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ORIGINAL ARTICLE

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Conditioned media of carcinoma cells cultured in hypoxic microenvironment stimulate angiogenesis in vitro; relationship to basic fibroblast growth factor

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Abstract Conditioned media (CM) harvested from human pulmonary squamous cell carcinoma (QG56), pulmonary small cell carcinoma (QG90) and gastric adenocarcinoma (MKN28) cultivated under hypoxic conditions (3% oxygen), enhanced the angiogenic activity in vitro more than those obtained under normoxic cultivation (20% oxygen). The total length of the tube structures formed by bovine capillary endothelial cells (BCEs) in the CM cultured at 3% oxygen was about 1.5 (QG56 and MKN28) or 1.9 (QG90) times longer than that at 20% oxygen. Tube formation was diminished by the preincubation of CM with anti-basic fibroblast growth factor (bFGF) IgG. After performing the fractionations of the CM and the crude extracts of cell lysates cultured using a heparin-Sepharose column, the mitogenic activity in the CM from all cancer cells at 3% oxygen was about twice that of CM at 20% oxygen, while it decreased in the cell lysates at 3% oxygen to about 40% of those at 20% oxygen. This mitogenic activity of BCEs in the CM from all cancer cells was almost totally suppressed by anti-bFGF IgG, but not with anti-vascular endothelial growth factor IgG. Hypoxia is an important factor in tumour angiogenesis by bFGF or bFGF-like molecule(s) derived from tumour cells.

Key words Angiogenesis · Fibroblast growth factor basic · Endothelial cells · Hypoxia · Neoplasms

Introduction

Angiogenesis plays a critical role in solid tumour growth, which has been postulated to be fundamentally dependent on the induction of stromal neovascularization [2, 6, 9, 10, 39]. Some chemical substances produced by tumour cells or endothelial cells have also been known to stimulate the angiogenic process in tumour tissue in either a paracrine or autocrine manner [13, 20, 27]. The growth of solid tumours has been thought to be initially limited to a few cubic millimetres before the infiltration of newly formed blood vessels from the stroma, but can continue after vascularization from the surrounding tissue [9]. Therefore, it is assumed that there are two stages in the early stage of solid tumour growth, namely, the avascular and the vascular phases. Freitas et al. [15] examined the induction process of vascularization in the stroma of transplanted Ehrlich carcinoma using both histochemical and ultrastructural methods, and his findings suggested that the stromal neovascularization in tumour tissue was an outcome of hypoxia.

Since Folkman and Haudenschild [11] reported that isolated and cultured endothelial cells formed tubular structures in vitro, which were similar to capillary structures in vivo, many authors have studied the angiogenic processes modified by several factors in vitro [12, 26, 31, 44, 48].

In the present study, we examined the regulating mechanism of angiogenesis by the interaction between endothelial cells and cancer cells, specifically the influence of hypoxia on cancer cells, using an in vitro angiogenic assay be quantitative methods to measure the total length of the tubular structures formed by cultured endothelial cells on type I collagen gels [44, 48], and the assay for DNA synthesis of bovine capillary endothelial cells (BCEs). We demonstrated that the conditioned media (CM) of cancer cells cultured under hypoxic conditions promoted tube forming activity and DNA synthesis of BCEs when compared with CM from normal conditions. These enhancing effects of the hypoxic CM on angiogenic activity were closely related to the function of basic fibroblast growth factor (bFGF); the hypoxic state probably induced the cancer cells to release bFGF without any apparent enhancement of bFGF mRNA.

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Materials and methods

BCEs were isolated from the bovine adrenal cortex [14, 44, 48]. Seventh to twelfth-passage cultures were used for the experiments described later in this report.

The cancer cells examined in this study were QG56 established from human pulmonary squamous cell carcinoma, well-differentiated [47], QG90 from pulmonary small cell carcinoma [47] and MKN28 from gastric adenocarcinoma, well-differentiated [21]. We have previously reported the coagulant and fibrinolytic properties of these cancer cell lines [23, 40], and showed that they possess a relatively high amount of fibrinolytic activity.

All cancer cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Flow Laboratories, Stanmore, Australia), penicillin (100 units/ml) and streptomycin (100 μg/ml) under either normo- (20% oxygen) or hypoxic (3% oxygen) conditions balanced with nitrogen in a humidified 5% carbon dioxide incubator at 37° C oxygen carbon dioxide Multi-Gas incubator BL3200, Astec Co., Fukuoka, Japan). The CM after the cultivations of cancer cells were harvested 3, 6, 12, 24 and 48 h later, and were centrifuged at 5500 xg for 10 min to avoid cell debris. The supernatants were sterilized through a 0.22 μm-pore filter, divided into aliquots and were measured for lactate dehydrogenase activity, and then frozen until use.

bFGF purified from bovine brain, active transforming growth factor-β_1 (TGF-β_1) derived from human platelets and anti-pocrine TGF-β_1 polyclonal IgG were purchased from R&D Systems (Minneapolis, Minn., USA), and the rabbit anti-human recombinant bFGF IgG from Biomedical Technologies (Stoughton, Mass., USA). The human recombinant vascular endothelial growth factor (VEGF) and rabbit anti-human VEGF IgG were obtained from Santa Cruz Biotechnology (Satan Cruz, Calif., USA). The biological activities of bFGF and TGF-β_1, and immunologically neutralizing activity of their antibodies were assessed by a DNA synthesis assay as described previously [44]. The lactate dehydrogenase assay kit was obtained from the Sigma (St. Louis, Mo., USA), and the bovine type I collagen was from Koken (Tokyo, Japan).

The preparation and immunological characterization of rabbit anti-human tissue type plasminogen activator (tPA) or urokinase type plasminogen activator (uPA) IgG have been previously reported [16, 40]. The rabbit anti-uPA IgG inhibited human uPA activity (50% inhibitory IgG concentration for 100 mIU/ml of human uPA: 0.6 μg/ml) but did not inhibit human tPA, while the rabbit anti-tPA IgG inhibited human tPA activity (50% inhibitory IgG concentration for 100 mIU of human tPA: 0.03 μg/ml).

For quantitative assay of tube formation of BCEs 100 μl of type I collagen solution (0.3%), containing 25 μg/ml of plasminogen, which are purified from human plasma by an affinity chromatography on a L-lysine-Sepharose column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) [7], and 5 μg/ml of human fibronectin (Collaborative Research, Bedford, Mass., USA), was put on the filter of Millicell-CM inserts (12 mm in diameter, Millpore Co., Bedford, Mass., USA), which were placed in 12 well plates (Corning Glass). After the gelation of collagen, the plates were filled and incubated with 3 ml of serum-free RPMI for 7 days before use to allow for permeation and equilibration with the culture media.

To evaluate the in vitro angiogenic activity by measuring the total length of tubes formed by BCEs, 5.3×10^4 cell suspension of BCEs were placed on the surface of type I collagen gels in the inner side of the Millicells and cultured in RPMI 1640 containing 10% FBS. The cultures reached confluence about 24 h later after seeding, when the media in the outer wells were replaced by 1.5 ml of CM that were then appropriately diluted, and the fluid levels of the inner and outer wells were equalized by applying 0.3 ml of RPMI 1640 with 10% FBS into the inner wells. The BCEs were cultured for 10 days at 37° C in a humidified atmosphere with 5% carbondioxide and both media were changed every 2 days.

On the tenth day the BCEs were fixed with 10% formalin in phosphate buffer, pH 7.4. Tubular structures organized by the BCEs could be observed in type I collagen gels under a phase contrast microscope. Nine areas selected at random in each well of triplicate cultures were then videotaped. The length of the tubular structures observed in the videos was measured with a Cosmozone 1S image analyser, and the total length of tube formation was calculated for quantitative in vitro assessment of angiogenesis.

The gels fixed with formalin were embedded in paraffin. Perpendicular sections of 4 μm-thickness were cut at their maximal diameter and stained with haematoxylin-eosin.

The 50% neutralization doses of anti-bFGF IgG for thymidine incorporation of BCEs promoted by 0.8 ng/ml of bFGF and anti-TGF-β_1 IgG for thymidine incorporation inhibited by 0.1 ng/ml of TGF-β_1 were about 0.5 μg/ml and 2 μg/ml, respectively. Therefore, anti-bFGF IgG was used at 20 μg/ml and anti-TGFβ_1 was at 30 μg/ml in the following experiments. The CM were preincubated with anti-bFGF IgG (20 μg/ml) or anti-TGF-β_1 IgG (30 μg/ml) at 37° C for 30 min, and were placed in the applied into outer wells to examine the effects of these anti-IgGs on tube formation induced by the CM. Preincubated CM with rabbit non-immune IgG were used as a control.

To examine whether anti-bFGF or TGF-β_1 IgG modified the effects of CM on the DNA synthesis of BCEs, the CM harvested 24 h after the cultivation of cancer cells at either 20% or 3% oxygen were preincubated with these IgGs or non-immune IgG at a final concentration of 20 μg/ml for 2 h at room temperature. After removing the precipitates be centrifugation at 5,500 xg for 15 min, the supernatants were sterilized by filtration, and then a DNA synthesis assay was performed.

The effects of heparin on tube formation by BCEs were also examined by adding heparin to CM to make a final concentration ranging from 0.01 to 100 ng/ml.

To examine the effects of the CM on DNA synthesis of BCEs, the incoporation of tritiated-thymidine by BCEs was measured as described previously [44]. Subconfluent BCEs were incubated with tritiated-thymidine (5.0 Ci/mM, Amersham Co., Arlington Heights, Ill., USA) at a concentration of 0.5 μCi/ml of CM or fresh medium. The BCEs were then washed and treated with cold 5% trichloroacetic acid (TCA). The resulting precipitates were washed with ethanol-ether (3:1 mixture) and solubilized by 1.0 M sodium hydroxide. Then the radioactivity of the precipitates was examined with a liquid scintillation counter (LSC-3500, Aloka, Co., Tokyo, Japan).

To partially characterize the in vitro angiogenesis enhancing factor(s) synthesized and/or released by the cancer cells, the CM and cell lysates of cancer cells, cultivated in FBS-free RPMI 1640 for 24 h under either 20% or 3% oxygen, were prepared as described below. The cells, detached with 0.25% trypsin (Mochida, Tokyo, Japan) and 0.02% ethylenediamine tetra-acetic acid (EDTA) solution, were washed with PBS and resuspended in 20 ml of 10 mM TRIS-HCl, pH 7.4, containing 1 M sodium chloride, then they were frozen and thawed three times, homogenized with a teflon-glass homogenizer, and then centrifuged at 5,000 xg for 20 min. The supernatants were frozen until use.

The CM or cell lysates were diluted with 10 mM TRIS-HCl, pH 7.4, to 15 times (300 ml), passed through a Millipore filter with 0.22 μm pore size and then applied to a heparin-Sepharose column (Pharmacia Line Chemicals AB). The column was washed with 10 mM TRIS-HCl, pH 7.4, and eluted with a linear gradient of 0–3.0 M sodium chloride in 10 mM TRIS-HCl, pH 7.4. The eluates were then dialysed against RPMI 1640 for 24 h at 4° C in a dialysis tube with a molecular weight cut-off of 2000 (Spetrum Medical Industries, Inc., Los Angeles, Calif., USA) and next an assay was performed for the DNA synthesis of BCEs by measuring the incorporation of tritiated-thymidine. The DNA synthetic activity was expressed in comparison to the non-conditioned fresh medium of RPMI containing 10% FBS as a control.

The CM harvested 24 h after the cultivation of cancer cells at either 20% or 3% oxygen were pretreated with anti-bFGF or anti-VEGF IgG at final concentration of 20 μg/ml for 2 h at room temperature. The 50% neutralizing dose of anti-human VEGF IgG for 0.5 ng/ml of recombinant human VEGF, which was the medium effective dose for the incorporation of thymidine by BCEs, was almost 0.1 μg/ml. The effect of anti-bFGF or VEGF IgG on the DNA synthetic activity of CM was compared with non-treated CM under 20% oxygen condition.

The CM or non-conditioned and fresh media containing 10% FBS were preincubated with anti-tPA (50 μg/ml), uPA (100

μg/ml) or non-immune IgG at 37° C for 30 min, sterilized by filtration, and then applied to the outer wells of Millicell-CM to determine the effects on tuba formation. The inner wells were filled with non-conditioned fresh media also containing the same concentration of anti-tPA, uPA or non-immune IgG in the outer well.

For the Northern blot analysis, the *Hin*dIII-*Eco*RI fragment derived from human bFGF cDNA (engineered gene coding for mature human bFGF, which was a kind gift from Mochida [18, 28] was cloned onto the polylinker of a pBluescript SK+ to serve as the template. A cRNA probe specific to human bFGF was generated by in vitro transcription using digoxigenin(DIG)-11-UTP (Boehringer Mannheim Biochemica, Mannheim, Germany) and T3 polymerase.

Total cellular RNA of cancer cells cultured at 3% or 20% oxygen were examined for bFGF mRNA expression by a Northern blot analysis as described before [4, 28]. A human β-actin probe was purchased from Wako (Tokyo, Japan) and used as a control probe.

Results were expressed as the mean±standard deviation (SD). The statistical comparison of the multiple means was examined by an analysis of variance, and a comparison of the two means was done with Student's unpaired *t*-test. All *p* values were analysed on two sides.

Results

Condition of cancer cells under hypoxia

None of the cancer cells examined showed either any apparent morphological alteration or viability after hypoxic cultivation for 24 h. In addition, neither cell debris nor the detachment of cells increased under the hypoxic conditions. The lactate dehydrogenase levels in the CM after a 24 h cultivation at 20% or 3% oxygen also showed no statistical difference in regard to the oxygen concentration (data not shown). The number of cancer cells after normo- and hypoxic cultivation for 24 h was not statistically different from each oxygen concentration for cultivation. The pH of the CM harvested after a 24 h cultivation at 20% or 3% oxygen was 7.10±0.08 and 6.98±0.08 (mean±SD, n=3), respectively. Thus the CM used were all buffered with 20 mM HEPES, pH 7.2, in the following studies.

Tube formation of BCEs

After becoming confluent on the surface of the collagen gels, the seeded BCEs started up sprout into the gels and formed tubular structures beneath the confluent monolayer on the second day after the application of the CM. Within a week these tubular structures gradually became elongated and formed networks (Fig. 1a). The tubular structures were observed to possess a lumen formed by several BCEs in the gels under light microscopy of the formalin-fixed and paraffin-embedded sections (Fig. 1b).

The extent of the tube formation of BCEs was low in the fresh media (0.42±0.09 mm/mm^2, n=3). The effect of the CM on tube formation was recognized even in the CM with a 6 h cultivation of cancer cells at 20% oxygen and was enhanced in a duration-dependent manner (Fig. 2). The total length of tube formation by BCEs reached a plateau in the CM obtained 24 h after the cancer cell cultivations (QG56:1.48±0.21; QG90: 1.69±0.26 and

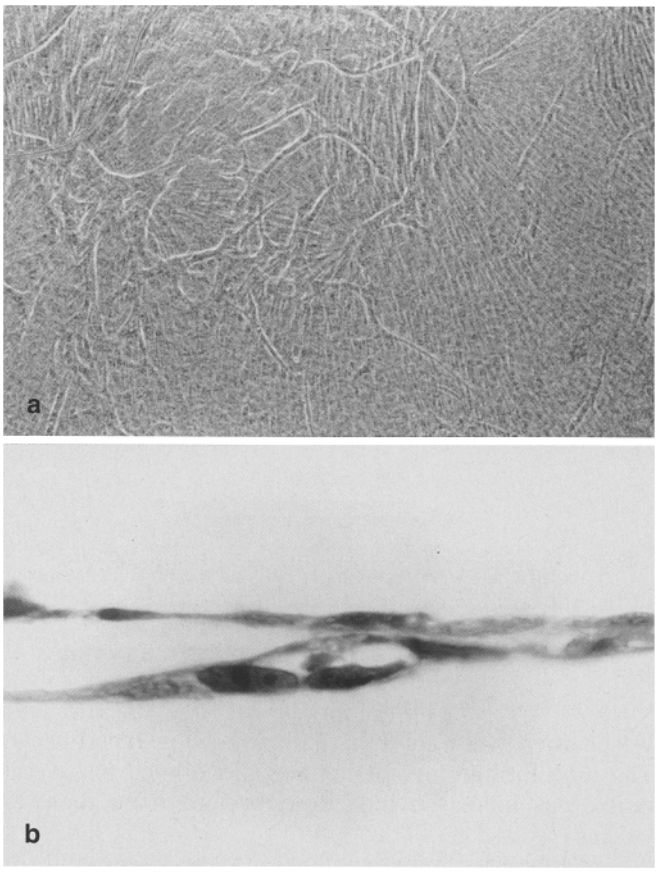

Fig. 1 **a** Representative phase-contrast microscopic findings of bovine capillary endothelial cells (BCEs) grown on and in type I collagen gel on the seventh day. The tubular structures are recognized as triplet layered bands composed of an inner dark area between two outer bright lines. BCEs were cultured in the conditioned media (CM) (20% oxygen) of QG56 ×100. **b** A light microscopic finding of tubular structures formed in a collagen gel of **a**. A tubular structure is formed by several BCEs. Hematoxylin and eosin staining, ×388

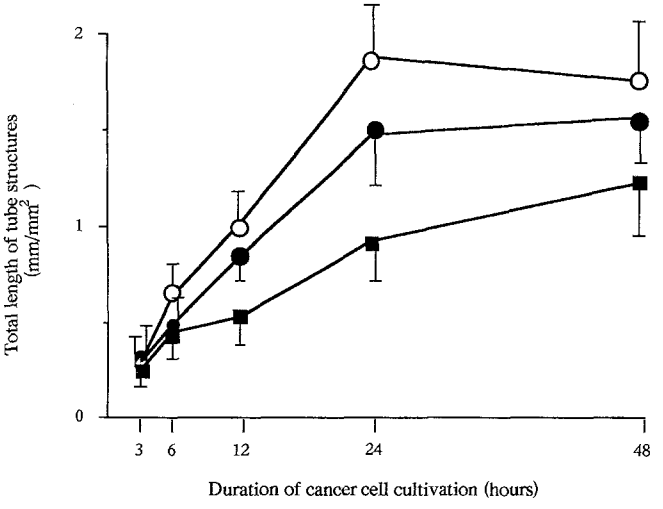

Fig. 2 Time-dependent effect of the CM of cancer cells (QG56 (-●-), QG90 (-○-) and MKN28 (-■-)) on tube formation of BCEs. The tube forming activity of the CM becomes apparent when cancer cells have been cultured at 20% oxygen for more than 12 h and reached a plateau after 24 h. The mean values (±SD) of three experiments are shown

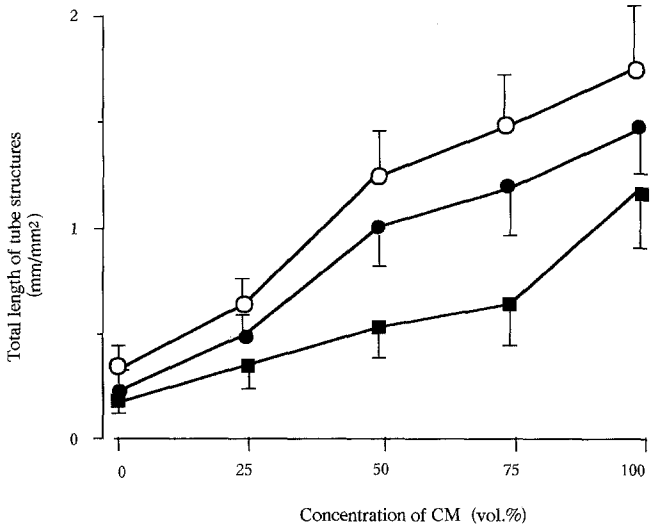

Fig. 3 The dose-dependent effect of CM (QG56 (-●-), QG90 (-○-) and MKN28 (-■-)) of cancer cells on the tube formation of BCEs. The tube forming activity of all CM obtained at 20% oxygen for 24 h appeared at a dose ratio of more than 25 vol.%. The mean values (±SD) of three experiments are shown

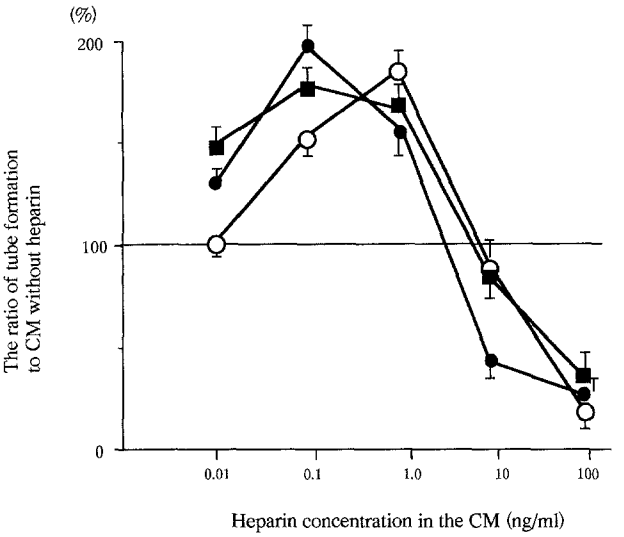

Fig. 5 The effect of heparin on tube forming activity of the cancer cell-CM (QG56 (-●-), QG90 (-○-) and MKN28 (-■-)). The exogenous addition of heparin to the CM generally enhances the tube forming activity of the CM at lower concentrations (0.01, 0.1 and 1.0 ng/ml), but reversely tends to reduce it at the higher concentrations (10 and 100 ng/ml) as compared with the heparin(-)-CM

MKN28: 1.15±0.24 mm/mm^2 (n=3)), thus the CM harvested after 24 h were used in the following experiments. The tube forming activity of the CM diluted to several ratios was increased in a dose-ratio of more than 25 vol.% (Fig. 3).

The tube formation examined in the CM cultured for 24 h at 3% oxygen was 2.32±0.18 (QG56), 2.77±0.21 (QG90) and 1.91±0.19 mm/mm^2 (n=3) (MKN28), and was about 1.4 (QG56 and MKN28) or 1.9 (QG90) times longer than that at 20% oxygen (Fig. 4). The enhancement of tube formation by bFGF (10 ng/ml), which is known to be one of the most potent angiogenic factors [36], was 2.13±0.27 mm/mm^2 (n=3).

The effects of anti-bFGF and TGF-β_1 IgGs, and heparin on in vitro angiogenesis

The tube forming activity of the CM from all cancer cells cultured at 20% oxygen was diminished by preincubation with anti-bFGF IgG (20 μg/ml), resulting in 19.2% (QG56), 27.5% (QG90) and 26% (MKN28). Anti-bFGF IgG also diminished the tube forming activity of the CM under hypoxic conditions to 21.3% (QG56), 33.4% (QG90) and 31.5% (MKN28). Anti-TGF-β_1 IgG (30 μg/ml) showed no apparent effect on the tube forming activity of the CM at either 20% or 3% oxygen (Fig. 4). Preincubated CM with rabbit non-immune IgG also revealed no apparent effect (data not shown).

The extent of tube formation generally increased by the addition of low amounts of heparin (0.01, 0.1 and 1.0 ng/ml) to the CM in a dose-dependent manner, but conversely tended to decrease by an excessive addition of heparin (10 or 100 ng/ml) as compared with that in the CM without heparin (Fig. 5).

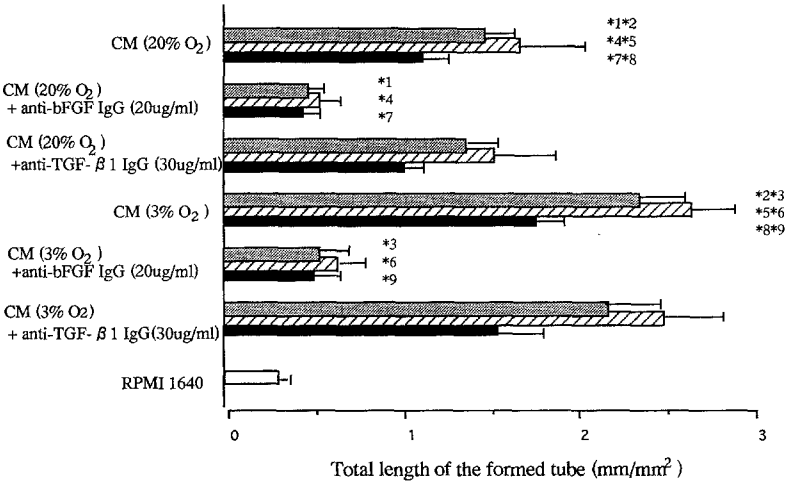

Fig. 4 The effect of CM obtained at normo- and hypoxic conditions with or without antibodies on the tube formation of the BCEs. The CM of cancer cells cultivated at hypoxia (3% oxygen) significantly promote the tube formation of BCEs. The preincubation of all CM (QG56 (▒), QG90 (▨) and MKN28 (■)) with anti bFGF-IgG (20 μg/ml) remarkably reduces the tube forming activity of the normo- and hypoxic CM, whereas anti TGF-β_1 IgG (20 μg/ml) does not. The mean values (±SD) of three experiments are shown

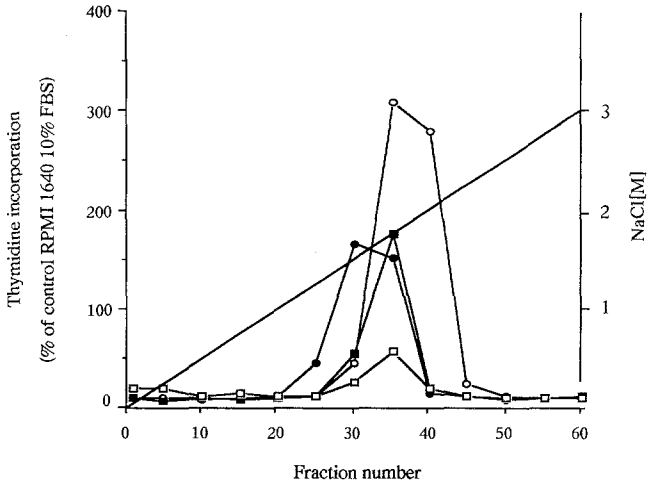

Fig. 6 The mitogenic effect of the eluates after the heparin-Sepharose column chromatographies of the QG56-CM and cell lysates on BCEs. The CM and cell lysates were obtained after the cultivation of cancer cells at either 20% or 3% oxygen (see the text). The mitogenic activity of the CM and cell lysates to BCEs was recognized in eluates obtained at 1.5–1.8 M sodium chloride. The CM obtained at 3% oxygen (-○-) enhanced the DNA synthesis of BCEs by about twofold compared with that of the CM cultured at 20% oxygen (-●-). The cell lysates obtained from cancer cells cultivated under 3% oxygen (-□-) also possess an enhancing effect of DNA synthesis of BCEs, but was less than 40% of that at 20% oxygen (-■-)

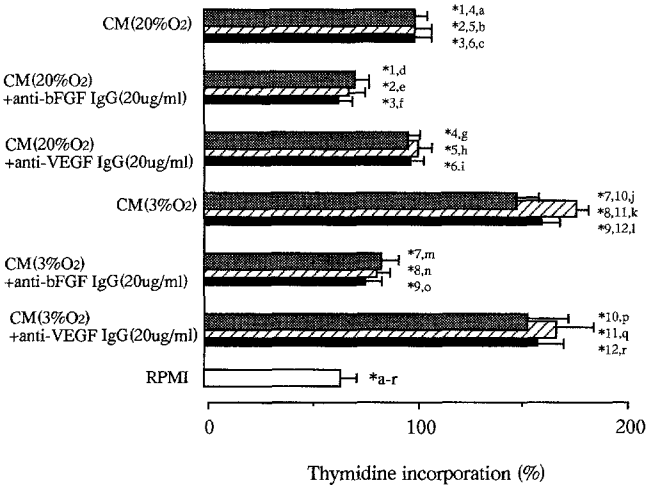

Fig. 7 The effects of the pretreatment of the CM (QG56 (▒▒), QG90 (▨), and MKN28 (■)) with anti-bFGF IgG (20 μg/ml) or anti-VEGF IgG (20 μg/ml) on thymidine incorporation by BCEs. The effects of CM at 20% oxygen on thymidine incorporation by BCEs was expressed as 100% and compared with DNA synthetic activity of the respective CM obtained at either 20% or 3% oxygen and with or without the antibody pretreatment. The pretreatment of CM with anti-bFGF IgG significantly suppressed DNA synthetic activity of the CM, but not with anti-VEGF IgG. The mean values (±SD) of three experiments are shown. *1–3, 7–9: $p<0.01$, *4–6, 10–12: NS *a–c, g–l, p–r: $p<0.01$, *d–f, m–o: NS

The effects of the eluates from heparin-sepharose column on DNA synthesis of BCEs

The thymidine incorporation of the BCEs increased by eluates at 1.5–1.8 M sodium chloride after heparin-Sepharose chromatographies of both the CM and cell lysates of all cancer cells cultured at 20% and 3% oxygen when compared with that of the non-conditioned RPMI 1640 containing 10% FBS. A representative elution profile of BCEs-mitogenic activity of QG56-CM or -cell lysates cultivated at 20% or 3% oxygen from the heparin-Sepharose column is shown in Fig. 6. The DNA synthetic enhancing effect of CM obtained after 3% oxygen cultivation was about twice that compared to the CM at 20% oxygen, whereas the DNA synthesis level of the BCEs in the cell lysates obtained after 3% oxygen cultivation decreased to 40% of that at 20% oxygen. The elution profiles after the heparin-Sepharose column chromatographies of the other cancer cells (QG90 and MKN28) were almost the same as those of QG56 (data not shown).

The DNA synthetic activity of CM and cell lysates was also markedly suppressed by preincubation with anti-bFGF IgG (data not shown).

The effects of anti-bFGF or VEGF IgG on DNA synthesis of BCEs

The CM of all cancer cells at either 20 and 3% oxygen stimulated thymidine incorporation of BCEs compared with non-conditioned fresh media. The CM obtained at 3% oxygen stimulated BCEs to incorporate thymidine from 1.4 (QG56) to 1.7 times (QG90) compared with those at a 20% oxygen cultivation. The pretreatment of CM with anti-bFGF IgG apparently inhibited this enhancing effect of thymidine incorporation, while no apparent effect of anti-VEGF IgG was detected (Fig. 7).

The effects of anti-tPA and uPA IgGs on in vitro angiogenesis

Tube formation was not affected by the preincubation of the CM of all cancer cells with anti-tPA IgG (50 μg/ml), but apparently was suppressed by preincubation with anti-uPA IgG (100 μg/ml) and was 17.9% (QG56), 14.8% (QG90) and 18.0% (MKN28) (n=3) (Fig. 8).

The expression of bFGF mRNA in cultured cancer cells

The average yields of the total RNA isolated from cancer cells (about 1.2×10^7 cells) cultured at either 20 or 3% oxygen for 24 h were 4.15 mg (QG56), 3.97 mg (QG90) and 2.98 mg (MKN28). A 10 μg/ml aliquot of the RNA samples was applied to each lane and Northern blot hybridization was simultaneously carried out by using an RNA probe of a bFGF or a DNA probe of β-actin. The specified mRNA bands of bFGF were apparent at 3.7 and 7.0 kDa in all lanes. bFGF mRNA expressed by QG56 and MKN 28 cultured at 20% oxygen was rela-

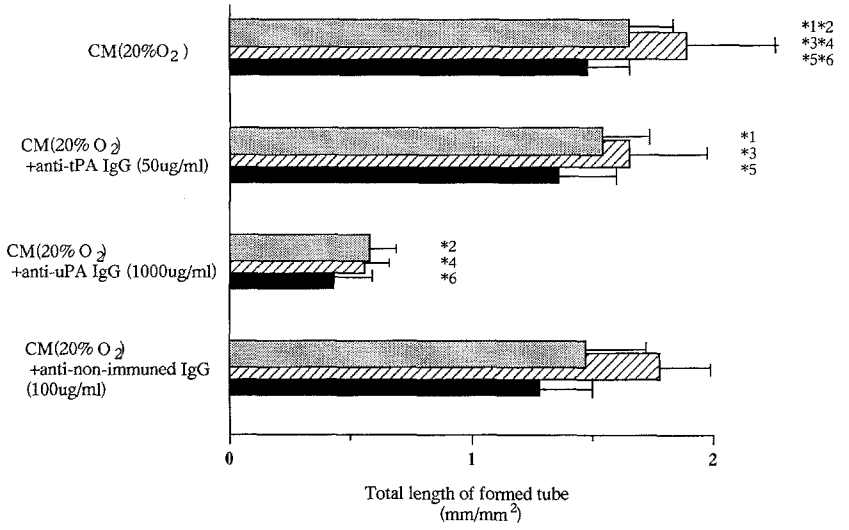

Fig. 8 The effects of anti-tPA and anti-uPA IgGs on tube formation of BCEs in the CM of cancer cells (QG56 ( ), QG90 ( ), and MKN28 ( )). The tube forming activity apparently decreased by the addition of anti-uPA IgG (100 μg/ml), whereas the addition of anti-tPA (50 μg/ml) or non-immune IgG (100 μg/ml) did not show such a decrease. The mean values (±SD) of three experiments are shown

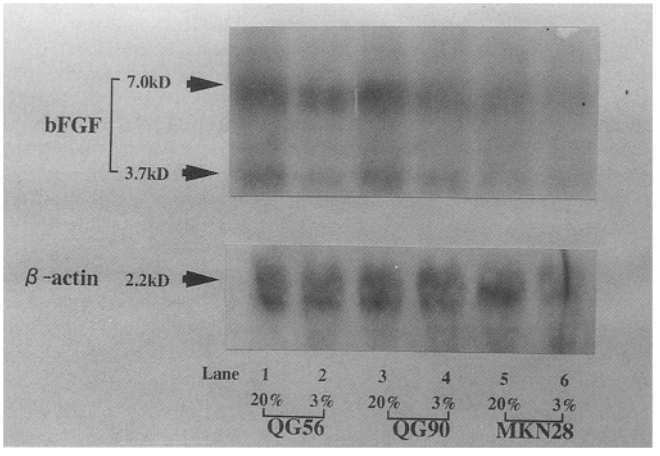

Fig. 9 A Northern blot analysis of the cancer cells for bFGF-specific mRNA. Total RNA was isolated from the cancer cells cultures at 20% oxygen (QG56 (lane 1), QG90 (lane 3) and MKN28 (lane 5)) or 3% oxygen (QG56 (lane 2), QG90 (lane 4) and MKN 28 (lane 6)) for 24 h. Of every total RNA 10 μg/ml was electrophoresed in 1.2% agarose gel, transferred to nitrocellulose membranes, and hybridized with digoxigenin(DIG) labelled bFGF mRNA. Anti DIG antibody conjugated with alkaline-phosphatase(ALP) was applied to the membrane. AMPPD was reacted to ALP, and then the membrane was exposed to radiographic film. The expression of Human β-actin mRNA was examined as a control

tively higher than that of QG90. However, hypoxic cultivation did not apparently increase the amount of bFGF-specific mRNA after 24 hours of cultivation (Fig. 9).

Discussion

Under pathological conditions, as in normal tissues, the angiogenic process is well regulated by specific initiating and inhibitory factors. However, in neoplasmia continuous stimulation of endothelial cells leads to a highly vascularized stroma. Recent findings support the hypothesis that tumour growth and metastasis are angiogenesis-dependent [9, 10, 39]. In rapidly growing tumours the central portion frequently undergoes necrosis probably due to hypoxia and nutritional deprivation.

We assumed that hypoxia would modulate the release and/or synthesis of angiogenic factor(s) by cancer cells to develop the collateral vascular channels. Our study showed that the exposure of human cultivated cancer cells, QG56, QG90 and MKN28, to hypoxia enhanced in vitro angiogenic activity in the CM of three cancer cells, and this enhancement was dependent upon the duration of a hypoxic state up to 24 h. This finding supports the hypothesis that hypoxia stimulates cancer cells to influence neovascularization in the cancerous stroma.

Hypoxia-dependent angiogenesis has also been observed in other conditions [25, 37]. In a previous study [44], we found that bovine aortic smooth muscle cells (SMCs) cultured under hypoxic conditions released active angiogenic factor(s) into the CM and the degree was dependent upon the hypoxic state which used similar in vitro angiogenesis models to the present study. The angiogenic factor(s) derived from smooth muscle cells seemed to be similar to TGF-β_1 and exposure of SMCs in the atherosclerotic intimas to hypoxia may be a mechanism inducing neovascularization in atherosclerotic plaques.

In the present study, however, the in vitro angiogenic factor(s) derived from cultured cancer cells examined seemed to be functionally and immunologically related to bFGF or bFGF-like molecule(s), because the tube forming activity observed in normo- and hypoxic cancer cell-CM was significantly suppressed by the pretreatment of CM with anti-bFGF IgG. BCEs-mitogenic activity of CM and cell lysates from these cancer cells was eluated at 1.5–1.8 M sodium chloride from a heparin-Sepharose column and their elution profile was almost the same as that of the bFGF reported previously [24], but was different from that of acidic fibroblast growth factor (aFGF) [8] and VEGF [43]. In addition, the promoting effect of CM from all cancer cells examined on DNA synthetic activity of BCEs was almost totally suppressed

by the pretreatment of the CM with anti-bFGF IgG, but not with anti-VEGF IgG.

We studied the expression of mRNA of bFGF using a Northern blot analysis. All cancer cells examined expressed mRNA of bFGF, but no significant increase of bFGF mRNA was recognized in cancer cells exposed to hypoxia. In addition, the elution profile of bFGF-related activity after a heparin-Sepharose column chromatography of CM from all cancer cells at hypoxic state was apparently increased, while it decreased in the cell lysates at hypoxic state, when compared with that of CM at 20% oxygen, respectively. Therefore, the enhancing effect of hypoxia on the expression of angiogenic activity by cancer cells examined seemed to result from the increase of bFGF release from cancer cells, but not the enhancement of bFGF synthesis. These findings suggest that bFGF synthesized and released by these cancer cells is involved in tumour growth probably in a paracrine manner, and the hypoxic microenvironment plays a critical role in the induction of stromal angiogenesis in solid tumours by enhancing the release of angiogenic factor(s) from the cancer cells. However, the autocrine mechanism of in vitro angiogenesis may be also affected through the up-regulation of bFGF synthesis or bFGF-receptor expression by BCEs, possibly via the effects of nucleotides metabolites such as adenosine and nicotinamide derived from cultured cancer cells at hypoxia [37].

bFGF is well known to have no signal peptide for its secretion [1], and may be passively released by cell damage or death [3, 17, 33, 38, 42]. In this study, as the LDH levels in the CM were not increased and no cell damage or death of cancer cells was apparent under hypoxic cultivation. Recent reports have revealed that some proteins such as yeast mating pheromone factor [32], haemolysin [30], muscle lectin [5] and interleukin-1 β_1 [49] which lacks a signal peptide, were actively exported without any apparent cell damage. Mignatti et al. [35] suggested that bFGF might be secreted via a pathway independent of the endoplasmic reticulum-Golgi complex. Further studies will be necessary to clarify not only the detailed mechanism of bFGF release by cancer cells but also the effect of hypoxia on the exocytosis of bFGF.

VEGF, also known as vascular permeability factor, is a specific growth factor for endothelial cells and a hypoxia-inducible angiogenic factor [45]. Pepper et al. [41] reported that the angiogenic activity of VEGF was synergistic with bFGF. Therefore, although bFGF seemed to play a major role in the enhancement of tube forming activity and the thymidine incorporation of BCEs by the CM of cancer cells cultured in a hypoxic state in this study, further study including the mRNA expression of VEGF in both normal and hypoxic states will be necessary.

Glycosaminoglycans, especially heparin, heparan sulphate and related polysaccharides possessing a high affinity for bFGF, appear to participate intimately in the regulation of bFGF function. They affect binding of bFGF to its high receptor on the cell surface [22], support the protective effect of heparin from the degradation of bFGF by proteases [19, 29] and develop a reservoir of biologically active bFGF in the extracellular matrix and on the cell surface. However, there are still several unresolved problems concerning the specific heparin binding domain of bFGF and the effect of soluble heparin on bFGF function. The effect of heparin exogenously added to the cancer cell CM on angiogenesis in vitro was biphasic, and the enhancing effect of these CM on angiogenesis was potentiated at lower concentrations (0.1 and 1.0 ng/ml) of soluble heparin, while it was suppressed it at higher concentration (10 or 100 ng/ml). This biphasic effect of soluble heparin may play an important role in the development of neovascularization in cancer by bFGF release from cancer cells, though the mechanisms remain unclear.

We have previously reported that the tube forming activity of BCEs was apparently enhanced by the addition of human plasminogen into collagen gels in a dose dependent manner and was inhibited by anti-plasmin reagents [48]. The tube forming activity of the BCEs stimulated by the CM of cancer cells was also enhanced by the addition of plasminogen into the gels and decreased by the addition of specific IgG against uPA and anti-plasmin reagents. Therefore, the fibrinolytic system, especially uPA expressed by endothelial cells, may play an important role in the angiogenesis of cancer tissue. It has also been reported that the expressions of bFGF mRNA and uPA receptor by endothelial cells are enhanced by exogenous bFGF [34, 46]. Based on these findings, bFGF released by cancer cells may therefore induce stromal angiogenesis by enhancing both the mitogenic activity and migration of endothelial cells.

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