concluded to be the pharmacologically active one, as judged from tests relating chemical structure to biological activity (*6*). This contradiction could be caused by (i) the chemical studies being inconclusive, or (ii) the 3′-iodine distally oriented conformation being necessary for hormonal activity and the total energy of T_3-receptor complex being favorable enough to easily effect rotation of the β ring.

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Tissue Factor (Thromboplastin): Localization to Plasma Membranes by Peroxidase-Conjugated Antibodies

Abstract. *Peroxidase-conjugated antibodies were used to determine the histologic and cytologic localization of bovine and human tissue factor (thromboplastin). Tissue factor antigen was found in highest concentration in the intima of blood vessels, particularly in the plasma membranes of endothelial cells and in human atheromatous plaques. Tissue factor was also found limited to the plasma membranes of many cell types. The presence of tissue factor in the plasma membranes of endothelial cells and atheromata suggests that it may play a significant role in hemostasis and thrombosis.*

Blood coagulation proceeds by two mechanisms in which proteins, lipids, and calcium ions interact to yield a fibrin clot (*1*). The intrinsic system is initiated in vitro by contact with any of a number of surfaces including collagen, although the nature of the physiological activator is uncertain. The extrinsic or tissue factor system is activated by a specific particle-bound lipoprotein found in extracts of many tissues. Nemerson and Pitlick have purified a protein solubilized from delipidated bovine lung powder; this protein, when combined with certain phospholipids, has the biological characteristics of tissue factor (*2*). We have now prepared specific rabbit antiserums to purified bovine lung and human placenta tissue factor. Using the techniques of Nakane and Pierce (*3*) and Avrameas and Bouteille (*4*) for localizing tissue antigens with peroxidase-conjugated antibodies, we have shown that tissue factor is present in the plasma membranes of many cell types. Significantly, it is in highest concentration in the plasma membranes of endothelial cells and in atheromata where it may interact with circulating clotting factors to initiate coagulation.

Antibodies were produced as follows. White New Zealand rabbits were given an initial intradermal injection of 1 mg of purified human or bovine tissue factor with complete Freund's adjuvant, and subsequent biweekly injections of 100 μg of the antigen; the antiserum was collected for the first time 2 weeks after the third injection, and then 2 weeks after subsequent injections. Gamma globulin was precipitated and washed with 33 percent saturated ammonium sulfate and dialyzed in phosphate-buffered saline, *p*H 7.2, ionic strength, 0.15. Serum from unimmunized rabbits was processed in the same way and used for controls.

The rabbit antibody to bovine tissue factor neutralized the coagulant activity of preparations derived from lung, brain, kidney, spleen, and liver. The antibody to human tissue factor neutralized tissue factor from human brain and placenta. When the soluble form of tissue factor from bovine lung and human placenta was studied by immunodiffusion (*5*), two precipitin lines were seen. These most likely correspond to the two species of tissue factor previously noted (*2*).

Horseradish peroxidase (Miles Laboratories) was conjugated to the γ-globulin fraction of sheep antiserum to rabbit globulin (GIBCO) by a slight modification of the technique of Avrameas and Bouteille (*4*). Sections (6 μm) were dried in air and fixed in 10 percent formaldehyde in phosphate-buffered saline for 5 to 10 minutes. Tissue sections were incubated for 30 minutes at room temperature in γ-globulin (1 mg/ml) either from unimmunized rabbits or from rabbits immunized against tissue factor; the sections were incubated with the peroxidase–sheep globulin conjugate and stained for peroxidase activity (*6*). An easily recognizable brown-black reaction product was found in sections incubated with globulin from immunized rabbits. A faint brown coloration of membranes and cytoplasm in controls was attributed to nonspecific binding of conjugated protein.

In all tissues studied, the intense brown staining of the tissue factor antigen was located on the plasma membranes of most cells (Fig. 1A). Blood vessels showed a typical staining regardless of their size or location. There was marked endothelial staining, particularly of the plasma membranes, with lesser amounts in the media (Fig. 1, C, D, and F). The adventitia was essentially devoid of antigen. The findings were identical in human and bovine tissues. Of particular interest was the concentration of tissue factor in the intimal and subintimal tissues of human atheromata (Fig. 1, H and I). Cholesterol crystals were completely surrounded by intensely staining cells.

Because tissue factor may initiate coagulation in vivo, the vascular localization of this substance is of interest. Previous studies, particularly by Astrup and Buluk (*7*) and Kirk (*8*), have shown that large vessels contain tissue factor and that it is mainly in the intima. Our data confirm these findings and extend them to include small vessels. Indeed, even vasa vasorum are shown to contain intimal tissue factor (Fig. 1F).

The resolution of this technique is sufficient to demonstrate the intracellular localization of tissue factor. Thus, the finding that tissue factor is located on the plasma membranes of most cells is entirely new. In the kidney, cell membranes of all tubular cells in the medulla and cortex were stained. The glomeruli showed weak, diffuse staining. Hepatocytes showed staining of plasma membranes as well as some intracellular granules (Fig. 1, A and B). The latter, however, which are believed to represent endogenous peroxidases, were present in control specimens and do not reflect the distribution of tissue factor.

In the myocardium, myofibrils were free of reaction product, but the sarcolemma stained positively. Intramural arteries, as well as the major coronary arteries, showed typical intimal staining. The aortic and ventricular surfaces of the aortic valve were positive. The ventricular surface showed more intense staining which included the subendothelial endocardium. The aortic surface had a monocellular layer of endothelial staining.

In the lung, plasma membranes of the alveolar cells stained. Small pieces of cartilage and pulmonary macrophages were unreactive. Endothelial staining was present in the pulmonary vessels.

Although frequently surmised, it has not been previously shown that tissue factor is located so that it can readily interact with plasma coagulation factor VIII after vascular injury. Our findings demonstrate that even minimum trauma to the vascular endothelium might allow coagulation to be initiated via the extrinsic system, since tissue factor is found on plasma membranes of all vascular endothelia. Ashford and Freiman (*9*) have demonstrated fibrin formation at the site of minimum endothelial injury. The substance initiating coagulation was not defined, but our finding that tissue factor is in the plasma membranes of endothelial cells

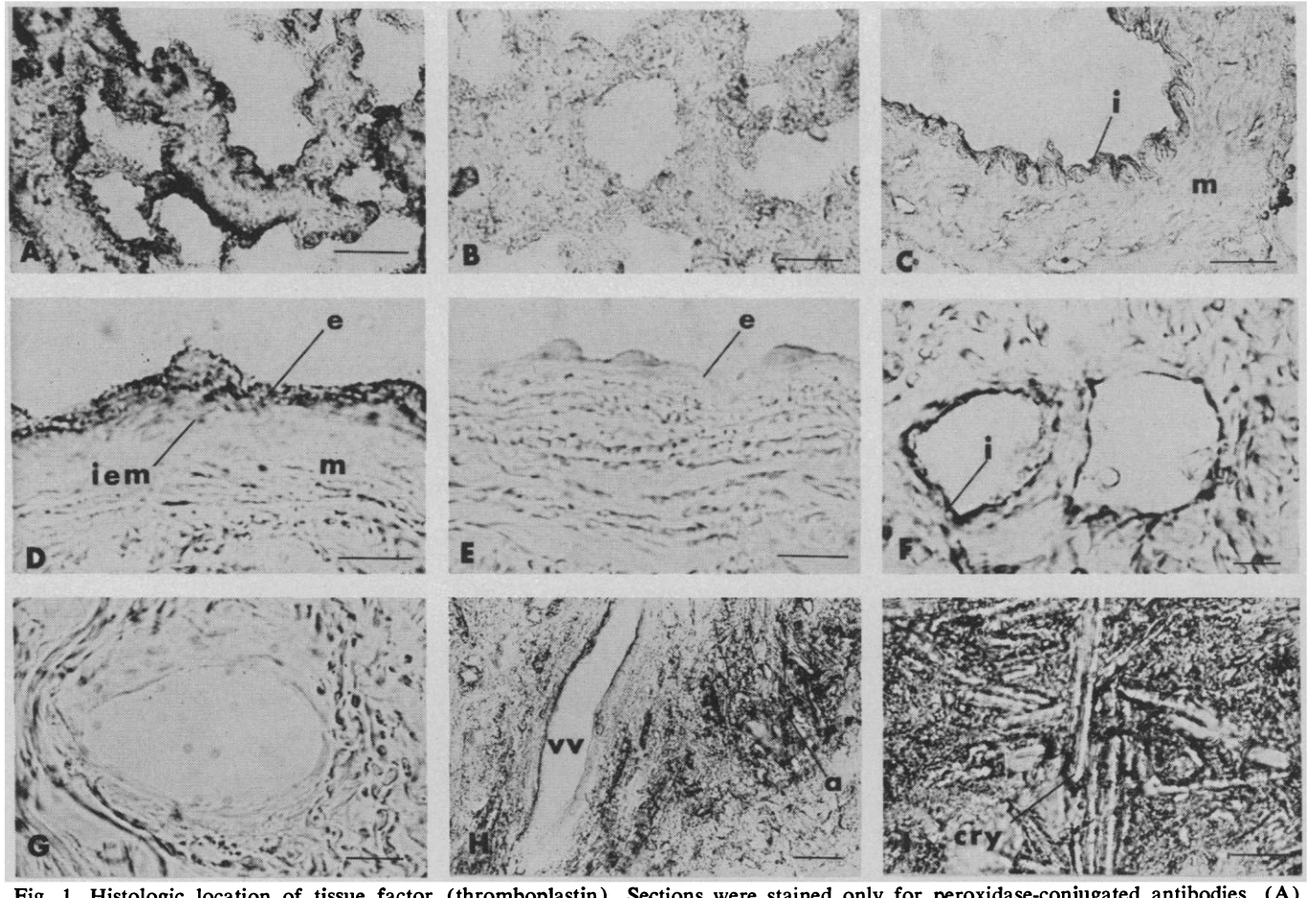

Fig. 1. Histologic location of tissue factor (thromboplastin). Sections were stained only for peroxidase-conjugated antibodies. (A) Bovine liver cells showing specific staining of cell membranes (× 1020); scale equals 10 μm. (B) Bovine liver control (incubated with globulin from unimmunized rabbits) showing no staining of the membrane but nonspecific staining of intracellular granules (× 1020); scale equals 10 μm. (C) Muscular artery in bovine kidney showing intimal staining (*i*) and weak, diffuse medial staining (*m*) (× 500); scale equals 5 μm. (D) Bovine aorta showing intense endothelial (*e*) staining with no reactivity in the internal elastic membrane (*iem*) (× 1660); scale equals 6 μm. (E) Aorta control showing absence of endothelial staining (*e*) (× 1660); scale equals 6 μm. (F) Bovine pulmonary artery vasa vasorum revealing intense intimal staining (*i*) (× 1350); scale equals 10 μm. (G) Pulmonary artery vasa vasorum control without staining of the intima (*i*) (× 1560); scale equals 6 μm. (H) Human aorta showing intense staining of the intima of a vas vasorum (*vv*) and the thickened intima surrounding the cholestrol crystals in an atheroma (*a*) (× 600); scale equals 1.25 μm. (I) Higher-power view of a human atheroma showing intense reactivity surrounding the cholesterol crystals (*cry*) (× 1400); scale equals 6 μm.

could readily explain the occurrence of fibrin at sites where the endothelium is nominally intact.

The thrombogenic potential of tissue factor is illustrated by the high concentrations found in human atheromata. Ulceration of a plaque would then place tissue factor in contact with factor VII, thereby allowing initiation of the extrinsic system.

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1,25-Dihydroxycholecalciferol: A Potent Stimulator of Bone Resorption in Tissue Culture

Abstract. *1,25-Dihydroxycholecalciferol (DHCC), isolated from kidney homogenates incubated with 25-hydroxycholecalciferol (HCC), stimulated the release of previously incorporated ^{45}Ca from fetal rat bones in organ culture, at concentrations of 10^{-10} to 10^{-8}M. The dose response curves for 1,25-DHCC and 25-HCC, the parent compound, are parallel, but 1,25-DHCC is about 100 times as potent on a weight basis. Brief exposure to maximum doses of either agent leads to prolonged bone resorption.*

Vitamin D_3 is hydroxylated in the liver to produce 25-hydroxycholecalciferol (HCC), which then enters the circulation and is taken up in target tissues (*1*). The active form of vitamin D was considered to be 25-HCC because it could stimulate intestinal transport of calcium and bone resorption directly in isolated systems (*2*). However, 25-HCC is further transformed to other active metabolites, one of which has now been identified as 1,25-dihydroxycholecalciferol (DHCC) (*3*). The second hydroxylation is carried out largely in the kidney (*4*). On the assumption that it is the active component of the more polar products than 25-HCC previously identified as peaks V, 4-B, or P (*5*), 1,25-DHCC appears to act on the gut more rapidly than 25-HCC but is less effective in curing rickets in rats. The experiments reported here were designed to test whether the second hydroxylation affects direct stimulation of bone resorption in organ culture.

Table 1. Relative potency of 1,25-dihydroxycholecalciferol (DHCC) and 25-hydroxycholecalciferol (HCC). Values for potency ratios (with 95 percent fiducial limits in parentheses) and for the index of precision were obtained with the use of a computer program for parallel line bioassay (*14*).

Assay	Potency ratio DHCC/HCC	Index of precision
3 × 3	103 (42–266)	0.45
2 × 2	332 (157–790)	0.30

The culture methods have been described (*6*). Bone shafts from 19-day rat fetuses labeled with ^{45}Ca in vivo were first incubated for 24 hours in a chemically defined medium supplemented with 5 percent human serum inactivated at 60°C for 30 minutes. Paired bones were transferred to vessels containing the same medium with or without 1,25-DHCC or 25-HCC dissolved in ethanol. Equal amounts of ethanol were added to control cultures. The final concentration was less than 0.5 percent.

1,25-DHCC was prepared by incubation of 25-[26,27-^{3}H]HCC (320 dpm/ng) with kidney homogenates from rachitic chicks (*4*). The products were isolated by Sephadex LH-20 chromatography (*7*). 25-HCC was prepared by chemical synthesis (*8*).

1,25-DHCC produced a graded increase in ^{45}Ca release from fetal bones in culture at concentrations of 0.025 to 10 ng/ml, or 10^{-10} to 10^{-8}*M* (Fig. 1). The dose response curves for pooled data indicate that a much higher concentration of 25-HCC was required to produce the same response. In two individual bioassays, the mean potency ratios for 1,25-DHCC to 25 HCC were 103 and 332 (Table 1). The higher ratio in the second assay was ascribable to a diminished response to 25-HCC.

1,25-DHCC shares with parathyroid hormone (PTH) and 25-HCC the ability to produce a prolonged increase in bone resorption, which we have termed induction (*9*). Significant stimulation of 48-hour ^{45}Ca release was obtained with as little as 30-minute exposure, although the response was less than to continuous exposure (Table 2). Six-hour exposure to either 1,25-DHCC at 0.025 μg/ml or 25-HCC at 80 times the concentration gave nearly maximum resorption.

The greater potency of 1,25-DHCC as compared to that of 25-HCC in mobilizing calcium in vitro, but not in vivo, could be due to differences in the concentrative uptake by bone cells. Labeled 25-HCC is taken up by bone

Table 2. Effect of brief exposure to 25-hydroxycholecalciferol (HCC) or 1,25-dihydroxycholecalciferol (DHCC) on subsequent release of ^{45}Ca from fetal rat bones in 48-hour culture. Paired bones were incubated with or without the 25-HCC or 1,25-DHCC for the indicated times and then transferred to fresh medium. Values are mean ± S.E. for ratio of treated to control cultures for the cumulative 48-hour ^{45}Ca release from four pairs of cultures.

Dose (μg/ml)	Time (hours)	^{45}Ca release (treated/control)
	25-HCC	
2	6	2.60 ± 0.29*
	1,25-DHCC	
0.025	½	1.65 ± 0.15*†
0.025	2	1.84 ± 0.13*†
0.025	6	2.11 ± 0.15*
0.025	48	2.82 ± 0.20*

* Significantly different from 1.0; $P < .02$. † Significantly different from response in 48-hour continuous culture; $P < .05$.