Binding of Thrombin to Cultured Human Fibroblasts: Evidence for Receptor Modulation

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ABSTRACT Previous work from this laboratory has indicated that thrombin's influence on cell growth can be negative as well as positive. Addition of enzyme to actively growing or confluent cultures of human skin fibroblasts produced growth stimulation, whereas cultures receiving thrombin at the time of subculture displayed inhibited DNA synthesis and mitosis. The specific binding of [^{125}I]thrombin to cells under stimulatory and inhibitory conditions has been studied. Fibroblasts receiving enzyme at subculture bound about two times more [^{125}I]thrombin than those processed in the same way several hours later. The apparent dissociation constant for both groups was $\sim 1.5 \times 10^{-8}$ M. In each case binding was saturable, although cells receiving enzyme at subculture showed a much higher rate of binding. Experiments were conducted in which enzyme was added to cells at various times after subculture. It was found that the ability of these fibroblasts to specifically bind [^{125}I]thrombin decreased progressively over a 2-h period after subculture and then remained constant for at least 24 h. Evidence is also presented indicating that the binding of [^{125}I]thrombin in both experimental groups was inversely dependent upon the culture density. The biological effects of elevated thrombin binding in cells receiving enzyme at subculture were examined. It was found that inhibited DNA synthesis and altered cellular morphology were directly related to this parameter. This study suggests that fibroblasts may possess cryptic thrombin receptors that become exposed during subculture or after injury in vivo. These possibilities and the relationship of cell shape to the availability of thrombin receptors are discussed.

A characteristic of many nontransformed animal cells is their ability to cease growth at certain critical culture densities. This effect is known as density-dependent inhibition of growth and seems to reflect a basic form of growth control. Reinitiation of cell division in density-arrested cultures can often be accomplished by the addition of certain proteolytic enzymes. A notable example of such an enzyme is the serine protease thrombin (3, 4, 17–20). This enzyme is of particular biological interest because of its limited substrate specificity (12) and its involvement at sites of tissue regeneration after injury in vivo. Thrombin is known to potentiate the growth-promoting effects of serum and other mitogenic factors (22) or to act in the absence of serum with some cell types (2). After vascular injury, thrombin is known to bind to the surface of platelets, causing them to aggregate and release certain internal components (6). Recent work has shown that this enzyme also specifically binds to fibroblasts (2, 13, 21). This binding is saturable and is followed by endocytosis. Studies using thrombin covalently linked to carboxylate-modified polystyrene beads have demonstrated that action of this protease at the cell surface is sufficient to initiate growth (1).

We have recently reported that thrombin's influence on cell growth can be negative as well as positive (9, 10). Cultures of human fibroblasts exposed to thrombin 24 h after subculture or at confluence showed levels of growth stimulation comparable to that reported by others for human cells (17, 18). In contrast, thrombin added at the time of cell plating produced morphologically altered cells unable to synthesize DNA or to grow. This inhibitory effect was reversible and was found to be produced, at least in part, by direct action of thrombin on serum components present in the growth medium. To further characterize this effect, we examined thrombin's specific association with cells under various conditions. We now report that binding is also a function of the culture state at the time of enzyme addition. Cells receiving [^{125}I]thrombin at the time of subculture specifically bound up to two times more of this enzyme than parallel cultures allowed to establish for several hours before thrombin addition. This binding was found to be saturable and could be blocked by the addition of hirudin or excess unlabeled thrombin. Under serum-free conditions, ele-

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vated binding was found to correlate directly with inhibition of DNA synthesis. This study suggests that fibroblasts possess cryptic thrombin receptors that become exposed after subculture or injury in vivo. The physiological significance of this finding and its relationship to thrombin's growth altering properties are discussed.

MATERIALS AND METHODS

Cell Cultures

Human diploid embryonic skin fibroblasts (HF) between the tenth and twentieth passage were used in this study. Stock cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Stock medium consisted of Eagle's Minimum 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). All culture reagents were purchased from Grand Island Biological Company (GIBCO), Grand Island, New York.

Growth Assays

Assay cultures were prepared from trypsinized stocks (0.25% [GIBCO]; 1:250 trypsin in Puck's Saline D [PSD], pH 7.45). Trypsin was removed by centrifugation and the cells resuspended in FCS medium before plating in Falcon multiwell tissue culture plates (2.1 cm^2 growth area/well; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). Thrombin was added at the time of cell plating or at various times after plating in small aliquots. Cell counts were determined daily or at designated times with a Coulter counter (model ZBI with channelizer; Coulter Electronics Inc., Hialeah, Fla.). All determinations were done in duplicate or triplicate, and standard deviations were usually 5% or less of the mean.

Measurement of DNA Synthesis

The incorporation of [^{3}H]thymidine into an acid-insoluble product was used as a measure of DNA synthesis. [^{3}H]thymidine (New England Nuclear; Boston, Mass.) was added to cultures at a final concentration of 2–10 μCi/ml, with incubation periods of 2–6 h. The reaction was stopped by the addition of unlabeled thymidine to a final concentration of 0.5 mM. The medium was removed, and aliquots of ice-cold 5% TCA were added to all cultures. Cells were incubated at 4°C for 30 min to ensure complete precipitation of DNA. The cultures were then washed twice with cold acid, and the DNA was solubilized in 1 M NaOH. ^{3}H radioactivity was determined in a liquid scintillation counter.

Clotting-time Assays

Thrombin activity was determined before all growth and binding assays by the following method. Tissue culture medium and isotonic Tris-saline (0.025 M Tris and 0.125 M NaCl) were mixed in equal amounts, giving a solution of pH 7.45 with an FCS concentration of 3%. Thrombin or [^{125}I]thrombin was added to this solution and 250-μl aliquots removed. 50 μl of 2% fibrinogen (grade L; AB Kabi, Stockholm, Sweden) were added to each 250-μl test sample and the time required for clot formation was measured in a fibrometer (FibroSystem; Bio-Quest, Cockeysville, Md.). Activity was determined by comparison with a standard curve.

Proteins

For all binding and growth assays purified bovine thrombin (11) with a minimum specific activity of 2,000 NIH U/mg was used. Hirudin with a specific activity of 1,790 U/mg protein was purchased from Sigma Chemical Co., St. Louis, Mo.

Bovine thrombin was iodinated by the chloramine T method as previously described (6). Unbound label was removed by gel filtration. Enzyme labeled in this manner retained at least 90% of its clotting activity and corresponding amounts of its stimulatory and inhibitory activities. Specific activities ranged from 1.5–6.5 × 10^6 cpm/μg.

Binding Assays

The binding of [^{125}I]thrombin to HF was measured in cultures grown in Falcon multiwell plates at densities of 2–6 × 10^4 cells/well. Assay medium consisted of 3% FCS medium supplemented with 1% bovine serum albumin (BSA) to reduce nonspecific binding (2). The pH of this medium was adjusted so that a value of 7.25–7.30 would be maintained under assay conditions at 37°C in a 5% CO_2 atmosphere. [^{125}I]thrombin was added at subculture or at other designated times at concentrations ranging from 3.0 × 10^5–1.5 × 10^6 cpm/well. After the incubation period, binding medium was removed, and each well was filled with harvest buffer (0.025 M Tris, 0.125 M NaCl, 0.5% BSA, and 0.05% EDTA, pH 7.24). After a 5-min incubation at room temperature, cells were removed from the substrate by a gentle up and down pipetting of the solution. Cells were transferred to 15-ml conical Falcon centrifuge tubes and washed three times by centrifugation, using 14–15 ml of harvest buffer/tube. The radioactivity of the final cell pellet was determined in a Packard Auto-Gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Cell numbers were determined for each group from parallel cultures. Nonspecific binding was determined at each test point from samples containing excess hirudin added simultaneously with [^{125}I]thrombin and generally ranged from 10–25% of the total. Both test and nonspecific values were determined in duplicate or triplicate and averaged. Standard deviation for all data presented ranged from 3–14% of the mean and averaged ~10%.

Binding measurements in this study were performed on two general types of experimental groups: cells receiving thrombin at subculture and those allowed to establish in culture for up to 24 h before enzyme addition. To minimize the biochemical differences between these two test groups, we felt it appropriate to use binding periods of up to 24 h. This procedure allowed freshly plated cells to fully establish and begin DNA synthesis before the termination of the binding incubation. Time-course binding experiments indicated that the level of cell-associated [^{125}I]thrombin remained constant from ~8 h to at least 29 h after enzyme addition for both types of experimental groups.

Most binding measurements were done in the presence of 3% FCS medium. Previous work (10) indicated that this serum lot (A984218) contained very low amounts of thrombin inhibitors. Fibrinogen coagulation time was not altered at this serum level with the thrombin concentrations used in this study.

RESULTS

Binding Experiments

During the initial development of the binding assay, the disposition of radioactivity at each processing step was determined. Generally 95–98% of the total radioactivity present was removed in the first centrifugation. The amount of [^{125}I]thrombin remaining changed very little from the second to the third centrifugation, indicating that washing was complete. Initial experiments indicated that the presence of EDTA in the harvest buffer did not cause dissociation of radiolabeled thrombin from the cell surface but did facilitate the removal of the cells

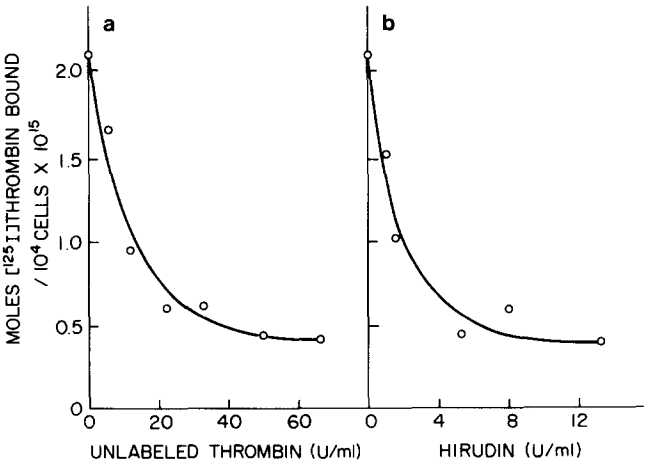
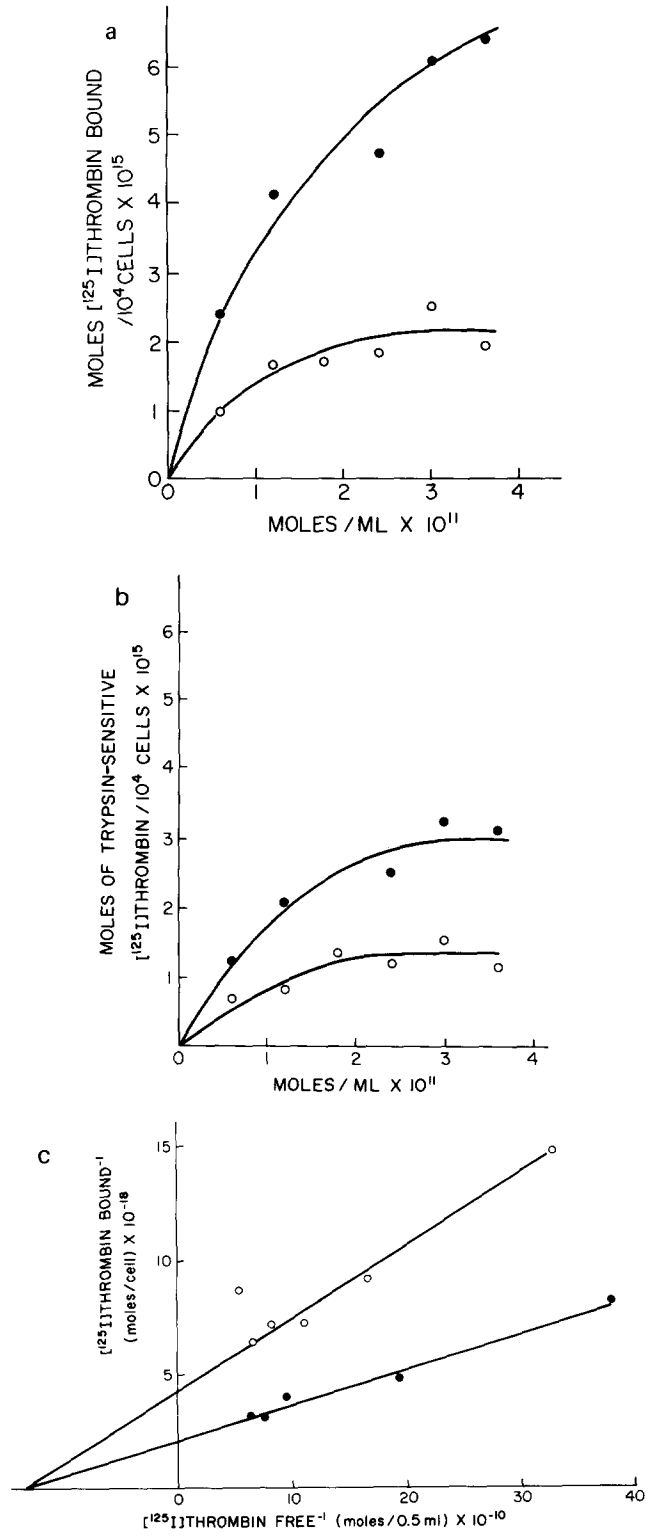


FIGURE 1 The effects of increasing concentrations of unlabeled thrombin (*a*) and hirudin (*b*) on the binding of [^{125}I]thrombin to HF. Cells were plated in media containing 7.2% FCS and allowed to establish in culture for 5 d. At this time, the old media was removed and binding media containing 9.1 × 10^{-12} mol/ml of [^{125}I]thrombin and various concentrations of unlabeled thrombin or hirudin was added. Cultures were harvested and the amount of cell-associated radioactivity determined 19 h later. Each well contained ~53,819 cells at this time.

from the plastic substrate. This agent has been used by others in thrombin binding studies (13). Fig. 1 demonstrates that excess unlabeled thrombin and hirudin were equally effective at reducing the amount of specific cell-associated [^{125}I]thrombin. Although hirudin has been used for nonspecific binding determinations in platelets (6), we are unaware of its use with fibroblasts. Hirudin was chosen for use in this study because it had no effect upon cellular morphology. In contrast, cells receiving high amounts of unlabeled thrombin (>20 U/ml) at subculture failed to spread and establish properly in culture. Initial experiments indicated that heat-inactivated thrombin could be successfully used for nonspecific binding measurements, but hirudin was chosen for its convenience.



Thrombin Binding in Freshly Plated and Established Cultures

Cells exposed to thrombin at subculture show growth inhibition not observable in parallel cultures receiving the enzyme at 24 h (10). Previous evidence indicated that this effect was at least partially mediated by thrombin-induced serum changes. Experiments were conducted to determine whether these two experimental cell groups also differed in their ability to bind thrombin specifically. From the data presented in Fig. 2*a*, it is obvious that cells receiving labeled enzyme at subculture bound significantly more thrombin than those to which enzyme was added at the later time. Nonspecific binding measured at each enzyme concentration was the same for both test groups (see also Fig. 4).

The amount of trypsin-sensitive [^{125}I]thrombin associated with the cell surface for each group of samples (Fig. 2*a*) was determined by previously described methods (13). In both groups, the amount of [^{125}I]thrombin bound at the cell surface was 40–50% of the total (Fig. 2*b*), and both curves plateaued, indicating an apparent saturation of available receptors. It should also be noted that cell surface enzyme in both groups was directly related to the total amount of cell-associated thrombin. These factors suggest that internalization was proportioned to the amount of enzyme at the cell surface in both experimental groups.

Before the harvest of each culture (Fig. 2*a*), aliquots of media were removed from each experimental group and the radioactivity determined. From these data and the surface binding information, a double reciprocal plot (bound enzyme^{-1} vs. free enzyme^{-1}) was prepared (Fig. 2*c*). This figure indicates that the binding capacity of freshly subcultured cells was about

FIGURE 2 (*a*) Net binding of thrombin in freshly plated and established cultures. Assay cultures were prepared from trypsinated stocks and to half of these cultures [^{125}I]thrombin at 0.242–1.45 μg/ml was added (●). Hirudin at 14 U/ml was included in some samples at each enzyme concentration for determination of nonspecific binding. 18 h later this group of samples was harvested and radioactivity determined. The cell number at this time was 54,085 cells/well. To the other half of the original cultures (○) enzyme or enzyme plus hirudin was added 24 h after subculture. As before, harvesting was begun 18 h after enzyme addition (42 h after subculture). These cultures contained 57,508 cells/well. Nonspecific binding was about 0.77 × 10^{-15} mol of [^{125}I]thrombin/10^4 cells for both experimental groups at the highest media enzyme concentration. Nonspecific binding at each enzyme concentration has been subtracted from each test value to give the net cell-associated [^{125}I]thrombin shown in this figure. (*b*) Net surface-bound (trypsin-sensitive) thrombin in freshly plated and established cultures. After determination of total cell-associated [^{125}I]thrombin (*a*) cell pellets were incubated with 0.25% trypsin (GIBCO; 1:250 in PSD, pH 7.5) for 45 min at 37°C. The pellets were rewashed by centrifugation and the remaining ^{125}I radioactivity determined. These values, which represented internalized (trypsin-insensitive) [^{125}I]thrombin were substrated from total cell-associated radioactivity to obtain surface bound enzyme. ●, Cells receiving enzyme at subculture; ○, cells receiving enzyme 24 h after subculture. (*c*) Double reciprocal plot of [^{125}I]thrombin binding. ●, Cultures receiving [^{125}I]thrombin at subculture. ○, Cultures receiving [^{125}I]thrombin 24 h after subculture. Details of this experiment were given in the legend to *a* and in the text.

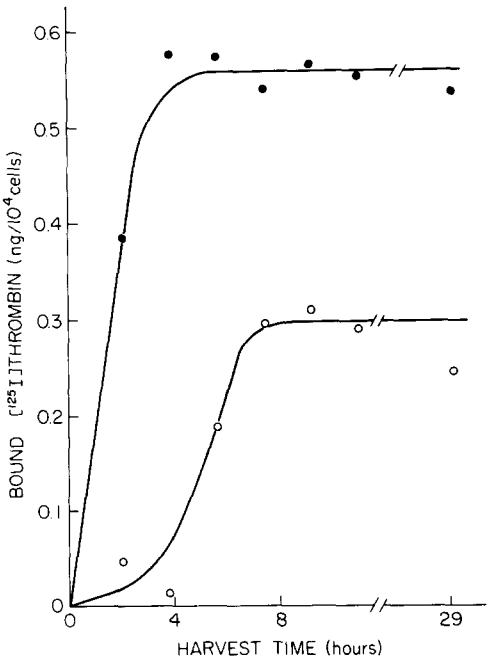


FIGURE 3 Time-course studies on the binding of [^{125}I]thrombin to fibroblasts. ●, Cultures receiving [^{125}I]thrombin at subculture. ○, Cultures receiving [^{125}I]thrombin 13.3 h after subculture. Nonspecific binding samples contained 24 U of hirudin/ml in addition to [^{125}I]thrombin. These values did not change with time and were identical for both experimental groups (0.052 ng of [^{125}I]thrombin/10^4 cells). Nonspecific values have been subtracted at each time-point.

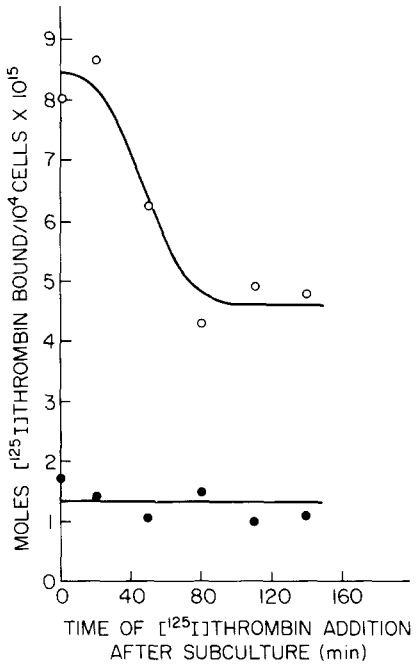


FIGURE 4 Net thrombin binding to HF as a function of time after subculture. ○, Cultures receiving 1.1 μg/ml of [^{125}I]thrombin. ●, Cultures receiving 1.1 μg/ml of [^{125}I]thrombin and 16 U/ml hirudin. The cell density at harvest was 31,076 cells/well in each group.

two times greater than that observed in established cultures, but the affinity of the receptor for thrombin remained the same.

Kinetics of Thrombin Binding

We examined the time-course of binding of [^{125}I]thrombin to HF receiving enzyme at subculture and those exposed to enzyme several hours after subculture. One set of assay cultures was prepared by the standard method at 30,635 cells/well and incubated without thrombin. 13.3 h later, a second group of assay cultures was prepared from a parallel stock culture at a density of 30,651 cells/well. At this time, 266 ng of [^{125}I]-thrombin was added to each of the wells of both groups. At various times after thrombin addition, cultures from both groups were removed and processed to determine specific and nonspecific enzyme binding and cell numbers. At saturation, cells receiving [^{125}I]thrombin at subculture showed about two times the specific binding of those exposed to this enzyme 13.3 h after plating (Fig. 3). These findings are in general agreement with those presented in Fig. 2*a*. In addition, this experiment indicates that the rate at which maximal binding was achieved in each group was substantially different. It is also of interest that both levels of binding remained reasonably constant throughout the entire 29-h experiment.

Transition from High to Low Thrombin-binding States

The results presented to this point indicate that a dramatic reduction in thrombin-binding capacity of cells occurs within

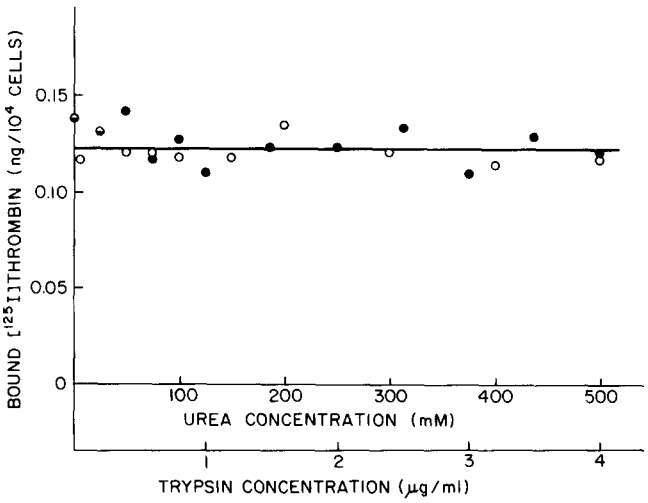


FIGURE 5 Net binding of [^{125}I]thrombin to fibroblasts after pretreatments with trypsin or urea. Cells were plated in 10% FCS media and allowed to grow for 4 d. At that time the medium was removed, the monolayers washed with pretreatment buffer (one part 0.025 M Tris, 0.125 M NaCl to one part PSD, with BSA to a final concentration of 0.05%, pH 7.48) and pretreatment buffer with either trypsin (Worthington Biochemical Corp., Freehold, N. J.; 3 × crystallized) or urea (American Chemical Society grade; Fisher Scientific Co., Fair Lawn, N. J.) was added. Each group of cultures was incubated at room temperature for 5 min and observed microscopically during this period. After this incubation, the pretreatment solution was removed, each culture washed, and binding medium (3% FCS) containing 4.56×10^{-11} mol/ml of [^{125}I]thrombin added. 16 h later, cultures that contained ~60,000 cells each were harvested as described in Materials and Methods, and the associated radioactivity was determined. The concentration of trypsin and urea were selected so that an estimated 5–10% of the cells in each well would show slight rounding at the highest concentration of each pretreatment material. ○, Urea concentration; ●, trypsin concentration.

a few hours of subculture. During this period, some process or cellular change occurs that appears to reduce the number of available thrombin receptors. Experiments were conducted to determine the time required for cells to make this transition to a lower thrombin-binding capacity after subculture. Assay cells were prepared in the standard way. To one group of cultures [^{125}I]thrombin was added at this time. Other cultures received the enzyme at the indicated times after subculture. Each set of cultures was harvested 16.25 h after the addition of thrombin. The data in Fig. 4 indicate that by ~80 min after subculture a complete transition to the lower binding state had occurred. Other experiments have indicated that this period is lengthened at higher enzyme concentrations. Nonspecific binding did not change as specific binding transitioned from higher to lower levels. These findings indicate that cellular changes occur very

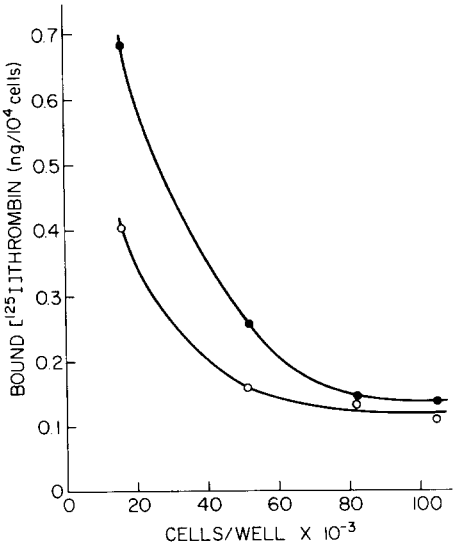


FIGURE 6 Net thrombin binding as a function of cell density. ●, Cells receiving [^{125}I]thrombin at subculture (0.572 μg/ml). ○, Cells receiving [^{125}I]thrombin 4 h after subculture (0.572 μg/ml). Nonspecific determinations were made for each group at every cell density, using 18 U of hirudin/well. These values were identical for each experimental group and have been subtracted from the gross binding values. Nonspecific binding averaged 16.7 ± 2.0% of the gross for each sample receiving enzymes at subculture and 12.2 ± 1.1% for those samples treated 4 h later with [^{125}I]thrombin.

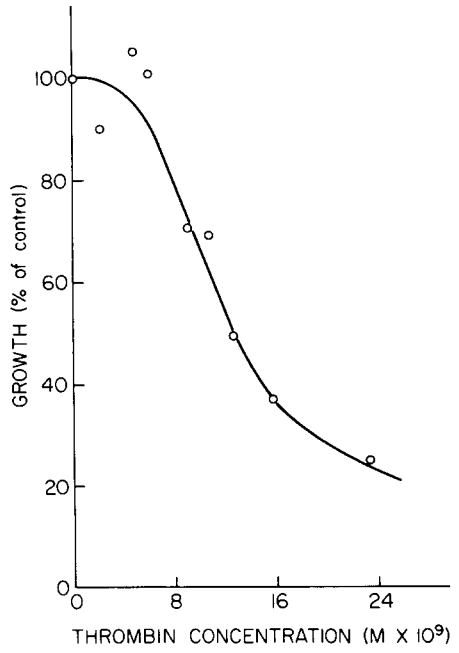


FIGURE 7 Growth inhibition in fibroblasts receiving thrombin at the time of subculture as a function of enzyme concentration. Cells were plated in 2.7% FCS medium containing various amounts of thrombin at an initial density of 22,001 cells/well. 48 h later, cultures were harvested, and cell numbers were determined. Controls without thrombin reached a density of 37,140 cells/well. Growth of test samples is expressed as percent of control. These thrombin concentrations are stimulatory for HF grown under the same experimental conditions if added 24 h after subculture (10).

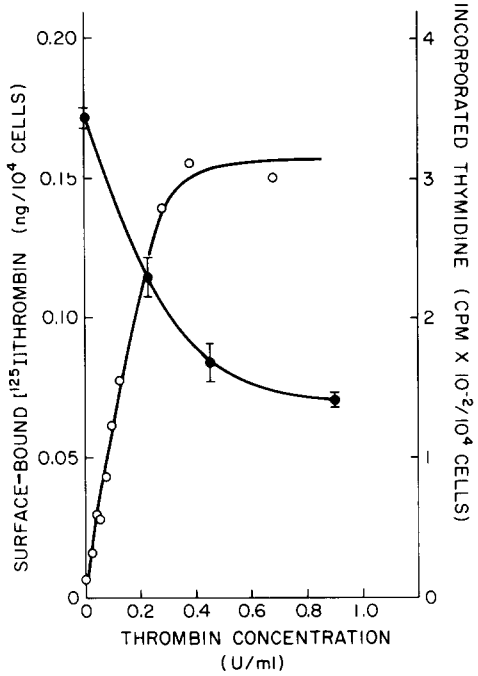
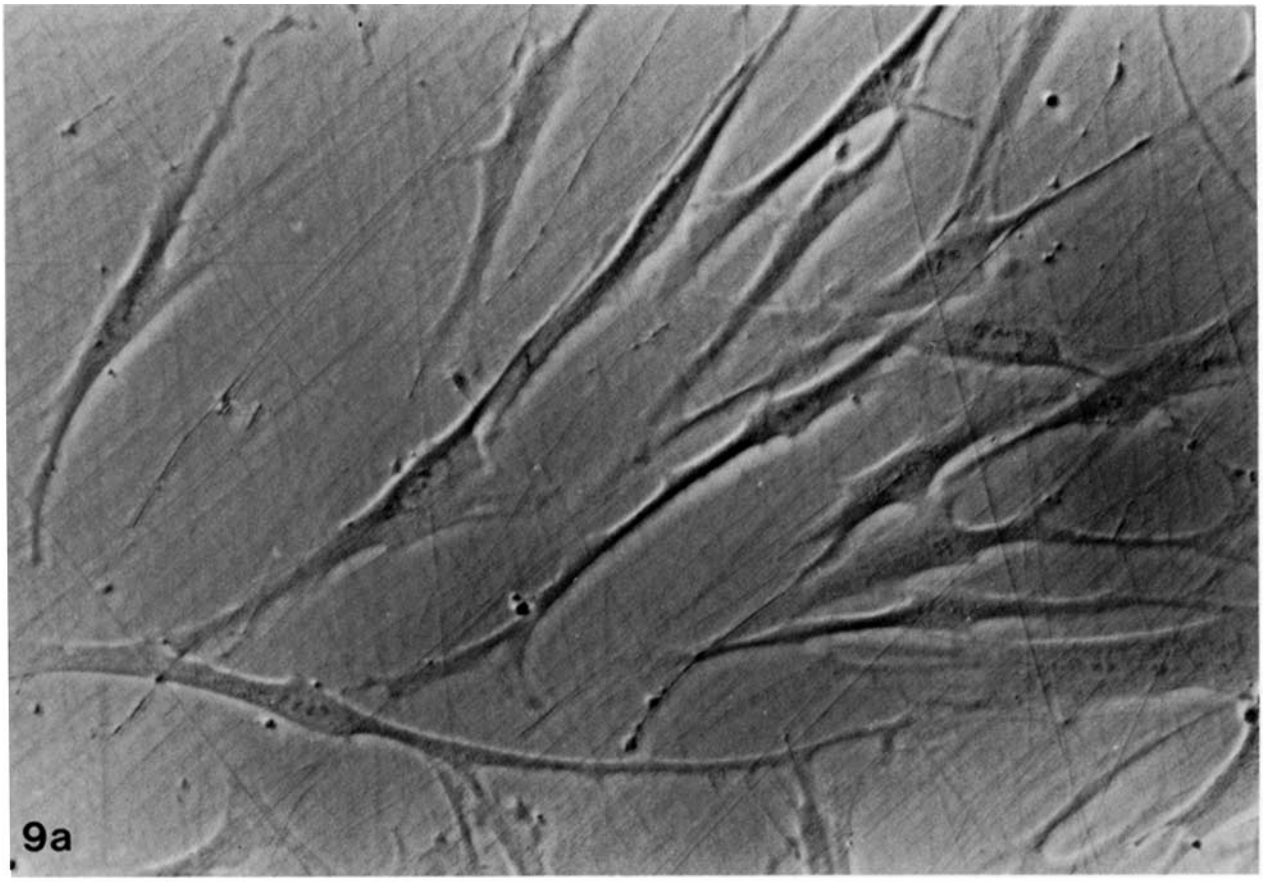


FIGURE 8 The relationship between binding and DNA synthesis in fibroblasts plated in serum-free medium containing thrombin. HF from a trypsinized stock culture were suspended in 20 ml of serum-free MEM containing 0.1% BSA, and centrifuged, and the medium was aspirated. This step was repeated four more times to ensure complete separation of trypsin and residual serum from the cell pellet. We have observed that even trace amounts of trypsin will inhibit cell spreading in the absence of serum. Fig. 9 confirms that cytoplasmic spreading was complete, suggesting that virtually all trypsin was removed by this washing procedure. In addition, this observation suggests that the cell pellet was essentially free of residual serum. After the final centrifugation cells were resuspended in serum-free MEM containing 0.1% BSA, thrombin (or [^{125}I]thrombin), and serum components below 10,000 daltons, and plated into assay wells. This serum fraction was prepared by ultrafiltration of whole FCS and was added to cultures at a concentration equivalent to that found in 3% FCS medium. Separate experiments indicated that this fraction did not contain the serum component responsible for thrombin-induced growth inhibition (the thrombin-sensitive component is >200,000 daltons). Experiments performed in the absence of this 10,000-dalton fraction yielded equivalent results as presented here, but the overall levels of DNA synthesis were lower. 17 h after subculture, each DNA synthesis culture received 5 μCi of [^{3}H]thymidine. Incubation was terminated 5 h 35 min later, and samples were processed as described in Materials and Methods. At 19 h 40 min after subculture, the binding incubation was terminated and the amount of ^{125}I radioactivity determined. In this experiment, the specific activity of thrombin was 3,100 U/mg. ○, Surface-bound [^{125}I]thrombin; ●, incorporated thymidine.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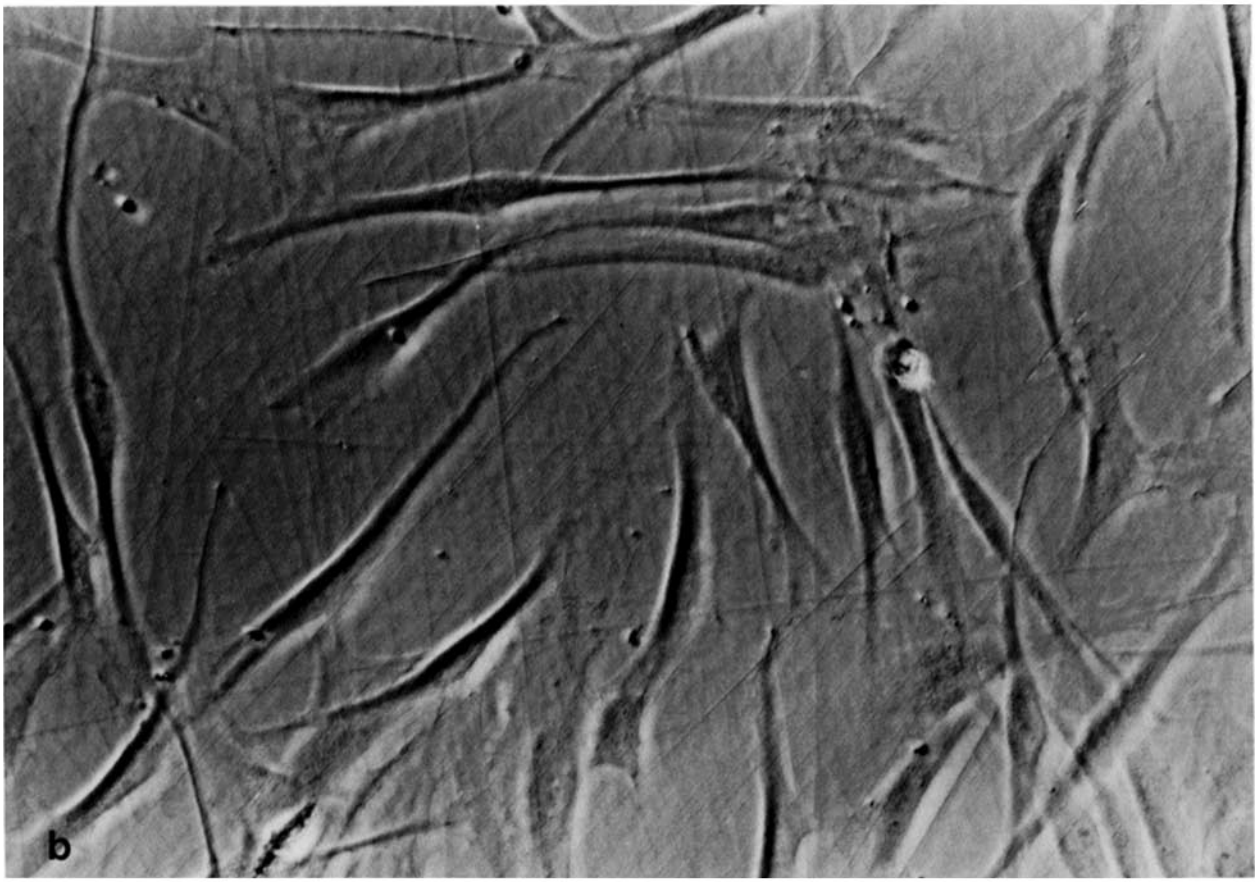
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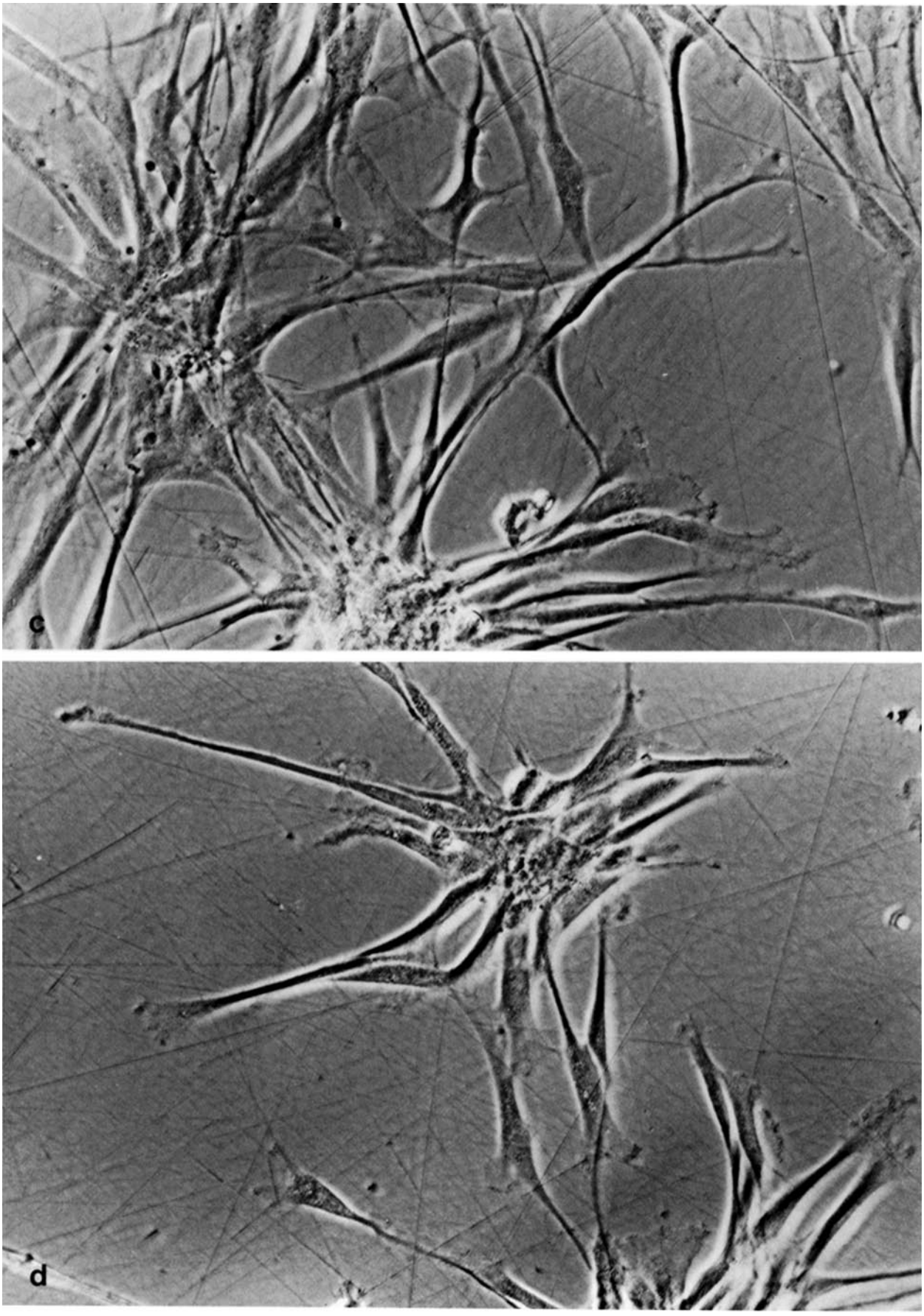
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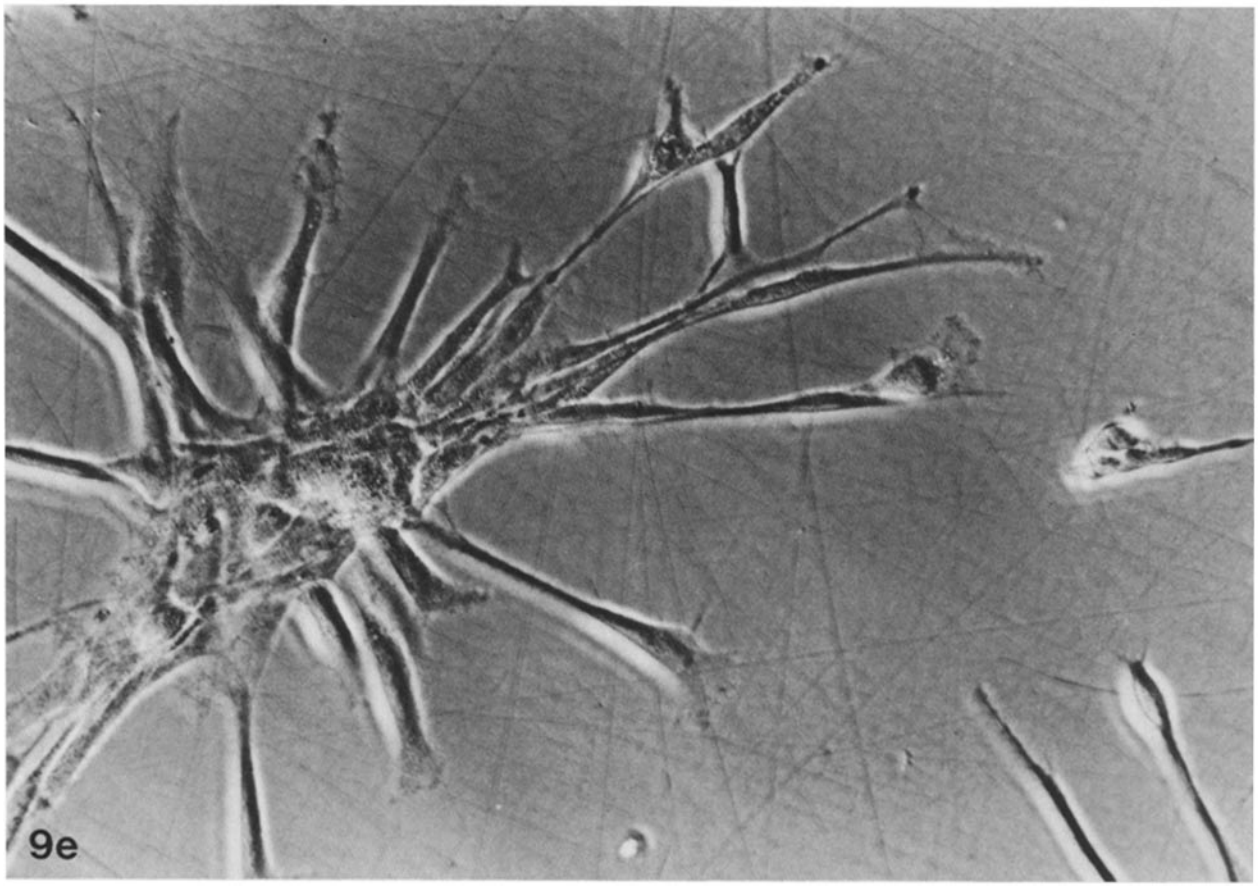

FIGURE 9 Appearance of cells plated in serum-free medium containing thrombin. The photographs presented here are of the cultures used for measurement of DNA in the previous figure. Binding cultures appeared the same at equivalent enzyme concentrations. Photographs were taken with a Zeiss Photomicroscope III (phase contrast), using Kodak Panatonic X film. A photograph of a culture containing 3% FCS medium at the same cell density is included for comparison. (*a*) Cells plated in 3% FCS medium. (*b*) Control cells of Fig. 8 (no thrombin). (*c*) Cells with 0.225 U/ml thrombin. (*d*) Cells with 0.450 U/ml thrombin. (*e*) Cells with 0.900 U/ml thrombin. (*a–e*) × 200.

rapidly after subculture that reduce the number of specific [^{125}I]thrombin receptors available for binding.

Mild Treatment of the Cell Surface by Trypsin and Urea

It seems probable from the data presented that fibroblasts possess cryptic thrombin-binding receptors that become exposed during the subculture process. If this is true, then limited removal of cell surface components might also increase the observed binding of thrombin by revealing hidden receptors. To test this hypothesis, established cultures were pretreated with either trypsin or urea and the binding of [^{125}I]thrombin was measured. These two agents were chosen because they are chemically dissimilar and act upon the cell surface in totally different ways. Fig. 5 shows the results of such an experiment. From these data it is obvious that neither type of pretreatment caused any significant increase in cell-associated [^{125}I]thrombin. Under these conditions, no visible alteration in cellular morphology was observed except at the highest concentration of each agent. In those samples an estimated 5–10% of the cells showed some slight degree of rounding during the 5-min pretreatment. At higher concentrations of each material (data not shown), cell rounding increased and so did specific enzyme binding. Unfortunately, this effect was difficult to quantitate because many rounded cells were lost during removal of the pretreatment agent and the subsequent monolayer wash. From the data presented it is evident that a mild, nondisrupting treatment of the cell surface, under these conditions, is not sufficient to expose cryptic thrombin receptors.

Thrombin Binding as a Function of Cell Density

Throughout this study, we observed that the amount of [^{125}I]thrombin bound per cell seemed to be related in some way to the culture density employed. Experiments were conducted to investigate this observation. Fig. 6 indicates that [^{125}I]thrombin binding was inversely related to cell density and varied severalfold for both test groups over the range of densities employed. These data indicate that HF undergo a second form of receptor modulation related to culture density. It is of interest that both freshly plated as well as established cells showed this binding sensitivity.

Biological Effects of Thrombin Addition at Subculture—Relationship to Binding

Fig. 7 shows the dose-dependent ability of thrombin to inhibit growth in freshly subcultured cells. Previous work has shown that DNA synthesis is also inhibited under these conditions and that cellular morphology is greatly altered compared to controls without thrombin (10). These effects were found to be at least partially mediated through thrombin-

induced serum changes.

To more directly assess thrombin's effects upon HF growth, we conducted experiments in serum-free medium. With only a small amount of BSA as the protein supplement, these cells attach, spread, and appear morphologically indentical to cells incubated with serum (see Fig. 9). Their viability remains high for at least 48 h, and they will synthesize DNA if supplemented with those components of serum below 10,000 daltons. This fraction is known to contain many low molecular weight growth-stimulating agents but does not contain the thrombin-sensitive element responsible for growth inhibition (W. M. Hall and P. Ganguly, manuscript in preparation). This fraction was used in our experiments to stimulate DNA synthesis in the absence of whole serum. Fig. 8 is a representative example of thrombin's effect upon this parameter when added at the time of subculture. This figure also shows the specific cell surface binding of [^{125}I]thrombin under the same experimental conditions. It is clear from these data that higher levels of thrombin binding produced a dose-dependent inhibition of DNA synthesis that was accompanied by an alteration in cellular morphology (Fig. 9). Samples receiving hirudin in addition to enzyme showed control levels of DNA synthesis and unaltered morphology (data not presented). It is also of interest that HF in the absence of serum formed tight clusters or "islands" in response to thrombin. In the presence of FCS, this clustering occurred to a lesser extent (10), but the reduced cytoplasmic spreading exhibited by individual cells was produced under both sets of experimental conditions. Both inhibition of DNA synthesis and altered morphology appear to be the direct result of a thrombin-cell interaction and suggest that growth inhibition in serum medium may be partially mediated by high levels of thrombin binding at the cell surface.

To further investigate this possibility, we measured DNA synthesis as a function of the time of thrombin addition after subculture in medium containing serum. The binding of thrombin under these conditions is shown in Fig. 4. We reasoned that if cellular binding were a contributing factor to growth inhibition produced by thrombin in the presence of serum, then some correlation should be seen in the levels of DNA synthesized. Fig. 10 shows that such a correlation was observed. A return to control levels of DNA synthesis was found to be dependent upon the thrombin concentration used as well as upon the times of enzyme addition and to be generally compatible with enzyme binding. From these data, it appears that direct cellular enzyme association may also contribute to growth inhibition in the presence of serum.

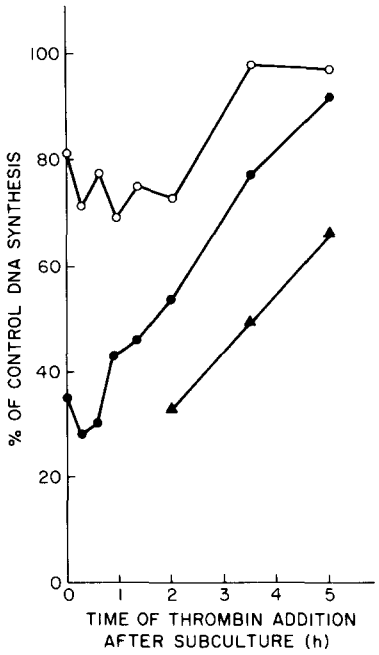

FIGURE 10 DNA synthesis in fibroblasts as a function of time of thrombin addition after subculture. At 21.75 h after subculture, [^{3}H]thymidine was added to cultures at a final concentration of 8 μCi/ml. Cells were incubated for 2.5 h, and the amount of acid-insoluble ^{3}H radioactivity was determined. Thrombin added at the designated times had the following final concentrations: ○, 1.9 U/ml; ●, 3.8 U/ml; ▲, 9 U/ml.

DISCUSSION

Previous work from this laboratory has indicated that thrombin's influence on cell growth can be negative as well as positive (9, 10). Fibroblasts incubated with thrombin at subculture showed inhibited growth and altered morphology, whereas those receiving enzyme several hours after plating displayed growth stimulation typical of human cells (17, 18). In this work, the specific binding of thrombin to HF under both sets of experimental conditions was studied. The data presented indicate a major quantitative difference in the amount of thrombin that becomes cell associated in the two groups and suggest that cryptic receptors may account for this phenomenon.

Cells exposed to [^{125}I]thrombin at subculture (Fig. 2*a*) specifically bound more enzyme than parallel cultures treated in the same way 24 h later. Double reciprocal plot analysis of the surface binding data (Fig. 2*c*; internalized thrombin was not included in calculations of K_d) showed a similar affinity for both groups (1.5×10^{-8} M). From this evidence, it seems likely that the differential binding observed was probably attributable to a difference in receptor number for the two sets rather than to a change in affinity. It should be noted that a true state of equilibrium binding may not exist in fibroblasts because much of the surface enzyme is internalized. This is a problem encountered by many agents that stimulate growth in fibroblasts through surface binding. Because both experimental cell groups in Fig. 2*c* internalized thrombin at ratios proportional to the amount of surface-bound enzyme, a direct comparison of their K_ds would appear to be valid. Fig. 2*c* indicates that cells receiving [^{125}I]thrombin at subculture bound ~2.9×10^5 molecules of enzyme each, whereas those treated 24 h after subculture bound 1.4×10^5 molecules/cell. This relative difference can be seen in most figures.

The kinetic data presented in Fig. 3 indicate that cells incubated with [^{125}I]thrombin at subculture not only bound about twice the enzyme as established cultures but reached this level much more rapidly. This observation suggests that thrombin receptors may have been insensitive to subculture trypsin. Under these conditions, enzyme-receptor association might have occurred in the relative absence of many normally present trypsin-labile membrane components. In this altered state, rapid enzyme binding may have been facilitated.

Fig. 4 shows that the number of available [^{125}I]thrombin receptors quickly decreased after subculture. Within 2 h the ability of thrombin to become cell-associated dropped to about half the level observed in cells receiving enzyme at subculture. During this period, cell shape progressed from a fully spherical to a partially stretched and spread configuration. This time-dependent reduction in binding could be the result of a rearrangement or masking of receptors. Carney and Cunningham

(2) have suggested that [^{125}I]thrombin receptors on mouse embryo cells are masked or shielded in some way by serum components. Studies using different serum concentrations including information on surface vs. internalized and/or degraded enzyme would be helpful in a further analysis of the phenomena. Although the nature of this process is obscure, it is apparent that new receptors become available during subculture.

Mildly disrupting treatments of the cell surface were insufficient to reveal cryptic receptors (Fig. 5). Only when cellular morphology was altered, i.e., at subculture, was any increase in binding observed. This suggests that binding may be related to cell shape; i.e., the degree of rounding exhibited by a cell may be one of the factors that determine its ability to bind thrombin. Recent work from several laboratories has suggested that this parameter is directly related to cellular growth and function in several cell lines (5, 7, 8). Cultures maintained on a variety of growth substrates exhibited different morphologies and rates of proliferation. Cell shape may therefore determine the specific binding of critical serum components necessary to promote cell attachment and spreading. These views are strengthened by the finding that binding of thrombin and culture density were inversely related (Fig. 6). At lower densities, where cells appeared long and well spread, binding was higher than in confluent cultures where cells were noticeably thinner and taller. That both established cultures, as well as those receiving thrombin at subculture, displayed this density-dependent binding is consistent with this hypothesis. It may also be significant that the relative difference in binding between the two groups decreased at higher densities where similar morphology was observed.

In a previous communication (10), it was established that pre-exposure of serum medium to thrombin is sufficient to produce growth inhibition as well as morphological alteration in HF. The results of the present work show that elevated binding can also produce these negative effects on DNA synthesis and cellular structure. Thrombin-induced growth inhibition would therefore appear to result from a combination of these two factors. Fibronectin is known to be a substrate for thrombin (14), and some cell lines apparently require the serum form of this molecule for a short time after plating (16). Proteolysis of this molecule either in serum or at the cell surface might provide the basis for the altered morphologies we have observed. In the absence of functional fibronectin, freshly subcultured cells might be incapable of achieving a physical structure compatible with further growth. Although the hydrolysis of fibronectin by thrombin is known to be a slow reaction (14), it should be pointed out that cells were exposed to enzyme for a number of hours under our experimental conditions. This hypothesis reconciles both of the observed ways in which thrombin inhibits growth in HF and is consistent with the relationship between cell shape and growth reported by Folkman and Moscona (5).

The purpose of cryptic thrombin receptors at the surface of HF is not known. After injury in vivo these sites may become exposed on rounded or morphologically altered cells, which may facilitate blood coagulation by localizing thrombin to areas of cellular disruption. The ability of fibroblasts to retract fibrin (15) may benefit from higher levels of thrombin binding. Finally, the ability of a cell or its surroundings to alter the number of thrombin receptors may influence the cell's rate of growth. A system of this type may impart considerable flexibility to a cell population at the site of an injury where multiple cell functions are required.

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