Platelet Effects on Tissue Factor and Fibrinolytic Inhibition of Cultured Human Fibroblasts and Vascular Cells

By Paulette E. Smariga and James R. Maynard

Platelets stimulate tissue factor, the initiator of the extrinsic coagulation pathway, and increase fibrinolytic inhibition in fibroblasts grown in vitro. Cellular tissue factor increases an average of 2.8-fold over the control levels after a 6-hr incubation with platelets, and no activity is present in the media. Fibrinolytic inhibition is stimulated in both the fibroblasts and their media in the presence of platelets and accumulates throughout a 24-hr incubation. Neither leukocytes nor erythrocytes stimulate these changes. Both tissue factor and fibrinolytic inhibition increases are dependent on platelet concentration and are blocked by inhibitors of RNA or protein synthesis. Control smooth muscle cells have higher tissue factor and fibrinolytic inhibition than fibroblasts, but their response to the presence of platelets is similar. Confluent monolayers of endothelial cells have very low levels of tissue factor that are not altered by the presence of platelets. However, the ability of endothelial cells to inhibit fibrinolysis is enhanced by the presence of platelets. The fraction that stimulates tissue factor and fibrinolytic inhibition is distinct from platelet-derived growth factor and from the fraction that enhances leukocyte tissue factor. It is associated with an insoluble, nonmitogenic fraction that is not inactivated by phospholipase C, or diisopropylfluorophosphate, nor is it chloroform:methanol extractable. Platelets are a physiologic modulator for both cellular tissue factor and the fibrinolytic system in vitro.

CELLULAR ENZYMES and cofactors are potent initiators of several competing processes which, when balanced, result in normal hemostasis. Limiting the expression of these initiators is therefore important in controlling hemostasis. Both coagulation and fibrinolysis initiators are normal cellular constituents of cells grown in vitro, so these cells can be used to study hemostatic regulatory processes. The level of cellular tissue factor, the initiator of the extrinsic coagulation system, varies in response to changes in the growth state and can be further influenced by the presence of exogenous drugs. Cellular constituents can also influence fibrinolysis, since both plasminogen activators, the initiator of fibrinolysis, and fibrinolytic inhibitors are present. The ability of in vitro cells to affect fibrinolysis is also not constant but varies with changes in the growth state and in the presence of drugs. Although the levels of both tissue factor and several factors that affect fibrinolysis do vary, possible physiologic stimuli for these changes have not been identified. The present studies examine the possibility that platelets play such a regulatory role for both of these activities.

MATERIALS AND METHODS

Materials

N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES), type III bovine trypsin, type IV bovine fibrinogen, bovine thrombin (2000U/mg), cephalin, endotoxin (E. coli 026B6), phospholipase C (C. perfringens), phosphatidyl choline, Russell’s viper venom, cycloheximide, actinomycin D, and Ficol-Hyphaque were purchased from Sigma Chemical Company, St. Louis, Mo. Diisopropylfluorophosphate (DFP) and dimethylaminocinnamaldehyde were obtained from Aldrich Chemical Company, Milwaukee, Wisc. Other chemicals were Mallincrodt analytical grade. Bovine factor X, the generous gift of Dr. Sidonie Silverberg, Stony Brook, N.Y. Rabbit brain thromboplastin, urokinase substrate S-2444, and plasmin substrate S-2251 were obtained from Ortho Diagnostics, Raritan, N.J.

Stock urokinase solutions were prepared from human urine urokinase (Sigma) or Abbokinase (Abbott Pharmaceutical Company, Chicago, Ill.). Plasminogen was prepared by affinity chromatography from human plasma. Plasminogen-free fibrinogen was prepared by the method of Mosesson. Fetal bovine serum, nonessential amino acid supplement (100 x concentrate), trypan blue, medium 199 with Earle’s salts, glutamine, streptomycin, penicillin, and Puck’s saline A were products of Gibco, Grand Island, N.Y. Puck’s saline A was supplemented with 20 mM HEPES, pH 7.4, in all experiments. Bovine plasma was obtained from Pel-Freez Biologicals, Rogers, Ark. Endothelial growth substance and platelet-derived growth factor were obtained from Collaborative Research, Waltham, Mass. Rabbit antiserum to human factor VIII-associated protein was obtained from Calbiochem, La Jolla, Calif. Goat-FITC anti-rabbit IgG was the product of Miles Laboratories, Elkhart, Ind.

Plastic Petri dishes and MultiWell dishes were products of Falcon Plastics, Division of Bioquest, Oxnard, Calif. Sterile 0.2 µm filters were products of the Nalge Company, Rochester, N.Y.

Sodium 125I-iodide and 6-3H-thymidine were obtained from New England Nuclear, Boston, Mass.

Platelet Preparation

Fresh platelets were obtained from 80 ml of blood drawn by venipuncture from volunteers who had not taken aspirin for at least 10 days prior to donation. Blood was collected into plastic tubes and anticoagulated with 1/9 volume of 3.8% (w/v) sodium citrate. Platelet-rich plasma was centrifuged at 1000 g for 20 min and the pellet was then washed by the method of Miller et al. All washings were carried out at 4°C.

Expired platelet concentrates were obtained within 3 days of expiration from the John Elliot Blood Bank, Miami, Fla. These platelet concentrates were washed by the method of Kohler and Lipton and stored in 4 ml of 0.08M NaCl, 0.01M sodium bicarbonate. Platelet concentrates are not. From www.bloodjournal.org by guest on September 23, 2017. For personal use only.
phosphate buffer, pH 7.4, per unit of original whole blood at −70°C.

**Cell Culture Techniques**

Human foreskin fibroblasts were grown in Earle’s medium 199 containing 10% fetal bovine serum and supplemented with glutamine (2.92 mg/liter). Human endothelial and smooth muscle cells were prepared from fresh umbilical cords by the methods of Jaffe et al. The endothelial cells were grown in medium 199 containing 20% fetal bovine and 5% human type AB serum or by inclusion of 200 μg/ml endothelial growth substance in medium 199 supplemented with 20% fetal bovine serum. All cells had the morphological characteristics previously described for the respective cell type, and endothelial cells had factor VIII antigens, as determined by immunofluorescence.

**Experimental Protocol**

Prior to an experiment, cells were subcultured into fresh complete serum-containing medium at a concentration of 2 × 10^7/35-mm Petri dish. The cells were allowed to grow to at least 90% confluence before being used in an experiment.

At the beginning of an experiment, the growth medium was removed from a confluent monolayer, the cells were washed with saline A, and 1 ml of serum-free medium 199 with or without additions as indicated in the text and figure legends was added to each 35-mm dish. The cells were then incubated at 37°C in a CO2 incubator. At subsequent times, the incubation media were removed, centrifuged to remove any debris, and stored at −70°C in plastic tubes. These growth media were subsequently assayed for both fibrinolytic and fibrinolytic inhibitory activities (see below).

After incubation and removal of medium, the remaining cell monolayer was washed once and then scraped into 2 ml of saline A using a rubber policeman. The cell suspension was centrifuged at 1000 g for 10 min at room temperature (RT). Each cell pellet was then osmotically shocked in 1 ml of distilled water, vortexed, and stored at −70°C in a plastic tube. The cell pellets were freeze-thawed 3 times before assay for tissue factor, fibrinolytic, and fibrinolytic inhibitory activities (see below).

All time points are the average of at least duplicate assays of duplicate dishes for each experiment. The cells from a third dish incubated under identical conditions to each treatment were suspended with 0.02% trypsin and the viable cell count using trypsin blue exclusion was determined. Cell viability was also assessed by measuring the loss of 3H-thymidine from prelabeled cells. Cells treated with trypsin could not be assayed, since trypsin interfered with both the coagulant and fibrinolytic assays.

**Tissue Factor Procoagulant Assay**

A one-stage coagulant assay was used to measure tissue factor activity of freeze-thawed cell suspensions. After disruption, the fibroblast or smooth muscle cell suspensions were diluted between 1:4 and 1:10, then 100 μl was added to 100 μl of 25 mM CaCl2 in saline A at 37°C. The clotting reaction was started by the addition of 100 μl of bovine plasma that contained 4 μl of cephalin. Endothelial cell suspensions and media were assayed without dilution.

Units of activity, equivalent to EDTA brain units, were calculated from a standard curve prepared using rabbit brain thromboplastin. The thromboplastin and the cell suspensions gave identical dilution curves. Each dish was assayed at least in quadruplicate. The variation between the calculated activity for duplicate sample dishes was less than 10%. This assay gave identical results (within assay error) when compared with a two-stage assay previously described.

Specificity was determined by assaying undiluted samples using bovine plasma that had been incubated at RT for 3 hr with 5 mM DFP to remove factor VII. The DFP-treated plasma gave identical dilution curves with either thrombin, purified factor X, or Russell’s viper venom when compared to nontreated bovine plasma. Therefore, the extrinsic coagulation pathway factors other than factor VII were not altered by the DFP treatment. For samples ± platelets, less than 100 U/10^9 cells was measured in this factor-VII-deficient plasma. This activity showed no increase during the incubations.

**Fibrinolytic Inhibition**

Fibrinolytic inhibition was measured by one of two methods which gave identical results. First, the release of fibrin degradation peptides from 125I-labeled fibrin-coated wells was measured. Fibrinogen was labeled with 125I using the method of Hawker and Hawker. MultiWell plates containing 125I-labeled fibrin at 100,000 cpm/well were prepared as described by Unkeless et al. Each assay contained 4 μg of human plasminogen, 50 μl of media or cell suspension, 0.5 U of urokinase, and 0.1M Tris-HCl buffer at pH 8.1, in a final volume of 500 μl. Samples of 100 μl were counted at two time points to insure assay linearity. Results were calculated by comparison to a dilution curve of urokinase run simultaneously. All cell extracts or media were assayed in duplicate wells for each incubation point. The variation between the original duplicate incubations was 10% or less.

The urokinase concentration was determined from the initial rate of hydrolysis of 0.3 mM pyro-Glu-Gly-Arg-p-nitroanilide (S-2444) in 50 mM Tris, 38 mM NaCl, pH 8.8 at 37°C. Under these conditions, 25 Ploug U/ml produced a change in absorbance of 0.05/min at 405 nm. When adjusted to the same concentration, urokinase from Sigma or Abbott gave identical assay results.

Second, a coupled colorimetric assay utilizing the chromogenic substrate for plasmin, d-Val-Leu-Lys-p-nitroanilide (S-2251), was used. The standard assay contained up to 50 μl of sample plus 0.5 U of urokinase, 4 μg plasminogen, 0.3 mM substrate S-2251, and 0.1 M NaCl, 0.05M Tris, pH 7.4 in a final volume of 250 μl. The reaction was carried out for 60–90 min at 37°C and terminated by addition of 250 μl of cold 40% trichloroacetic acid, then centrifuged 1000 g for 10 min. To increase the assay sensitivity, 400 μl of the supernatant was transferred to tubes containing 200 μl of 8 mg/ml dimethylaminocinnamaldehyde in ethanol. The absorbance at 570 nm was determined after a 15-min incubation. At the levels used, all cell extracts or media samples showed background activity less than 0.05 U when measured in the absence of urokinase. Both fibrinolytic assays had an absolute dependence on plasminogen for the expression of urokinase activity.

**Leukocyte and Erythrocyte Preparation**

Leukocytes and erythrocytes were prepared from freshly drawn blood. Leukocytes were separated from a washed buffy coat on a Ficoll-Hypaque gradient. The lymphocyte/monocyte fraction was used and the granulocytes were discarded. The platelet contamination in these preparations was less than 20% of the leukocyte concentration. Washed erythrocytes from the same blood were prepared by the method of Cameron and Smariga. Platelet, erythrocyte, and leukocyte concentrations were determined using a Coulter Model S electronic counter and/or by standard hemocytometer counting methods.

**Phospholipid Treatments**

Phospholipase C treatment and chloroform:methanol extraction of platelets followed the methods of Niemetz and Marcus. The phospholipase cleaved both platelet phospholipids and phosphatidylcholine as determined by thin-layer chromatography.
Preparation of Platelet-Derived Growth Factor (PDGF)

Washed platelets were freeze-thawed, sonicated, heated to 100°C for 3 min and centrifuged at 7500 g for 30 min. The platelets were washed with 1M NaCl, and the combined supernatants were filtered through a 0.22-μm filter to prepare the crude PDGF. The remaining insoluble platelet fraction was resuspended in saline A and tested for its ability to stimulate cell division and to enhance both procoagulant and fibrinolytic inhibition.

RESULTS

Platelet Effects on Fibroblast Tissue Factor

Confluent monolayers of fibroblasts had measurable levels of tissue factor that showed only minimal changes when the cells were incubated in serum-free medium alone. The presence of platelets increased the peak level of activity approximately 2.8-fold 6 hr after the incubation began. All of the activity remained cell-associated, and no measurable tissue factor activity was found in the media (Fig. 1). The increase in cell-associated activity was concentration dependent in the range of 3.5–17.5 x 10⁷ platelets/35-mm dish. At higher platelet concentrations, no further enhancement was observed. Two methods confirmed that the procoagulant activity was indeed due to tissue factor. First, little activity could be measured in any of the samples in factor VII-deficient plasma. Second, when the samples were incubated for 3 hr with 5 mM DFP prior to being assayed, none of the procoagulant activity was lost and the same stimulation by platelets was observed for these samples as for samples not exposed to DFP. Although the serum-free medium was not adequate to support fibroblast growth and division, cell viability throughout the 24-hr incubation did not change in the presence or absence of platelets (Fig. 1). A second method confirmed that cell viability was not being altered by exposure to platelets. Fibroblasts prelabeled with 3H-thymidine lost only 2% ± 1% of their thymidine during the 24-hr incubation. There was no difference between fibroblasts incubated in the presence or absence of platelets.

Experiments similar to that shown in Fig. 1 have been carried out with fibroblasts isolated from 4 individuals. Basal levels for tissue factor between 500 and 1800 U/10⁶ fibroblasts after 6-hr incubation were observed. In spite of this variation, incubation of the fibroblast isolates with various platelet preparations for 6 hr increased cellular tissue factor a relatively constant 2.8 ± 0.6-fold (n = 24) when compared with control cells. Fibroblasts with high basal levels were stimulated equally as effectively as those with low basal levels. Paired observation statistics of each isolate at 6 hr showed samples plus platelets to be statistically different from control fibroblasts, p < 0.01. Fresh platelets or platelets obtained from expired concentrates were able to maximally stimulate tissue factor levels.

Platelets were left in contact with the fibroblasts throughout the experiment, and some platelets became detached to the cell monolayer. Since platelets have procoagulant activity, including the ability to enhance factor Xa activity, control experiments were performed to eliminate the possibility that the activity changes were due solely to the activity of platelets. After 5 x 10⁷ platelets/ml medium 199 were incubated alone for 3, 6, or 24 hr in a 35-mm dish, any adherent platelets were resuspended in the incubation medium. None of these suspensions had activity greater than the buffer blanks (Table 1). Although no activity was measurable in the platelet suspensions, it was possible that they might develop a latent activity during incubation which could then be expressed upon
Contact with fibroblasts. To test this possibility, platelets and fibroblasts were incubated separately and then combined after 3, 6, and 24 hr incubations. In each case, there was no increase in tissue factor above the level of fibroblasts incubated for the same time and not mixed with platelets (Table 1).

Platelet Effects on Fibroblast Fibrinolytic Inhibition

The fibroblasts and media shown in Fig. 1 were also tested for fibrinolytic inhibition. Throughout the incubation period, fibroblast control levels were increased in a concentration-dependent manner by platelets (Fig. 2A). Unlike tissue factor, this activity also increased in the media (Fig. 2B). This is apparently a general phenomenon, since medium from the 4 different fibroblast isolates inhibited fibrinolysis and the inhibition was increased in the presence of platelets. After a 24-hr incubation, 1 ml of medium from unstimulated fibroblasts inhibited 2.0 ± 0.9 U of urokinase, while the same cells exposed to platelets inhibited 8.1 ± 1.8 U (n = 24). The calculated stimulation, 4.7 ± 2.0-fold, was more variable in the fibrinolytic inhibition assay than in the tissue factor assay, but again, all isolates were stimulated and there was no trend toward lower stimulation in fibroblasts that had higher initial inhibitor(s) levels. Paired observation statistics of each isolate at 24 hr showed media samples plus platelets to be statistically different from controls, p < 0.01. Fresh or expired platelets both were able to stimulate maximum inhibition levels.

Platelets have been shown to have inhibitors directed against both plasmin and urokinase. Platelets alone were therefore tested for fibrinolytic inhibition. At the concentration employed in the present experiments, virtually no inhibition was observed (Table 1). At 10 × higher concentration, fibrinolytic inhibition was measurable, but it did not change upon incubation (data not shown).

Platelets and fibroblasts might interact to enhance the expression of a latent inhibitory activity within the platelets. This possibility was eliminated by incubating platelets and fibroblasts separately for 3, 6, or 24 hr and mixing them prior to assay. No increase in inhibition above that of control fibroblasts was observed (Table 1).

Effects of Blockers of RNA and Protein Synthesis

The increase in tissue factor in fibroblasts in the presence of platelets was completely blocked by either 4 μg/ml actinomycin or 50 μg/ml cycloheximide when the inhibitors were added at the same time as the platelets (Fig. 3A). These blockers had a similar effect on the levels of media fibrinolytic inhibition (Fig. 3B). Similar results were observed when the two blockers were added to fibroblasts after a 2-hr incubation with platelets. Instead of continuing to increase, tissue

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Tissue Factor (U/10^7 Cells*)</th>
<th>Fibronolytic Inhibition (U Inhibited/ml Media)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-5 min</td>
<td>3 hr</td>
</tr>
<tr>
<td>(A) Medium 199</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>(B) A + Platelets</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>(C) A + Fibroblasts</td>
<td>1.051 ± 46</td>
<td>1.078 ± 102</td>
</tr>
<tr>
<td>(D) B and C incubated together</td>
<td>1.065 ± 30</td>
<td>1.742 ± 323</td>
</tr>
<tr>
<td>(E) B and C mixed after incubation</td>
<td>1.072 ± 53</td>
<td>1.063 ± 79</td>
</tr>
</tbody>
</table>

Medium 199 (1 ml) ± platelets (5 × 10^5) was incubated for the indicated times in 35-mm sterile dishes with confluent monolayers of fibroblasts (7.0 ± 1.0 × 10^5) or dishes that had previously contained medium 199 ± 10% fetal bovine serum. Fibrinolytic inhibition of the media was measured by the colorimetric assay described in Materials and Methods. Data are the mean ± SD of triplicate experiments.

*A and B based on the entire contents of scraped dishes at the indicated time points.
factor activity remained near that of control fibroblasts with either substance (Fig. 3A). Actinomycin, added after 2 hr, resulted in a decrease in media fibrinolytic inhibition to levels slightly below the control values, while cycloheximide prevented the full increase in inhibition observed for fibroblasts exposed to platelets (Fig. 3B). When either actinomycin or cycloheximide was added to fibroblasts in the absence of platelets, both tissue factor and fibrinolytic inhibition levels were the same as control fibroblasts. Fibroblast-associated inhibition was also not elevated above control values when these agents were present. The blocked stimulation, therefore, was not due to an accumulation of inhibitor(s) in the cells (data not shown). Thus, new RNA and protein synthesis are required for the increased expression of fibroblast tissue factor and fibrinolytic inhibition in the presence of platelets.

Platelet Effects on Vascular Cells

The effect of platelets on smooth muscle and endothelial cells was studied. Smooth muscle cells had higher basal tissue factor levels than fibroblasts, however, the stimulation of this activity in the presence of platelets was less pronounced. Data from three different isolates showed a 2.2 ± 0.8-fold stimulation at 3 hr. A typical experiment for one isolate is shown in Fig. 4A. Even though control levels of media fibrinolytic inhibition from smooth muscle cells were higher than fibroblasts, they were readily stimulated by the presence of platelets (Fig. 4B). These vascular cells behave in a manner similar to the nonvascular fibroblasts showing a 2.6 ± 0.5-fold increase at 24 hr. Endothelial cell tissue factor differs in several respects from that of fibroblasts and smooth muscle cells. The basal tissue factor levels from three different isolates of endothelial cells were much lower than that of the other two cell types. In confluent monolayers, the activity was indistinguishable from blanks, and no increase was observed in the presence of platelets (Fig. 4A).

Human endothelial cells are able to inhibit fibrinolysis. Their inhibition was further enhanced 1.9 ±

3000
and fibrinolytic inhibition (\(\text{fib}^{+}\)) was stimulated by the presence of platelets. Addition of actinomycin (\(\text{Actom}^{+}\)) or cycloheximide (\(\text{Cyclh}^{+}\)) to platelets (Fig. 3B). When either actinomycin or cycloheximide was added after 2 hr, resulted in a further increase in activity. Control fibroblasts, not exposed to platelets showed little change in activity (\(\text{Control}^{+}\)). The fibrinolytic activity was measured using the coupled colorimetric assay. The fibroblast monolayer cell counts ranged from 7.8 to 8.1 \(\times 10^6\)/35-mm dish with >95% viability.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** The stimulatory effects of platelets on tissue factor and fibrinolytic inhibition can be blocked by RNA or protein synthesis inhibitors. (A) The presence of 5 \(\times 10^5\) platelets/dish (\(\text{P}^{+}\)) increased tissue factor activity. This increase was suppressed when either actinomycin (\(\text{Actom}^{+}\)) or cycloheximide (\(\text{Cyclh}^{+}\)) were added with the platelets. Addition of actinomycin (\(\text{Actom}^{+}\)) or cycloheximide (\(\text{Cyclh}^{+}\)) to platelets after a 2-hr incubation with platelets suppressed any further increase in activity. Control fibroblasts, not exposed to platelets showed little change in activity (\(\text{Control}^{+}\)). (B) Control medium fibrinolytic inhibition (\(\text{Fib}^{-}\)) was stimulated by the presence of platelets (\(\text{P}^{+}\)). The inhibition in medium from fibroblasts exposed to platelets was completely suppressed by actinomycin (\(\text{Actom}^{+}\)) and partially suppressed when added 2 hr after the start of the incubation with platelets (\(\text{Actom}^{-}\)). Fibroblasts exposed to cycloheximide plus platelets (\(\text{Cyclh}^{+}\)) developed inhibition similar to that of the control fibroblasts. When cycloheximide was added 2 hr after the start of the incubation with platelets, full expression of inhibition was suppressed (\(\text{Cyclh}^{+}\)). The fibrinolytic activity was measured using the coupled colorimetric assay. The fibroblast monolayer cell counts ranged from 7.8 to 8.1 \(\times 10^6\)/35-mm dish with >95% viability.

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** Comparison of the stimulatory effects of 8 \(\times 10^6\) platelets/dish on human umbilical vein endothelial cells, smooth muscle cells, and foreskin fibroblasts. (A) Control endothelial cells (\(\ldots\)) have very low levels of tissue factor (\(\text{TF}^{-}\)), which does not change in the presence of platelets (\(\text{P}^{+}\)). Control smooth muscle tissue factor (\(\text{SM}^{+}\)) is high and is transiently increased by the presence of platelets (\(\text{P}^{+}\)). Control fibroblast activity shows little change (\(\text{Fib}^{-}\)), as in the other figures, unless platelets are present (\(\text{P}^{+}\)). Fibrinolytic inhibition in the media (\(\text{Fib}^{-}\)) from control endothelial cells (\(\ldots\)) increased during the 24-hr incubation (\(\text{P}^{+}\)). The accumulation is enhanced by the presence of platelets (\(\text{P}^{+}\)). Control smooth muscle cells also accumulate high levels of inhibitor(s) in their media (\(\text{SM}^{+}\)). This accumulation is further stimulated by the presence of platelets (\(\text{P}^{+}\)). Fibroblasts, included for comparison, behave in a manner similar to that shown in the other figures. There is an increase in inhibition in the media (\(\text{Fib}^{-}\)), which is enhanced by the presence of platelets (\(\text{P}^{+}\)). The fibrinolytic activity of the media was determined using the coupled colorimetric assay. Each 35-mm dish of endothelial cells contained 2.9 \(\pm\) 0.2 \(\times 10^5\) cells; smooth muscle cell dishes contained 5.7 \(\pm\) 0.3 \(\times 10^5\) cells, and fibroblast dishes contained 8.1 \(\pm\) 0.2 \(\times 10^5\) cells. Cell viability was >95% for all cell types at all time points.
PLATELETS AFFECT FIBRINOLYSIS/TISSUE FACTOR

0.2-fold \((n = 3)\) by the presence of platelets. The results from one isolate are shown in Fig. 4B. In analogy with fibroblasts, both smooth muscle and endothelial cells had cell-associated fibrinolytic inhibition that increased with time and was stimulated by the presence of platelets (data not shown).

Effects of Leukocytes and Erythrocytes

The platelet preparations employed sometimes contained low concentrations of leukocytes. Leukocytes are known to interact with endothelial cells in culture\(^3\) and have tissue factor procoagulant activity.\(^3\) Leukocytes were tested to see if they were responsible for the observed activity changes. Leukocyte suspensions added at \(10^6\) the contaminating concentration found in the platelet preparation did not alter either tissue factor or fibrinolytic inhibition of fibroblast monolayers. Erythrocytes were added at \(10^6\) their concentration in the platelet preparation and were also without effect (Table 2).

Table 2. Comparison of Nonstimulatory and Stimulatory Materials on Fibroblast Tissue Factor and Fibrinolytic Inhibition

<table>
<thead>
<tr>
<th></th>
<th>Tissue Factor*</th>
<th>Fibrinolytic Inhibition†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Nonstimulatory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium 199</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Leukocytes ((5 \times 10^6)/\text{ml})</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Erythrocytes ((1.3 \times 10^6)/\text{ml})</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Chloroform:methanol-soluble fraction from (5 \times 10^7) platelets</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Endotoxin ((5 \mu\text{g}/\text{ml}))</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Platelet-poor plasma ((85 \mu\text{g}/\text{ml}))</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>PDGF ((34 \mu\text{g}/\text{ml}))</td>
<td>1.3 ± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td><strong>(B) Stimulatory</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets ((5 \times 10^7)/\text{ml})</td>
<td>3.2 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Chloroform:methanol-extracted platelets</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Phospholipase C-treated platelets</td>
<td>3.1 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Platelets + endotoxin ((5 \mu\text{g}/\text{ml}))</td>
<td>3.1 ± 0.1</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Insoluble fraction from PDGF ((34 \mu\text{g}/\text{ml}))</td>
<td>2.8 ± 0.6</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>DFP-treated platelets ((5 \times 10^7)/\text{ml})</td>
<td>3.2 ± 0.1</td>
<td>3.1 ± 0.2</td>
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</tbody>
</table>

* Value of tissue factor obtained after 6-hr incubation divided by the tissue factor of control fibroblasts after 6 hr.
† Value of fibrinolytic inhibition of media after 24-hr incubation divided by control fibroblast media inhibition at 24 hr. Inhibition levels were determined using the coupled colorimetric assay.

Data are the mean ± SD of 3 experiments.

The platelet-derived stimulatory material could be due to a plasma protein contamination in the platelet preparation. When platelet-poor plasma was added at the same protein concentration as the platelets \((85 \mu\text{g}/\text{ml})\), no stimulation of either tissue factor or fibrinolytic inhibition above control values was observed (Table 2). Platelets are known to bind the coagulation enzymes thrombin and factor Xa.\(^29\) Low concentrations of these or other serine proteases might be responsible for the platelet stimulatory effects. To test this possibility, platelets were pretreated for 3 hr with 10 mM DFP then incubated with fibroblasts. This treatment did not affect the stimulatory ability of the platelets (Table 2), so the stimulatory fraction from platelets does not appear to be a serine protease.

Platelets contain platelet-derived growth factor (PDGF).\(^35\) When 34 \(\mu\text{g}/\text{ml}\) of crude soluble PDGF, derived from \(5 \times 10^7\) platelets, was added to fibroblasts in medium containing 0.5% fetal bovine serum, cell multiplication was stimulated to an extent similar to that produced by 10% fetal calf serum. This PDGF preparation produced little change in either tissue factor or fibrinolytic inhibition (Table 2). The remaining insoluble fraction from \(5 \times 10^7\) platelets contained the same amount of protein as the extracted PDGF and was able to stimulate both tissue factor and media fibrinolytic inhibition (Table 2). In contrast to the PDGF fraction, it was unable to stimulate cell multiplication even in the presence of 0.5% fetal bovine serum.

DISCUSSION

Coagulation and fibrinolysis are the result of coupled enzymic processes that are initiated through the interaction of systemic cellular constituents with circulating zymogens. The sequential production of enzymes by these two systems allows them to respond dramatically to a stimulus, but also implies that care-
ful control is essential to elicit an appropriate response.

Platelets play a central role in a number of hemostatic processes. The present studies were undertaken to assess if platelets may also play a role in the control of tissue factor and the fibrinolytic system. Fibroblast-associated tissue factor increased when the cells were exposed to medium containing platelets, while no activity was observed in the media (Fig. 1). There was a simultaneous increase in fibrinolytic inhibition in both the fibroblasts and their media (Fig. 2). Even though control levels varied with different fibroblast isolates, all isolates tested were stimulated by platelets. These changes were specifically due to platelets rather than other two blood cells, leukocytes or erythrocytes (Table 2), and were not the result of fibroblast degeneration or death (Fig. 1).

Platelets remained in contact with the fibroblasts throughout the incubation period, so could be directly contributing to either activity. These possibilities have been ruled out, since at the concentrations necessary to stimulate fibroblast tissue factor, platelets did not contribute to the observed procoagulant activity. A direct platelet-derived fibrinolytic inhibition could be measured at higher platelet concentrations, but it neither increased upon incubation nor was it measurable at the concentrations of platelets necessary for fibroblast stimulation (Table 1). From these results, and the requirement for both new RNA and protein synthesis for the expression of activity (Fig. 3), it is concluded that both tissue factor and fibrinolytic inhibition increases are the result of platelet-stimulated de novo synthesis by fibroblasts and not from platelets alone.

Tissue factor of smooth muscle cells is high and is transiently increased by the presence of platelets, so this normal vascular cell type responds in a manner similar to fibroblasts. Endothelial cells, on the other hand, are unique since the tissue factor of confluent endothelial cells is very low and is only minimally altered by the presence of platelets (Fig. 4A). Fibrinolytic inhibitory activity of both the vascular cell types is pronounced and was further enhanced by the presence of platelets (Fig. 4B).

The present studies show that the material that increases tissue factor and fibrinolytic inhibition is distinct from two other previously described stimulatory materials. It was not chloroform:methanol soluble nor was it inactivated by phospholipase C (Table 2) so it does not appear to be the phospholipid fraction that stimulates leukocyte tissue factor. It also differs from soluble PDGF since it is associated with a nonmitogenic insoluble fraction (Table 2). These observations show that this stimulatory substance(s) is unique from these other important platelet-derived macromolecules. DFP did not impair the ability of platelets to stimulate the fibroblasts, therefore, a serine protease, such as surface-bound thrombin or factor Xa, is not the source of the stimulatory activity. Preliminary studies on the nature of the stimulatory fraction have raised the interesting question of how an insoluble platelet substance is able to influence cellular functions. Studies are in progress to purify the material from platelets and then to study its interaction with cells in culture to help answer this question.

In summary, platelets are capable of stimulating cellular tissue factor transiently while increasing the ability of both the cells and their media to inhibit fibrinolysis. If these effects also occur in vivo, then platelet interaction with systemic cells would lead to a more thrombogenic state by simultaneously increasing their procoagulant activity, while they also become more antifibrinolytic. Such an effect would be consistent with the other prothrombogenic properties previously reported for platelets.

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