Characterization of Tumor-Induced Inflammation and the Effect of Some Anti-Inflammatory Drugs on the Increased Vascular Permeability

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Abstract—The vascular bed in a murine dermal tissue responded to 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 inflammation was sensitive to glutathione (GSH). Glucocorticoids reduced the increased vascular permeability, but neither acetylsalicylic acid nor indomethacin did. The later vascular response was produced by a growing solid tumor in a continuous mode beginning at 5th to 10th day post inoculation. The degree of the increased vascular permeability in this chronic phase was in direct proportion to the wet weight of the solid tumor, and the inflammation was insensitive to glutathione. Glucocorticoids reduced the increased vascular permeability, but neither acetylsalicylic acid nor indomethacin did. The action of glucocorticoids on the tumor-induced vascular hyper-permeability was discussed in connection with a tumor factor possibly responsible for the vasoexudation.

Tumor cells are usually transferred to syngeneic or athymic animals to maintain a progressive growth of the transplantable tumors. In the experimental models, the body part bearing tumor cells become inflamed. A variety of leucocytes aggregate at the inflamed site (1), and some of them such as natural killer cells, cytotoxic T lymphocytes and macrophages have been the object of discussion in connection with the host defense mechanism against tumor cells (2–6). On the other hand, neovascularization into tumor implants has also been an attractive topic from the standpoint of nutritional supply for the tumor growth (7).

The characteristics of tumor-induced inflammation seems to be different to some extent from those induced by various phlogistic agents such as cotton pellet, carageenin, complete adjuvant, etc., because tumor cells escape acute and chronic rejection and proliferate in the animal host. The present experiment was undertaken in an attempt to characterize the tumor-induced

inflammation in mice, especially concerning changes in the vascular permeability in response to transplanted tumor cells and the effect of some anti-inflammatory drugs on the inflammatory process.

Materials and Methods

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.

Chemicals: Evan's blue dye was purchased from Sigma (Missouri, U.S.A.). Hydrocortisone acetate, prednisolone and glutathione (reduced form) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Acetylsalicylic acid was purchased from Yoshida Pharmaceutical Industries (Tokyo, Japan), and indomethacin was from

Sumitomo Chemicals Industries (Osaka, Japan).

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 (BAMC-1, ascites tumor cells) in BALB/c mice was kindly donated by Dr. H. Mitsui, Research Laboratories, Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), and 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); 4×10^6 – 4×10^7 cells were inoculated subcutaneously into the central portion of the dorsal skins of mice.

Measurement of extravasated dye content: One percent Evan's blue in physiological saline solution was intravenously injected into tumor-bearing mice by their tail veins at a volume of 0.1 ml per 10 g body weight. Twenty four hours after the dye injection, mice were killed. The dorsal skin containing the colored tumor tissue was cut into pieces after measuring the wet weight of the solid tumor, and the extravasated dye was extracted from the pieces in a medium composed of 14 ml of acetone and 6 ml of a 0.5% aqueous solution of sodium sulphate according to the method of Harada et al. (8). The extracted dye content was measured by the absorbance of the supernatant at 620 nm.

Effect of anti-inflammatory drugs on the increased vascular permeability: Ehrlich ascites tumor cells (1.4×10^7) were inoculated into dorsal skins of male ddY mice. One day or two weeks after the tumor cells inoculation, the tumor-bearing mice were divided at random into groups. Ten mice were used for each group. Anti-inflammatory drug was emulsified in a 2% solution of gum arabic. Non-steroidal drugs were administered orally 30 min before intravenous injection of 1% Evan's blue. Steroids were injected subcutaneously 60 min before dye injection. The control group of mice was given only the vehicle instead of the drug. Three hours after the dye injection, extravasated dye content at the site of tumor implants was measured.

Effect of glutathione on the increased

vascular permeability: One-tenth ml of glutathione (reduced form) of 0.1 and 1.0 mM concentration was injected subcutaneously to the tumor implants immediately after intravenous injection of Evan's blue dye. Mice were killed after 24 hr to measure the extravasated dye content as described above.

Isolation of vascular permeability factor from tumor tissues: Ehrlich solid tumors (50 g) were sliced and treated with 200 ml of acetone overnight at -20°C . After decanting the acetone, the freeze-dried tumor tissues were suspended in 200 ml of 10 mM phosphate buffer (pH 7.4) containing 0.5 M KCl for 24 hr at 2°C . The supernatant was obtained by $1500 \times g$ centrifugation, and it was fractionated by a micro-thin-channel ultrafiltration system (Amicon, model TFC-2; Massachusetts, U.S.A.). A component of high molecular weight (M.W. $> 300,000$, with Diaflow membrane XM 300) was precipitated in chilled 80% ethanol. After evaporating the ethanol using a freeze-drying system (Refrigeration for Science, Inc., model 5000B; New York, U.S.A.), the dried material was dissolved in 20 mM Tris-HCl (pH 7.5). After centrifuging at $1500 \times g$, the resultant supernatant was adjusted to give a protein concentration of 20 mg/ml and kept at -20°C until use.

Results

Changes in vascular permeability induced by tumor cells: Ehrlich tumor cells (1.4×10^7) or BAMC-1 tumor cells (4.0×10^7) were inoculated subcutaneously into the dorsal skins of male ddY mice. Ehrlich tumor cells progressed rapidly in this strain. The tumor implants began to form solid tumors about a week after inoculation and the size reached to 0.44 ± 0.321 g (mean $\pm$ S.D., $n=8$), 1.17 ± 0.475 g ($n=8$) and 1.67 ± 0.350 g ($n=8$) in wet weight on 10th, 15th and 19th days post inoculation. On the contrary, BAMC-1 tumor cells which are allogeneic to ddY mice regressed completely in this strain in spite of the inoculation of a larger amount of cells. At various stages of tumor progression or regression, 1% Evan's blue dye was injected intravenously into the tumor-bearing mice. The dye leaked out from the vascular bed surrounding tumor implants and accumulated

gradually into the tissues. The extravasated dye content at each stage is shown in Fig. 1. The vascular bed showed a two-phased response to the inoculated Ehrlich tumor cells: An initial increase in the vascular permeability at the site of tumor implant was seen in the early stage of 1 to 3 day post inoculation, and this was followed by a gradual increase in the vascular permeability beginning on 5th to 10th day post inoculation. In the cases bearing fully progressed Ehrlich solid tumors, about 60% of the injected dye leaked out and accumulated. In the mice inoculated with BAMC-1 tumor cells, there were only initial increases in the vascular permeability. The enhanced vascular permeability was decreased gradually from 5th day post inoculation and diminished according to the regression of the inoculated tumor cells.

BAMC-1 tumor cells (4×10^6) or Ehrlich tumor cells (1.4×10^7) were inoculated subcutaneously into dorsal skins of BALB/c mice. BAMC-1 tumor cells are syngeneic to this strain of mice. Ehrlich tumor cells are virulent also in this strain. Both tumor cells

progressed and formed solid tumors. The size of BAMC-1 solid tumors reached to 0.12 ± 0.009 g (mean $\pm$ S.D., $n=8$), 0.49 ± 0.318 g ($n=8$) and 0.76 ± 0.382 g ($n=8$) at 10th, 15th and 19th days post inoculation respectively. That of Ehrlich solid tumors reached to 0.18 ± 0.104 g ($n=8$) at the 13th day and 0.58 ± 0.374 g ($n=8$) at the 20th day. Changes in the vascular permeability at each stage are shown in Fig. 2. Initial increase in the vascular permeability was high in inoculation of Ehrlich tumor cells and was slight in that of BAMC-1 tumor cells, while gradual increases in the later stage were dependent on the degree of tumor progression in both kinds of cells.

To athymic nude mice, Ehrlich tumor cells (1.4×10^7) or BAMC-1 tumor cells (1.4×10^7) were inoculated subcutaneously into the dorsal skins. Both tumor cells progressed and formed solid tumors. The size of Ehrlich solid tumors reached to 0.27 ± 0.072 g (9th day, mean $\pm$ S.D., $n=8$), 0.89 ± 0.400 g (13th day, $n=8$) and 1.58 ± 0.381 g (17th day, $n=8$).

Fig. 1. Changes in vascular permeability at the site bearing tumor cells in dorsal skins of male ddY mice. ●: Inoculation of Ehrlich tumor cells (1.4×10^7), ○: Inoculation of BAMC-1 tumor cells (4.0×10^7). Mean extravasated dye content $\pm$ S.D. (N=8).

Fig. 2. Changes in vascular permeability at the site bearing tumor cells in dorsal skins of male BALB/c mice. ●: Inoculation of Ehrlich tumor cells (1.4×10^7), ○: Inoculation of BAMC-1 tumor cells (4.0×10^6). Mean extravasated dye content $\pm$ S.D. (N=8).

That of BAMC-1 reached to 0.04 ± 0.013 g (13th day, $n=8$) and 0.14 ± 0.026 g (20th day, $n=8$). Changes in the vascular permeability at each stage are shown in Fig. 3. Initial increases in the vascular permeability at an early stage (1–3 day) were lower in these combinations than those in Ehrlich tumor cells-ddY mice, Ehrlich tumor cells-BALB/c mice, or of BAMC-1 tumor cells-ddY mice, though the increases in the vascular permeability at the later stage were dependent on the degree of tumor progression in both types of tumor cells.

Through these experiments, it is noteworthy that there was a significant linear correlation between the degree of dye extravasation in the later chronic stage and the wet weight of the solid tumor in all hosts versus tumors combinations as shown in Fig. 4.

Effect of anti-inflammatory drugs on the increased vascular permeability induced by tumor cells: To evaluate effect of anti-inflammatory drugs on the increased vascular permeability induced by tumor cells, ddY mice

bearing Ehrlich tumor implants were chosen because of the severity of the inflammation. Initially, effects of hydrocortisone, prednisolone, acetylsalicylic acid and indomethacin were examined on the acute phase of vascular response in mice bearing 1-day-old tumor implants. As shown in Table 1, both glucocorticoids reduced the dye extravasation, but neither acetylsalicylic acid nor indomethacin did. Secondly, effects of the same doses of drugs were examined on the chronic phase of vascular response in mice bearing 15-day-old solid tumors. In this case, the effect of drugs on dye extravasation was evaluated by comparison of extravasated dye content (mg)/g tumor wet weight. The results are summarized in Table 2. Both glucocorticoids reduced the dye extravasation, but neither acetylsalicylic acid nor indomethacin did.

Effect of glutathione on the increased vascular permeability induced by tumor cells: To see the effect of sulphhydryl agent on the process of tumor-induced inflammation, 0.1 ml of 0.1 and 1.0 mM glutathione (GSH) was injected subcutaneously into the part bearing one-day-old Ehrlich tumor implants in ddY mice or into the site where allogeneic BAMC-1 tumor cells had regressed after 8 day post inoculation in ddY mice. The same doses of glutathione were also injected to the part bearing 15-day-old Ehrlich solid tumor in ddY mice. Extravasated dye contents during the following 24 hr are shown in Table 3. Glutathione reinforced the increased state of the vascular permeability in the acute phase induced by inoculated Ehrlich tumor cells, and it reproduced the vascular hyper-permeability state at the site where BAMC-1 tumor cells had regressed. Glutathione, however, did not show a significant effect on the chronic stage of the enhanced vascular permeability induced by developed solid tumors.

Effect of anti-inflammatory drugs on the increased vascular permeability induced by tumor permeability factor: A linear correlation between the degree of dye extravasation at the tumor-bearing site and the weight of the solid tumor suggested a possible participation of a tumor factor responsible for the vasoexudation. We used a soluble component of high molecular weight (M.W. >300,000) isolated

Fig. 3. Changes in vascular permeability at the site bearing tumor cells in dorsal skins of athymic nude mice. ●: Inoculation of Ehrlich tumor cells (1.4×10^7). ○: Inoculation of BAMC-1 tumor cells (1.4×10^7). Mean extravasated dye content $\pm$ S.D. ($N=8$).

Fig. 4. Individual correlation between wet weight of a developed solid tumor and a dye extravasation. A: Ehrlich tumor cells (1.4×10^7) was inoculated into the dorsal skins of male ddY mice. The regression line was: $y = 1.12x + 0.33$, $r = 0.876$, $t = 7.493$ ($P < 0.001$). B: BAMC-1 tumor cells (4.0×10^6) was inoculated into the dorsal skins of male BALB/c mice. The regression line was: $y = 0.53x + 0.27$, $r = 0.864$, $t = 7.288$ ($P < 0.001$). C: Ehrlich tumor cells (1.4×10^7) was inoculated into the dorsal skins of male athymic nude mice. The regression line was: $y = 0.86x + 0.27$, $r = 0.833$, $t = 6.903$ ($P < 0.001$)

Table 1. Effect of anti-inflammatory drugs on the increased vascular permeability induced by tumor cells

Drug	Dose (mg/kg)	Dye content (mg $\pm$ S.D.)	Inhibitory ratio (%)
Control	—	0.14 $\pm$ 0.045	
Acetylsalicylic acid	300, p.o.	0.13 $\pm$ 0.043	N.S.
Indomethacin	5, p.o.	0.18 $\pm$ 0.093	N.S.
Control	—	0.11 $\pm$ 0.038	
Hydrocortisone	5, s.c.	0.08 $\pm$ 0.012***	27.3
	20, s.c.	0.06 $\pm$ 0.021***	45.5
Prednisolone	5, s.c.	0.06 $\pm$ 0.011***	45.5

ddY Mice bearing 1-day-old Ehrlich tumor implants were used. Statistically significant at *** $P < 0.001$ by Student's *t*-test ($N = 10$). N.S.: not statistically significant.

from a solid tumor as a candidate for the factor because of its high potency. It contained approximately 5% nucleic acid, 47% protein and 7% carbohydrate. Subcutaneous injection of 0.1 ml of the sample (at 20 mg/ml of protein concentration) produced severe vascular hyper-permeability with 1 to 2 hr duration as shown in Fig. 5. Ac-

cordingly, effects of anti-inflammatory drugs and glutathione were evaluated in this model as described before. As shown in Table 4, hydrocortisone, prednisolone and a higher dose of acetylsalicylic acid reduced the dye extravasation, but indomethacin did not. Glutathione was inert and had no effect on the changed vascular permeability.

Table 2. Effect of anti-inflammatory drugs on the increased vascular permeability induced by growing solid tumor

Drug	Dose (mg/kg)	Dye content/g tumor wt. (mg±S.D.)	Inhibitory ratio (%)
Control		0.70±0.141	
Acetylsalicylic acid	300, p.o.	0.86±0.156	N.S.
Control		0.66±0.149	
Indomethacin	5, p.o.	0.98±0.464	N.S.
Hydrocortisone	20, s.c.	0.38±0.090***	42.4
Prednisolone	5, s.c.	0.42±0.075**	36.4

ddY Mice bearing 15-day-old Ehrlich tumor implants (solid tumors) were used. Statistically significant at **P<0.01, ***P<0.001 (N=10). N.S.: not statistically significant.

Table 3. Effect of glutathione on the increased vascular permeability induced by tumor cells

Tumor implant in ddY mice	Glutathione (mM)	Extravasated dye content	
		(mg/site)	(mg/g tumor wt.)
Ehrlich tumor cells (1-day-old, progressing)	—	0.36±0.021	
	0.1	0.43±0.073	
	1.0	0.52±0.064***	
BAMC-1 tumor cells (8-day-old, regressed)	—	0.17±0.030	
	0.1	0.15±0.019	
	1.0	0.25±0.024***	
Ehrlich tumor cells (15-day-old, solid tumor)	—	1.28±0.454	1.90±0.377
	0.1	1.29±0.639	2.04±0.616
	1.0	1.22±0.656	1.89±0.699

Glutathione (reduced type, 0.1 ml/body) was injected subcutaneously to tumor implant. Statistically significant at ***P<0.001 (N=10)

Fig. 5. Dye extravasation induced by a permeability factor extracted from tumor tissues. Each 0.1 ml of the crude permeability factor (20 mg/ml protein concentration) was injected subcutaneously into the central portion of dorsal skins of male ddY mice. At 0, 1, 2, 3 or 4 hours after the permeability factor injection, 0.1% Evan's blue was injected intravenously to each group of mice, respectively, in a dose of 0.1 ml/10 g b.w. In all groups, mice were killed one hour after the dye injection to measure the extravasated dye content within each one hour's interval at the inflamed skin. Results are means±S.D. (n=5).

Table 4. Effect of anti-inflammatory drugs and glutathione on the increased vascular permeability induced by tumor permeability factor

Drugs	Doses	Dye extravasation (%)
		100.0
Acetylsalicylic acid	100 mg/kg (p.o.)	84.8
	300 mg/kg (p.o.)	51.1*
Indomethacin	1 mg/kg (p.o.)	114.1
	5 mg/kg (p.o.)	87.4
	10 mg/kg (p.o.)	86.2
	20 mg/kg (p.o.)	69.6
Hydrocortisone	20 mg/kg (s.c.)	41.4***
Prednisolone	5 mg/kg (s.c.)	53.4***
Glutathione	1 mM (0.1 ml, s.c.)	108.3

1% Evan's blue was injected intravenously to ddY mice. Immediately after the dye injection, acetylsalicylic acid, indomethacin or glucocorticoids were given as described in the Table. One-tenth ml of a tumor permeability factor (crude, 20 mg/ml protein concentration) was injected subcutaneously into dorsal skins of mice 30 min after the nonsteroidal drugs and 60 min after glucocorticoids administration. Glutathione was included in the solution of tumor permeability factor. Three hours thereafter, mice were killed to measure extravasated dye content. Statistically significant at * $P < 0.05$ (N=5), *** $P < 0.001$ (N=5).

Discussion

Initial vascular response to tumor cells was maximum on 1 to 3 day post inoculation. Generally speaking, as dye extravasation in the initial phase was greater in the allogeneic host versus tumor combination, a major part of the inflammation may be produced by alloantigen. This initial stage of inflammation exceptionally showed sensitivity to glutathione for an unknown reason. In the Arthus-type inflammation, sensitivity to glutathione was well characterized by the participation of thiol-protease in the inflammatory process (9).

The vascular response in the chronic phase seems essentially different from the initial one and was particularly impressive as tumor-growth related inflammation. The degree of dye extravasation related more closely to the degree of progression of a solid tumor, and it did not show sensitivity to glutathione. The present results suggest a possible participation of a tumor factor responsible for the vasoexudation in this chronic phase. A soluble component with high molecular weight which was isolated from the solid tumor seems to include a vasoactive substance as a permeability

factor. The component contains approximately 5% nucleic acid, 47% protein and 7% carbohydrate. Its activity to increase vascular permeability is diminished by pronase treatment. From Walker 256 carcinoma and chondrosarcoma (rats), tumor-derived angiogenic factors with low molecular weight were isolated (10–12). It is probable that transplanted tumor cells derive larger amount of nutrition for their progressive growth by increasing vascular permeability concomitantly with induced neovascularization. In earlier works, it was shown that tumor cells preferentially use plasma protein for their nitrogen source (13, 14).

A single injection of glucocorticoids reduced both tumor-induced and a permeability factor (TPF)-induced vascular hyper-permeability. Effect of glucocorticoids seems to depend on the direct local action of the steroids. Recently, glucocorticoids were shown to suppress serotonin-induced oedema by bringing about production of a specific protein through gene expression (15). It is possible that glucocorticoids might exert an effect on TPF activity by inducing an inhibitory protein or directly suppress TPF production. It is, therefore, of particular interest to examine the effect of addition of

an inhibitor of protein biosynthesis to the steroids treatment of TPF-induced inflammation. A higher dose of acetylsalicylic acid suppressed TPF-induced vascular hyperpermeability, although it was inert in suppressing tumor-induced ones. The reason for the discrepancy is not clear. Since TPF is still in a complexed form of protein, polysaccharides, nucleic acid, etc., the effect of acetylsalicylic acid on the TPF-induced inflammation might have relation to an inflammation induced by an additional factor which may contaminate the TPF preparation. Further purification of TPF is necessary to define the chemical and biological nature concerning its molecular aspects, antigenicity or in vivo localization.

Indomethacin was inert in suppressing both tumor-induced and TPF-induced vascular hyperpermeability. This may suggest, as far as the present experimental model is concerned, that tumor cells provoke vascular hyperpermeability apart from the prostaglandin generation system. There is additional data that esculetin and caffeic acid, which were reported to inhibit 5-lipoxygenase (16, 17), are inert in suppressing tumor-induced inflammation (data not shown). The participation of leukotriene may also be negligible in tumor-induced inflammation in the present experiment.

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