposed to plasma and FGF and that of cultures maintained on an ECM and exposed to plasma but not to FGF were similar. A noticeable but small difference was that the final cell density of cultures maintained on an ECM and exposed to plasma alone was 50% higher than that of cultures maintained on plastic and exposed to plasma and FGF. Addition of FGF to cultures maintained on an ECM and plasma did not affect their growth rate. It resulted instead in a final cell density slightly lower than that observed with cultures not exposed to FGF. When the growth rate and final cell density of cultures exposed to serum and FGF were compared as a function of the substrate upon which cells were maintained, addition of FGF to cultures maintained on plastic and exposed to serum resulted in a decrease in the mean doubling time of the cultures (from 30 to 16 hr) as well as in a 2- to 3-fold increase in the final cell density (Fig. 3*B*). When FGF was added to cultures maintained on ECM, it did not affect their growth rate, which was already maximal (mean doubling time, 15 hr), nor did it affect their final cell density. One could therefore conclude that, although FGF greatly increases the growth rate of cultures exposed to plasma and, to a lesser extent, that of cultures exposed to serum when the cultures are maintained on plastic, it does not affect the growth rate of cultures maintained on an ECM and exposed to either plasma or serum.

Cells Maintained on ECM Depend on Factors Present in Plasma for their Proliferation. The increased rate of proliferation of cells maintained on ECM and exposed to plasma or serum could either be the result of (*i*) a direct mitogenic effect of the ECM, with the plasma or serum having a permissive role, or (*ii*) a direct mitogenic effect of plasma or serum, with the ECM having a permissive role. To distinguish between these

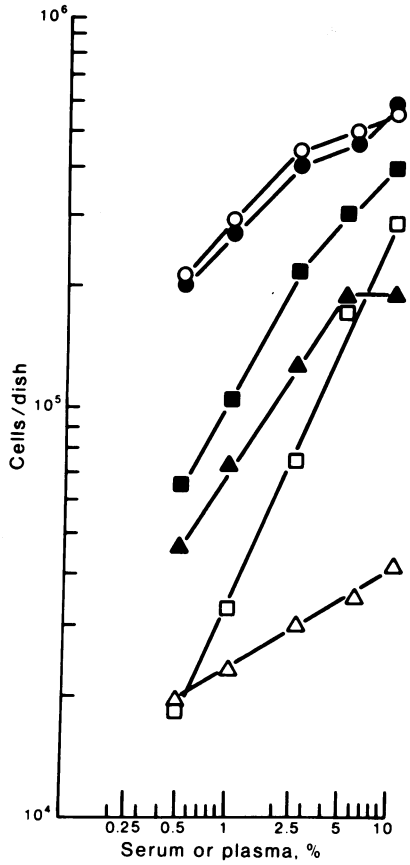

FIG. 4. Dependence of vascular smooth muscle cells on plasma for proliferation when the cells are maintained on an ECM. Vascular smooth muscle cells (second passage) were plated at 2×10^4 cells per 35-mm dish on plastic or on an ECM. Cultures were maintained for 8 hr in the presence of DME medium supplemented with either 10% plasma or serum. After 8 hr, the medium was removed and the cultures were washed once with fresh unsupplemented medium. Then, DME medium supplemented with 2.5 μg of Fungizone and 50 μg of gentamycin per ml and containing the indicated concentrations of serum or plasma was added to the dishes. FGF (100 ng/ml) was added every other day to some of the dishes. After 5 days, the cultures were trypsinized and the cells were counted. Conditions: cultures maintained on plastic and exposed to plasma (△) or to plasma and FGF (▲); cultures maintained on plastic and exposed to serum (□) or to serum and FGF (■); cultures maintained on ECM and exposed to plasma (●) or to serum (○).

two possibilities, cells maintained on ECM were exposed to increasing concentrations of plasma or serum and the final cell densities were compared (Fig. 4). If the ECM were the mitogen and the plasma or serum had a permissive effect, one would expect little difference in the rates of proliferation between high (10%) and low (0.5%) plasma or serum concentrations. However, final cell density of cultures maintained on an ECM and exposed to either plasma or serum was found to be a direct function of the serum or plasma concentration. It is therefore likely that the proliferation of cells maintained on an ECM is controlled by factors present in plasma and that the ECM has only a permissive role.

DISCUSSION

Cell proliferation *in vivo* is controlled by both short- and long-range interactions. During the embryonic development, normal growth and differentiation of the epithelium are dependent upon the surrounding mesenchyme (16, 17). It has been shown by others that the primary mechanism is the provision of the epithelium with a proper substratum (16). Therefore, the ECM produced by mesenchymal cells in response to their close contact with epithelial cells or that produced by epithelial cells in response to a similar contact could indirectly or directly modulate epithelial cell proliferation and differentiation (short-range interaction), which lead to the development of various organs in embryonic life (15, 16). In the neonate and the adult, a second type of control of cellular proliferation is superimposed on the control already exerted by the ECM. It corresponds to the development of endocrine organs and involves humoral factors (hormones) which are involved in long-range interactions with various tissues. This second type of control is best seen when bursts of mitotic activity have to take place in a precise and highly coordinated time sequence in organs located at a great distance from one another (18). Such is the case for the cell proliferation that occurs during the ovarian cycles in organs as different as the ovaries, mammary glands, and uterus and that is controlled by hormones as different in structure as steroids (estrogen, progesterone) and polypeptides (follicle-stimulating hormone or prolactin).

One of the paradoxes arising when the situation *in vitro* is compared to that *in vivo* has been that, although hormones are potent mitogens *in vivo*, they have no effect or the opposite effect (antimitotic) on their target cells *in vitro*. For example, prolactin, growth hormone, follicle-stimulating hormone, and estrogens do not stimulate replication of cells *in vitro*, although *in vivo* they can induce spectacular hyperplasia of their target organs (for a review, see refs. 19 and 20). Corticotropin, a potent mitogen for adrenal cortex cells *in vivo*, has exactly the opposite effect *in vitro* (21, 22). This paradoxical difference between *in vivo* and *in vitro* control of cell proliferation is even more evident at the level of the mechanisms involved in mediating cellular proliferation. *In vivo*, cellular proliferation triggered by tropic hormones results in an increased cellular cyclic AMP content in the target cells (23). *In vitro*, with cells whose growth is anchorage dependent, exposure to agents such as corticotropin (which increases the cellular cyclic AMP content of adrenal cells) or direct exposure of cells to cyclic AMP leads to inhibition of cell growth (21, 22). In contrast, in the case of cells such as lymphocytes, which have no need for an ECM and whose growth is not anchorage dependent, cyclic AMP as well as hormones capable to activating adenylate cyclase are mitogenic (24). One is therefore led to wonder what the main difference is that is responsible for these discrepancies between the *in vivo* and *in vitro* conditions.

A common explanation for the lack of activity of hormones *in vitro* has been that their action *in vivo* is indirect and that they generate a second generation of factors (growth factors) that are directly responsible for the control of proliferation of target cells. Equally likely, however, is the possibility that cells maintained on plastic are prevented from responding to the physiological agents normally present in plasma. That this could be the case is suggested by our results. Vascular smooth muscle cells maintained on plastic require mitogens such as platelet-derived growth factor(s) (6), FGF (14), or serum (6) in order to proliferate; when exposed to plasma they do not proliferate (4, 15). In contrast, when maintained on an ECM they no longer require either serum or FGF and they respond to plasma factors. If, in the case of vascular smooth muscle cells, one were to extrapolate to the *in vivo* situation, maintaining them on an ECM and plasma is clearly a closer approximation to physiological conditions than exposing them to plastic and serum.

Because vascular smooth muscle cells proliferate at a maximal rate when maintained on an ECM and exposed to plasma, it is likely that they are responding to mitogen(s) already present in plasma rather than to FGF or to mitogen(s) generated during the coagulation process.

We thank Mr. Harvey Scodel for his invaluable assistance in the preparation of this manuscript. This work was supported by Grants HL 23678 and HL 20197 from the National Institutes of Health.

1. Carrel, A. J. (1912) *Exp. Med.* **15,** 516–528.
2. Balk, S. D. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 271–275.
3. Balk, S. D., Whitfield, J. F., Youdale, T. & Braun, A. C. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 675–679.
4. Ross, R., Glomset, J., Kariya, B. & Harker, L. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1207–1211.
5. Kohler, N. & Lipton, A. (1974) *Exp. Cell Res.* **87,** 297–301.
6. Ross, R. & Vogel, A. (1978) *Cell* **14,** 203–210.
7. Gospodarowicz, D., Delgado, D. & Vlodavsky, I. (1980) *Proc. Natl. Acad. Sci. USA,* **77,** in press.
8. Gospodarowicz, D., Ill, C. R., Hornsby, P. J. & Gill, G. N. (1977) *Endocrinology* **100,** 1080–1089.
9. Gospodarowicz, D., Ill, C. R. & Birdwell, C. R. (1977) *Endocrinology* **100,** 1108–1122.
10. Gospodarowicz, D., Brown, K. S., Birdwell, C. R. & Zetter, B. R. (1978) *J. Cell Biol.* **77,** 774–789.
11. Gospodarowicz, D., Mescher, A. L. & Birdwell, C. R. (1977) *Exp. Eye Res.* **25,** 75–91.
12. Gospodarowicz, D., Bialecki, H. & Greenburg, G. (1978) *J. Biol. Chem.* **253,** 3736–3743.
13. Gospodarowicz, D. & Greenburg, G. (1979) *Exp. Eye Res.* **28,** 249–266.
14. Gospodarowicz, D., Moran, J. & Braun, D. (1977) *J. Cell Physiol.* **91,** 377–385.
15. Ross, R., Nist, C., Kariya, B., Rivest, M. J., Raines, E. & Callis, J. (1978) *J. Cell Physiol.* **97,** 497–508.
16. Grobstein, C. (1967) *Natl. Cancer Inst. Monog.,* Vol. 26, pp. 279–294.
17. Wessels, N. K. (1977) in *Tissue Interaction and Development,* (Benjamin, New York), pp. 213–229.
18. Hay, E. D. (1978) in *Birth Defects,* Int. Cong. Series 432, eds. Littlefield, J. W. L., de Grouchy, J. & Ebling, F. J. G. (Excerpta Medica, Amsterdam), pp. 126–140.
19. Gospodarowicz, D. & Moran, J. (1976) *Annu. Rev. Biochem.* **45,** 531–552.
20. Gospodarowicz, D., Mescher, A. L. & Birdwell, C. R. (1978) *Natl. Cancer Inst. Monog.* Vol. 48, pp. 109–131.
21. Masui, H. & Garren, L. D. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 3206–3210.
22. Ramachadran, J. & Suyama, A. T. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 113–117.
23. Robinson, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP,* (Academic, New York), pp. 17–46; 339–398.
24. Whitfield, J. F., MacManus, J. P. & Gillan, D. J. (1970) *J. Cell. Physiol.* **76,** 65–82.