Characterization of Vascular Permeability-Increasing Component Isolated from Solid Tumors and the Effect of Highly Polymerized Dextran Sulfate on Its Activity

Norimitsu L. SATO, Sueo NIIMURA, Nobuyoshi FUJISAWA
and Yoshitaka MAEDA

Facilities for Comparative Medicine & Animal Experimentation,
Niigata University School of Medicine,
Ichibancho, Asahimachi-dori, Niigata 951, Japan

Accepted February 17, 1986

Abstract—Increase in vascular permeability is usually seen at the growth site of a tumor implant in murine dermal tissue. Increased vascular permeability was inducible by the subcutaneous injection of a solid tumor extract rich in protein precipitable at 20–50% saturation of ammonium sulfate. The vascular permeability-increasing activity of the tumor extract was reducible in the presence of highly polymerized dextran sulfate (DS-500) which showed a strong anticomplementary activity, but not by other substances such as dextran sulfate with a low molecular weight, non-sulfated dextran, chondroitin sulfate or heparin. As the tumor extract includes γ -globulins in aggregated or bound form and adsorbs complements, it is probable that the aggregated γ -globulins increase vascular permeability by triggering the complement activation system in the skin. DS-500 might antagonize the process.

Abnormal accumulation of fluid commonly accompanies solid and particularly ascites tumor growth (1). This phenomenon relates to the increase in vascular permeability at the inflamed site. In 1979, Dvorack et al. (2) first reported that the guinea pig line 10 hepatocarcinoma cells released a vascular permeability-increasing substance in serum-free culture. They next isolated permeability factors with an apparent molecular weight of 34,000 to 42,000 from both the tumor ascites and tissue culture medium (3).

We have recently characterized the increased vascular permeability induced by transplanted tumor cells in murine dermal tissue (4). The vascular bed in murine dermal tissue responded to the inoculated tumor cells by two-phased changes in the vascular permeability. The initial increase in the vascular permeability was seen in an early stage (1 to 3 day post tumor cells inoculation), and the degree of the increased vascular permeability was greater in the allogeneic host

versus tumor combination. The later vascular response was produced by a growing solid tumor in a continuous mode beginning at the 5th to 10th day post inoculation. The degree of the increased vascular permeability in the later phase was in direct proportion to the wet weight of the solid tumor. Glucocorticoids reduced the increased vascular permeability. Initially, we tried to isolate a permeability factor from culture medium of the tumor cells, but it was unsuccessful. We then attempted to isolate a factor from the inflamed site involving a developing solid tumor in the later phase.

This report concerns some physico-chemical and biological properties of the extracted material from solid tumors which shows a vascular permeability-increasing activity by subcutaneous injection into murine dermal tissue.

Materials and Methods

Chemicals: Bovine pancreatic DNase I and

RNase A, trypsin, micrococcal protease and soybean trypsin inhibitor were purchased from Sigma (Missouri, U.S.A.). Hyaluronoglucosidase and chondroitinase ABC were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Dextran (abt. M.W. 199,000) and dextran sulfate (abt. M.W. 500,000) were from Nakarai Chemicals, Ltd. (Kyoto, Japan). Dextran sulfates with low molecular weights (abt. M.W. 8,000 & 5,000) were from Sigma. Heparin (abt. M.W. 5,000) and chondroitin sulfate (abt. M.W. 50,000) were from Wako Pure Chemicals Ind. (Osaka, Japan). Mouse albumin (Fr. V) was from Sigma. Mouse γ -globulin (Fr. II) was from Miles (Illinois, U.S.A.). Evan's blue dye was from Sigma.

Experimental animals: Five week-old ddY mice (male, closed colony), BALB/c mice (male, inbred strain) and ICR nu/nu mice (male, closed colony) were used. They were housed in plastic cages and given a cubic diet, MF-1 (Oriental Co., Tokyo, Japan) and water ad libitum. Constant temperature ($23 \pm 1^\circ\text{C}$) and humidity (45–75%) were maintained.

Tumor implantation: Ehrlich ascites tumor cells were maintained in male ddY mice by weekly transfers by intraperitoneal injection of 0.1 ml of ascites fluid. Methylcholanthrene-induced fibroma in BALB/c mice (BAMC-1, ascites tumor cells) were maintained in male BALB/c mice by weekly transfers by intraperitoneal injection of 0.1 ml of ascites fluid. The tumor cells were harvested at 7th to 10th day post transfers and washed in PBS (phosphate-buffered saline, pH 7.4); 2×10^7 cells were inoculated subcutaneously into the central portion of the dorsal skins of mice.

Isolation of a vascular permeability-increasing components: Solid tumors (50 g in total), isolated from mice at 20th day post inoculation, were sliced and suspended overnight in 200 ml acetone at -20°C to remove lipids which were not effective to increase vascular permeability. After evaporating the acetone using a freeze-drying system (Refrigeration Science, Inc., model 5000B, New York, U.S.A.), the dried tumor pieces were suspended overnight in 200 ml of sterilized 10 mM phosphate buffer (pH 7.4) containing 0.5 M KCl at 2°C . The super-

natant was obtained by $1500 \times g$ centrifugation, and it was subjected to a precipitation with ammonium sulfate. The precipitate at 20–50% saturation was dissolved in 10 mM Tris-HCl (pH 7.5) and further fractionated by gel filtration using Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden).

Assay of vascular permeability-increasing activity: One percent Evan's blue in physiological saline solution was intravenously injected into mice by their tail veins at a volume of 0.1 ml per 10 g body weight. One-tenth ml of fractionated sample (20 mg protein/ml) after dialysis against PBS was subcutaneously injected into the central portion of the murine dorsal skin immediately after the dye injection. Three hours thereafter, mice were killed. The part of the dorsal skin containing extravasated dye was cut into pieces and immersed for 24 hr in a medium composed of 14 ml of acetone and 6 ml of a 0.5% aqueous solution of sodium sulfate to extract dye according to the method of Harada et al. (5). The extracted dye content was measured by the absorbance of the supernatant at 620 nm.

Determination of γ -globulin and albumin content: Antibody (Ab) to mouse γ -globulin in rabbit (UCB-Bioproductions, Brussels, Belgium), Ab to mouse albumin in rabbit (Miles Biochemicals, Indiana, U.S.A.) and peroxidase labelled anti-rabbit IgG in goat (Kirkegaard & Perry Laboratories Inc., Maryland, U.S.A.) were used. Contents of γ -globulin and albumin in the fractionated samples of tumor extract were detected by the ELISA technique applying the F. A. S. T. system (Becton Dickinson Overseas Inc., Tokyo, Japan).

Determination of cholesterol content: Total cholesterol in the fractionated samples of tumor extract was measured enzymatically by the RaBA system (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan).

Hemolytic complement activation: Erythrocytes (sheep), hemolysin (antibody to sheep erythrocytes in rabbit, Denka-Seiken, Niigata, Japan) and complements (guinea pig serum) were used. Erythrocytes were at first incubated at 25°C for 20 min with a 1:6000 dilution of hemolysin. After washing the activated erythrocytes with PBS, 1.0×10^8

cells were reacted with appropriate concentrations of complement at 37°C for 30 min. The hemolytic reaction was measured by the absorbance of the supernatant at 541 nm.

Results

Fractionation of tumor extract and some physico-chemical properties: A supernatant extracted from solid tumor with PBS containing 0.5 M KCl showed a vascular permeability-increasing (VPI) activity. Initially, effect of hydrolytic enzymes on VPI activity of the crude extract was examined to estimate which component was the major one to increase vascular permeability. The crude extract was digested in the presence of hydrolytic enzymes under the appropriate conditions. Each 0.1 ml of a mixture of crude extract and enzyme was injected subcutaneously into the dorsal skin of mice. Extravasated dye content at the inflamed site was determined 3 hr after the injection and compared with those of non-treated extract or of enzyme alone in the reaction solution. As shown in Table 1, VPI activity of the crude extract was destroyed by treatment with micrococcal protease, but not with trypsin, DNase I, RNase A, hyaluronoglucosidase or chondroitinase ABC. This suggested that VPI activity of the tumor extract was associated with protein, although it was resistant to trypsin. By precipitation of protein

in the tumor extract with ammonium sulfate, the most active precipitate was obtained at 20–33% saturation of ammonium sulfate (γ -fraction) in both Ehrlich and BAMC-1 solid tumors as shown in Table 2. A precipitate obtainable at 33–50% saturation (β -fraction) showed a moderate activity. A precipitate at 50–66% saturation (α -fraction) showed low activity. The VPI activity of the γ -fraction was fractionated to two peaks of activity, TPF-I with a large molecular weight and TPF-II with a small molecular weight, by Sepharose 4B chromatography as shown in Fig. 1. The β -fraction was also fractionated to TPF-I and TPF-II (data not shown), suggesting that the γ - and β -fraction included the same active components. VPI potency of TPF-II was higher than that of TPF-I. TPF-II was relatively unstable at –20°C. Content of γ -globulin, assayed immunologically by ELISA, was high in the γ -fraction, TPF-I and TPF-II, and moderate in the β -fraction. Content of albumin was high in the α -fraction, and it was low in the γ -fraction, β -fraction, TPF-I and TPF-II. In this respect, we examined VPI activity of serum albumin and γ -globulin. Neither albumin (Fr. V) nor γ -globulin (Fr. II) from normal mice showed a significant VPI activity at concentrations of 10–20 mg/ml (data not shown). A lipid, assayed as cholesterol, was slightly detectable in the β - and α -fraction.

Table 1. Effect of hydrolytic enzymes on VPI activity of tumor extract

Enzymes	Concentration	Dye extravasation (%)
—	—	100.0 ¹
DNase I	162 Kunitz units/ml	93.8
RNase A	25 Kunitz units/ml	87.8
Hyaluronoglucosidase	250 units/ml	97.5
Chondroitinase ABC	1.25 units/ml	109.1
Trypsin	5 mg/ml of 1:250 sample	92.3
Pronase ²	10 units/ml	44.5*

Tumor extract (20 mg protein/ml of 80 mM Tris-HCl, pH 7.5) was incubated in the presence of enzyme at 37°C for 60 min. DNase I solution included 4 mM MgSO₄. Each 0.1 ml of a mixture of tumor extract and enzyme was injected subcutaneously into the dorsal skin of mice immediately after intravenous injection of 1% Evan's blue dye. Extravasated dye content at the inflamed skin was determined 3 hours after the injection. Enzymes alone except pronase did not show a significant increase in vascular permeability in the concentrations used. ¹Mean extravasated dye content induced by non-treated tumor extract was 260 μ g and is expressed as 100% control. ²Pronase-treated tumor extract was heated before injection at 100°C for 15 min to destroy completely the strong irritability of pronase itself (tumor extract was stable against the heating). *Statistically significant at $P < 0.01$ by Student's t -test ($N = 8$).

Table 2. Relative VPI potencies of fractionated samples and some physico-chemical properties

Fractions	Relative VPI potency ¹	γ -Globulin ²	Albumin ²	Cholesterol ³
Ehrlich solid tumor grown in ddY mice				
Crude extract	118.6	4,096	1,024	6.6
γ -Fraction (20–33%)	330.1	4,096	64	2.8
β -Fraction (33–50%)	119.5	1,024	128	13.4
α -Fraction (50–66%)	84.0	64	4,096	22.0
TPF-I	132.6	4,096	64	1.8
TPF-II	820.8	8,192	128	1.6
Ehrlich solid tumor grown in ICR/nu/nu mice				
Crude extract	123.0	4,096	1,024	5.2
γ -Fraction (20–33%)	229.3	4,096	64	1.4
β -Fraction (33–50%)	184.5	1,024	256	10.0
α -Fraction (50–66%)	25.8	32	2,048	15.3
TPF-I	202.2	2,048	64	0.6
TPF-II	404.9	4,096	256	1.3
BAMC-1 solid tumor grown in BALB/c mice				
Crude extract	128.2	4,096	1,024	4.8
γ -Fraction (20–33%)	265.6	2,048	32	2.0
β -Fraction (33–50%)	150.7	1,024	512	8.5
α -Fraction (50–66%)	31.1	64	2,048	14.1
TPF-I	180.3	2,048	32	2.0
TPF-II	381.0	4,096	512	2.1

¹Relative VPI potency is expressed as extravasated dye content (μ g) at the inflamed site by injection of 1 mg protein of fractions. ²Contents of γ -globulin and albumin were assayed immunologically by ELISA and expressed as final dilution titer of fractions which reacted with fixed anti-serum to γ -globulin or to albumin. ³Cholesterol (mg/dl) was measured enzymatically using the RaBA system.

Fig. 1. Sepharose 4B chromatography of γ -fraction of Ehrlich solid tumor extract. One ml of γ -fraction (20 mg protein/ml) was put on a column (1.5×50 cm) and eluted with PBS (25 ml per hour).A280. A solid bar indicates vascular permeability-increasing activity/mg protein of fractionated portion. alb: albumin eluted in this portion.

As described above, there was no essential difference in characteristics of VPI components between Ehrlich and BAMC-1 solid tumors origin, and active samples produced substantial increase in vascular permeability with one to two hr's duration when injected subcutaneously into murine dorsal skin. In addition, it was noteworthy that TPF-I and TPF-II showed the same VPI activity in a different strain of mice as did in syngeneic mice. From these observations, we used the TPF preparations from Ehrlich solid tumor grown in ddY mice in the following experiments because of the rapid progression of the tumor and the severity of the inflammation.

Effect of trypsin inhibitor on VPI activity of TPF-I and TPF-II: Plasma exudes and accumulates at the inflamed site around and in the growing solid tumor in murine dermal tissue. TPF preparations from the solid tumor therefore seemed to include also the ex-

travasated blood proteins. We examined the effect of soybean trypsin inhibitor on VPi activity of TPF-I and TPF-II to exclude the possibility that the TPFs might be PF/Dil. Soybean trypsin inhibitor (10–30 μ g/ml of TPF sample) was injected subcutaneously into murine dorsal skin immediately after dye injection. Three hours after the injection, extravasated dye content at the inflamed site was measured and compared with that of TPF alone. As shown in Table 3, soybean trypsin inhibitor did not reduce VPi activities of any TPF preparations at all.

Anticomplementary activity of fractionated samples: The outstanding physico-chemical property of VPi components suggested their ability as a complement activator because active fractions consistently contained γ -globulin. Using an in vitro assay system, we examined the antagonizing effect of fractionated samples on hemolytic com-

plement. As shown in Table 4, the γ -fraction, β -fraction and TPF-I showed a strong anticomplementary activity and suppressed the hemolytic reaction triggered by hemolysin and complement at 1:2400 dilution. TPF-II, however, showed a very weak inhibition (1:150 dilution) in spite of its high VPi activity. This showed that TPF-II might not include immune complex to adsorb complement. The α -fraction also had a weak inhibition (1:300 dilution). Inhibition of the hemolytic reaction by the γ -fraction, β -fraction and TPF-I was not by direct action on the erythrocyte membrane or on the first step of hemolysin binding with erythrocytes because the effect of the fractions on the hemolytic system was observed when they acted on the stage of complement binding with the erythrocyte-hemolysin complex, and it was competitive with complement.

Effect of dextran sulfate 500 on VPi

Table 3. Effect of soybean trypsin inhibitor on VPi activity of γ -fraction, TPF-I and TPF-II

TPF samples ¹	Concentration of inhibitor (μ g/ml)				
	0	10	30	100	300
γ -Fraction	100.0(%)	115.5	96.7	115.6	104.7
TPF-I	100.0	110.0	98.2	107.7	109.1
TPF-II	100.0	99.3	100.5	96.4	107.5

¹Protein concentration of TPF samples was adjusted to each 20 mg/ml. The γ -fraction includes TPF-I, TPF-II and other unidentified substances. One-tenth ml of TPF samples was injected subcutaneously into murine dorsal skin. Extravasated dye contents (μ g) induced by the γ -fraction, TPF-I or TPF-II without soybean inhibitor were 485.2, 257.2 or 763.0, respectively, and they are expressed as 100% controls. Soybean trypsin inhibitor alone did not show a significant change in vascular permeability at any concentrations used.

Table 4. Antagonizing effect of fractionated samples of tumor extract on hemolytic complement

Fractions ¹	Dilution of TPF samples					
	1:150	1:300	1:600	1:1200	1:2400	1:4800
	(Optical density at 541 nm)					
γ -Fraction	0.070	0.030	0.101	0.163	<u>0.332</u>	0.482
β -Fraction	0.050	0.044	0.124	0.156	<u>0.345</u>	0.483
α -Fraction	0.156	<u>0.320</u>	0.481	0.482	0.480	0.480
TPF-I	0.070	<u>0.056</u>	0.126	0.172	<u>0.356</u>	0.481
TPF-II	<u>0.330</u>	0.408	0.480	0.478	0.481	0.478

¹Protein concentration of fractions was each 20 mg/ml. Optical density of 541 nm of control hemolytic reactant (without tumor extract) was 0.480. Underlines indicate final dilution titers of fractions showing anticomplementary activity.

activity of TPF-I and TPF-II: It was found unexpectedly that dextran sulfate 500 reduced the VPi activity of TPF-I. When dextran sulfate 500 was included at a final concentration of 83 μ M in the γ -fraction or in the TPF-I solution to inject subcutaneously into murine dorsal skin, they lost 70 to 80% of their initial potency as shown in Table 5. TPF-II lost only about 20% of its initial potency by the same concentration of dextran sulfate 500. Dextran sulfates with low molecular weight were almost inactive. Non-sulfated dextran 199, chondroitin sulfate and heparin were inert (Table 6). TPF solutions in the presence of dextran sulfate 500 did not show appreciable changes such as pH differentiation, precipitation, etc. There was no correlation between the anti-coagulant activity of polysaccharides and their anti-TPF

activity.

A possible role of dextran sulfate 500 in the inhibitory process against TPF-I activity was assumed to be interference with the complement activation system because highly polymerized dextran sulfate has been known as a potent inhibitor of complement in the classical pathway. To assure the activity, the effect of polysaccharides on hemolytic complement was then examined using an in vitro system. As shown in Fig. 2, dextran sulfate 500 strongly inhibited the hemolytic reaction. Its inhibition was seen at pM concentration and was highly competitive with complement at an earlier stage of reaction. Dextran sulfate 8 and 5 showed a weak activity. Dextran 199, chondroitin sulfate and heparin were almost inert. There was a good correlation between anti-complementary activity of polysac-

Table 5. Effect of dextran sulfate 500 on VPi activity of γ -fraction, TPF-I and TPF-II

Polysaccharide	abt. M.W.	Concentration	γ -Fraction	TPF-I	TPF-II
—	—	mg/ml (M)	100.0(%)	100.0	100.0
Dextran sulfate 500	500,000	2.5 (0.5×10^{-4})	101.3	100.0	105.2
		10.4 (2.1×10^{-5})	78.5*	69.7*	98.6
		41.6 (8.3×10^{-5})	28.1**	30.0**	82.4*

Dextran sulfate 500 (DS-500) was included in TPF samples at the concentration indicated and injected subcutaneously into murine dorsal skin. Three hours after the injection, extravasated dye content at the inflamed skin was measured and compared with that of the γ -fraction, TPF-I or TPF-II alone. Extravasated dye contents (μ g) at the inflamed skin by TPFs alone were 324.3 (γ -fraction), 238.0 (TPF-I) and 541.6 (TPF-II) and are expressed as 100% controls. Statistically significant at * $P < 0.05$ (N=8), ** $P < 0.01$ (N=8) by Student's *t*-test.

Table 6. Effect of other polysaccharides on VPi activity of γ -fraction

Polysaccharides	abt. M.W.	Concentration	Dye extravasation
—	—	mg/ml (M)	100.0 (%)
Dextran sulfate 8	8,000	20.0 (2.5×10^{-3})	100.0
		80.0 (1.0×10^{-2})	91.3*
Dextran sulfate 5	5,000	12.5 (2.5×10^{-3})	100.0
		50.0 (1.0×10^{-2})	98.8
Dextran 199	199,000	16.5 (8.3×10^{-5})	104.0
		66.0 (3.3×10^{-4})	115.4
Chondroitin sulfate	500,000	12.5 (2.5×10^{-4})	101.3
		50.0 (1.0×10^{-3})	97.6
Heparin	5,000	12.5 (2.5×10^{-3})	97.1
		50.0 (1.0×10^{-2})	133.5

Polysaccharide at the concentration indicated was included in the γ -fraction which contains TPF-I and TPF-II and injected subcutaneously into murine dorsal skin. Three hours after the injection, extravasated dye content (μ g) at the inflamed skin was measured and compared with that of the γ -fraction alone. Extravasated dye content induced by the γ -fraction alone was 336.1 μ g and is expressed as 100% control. Statistically significant at * $P < 0.05$ (N=7).

Fig. 2. Anticomplementary activities of polysaccharides. DS: dextran sulfate with abt. M.W. 500,000 (DS-500), 8,000 (DS-8) and 5,000 (DS-5). H: heparin (abt. M.W. 5,000). CS: chondroitin sulfate (abt. M.W. 50,000). D: dextran (non-sulfate, abt. M.W. 199,000).

charides and their anti-TPF activity described in Tables 5 and 6 except for the difference in the order of effective doses.

Discussion

The vascular permeability-increasing components (TPF-I and TPF-II) were extracted from growing solid tumors with PBS containing 0.5 M KCl. In the sera of all mammals, the presence of a highly potent permeability factor called as PF/Dil has been known (6). PF/Dil was a large molecular weight substance identified as α -globulin and had fibrinolytic activity. It was strongly inhibited by soybean trypsin inhibitor. The possibility that VPi components in the present experiment might be PF/Dil was excluded because soybean trypsin inhibitor did not reduce the VPi activity of TPFs at all. The exact nature of TPF-I and TPF-II still remains obscure, but in all probability, TPF-I represents several categories of complement activators. Evidences favoring this hypothesis are: 1) TPF-I includes γ -globulin in aggregated form, 2) TPF-I antagonizes the hemolytic reaction triggered by hemolysin and complement in the *in vitro* assay system, and 3) dextran sulfate 500 (DS-500) abolishes the VPi activity of TPF-I.

It has been well-known that immune complex including aggregated γ -globulins absorbs Clq and activates the C system of the classical pathway releasing anaphylatoxins such as C3a and C5a (7-12). These factors are known as mediators directly or indirectly responsible for the increase of vascular permeability (13-15). It is probable, therefore, that TPF-I increases vascular permeability by triggering the complement activation system in the skin. Since DS-500 is a potent inhibitor of complements in the classical pathway (16, 17), it seemed to reduce VPi activity of TPF-I by interfering with the complements in the process. However, we can not completely deny the participation of another pathway. Some types of tumor cells are well-known to activate the alternative pathway possibly releasing C3a and C5a (18, 19). We could not determine how TPF-I participated in the dermal inflammation through the alternative pathway mechanism because Ca^{++} chelators such as EGTA and EDTA, inhibitors of the classical pathway, showed irritability in the skin and disturbed evaluation of their inhibitory effects on TPF-I activity. In any case, it will be quite important to see the *in situ* localization of activated C3a and C5a at the growth site of the tumor implant.

There was an apparent difference in molecular aspects between TPF-I and TPF-II. TPF-I had a large molecular weight (M.W. $>3 \times 10^5$) with a positive reaction to Ab to γ -globulin. TPF-II eluted from the Sepharose 4B column just before albumin. TPF-II also reacted with Ab to γ -globulin, but it did not absorb complement. As TPF-II was separated from the tumor extract after subjection to precipitation with ammonium sulfate, it might be segregated components from large-molecular TPF-I containing γ -globulin and probably activated anaphylatoxins. It is not known at present, however, whether γ -globulins in the TPF preparations bind tumor-associated antigen or not. For the analysis, further purification of TPF molecules and studies on the structure will be quite necessary.

A preliminary observation showed that the bulk of TPF-I and TPF-II seems to be isolated from the necrotic area in a solid tumor tissue

Fig. 3. Staining of the necrotic areas of Ehrlich solid tumor by the indirect immunofluorescent antibody technique. A: a thin tumor section (6 μ m thick) was treated with anti-TPF-I serum (rabbit) and secondly with FITC-labelled anti-rabbit IgG. $\times 100$. B: treated with anti-TPF-II serum (rabbit) and secondly with FITC-labelled anti-rabbit IgG. $\times 100$. C: treated with anti- γ -globulin serum (rabbit) and secondly with FITC-labelled anti-rabbit IgG. $\times 100$.

(cf. Fig. 3). At the necrotic site, various types of degenerated cells such as dead tumor cells, dead leucocytes and macrophages are abundant. When tumor cells are transplanted into murine dermal tissue, they grow in densely packed populations that develop into spheroid aggregates (solid tumor). This configuration seems to limit the absorption of oxygen and nutrients and the disposal of wastes particularly at inner site of a solid tumor. In and around the solid tumor, a gradual neovascularization and/or abnormal accumulation of fluid are common in a variety of host versus tumor combinations. Some investigators have suggested a participation of tumor-derived angiogenic factor in the process (20–22). A participation of tumor-derived permeability factor has also been reported previously (2, 3). It is interesting to see a participation of tumor-associated antigen in the process of TPF formation *in vivo*. In the present experimental model, a large amount of tumor cells particularly at the inner site of growing solid tumor degenerated in spite of the presence of neovascularization. At the necrotic site, there was considerable accumulation of γ -globulin immunologically or pathologically.

As far as mice strains in the present experiment are concerned, however, they could not produce antibody to any TPF preparations. This makes it difficult to distinguish a tumor-derived antigen binding with γ -globulin in TPF preparations.

Acknowledgements: We thank Mrs. A. Kato for technical assistance and Miss T. Tashiro and Miss Y. Nagano for the secretarial work.

References

- 1 Gullino, P.M.: A comprehensive treatise. *In* Cancer 3, Edited by Becker, F.F., p. 327–354, Plenum, New York (1975)
- 2 Dvorak, H.F., Orenstein, N.S., Carvalho, A.C., Churchill, W.H., Dvorak, A.M., Galli, S.J., Feder, J., Bitzer, A.M., Rypsc, J. and Giovenco, P.: Induction of a fibrin-gel investment—An early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J. Immunol.* **122**, 166–174 (1979)
- 3 Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F.: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983–985 (1983)
- 4 Sato, N.L., Fujimaki, M. and Niimura, S.: Characterization of tumor-induced inflammation and the effect of some anti-inflammatory drugs

- on the increased vascular permeability. Japan. J. Pharmacol. **37**, 31–38 (1985)
- 5 Harada, M., Takeuchi, M., Fukao, T. and Katagiri, K.: A simple method for the quantitative extraction of dye extravasated into the skin. J. Pharm. Pharmacol. **23**, 218–219 (1971)
 - 6 Wilhelm, D.L., Miles, A.A. and Mackay, M.E.: Enzyme-like globulins from serum reproducing the vascular phenomena of inflammation. II. Isolation and properties of the permeability factor and its inhibitor. Br. J. Exp. Pathol. **36**, 82–104 (1955)
 - 7 Becker, E.L.: Concerning the mechanism of complement action. II. The nature of the first component of guinea pig complement. J. Immunol. **77**, 469–478 (1956)
 - 8 Rapp, H.J. and Borsos, T.: Effects of low ionic strength on immune hemolysis. J. Immunol. **91**, 826–832 (1963)
 - 9 Müller-Eberhard, H.J., Polley, M.J. and Calcott, M.A.: Formation and functional significance of a molecular complex derived from the second and the fourth component of human complement. J. Exp. Med. **125**, 359–380 (1967)
 - 10 Cooper, N.R. and Müller-Eberhard, H.J.: The reaction mechanism of human C5 in immune hemolysis. J. Exp. Med. **132**, 775–793 (1970)
 - 11 Shin, H.S., Pickering, R.J. and Mayer, M.M.: The fifth component of the guinea pig complement system. II. Mechanism of SAC1, 4, 2, 3, 5b formation and C5 consumption by EAC1, 4, 2, 3. J. Immunol. **106**, 473–479 (1971)
 - 12 Hughes-Jones, N.C.: Functional affinity constants of the reaction between ¹²⁵I-labelled Clq binders and their use in the measurement of plasma Clq concentrations. Immunology **32**, 191–198 (1977)
 - 13 Dias da Silva, W. and Lepow, I.H.: Complement as a mediator of inflammation. II. Biological properties of anaphylatoxin prepared with purified components of human complement. J. Exp. Med. **125**, 921–946 (1967)
 - 14 Jensen, J.: Anaphylatoxin in its relation to the complement system. Science **155**, 1122–1123 (1967)
 - 15 Bokish, V.A., Müller-Eberhard, H.J. and Cochrane, C.G.: Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. J. Exp. Med. **129**, 1109–1130 (1969)
 - 16 Hack, C.E., Huijbregts, C.C.M. and Paardekooper, J.: Influence of ionic strength, EDTA concentration, endogenous Clq and polyanions on the ¹²⁵I-Clq-binding test. J. Immunol. Methods **72**, 197–206 (1984)
 - 17 Hughes-Jones, N.C. and Gardner, B.: The reaction between the complement subcomponent Clq, IgG complex and polyionic molecules. Immunology **34**, 459–463 (1978)
 - 18 Okada, H. and Baba, T.: Rosette formation of human erythrocytes on cultured cells of tumor origin and activation of complement by cell membrane. Nature **248**, 521–522 (1974)
 - 19 Budzko, D.B., Lachmann, P.J. and MacConell, I.: Activation of the alternative complement pathway by lymphoblastoid cell lines derived from patients with Burkitt's lymphoma and infectious mononucleosis. Cell. Immunol. **22**, 98–109 (1976)
 - 20 Folkman, J., Merler, E., Abernathy, C. and Williams, G.: Isolation of a tumor factor responsible for angiogenesis. J. Exp. Med. **133**, 275–288 (1971)
 - 21 Fenselau, A., Watt, S. and Mello, R.J.: Tumor angiogenic factor; Purification from the Walker 256 rat tumor. J. Biol. Chem. **256**, 9605–9611 (1981)
 - 22 Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M.: Heparin affinity; Purification of a tumor-derived capillary endothelial cell growth factor. Science **223**, 1296–1299 (1984)