Korean J. Toxicol.
Vol. 11, No. 2, pp. 169-173, 1995

Effects of Tumor Necrosis Factor Alpha on Growth and Tube Formation of Bovine Vascular Endothelial Cells *in vitro*

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(Received October 11, 1995)
(Accepted October 20, 1995)

ABSTRACT : *The effects of tumor necrosis factor alpha (TNF-α) on growth and tubular formation of bovine aortic endothelial cells were examined using an in vitro angiogenesis model system. The growth of endothelial cells was enhanced in a dose-dependent manner when the cells were cultured with TNF-α for 3 days, but TNF-α, at the concentration of 1 nM or higher, produced a growth inhibition of endothelial cells when the cells were cultured for 8 days. The endothelial cells incubated with TNF-α for 48-h exhibited a typical morphologic change. Then, they showed a fibroblastoid organization of overlapping, elongated, and spindle-shaped cells. TNF-α, at the concentration of 0.1 nM or higher, inhibited the tubular formation of vascular endothelial cells in an in vitro angiogenesis model using a 3-dimensional culture system.*

Key Words : *Bovine aortic endothelial cells, Tumor necrosis factor alpha, Angiogenesis, Collagen, growth inhibition*

I. INTRODUCTION

Angiogenesis, the proliferation and migration of endothelial cells that result in the formation of new blood vessels, is an essential event in a wide variety of not only normal phenomena such as embryonic development, pregnancy, formation of corpus luteum, and menstration but also pathologic conditions such as wound healing, psoriasis, diabetic retinopathy, rheumatic arthritis, and tumor formation (Folkman, 1985; Folkman and Brem, 1992). Therefore, understanding the detailed cellular and biochemical mechanisms involved in angiogenesis is expected to lead to rational therapeutic approaches for many diseases. Although TNF-α has tumoricidal and tumorstatic properties *in vivo*, the mechanism of its antitumor action is not fully understood. TNF-α has been shown to affect vascular endothelial cells, inducing surface antigens (Doukas and Pober, 1990; Delomenie *et al.*, 1993) and morphologic changes (Sato *et al.*, 1986), and inhibiting proliferation (Sato *et al.*, 1986) and migration (Mano-Hirano *et al.*, 1987). Recently, TNF-α has been reported to have dual roles in *in vivo* angiogenesis; low doses of TNF-α induced angiogenesis, whereas high doses inhibited it (Fajardo *et al.*, 1992). Up to now, the effects of TNF-α for *in vivo* or *in vitro* angiogenesis have been incompletely understood. In the present study, we examined *in vitro* effects of recombinant human TNF-α on growth and capillary-like tube formation of vascular endothelial cells.

II. MATERIALS AND METHODS

1. Endothelial cell culture

Bovine aortic endothelial (BAE) cells were isolated from the bovine thoracic aorta and cultured as described by Gospodarowicz *et al.* (1976). Briefly, the endothelial cell layer was removed by gently scraping the intimal surface with a scalpel. The cells were cultured in 20% FBS-MEM supplemented with 15 μg/ml gentamycin, 2 μg/ml amphotericin B, 1 μg/ml minocycline, and 50 μg/ml ampicillin. When the primary cultures reached confluence, the cells were trypsinized, and cultured in MEM containing 10% FBS, 100 μg/ml penicillin G, 100 U/ml streptomycin, and 2 mM L-glutamine

for further passages. The cells from 3 to 8 passages were used for these experiments.

2. Materials

N-methyldibenzopyrazine methyl sulfate salt (phenazine methosulfate: PMS) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human tumor necrosis factor alpha (rh TNF-α, 10 μg/ml) was purchased from R & D systems (Minneapolis, MN, U.S.A.). Type I collagen solution was purchased from Koken (Tokyo, Japan).

3. Growth Inhibition Assay

XTT-microculture tetrazolium assay for the growth inhibition was performed. In brief, BAE cells were dispensed within 96-well culture plates by the numbers of $5x10^3$ cells/well in 100 μl medium. After overnight incubation at 37°C in a 5% CO_2 condition, each 50 μl of culture medium containing different concentrations of TNF-α was added within appropriate wells. The cultures were incubated for 3, 5, or 8 days with TNF-α prior to the addition of tetrazolium reagent. XTT was prepared at 1 mg/ml in prewarmed (37°C) medium without serum. PMS was prepared at 5 mM (1.53 mg/ml) in PBS. For a 0.025 mM PMS-XTT solution, 25 μl of the stock 5 mM PMS was added per 5 ml of XTT (1 mg/ml). Fifty μl of this mixture was added to each well after incubation with TNF-α. After a 2-hr incubation at 37°C, the plates were measured at 492 nm absorbance wavelength using a microplate reader (Navapath Mini Reader, Biorad, Japan). For a morphological examination, the cultures were photographed with a phase contrast microscope (IMT 2, Olympus, Japan).

4. Tube Formation of BAECs Between Type I Collagen Gels and Quantitative Analysis

Experiments were conducted in duplicate in 24-well culture plates using the sandwich method (Ono *et al.*, 1992) with some modifications. Collagen gel solution (0.5 ml) consisting of a mixture of 8 volumes of type I collagen solution, 1 volume of 10×DMEM, and 1 volume of 0.05 N NaOH, 200 mM HEPES and 260 mM $NaHCO_3$, was poured into each well of the culture plates, and allowed to gel at 37°C. BAECs were inoculated at a density of 1×10^5 cells/well with 1 ml of medium containing 10% FBS. After overnight incubation the medium was aspirated, 0.5 ml of collagen gel was overlayered, and 1 ml of medium supplemented with 2% FBS and different concentrations of TNF-α was applied to each appropriate well. On day 8 after TNF-α treatment, the cultures were photographed with a phase contrast microscope, and the degree of tube formation was measured by a computer-assisted image analyser (MCID, Imaging Research Inc.).

III. RESULTS AND DISCUSSION

In the present study, we determined the effects of recombinant human TNF-α on the growth and capillary-like tubular formation of bovine aortic endothelial cells. The effect of TNF-α on cells may be antiproliferative (BT-20, ME-180, MCF-7, and SK-MEL-109 cell lines) or growth-enhancing (WI-38, CCD-18Co, and Detroit 551 cell lines), thus, TNF-α has been known to have divergent effects on cell growth (Sugarman *et al.*, 1985). It is unclear how

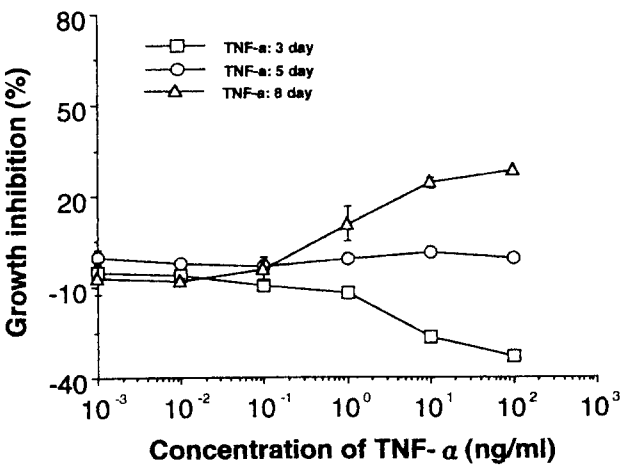

Fig. 1. Effect of TNF-α on the growth of bovine aortic endothelial cells. The cells were seeded in 96-well culture plate and incubated with TNF-α at different concentrations. The cell growth was determined by XTT assay on day 3, 5 and 8 after TNF-α addition.

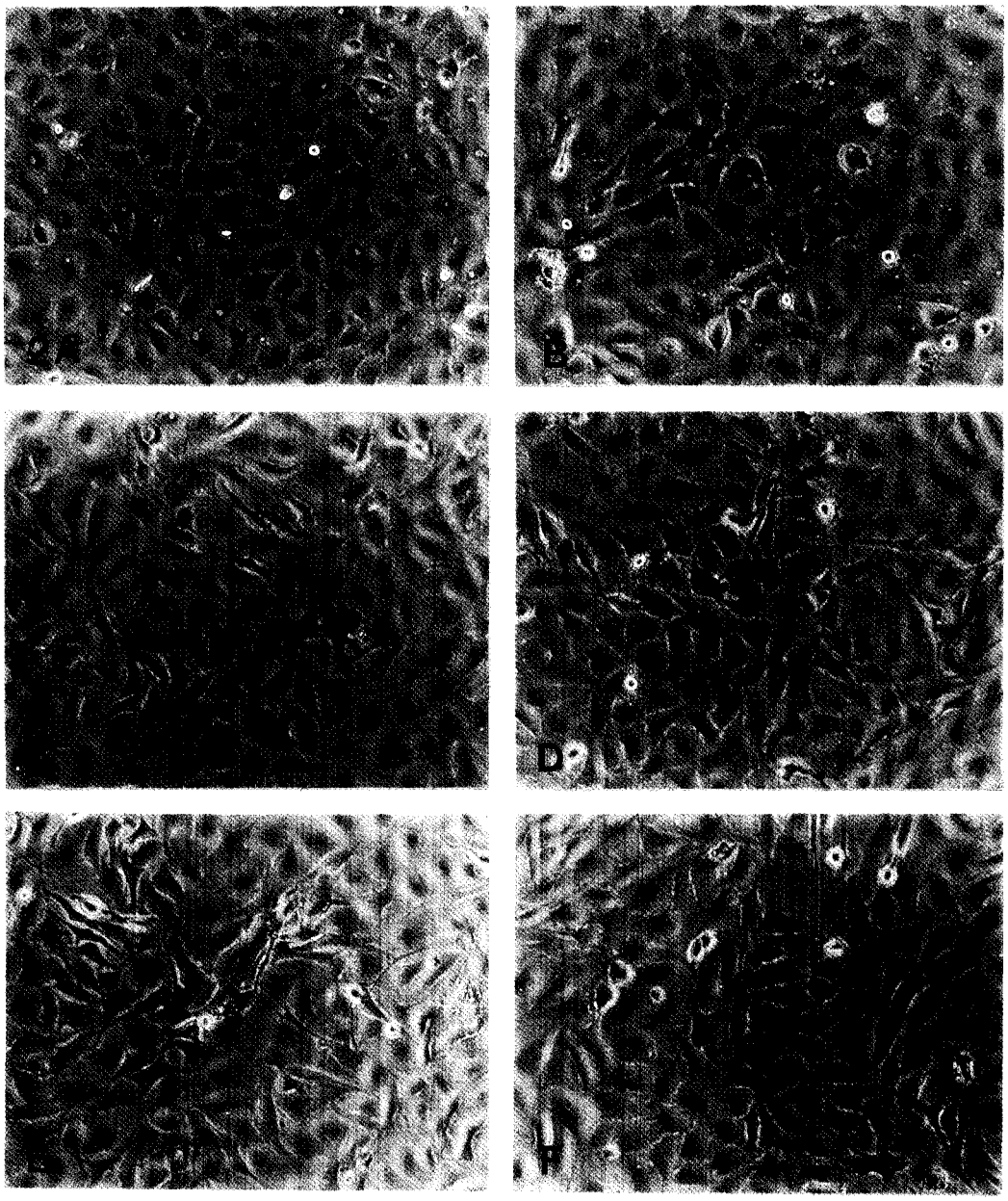

Fig. 2. Phase contrast micrographs of TNF-α-induced bovine aortic endothelial cells. The cells were inoculated into 96-well culture plates as the number of 5×10^3 cells/well. After overnight incubation, the cells were cultured for 48 hours in the absence or presence of TNF-α. (A) control; (B) 1 ng/ml TNF-α; (C) 5 ng/ml TNF-α; (D) 10 ng/ml TNF-α; (E) 50 ng/ml TNF-α; (F) 100 ng/ml TNF-α. ×200.

TNF-α can stimulate the growth of certain cell lines while inhibiting the growth of others. In our study, the growth of endothelial cells was enhanced in a dose-dependent manner when the cells were cultured for 3 days with various concentrations of TNF-α, however, it was progressively inhibited in a dose-dependent manner when the cells were cultured for 8 days with TNF-α (Fig. 1). Then, the growth proportion of endothelial cells was increased in the presence of 1, 10, and 100 ng/ml TNF- by 11.9%, 26.8%, and 33.1%, respectively, after 3-day incubation with TNF-α, however, it was inhibited in the presence of 1, 10, and 100 ng/ml TNF-α by 10.8%, 24.3%, and 28.2%, respec-

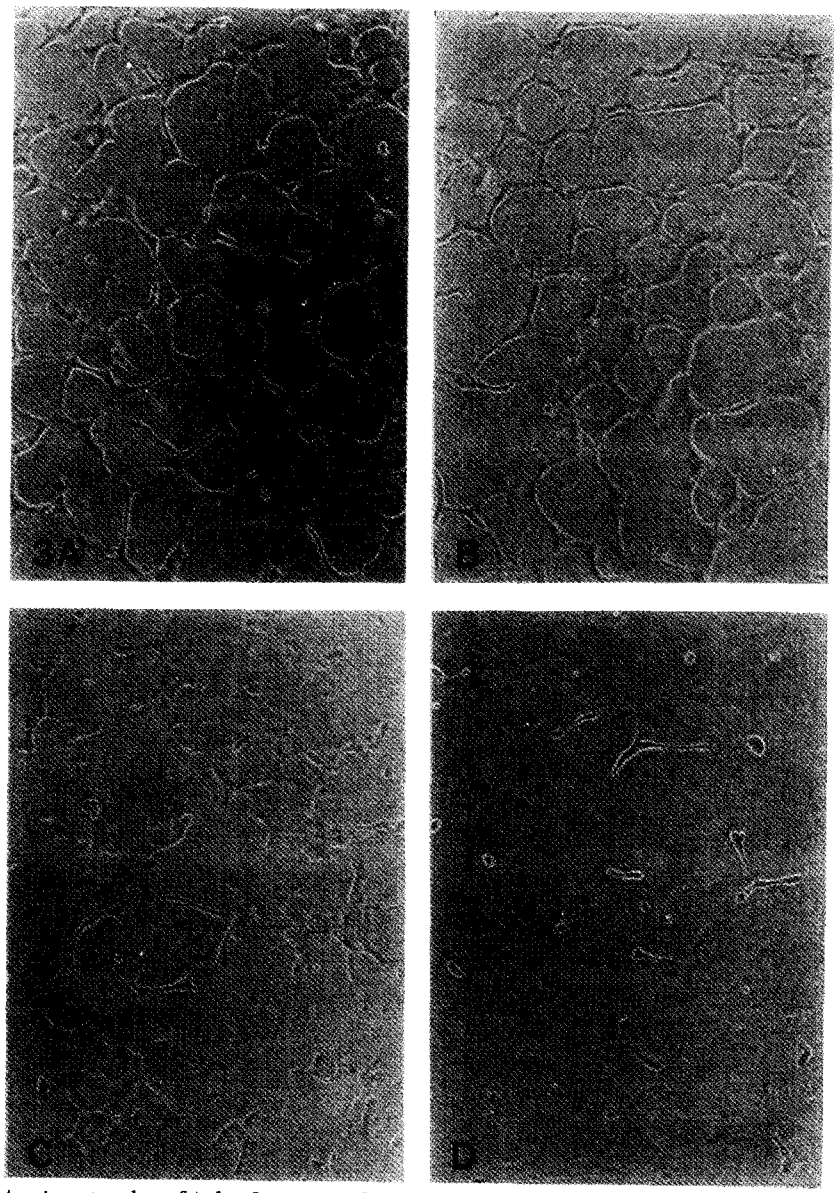

Fig. 3. Phase contrast micrographs of tube formation by bovine aortic endothelial cells after TNF-α treatment. The cells were cultured between two layers of collagen gel for 8 days at various concentrations of TNF-α. (A) control; (B) 0.01 ng/ml TNF-α; (C) 0.1 ng/ml TNF-α; (D) 1 ng/ml TNF-α. ×100.

tively, when the cells were cultured for 8 days with TNF-α. Bovine aortic endothelial cells exhibited a characteristic cobblestone polygonal morphology when reached a confluence (Fig. 2A). Cytokines such as IL-1α, IL-1β, IFN-γ, and TNF-α have been reported to modulate the expression of adhesion molecules on the endothelial cell surface, and alter endothelial cell morphology (Stolpen *et al.*, 1986; Pober, 1988). In the present study, bovine aortic endothelial cells exposed to TNF-α for 48-h were converted from their usual contact-inhibited epithelioid organization to a fibroblastoid organization of overlapping, elongated, spindle-shaped cells (Fig. 2C, 2D, 2E, and 2F). Then, the morphologic changes of endothelial cells induced by TNF-α may be a result of endothelial activation (Pober, 1988). In order to determine the effect of TNF-α for *in vitro* angiogenesis of vascular endothelial cells, we cultured the endothelial cells between the type I collagen gel layers overlayed

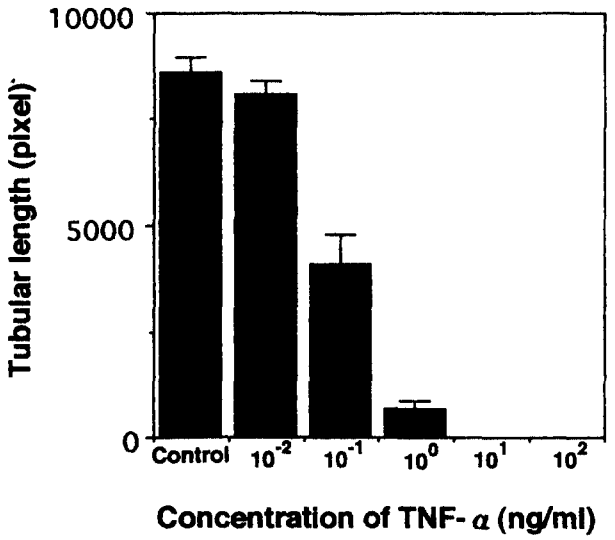

Fig. 4. Effect of TNF-α on tube formation of bovine aortic endothelial cells grown between collagen gel layers. The lengths of formed tubes were measured on day 8 after TNF-α treatment using a computer-assisted image analyser. Each value is the mean±SD of triplicate determinants.

with various concentrations of TNF-α. To quantify the degree of tube formation, we measured the total lengths of tubular structure using computer-assisted image analyser. In the presence of TNF-α (0.1 and 1 ng/ml), as shown in Fig. 3C and 3D, the endothelial tube formation was markedly inhibited. Then, the total lengths of tubular structure were inhibited in a dose-dependent manner as shown in Fig. 4. TNF-α inhibits the proliferation of endothelial cells *in vitro*, whereas it has dual role in angiogenesis *in vivo* (Fajardo *et al.*, 1992). Thus, the role of TNF-α in angiogenesis, up to now, has been controversial. In the present study, endothelial cells treated with TNF-α were converted from epithelioid cells to overlapping and spindle-shaped cells. TNF-α not only inhibited the growth of endothelial cells, but also inhibited the tube formation of endothelial cells in an *in vitro* angiogenesis model using a 3-dimensional culture system. The precise mechanism for the inhibitory action of TNF-α on the capillary-like tube formation of endothelial cells is not yet clear.

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