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INHIBITION OF IN VITRO ANGIOGENESIS BY LYMPHOTOXIN AND INTERFERON-γ

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The effects of lymphotoxin (LT) and interferon (IFN)-γ on the capillary formation was examined using an in vitro angiogenesis model system. Both LT and IFN-γ inhibited the capillary formation in a dose dependent manner. To elucidate the mode of action, effects of the lymphokines on endothelial and myofibroblastic cells were studied. We found that the lymphokines inhibited not only the growth of endothelial cells but also the production of collagen by myofibroblastic cells. These results suggest that the pleiotropic effects of the lymphokines on different types of cells might result in the inhibition of the capillary formation.

Several cytokines, such as TNF, LT, and IFN-γ have been shown to have anti-tumor activity in vivo (1). However, the mechanism underlying this effect is still unclear. There is some discrepancy between in vivo and in vitro tumoricidal activities, suggesting that anti-tumor activity in vivo might be, in part, indirect (2).

On the other hand, it is well known that angiogenesis inhibitors suppress tumor growth by restricting neovascularization in animal experiments (3). In this

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Abbreviations: IFN, interferon; TNF, tumor necrosis factor; LT, lymphotoxin; BCE cells, bovine capillary endothelial cells; HUVE cells, human umbilical vein endothelial cells.

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communication, we show both LT and IFN-γ inhibit the *in vitro* capillary formation. Our results suggest that inhibition of angiogenesis by the lymphokines might contribute to the suppression of tumor growth *in vivo*.

MATERIALS AND METHODS

Cells BCE cells were obtained from the adrenal cortex by the method of Folkman et al (4), and HUVE cells were prepared according to the method of Mano et al (5).

Lymphokines Both human recombinant LT (spec. 2 X 10^6U/mg) and IFN-γ (spec. 5 X 10^6U/mg) were produced in *E.coli* and were purified to homogeneity at Suntory Institute for Biomedical Research (6). Rat IFN-γ (spec. 4 X 10^6U/mg) was obtained from Holland Biotechnology (The Netherland).

Preparations of Microvessels Microvessels were prepared from rat epididymal fat pads as described previously (7). In brief, the tissue was rinsed twice with ice-cold PBS (pH7.4), cut into pieces, and then digested by 0.1% collagenase (type II, Sigma Chemical Co., St. Louis, MO), in the Hepes buffer containing 5% fetal calf serum for 20min. at 37 °C. After the digest was suspended by pipetting, microvessels were collected by centrifugation (250 X g, 10min.), and the pellet was resuspended in medium 199 containing 20% fetal bovine serum. The suspension was filtered through a 140μm pore size mesh to remove undigested tissue debris. Microvessel fragments thus obtained were further fractionated by centrifugation(15000 X g, 20min.) using a 45% Percoll solution (Pharmacia, Sweden) and then plated in culture dishes.

Cell Proliferation Endothelial cells were seeded in 96-well plates and incubated in a CO_2 incubator at 37°C in the presence of desired reagents. At intervals, plates were removed from the incubator and medium (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum) was removed by rapid decantation. Then the cells were harvested by trypsinization and enumerated with a cell counter.

Measurement of Collagen Synthesis Collagen synthesis was measured as described by Peterkofsky and Diegelmann (8). Namely, cells were cultured in MEM (1ml) containing 50μg each of ascorbic acid and β-aminopropionitrile and labeled for 3h with 5μCi of L-[3,4-^{3}H] proline. The medium was separated, and cells were scraped into 1ml of 0.2N NaOH and homogenized. After suspended in the medium, proteins were precipitated by adding 0.4ml of 50% TCA containing 5% tannic acid and then dissolved in 1ml of NaOH. The solution was divided into two equal portions, and added to an equal volume of 0.2M Tris-HCl buffer pH7.5 containing 100mM $CaCl_2$ and 2mM N-ethylmaleimide, and the mixture was incubated for 3h at 37° C with or without 25 units of purified collagenase form III (Advance Biofactures Co, New York, NY). After incubation, TCA was added to the mixture to a final concentration of 10% and proteins were precipitated. The radioactivity of proteins in the supernatant solubilized by collagenase was measured for determination of collagen synthesis.

RESULTS AND DISCUSSION

Because LT was shown to induce the hemorrhagic necrosis of some tumors (9), we first examined the effect of this material on the in vitro angiogenesis which we established previously (7). When the microvessel fragments and myofibroblastic cells from rat epididymal fat pads were co-cultured, myofibroblastic cells initially began to grow and reached confluence. A few days later, endothelial cells started to sprout from the vessel fragments, forming cellular cord networks on and in the multilayered myofibroblastic cells within a week. To test the effect of LT, freshly prepared microvascular fragments were seeded on the monolayered myofibroblastic cells and cultured in the presence or absence of LT. At day 7 after the addition of LT, the formation of the cellular cords was measured under the microscope. As shown in Fig.1, cord elongation was significantly suppressed by LT.

To quantitate the growth of cellular cords, the density of extending cellular cords per a definite area was measured with the aid of grids. As shown in Fig.2, LT inhibited the formation of cellular cords in a dose dependent manner. These results

Inhibition of capillary growth by lymphotoxin, day 7

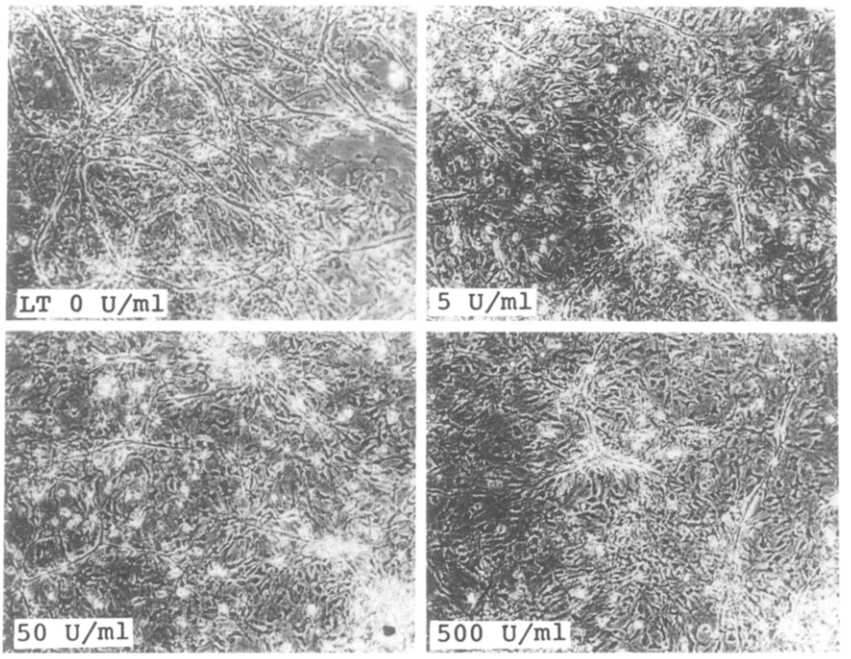


Fig.1 Capillary cords in culture and effect of recombinant human LT on their growth.

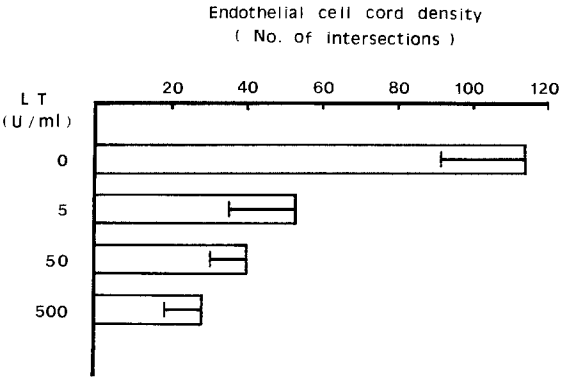


Fig.2 Inhibition of angiogenesis in vitro by human lymphotoxin. Capillary formation was quantitated at day 7 by measuring the density of endothelial cellular cords as described in the text.

correlated with our previous observation that TNF, which shared the same receptor with LT (10), inhibited the angiogenesis in vitro (11).

Next, we examined the effect of IFN-γ, another tumoricidal lymphokine, on the formation of the capillary in vitro (Fig.3). The growth of the control cords was significantly inhibited by as low as 1U/ml of IFN-γ. Apparent inhibition was observed at higher concentrations of IFN-γ, especially at the late phase of the incubation period (14 days, data not shown). These results indicated that IFN-γ as well as LT inhibited the in vitro formation of capillary cords.

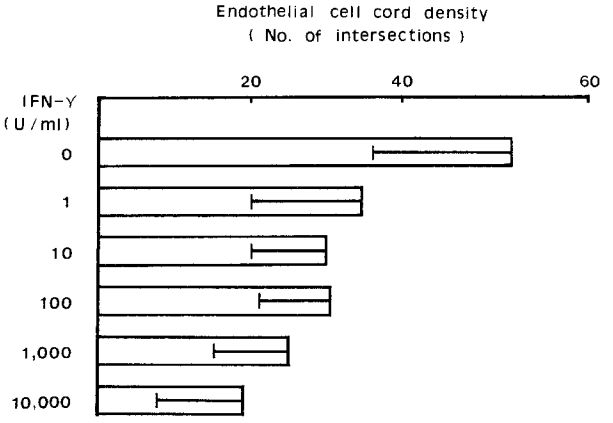
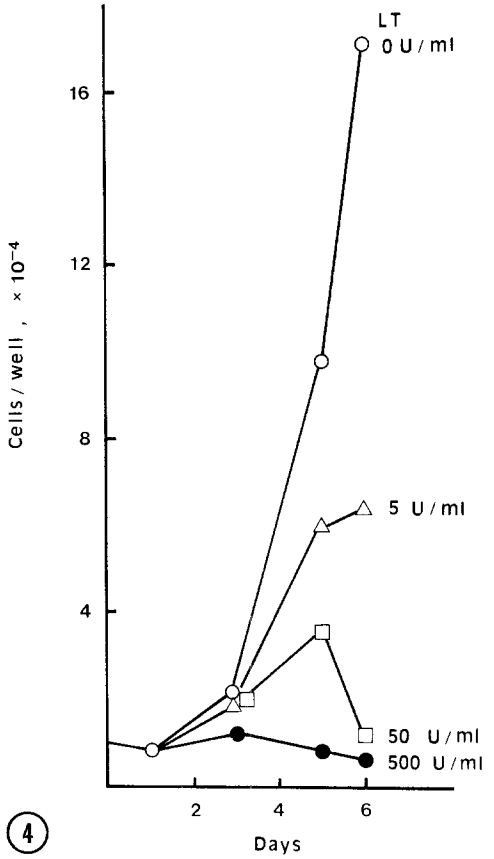


Fig.3 Effect of rat IFN-γ on capillary formation in vitro. Capillary formation was quantitated at day 7 by measuring the density of endothelial cellular cords as described in the text.



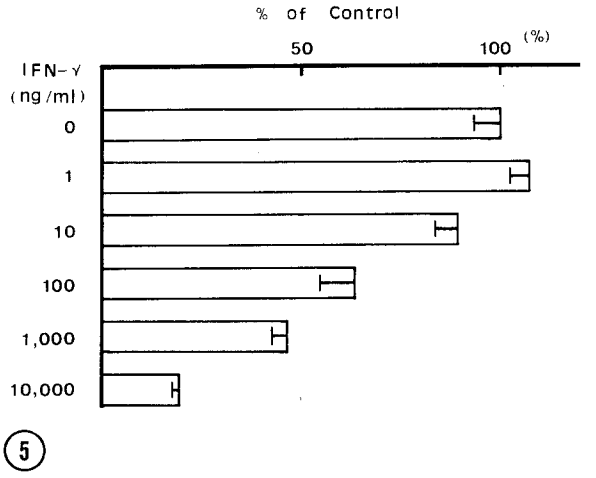


Fig.4 Effect of human LT on proliferation of BCE cells. Cells were seeded at a density of 10 X 10^4 cells/well in 24-well plates. Next day, culture medium was replaced with fresh medium with or without LT. Cells were harvested by trypsinization and counted at indicated times.

Fig.5 Effect of human IFN-γ on proliferation of HUVEC cells. Cells were seeded at a density of 5 X 10^3 cells/well in 96-well plates and were cultured in the presence of medium conditioned to rat myofibroblastic cells (25%). At day 5 after addition of IFN-γ, cells were harvested by trypsinization and counted.

To demonstrate the target cells of these two lymphokines, we examined the effects of the lymphokines on the growth of endothelial cells. As shown in Fig.4, LT inhibited the growth of BCE cells. LT at higher concentration, showed the cytotoxic effect on BCE cells, which correlated with our previous finding that TNF exerted cytostatic and cytotoxic effects on cultured BCE cells (12).

As is shown in Fig.5, IFN-γ also inhibited the growth of HUVE cells. We performed these experiments using HUVE cells and human IFN-γ because of the limiting availability of rat capillary endothelial cells and apparent species specificity of

Table I. Effect of human LT and rat IFN-γ on production of collagen by rat myofibroblastic cells

	Collagen Synthesis	
	cpm/well	cpm/10^5cells
Control	11,100±1,900	1,210±120
IFN-γ 1000U/ml	2,020±332	243±23
LT 500U/ml	3,030±883	361±61
IFN-γ + LT	227±68	19±3

Production of collagen was measured as described in "Materials and Methods".

IFN-γ. In separate experiments, we have shown the cord formation using HUVEC and rat myofibroblasts (data not shown). On the other hand, both lymphokines had little effect on growth of myofibroblastic cells. These results suggest that both LT and IFN-γ inhibit the cord formation by directly inhibiting the growth of endothelial cells, but not myofibroblastic cells.

Extracellular matrix surrounding endothelium is known to be important in the regulation of cellular phenotype and proliferation (13). Because rat myofibloblastic cells are essential component of *in vitro* cord formation (7), we next examined the effect of the lymphokines on the production of collagen, one of the major components of extracellular matrix. As shown in Table I, both LT and IFN-γ inhibited the production of collagen. Moreover, synergistic effect of LT and IFN-γ on inhibition of collagen production was also observed. These results suggest that lymphokines exert the inhibitory effect on cord formation *via* the modulation of the production of extracellular matrix.

In this study, we have shown that both LT and IFN-γ markedly inhibit the capillary formation *in vitro*. In our system, rat myofibroblastic cells induce the formation of capillary networks *in vitro*, by producing migration and proliferation stimulating factors for endothelium and secreting an extracellular matrix, which modulates the endothelial cell shape (7,12). Both lymphokines show not only marked inhibition of endothelial cell growth but also inhibition of the production of collagen by myofibroblastic cells. These findings suggest

that pleiotropic effects of these lymphokines on several types of cells synergistically contribute to the inhibition of neovasculation, resulting in the regression of tumors in vivo.

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