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## Tumor Shedding and Coagulation

Abstract. *Three syngeneic carcinomas from two species shed plasma membrane vesicles when cultured in vitro or grown in the ascites tumor form in vivo. Shed vesicles carry procoagulant activity that can account for the activation of the clotting system and the fibrin deposition associated with these and many other types of malignancy in animals and man.*

Clotting abnormalities are commonly observed in patients with malignant neoplasms (*1*), and fibrin deposits have been reported to surround many primary and transplanted tumors in man and animals (*2*, *3*). However, the biological significance of tumor-associated coagulation and its pathogenesis are not understood. Among other possibilities, a fibrin gel deposited about tumors could serve as a physical barrier or "cocoon" that isolates tumor cells from the host's immunological defense mechanisms (*4*). Also, there is evidence that fibrin itself, or perhaps certain of its breakdown products, may induce angiogenesis, a characteristic and essential feature of solid tumor growth (*4*).

We report that guinea pig syngeneic line 1 and line 10 hepatocarcinomas (*5*) and mouse breast TA3-St carcinomas (*5*) release procoagulant activity (PCA) in tissue culture and in vivo. In all three tumors PCA is associated with sedimentable, ultramicroscopic plasma membrane–derived vesicles. Others have reported that tumor cells shed membrane fragments that contain various cell surface antigens and, in murine systems, virus-like structures (*6*), but such shedding has not previously been related to activation of the clotting system.

Earlier studies (*7*) demonstrated that guinea pig line 1 and line 10 hepatocarcinomas, when cultured for 4 hours in serum-free medium, release at least four distinct mediators with important biological activities: PCA, plasminogen activator, a factor that enhances vascular permeability, and an inhibitor of macrophage migration. Mediator release decreased 67 to 98 percent when cells were cultured at 4°C instead of 37°C. Puromycin ($2 \times 10^{-5} M$) decreased the release of plasminogen activator and vascular permeability factor by 66 and 45 percent, respectively, but had little effect on PCA (< 10 percent), suggesting that PCA release does not depend significantly on new protein synthesis.

In the present experiments, ascites tumor cells were harvested from strain 2 guinea pigs (tumor lines 1 and 10) or A/Jax mice (tumor line TA3-St), washed three times in Hanks balanced salt solution, and cultured at a density of $5 \times 10^6$ cells per milliliter in serum-free, antibiotic-supplemented Hanks minimal essential medium under a humidified atmosphere containing 5 percent $CO_2$. After 4 hours, the cultures were centrifuged for 10 minutes at 160*g* and then 10,000*g* and the cell-free supernatants were collected for procoagulant studies.

Substantial PCA was found in the cell-free culture supernatants of all three tumors (Table 1). Whereas plasminogen activator and other mediators secreted by these tumors remained soluble, little or no PCA remained in the supernatant after ultracentrifugation for 90 minutes. Recovery of PCA in the pellets varied from 50 to 95 percent. The PCA from line 10 tumors ($PCA_{10}$) could also be concentrated by membrane ultrafiltration (Amicon XM-50) without significant loss of activity. When passed over Sephadex G-200 columns, $PCA_{10}$ emerged in the void volume with nearly complete recovery and with a 20- to 40-fold increase in specific activity over that of cell-free culture fluids.

Procoagulant activity was not inhibited by up to 10 m*M* diisopropyl fluorophosphate (DFP) under conditions that totally inhibited trypsin (37°C, 2 hours, *p*H 8.0). DFP (10 m*M*) completely inactivated 30 μg of trypsin (in a reaction volume of 1.0 ml), whereas PCA containing 25 μg of total protein was not inactivated. In addition, PCA was relatively heat-stable, retaining 60 percent of its activity after 30 minutes at 70°C. PCA function was not reduced by proteolytic digestion with either trypsin or papain.

To determine whether PCA was released in vivo, ascites tumor fluid was collected from guinea pigs and mice and rendered cell-free by low-speed centrifugation. Ascites fluid from both species contained abundant PCA, all or most of which sedimented at 100,000*g* (Table 1). That PCA may have biological significance is suggested by the extensive fibrin deposits found in the peritoneal walls of guinea pigs bearing ascites tumors (Fig. 1a).

Table 1. Procoagulant activity shed by tumor cells in short-term tissue culture and in ascites fluids. Data were obtained from four to seven separate experiments. Cell viability was 95 to 99 percent, as judged by trypan blue exclusion and by lack of release of succinate dichlorophenolindophenol oxidoreductase. Tumor cells accounted for ≥ 98 percent of nucleated cells, the remainder being lymphocytes or macrophages. Identical results (not shown) were obtained with line 10 tumor cells carried in continuous culture. For assay of ascites fluids, citrated Hanks balanced salt solution was injected intraperitoneally into strain 2 guinea pigs bearing 7-day-old tumors (20 ml) or into A/Jax mice (5 ml). The peritoneal fluid was harvested and cell-free fluids, ultracentrifuge pellets, and supernatants were prepared as for culture fluids. Aliquots of cell-free culture or ascites fluids were centrifuged at 4°C for 90 minutes at 100,000*g*, the supernatant was harvested, and the pellet was reconstituted to the original volume. PCA (0.1 $cm^2$) was assayed by its ability to shorten the recalcification time of citrated, platelet-poor guinea pig or mouse plasma.

| Tumor cells | Mean shortening of recalcification time (%) | | Sedimentable PCA (%)* |
|---|---|---|---|
| | Cell-free fluids | Reconstituted pellets | |
| Four-hour tissue culture | | | |
| Line 10 | 72.4 ± 1.1 | 63.8 ± 5.7 | 97 |
| Line 1 | 37.6 ± 2.6 | 30.4 ± 2.7 | 67 |
| TA3-St | 40.9 ± 3.6 | 22.4 ± 3.7 | 64 |
| Ascites fluids | | | |
| Line 10 | 52.8 ± 3.8 | 47.2 ± 2.6 | 100 |
| Line 1 | 29.2 ± 6.1 | 16.6 ± 4.1 | 61 |
| TA3-St | 27.0 ± 3.4 | 26.4 ± 1.8 | 98 |

*Determined by multiplying by 100 the quotient of the amount of PCA in the centrifuge pellet divided by the total amount of PCA (pellet plus supernatant).



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Ultracentrifuge pellets derived from cell-free line 1 or line 10 culture supernatants or from cell-free ascites fluid consisted largely of membrane vesicles varying widely in diameter from 15 to 800 nm (median, 60 nm) (Fig. 1b). Cytoplasmic organelles were rare but more common in ascites than in culture fluids. Virus-like structures were not seen (*8*). Ultracentrifuge pellets from cultured or ascites TA3-St cells contained similar vesicles but included numerous virus-like structures as well. We recently found vesicular structures similar to those described here in malignant pleural effusions in patients, and these also had PCA.

Since it appeared that all three tumors shed membrane-associated PCA, various marker enzymes were measured to define the intracellular source. The specific activities of $PCA_{10}$ and of several plasma membrane–derived enzymes—5′-nucleotidase, ouabain-sensitive $Na^+$-$K^+$-adenosinetriphosphatase, and phosphodiesterase I—were much greater in ultracentrifuge pellets of culture supernatants than in line 10 cell homogenates (10, 70, 11, and 122 times greater, respectively) or cell-free culture supernatants (4, > 315, 6, and 7 times greater, respectively). Enzyme markers for endoplasmic reticulum (NADH oxidoreductase) and lysosomes (β-D-glucuronidase) were only detected at low levels in culture fluids and remained in the 100,000*g* supernatant. Succinate dichlorophenolindophenol oxidoreductase, a mitochondrial enzyme, was not detectable in culture fluids. We conclude that PCA is associated with vesicles derived from the tumor cell plasma membrane and that the shedding of such vesicles by tumors in vivo and in vitro cannot be attributed to cell death in culture.

Shedding of PCA is apparently not unique to malignant cells. A small amount of vesicle-associated PCA, < 10 percent of that in line 10 ascites fluid, was also found in the peritoneal washings of normal guinea pigs. Cell-free supernatants from cultured peritoneal exudate cells (75 percent macrophages, 20 percent lymphocytes, 5 percent neutrophils) generally lacked detectable PCA. However, vesicular structures with PCA were detected in ultracentrifuge pellets obtained from such fluids.

Detailed clotting studies revealed that $PCA_{10}$ clots platelet-poor human plasma, but only about half as efficiently as it clots guinea pig plasma; is not itself factor II, V, or VII–XII; does not activate factor X directly; is effective in human plasmas singly deficient in factors VII, VIII, IX, XI, and XII; and requires factors II, V, X, and calcium for expression. Expression of $PCA_{10}$ activity in factor VII–deficient human plasma and in guinea pig plasma that is normally deficient in factor VII argues that $PCA_{10}$ is not thromboplastin. However, $PCA_{10}$ resembles thromboplastin in other respects, including derivation from the plasma membranes (*9*).

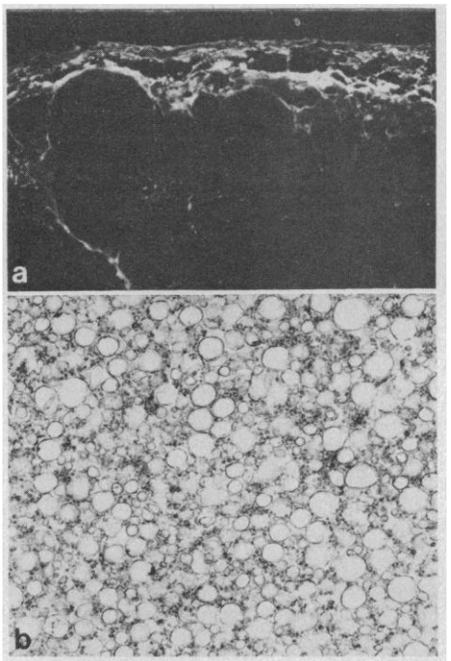


Fig. 1. (a) Photomicrograph illustrating fibrin deposits in the peritoneal wall of a strain 2 guinea pig bearing line 10 ascites tumor. Stain: fluorescein-labeled rabbit antibody to guinea pig fibrinogen (×200). (b) Electron micrograph of ultracentrifuge pellet of line 10 culture supernatant, illustrating membrane vesicles lacking visible content (×16,000).

Several different procoagulant activities have been reported in tumor cell homogenates and culture supernatants. O'Meara (*2*) and subsequently many others (*10*) described thromboplastin-like procoagulants in various tumor cells. Gordon *et al.* (*11*) found in supernatants of cultured cell lines a DFP-inhibitable procoagulant capable of activating factor X. Others have described tumor procoagulants that may also act at this step (*12*). Finally, Laki *et al.* (*13*) reported a mouse tumor–derived, factor XIII–like clot-stabilizing activity. $PCA_{10}$ is apparently distinct from other reported tumor procoagulants in that it is associated with plasma membrane vesicles and primarily acts late in the clotting sequence at the level of prothrombinase generation. Phospholipid vesicles and platelet surface membranes greatly facilitate prothrombinase generation by providing surface receptors for factors V, Xa, and II (*14*). The tumor vesicles described here may act in similar fashion by providing binding sites for soluble clotting components.

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