Journal of Supramolecular Structure 11:361–370 (1979)
Tumor Cell Surfaces and Malignancy 241–250

Stimulation of Human Vascular Endothelial Cell Growth by a Platelet-Derived Growth Factor and Thrombin

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Repair of a vascular wound is mediated by migration and subsequent replication of the endothelial cells that form the inner lining of blood vessels. We have measured the growth response of human umbilical vein endothelial cells (HuE) to two polypeptides that are transiently produced in high concentrations at the site of a wound; the platelet-derived growth factor (PDGF) and the protease thrombin. When 10^4 HuE cells are seeded as a dense island (2-mm diameter) in the center of a 16-mm tissue culture well in medium containing 20% human serum derived from platelet-poor plasma (PDS), no increase in cell number or colony size is observed. With the addition of 0.5 ng/ml partially purified PDGF, colony size increases and the number of cells after 8 days is 4.8×10^4. When human thrombin (1 μg/ml) is added along with the PDGF, the cell number rises to 9.2×10^4. Thrombin alone stimulates no increase in cell number. Although partially purified PDGF stimulates endothelial cells maintained in PDS as well as those maintained in whole blood serum (WBS), pure PDGF is active only when assayed in medium that contains WBS and is supplemented with thrombin. These results suggest the existence of a second class of platelet-derived factors that enable HuE cells to respond to the mitogenic activity of the purified platelet mitogen and thrombin.

Key words: endothelial cells, platelet-derived growth factor, thrombin, wound healing

The endothelial cells that form the inner lining of blood vessels have an extremely low turnover rate and rarely undergo mitosis in the normal adult vasculature in vivo [1–3]. These cells do, however, proliferate during the healing process that follows a vascular injury [4–6]. In tissue culture, endothelial cells also exhibit a stringent growth control in which cells in a confluent monolayer fail to proliferate in response to serum growth factors unless gaps or wounds are first made in the monolayer [7–9].

Received April 25, 1979; accepted July 16, 1979.

0091-7419/79/1103-0361$02.00

In recent years, considerable progress has been made in identifying mitogens present in the circulation that can stimulate division of cells in culture [10–12]. Two classes of polypeptides have been identified that can be expected to be produced in high concentration at a wound site and that might be relevant to the endothelial cell proliferation that occurs in wound healing. The first class of these polypeptides is the growth factors released from platelet granules during platelet activation. Such platelet-derived growth factors have been found to induce proliferation of several different cell types including smooth muscle cells [13, 14], human and mouse fibroblasts [15–22], glial cells [23, 24], and mammary tumor cells [25]. Recently, a polypeptide growth factor derived from human platelets has been purified to homogeneity [26]. The platelet-derived growth factor (PDGF) is a heat-stable polypeptide with an isoelectric point of 9.8 and a molecular weight of approximately 13,000 as judged by analytical SDS-polyacrylamide gel electrophoresis. The properties of PDGF are identical to those of the serum-derived growth factor previously isolated from whole human serum [19].

The second polypeptide of potential interest for vascular wound healing is thrombin, a highly specific protease that is produced in high concentration at a wound site as a result of coagulation [27]. Thrombin has been found to directly stimulate cell division or to potentiate the mitogenic response of cells to other growth factors in systems as diverse as the chick embryo fibroblast [28–31], mouse splenocyte [32], mouse, rabbit, and human fibroblast [33–36], and human but not bovine endothelial cells [37, 38]. In the studies described herein, we have investigated the possibility that thrombin and platelet-derived growth factors might act *together* to simulate endothelial cell proliferation. Our results support this conclusion.

METHODS

Purification of Platelet-Derived Growth Factors

The platelet-derived growth factor (PDGF) was purified according to previously published techniques [26]. In the present report, experiments described as being performed with "partially purified" PDGF employed material prepared by heat treatment of washed human platelets followed by ion-exchange chromatography, gel filtration in 1 M acetic acid, and isoelectric focusing, whereas experiments performed with "purified" PDGF employed material that had been further fractionated using preparative SDS-polyacrylamide gel electrophoresis [26]. Using Balb/c 3T3 cells as an assay, the pure PDGF was estimated to have a specific activity of about 5×10^6 units per milligram protein. One unit is defined as the amount of PDGF capable of inducing DNA synthesis in 50% of a population of quiescent Balb/c 3T3 (clone A31) cells.

Preparation of Whole Blood Serum and Plasma-Derived Serum

Human whole blood serum (WBS) and serum derived from platelet-poor plasma (PDS) were prepared according to the method of Ross et al [22] with the omission of the final chromatography of PDS on CM-Sephadex. PDS preparations were discarded if they were found to induce proliferation when added at a concentration of 20% (v/v) to sparse cultures of Balb/c 3T3 cells.

Maintenance of Human Vascular Endothelial Cells in Tissue Culture

Primary cultures of human umbilical vein endothelial cells were isolated in the laboratory of Dr. M. Gimbrone (Harvard Medical School) according to established procedures [39]. Cultures were discarded if they contained any smooth muscle colonies as detected by morphology or by the presence of thrombogenic cells to which exogenously added platelets would adhere [40]. Cells were trypsinized and split at a ratio of 1:5 into gelatin-coated Falcon T25 tissue culture flasks. To prepare the gelatin-coated substratum, the flasks were flooded with 5 ml of 1% (w/v) Difco gelatin in calcium- and magnesium-free phosphate-buffered saline. The flasks were allowed to stand at 4°C over night. Just before use, the gelatin was warmed to room temperature and aspirated, and the dishes were washed twice with medium. The culture medium was HEPES-buffered medium 199 (H199, Microbiological Associates) supplemented with 25 μg/ml endothelial cell growth supplement (ECGS, Collaborative Research), 250 ng/ml fibroblast growth factor (courtesy of Dr. D. Gospodarowicz, University of California, San Francisco), and 1 μg/ml human alpha-thrombin (generously provided by Dr. J. Fenton, Albany). After ten passages, the cultures were discarded and new primary cultures isolated.

Cell Growth Assays

All growth assays were carried out in gelatin-coated Falcon 24-well culture dishes. For studies on sparse cultures, 10^4 cells were dispersed randomly into each well. For studies on outgrowth from dense cultures, 10^4 cells were seeded as a dense island in the center of each well according to the method of Folkman and Butterfield (manuscript submitted for publication). In this method, the wells are first partially filled with 0.7 ml of agar solution (1% Difco agar in medium 199 with 10% human serum or PDS) which is allowed to harden in the well. With a cork borer, a 2-mm hole is punched into the agar in the center of each 16-mm well. Then 10^4 cells are seeded into each hole in a total volume of 10 μl and allowed to settle for 10 min before the entire well is flooded with 0.5 ml H199 medium containing 20% PDS and incubated over night at 37°C. Each well now contains a 2-mm colony of 10^4 cells still surrounded by an agar plug. When the agar plug is removed by means of a sterile curved spatula, the small, dense colony can grow out to fill the entire 16-mm well. After removal of the agar plug, the wells are refilled with 0.8 ml of fresh medium containing either PDS or WBS and the growth factor to be assayed. Fresh medium and fresh growth factors are added every 2 days until the conclusion of the experiment, at which time the cells are removed with 0.25% trypsin and counted on a Coulter Zf particle counter.

RESULTS

Platelet-Derived Growth Factors Stimulate Endothelial Cell Proliferation

When partially purified human PDGF is added to cultures of human umbilical vein endothelial cells that have been seeded as dense islands with room to outgrow onto the culture dish, cell proliferation is stimulated (Fig. 1). The amount of cell proliferation stimulated by PDGF increases as the concentration of either PDS or whole blood serum is in-

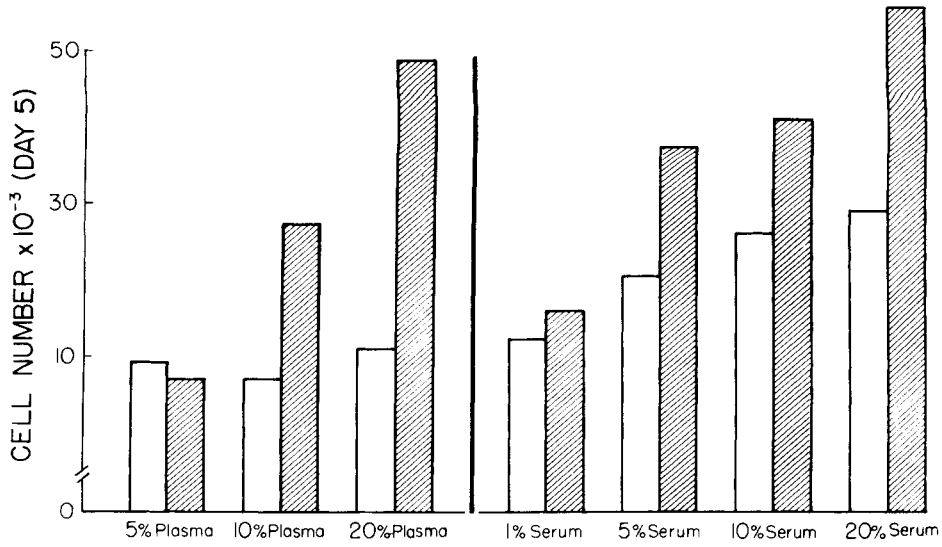

Fig. 1. Response of human umbilical vein endothelial (HuE) cells to PDGF. HuE cells (10^4) were seeded as a dense 2-mm island in the center of a 12-mm tissue culture well in medium containing 5% PDS. After 18 h, the medium was replaced with fresh medium containing either plasma (PDS, left panel) or whole blood serum (WBS, right panel) at the concentrations indicated. Shaded columns represent cultures supplemented with 5 ng/ml partially purified PDGF prepared as described in Methods.

creased. In some experiments, WBS alone stimulated a small increase in cell number. For example, in Figure 1, cell number can be seen to increase from 1×10^4 to 1.9×10^4 after 3 days in WBS alone. When experiments are carried out over longer time periods, WBS is found to stimulate proliferation only transiently over a period of 2–3 days (Fig. 2), and in many experiments no increase at all is seen in the presence of WBS alone. Stimulation by PDGF, on the other hand, was consistently observed in every experiment performed.

Thrombin Potentiates PDGF-Induced Endothelial Cell Proliferation

We have previously demonstrated that although thrombin cannot by itself stimulate endothelial cell proliferation, it can potentiate the growth response to other mitogens [33, 37, 38]. As shown in Figure 2, thrombin potentiates the PDGF-induced proliferation of human endothelial cells. When 10^4 cells were seeded as a dense island in medium H199 supplemented with 20% human WBS, only a small increase in cell number was observed within 8 days in the presence or absence of 1 μg/ml purified human thrombin. In the presence of 5 ng/ml partially purified PDGF, the cell number after 8 days was 4.2×10^4. However, with the addition of PDGF and thrombin, the cell number after 8 days was 9.2×10^4. The pattern of the growth curve indicates that although the initial growth rate is the same for cells growing in PDGF as for those growing in PDGF plus thrombin, the presence of thrombin allows the cells to continue dividing at this rate for a longer period of time. Since the experiment is terminated before the expanding colony totally fills the dish, the increase in cell number observed is not simply the result of a higher saturation density in PDGF plus thrombin.

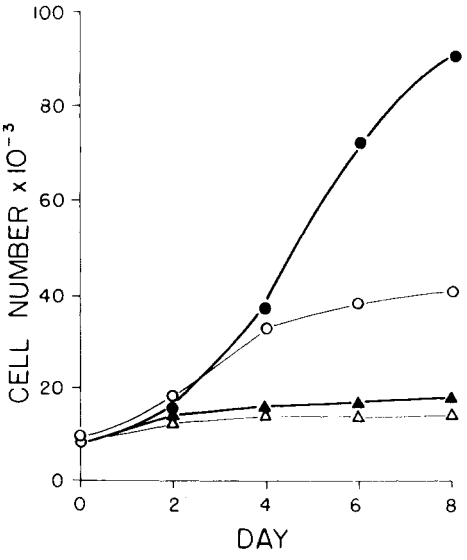

Fig. 2. Response of HuE cells to PDGF and thrombin. HuE cells were seeded as described in the legend to Figure 1, and maintained in medium containing 20% WBS alone (△—△), or supplemented with either 2 μg/ml human thrombin (▲—▲), 5 ng/ml partial purified PDGF (○—○), or 5 ng/ml partially purified PDGF plus 1 μg/ml human thrombin (●—●). Fresh medium and growth factors were added every 2 days.

Diminished Response of Sparse Endothelial Cell Cultures

The experiments described above employed a protocol in which 10^4 cells were seeded in a 2-mm circle in the center of a 16-mm well so that the increase in cell number observed represents outgrowth from the dense island of cells. In contrast, when the same number of cells are dispersed as a sparse culture in the same well, the cells display a diminished responsiveness to the mitogenic activity of PDGF and thrombin (Table I). After 6 days, the cell number in cultures supplemented with 5 ng/ml partially purified PDGF and 1 μg/ml thrombin is 62,428 if the cells were seeded as a dense island, but only 14,107 in cultures where 10,000 cells had been seeded sparsely. The sparse cells fail to proliferate even when plated in wells previously filled with an agar plug. It is of interest that as the cells proliferate in the dense culture, they tend to maintain contact with the other cells in the island rather than migrating away from the island and then dividing. This results in a circle of growing cells that increases in diameter daily.

Response to Increasing Concentration of Partially Purified PDGF

Figure 3 shows the proliferative response of human endothelial cells to increasing concentration of partially purified PDGF in the presence or absence of 1 μg/ml human thrombin. This material stimulates endothelial cell proliferation whether the experiment is carried out in PDS (left panel) or in WBS (right panel) and the response is stimulated by thrombin in both cases. It is important to note that the dose response to PDGF or to PDGF plus thrombin is described by a bell-shaped curve. High concentrations of PDGF are inhibitory to endothelial cell proliferation.

TABLE I. Effect of Cell Density on Endothelial Cell Response to PDGF and Thrombin*

	10^4 Cells sparsely seeded	10^4 Cells seeded as dense island
Control	8,423[a] ± 162	9,686 ± 274
+ 1 μg/ml thrombin	7,760 ± 384	12,402 ± 270
+ 5 ng/ml PDGF	11,212 ± 566	29,566 ± 946
+ 5 ng/ml PDGF + 1 μg/ml thrombin	14,107 ± 322	62,428 ± 1,242

*All values represent the mean of three samples.
[a]Cell numbers after 6 days.

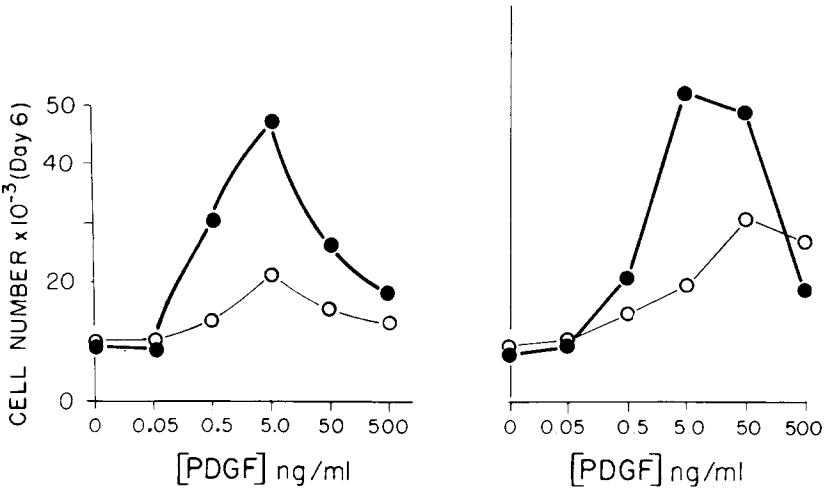

Fig. 3. Dose response of HuE cells to partially purified PDGF. HuE cells, seeded as dense islands, were treated with increasing concentrations of partially purified PDGF (○—○), or partially purified PDGF plus 1 μg/ml thrombin (●—●) in medium supplemented with either 20% PDS (left panel) or 20% WBS (right panel). Fresh medium and factors were added every 2 days.

Response to Increasing Concentration of Purified PDGF

The response of endothelial cells to the purified mitogen (Fig. 4) is surprisingly different from that of the partially purified material. Purified PDGF stimulates human endothelial cell proliferation only when assayed in whole blood serum supplemented with 1 μg/ml of pure human thrombin. It has no effect on endothelial cell proliferation when assayed in platelet-poor PDS. The maximal response is observed with 0.5 ng/ml of the pure mitogen in WBS supplemented with thrombin. Higher concentrations are again inhibitory to cell growth. These results differ from the situation with mouse fibroblasts [26], in which the pure factor has been shown to be mitogenic when assayed in medium containing platelet-poor plasma.

DISCUSSION

The mitogenic activity of factors secreted by platelets is now well documented [12–25]. Since these factors are secreted during the platelet activation that occurs during wound healing, it is not surprising that cells such as fibroblasts and vascular smooth muscle cells

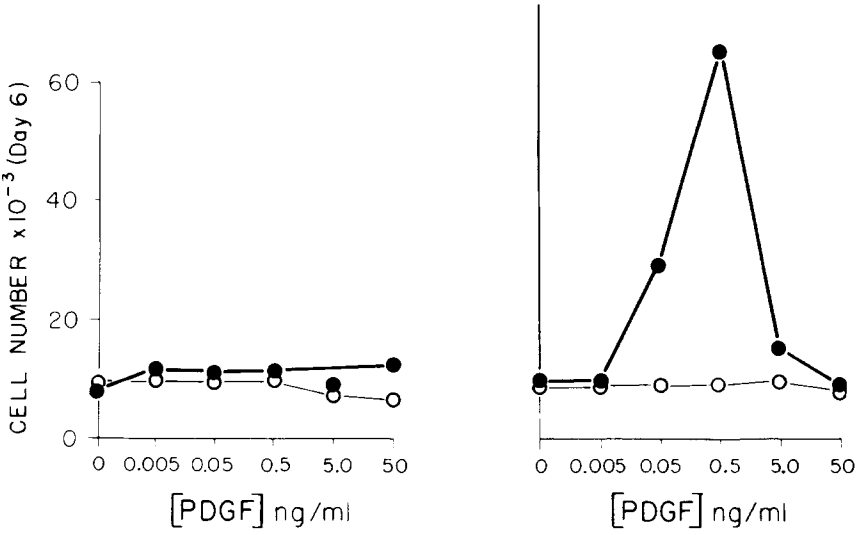

Fig. 4. Response of HuE cells to pure PDGF. HuE cells, seeded as dense islands, were treated with increasing concentrations of pure PDGF (○–○) or pure PDGF plus 1 μg/ml thrombin (●–●) in medium supplemented with either 20% human PDS (left panel) or 20% human WBS (right panel). Fresh medium and factors were added every 2 days.

that regenerate during wound healing are among those that respond to PDGF as a mitogen in vitro. The endothelial cells that line the inner surfaces of blood vessels are also required to regenerate after a vascular wound is made, yet the literature regarding the effect of PDGF on endothelial cell proliferation is not conclusive. Although endothelial cell proliferation in vitro has been reported to be stimulated by the inclusion of whole platelets in the growth medium [41, 42], platelet extracts and partially purified platelet factor preparations have not previously been found to be active [43–45].

We now report that under certain experimental conditions, human umbilical vein endothelial cells are stimulated to divide by human platelet-derived growth factors and that this stimulation is markedly enhanced by addition of the protease thrombin to the cultures. Thrombin alone is not mitogenic for these cells. Our results indicate that endothelial cells will respond to PDGF and thrombin if the cells are plated onto gelatin-coated dishes in the form of a small, dense island of cells that has room to outgrow into a comparatively large area. On the other hand, the same number of cells seeded sparsely onto the same size culture dish will not respond to the growth factors. This suggests that endothelial cell proliferation may be influenced by factors such as cell density, cell–cell contact, or other forms of metabolic cooperation that are more likely to occur when the cells are seeded as a dense island. It should be noted that in veins and arteries in vivo, endothelial cells are virtually never found at sparse densities and that the repair of a vascular wound generally involves outgrowth from a dense cell population proximal to the wound. Outgrowth from a dense culture of endothelial cells may, therefore, be more relevant to physiologic conditions than growth from sparse cultures. This type of growth control may be more relevant for endothelial cells than for other cell types that grow in different configurations in vivo.

Since whole blood serum contains material secreted by platelets during clotting, WBS contains PDGF and this may account for the initial observation made by several groups that fibroblasts and smooth-muscle cells will proliferate in WBS, but not in plate-

let-poor plasma or plasma-derived serum [13, 15, 18, 21]. Since our results demonstrate that PDGF can stimulate endothelial cell proliferation even when the cells are maintained in 20% WBS, we conclude that this concentration of WBS contains an amount of PDGF that is subsaturating for endothelial cell growth. It is conceivable, however, that the amount of PDGF produced locally by a platelet aggregate in vivo may be higher than that present in WBS.

An intriguing possibility raised by these results is that platelets may produce more than one factor that affects endothelial cell proliferation. The evidence for this is that pure PDGF is mitogenic for endothelial cells only when assayed in WBS. In PDS, pure PDGF is inactive. Since WBS contains other platelet-derived material in addition to subsaturating levels of PDGF, these other platelet-derived factors may be conditioning factors essential for endothelial cell proliferation. Since pure PDGF stimulates 3T3 cell proliferation in platelet-poor plasma [26], these other factors must not be essential for the growth of fibroblasts. Further evidence of possible secondary platelet-derived growth factors is provided by the observation that *partially purified* preparations of PDGF will support endothelial cell growth in PDS whereas *pure* PDGF will not. One explanation for this observation is that the partially purified PDGF contains the secondary factors necessary for endothelial cell proliferation that are absent in PDS but present in WBS. Thrombin itself is not likely to be an active component of the partially pure PDGF, since this material undergoes a heat treatment that renders thrombin inactive [26].

In summary, maximal stimulation of human endothelial cell proliferation is observed when cells are seeded as a dense island with room to outgrow on a gelatin-coated substratum and incubated in medium supplemented with human WBS, human PDGF, and human alpha-thrombin. A failure to observe an effect of PDGF on endothelial cell proliferation might, therefore, occur if the cells are seeded at sparse population density, if thrombin is omitted from the reaction mixture, or if the various components of the system (cells, serum, thrombin, and platelet factor) are not all derived from the same species.

Whereas thrombin and platelet-derived growth factors are both produced in high concentration at the site of a vascular wound, we would propose a model for wound healing in which blood clotting and cell proliferation are mechanistically interrelated (Fig. 5). The platelet adhesion and aggregation that occur during wound healing play an important role in the closure of the wound by the platelet plug. At the same time, platelet-derived growth factors are released which will stimulate proliferation of vascular endothelial cells as well as smooth-muscle cells [13]. Thrombin is formed during the coagulation cascade that occurs in response to vascular injury. Thrombin plays a crucial role in clot formation by converting soluble fibrinogen to a polymeric fibrin clot. At the same time, thrombin will potentiate the response of endothelial cells to the mitogenic properties of the platelet-derived growth factors. In a recent report, Pohjanpelto has demonstrated a synergistic effect of thrombin and platelet extract on the growth of human fibroblasts [46]. Further work will be necessary to determine if the combination of PDGF and thrombin may indeed be relevant to the proliferation of other cell types that regenerate after injury, including the fibroblasts of vascular adventitia and vascular smooth-muscle cells.

ACKNOWLEDGMENTS

We wish to thank Dr. J. Buchanan for stimulating us to undertake these studies and Dr. J. Folkman for advice and support. We also thank Dr. M. Gimbrone for providing primary cultures of human umbilical vein endothelial cells, Dr. J. Fenton for his generous

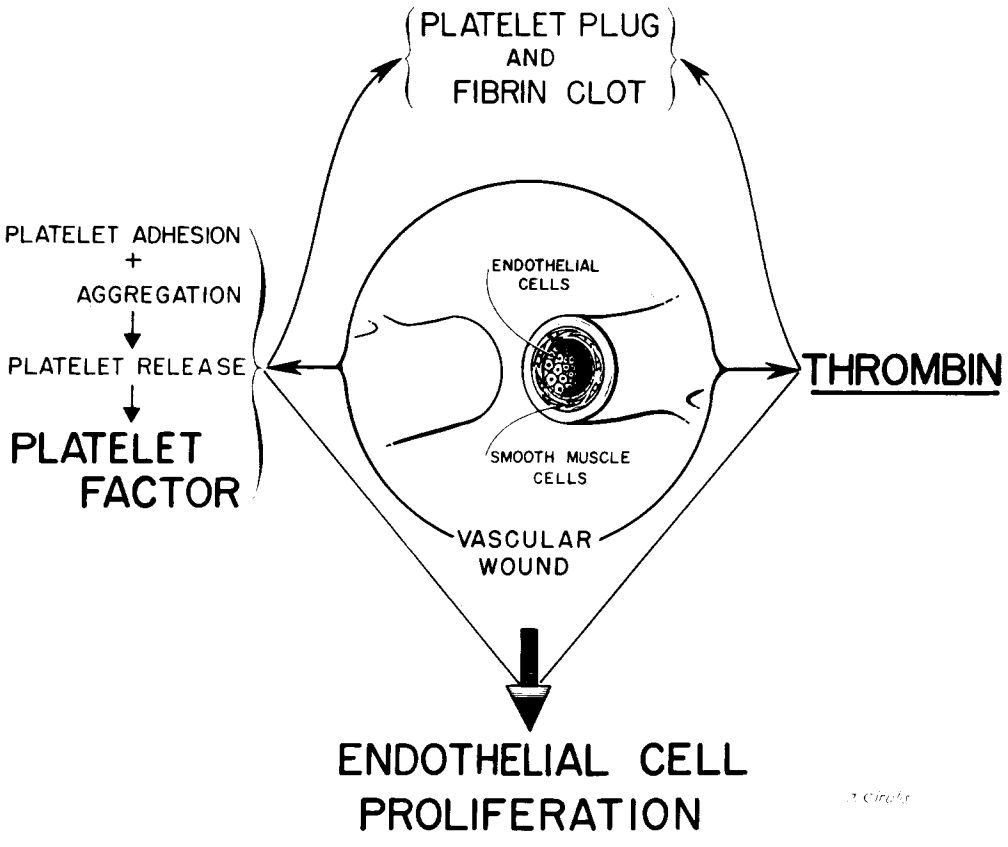

Fig. 5. Wound healing: Interrelationships of coagulation and cell proliferation.

gift of pure human alpha-thrombin, and Drs. C. Scher, C. Stiles, and M. Simons for their helpful advice, as well as M. J. Canavan for her editorial assistance. This work was supported by grant Nos. 5R01CA14019 and CA15388 from the National Institutes of Health.

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