[CANCER RESEARCH 48, 2179–2183, April 15, 1988]

Toxic Effect of Tumor Necrosis Factor on Tumor Vasculature in Mice

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ABSTRACT

Stereoscopic observation via an implanted sight glass in mice bearing transplanted methylcholanthrene-induced A-cells showed tumorivascular hemorrhage at 1–2 h after tumor necrosis factor (TNF) administration, congestion at 4–6 h, and hemorrhage, congestion, and blood circulation blockage at 24 h.

Histological examination after TNF administration to mice bearing similar methylcholanethrene-induced A-cell transplants showed thrombus formation in the tumor vasculature at 4 h and thereafter. Suppression of this thrombus formation with heparin had no apparent influence on the necrotic response, tumor growth inhibition or complete cure rate following TNF administration to mice bearing the methylcholanethrene-induced A-cell tumors. The results suggest that direct toxicity of TNF on tumor vasculature is a factor in the overall antitumor mechanism of TNF.

INTRODUCTION

TNF[1] is an anticancer cytokine derived from monocytes and macrophages (1–3) and is known to exert a strong antitumorigenic effect against tumor cells both *in vivo* (1, 4) and *in vitro* (5). Its direct effect on tumor cells has been demonstrated *in vitro* and undoubtedly plays a part in its antitumor effect. Many aspects of the overall antitumor mechanism nevertheless remain unclear. One of these is the possible involvement of reactions affecting the tumor blood vessels, which is suggested by the hemorrhagic necrosis which is usually observed in the course of transplanted tumor regression following administration of TNF in mice (1). *In vitro* studies have provided evidence of such reactions. TNF reportedly exerts a cytotoxic effect on endothelial cells from bovine arteries (6) and human umbilical veins (7), and promotes thrombus formation by acting on vascular endothelial cells and stimulating production of a procoagulant factor (8, 9). No direct evidence of their (6-9) occurrence *in vivo* has yet been reported.

The present study is a more direct investigation of the effect of TNF on tumor vasculature. To allow visual observation of changes in the tumor vasculature under the influence of TNF, we implanted a sight glass in mice before their inoculation with Meth-A cells. We also investigated the influence of thrombus suppression with heparin on the antitumor effect of TNF.

MATERIALS AND METHODS

Materials. Human recombinant TNF (2.37×10^6 units/mg protein) (10) was provided by Asahi Chemical Industry Co., Ltd. Heparin (Midori Juji, Osaka, Japan) was used as anticoagulant. BALB/c mice (female, 5 weeks of age; Clea Japan Co., Ltd.) were used.

Cell Culture. CPAE cells (bovine endothelial cells of the pulmonary artery, L-M cells (mouse tumorigenic fibroblast) and Meth-A cells (mouse fibrosarcoma) were used. CPEA and L-M cells were maintained in Eagle's MEM (Nissui Pharmaceutical) containing 10% fetal bovine serum (Flow Laboratories) in a 5% CO_2 incubator at 37°C. All incubations were performed under the same conditions except as otherwise noted. Meth-A cells were passaged i.p. in mice.

Observation of Tumor Vasculature. Observations were made under a stereoscopic microscope, through a sight glass consisting of two stainless steel plates (11), one containing a drilled hole 13 mm in diameter which was covered by a clear glass plate affixed to the outside surface.

On day 0, the sight glass was affixed with surgical thread to a raised portion of the dorsum as shown in Fig. 1 with an excised dermal area facing the drilled hole, and Meth-A cells (1×10^6 cells/0.1 ml Eagle's MEM) were then injected into the region under the drilled hole. On day 9, TNF (1×10^4 units/0.1 ml 0.1% gelatin-phosphate buffered saline, pH 7.4) or the TNF diluent as control was administered i.v., and the tumor vasculature under the sight glass was observed 1, 2, 4, 6, and 24 h later with the mouse under secobarbital anesthesia (0.6 mg, i.p.; Yoshitomi Pharmaceutical). Prophylactic penicillin (0.8 mg; Toyama Chemical Industry Co.) was administered on days 0, 1, 2, 4, and 6.

Evaluation of Antitumor Effect. Meth-A cells (1×10^6 cells/0.1 ml Eagle's MEM) were injected i.d. in each mouse on day 0, and on day 6 TNF (1×10^3–1×10^4 units/0.1 ml) was administered i.v. with or without prior injection of heparin (3 units/0.1 ml) via the caudal vein. Control mice each received TNF diluent (0.1% gelatin-phosphate buffered saline, pH 7.4, 0.1 ml) instead of TNF. Tumor weight was estimated from measurements on days 6 and 8 of the minor (a) and major (b) axes, as $a^2 \times b/2$. Necrotic response was graded on day 8 as +++ (necrosis over entire tumor), ++ (necrosis over 50% or more of tumor), + (necrosis over less than 50% of tumor), and − (no apparent necrosis). Occurrence of complete cure was judged on day 97. Tumor sections were obtained from 8 mice 4 h after TNF administration with and without heparin and periodically thereafter, embedded in paraffin, and subjected to histological examination by optical microscope after hematoxylin and eosin staining.

Cytotoxic Assay. One hundred μl of CPAE or L-M cells (1×10^5 cells/ml) and 100 μl of TNF at various concentrations were added to the wells of a 96-well microculture plate and incubated for 48 h. Cytotoxicity was then assessed by dye uptake method (12–14).

RESULTS

Effect of TNF on Tumor Vasculature. Stereomicroscopic observation of the newly formed blood vessels of the Meth-A tumor under the sight glass showed hemorrhage from capillary vessels at 1 and 2 h after TNF administration, both hemorrhage from capillary vessels and congestion of nutrient vessels at 4 and 6 h, and congestion of entire vasculature and complete loss of blood circulation due to blocking at 24 h (Fig. 2). None of these effects was observed in the control group. Histological examination of the Meth-A tumors resected from mice 4 h after administration of TNF revealed extensive thrombus formation in the tumor vessels of those receiving TNF alone (Fig. 3*c*, *arrow*), but none in those receiving both TNF and heparin (Fig. 3*d*).

Influence of Thrombus Suppression on TNF Antitumor Effect. Despite the histologically observed suppression of thrombus formation by heparin in combination with TNF, no significant difference in Meth-A tumor growth inhibition, necrotic response, or complete cure rate was observed between the mice given TNF alone and those given both TNF and heparin (Table 1).

CPAE Cell Susceptibility. TNF showed dose-dependent cytotoxicity against CPAE cells (Fig. 4) but the ID_{50} of 1.6×10^4

Received 9/14/87; revised 12/28/87; accepted 1/22/88.

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[1] The abbreviations used are: TNF, tumor necrosis factor; Meth-A, methylcholanthrene-induced A-cells; MEM, minimal essential medium; i.d., intradermal(ly); ID_{50}, 50% inhibitory dose.

units/ml was much higher than the ID_{50} of 1.0 unit/ml observed for L-M cells.

Effect of TNF on Normal Blood Vessels. The skin vessels, pulmonary aorta, and abdominal vena cava of mice bearing Meth-A cells 24 h after administration of TNF (1×10^4 units/mouse) or the TNF diluent as control were excised. Examination by optical microscope showed no difference between the excised tissue of the TNF group and that of the control group (Fig. 5).

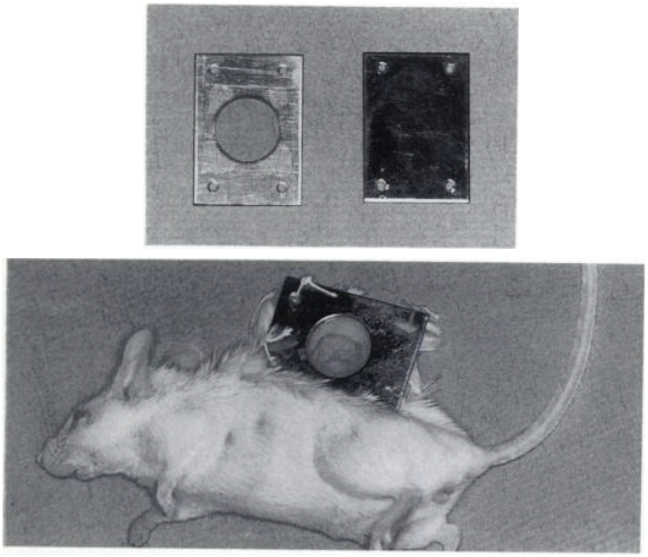

Fig. 1. Parts of the sight glass for a mouse.

DISCUSSION

Various reports have suggested that reactions affecting the tumor vasculature may be involved in the overall antitumor mechanism of TNF, in addition to its proven direct cytotoxicity against tumor cells and activities mediated by neutrophils (15) or macrophages (16). This is based on observation of cytotoxic effects by TNF on vascular endothelial cells derived from bovine aorta (6) and from human umbilical cord (7). In the present study with CPAE cells, a cytotoxic effect was similarly observed.

The effect was dose dependent in the range of $1–1 \times 10^5$ units/ml. The level of cytotoxicity against these cells was nevertheless much lower than that against various tumor cell lines as shown by the ID_{50} of 1.6×10^4 units/ml in the 48-h assay.

The observation of *in vitro* cytotoxicity against vascular cells, however, does not necessarily imply significant alteration of tumor blood vessels by TNF. As shown in Fig. 6, the clearance of TNF from the blood is relatively rapid. In this experiment, the TNF blood level 30 min after i.v. administration of 1×10^4 units/mouse was 4485 units/ml, and the concentration half-life

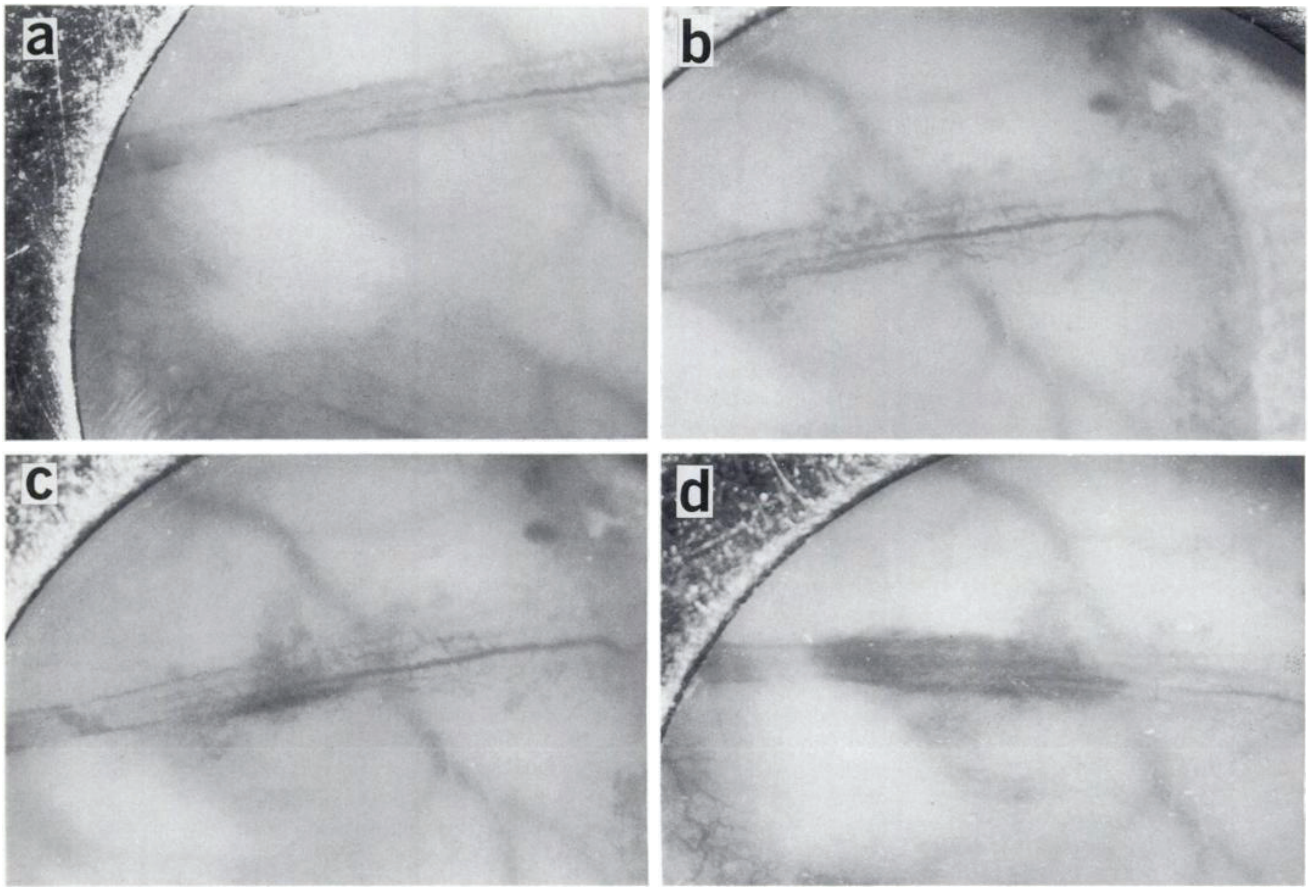

Fig. 2. Effect of TNF on tumor vasculature. Meth-A cells (1×10^6 cells/mouse) were injected into the region under the drilled hole of sight glass. On day 9, TNF (1×10^4 units/0.1 ml 0.1% gelatin-phosphate buffered saline, pH 7.4) or the TNF diluent as control was administered i.v. and the tumor vasculature under the sight glass was observed. *a*, tumor vasculature at 24 h after i.v. injection of the TNF diluent; *b–d*, tumor vasculature at 1, 5, or 24 h, respectively, after i.v. injection of TNF.

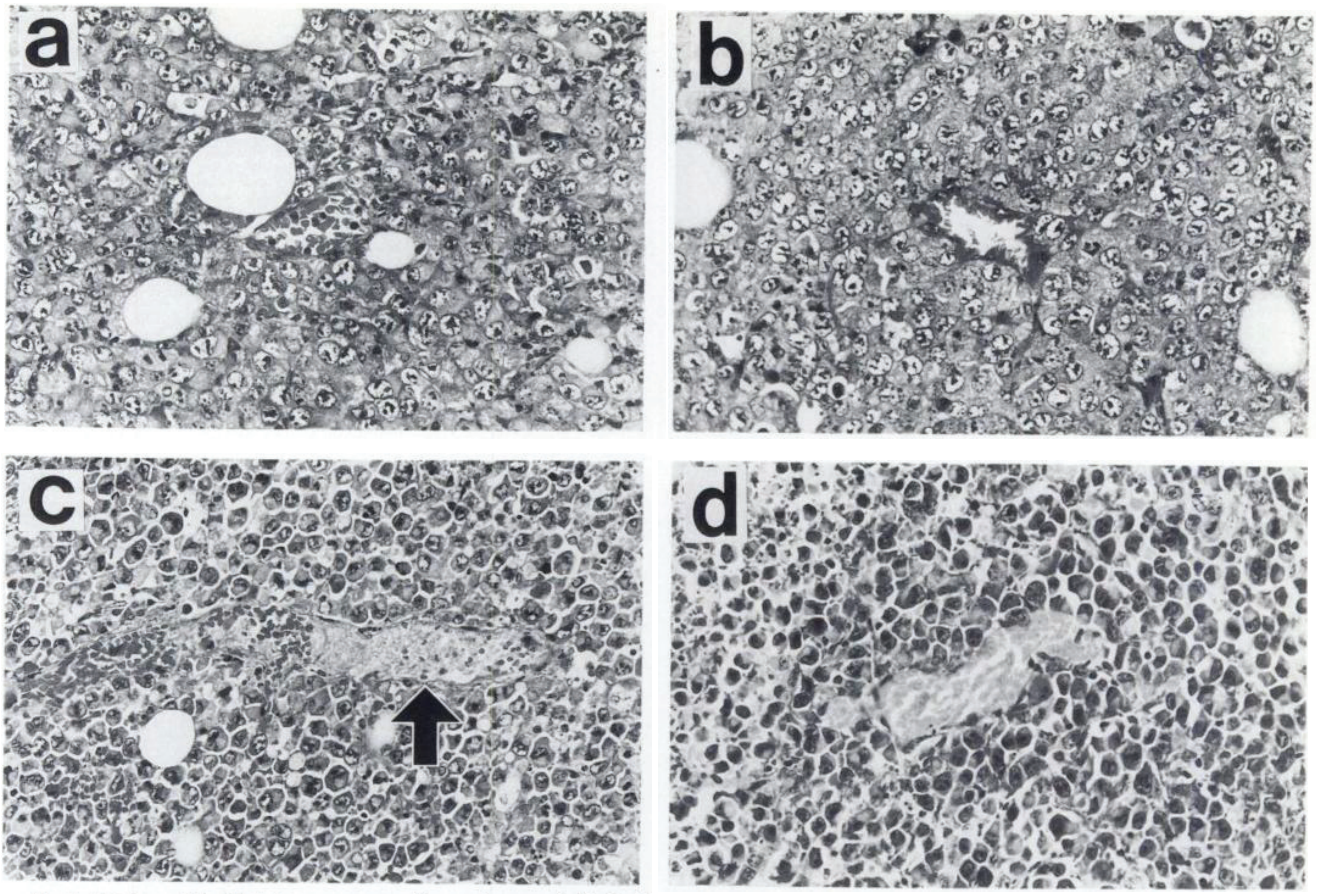

Fig. 3. Histology of the Meth-A tumors resected from mice treated with the diluent solution of TNF (*a*), heparin (*b*), TNF (*c*), and TNF-heparin (*d*). Meth-A cells (1×10^6 cells/mouse) were inoculated i.d. on day 0, and on day 6 TNF (3×10^3 units/0.1 ml) was administered i.v. with (*d*) or without (*c*) prior injection of heparin (3 units/0.1 ml) via the caudal vein. The TNF diluent (0.1% gelatin-phosphate buffered saline, pH 7.4, 0.1 ml) (*a*) or heparin (3 units/0.1 ml) (*b*) was administered i.v. as control. The histology of the tumor vessels was examined 4 h after each administration. *Arrow*, thrombus formation.

Table 1 *Effect of heparin on antitumor effect of TNF against Meth-A fibrosarcoma cells*

Meth-A cells (1×10^6 cells/mouse) were inoculated i.d. and on day 6, TNF (1×10^3 - 1×10^4 units/mouse) and heparin (3 units/mouse) were administered i.v.; 0.1% gelatin–phosphate buffered saline, pH 7.4, the diluent solution of TNF, was administered as control. Necrotic response was judged on day 8, with grades as described in "Materials and Methods." Cured ratio was judged on day 97.

	TNF (units/mouse)	Necrotic response (*n*)				Mean tumor growth, days 6–8	Cured ratio
		−	+	++	+++		
Experiment 1 (TNF)	1×10^4	0	0	5	2	−11	6/7
	3×10^3	0	2	5	0	+4	5/7
	1×10^3	0	6	1	0	+9	4/7
	Control	4	3	0	0	+71	0/7
Experiment 2 (TNF + heparin)	1×10^4	0	0	5	2	−38	5/7
	3×10^3	0	0	7	0	+21	7/7
	1×10^3	0	5	2	0	+8	4/7
	Control	6	1	0	0	+53	0/7

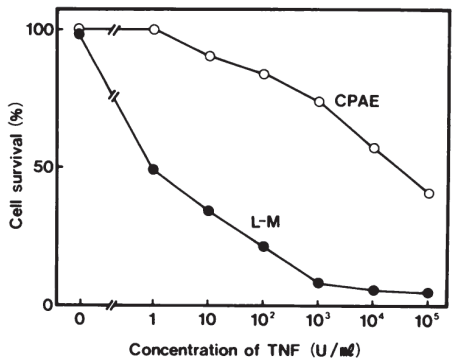

Fig. 4. Cytotoxic activity of TNF against CPAE cells and L-M cells. Cell survival (%) was measured by dye uptake assay after incubation of CPAE and L-

was approximately 27 min. Tumor vasculature, moreover, is known to be structurally different from that of normal blood vessels (17, 18).

The sight glass observations in the present study provided clear and direct evidence of toxic effects by TNF on newly formed tumor vasculature. These included hemorrhaging at 1–2 h, congestion at 4–6 h, and complete loss of circulation in the blood vessels of tumors in mice after TNF administration. None of these effects was observed under the same conditions in the mice that received no TNF.

Histological examination of vascular tissue from the Meth-A tumors following TNF administration showed thrombus formation at 4 h and thereafter.

This is in accord with reports by Nawroth and Stern (8) and Bevilacqua *et al.* (9) which show that TNF promotes thrombus

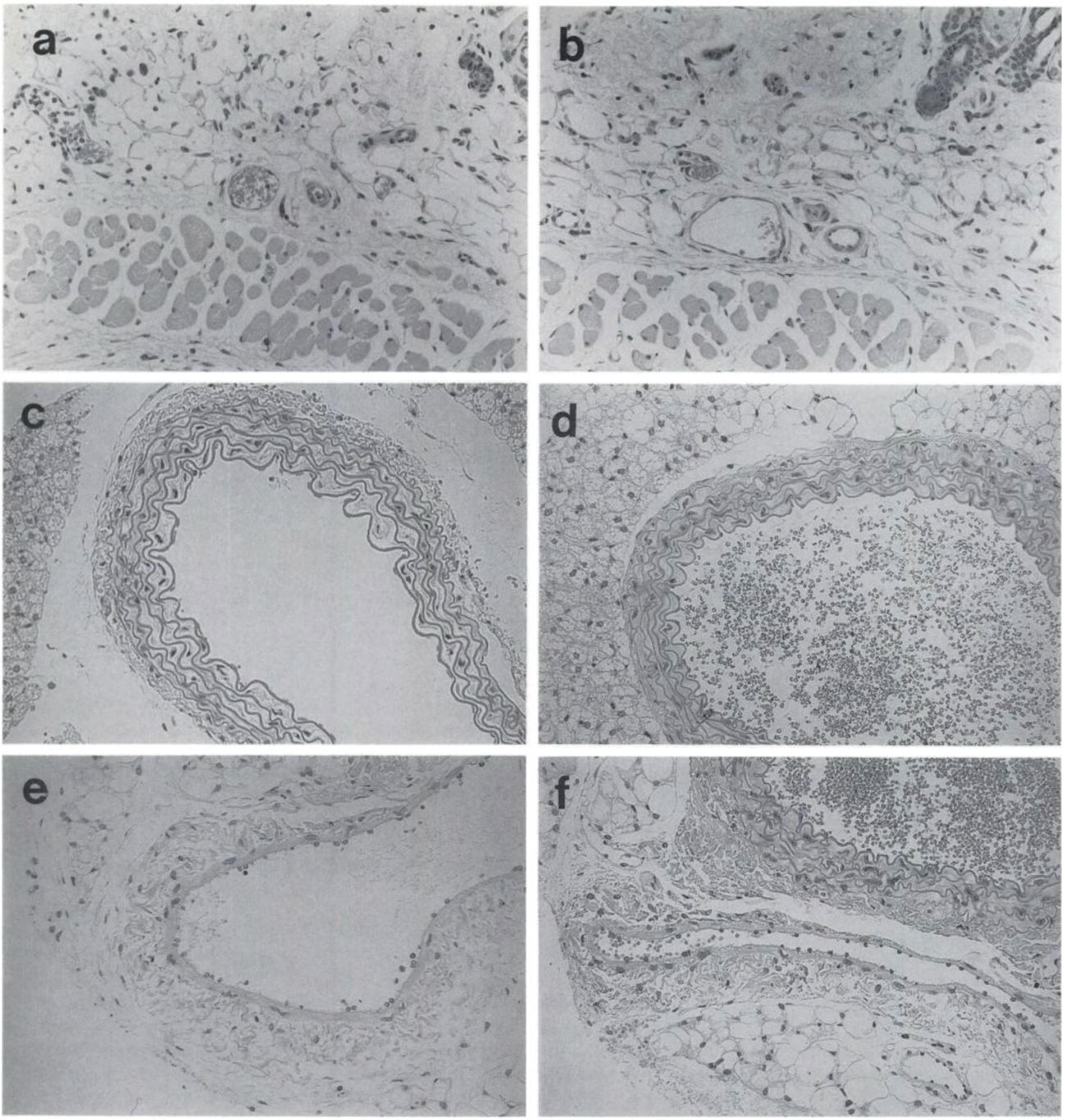

Fig. 5. Histology of the skin vessels (*a*, *b*), aorta (*c*, *d*), and vena cava inferior (*e*, *f*) in BALB/c mice treated with or without TNF. Meth-A cells (1×10^6 units/mouse) were inoculated i.d., and TNF (1×10^4 units/mouse) (*a*, *c*, *e*) or the TNF diluent as control (*b*, *d*, *f*) was administered i.v. on day 9 following transplanting. Histology was examined 24 h after TNF injection. H & E, × 100.

ing the production of procoagulant factor *in vitro*. Thrombus formation is apparently not essential to the antitumor effect, however, since no lowering of tumor growth inhibition, necrotic response, or cure rate occurred under complete thrombus suppression with heparin.

It may further be noted that, at least in the dose range used in this study, TNF apparently had no toxic effect on normal blood vessels.

Histological examination of the skin vessels, pulmonary aorta, and abdominal vena cava from mice in which tumor necrosis or complete cure had been observed showed no abnormality in any of the vascular endothelial cells. These results as well as those of the *in vitro* investigation with CPAE cells suggest that the endothelial cells of normal vasculature are far less susceptible to TNF than are those of newly formed tumor vasculature.

ACKNOWLEDGMENTS

We wish to thank Orville M. Stever for his help in preparation of this manuscript.

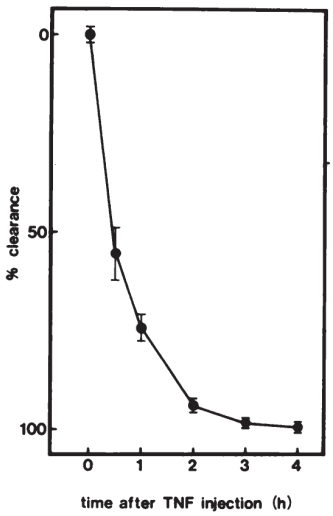

Fig. 6. Clearance of TNF in BALB/c mouse. TNF (1×10^4 units/mouse) was injected into the caudal vein, and TNF activity of serum was measured by dye uptake assay using L-M cells as target. *Bars*, SD.

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