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Tumor Cells Secrete a Vascular Permeability Factor That Promotes Accumulation of Ascites Fluid

Abstract. *Tumor ascites fluids from guinea pigs, hamsters, and mice contain activity that rapidly increases microvascular permeability. Similiar activity is also secreted by these tumor cells and a variety of other tumor cell lines in vitro. The permeability-increasing activity purified from either the culture medium or ascites fluid of one tumor, the guinea pig line 10 hepatocarcinoma, is a 34,000- to 42,000-dalton protein distinct from other known permeability factors.*

Abnormal accumulation of fluid commonly accompanies solid and particularly ascites tumor growth (*1*). To investigate the mechanism of tumor ascites formation, we measured the rates of influx and efflux of ^{125}I-labeled human serum albumin (HSA) at various times after the implantation of tumor cells in the peritoneal cavities of guinea pigs. We detected a markedly increased influx of HSA as early as 1 hour after intraperitoneal injection of guinea pig line 10 hepatocarcinoma cells, which provoke a substantial accumulation of ascites fluid (Table 1). In contrast, efflux of HSA from the peritoneal cavities of animals bearing line 10 tumors did not change significantly, even with progressive tumor growth (*2*).

To establish whether the increased influx of fluid induced by tumor cells reflects an alteration in vessel permeability, we injected animals intravenously with colloidal carbon. Examination of the peritoneal cavities of strain 2 guinea pigs, Syrian hamsters, and A/Jax mice bearing syngeneic ascites tumors (line 10, HSV-NIL8, and TA3-St, respectively) revealed that many venules of the peritoneal wall, diaphragm, mesentery, and gastrointestinal serosal surfaces were heavily labeled with colloidal carbon, indicating increased permeability; comparable vessels in control animals were not labeled.

These observations suggest that tumor ascites may be attributable to alterations in the permeability of vessels lining the peritoneum. To investigate the basis for this increased permeability, we used the Miles assay (*3*) to test ascites fluid for the presence of factors that increase vascular permeability (Table 2 and Fig. 1). Ascites fluid from line 10 guinea pig and TA3-St mouse carcinomas and the HSV-NIL8 hamster sarcoma all markedly increased local cutaneous vascular permeability. The increase was evident after 1 minute and maximal within 5 to 10 minutes. By contrast, platelet-poor plasma samples from the same species (Table 2 and Fig. 1) and oil-induced peritoneal exudate fluids (*4*) had little or no activity. The tumor ascites permeability-increasing activity was not inhibited by soybean trypsin inhibitor (1000 μg/ml); therefore, it is not PF/dil (*5*), a permeability factor unmasked when serum is diluted ≥ 1:100 (*6*).

We previously reported that line 10 tumor cells release a vascular permeability–increasing activity in serum-free culture (*7*). This activity is not inhibited by soybean trypsin inhibitor (200 μg/ml), and its production by cells in vitro requires protein synthesis (complete inhibition by 20 μg of cycloheximide per milliliter). Many other tumor cell lines also release permeability-increasing activity in serum-free culture, including guinea pig 104 C1 fibrosarcoma, hamster HSV-NIL8 sarcoma, rat sarcomas B77 Rat 1 and RR 1022, and mouse TA3-St carcinoma, MOPC 21 myeloma, and polyoma BALB/c 3T3 sarcoma. Line 1 guinea pig hepatocarcinoma cells release one-fourth the activity released by line 10 cells, a finding that may explain the relative ability of these cells to promote HSA influx (Table 1) and ascites fluid accumulation (the volume of line 1 ascites fluid was routinely one-fourth that of line 10). Oil-induced guinea pig peritoneal exudate cells (> 70 percent macrophages) neither increase the influx of HSA into the peritoneum (Table 1) nor secrete detectable permeability-increasing activity in vitro. Guinea pig fibroblasts and smooth muscle cells release approximately one-eighth the activity released by comparable numbers of line 10 cells (*8*).

Table 1. Peritoneal vessel permeability. Guinea pigs (400 g) were injected intraperitoneally with 3×10^7 line 1 or line 10 tumor cells (*17*) or with peritoneal exudate cells in Hanks balanced salt solution (HBSS) and immediately thereafter were injected intravenously with ^{125}I-labeled HSA (5×10^6 dis/min). One hour later the animals were exsanguinated under ether anesthesia, and peritoneal fluid was collected following intraperitoneal injection of 20 ml of heparinized (10 U/ml) HBSS. For each animal total radioactivity in the ascites fluid was determined and normalized for that in the blood: influx of HSA was computed as the ratio of total disintegrations per minute in peritoneal fluid to those per milliliter of blood. Net influx was determined by subtracting influx values for control animals. Values are means ± standard errors ($N = 4$).

Type of cells injected intraperitoneally	Net peritoneal influx of HSA
Line 1	0.09 ± 0.04
Line 10	0.41 ± 0.08
Line 10 + immune IgG (2 mg)	0.11 ± 0.03
Peritoneal exudate	0

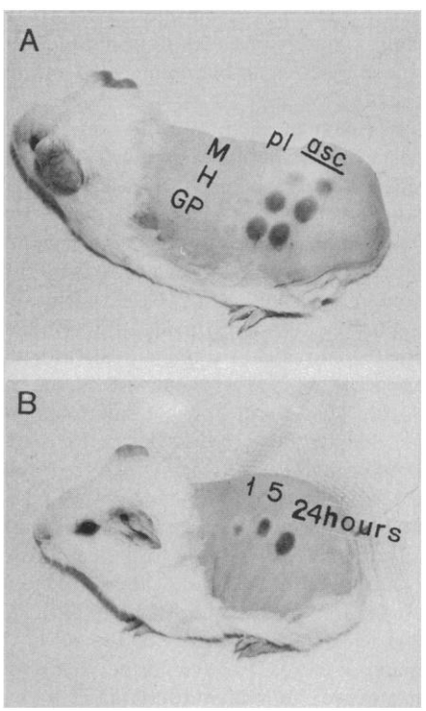
We next purified both the ascites and tissue culture permeability factors from a single tumor, the line 10 guinea pig carcinoma. Permeability-increasing activities from both sources chromatographed identically as single peaks on columns containing Sephadex G-150, heparin-Sepharose, or hydroxylapatite (*9*) and electrophoresed as a single peak with an apparent molecular weight of 34,000 to 42,000 on sodium dodecyl sulfate–polyacrylamide gels (Fig. 2). Using the heparin-Sepharose, hydroxylapatite, and electrophoretic steps in tandem, we purified the permeability-increasing activity approximately 1200-fold from serum-free conditioned medium and approximately 10,000-fold from ascites fluid. As little as 200 ng (5×10^{-12} mole) of the purified material increased vascular permeability to a degree equivalent to that induced by 1.25 μg (4×10^{-9} mole) of histamine.

Rabbits immunized with the purified permeability factor secreted by line 10 cells in vitro produced an immunoglobulin G (IgG) that bound and neutralized virtually all the permeability-increasing activity in undiluted line 10 and line 1 tumor ascites fluids (Table 2) and in line 10 and line 1 culture media. This antibody also blocked the peritoneal influx that follows intraperitoneal injection of line 10 tumor cells (Table 1). In every

Table 2. Dermal vessel permeability, as determined by the Miles assay (*3*). Depilated guinea pigs were injected intravenously with ^{125}I-labeled HSA (1.3×10^7 dis/min) in 1 ml of saline containing 0.5 percent Evans Blue dye. Samples to be tested for permeability-increasing activity, in isotonic solution and at neutral *p*H, were injected intradermally in a volume of 0.1 ml. After 20 minutes the animals were exsanguinated under ether anesthesia. Test sites were excised and quantitated for ^{125}I in a gamma counter. The number of net disintegrations per minute extravasated was determined by subtracting values for control sites injected with saline. Each animal also received a series of intradermal histamine injections; these sites served as reference points for the calculation of histamine equivalents. B.L., below limit of quantitation (0.6 μg histamine).

Substance injected intradermally	Net disintegrations per minute ^{125}I-HSA extravasated (mean ± standard error) (N = 3 to 7)	Histamine equivalent* (μg)
Hamster plasma	70 ± 176	B.L.
Hamster ascites (HSV-NIL8)	15,309 ± 1,508	1.3
Guinea pig plasma	1,989 ± 1,070	B.L.
Line 1 ascites	69,609 ± 6,850	5.5
Line 1 ascites + preimmune IgG (80 μg)	70,321 ± 2,567	5.5
Line 1 ascites + immune IgG (80 μg)	3,935 ± 1,568	B.L.
Line 10 ascites	92,472 ± 4,886	10.0
Line 10 ascites + preimmune IgG (80 μg)	93,756 ± 1,171	10.0
Line 10 ascites + immune IgG (80 μg)	7,187 ± 930	B.L.
Line 10 serum-free culture supernatant†		
After 1 hour of culture	1,054 ± 60	B.L.
After 5 hours of culture	3,610 ± 295	0.7
After 24 hours of culture	21,565 ± 617	2.5

*A plot of net disintegrations per minute extravasated in response to histamine versus the logarithm of the number of micrograms of histamine injected generated a straight line (histamine range, 0.6 to 10 μg). †Derived from cultures containing 2.5×10^6 cells per milliliter.



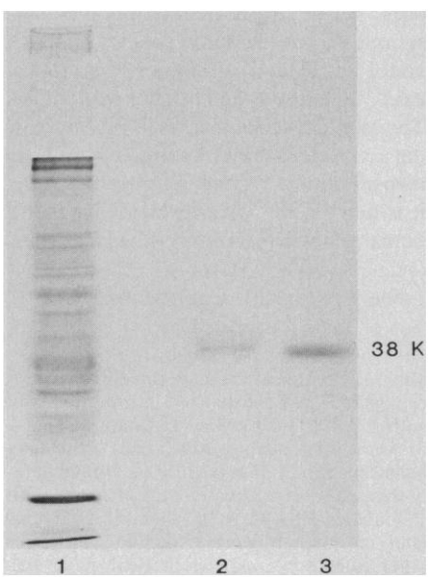


Fig. 1 (left). Miles assay for measuring increased vascular permeability in the skin (see legend to Table 2). Mediators of increased permeability cause bluing at the site of intradermal injection within 5 minutes, whereas control substances such as saline elicit no response. Abbreviations in (A): *pl*, control plasma; *asc*, ascites fluid; *M*, mouse TA3-St tumor; *H*, hamster HSV-NIL8 tumor; and *GP*, guinea pig line 10 tumor. In (B), line 10 cells were cultured (1×10^6 cell/ml) in serum-free Dulbecco's modified Eagle's medium and conditioned media was harvested at 1, 5, and 24 hours as indicated. Fig. 2 (right). Resolution of the permeability-increasing activity on sodium dodecyl sulfate–polyacrylamide slab gels (*16*). Samples were electrophoresed without reduction at 4°C in a 7.5 percent polyacrylamide gel containing 0.1 percent sodium dodecyl sulfate and washed for 1 hour at 4°C in 2.5 percent Triton X-100 and then in phosphate-buffered saline for 1 hour. The gel was sliced, individual slices were extracted, and dialyzed extracts were tested for activity by the Miles assay. Track 1 shows the stained pattern of concentrated line 10 serum-free culture medium. All the activity in track 1 (total recovery was regularly 50 percent) was confined to two adjacent slices (reelectrophoresed in tracks 2 and 3) composing the 34,000 to 42,000 molecular weight region. Line 10 ascites fluid permeability-increasing activity was found to electrophorese identically (molecular weight 34,000 to 42,000) with the activity in line 10 culture medium.

case, IgG from animals before their immunization (preimmune IgG) was without effect. The IgG from immunized animals (immune IgG) also neutralized the permeability-increasing activity released in culture by an unrelated tumor, the 104 C1 guinea pig fibrosarcoma (*10*), but not the activity of guinea pig PF/dil or the low levels of activity released by guinea pig fibroblasts or smooth muscle cells.

As determined by light and electron microscopy, line 10 permeability factor did not cause endothelial cell damage or mast cell degranulation. Vessels responded equally well to multiple challenges with equivalent doses of line 10 permeability factor administered 30 minutes apart; the effect of a single intradermal injection was rapid (evident within 5 minutes) and transient (little residual increased permeability was detectable 20 minutes after injection), providing further evidence that line 10 permeability factor is not toxic to blood vessels. It does not resemble bradykinin (molecular weight, 1200), plasma kallikrein (108,000), or leukokinins (2500). Leukokinins (*11*) are generated in ascites fluids under nonphysiological conditions (*p*H 3.8 and 37°C for 24 hours) by a mechanism sensitive to 1 μ*M* pepstatin A. Line 10 permeability factor is present in fresh, unmanipulated ascites fluid (*p*H 6.4 to 6.9), and its action is unaffected by 20 μ*M* pepstatin A. Lymphocyte permeability factors with molecular weights of 12,000 (*12*) and 39,000 (*13*) have been reported; however, unlike line 10 permeability factor, the latter increases vascular permeability only after a latent period of 20 minutes. The effects of line 10 permeability factor are not mediated by histamine. Guinea pigs treated with the antihistamines mepyramine (5 μmole/kg, subcutaneously) plus cimetidine (500 μmole/kg) (*14*) responded normally to line 10 permeability factor, although the action of 20 μg of histamine was blocked. It is also unlikely that the effects of this factor are mediated through prostaglandin synthesis. Neither systemic (14 μmole/kg, intraperitoneally) nor local (2 nmole, intradermally) treatment with indomethacin (*15*) affected the response of vessels to the permeability factor.

In conclusion, vessels lining the peritoneal cavities of guinea pigs, hamsters, and mice bearing ascites tumors display markedly greater permeability than do the same vessels in control animals. This increased permeability is apparently due to the presence in ascites fluid of a potent permeability factor not found in normal plasma or serum. The permeability factors found in guinea pig line 10

culture medium or ascites fluids appear to be identical. In addition, they are antigenically related to permeability factors produced by guinea pig line 1 or 104 C1 tumor cells. Secretion of permeability-increasing activity appears to be a common feature of tumor cells, and may contribute to the abnormal accumulation of fluid associated with neoplastic disease.

Note added in proof: The immune IgG raised against line 10 permeability factor also neutralizes the rat dermal vessel permeability-increasing activity released by Walker rat carcinoma cells in culture. Preimmune IgG has no neutralizing effect.

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2. Clearance of labeled HSA from the peritoneal cavity was not impaired in tumor-bearing animals at any interval. For example, 7 days after intraperitoneal injection of 3×10^7 line 10 tumor cells, 58.4 ± 4.2 percent (mean ± standard error) of the HSA (90 to 95 percent precipitable with trichloroacetic acid) remained in the peritoneal cavities after 2 hours; 56 ± 1.15 percent remained in the controls.
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Yolk Pigments of the Mexican Leaf Frog

Abstract. *Eggs of the Mexican leaf frog contain blue and yellow pigments identified as biliverdin and lutein, respectively. Both pigments are bound to proteins that occur in crystalline form in the yolk platelet. The major blue pigment is biliverdin IX α. The eggs vary in color from brilliant blue to pale yellow-green depending on the amount of each pigment. These pigments may provide protective coloration to the eggs.*

While studying the lipid composition of the eggs and embryos of the Mexican leaf frog, *Agalychnis dacnicolor* (*1*), we observed that their green coloration was due to the presence of two pigments, one blue and one yellow, which together produce blue, blue-green, or yellow-green eggs. We have now identified the major blue pigment as biliverdin IX α and the major yellow pigment as lutein. The presence of the latter pigment is not surprising since lutein is widely distributed among plants and animals (*2*). Biliverdin occurs less often as a pigment, although it has been found in the dog placenta, in the shells of bird eggs, in the skin of fishes and amphibians (*2, 3*), in the eggs and larvae of the tobacco hornworm (*4*), and in the serum and eggs of *Xenopus* (*5*). It seems likely that the utilization of these two pigments by *A. dacnicolor* evolved as a mechanism for producing green eggs. The green coloration of leaf frog eggs, which are laid on green vegetation, may afford camouflage to protect eggs and embryos from predation. However, as far as we can ascertain, there have been no definitive studies on the adaptive value of green eggs, although the ecological implications deriving from a two-pigment system for egg coloration are apparent.

Six different batches of *A. dacnicolor* eggs (100 to 250 eggs) varying in color from brilliant blue to yellow-green were extracted with a 1:1 mixture of chloroform and methanol and a mixture of acetone and hydrochloric acid to obtain the yellow and blue pigments. The pigments were separated by column chromatography on silicic acid. Chloroform eluted the yellow pigment, and acetone eluted the blue pigment. The pigments were further purified by preparative thin-layer chromatography (TLC). The yellow and blue pigments were localized in lipid-rich yolk platelets.

Yolk platelets, which were pale blue-green or pale yellow, were obtained by collagenase treatment of homogenized eggs, followed by differential centrifugation. Analysis by light microscopy of the isolated fresh yolk platelets revealed rounded rectangular platelets of different sizes, and electron microscopy showed that the platelets consisted of closely stacked crystalline arrays about 70 Å thick.

The ultraviolet to visible spectra of the silicic acid column–purified pigments from different eggs are given in Fig. 1, a and b. The blue pigment has major bands at 372 to 376 nm and 640 to 690 nm. The yellow pigments have major bands at 442 to 444 nm and 470 to 471 nm. The relative amount of the yellow and blue pigments in the various eggs was determined by the ratio of the absorbance at 442 nm to that at 650 nm. This ratio was correlated with the color of the egg. The ratio of brilliant blue eggs was 1.15, that of blue eggs was 1.7 to 2.3, that of green eggs was 3.4 to 3.6, and that of yellow-green eggs was 10.4.

The blue pigment has properties consistent with a biliverdin. Both the blue pigment and biliverdin (Sigma) were converted to methyl esters by treatment with methanolic HCl (Supelco). The dimethyl esters were purified by preparative TLC (Merck-Darmstadt silica gel 60 plates) using chloroform and methanol 9:1. Both had identical relative mobility (R_f) values of 0.62, and gave a purple