Virchows Archiv A Pathol Anat (1990) 417:473–476

Virchows Archiv A
Pathological Anatomy and Histopathology

Effect of tumour angiogenesis factor on proliferation of endothelial cell and tube formation

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Received March 3, 1990 / Received after revision April 25, 1990 / Accepted July 5, 1990

Summary. The effect of a tumour angiogenesis factor on proliferation of various kinds of cells was examined in vitro. The factor (TAF) a polypeptide of 14000 molecular weight, was extracted and purified from the conditioned medium of ovarian clear cell carcinoma cell line (HUOCA-II). TAF at concentrations of 10 ng/ml and 100 ng/ml promoted proliferation of the endothelial cells and induced tube formation. However, it had no stimulatory effect on the proliferation of fibroblasts, endometrial columnar cells, squamous epithelial cells or cancer cells.

Key words. TAF – Endothelial cell – Tube formation

Introduction

In order that a tumour may proliferate, neovascularization is indispensable, and chemical substances produced by the tumour are believed to induce the entry of blood vessels into it from the surrounding tissues. One substance was named as tumour angiogenesis factor (TAF) by Folkman et al. (1971), and several TAFs have since been isolated and characterized (Shing et al. 1984; Fett et al. 1985). In an earlier study, we transplanted 24 cell lines of various kinds of gynaecological tumours on nude mice and examined their transplantability, proliferating speed, and number of blood vessels in the mouse grafts. We also placed methylcellulose (1%) pellets containing the conditioned medium of cell lines on Chorioallantoic membranes (CAMs) of chick embryos to observe the degree of angiogenesis (Ishiwata et al. 1988). As a result, it was revealed that the conditioned medium of HUOCA-II line showed a strong angiogenic activity. Therefore, we cultured HUOCA-II cells in serum-free Ham's F-12 culture medium and extracted and purified a TAF polypeptide of 14000 molecular weight (Ishiwata et al. 1987) from the conditioned medium by the method of Fett et al. (1985). In this study, the effect of this purified TAF on endothelial cells, fibroblasts, endometrial columnar cells, squamous epithelial cells and cancer cells was examined in vitro with a view to elucidating its role in the proliferation of endothelial cells and tube formation.

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Materials and methods

Endothelial cells were obtained from human umbilical vein (HUEC) and from the pulmonary artery (CPAE) of a young cow (Flow Laboratories, Virginia, USA). The umbilical cord was washed several times with phosphate-buffered saline (PBS), and the umbilical vein was filled with PBS solution supplemented with 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), ligated at either end of the cord with silk and incubated at 37° C for 30 min. The PBS solution containing HUECs was centrifuged at 300 *g* for 10 min and the sediment was resuspended in the culture medium (Ham's F-12; Gibco, New York) containing 15% fetal calf serum, and cultured in 5% CO_2 incubator at 37° C.

Fibroblasts and squamous epithelial cells were obtained from the lung and skin of fetuses (14 weeks old; spontaneous abortion), respectively. The endometrial columnar cells were obtained from three patients (aged 40, 41 and 45 years) who underwent hysterectomy for myoma of the uterus. Tissue from lung and endometrium was rinsed twice with PBS, minced with a pair of sharp scissors, digested with PBS solution supplemented with 0.1% trypsin and 0.02% EDTA for 30 min at 37° C and centrifuged at 300 *g* for 10 min. In contrast, the squamous epithelial cells were obtained by explant culture; human fetal skin was cut into $2 \times 2 \times 2$ mm fragments with a pair of sharp blades and placed directly in 3.5 cm plastic dishes. The squamous cells migrated and proliferated in monolayer from the explant 3 days after culturing. Four cell lines (HHUA, endometrial adenocarcinoma cell line; HTOA, ovarian serous-cyst adenocarcinoma cell line; SKG-II, uterine cervical epidermoid carcinoma cell line; and SKN, uterine leiomyosarcoma cell line) established by us were also used for this experiment (Ishiwata et al. 1988).

To evaluate the effect of TAF on cell proliferation approximately 5×10^4 single-suspension cells (except for the squamous epithelial cells) were placed in 3.5 cm plastic dishes (Terumo, Tokyo,

Japan) and cultured for 9 days at 37° C in a 5% CO_2 incubator. The medium, which contained either 10 ng or 100 ng TAF/ml, was changed the day following inoculation. The average number of cells was determined every 2 days by counting the cells in three dishes in each concentration of TAF. The medium with or without (control) TAF was changed every 2 days. The effect of TAF on cellular proliferation was studied by the growth curve and the population doubling time.

To determine the effects of TAF on the growth of squamous epithelial cells, the longest and the shortest distances from the edge of the fragment to the margin of the culture were measured every 2 days (Ishiwata et al. 1978).

To determine the effect of TAF on tube formation the HUEC and CPAE cells at sub-confluence were treated with 10 ng TAF/ml. The medium containing TAF was changed every 2 days. Morphological changes in the cells were examined with both phase contrast and electron microscopes during the 10 days following administration of TAF (Ishiwata et al. 1977).

Table 1. Effect of tumour angiogenesis factor (TAF) on cellular growth

Concentrations of TAF (ng/ml)	Population doubling time (h)				
	HUEC	CPAE	F	EM	HHUA
0	105±11	132±17	71±6	86±7	31±5
10	72± 7	85± 9	69±7	83±9	31±4
100	61± 7	81± 8	70±6	84±9	32±3

Mean±SD; HUEC, human umbilical vein endothelial cell; CPAE, pulmonary artery endothelial cell; F, fibroblast; EM, endometrial columnar cell; HHUA, endometrial adenocarcinoma cell

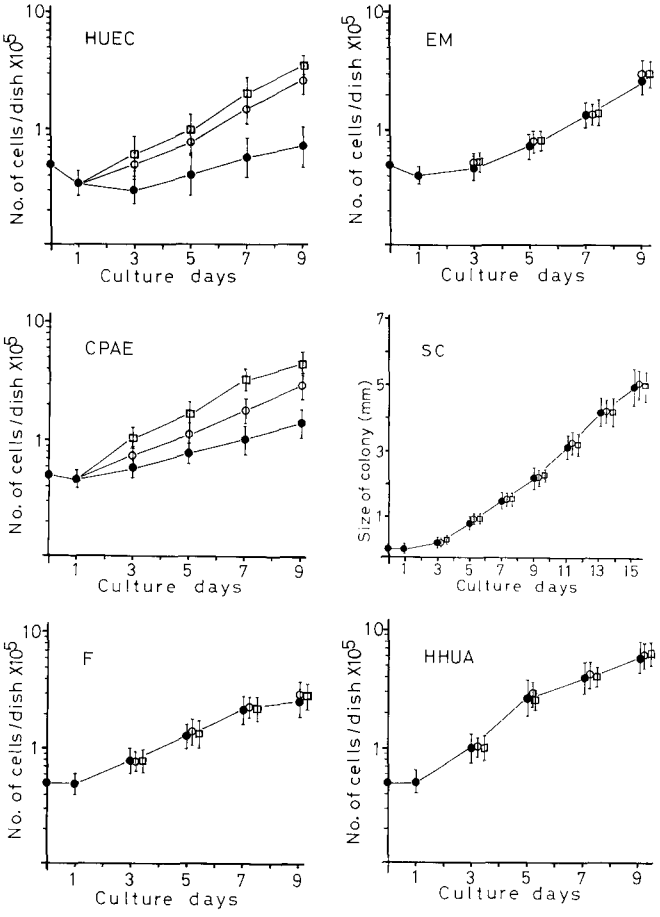

Fig. 1. Effects of 10 ng/ml and 100 ng/ml of tumour angiogenesis factor (TAF) on growth curves of HUEC, CPAE, fibroblast (*F*), endometrial columnar cell (*EM*), squamous epithelial cell (*SC*) and endometrial adenocarcinoma cell (*HHUA*). ●—●, Control; ○—○, 10 ng/ml TAF; □—□, 100 ng/ml TAF. Note that the TAF promoted the rate of growth of endothelial cells (*HUEC* and *CPAE*), and at both the concentrations there was significant difference from the control values. In the case of the F, EM, SC and HHUA; however, there was no difference in the number of cells between the TAF treated ones and the respective controls

Results

The TAF (10 ng/ml, 100 ng/ml) promoted the rate of growth of endothelial cells (both HUEC and CPAE) as shown in Table 1 and Fig. 1, but had no effect of the growth of other cells (fibroblasts, endometrial columnar cells and squamous epithelial cells), including the cancer cells (HHUA, HTOA, SKG-II and SKN).

TAF directly affected the endothelial cells (both HUEC and CPAE) and promoted tube formation. The monolayer cultures formed mount-like elevations 12 h after administration of TAF, and tube-like structures 48 h after administration. The tube-like structures extended, anastomosed each other, and finally formed networks of tubular structures after 7 days (Fig. 2). Cross-sections through the tubular structures showed that they were formed by 3–15 cells. Complete tubular structures were not formed without TAF. Electron micrographs showed the structures to be hollow tubes composed of endothelial cells with overlapping and interdigitating cytoplasmic processes typical of those seen in in vivo capillaries. The lumen of these tubes generally contained fibrous material (Fig. 2).

Discussion

Angiogenesis is indispensable in the processes of healing of wounds, inflammation, regeneration, embryogenesis, proliferation and metastasis of cancer. Capillaries are composed of a layer of endothelial cells and in the areas of active proliferation of cells, communication between capillaries and the formation of networks and sinuses are quite common. An abundant blood supply in such areas is well documented. The process of angiogenesis, including that of the capillaries, involves: a melt down of the endothelial matrix of blood vessel, migration of endothelial cells, proliferation of endothelial cells, and formation of tubes of endothelial cells.

We have examined the angiogenic activity of a TAF isolated and purified from the conditioned medium of ovarian clear cell carcinoma line (HUOCA-II) using CAM in an in vivo bioassay system at 1 ng/ml concentration (Ishiwata et al. 1987). Pilot studies for evaluating the effects of TAF on the rate of growth of CPAE revealed that TAF enhanced the growth of CPAE at concentrations higher than 10 ng/ml, but had no effect at less than 1 ng/ml in in vitro culture. Therefore, we exam-

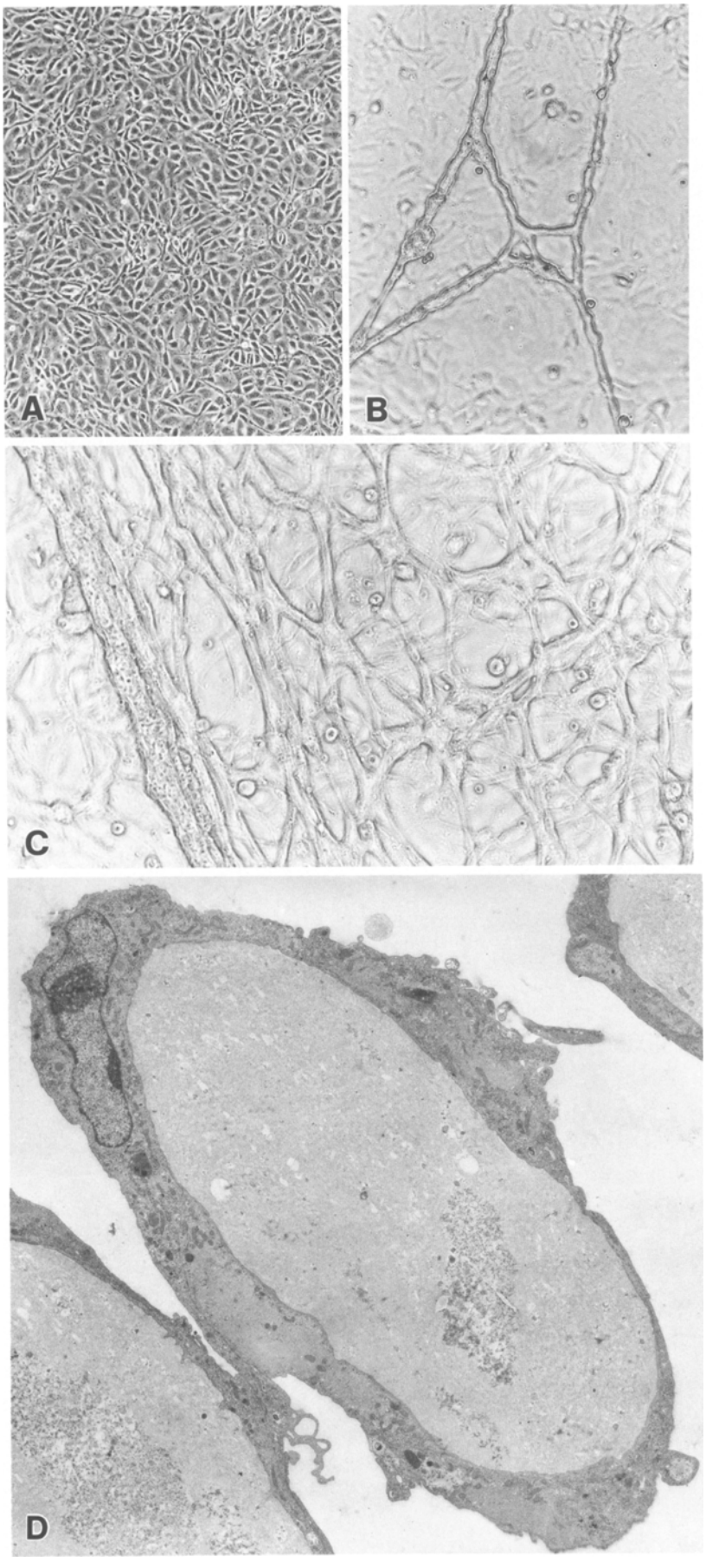

Fig. 2A–D. Effects of TAF (10 ng/ml) on tube formation of CPAE. **A** Control. CPAEs showed a pavement cell-arrangement. **B** (10 ng TAF/ml) CPAEs formed a tubular structure 48 h after administration of TAF. **C** (10 ng TAF/ml) CPAEs formed network of tubular structure 7 days after administration of TAF. Phase contrast microscopy. $\times 100$. **D** Electron micrograph of cross-sectioned tubular structure cultured CPAE with 10 ng/ml TAF containing medium. The lumen of the hollow tubes contained fibrous materials. $\times 2836$

ined the effects of TAF on various kinds of cells at 10 ng/ml and 100 ng/ml concentrations in the present in vitro bioassays. Our results indicate that TAF has an activity for proliferation of endothelial cells (HUEC and CPAE) in vivo as well as in vitro.

In addition to the TAF, several growth factors are known to promote proliferation of endothelial cells in blood vessels; these are endothelial cell growth factor (ECGF), fibroblast growth factor (FGF), macrophage-derived growth factor (MDGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), cartilage growth factor, insulin-like growth factor-1, and so on (Mitsui 1988). In general, growth factors are capable of promoting proliferation of various types of cells, indicating that the target cells can be diverse. For example, EGF promotes proliferation of various epithelial cells, cells of mesodermal origin, and cancer cells; FGF that of fibroblast, cartilage cells, and myoblast cells; ECGF that of endothelial cells and fibroblasts; and PDGF that of smooth muscle cells, fibroblasts, and glia cells. TAF is a kind of growth factor, but little information is available regarding its effect on proliferation of cells other than endothelial cells. Therefore, we examined the effect of TAF on proliferation of endothelial cells (HUEC, CPAE), fibroblasts, squamous epithelial cells, endometrial columnar cells, and various cancer cells. TAF promotes proliferation of endothelial cells, but has no effect on the other cells studied.

Clinically TAF might be applied locally to an infarcted part to induce capillary blood vessels grow from the surrounding healthy vessels, resulting in the disappearance of the necrosed tissue. The inside of a substitute blood vessel can be coated with TAF and covered with endothelial cells to simulate the physiological functions of a real blood vessel. If a monoclonal antibody against TAF is prepared and is tagged with a substance having a high affinity for cancer cells, growth of blood vessel into the cancerous tissue may be checked and the proliferation of cancer stopped. The fact that TAF has no direct action to promote proliferation of cancer relieves us from the concern against promoting proliferation of any cancer that may possibly exist in the living body; and this fact makes it easy to apply TAF clinically. In contrast, EGF promotes proliferation of cancer cells (Hirata 1988) and transforming growth factor may cause transformation of normal cells in the body (Umeda 1988). Therefore, some of the growth factors cannot be administered in this way.

We also examined the effect of TAF on tube formation by endothelial cells in vitro. If the tubular structures observed in vitro are the same as capillary blood vessels, then their formation reflects their ability to induce formation of capillary blood vessels in the living body. Consequently we examined the possible structural similarity between the tubular structures formed in vitro and the capillary blood vessels under optical and electron microscopes. The tubular structures are formed by a layer of loosely connected endothelial cells and closely resemble capillary blood vessels. The presence of fibrous materials in the lumen of the tube suggests that the orientation of the endothelial cells in the tube is the reverse of that normally found in in vivo capillaries, as has been mentioned by Feder et al. (1983). Thus, we have confirmed that TAF is capable of promoting proliferation and connection of endothelial cells resulting in formation of tubular structures. However, it appears that other factor(s) are necessary for the proper orientation of endothelial cells in the capillaries. The present system of experiments can be useful for quantitative determination of TAF on melt down of matrix and wandering of endothelial cells.

Acknowledgements. We wish to thank Dr. M. Furusato and Dr. S. Wakui for their assistance in the preparation of the electron micrograph.

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