Human Fibroblast Tissue Factor Is Inhibited by Lipoprotein-Associated Coagulation Inhibitor and Placental Anticoagulant Protein but not by Apolipoprotein A-II

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Studies of proteins that inhibit tissue factor activity have generally been conducted using either an extracted tissue homogenate ("thromboplastin") or tissue factor protein reconstituted into phospholipid vesicles rather than with tissue factor expressed in cell membranes (its physiological environment). In the present study, a human fibroblast cell strain was used to evaluate the effects of lipoprotein associated coagulation inhibitor (LACI), placental anticoagulant protein (PAP), and apolipoprotein A-II (apo A-II) on human tissue factor in cell membranes. LACI was tested from 7.8 to 500 pmol/L on fibroblasts cultured at cell densities ranging from 3,500 to 9,925 cells/well, and caused a progressive inhibition of tissue factor activity. PAP was tested from 3.9 nmol/L to 1 μmol/L at cell densities ranging from 4,500 to 15,400 cells/well and caused up to 83% inhibition of tissue factor activity. Inhibition by these proteins appeared to be influenced by cell density as well as whether the cells were intact or disrupted. Apo A-II, up to 1 μmol/L, did not inhibit the tissue factor activity of intact or disrupted fibroblasts at any cell density examined even though it did inhibit the activity of tissue factor in phospholipid vesicles. Of these inhibitors of tissue factor-dependent activation of factor X, LACI was the most effective in suppressing the generation of factor Xa activity. The effects obtained with apo A-II are clearly dependent on the nature of the tissue factor preparation with which it is tested. The disparity between the inhibitory effect of apo A-II on the activity of tissue factor reconstituted into lipid vesicles and the absence of effect on the activity of tissue factor remaining in cell membranes serves to reemphasize the necessity of reexamining results obtained with model systems using as nearly physiological reagents as possible.

TISSUE FACTOR is a membrane protein that initiates blood coagulation by serving as a cofactor for factor VIIa in the activation of factors IX and X.[1,2] Recently, interest in tissue factor-initiated coagulation has turned to the identification and characterization of physiologically important tissue factor inhibitors. Apolipoprotein A-II (apo A-II),[3] placental anticoagulant protein (PAP),[4-6] and lipoprotein-associated coagulation inhibitor (LACI, or extrinsic pathway inhibitor [EPI])[7,8] have all been reported to inhibit tissue factor activity. The experiments reported here were performed to establish whether these proteins were inhibitory toward tissue factor in cell membranes and their relative effectiveness for suppressing the generation of factor Xa activity. We report here the further characterization of the inhibitory activity of these proteins using tissue factor in nondisrupted (intact) and disrupted (freeze/thaw/sonicated) cultured human fibroblasts.

MATERIALS AND METHODS

Human factors X and VII were purified by established methods.[9] Bovine factors X, VII, and XII were provided by Drs A. Guha and Y. Nemerson (Mt Sinai Medical School, New York). Factor VII was activated by incubation with factor XIIa and 5 mmol/L calcium overnight in a glass tube at 25°C (protocol provided by Dr A. Guha).[10] These enzymes were stored at −20°C in tris/saline (0.05 mol/L tris, 0.1 mol/L NaCl, 0.02% NaN_3, pH 7.6) containing 50% glycerol and 5 mmol/L benzamidine.

Human plasma was obtained from Belle Bonfils Memorial Blood Center (Denver). Apo A-II was chromatographically purified from human plasma using phenyl-Sepharose.[11] The dialyzed sample obtained from the phenyl-sepharose column was applied to a PBE 94 column (24.5 cm × 1.5 cm) that was previously equilibrated with 0.02 mol/L histidine, 6 mol/L urea, pH 6. The apo A-II–rich fractions were pooled and applied to a column of factor Xa Affi-Gel to remove any residual LACI.[12] The resultant apo A-II was judged to be pure by silver staining (Bio-Rad kit, Bio-Rad, Richmond, CA) of the sample electrophoresed in a 12.5% polyacrylamide gel and by absence of effect by an anti-LACI antibody on the inhibition of tissue factor in phosphatidylserine/phosphatidylcholine (PS/PC) vesicles (30/70) by the apo A-II.

The purification of PAP was performed as described by Funakoshi et al[4] with the following modifications. The separation of PAP by DEAE-Sepharose was done by column chromatography instead of by batch adsorption. The PAP was eluted from the DEAE-sepharose and collected into fractions as described.[4] The fractions were assayed for their ability to inhibit tissue factor activity. The inhibitory fractions were pooled, made to 25% saturation with ammonium sulfate, and added to a phenyl-sepharose column (16.5 cm × 2.5 cm). After the sample was applied, the column was washed with 10 column volumes of 25% saturated ammonium sulfate and then eluted with a 0% to 70% propylene glycol gradient in tris/saline. Fractions were collected and assayed for their tissue factor inhibitory activity. Inhibitory fractions were pooled and dialyzed against 3 L of tris/saline, with four changes over 72 hours at 4°C. The pools were then concentrated by ultrafiltration on an Amicon YM-10 membrane (Amicon, Danvers, MA). A sample from each concentrated pool was analyzed on a 12.5% polyacrylamide gel under reducing and nonreducing conditions. The sample in which the predominant protein was 36,500 molecular weight (mol wt) was used as stock PAP. This pool also contained dimeric protein, which

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Submitted July 20, 1988; accepted November 2, 1988.

Supported in part by Grants No. HL 31408 and HL 34462, and RCDA HL 02072 from the National Institutes of Health.

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0006-4971/89/7304-0014$3.00/0

reduced to monomeric molecular weight as reported.[4] The PAP was apparently free of any LACI since an LACI-neutralizing rabbit antibody did not diminish the inhibitory activity of the PAP. LACI was purified as previously described.[12,13]

The cells used for these experiments were a human primary fibroblast cell strain, GM5756 (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ). Fibroblasts were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing penicillin (83 U/mL), streptomycin (83 μg/mL), and gentamicin (63 μg/mL), and supplemented with 5% NCTC 109 (Whittaker M. A. Bioproducts, Walkersville, MD). Cells were subcultured with trypsin-EDTA (Flow, McLean, VA), splitting each confluent culture in a 25 cm^2 T-flask into three flasks. Cells were maintained in a humidified atmosphere of 90% air and 10% CO_2.

For the determination of tissue factor activity in cells, the cells were subcultured into 96-well microtiter plates 24 hours before the tissue factor assay. Tissue factor activity was measured using a spectrophotometric assay and an automated ELISA reader as previously described.[14,15] Culture media was washed from the cells by gently rinsing each microtiter well twice with tris/saline. The cells were then covered with 40 μL of tris/saline per well containing 1 mg/mL of bovine serum albumin. For assays of disrupted cells, the culture plates were placed on a block of dry ice until the 40 μL of tris/saline within each microtiter well was frozen; the microplate was then removed from the dry ice and thawed. This freeze-thaw cycle was repeated twice. The microtiter plate was then covered with cellophane tape, the plate floated on water in a sonicating bath, and the microtiter plate was sonicated a total of eight times for ten seconds each, rotating the plate 90 degrees between each sonication. For assays of nondisrupted (intact) cells the freeze/thaw/sonication steps were omitted. Tissue factor activity in each microtiter well was determined by adding the following reagents, in order, to the 40 μL of tris/saline in each well: 20 μL of experimental inhibitor or buffer alone to give the appropriate final inhibitor concentrations; 20 μL of human factor VIIa (to give 100 pmol/L final assay concentration), 20 μL of human factor X (to give 49.4 nmol/L final assay concentration); 10 μL of 5 mmol/L Spectrozyme FXa (American Diagnostica, New York) and 50 μL of 25 mmol/L $CaCl_2$. In the assays the Spectrozyme FXa and $CaCl_2$ were combined and added together in 60 μL. Absorbance readings at 405 nm were recorded at one-minute intervals for 80 minutes.

The inhibitory activities of LACI reported in this manuscript are derived by averaging the tissue factor activity (change in absorbance per $minute^2$) during the first 20 minutes of the assay. This was done because the progress curves (absorbance ν $time^2$) from the LACI experiments had no linear portion from which to accurately derive a velocity, due to progressive factor Xa and tissue factor inhibition by LACI. Most assay reactions with LACI progressed to 0 absorbance units per $minute^2$ by the end of the 80-minute assay. Therefore, the inhibitory activities of LACI reported here are relative estimates.

The tissue factor activity at each cell density was determined in 16 replicate culture wells. Four culture wells were used for cell enumeration at each cell density. The time course experiments were conducted using 12 replicate wells at each time point. For all the inhibitor experiments, tissue factor activity was determined in 12 replicate culture wells at each cell density and inhibitor concentration. Cell number in the inhibitor experiments were determined in six replicate wells at each cell density.

Tissue factor activity at t = 0 in the time course experiment was determined by washing a sample of the trypsinized cells with tris/saline, centrifuging the cells in a 15 mL centrifuge tube at 180 g for ten minutes and resuspending the cells in tris/saline with 1 mg/mL bovine serum albumin at a cell concentration equivalent to the cell concentration subcultured into microtiter plates for tissue factor determination at later time points in the time-course experiment. Bovine factors X (100 nmol/L) and VIIa (1 nmol/L) were used for the cell density and time course experiments, and, to maintain a species-homologous assay system, human factors X and VIIa were used for the inhibitor experiments. For all the experiments, to achieve cell number reproducibility in each microtiter well, cell suspensions were delivered into each microtiter well with a P-200 pipetman.

Cell number was quantitated by hemocytometer counting using a Neubauer type chamber (American Scientific Products, McGaw Park, IL) as described by Absher.[16] Cells in 96-well microtiter plates were dispersed by adding 100 μL of trypsin-EDTA per well. A sample of this cell suspension was added to the Neubauer chamber and cells were counted using a phase contrast inverted microscope (Olympus). Cell viability was determined by trypan blue exclusion as described by Phillips.[17] Cells in 96-well microtiter plates were dispersed by adding 80 μL of trypsin-EDTA per well. To this, 20 μL of trypan blue (0.4%) was added per well. The cells were incubated with trypan blue for approximately ten minutes and then counted. The cell viability was determined and expressed as percent by dividing the nonstained cells by the total cells and multiplying by 100.

Dose-response curves of percent inhibition as a function of inhibitor concentration were fit to the data by an iterative least squares algorithm. The SIMP3 simplex program,[14] was used to fit the equation of a rectangular hyperbola, where the maximum inhibition was constrained to $\leq$100%.

RESULTS

Previous studies have demonstrated a transient increase in tissue factor activity in cells after subculturing.[18-20] Tissue factor activity was determined in the fibroblasts at various time intervals after trypsinization to establish an appropriate time for experiments with tissue factor inhibitors. Fibroblasts were subcultured into 96-well microtiter plates and, at predetermined times posttrypsinization, disrupted and intact cells were assayed for tissue factor activity. In agreement with previous work,[18,19] the fibroblasts underwent a "burst" of tissue factor activity following subculturing (Fig 1). The fibroblasts expressed a peak of tissue factor activity at about eight hours posttrypsinization in disrupted cells. The time course of tissue factor expression was similar at all three cell densities examined. The "burst" of tissue factor activity subsided after approximately 18 hours following subculturing. Extremely little tissue factor was expressed immediately following trypsinization as previously observed,[18,19] even in the disrupted cells, indicating that there is not a "protected" pool of tissue factor in these cells. The tissue factor activity per cell remained equivalent for each of the experimental cell densities for the first 18 hours, but as the burst of activity subsided, the tissue factor activity per cell was greatest in the sparse cultures and lowest in the dense cultures. In light of these findings subsequent experiments used fibroblasts at 24 to 28 hours after subculturing.

The cell density dependence of tissue factor activity was characterized in the fibroblasts by subculturing the cells at four cell densities in a 96-well microtiter plate. Intact and disrupted cells were assayed for tissue factor activity at 24 hours after subculturing. In agreement with previous work,[21] the fibroblast activation of factor X was dependent on added fVIIa. A monoclonal anti-tissue factor antibody (HTF1-7B8, 30 nmol/L)[22] completely eliminated factor X activa-

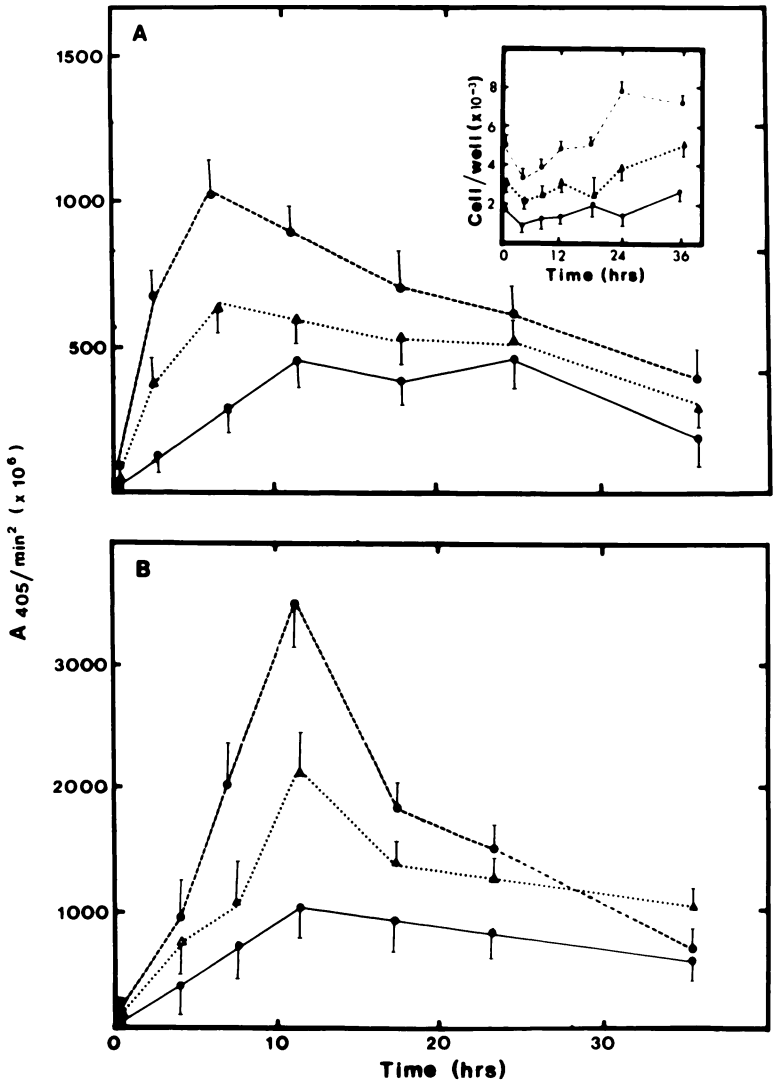

Fig 1. Tissue factor activity expressed in intact (A) and disrupted (B) fibroblasts following subculturing. Cells were seeded into microtiter wells at initial densities of 5,200 (--●--), 3,400 (···▲···), and 1,800 (—●—) cells/well and subsequently assayed for tissue factor activity at various times after seeding. The inset in A shows the cell counts per well at each assay time point. Error bars represent 1 SEM.

tion. Therefore, fibroblast cell activation of factor X in the presence of factor VIIa was due to tissue factor. As can be seen in Fig 2, the fibroblast expression of tissue factor was related to cell density as had been previously reported in fibroblasts[20] and HeLa cells.[15] Tissue factor activity in disrupted (freeze/thaw/sonicated) cells was always greater than nondisrupted (intact) cells. Experiments with tissue factor inhibitors were therefore conducted using fibroblasts at several cell densities, after they had passed the posttrypsinization peak of tissue factor activity (ca, 24 hours).

LACI is a potent inhibitor of tissue factor initiated coagulation,[7,8] but has not been studied for its effect on tissue factor in cell membranes. To assay LACI's inhibitory activity in cell membranes, fibroblasts were subcultured into 96-well microtiter plates and assayed for tissue factor activity with varied concentrations of LACI. We examined concentrations of LACI from 7.8 to 500 pmol/L using cell densities from 3,500 to 9,925 cells per well. Inhibition of tissue factor activity and factor Xa by LACI approached 100% at the higher LACI concentrations examined (Fig 3). There was a clear dose response between tissue factor activity and LACI concentration. The tissue factor activity curves (absorbance v minutes^2) generated in the presence of LACI were hyperbolic with complete inhibition of tissue factor and factor Xa by the end of the 80-minute assay at high LACI concentrations. LACI's inhibitory effect was consistently greater in intact cells compared with disrupted cells, except at the lowest cell density examined (Fig 3). The difference between effects on intact and disrupted cells diminished at high LACI concentration.

The effect of PAP on fibroblast tissue factor activity was examined from 3.9 nmol/L to 1 μmol/L at cell densities of 4,500 to 15,400 cells/well. As shown in Fig 4, PAP was consistently more effective in disrupted cells than intact cells, reaching the maximal inhibition at lower concentrations when tested on disrupted cells. The maximal inhibition attained with 1 μmol/L PAP was near 83% irrespective of whether the cells were intact or disrupted.

Apo A-II has been shown to inhibit tissue factor purified from bovine brain and reconstituted into lipid vesicles[3] as well as human tissue factor.[23] We examined the effects of apo A-II on tissue factor in cell membranes, using disrupted

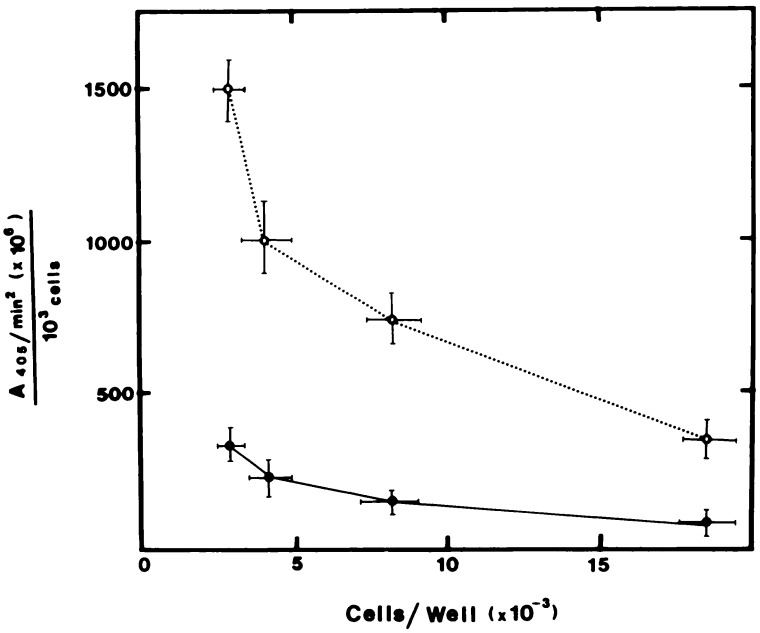

Fig 2. Tissue factor activity per cell at each of four cell densities 24 hours after subculturing. Intact (—●—) or disrupted (--O--). Error bars represent 1 SEM.

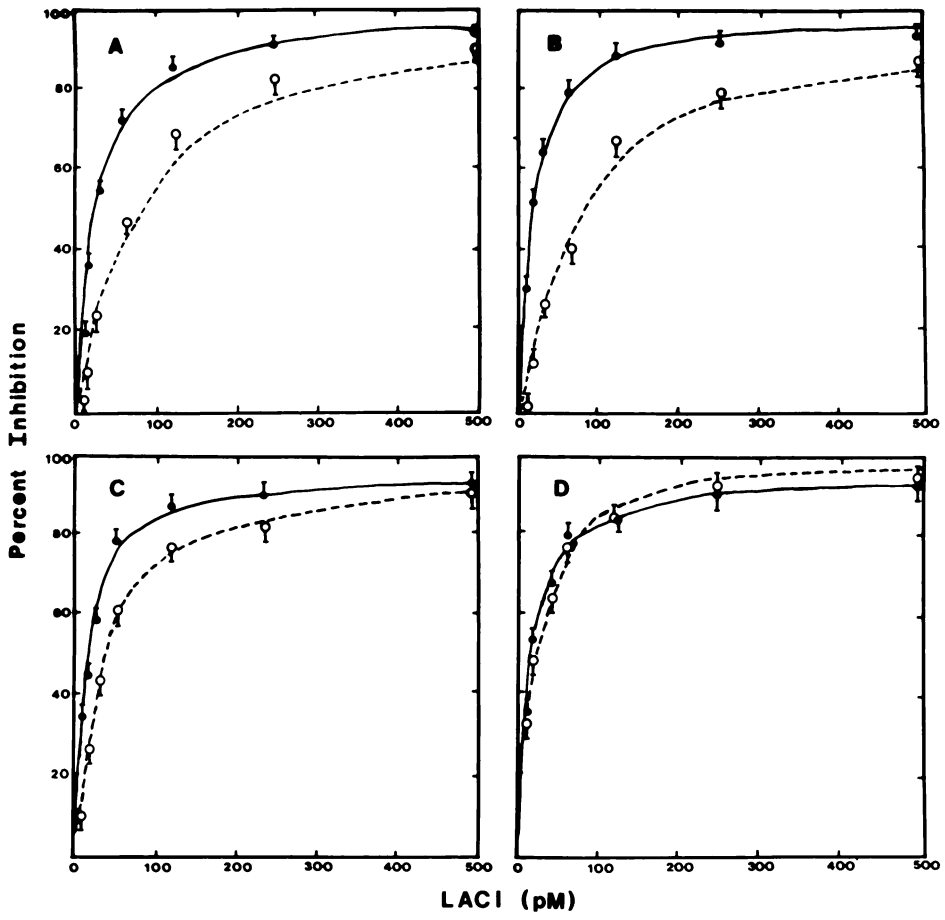

Fig 3. Inhibition of fibroblast tissue factor activity by LACI. Fibroblasts were either intact (—●—) or disrupted (--O--) and were used at densities of (A) 9,925 ± 1,047, (B) 8,700 ± 1,003, (C) 4,950 ± 130, and (D) 3,500 ± 420 cells/well. Error bars represent 1 SEM.

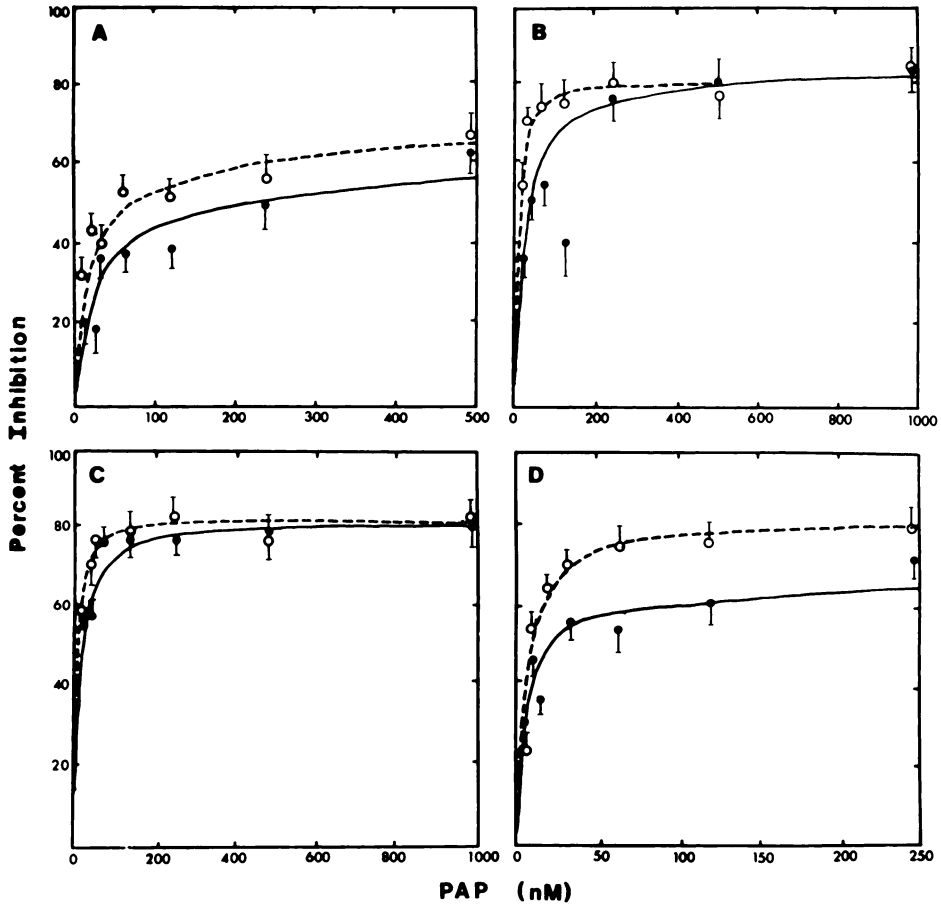

Fig 4. Inhibition of fibroblast tissue factor by PAP. Cells were either intact (—●—) or disrupted (--O--) and were examined at densities of (A) 15,400 ± 2,978, (B) 7,171 ± 1,558, (C) 5,450 ± 1,917, and (D) 4,500 ± 586 cells/well. Error bars represent 1 SEM.

(freeze/thaw/sonicated) and nondisrupted (intact) fibroblasts. The effects of apo A-II on cultured cells were examined from 0.07 to 1 μmol/L at cell densities of 5,600 to 11,875 cells/well. The effects of apo A-II fluctuated around 0% inhibition at every apo A-II concentration and every cell density examined regardless of whether the cells were disrupted or intact. To demonstrate that the apo A-II was inhibitory to tissue factor reconstituted in PS/PC model membrane vesicles as previously reported,[3] the apo A-II was concurrently tested on fibroblasts (scraped from confluent cultures in 25 cm^2 T-flasks suspended in tris/saline and sonicated) and human brain tissue factor reconstituted with PS/PC. As shown in Table 1, apo A-II at 1 μmol/L inhibited tissue factor approximately 63% in reconstituted vesicles at both tissue factor concentrations examined but had no effect on equivalent tissue factor activities expressed by the cellular membranes.

DISCUSSION

Since tissue factor is a membrane protein, it will be expressed physiologically in cell membranes or their fragments. It was therefore necessary to confirm the experimental results derived from studies with tissue extracts and model systems by using tissue factor in a more physiological environment. To this end, the expression of tissue factor in membranes of cultured fibroblasts was characterized with respect to density and time parameters, and then examined for the fibroblast tissue factor susceptibility to the effects of apo A-II, PAP, and LACI. The time-course experiments were performed to determine an appropriate time after subculturing to study the effects of inhibitors on fibroblast tissue factor activity. In agreement with earlier studies,[20,21] subculturing was followed by a time-dependent burst of tissue factor activity. There was a peak of tissue factor activity at eight hours in intact cells and 12 hours in disrupted cells following subculturing at all cell densities examined (Fig 1). It is interesting that the peak of tissue factor activity in intact cells preceded that of disrupted cells by four hours. The intact cells appear to be "down regulating" available surface tissue factor activity while the disrupted cells were still increasing in tissue factor activity, suggesting that the cells began to regulate the amount of expressed tissue factor while new tissue factor was still being

Table 1. Comparison of apo A-II Effect on Human Brain Tissue Factor in PS/PC Vesicles and Disrupted GM5756 Fibroblasts

Tissue Factor Source	Absorbance 405 nm/min² (× 10^6)	
	[apo A-II] = 0	[apo A-II] = 1 μmol/L
GM5756 fibroblasts (20 μL)	3,518 ± 317	3,531 ± 243
Brain TF (2.2×10^{-16} mol)	3,459 ± 270	1,284 ± 72
GM5756 fibroblasts (40 μL)	6,054 ± 404	5,734 ± 204
Brain TF (4.4×10^{-16} mol)	6,297 ± 444	2,344 ± 137

Assays were conducted in replicates of 5 and results are mean ± SD.

synthesized but not expressed. The tissue factor activity per cell was approximately the same up to 18 hours after subculturing regardless of cell density. After 18 hours, the tissue factor activity per cell changed with respect to cell density, with the lowest cell densities expressing the highest tissue factor activity per cell. At later times following subculturing, both intact and disrupted cells appeared to regulate expressed and total tissue factor activity at levels related to cell density.

The effect of cell density on fibroblast tissue factor activity was examined to confirm that the amount of tissue factor expressed in intact and disrupted cells was a function of cell density with this cell strain, as had been previously reported for other fibroblast strains.[20] The amount of tissue factor activity per cell 24 hours after subculturing was consistently higher in disrupted cells than in intact cells (Figs 1 and 2). This suggests that there is a "pool" of nonexpressed (or perhaps under-expressed) tissue factor activity in intact cells, consistent with other studies.[24] Furthermore, as the cell density increased, the amount of tissue factor activity per cell decreased, suggesting that these cells regulated their expression of tissue factor depending on their culture density. Because the tissue factor activity changed with respect to both cell density and whether the cells were disrupted or not, the effects of LACI, PAP, and apo A-II were examined with respect to these parameters.

The quantitation of fibroblast tissue factor activity inhibition by LACI was complicated in our assay due to the progressive inhibition of tissue factor by LACI and inhibition of factor Xa. The inhibitory activity of LACI toward tissue factor is dependent on the generation of factor Xa, since LACI and factor Xa form an inhibited complex, which then becomes the inhibitor of the factor VIIa-tissue factor catalytic complex.[7] The inhibition of factor Xa is competitive, with a final Ki of 660 pmol/L for the human enzyme.[25] The maximum LACI concentration used in these studies (500 pmol/L) was well below this Ki and even further below the plasma concentration of LACI (2.5 nmol/L).[13] Our assay estimated the inhibitory activity of LACI by averaging the rate of active factor Xa production over the first 20 minutes of the assay. This measurement included the removal of factor Xa by LACI, as well as inhibition of factor Xa generation by tissue factor-factor VIIa, and provided relative estimates of the LACI inhibition.

The inhibition of tissue factor activity by LACI at all cell densities examined was dramatic. The apparent half-maximal inhibition was obtained with picomolar LACI concentrations (Fig 3), and the maximal inhibition approached 100% between 250 and 500 pmol/L LACI. After the first 20 minutes used to estimate the LACI activity, there was frequently neither tissue factor nor factor Xa activity remaining in the reaction. The inhibition of tissue factor activity was consistently greater in intact cells when compared with sonicated cells (Fig 3). This may reflect differences in tissue factor sensitivity to inhibition under the different conditions, or, given the assay methodology, may be due to increased tissue factor levels in disrupted cells.

PAP also inhibited tissue factor in cultured fibroblasts. The apparent half-maximal inhibition to tissue factor activity, estimated from Fig 4, ranged from 5 to 27 nmol/L and the maximal inhibition reached 83% at approximately 1 μmol/L PAP. It is interesting that the inhibition of tissue factor activity by the lower concentrations of PAP in disrupted cells was greater than in intact cells. This may reflect the PAP binding to anionic lipids, which are generally present largely on the cytoplasmic leaflet of intact membranes.[26]

The lack of inhibition of fibroblast tissue factor by apo A-II was unexpected, since it has previously been shown to inhibit factor X activation by factor VIIa and bovine tissue factor in PS/PC vesicles[3] and human tissue factor in mixed brain lipids.[23] Apo A-II concentrations were examined from 0.07 to 1 μmol/L at cell densities ranging from 5,600 to 11,875 cells/well. Both intact and disrupted cells were examined, and in no experiment did apo A-II inhibit the tissue factor activity. To confirm the reported inhibitory activity of the apo A-II used, the apo A-II was concurrently tested on fibroblasts and human brain tissue factor reconstituted in PS/PC vesicles. As expected from previous work,[3] apo A-II inhibited tissue factor in vesicles as had been originally described, but failed to inhibit fibroblast tissue factor. These different effects may be due to differences in membrane composition or physical organization of PS/PC vesicles and cell membranes.

These experiments clearly indicate that LACI, PAP, and apo A-II vary widely in their effects on tissue factor activity. LACI and PAP are capable of inhibiting tissue factor in both reconstituted vesicles and fibroblast membranes, while apo A-II may be inhibitory only in model membrane systems. LACI appears to inhibit tissue factor activity to a greater extent and at lower concentrations than does PAP, although the assay system used in these studies does not permit a quantitative assessment of the relative potencies of these proteins. Given its combined effects, inhibition of factor Xa along with the subsequent inhibition of tissue factor-factor VIIa, LACI has been confirmed to be an extremely potent inhibitor of coagulation reactions initiated by tissue factor and is effective against tissue factor expressed in cell membranes.

ACKNOWLEDGMENT

The authors thank Sherman E. Ross and William F. Novotny for purification of apo A-II, PAP, and LACI used in these experiments.

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1989 73: 983-989

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.