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Enhancement of Blood Stasis and Vascular Permeability in Meth-A Tumors by Administration of Hyperthermia in Combination with Tumor Necrosis Factor

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Blood stasis and vascular permeability induced by tumor necrosis factor (TNF) in Meth-A tumors transplanted in BALB/c mice were significantly enhanced by hyperthermia at 40°C for 30 min immediately following TNF administration. A dose-dependent, sustained decline in the intratumoral blood flow rate occurred following the administration of TNF alone (i.v.; 5×10^3, 5×10^4, or 5×10^5 JRU/kg) and was enhanced by the administration of hyperthermia in combination with the TNF, even though no decline occurred with hyperthermia alone. The combination of TNF at 5×10^5 JRU/kg and hyperthermia resulted in a blood flow ratio (ratio of blood flow after administration to that before) of 0.47 at 1 h compared with a ratio of 0.65 at 1 h after TNF alone. The blood flow in normal skin sites did not decrease in any case. The permeability of the intratumoral vasculature similarly increased in a dose-dependent manner after the administration of TNF alone and was further increased by combination with hyperthermia, even though no increase occurred with hyperthermia alone. The mean permeability in mice receiving TNF alone at 5×10^5 JRU/kg was 1.35 times that in untreated mice. In mice receiving TNF at the same dose together with hyperthermia, the ratio was increased to 1.65. The results suggest that TNF selectively suppresses intratumoral blood flow, that this effect is enhanced by mild hyperthermia, and that the mechanism of the suppression by TNF with or without hyperthermia partly involves an increase in blood vessel permeability.

Key words: Tumor necrosis factor — Hyperthermia — Blood stasis — Vascular permeability — Meth-A tumor

TNF[2] is known to inhibit tumor growth and to cause tumor necrosis in tumor-bearing mice,[1-3] and various studies have shown injury to the tumor vasculature to be involved in its antitumor effect. We have previously reported the observation of vascular congestion in tumors at 1 h after TNF administration, hemorrhage at 4–6 h, and complete blood circulation blockage at 24 h,[4] via a sight glass implanted in the transplanted tumor region. Similar findings have been reported by Palladino *et al.*, Talmadge *et al.*, Kawai *et al.*, and Johnson *et al.*, based on their analyses of histological changes and ^{59}Fe-RBC accumulations in tumor tissue following TNF administration.[5-8] The nature of the vascular injury, however, has not been elucidated in *in vivo* investigations.

Hyperthermia was previously shown to enhance markedly the antitumor effects of TNF without itself showing such effects.[9-11] We therefore investigated, in the present study, the effects of TNF administered alone and in combination with hyperthermia on intratumoral blood flow and vascular permeability *in vivo*.

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[2] Abbreviations used: TNF, tumor necrosis factor; Meth-A, methylcholanthrene-induced A-cells; i.d., intradermal(ly); i.p., intraperitoneal(ly); i.v., intravenous(ly); SPF, specific pathogen-free; JRU, Japan reference unit.

MATERIALS AND METHODS

Animals Female BALB/c mice, purchased at 4 weeks of age from Charles River Japan, Inc. and then screened for abnormality under quarantine for 1 week prior to use, were maintained in SPF chambers with a 12 h daylight cycle (lights on at 7:00 a.m., off at 7:00 p.m.) and free access to sterilized feed and tap water.

Meth-A cell implantation Mouse fibrosarcoma Meth-A cells (gift from Asahi Chemical Ind. Co., Ltd., Tokyo) were implanted i.d. in the right abdominal aspect of BALB/c mice (0.1 ml suspension of 1×10^6 cells/mouse), and mice bearing resultant tumors of 6–8 mm on the 7th day with no sign of natural necrosis were randomly separated into 8 groups of 5 mice each for the investigation of blood flow and into 33 groups of 5 mice each for the investigation of vascular permeability.

Administration of TNF and hyperthermia Human recombinant TNF (gift from Asahi Chemical Ind. Co., Ltd.; relative activity, 2.3×10^6 JRU/mg protein; endotoxin, less than 10 pg/10^6 JRU)[12] was diluted in phosphate-buffered saline with 0.1% gelatin (pH 7.4) to prevent adhesion of TNF to syringe and container surfaces, to obtain the experimentally employed doses of 0, 5×10^3, 5×10^4 and 5×10^5 JRU/kg body weight, and injected via the tail vein after anesthetization of the animals

with pentobarbital sodium (30 mg/kg; i.p.; Dainippon Pharmaceutical Co., Ltd., Tokyo). Hyperthermia was then immediately administered by a previously described procedure which was confirmed to afford rectal and intratumoral temperatures of 40°C, the temperature of the water bath, within 5 min after initial immersion and thereafter for its duration.[9] Briefly, the mouse was fixed in a 50 ml plastic centrifuge tube (Falcon Labware) having 16 holes of 7 mm diameter, and was lowered into a water bath at 40°C for 30 min so as to immerse the whole mouse body including the tumor-bearing portion.

Measurement of blood flow Blood flow in the tumor region and that in the normal skin layer of the left abdominal aspect were measured at a depth of approximately 1 mm with an intratissue vascular blood circulation laser flowmeter (ALF 2100, Unique Medical Co., Tokyo) via a probe fixed with adhesive to the tumor surface or skin and left in place throughout the measurement period, at 0, 0.5, 1, 2, and 4 h after TNF administration. The blood flow ratio in each case was calculated as the post-administration flow value divided by the pre-administration flow value.

Determination of vasculature permeability Evans blue (Wako Pure Chemical Ind. Co., Ltd., Tokyo) was administered to mice i.v. at 50 mg/kg at the indicated times following TNF injection, and the mice were killed 10 min thereafter by cervical vertebral dislocation, followed by resection of the tumor. The resected tumor was weighed and the pigment was then extracted by immersion for 24 h in 1.2 ml of dimethyl sulfoxide (Wako Pure Chemical Ind. Co., Ltd.). The optical density was measured with an absorption meter (UV-160, Shimadzu Co., Tokyo) at 620 nm. The permeability ratio was calculated as the optical density per gram of tumor after TNF administration divided by that of tumor without TNF administration.

Statistical methods The significance of differences between pre- and post-administration blood flow ratio and between the ratios of permeability with and without TNF was evaluated by using Student's t test. Blood flow ratios and permeability ratios are reported here as mean ± SE.

RESULTS

Intratumoral blood flow A dose- and time-dependent decrease in intratumoral blood flow was observed after administration of TNF alone at 5×10^4 and 5×10^5 JRU/kg, but not at 0 or 5×10^3 JRU/kg (Fig. 1).

Hyperthermia alone had no significant effect on intratumoral blood flow, but its administration immediately after TNF injection at 5×10^3, 5×10^4, and 5×10^5 JRU/kg resulted in blood flow below that observed following injection of the same TNF doses alone (Fig. 2). This effect was particularly evident at the TNF dosage of 5×10^5 JRU/kg, as the combination of TNF and hyperthermia resulted in a mean blood flow ratio of 0.47 at 1 h ($P<0.05$), compared with the mean blood flow ratio of 0.65 at 1 h after administration of TNF alone, and virtually complete cessation of blood flow by 4 h.

Blood flow in normal skin site Administration of TNF alone (0, 5×10^3, 5×10^4, 5×10^5 JRU/kg) had no significant effect on blood flow in the normal skin site in the dermis on the opposite side of the abdomen from the

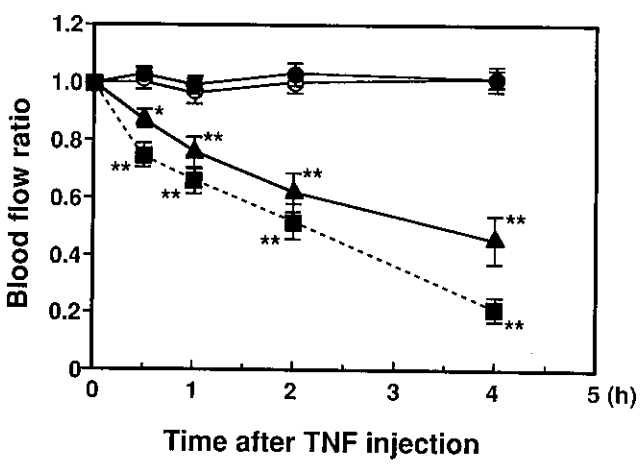

Fig. 1. Blood flow in Meth-A tumors following TNF i.v. injection. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean ± SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

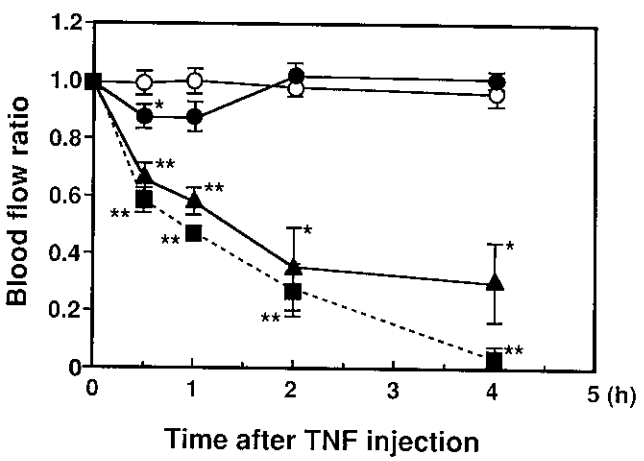

Fig. 2. Blood flow in Meth-A tumors following TNF i.v. injection and hyperthermia. Blood flow in the tumor was measured with a laser flowmeter. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean ± SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

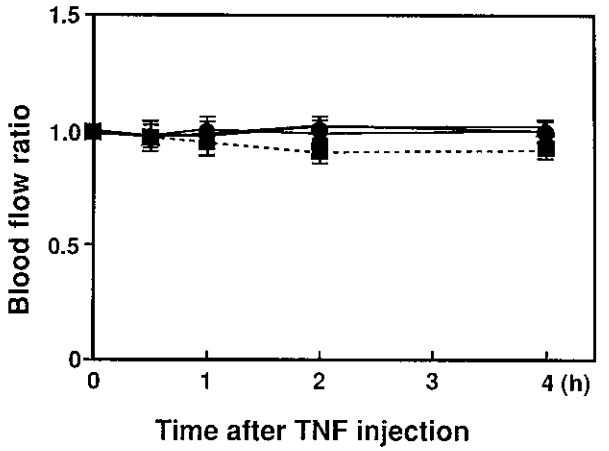

Fig. 3. Blood flow in normal dermis following TNF i.v. injection. Blood flow in the left abdominal dermis of the Meth-A tumor-bearing BALB/c mice described in Fig. 1 was measured with a laser flowmeter following TNF injection. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean±SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

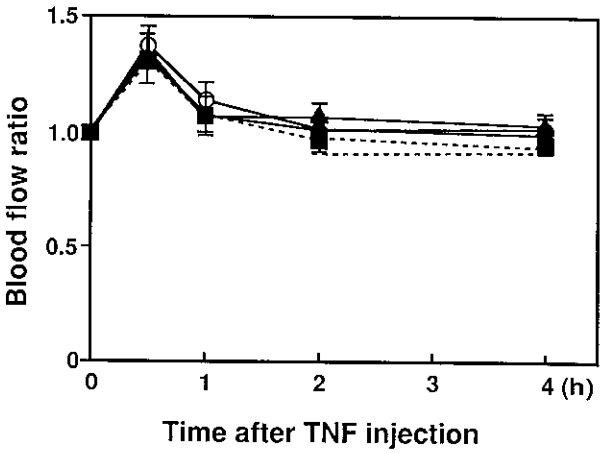

Fig. 4. Blood flow in normal dermis following TNF i.v. injection and hyperthermia. Blood flow in the left abdominal dermis of the Meth-A tumor-bearing BALB/c mice described in Fig. 3 was measured with a laser flowmeter following TNF injection and immersion in water at 40°C. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean±SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

tumor site (Fig. 3). In the group receiving hyperthermia alone, the mean blood flow in the normal dermis increased throughout the 30 min administration, but returned to the original rate in the following 30 min and remained constant thereafter. In the groups receiving both TNF and hyperthermia, the mean blood flow did not depart significantly from that observed in the group receiving hyperthermia only. Thus the combination therapy caused no decrease such as that observed in the intratumoral blood flow (Fig. 4).

Vasculature permeability Neither TNF alone at 0 or 5×10^3 JRU/kg nor hyperthermia alone showed any significant effect on the mean permeability of the tumor vasculature. TNF alone at 5×10^4 and 5×10^5 JRU/kg resulted in a significant and dose-dependent increase in permeability at both 60 min and 90 min after administration (Fig. 5). The maximum mean permeability in these groups, observed at 90 min after injection of 5×10^5 JRU/kg, was 1.35 times that of the control group receiving neither TNF or hyperthermia.

TNF at 5×10^3 JRU/kg with hyperthermia showed no significant effect on permeability in the tumor, but TNF at 5×10^4 or 5×10^5 JRU/kg with hyperthermia resulted in mean permeabilities higher than those at the same TNF doses without hyperthermia (Fig. 6). The maximum mean permeability with TNF and hyperthermia, again observed at 90 min after administration of 5×10^5 JRU/kg of TNF, was 1.65 times that of the control group receiving no TNF or hyperthermia ($P<0.05$).

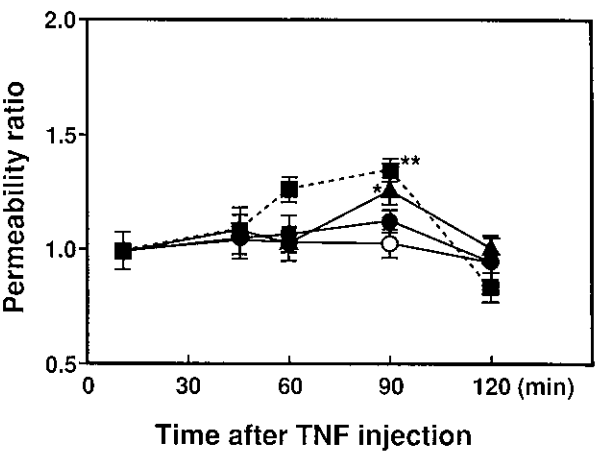

Fig. 5. Vascular permeability in Meth-A tumors following TNF i.v. injection. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean±SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

DISCUSSION

Since Carswell *et al.* first reported the appearance of necrosis and reduction in the size of transplanted tumors in mice following TNF administration,[1] several researchers have noted that injury to the tumor vasculature is related to the necrotic response.[2-8] In *in vitro* studies,

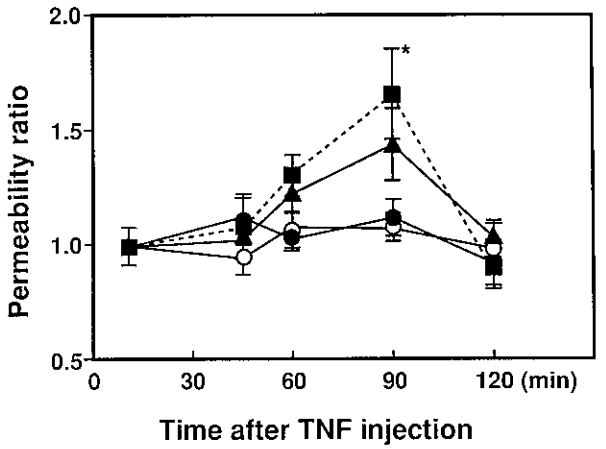

Fig. 6. Vascular permeability in Meth-A tumors with TNF i.v. injection and hyperthermia. TNF doses (JRU/kg): ○, 0; ●, 5×10^3; ▲, 5×10^4; ■, 5×10^5. Plots, mean±SE (bars) of 5 mice. *, $P<0.05$; **, $P<0.01$; by Student's t test in comparison with the 0 JRU/kg value.

the mechanism of this injury was reportedly related to changes in the endothelial cells,[13,14] induction of procoagulant activity,[15,16] and induction of cytotoxic radical production by leukocytes and tumor cells.[17–21]

Following our initial reports that hyperthermia markedly enhances the antitumor effect of TNF in mice,[9,10] various *in vitro* studies showed that elevated temperature enhances the antitumor effect of TNF,[22–24] and that it activates lysosomal enzymes and increases the production of oxygen radicals in cells, which effects were thought to be directly responsible for the necrotic effect of TNF.[25] Several *in vivo* studies have shown that TNF with hyperthermia clearly increases both tumor necrosis and intratumoral hemorrhage, as compared with TNF alone.[26,27]

The sustained decrease in intratumoral blood flow which was observed in the present study following the administration of TNF alone at high doses would in itself deprive the tumor cells of the levels of oxygen and nutrient supply and waste removal necessary for cell viability, and may thus be presumed to be at least a partial cause of tumor necrosis.

The absence of any significant suppression of the intratumoral blood flow by hyperthermia alone, at 40°C for 30 min, is in accord with Eddy's histological findings of intratumoral congestion, blockage, and hemorrhage following 30 min at 43°C and 45°C but not at 41°C,[28] and suggests that significant enhancement by this mild hyperthermia of the intratumoral blood flow suppression by TNF, as observed in the present study, was the result of a temperature dependence of TNF cytotoxicity which has been shown *in vitro* by Kull and Cuatrecasas,[22] Matthews and Watkins,[23] and Ruff and Gifford,[24] rather than a direct effect of the hyperthermia on the tumor or tumor vasculature.

The absence of any significant effect of TNF on the blood flow in the normal skin site, in contrast to its suppression of intratumoral blood flow, suggests that TNF may be selective for intratumoral vasculature. It is not yet clear, however, whether this apparent difference between the susceptibilities of intratumoral and normal vasculatures is attributable simply to the structural weakness of the intratumoral vasculature[29,30] or to differences between the two vasculatures in the number or activity of their TNF receptors.[31]

Hyperthermia had no appreciable effect on the blood flow of the normal skin site when administered either alone or following TNF injection, except for an increase which continued throughout the hyperthermia administration and was followed by a rapid return to the original levels.

A depressed intratumoral blood flow may generally be a result of a blockage of the vasculature leading into the tumor, a blockage of that leading out of the tumor, or a leakage of plasma or serum from the vasculature within the tumor and a concomitant increase in the intravascular blood viscosity, or to some combination of these three causes.

Blockage of the blood entry vasculature, however, would result in intratumoral ischemia and is therefore unlikely in the case of TNF, since various pathological analyses have shown a progressive intratumoral congestion to occur following TNF administration.[5–7] Shimomura *et al.* and Talmadge *et al.*, on the other hand, have shown that injection of TNF is followed by the formation of thrombi in the intratumoral vasculature.[32,33] This may be presumed to result in intravasculature stagnation and congestion, blockage of blood exit from the tumor, and thus in decreased blood flow through the tumor vasculature.

The results of the present investigation suggest that increased vasculature permeability is also involved in the decrease of intratumoral blood flow following TNF administration. The permeability, as measured by Evans blue uptake in the tumor tissue, clearly increased during the first hour, and reached a maximum at about 60–90 min after the administration of TNF. It is therefore distinguishable from intratumoral hemorrhaging, or leakage of red blood cells, which has been found to occur in Meth-A tumors some 4–6 h after TNF administration.[4] The permeability increase observed in the present study is therefore considered to be a specific effect of TNF administration, separate from and preceding the onset of intratumoral hemorrhaging. The decline in Evans blue uptake which was observed after attainment of maximum uptake at about 60–90 min, may be considered the effect

of decreasing blood entry into the tumor vasculature, rather than a reversal in the trend toward greater blood vessel permeability.

The administration of hyperthermia in combination with TNF resulted in significantly higher permeability and lower blood flow than with TNF alone, even though neither of these effects was observed when hyperthermia alone was administered, thus suggesting that the mild hyperthermia (40°C for 30 min) did not in itself directly increase the intravascular permeability or decrease the intratumoral blood flow, but rather enhanced these effects of TNF.

The results indicate, in conclusion, that the administration of TNF results in leakage of plasma or serum from the intratumoral vasculature and thus to increased viscosity and reduced flow of blood through the vasculature inside the tumor. They suggest, furthermore, that the previously observed enhancement by hyperthermia of TNF tumor growth suppression[9, 10] is at least partly attributable to enhancement of the increase in vasculature permeability induced by TNF, and that increased vasculature permeability, in addition to intravascular thrombi formation,[32, 33] is responsible for the suppression of intratumoral blood flow induced by TNF administration.

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