DIFFERENTIAL EFFECT OF THROMBIN ON THE GROWTH OF HUMAN FIBROBLASTS

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ABSTRACT

The ability of thrombin to alter the growth of human skin fibroblasts was studied under a variety of experimental conditions. In agreement with previous reports, we obtained a moderate level of cell growth in confluent cultures using 0.5–8.0 U/ml of thrombin. In subconfluent cultures, the effect was strikingly different and was found to be dependent upon the time in culture when the enzyme was added. Cultures exposed to thrombin 24 h after subculturing showed growth stimulation several days later. In contrast, thrombin added at the time of cell plating produced a complete block of DNA synthesis and cell growth that lasted for at least 3 d. Cells exposed to thrombin under these conditions were morphologically altered and smaller. These thrombin-induced effects were reversible and could be completely prevented by pretreatment of the enzyme with hirudin before it was added to the culture medium. Growth inhibition and altered morphology were found to be the result of changes generated in the growth medium by thrombin and could be blocked by higher serum concentrations. The results of this study indicate that thrombin's influence on cell growth can be stimulatory or inhibitory and suggest that the state of the cell surface determines the response.

The formation of a platelet-rich fibrin clot is central to the control of blood loss following injury. Thrombin mediates this process by converting fibrinogen to fibrin and by stimulating platelets to aggregate through specific binding at the cell surface. It is now known that this enzyme can stimulate growth in some types of nonproliferating, density-inhibited fibroblasts (2, 3, 16, 17, 19, 21). As is true of platelets, binding of the enzyme at the cell surface appears to be essential for expression of its biological activity (1, 14, 22). This binding is saturable and is followed by internalization of the enzyme through endocytosis. The extent of growth stimulation has been found to vary with the cell line and with culture conditions. In some systems, thrombin has been found to potentiate the mitogenicity of serum and other growth-promoting agents (23). Other reports indicate that even small amounts of serum repress thrombin activity, and growth studies have been conducted in chemically defined media (1). Despite a substantial amount of work, little is actually known of the mechanism(s) by which DNA synthesis and cell growth are triggered. By studying the effects of thrombin on growing cells and on cells near confluence, we hope to achieve a better understanding of the mitogenic nature of this enzyme. In this report, we show that thrombin can both stimulate and inhibit cell growth. Concentrations of this enzyme that promote confluent culture overgrowth completely block log-phase growth under certain conditions. The state of the cell surface when the enzyme is added seems to determine the response.

MATERIALS AND METHODS

Cell Cultures

The cells used in this study were human, diploid, embryonic skin fibroblasts (HF) maintained at 37°C in a humidified, 5% CO_2 atmosphere. The stock culture medium consisted of Eagle's Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (0.292 mg/ml), streptomycin (100 μg/ml), penicillin (100 U/ml), and Fungizone (0.25 μg/ml). Cultures between the 10th and 20th passage were used. All culture components were purchased from Grand Island Biological Co., Grand Island, New York.

Growth Assays

Cells used in growth assays were released from stock cultures with 0.25% trypsin in Puck's Saline D (PSD) (Grand Island Biological Co., 1:250). Trypsin was removed by centrifugation, and the cells were resuspended in 3% FCS medium before plating in MultiWell tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) (2.1 cm² growth area/well). In some experiments, thrombin was added to the growth medium before plating. In other experiments, thrombin was added immediately after plating or at 18–24 h. Cell counts and mean cell volumes were determined daily or at designated times with a Coulter counter (Model ZBI with channelizer, Coulter Electronics Inc., Hialeah, Fla.). All determinations were performed in duplicate or triplicate, and standard deviations were usually <5% of the mean.

Clotting Time Assays

Thrombin activity was determined in all growth experiments immediately before the enzyme was added to the cultures. Fresh tissue-culture medium and isotonic Tris-saline (0.025 M Tris and 0.125 M NaCl) were mixed in equal amounts to give a solution of pH 7.45, with an FCS concentration of 3%. Thrombin was mixed with this solution, and aliquots of 250 μl were removed. To each aliquot, 50 μl of 2% fibrinogen (Grade L, AB Kabi, Stockholm) was added, and clotting times were measured in a fibrometer (FibroSystem, BioQuest, Div. of Becton, Dickinson and Co., Cockeysville, Md.). Activity was determined by comparison with a standard curve. Because the activities of thrombin and other proteases have been reported to be essential for growth stimulation in cultured cells (3, 5, 16), thrombin concentrations in this study have been expressed in units. One National Institutes of Health unit of thrombin equaled about 0.326 μg of protein or 8.14×10^{-12} mol.

DNA Synthesis

The rate of DNA synthesis in HF was determined by incubation of cultures with [³H]thymidine for 2 h. Cells were released with trypsin, aspirated onto glass-fiber filters, and washed with cold PSD. DNA was precipitated by aspiration of cold, 5% trichloroacetic acid through the filters, followed by a wash of cold, 95% ethanol. Filters were air-dried, treated with NCS (Amersham Corp., Arlington Heights, Ill.) to solubilize DNA, and placed in counting vials containing Bray's scintillation fluid (New England Nuclear, Boston, Mass.). Radioactivity was determined in a Beckman Model L58000 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Proteins

Both commercial bovine thrombin (Parke-Davis, Div. of Warner-Lambert Co., Detroit, Mich.) and highly purified bovine thrombin (11), with a specific activity of about 3,000 U/mg, were used in this study. Each experiment was performed at least twice, usually once with Parke-Davis thrombin and once with the purified enzyme. Both thrombin preparations produced the same results.

Hirudin, with a specific activity of 1,790 U/mg of protein, was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

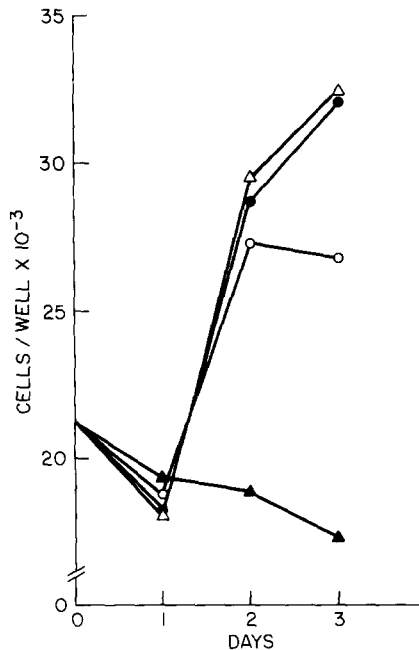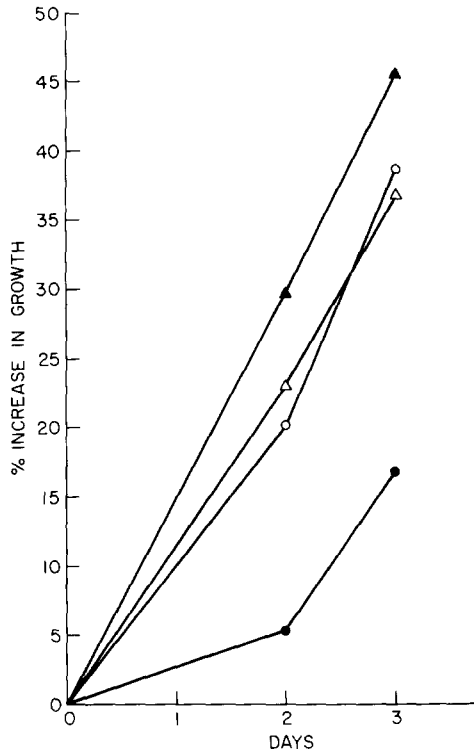### Effects of Thrombin on Culture Growth and Cellular Morphology

Fig. 1 a shows the effect of three concentrations of thrombin on the growth of quiescent cultures of confluent HF. The growth response is consistent with that reported by others with regard to human cells (16, 17). Fig. 1 b shows the effects of the same enzyme concentrations on the growth of subconfluent HF added at the time of culture plating (day 0). Under these conditions, cell growth was virtually blocked at enzyme concentrations that stimulated confluent cultures. The effect was dose dependent during the 3-d course of the assay. The cell count on day 1 of this assay (Fig. 1 b) indicates that thrombin did not quantitatively inhibit cell attachment.

In addition to being inhibited in their growth, cells incubated with thrombin that had been added at the time of subculturing appeared under the microscope to be smaller than cells in control cultures at 24 h (Fig. 2). This observation was confirmed with a Coulter counter channelizer. The cells shown in Fig. 2 a had a mean volume of 2,760 μm³, whereas those treated with thrombin (Fig. 2 b) had a mean volume of 1,830 μm³. The size of the cell was related to the thrombin concentration in the growth medium. The largest difference was usually detected 24 h after cell plating and may reflect the DNA content of the cell. In contrast to the findings described above, the addition of thrombin to cells 24 h after subculturing (day 1) produced no observable growth inhibition or morphological changes. Moreover, at higher initial plating densities, growth stimulation was observed. This differential effect of thrombin is clearly shown in Fig. 3. Cultures receiving thrombin at the time of cell plating showed dose-dependent growth inhibition 48 h later. Those receiving the same thrombin concentrations on day 1 showed enhanced growth when compared with controls without thrombin. These data indicate that the

ability of thrombin to stimulate or inhibit cell growth depends on the time in culture before enzyme is added.

In other experiments, thrombin was added dur-

ing subculture to cells plated over a wide range of initial densities. Growth inhibition and morphological change were found to be independent of initial density. Even cells plated at well above the confluent level for 3% FCS medium were sensitive to thrombin. The growth stimulated by this enzyme in established cultures did, however, appear to be density dependent. Actively growing, low-density cultures showed much less growth stimulation than those near confluence. It appears that freshly subcultured cells interact with thrombin differently than cells allowed to establish in culture for 24 h before enzyme is added.

Relationship of the Subculturing Process to Thrombin-induced Growth Inhibition

The growth inhibition observed in cultures receiving thrombin at the time of plating seems to be directly related to the subculturing process. To determine whether the trypsin used for cell release was involved in this effect, we prepared assay cultured from cells scraped from stock cultures with a rubber policeman. These cells had not been exposed to trypsin. Both test and control cultures were refed at 24 h to remove the cell debris that is a consequence of preparing cultures in this manner. Fig. 4 shows the results of a representative experiment. It is clear from these data that cultures prepared with "scraped" cells exhibit thrombin sensitivity similar to that of cultures prepared from trypsinized stocks. These results indicate that

FIGURE 1 *a* The effect of thrombin on the growth of confluent cultures. Cells were plated in MultiWell (Falcon Labware) plates at a density of about 15,000 cells/well in 10% FCS medium. 7 d later, one plate was harvested, and the cell density was found to be 47,688 cells/well. Medium from two more identical plates was removed and, without washing, 3% FCS medium that contained thrombin or control buffer (0.025 M Tris, 0.125 M NaCl, pH 7.4) was added. Cell numbers were determined 2 and 3 d later. Controls, ●; cultures with 0.5 U/ml thrombin, △; cultures with 1.5 U/ml thrombin, ○; cultures with 4.5 U/ml thrombin, ▲.

FIGURE 1 *b* The growth of subconfluent cultures in the presence of thrombin added at the time of cell plating. Cells were plated at a density of about 21,000 cells/well in 3% FCS medium that contained thrombin or control buffer. Growth was monitored for the next 3 d as described in Materials and Methods. Controls, ●; cultures with 4.5 U/ml thrombin, ▲; cultures with 1.5 U/ml thrombin, ○; cultures with 0.5 U/ml thrombin, △.

thrombin-induced growth inhibition is not trypsin dependent.

Specific Blockage of
Thrombin-induced Growth Inhibition
by Hirudin

Thrombin is a serine protease of very narrow substrate preference (12). Much of its specificity is undoubtedly related to a secondary binding site on this enzyme apart from the catalytic center. All known biological substrates for this enzyme require the participation of both sites for effective hydrolysis. Blockage of this secondary binding site by the highly specific peptide hirudin completely eliminates thrombin binding and catalysis. By utilizing hirudin-blocked thrombin, we have been able to completely reverse the growth-inhibiting effects of thrombin (Fig. 5).

As is shown by this experiment, growth is completely restored at thrombin to hirudin molar ratios of about 1:1. Thrombin is known to be completely inhibited through the formation of an almost irreversible complex ($K_n = 8 \times 10^{-11}$) at this molar ratio (13). In addition, cells exposed to hirudin-thrombin showed no morphological alteration and appeared identical to control cultures (Fig. 6). Coulter counter measurements showed these cells to be similar in size to controls without hirudin or thrombin. Hirudin alone had no effect on growth or cell morphology. The hirudin study presented here strongly suggests that thrombin-induced growth inhibition is mediated through specific binding to the cell or to a serum component. This peptide is known to block thrombin binding to fibrinogen and to the surface of platelets (7, 13).

DNA Synthesis in
Thrombin-inhibited Cells

Experiments described earlier in this paper have demonstrated that thrombin added at the time of subculturing markedly inhibits cell growth. We performed [^{3}H]thymidine incorporation studies to determine whether DNA synthesis is also inhibited under these conditions. Table I shows the results of a representative experiment. It is clear from these data that the inhibition of cell growth by thrombin is preceded by an inhibition of DNA synthesis. Comparison of the three time periods indicates a substantial reduction in both rate and amount of DNA synthesis in thrombin-treated cultures.

## Reversibility of Growth Inhibition
by Thrombin

Nontoxic biological agents that block growth usually act by specific and reversible mechanisms. As an inhibitor of growth, thrombin seems to meet the first criterion (Fig. 5). Fig. 7 shows that this effect is also reversible. Groups 2, 3, and 4 were plated on day 0 in medium that contained 7.2 U/ml thrombin. Group 1 received control medium without thrombin. At 24 h (day 1), cultures fed with thrombin showed the altered morphology typical of cells grown under these culture conditions (Fig. 2b). All cultures were refed at this time without washing (washing causes significant cell loss). Group 1 again received control medium without thrombin. Group 2 received medium that contained 1.5 U/ml thrombin. Group 3 was refed with control medium that contained no thrombin, and group 4 received medium with 1.5 U/ml thrombin and 18.8 U/ml hirudin. An excess of hirudin was used to compensate for residual thrombin from the plating medium. A lower thrombin concentration was also used in day-1 medium for this same reason.

Examination of these data indicates that cells receiving thrombin on both feeding days (group 2) showed approximately half the growth of controls (group 1). Cultures receiving thrombin medium at the time of subculturing and control medium on day 1 (group 3) showed significant recovery of growth when compared with group 2. The greatest recovery was observed in group 4. In this group, residual thrombin from the plating medium should have been inactivated by hirudin. Cells in

FIGURE 2a Control cultures containing no thrombin in 3% FCS medium. Cultures were photographed (phase-contrast, × 100) 24 h after subculture into assay wells. The culture density was 18,183 cells/well.

FIGURE 2b Cells in 3% FCS medium with thrombin. 4.25 U/ml thrombin was added at the time of subculture. Cultures were photographed (phase-contrast, × 100) 24 h later. The culture density was 19,995 cells/well.

2a

2b

W. M. HALL AND P. GANGULY   *Effect of Thrombin on Fibroblast Growth*   **75**
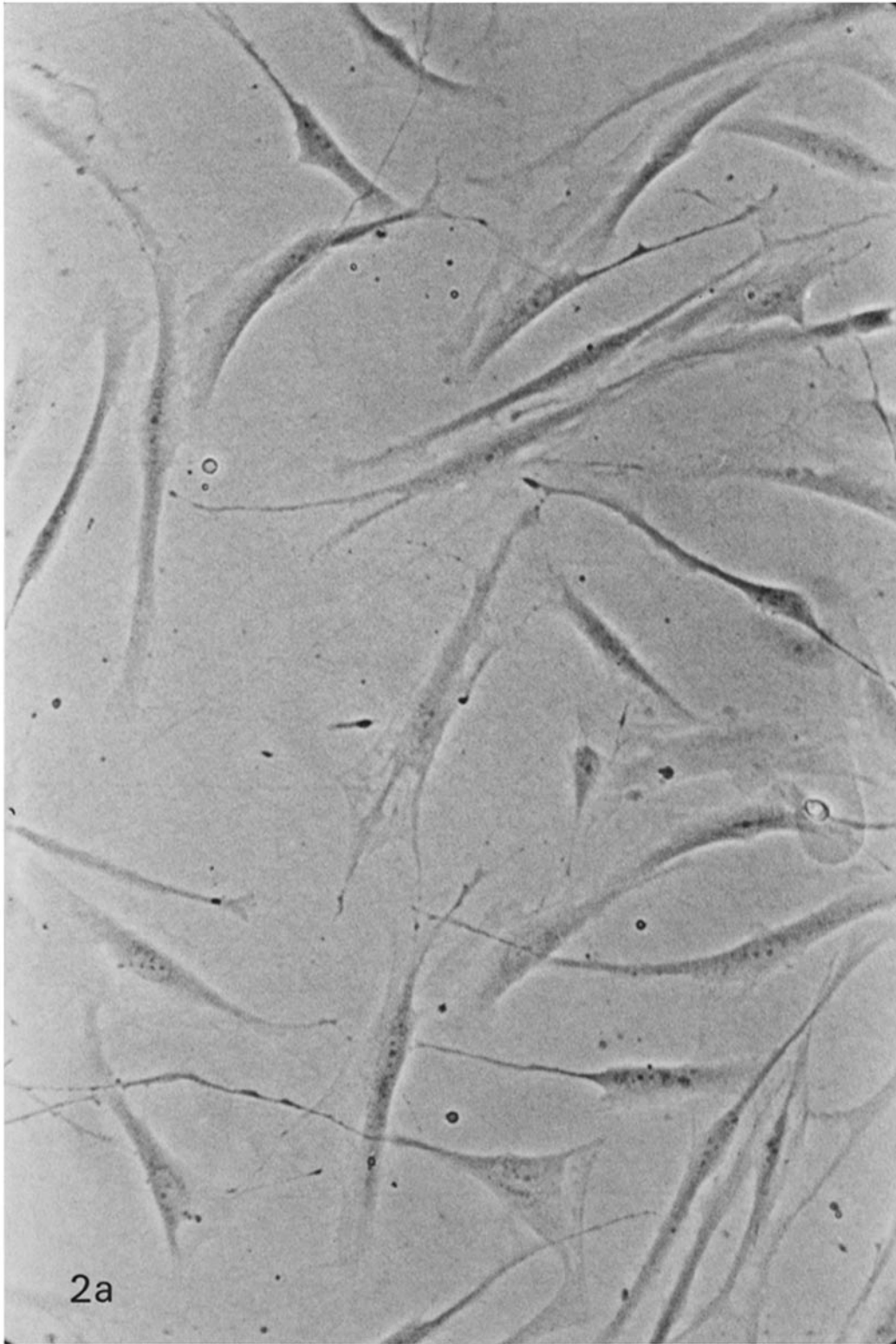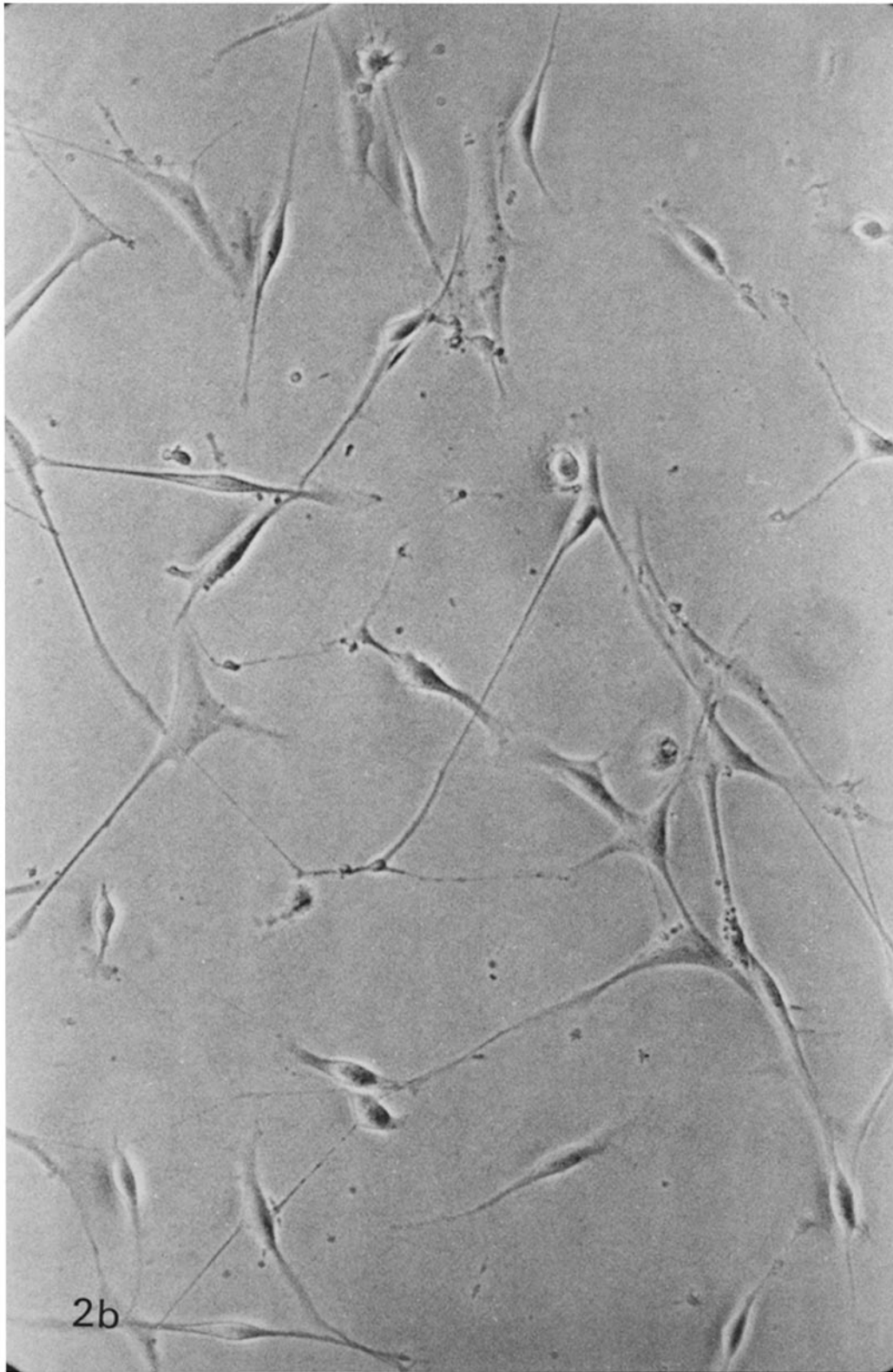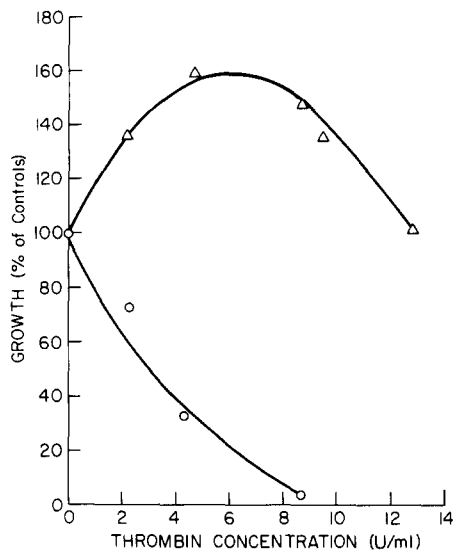
FIGURE 3 Thrombin growth alterations as a function of enzyme concentration. Cells were plated on day 0 at 20,410 cells/well in 3% FCS medium. To some cultures, thrombin was added to give the final medium concentrations shown. The thrombin cultures were harvested 48 h later (at day 2; O). On day 1, the remaining untreated cultures received thrombin in the same concentrations as on day 0. A set of control cultures was harvested at this time to determine the day 1 cell count (19,425 cells/well). 48 h later, cultures receiving thrombin on day 1 were harvested (on day 3; △). Percent growth of each assay set was determined by comparing it with its respective control without thrombin. Day 0 through 2 controls reached a density of 33,533 cells/well, and those for days 1 through 3 reached 38,573 cells/well. Control growth for each assay set was designated 100%, and test groups were normalized accordingly.
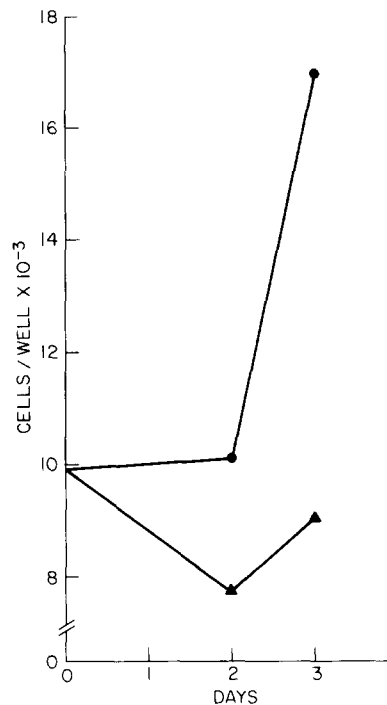

FIGURE 4 The effect of thrombin added at the time of subculture on "scraped" cells. Assay cultures were prepared from cells released from stock cultures with a rubber policeman and not exposed to trypsin. On day 1, all cultures were refed to remove debris which are a consequence of the subculturing processes. The day-1 medium was the same as that used on day 0 (3% FCS medium with or without thrombin). Control cultures, ●; test cultures with 4.5 U/ml thrombin, ▲.

groups 3 and 4, which showed strong morphological alteration at day 1, appeared normal by day 2. The mean volumes of these cells at day 3 (at harvest) showed a significant increase that correlated with recovery of growth. Although complete restoration of growth was not achieved by day 3, it should be pointed out that groups 2, 3, and 4 were inhibited by thrombin during the first 24 h of the assay and, therefore, had a shortened growth period when compared with the control group (group 1). These data demonstrate that thrombin-induced growth inhibition can be reversed and that this enzyme acts in an apparently nontoxic manner. Trypan blue exclusion studies have also indicated that thrombin is nontoxic under these conditions (unpublished data).

Effects of Media Preincubated with Thrombin on Cell Growth

Thrombin-induced growth in cultured cells appears to be the result of a direct enzyme-cell interaction. Serum in the growth medium is not thought to be primarily involved (2). A series of experiments was conducted to determine whether this is also true for growth inhibition induced by thrombin. Table II shows a representative experiment. It is clear from these data that culture media incubated overnight with thrombin and treated with hirudin before cell incubation (medium types 1', 2', and 3') produced growth inhibition and morphological change. Under these conditions, no free thrombin should have been present in the media at the time of cell incubation. Control media receiving thrombin and hirudin before the overnight incubation (medium types 1H, 2H, and 3H)
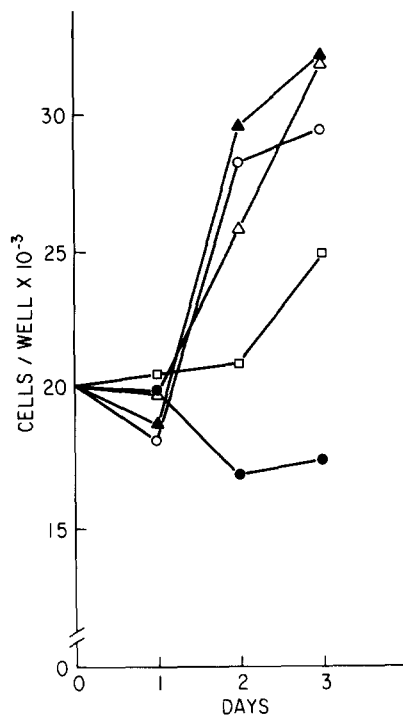
FIGURE 5 The growth of HF in 3% FCS medium receiving hirudin-blocked thrombin at the time of subculture. Hirudin was added to thrombin in various amounts to neutralize enzymatic activity. This was varified by measuring fibrinogen clotting times. The thrombin concentration added to all groups was 4.25 U/ml before neutralization. Control (no thrombin or hirudin), ○; thrombin control (4.25 U/ml thrombin without hirudin), ●; thrombin:hirudin unit ratio of 1:0.72, □; thrombin:hirudin unit ratio of 1:1.44, △; thrombin:hirudin unit ratio of 1:2.15, ▲. Free hirudin had no effect upon growth, as shown by separate experiments.

produced normal levels of growth without morphological change. These data seem to indicate that thrombin inhibits growth by the production of an inhibitory serum factor(s) rather than by direct action on the cell. We have also observed that serum must be present in the preincubation medium for inhibitory activity to develop. Thrombin incubated overnight in MEM that contained 2% bovine serum albumin to which hirudin and serum had been added before cell incubation produced no growth inhibition or morphological change.

In several experiments presented in this study, thrombin was added to growth media at the time of cell plating. Under these conditions, the growth inhibitory activity was produced in the presence

of cells. The media utilized in the experiment summarized in Table II contained pregenerated activity produced in the absence of cells. As a result of these experimental differences, part of the media utilized in the Table II experiment was tested for growth-altering activity on cells plated in normal 3% FCS medium and allowed to establish in culture. Addition of this medium to cultures 24 h after plating produced no growth inhibition or morphological change. These data are consistent with those presented earlier from experiments in which thrombin was added directly to cultures 1 d after they were subcultured (Fig. 3). Unlike the results obtained in this earlier experiment, no growth stimulation was observed when hirudin was utilized.

Effects of Serum on Growth Stimulation and Inhibition by Thrombin

Growth stimulation by thrombin is known to be influenced by serum in at least two ways. Under certain conditions, serum is known to inhibit the mitogenicity of thrombin without a corresponding reduction in enzymatic activity (1, 2). Under other conditions, the growth-promoting properties of serum appear to be potentiated by the presence of thrombin (23). In our system, both effects have been observed (Fig. 8). At a serum concentration of ~2.5-3.0%, thrombin-induced growth was maximal. We have found the level observed at this serum concentration to be dependent upon serum lot, initial cell density, and growth conditions. The results presented here suggest that thrombin potentiates the mitogenicity of serum at lower concentrations and is progressively less effective as the serum level increases. The reduced effectiveness of thrombin under these conditions did not correlate with inhibition of clotting activity, as will be discussed in the next paragraph.

Fig. 9 shows that thrombin-mediated growth inhibition is also influenced by the presence of serum in the culture medium. At higher serum concentrations, the effect was completely blocked, whereas lower concentrations were more permissive. No evidence of potentiation of serum was observed within the experimental concentration range. In addition, no inhibition of thrombin coagulative activity was found, even at the higher serum levels. This observation has been reproduced several times and seems to reflect a serum lot (A984218) of exceptionally low inhibitor con-

6

tent. Four other serum lots from the same supplier were found generally to be more effective in inhibiting coagulation, although a great deal of variation was observed. All serum lots were found to permit, at some concentrations, thrombin-induced growth inhibition. These data suggest that serum

TABLE I

Uptake of Tritiated Thymidine in Cultures Receiving Thrombin at Subculture

	^{3}H-TdR incubation periods		
	17.5–19.5 h	19.5–21.5 h	21.5–23.5 h
Control cultures cpm/10^3 cells	92.2	225.8	517.1
Thrombin cultures cpm/10^3 cells	26.0	44.3	74.5

On day 0, cells were plated at a density of 20,032 cells/ well in 3% FCS medium. Thrombin at a final concentration of 5.0 U/ml was added to test cultures. On day 1, at the indicated times after subculture, ^{3}H-TdR at 5 μCi/ ml final concentration was added to control and test wells. At the end of each 2-h incubation, cells were processed as described in the text.
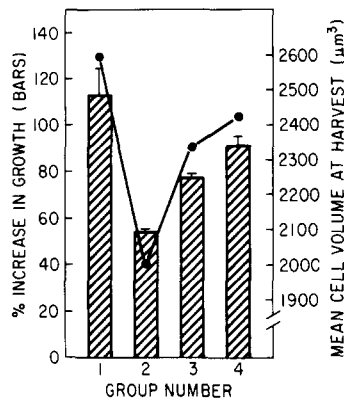

FIGURE 7 Growth and morphological recovery of cells incubated at subculture with thrombin. On day 0, cells were plated at a density of 20,541 cells/well. Thrombin was added to groups 2, 3, and 4 at a concentration of 7.2 U/ml. On day 1, all groups were refed as described in the text. Groups were harvested on day 3, and the percent increase in growth was determined.

represses both the stimulatory and inhibitory effects of thrombin by some mechanism other than the direct inhibition of the enzyme.

TABLE II

Effects of Media Preincubated with Thrombin on Cell Growth

Medium type	Thrombin concentrations	Growth	Cell volume	Morphological change
	U/ml	%	μm^3	
1	0.44	50.1	2,080	+
1′	0.44	52.8	2,160	+
1H	0.44	68.3	2,380	0
2	0.80	34.7	2,080	+
2′	0.80	39.0	2,120	+
2H	0.80	65.7	2,380	0
3	1.66	−15.4	1,810	++
3′	1.66	−7.4	1,980	++
3H	1.66	69.8	2,380	0

Three aliquots of 3.75% FCS medium were prepared with the thrombin concentrations indicated in the table. Each aliquot was then divided into three parts. Hirudin was immediately added to one part of each aliquot at a 1:2.5 thrombin to hirudin molar ratio (medium types 1H, 2H, and 3H). These medium types served as controls. All nine samples were then incubated overnight at 37°C in a 5% CO_2 atmosphere. After 24 h, the second part of each aliquot received an identical amount of hirudin (medium types 1′, 2′, and 3′). The third part of each aliquot received only control buffer (0.025 M Tris, 0.125 M NaCl, pH 7.4). These samples were designated as 1, 2, and 3 and still retained 5–15% of the original thrombin activity. Cells were prepared in serum-free MEM that contained 0.75% bovine serum albumin and added to assay plates in 100 μl/well aliquots. To each assay well, 400 μl of the appropriate medium type was added. This resulted in a final serum concentration of 3.0% and a corresponding reduction in the original thrombin concentration of 20%. At 48 h, cultures were harvested and the percent growth was calculated on the basis of the number of cells present after attachment was complete (24 h after plating). Before harvest, the morphological appearance of each culture was assessed by the following scale: 0, no visible morphological change (as shown in Fig. 2a); ++, full morphological change (as shown in Fig. 2b); and +, morphological change intermediate between 0 and ++. Cell volumes were determined as described in Materials and Methods.

FIGURE 6 The appearance of cells exposed to hirudin-neutralized thrombin at the time of subculture. Cells were plated on day 0 in 3% FCS medium that contained 4.25 U/ml thrombin and 9.14 U/ml hirudin (unit ratio of 1:2.15). On day 1, cultures were photographed (phase contrast, × 100) and harvested. The cell number was 18,708 cells/well, and the mean cell volume was 3,075 μm^3. Control cultures and cultures containing unneutralized thrombin are shown in Fig. 2.
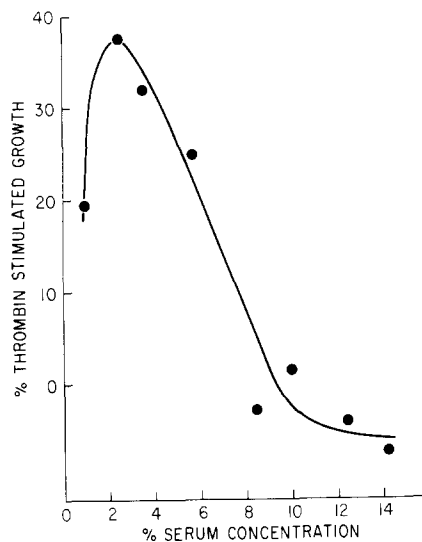
FIGURE 8 Growth stimulation by thrombin at various serum concentrations. On day 0, cells were plated at the serum concentrations shown. On day 1, cultures from each serum concentration were harvested, and the mean cell density was found to be 35,463 (± 4.8%) cells/well. Thrombin at a final concentration of 4.33 U/ml was added to test cultures. On day 4, all cultures were harvested. Percent increase in control growth was subtracted from percent increase in test growth at each serum concentration. At 2.5% serum, controls reached a density of 48,883 cells/well (37.8% increase in cell number), and the thrombin test samples were 62,250 cells/well (75.5% increase in cell number).

DISCUSSION

It is now well established that thrombin in moderate concentrations can stimulate confluent culture overgrowth in a number of fibroblast lines. Growth is associated with a specific saturable binding of the enzyme at the cell surface, followed by internalization. Our study of this phenomenon has revealed that thrombin can also inhibit cell growth under certain conditions. Fig. 3 illustrates this differential effect. Thrombin added to freshly plated HF produced a dose-dependent inhibition of growth 48 h later. Addition of this enzyme to parallel cultures 24 h after subculturing resulted in significant growth stimulation. Thrombin-inhibited cells exhibited altered morphology (Fig. 2), but this was not seen in control cultures or cultures receiving the enzyme at 24 h. The uptake of tritiated thymidine by DNA was also found to be greatly reduced in cells treated with thrombin at subculturing (Table I).

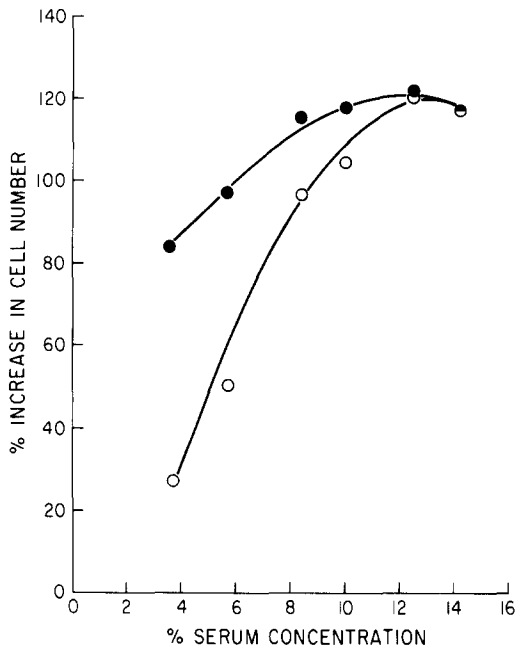The use of hirudin to block thrombin has proven

FIGURE 9 Growth inhibition by thrombin as a function of serum concentration. On day 0, cells were plated at a density of 21,990 cells/well in the serum concentrations shown. 4.21 U/ml thrombin was added to test cultures. Fibrinogen clotting activities were determined at this time as described in Materials and Methods. Cultures were harvested at day 3, and the percent growth was determined. The control cell number at 14.2% serum was 47,790 cells/well, and the thrombin test was 46,595 cells/well. Control (●) and thrombin (○) growth curves are shown.

to be an informative experimental technique. Enzyme treated with this specific inhibitor was unable to block growth or alter cellular morphology when added at subculturing (Fig. 5). Media incubated overnight with thrombin and treated with hirudin shortly before subculturing produced growth inhibition and morphological change even though no active thrombin was present (Table II). These data indicate that thrombin acts indirectly through products or changes generated in the growth medium. Thrombin's limited substrate specificity suggests that these effects are not the results of a random proteolytic destruction of serum components that are necessary for growth or cellular structure.

Little is known about the nature of the inhibitory changes generated in growth media by thrombin. Serum appears to be a necessary component, and inhibition seems to occur only in freshly

plated cells. Within minutes after subculturing, HF attach to the growth substrate, and by 12–18 h cytoplasmic spreading is complete. At this time, cells are no longer sensitive to growth inhibition by thrombin. These facts suggest that thrombin may produce or alter serum components essential to these early events. A number of serum factors that seem to hasten or facilitate initial cell-substrate adhesion have been investigated (4, 8–10, 20). Hydrolysis of one or more of these components by thrombin might impair cellular establishment following subculturing. Of particular interest is fibronectin, a major cell-surface and serum glycoprotein, which may be necessary for cell attachment and spreading. Thrombin is known to hydrolyze fibronectin (15), which might influence initial cellular attachment. The complexity of serum makes this only one of many possible explanations. The changes produced in serum by thrombin may be proteolytic, i.e., thrombin may activate a serum protease. Mild, continuous proteolysis of the cell surface could produce the altered morphology we have observed and might be sufficient to inhibit growth. This possibility seems less likely in light of our observation (data not presented) that even high concentrations of soybean trypsin inhibitor had no effect upon the development of morphological change or growth inhibition. It is also difficult to explain how a protease could selectively alter the properties of freshly plated cells but have no measurable influence on cells allowed to establish in culture for 24 h.

Even though morphological change and growth inhibition seem to parallel each other, their exact relationship is unknown. Folkman and Moscona (6) have demonstrated that cytoplasmic spreading in nontransformed cells is closely related to the rate of DNA synthesis. Factors that lessen a cell's affinity for its substrate may alter its morphology and potential growth rate. Other work has indicated that the hypothesis of Folkman and Moscona may not apply to all cell lines (18). Mutants of Balb/c3T3 fibroblasts displaying defective substrate adhesiveness and altered morphology have been isolated. Despite these changes, the growth rate and saturation density of these cells were not altered. The possibility cannot be ruled out that growth inhibition by thrombin is a secondary effect resulting from serum factors that alter or reduce cellular attachment and spreading.

The data presented in Figs. 8 and 9 indicate that both the stimulatory and inhibitory functions of thrombin are repressed at serum levels that do not block fibrinogen coagulation. Carney et al. (1, 2) have reported a similar effect with regard to growth stimulation in mouse embryo cells. In their system, serum at low concentrations blocked the specific binding of thrombin to the cell surface without inhibiting fibrinogen clotting. Our studies indicate that growth inhibition and growth stimulation probably occurs by different routes. Thrombin's ability to produce inhibitory changes in growth media suggests that direct cellular binding may not be the immediate cause of growth inhibition. That both processes are blocked at higher serum concentrations may be coincidental or may reflect some property common to both events.

The findings of this study may eventually lead to a better understanding of thrombin's interaction with cultured cells. Of potential importance is the relationship between growth stimulation and inhibition. Our results seem to indicate that the two processes operate at different times in culture and may, therefore, not oppose each other. Comparison of Figs. 8 and 9 indicates that growth stimulation can occur in established cultures at serum concentrations very permissive of growth inhibition in freshly subcultured cells. Even so, it seems unlikely that both events, mediated by this single enzyme, will prove to be totally independent. Establishment of a relationship would contribute to a fuller appreciation of thrombin's mitogenic properties.

Thrombin's ability to inhibit cell growth under certain experimental conditions is a surprising observation in light of its known mitogenic properties. The state of the cell surface at the time enzyme is added seems to be the determining factor in the response obtained. Our work indicates that growth inhibition is mediated by thrombin-generated serum changes. Thrombin's limited substrate specificity and its ability to inhibit growth in microgram quantities suggest that these changes may involve serum components central to cellular function. Isolation and identification of these factors should yield insight into the differential nature of thrombin activity, and this knowledge would be of value in the study of cellular growth regulation and its apparent loss in neoplastic disease.

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