Morphological Effects of Tumor Necrosis Factor-α on the Blood Vessels in Rat Experimental Brain Tumors

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Abstract

The morphological changes in the vascular endothelium caused by the administration of tumor necrosis factor-α (TNF-α) were studied in an experimental model of rat brain tumors. Wistar rats bearing implanted C6 glioma received human natural-type TNF-α (1.7 x 10^5 U/m^2) through the carotid artery and were sacrificed 3 or 24 hours later. The endothelial cells of the tumor blood vessels, demonstrated by the immunoreaction to factor VIII-related antigen, were enlarged after TNF-α administration. Morphometry demonstrated that the nuclei of these endothelial cells were also increased in size. The endothelial cells in the brain remote to the tumor were not affected. An in vitro binding study demonstrated that TNF-α binding sites were distributed in the vascular endothelial cells within the tumor but not in the brain remote to the tumor. The selective effect of TNF-α on the tumor blood vessels in experimental brain tumors may be related to the selective distribution of the TNF-α binding site.

Key words: tumor necrosis factor-α, tumor necrosis factor-α binding site, vascular endothelium, malignant glioma

Introduction

Tumor necrosis factor-α (TNF-α) is a cytokine with activity against a number of tumors including malignant gliomas of the brain.2,14,22 Malignant glioma cells as well as the tumor blood vessels may be the targets.4,8,19 TNF-α modulates procoagulant activity and expression of adhesion molecules in cultured endothelial cells.3,20 However, the *in vivo* effects on vascular endothelial cells are not well documented. Previously, the effects of TNF-α on cultured vascular endothelial cells were extensively studied,3,12,17,20 but the effects on the tumor blood vessels *in vivo* are little known.10,19 Intraluminal thrombus formation and leukocyte accumulation have been studied, but not the morphological changes of vascular endothelial cells.

The present study investigated the effects of TNF-α on the endothelial cells of tumor blood vessels and the distribution of TNF-α binding sites in a rat malignant glioma model.

Materials and Methods

Female Wistar rats weighing approximately 200 g were anesthetized with ketamine hydrochloride (80 mg/kg, i.p.). Under aseptic conditions, 1 x 10^5 C6 glioma cells suspended in 10 μl of Ham's F10 medium were injected stereotactically into the right caudate-putamen.13 Fourteen days after tumor cell inoculation, 5 x 10^5 U of human natural-type TNF-α (Mochida Pharmaceutical Co., Ltd., Tokyo; specific activity 2 x 10^6 U/mg protein) was infused into the internal carotid artery. Rats were sacrificed 3 or 24 hours after administration of TNF-α (both n = 4). Other rats bearing C6 glioma without any treatment were also sacrificed (n = 4). The brain was removed, immediately frozen in isopropyl alcohol, and stored at −80°C.

Immunohistochemical staining for factor VIII-related antigen (FVIIIIRAg) was carried out as described previously.7,13 Briefly, cryostat sections of 6 μm-thickness were fixed in 4% paraformaldehyde
and incubated in Tris-HCl buffer containing 0.3% hydrogen peroxide, then washed. Staining used the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC kit®; Vector, Burlingame, Cal., U.S.A.). Nonspecific binding was blocked by tissue conditioner (Biomeda, Foster, Cal., U.S.A.). Sections were incubated in prediluted goat anti-FVIII-RAg antibody (Biomeda) overnight at 4°C, in biotinylated anti-goat immunoglobulin G (Biomeda) for 30 minutes, and reacted with ABC complex for 30 minutes, then 3,3-diaminobenzidine tetrahydrochloride and counterstained in hematoxylin. Control sections were incubated in normal goat serum instead of the primary antibody. The nuclear size of the vascular endothelial cell was measured with an image processing system (NIH Image 1.47). At least 50 endothelial cells were analyzed in the five square regions of interest selected arbitrarily within the tumor and in the brain remote from the tumor (Fig. 1). The data were expressed as mean ± SD (,um²). Statistical differences were analyzed by the Mann-Whitney U test.

The receptor binding study demonstrated that the endothelial cells of almost all the blood vessels within the tumor were positive for TNF-α binding sites, but those in the brain remote to tumor were totally negative (Fig. 3). The tumor cells as well as the neuronal and glial cells of the brain were also negative for TNF-α binding sites.

Discussion

The present study showed that TNF-α caused increases in the size of both the endothelial cells and their nuclei in the blood vessels within the tumor. Swelling of endothelial cells is a rather nonspecific finding related to various kinds of cytotoxic events such as ischemia, reperfusion, and radiation. The morphological changes observed in this study may also be related to the cytotoxic process after exposure to TNF-α. Previous studies using the ex-
Experimental glioma model have demonstrated that thrombus formation in the tumor vessel and the adhesion of neutrophils to the tumor vessel are important in the cytotoxic effects of TNF-α.10,19 As the swelling of endothelial cells in the microvessels leads to narrowing or obliteration of the vascular lumen,15 the morphological changes observed in this study may contribute to the cytotoxic effects of TNF-α through the formation of intraluminal thrombus and the trapping of cytotoxic leukocytes.

The vascular endothelial cells in the brain remote to the tumor were not affected. The unresponsiveness of those endothelial cells agrees with previous findings of no histological changes in the blood vessels in the normal brain of mice after topical injection of TNF.21 Our in vitro binding study demonstrated that TNF-α binding sites were distributed in the endothelial cells of the blood vessels within the tumor but not in the brain remote to the tumor. The differences in the distribution of TNF-α binding sites between the tumor and the brain remote to the tumor could explain the selective effect of TNF-α on the tumor blood vessels.

The present study used a TNF-α dose of $5 \times 10^3$ U/animal (approximately $1.7 \times 10^5$ U/m²), which is comparable to those used clinically for intracarotid administration in patients with malignant gliomas (1–2 $\times 10^5$ U/m²).22 In this trial, the peritumoral edema was increased in several patients.22 Since endothelial cell damage leads to the increased permeability and aggravation of vasogenic edema,11 the increase in peritumoral edema in this trial could be related to the effect of TNF-α on the tumor blood vessels.

Fig. 2 Photomicrographs showing the vascular endothelium within the tumor (upper row) and in the brain remote to the tumor (lower row) demonstrated by the immunoreaction to FVIIIIRAg. Compared to those in the untreated rat (left column), the endothelial cells of the tumor blood vessels were increased in size 3 (center column) or 24 hours (right column) after administration of TNF-α, while those in the brain remote to tumor were not affected. Immunohistochemical staining for FVIIIIRAg, x 460.
Fig. 3 Photomicrographs showing the expression of TNF-α binding site in the vascular endothelium demonstrated by an in situ binding study for biotinylated TNF-α. TNF-α binding sites were present in the vascular endothelium within the tumor (left) but not in the brain remote to the tumor (right). Histochemical staining for biotinylated TNF-α, × 230.

The presence study focused on the effect of TNF-α on the endothelial cells of the tumor blood vessels, but the direct effect on the tumor cells is also of interest. In the present study, no direct effect on the tumor cells was expected since C6 glioma cells were negative for TNF-α binding sites. However, some human glioma-derived cell lines demonstrated an increased number of TNF-α receptors, so TNF-α may affect both the blood vessels and the tumor cells.

Malignant gliomas are difficult to control using all measures currently available, including surgery, radiotherapy, and chemotherapy. The selective cytotoxicity of TNF-α on the tumor blood vessels encourages application of this cytokine as an adjuvant treatment in managing this incurable malignancy.

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Commentary

Dr. Isaka and associates have made a very important in vivo observation. They have demonstrated that even in a tumor such as C6 which has no TNF-α binding sites, the vessels induced by the tumor not only contain such sites but express them at a substantially higher level than normal brain. Moreover these tumor vessels demonstrate changes in the endothelial cells in response to infused TNF-α. Both the therapeutic effects and unwanted side-effects of this drug may be explained at least in part by these findings. Not mentioned in the article but also of significance is that this provides evidence that may prove useful for using TNF-α and its receptors as a specific targeting strategy coupled with other agents for gene transfer, viral therapy, toxin-associated therapies, and/or angiogenesis inhibition.

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The article by Isaka et al. effectively demonstrates the morphological changes of vascular endothelial cells in the tumor following intra-arterial administration of TNF-α in glioma-bearing mice. The alteration observed by the immunohistochemistry using factor VIII-related antigen consisted of increase in size of both endothelial cells and their nuclei that is consistent to alteration seen in ischemia or irradiation. The alteration was observed selectively in the tumor as early as 3 hours after administration of TNF-α and remained so at 24 hours after administration. No alteration was seen in blood vessels of the normal brain infused with TNF-α. Various reports have demonstrated that TNF has cytotoxic effects against vascular endothelial cells in vitro and suggested that reactions affecting the tumor vasculature may be involved in the overall antitumor mechanism of TNF in addition to its direct effect against tumor cells. In vivo, TNF was shown to induce disruption of blood brain barrier, hemorrhage, thrombus formation, and circulation blockage in the tumor vasculature. This study has provided clear and direct evidence of toxic effects by TNF-α on vascular endothelial cells in the tumor in vivo. The authors also studied binding sites of TNF-α in the blood vessels by the autoradiographic receptor mapping technique and found that TNF-α binding sites were selectively distributed in vascular endothelial cells in the tumor. This is also the new information that might explain the previous and present observations that intravenous or intra-arterial TNF exerts a strong cytotoxic effect on newly formed tumor vasculature but no toxic effects on normal blood vessels. The authors have provided important informations concerning the effect of TNF-α in the treatment of gliomas.

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