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EFFECTS OF FIBRIN ON THE ANGIOGENESIS IN VITRO OF BOVINE ENDOTHELIAL CELLS IN COLLAGEN GEL

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SUMMARY

The effect of fibrin on angiogenesis in vitro was investigated using an experimental model of tube formation by bovine capillary endothelial cells (BCEs) in type I collagen gel. One milligram per milliliter of fibrin added into type I collagen gel significantly increased the length of the tubular structures formed by BCEs in the gel by about 180% compared with type I collagen only. The facilitating effect of fibrin on tube formation by BCEs was inhibited by either anti-basic fibroblast growth factor (bFGF) IgG (25 μg/ml) or anti-urokinase type plasminogen activator (uPA) IgG (10 μg/ml) added to the gel and culture medium, but not by anti-tissue type plasminogen activator (10 μg/ml) or non-immune IgG. The Arg-Gly-Asp (RGD) containing peptides (100 μg/ml) added to the culture medium also suppressed tube formation by BCEs in fibrin-containing type I collagen gel, but not in type I collagen gel. These results suggest that the increased release of bFGF and uPA by BCEs therefore plays a role in the angiogenic effect of fibrin in vitro, and the angiogenic effect of fibrin is mediated by the RGD sequence in fibrin, probably via the function of integrin receptor of the BCEs.

Key words: angiogenesis; fibrin; basic fibroblast growth factor; urokinase type plasminogen activator; RGD sequence.

INTRODUCTION

In vivo angiogenesis in adults is observed in some pathologic conditions such as chronic inflammation, wound healing, atherosclerosis, proliferative retinopathy in diabetes mellitus, and progressive growth of solid tumors. Fibrin deposition is commonly observed in these conditions and fibrin has also been reported to enhance angiogenesis *in vitro* (24,25,29) and *in vivo* (9), but the mechanism of the angiogenic effect of fibrin has not yet been fully clarified. Angiogenesis is a complex process which includes the destruction of basement membrane, migration, proliferation, and formation of tubular structures by endothelial cells, and this process is modulated by several growth factors (5,20,42), proteases (19,43), and extracellular matrices (16,21,26). So all these factors have to be taken into consideration when investigating the mechanism of angiogenesis.

Folkman and Haudenschild (10) revealed *in vitro* organization of capillary endothelial cells into tubular structures, and these tubes formed *in vitro* were also recently shown to be ultrastructurally similar to capillaries *in vivo* (14). This experimental model has thus been utilized to examine the role of various angiogenic factors *in vitro*. We previously developed a quantitive assay method of *in vitro* angiogenesis by morphometrically measuring the total length of the organized tubular structures that are formed by capillary endothelial cells in type I collagen gel with an image analyzer (23,35,43).

In this report, we demonstrate that the increased release of basic fibroblast growth factor (bFGF) and urokinase type plasminogen activator (uPA) from bovine capillary endothelial cells (BCEs) is related to the angiogenic effect of fibrin in vitro, and this effect is mediated by the Arg-Gly-Asp (RGD) sequence of fibrin probably via the integrin receptor of BCEs.

MATERIALS AND METHODS

Reagents. Bovine fibrinogen (fraction I, type IV) was purchased from Sigma (St. Louis, MO) and contaminating plasminogen was removed by lysine-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography. Bovine α-thrombin was purified by cationic ion exchange chromatography and gel filtration from bovine thrombin (Mochida Pharm. Co. Ltd., Tokyo, Japan). Bathroxobin was purchased from the Fujisawa Pharm. Co. Ltd. (Ohsaka, Japan), and the bovine bFGF came from R&D Systems (Minneapolis, MN) and the peptides of Arg-Gly-Asp-Ser (RGDS), Gly-Arg-Gly-Asp-Ser (GRGDS), and Arg-Gly-Glu-Ser (RGES) were from Sigma.

Antibodies. Anti-human recombinant bFGF polyclonal IgG was purchased from Biomedical Technologies Inc. (Stoughton, MA). The antisera against purified tPA from the conditioned media of melanoma cell line, and purified uPA, which was a kind gift from Mochida, were raised in rabbits (28,32). The IgG fractions were isolated by ammonium sulfate precipitation and protein A-Sepharose column (Pharmacia Fine Chemicals) chromatography from these antisera and non-immune sera.

Culture of endothelial cells. The primary culture of BCEs was isolated from the bovine adrenal cortex according to the method of Folkman et al. (11) and then was cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS, Flow Laboratories, Stanmore, Australia). The absence of any mycoplasmal contamination was confirmed with a Hoechst Stain Kit for the Detection of Mycoplasma in Cell Cultures (Flow Laboratories). Passage 10 to 20 cultures of BCEs were used in the experiments.

Preparation of gels. Collagen gel composed of 1 mg/ml of type I collagen (Koken Co., Ltd., Tokyo, Japan), RPMI 1640 medium (Life Technologies, Inc.-GIBCO Products, Grand Island, NY), and 0.2% of sodium bicarbonate was prepared as described previously (23,35,43) and used as a control gel. Fibrinogen plus collagen gel was prepared by adding 1 mg/ml of fibrinogen into the collagen gel. To investigate whether the effects of fibrin and des-A

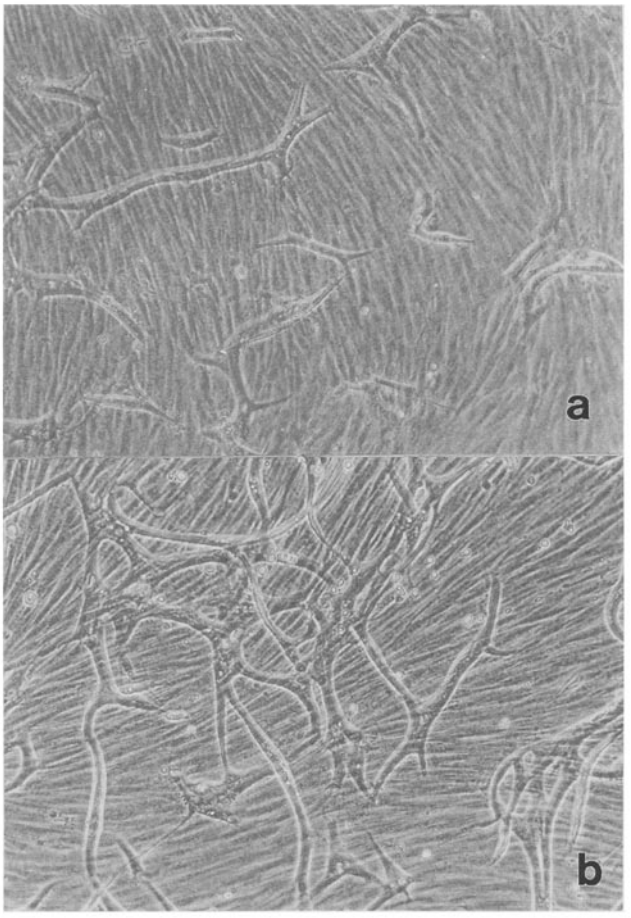

FIG. 1. Phase contrast micrographs of BCEs grown on collagen gel (*a*) and fibrin plus collagen gel (*b*) for 48 h. BCEs formed branching tubular structures which were recognized as parallel double bright lines. Total length of tubular structure was 2.36 mm/mm² (*a*) and 4.05 mm/mm² (*b*). ×100.

fibrin on the tube formation of BCEs *in vitro* are similar or not, des-A fibrin plus collagen gel and fibrin plus collagen gel were prepared by adding 1 BU/ml of bathroxobin or 1 U/ml of α-thrombin, respectively, into the fibrinogen plus collagen gel poured in a well of a 24-well plate (Corning, New York) followed by a rapid mixture. When bathroxobin acts on fibrinogen, fibrinopeptide A is released from fibrinogen and des-A fibrin is formed, while thrombin cleaves fibrinopeptide A and fibrinopeptide B from fibrinogen and des-A, B fibrin is formed. Fibrin is formed by polymerization of des-A, B fibrin, and is stabilized by factor XIII. Factor XIII is contaminated within the fibrinogen (fraction I, type IV) used in this study. One half milliliter of gel per well was prepared in 24-well plate, gelatinized at 37° C for 1 h and then washed with 1.5 ml/well of serum-free DMEM for 2 days in a humidified CO_2 incubator to eliminate either the unbound bathroxobin or α-thrombin in the gels.

Quantitative assay of tube formation by BCEs. Bovine capillary endothelial cells (11 × 10^4 cells/cm²) were seeded on each gel and cultured in DMEM supplemented with 10% FBS. In some experiments, antibodies or peptides were added in the gels and media. The tubular structures organized by the BCEs were observed under a phase-contrast microscope 48 h after BCEs were seeded. Nine fields randomly chosen in each well were recorded on a video floppy disc, and the length of the tubular structures were measured using a Cosmozone 1S image analyzer (Nikon, Tokyo, Japan). The total length of the tubular structures per area (mm/mm²) was calculated by summing the length of all tubular structures and dividing the summed length by the area of nine fields examined. The statistical analysis was made using Student's *t* test.

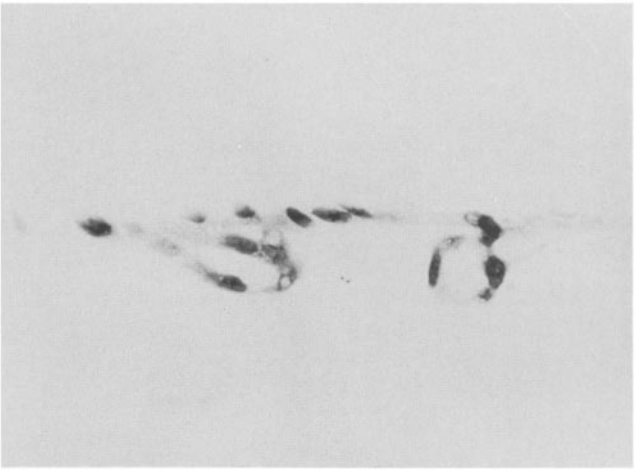

FIG. 2. Light micrograph of the tubular structures formed by BCEs in the fibrin plus collagen gel. BCEs formed tubular structures continuous with the monolayer of BCEs on the surface of the gel, and the cross sections of the tubular structures were formed by a few BCEs. Hematoxylin and eosin staining. ×200.

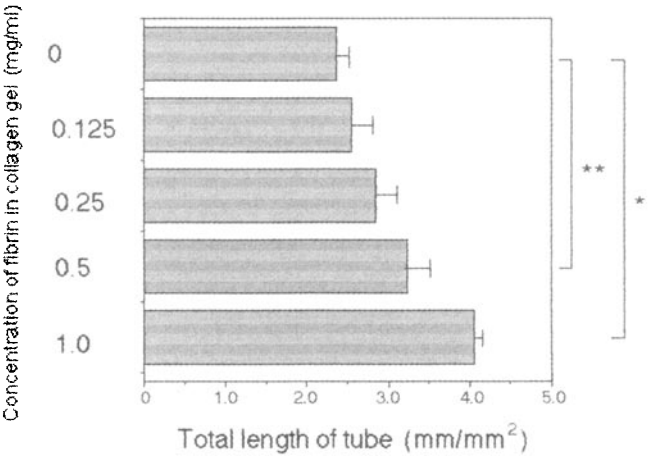

FIG. 3. Dose-dependent effect of fibrin on tube formation of BCEs in collagen gel. Length of the tubular structures formed by BCEs increased in proportion to the concentration of fibrin added to the collagen gel. Mean values (±SD) of three experiments are shown. $*P < 0.01$, $**P < 0.05$.

Light microscopy. For light microscopy, the BCEs plated on each gel were fixed with 10% formalin, embedded in paraffin, cut in 4-μm sections, and then were stained with hematoxylin and eosin.

RESULTS

Light microscopy. The BCEs grew to cofluency on collagen gels either with or without fibrin 24 h after the beginning of the cultivation and then formed tubular structures in the gels within further 24 h. Under phase contrast microscopy, the capillary-like branching tubular structures formed by BCEs in the gels were recognized as parallel double bright lines both in collagen gel (Fig. 1 *a*) and in fibrin plus collagen gel (Fig. 1 *b*). By light microscopy, the tubular structures seemed to be continuous with the monolayer of BCEs on the

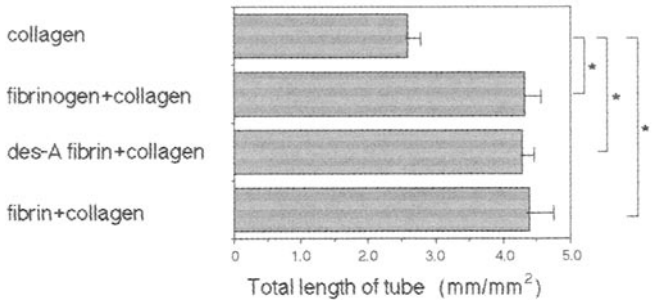

FIG. 4. Effects of fibrinogen, des-A fibrin, and fibrin on the tube formation of BCEs in the collagen gel. One milligram per milliliter of fibrinogen, des-A fibrin, or fibrin added to the collagen gel stimulated the tube formation of BCEs by about 180%, respectively. Mean values (±SD) of three experiments are shown. $*P < 0.01$.

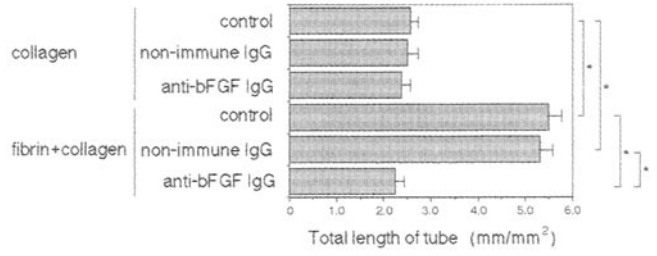

FIG. 5. Effect of anti-bFGF IgG on the tube formation of BCEs in the collagen gel and the fibrin plus collagen gel. Twenty five micrograms per milliliter of anti-bFGF IgG added to the gels and culture medium inhibited the tube formation of BCEs in the fibrin plus collagen gel, but did not inhibit the tube formation of BCEs in the collagen gel. Non-immune IgG did not inhibit the tube formation of BCEs in the collagen gel or the collagen plus fibrin gel. Mean values (±SD) of three experiments are shown. $*P < 0.01$.

surface of the gels, and the cross section of the tubular structures were usually composed of a few BCEs (Fig. 2).

The effects of fibrin on the tube formation of BCEs. When fibrin at varied concentrations was applied to collagen gel, the length of the tubular structures formed by the BCEs increased in a dose-dependent manner (Fig. 3), and 1 mg/ml of fibrin increased the length of tubular structures formed by the BCEs in the gel by about 1.8 times as compared with that in collagen gel only (Fig. 1 *a,b*). Fibrinogen and des-A fibrin (1 mg/ml, respectively) also increased the length of the tubular structures formed by the BCEs in collagen gel, and no significant difference was recognized among the angiogenic effects of fibrinogen, des-A fibrin, and fibrin (Fig. 4).

Effect of anti-bFGF IgG on the tube formation of BCE in the fibrin plus collagen gel. Anti-bFGF IgG (25 μg/ml) added to the gels and culture media significantly inhibited the tube formation by BCEs in fibrin plus collagen gel, but the tube formation by BCEs in collagen gel was not inhibited by the same dose of anti-bFGF IgG (Fig. 5). Non-immune IgG (25 μg/ml) did not show inhibitory effect on the tube formation by BCEs in fibrin plus collagen gel nor collagen gel.

Effect of Anti-PA IgGs on tube formation of BCEs in the fibrin plus collagen gel. Anti-uPA IgG (10 μg/ml) added to the gel and medium also decreased the total length of the tubular structures in the fibrin plus collagen gel. In contrast, anti-tPA IgG (10 μg/ml) showed a slight inhibitory effect on tube formation of BCEs in fibrin plus collagen gel, but this effect was not significant. Non-immune IgG (10 μg/ml) did not show any inhibitory effect on the tube formation of BCEs in the fibrin plus collagen gel (Fig. 6).

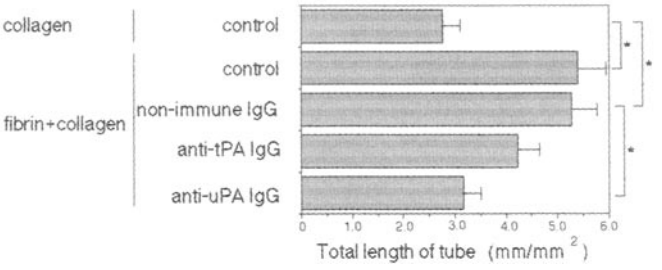

FIG. 6. Effects of anti-uPA IgG and anti-tPA IgG on the tube formation of BCEs in the fibrin plus collagen gel. Anti-uPA IgG (10 ng/ml) added to the gel and culture medium significantly inhibited the tube formation of BCEs in fibrin plus collagen gel. Anti-tPA IgG (10 ng/ml) added to the gel and culture medium did not significantly suppress the tube formation of BCEs in fibrin plus collagen gel. Mean values (±SD) of three experiments are shown. $*P < 0.01$, $**P < 0.05$.

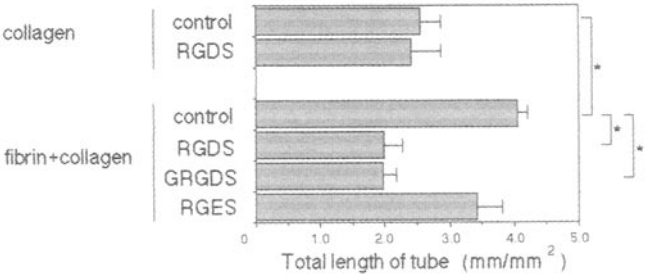

FIG. 7. Effects of RGD-containing peptides on the tube formation of BCEs in fibrin plus collagen gel. Tube formation of BCEs in the fibrin plus collagen gel was significantly inhibited by 100 μg/ml of GRDS or GRGDS added in the culture medium 12 h after the BCEs were seeded on the gel, although 100 μg/ml of RGES did not significantly inhibit the tube formation of BCEs in fibrin plus collagen gel. RGDS did not inhibit the tube formation of BCEs in the collagen gel. Mean values (±SD) of three experiments are shown. $*P < 0.01$.

Effect of RGD-containing peptides on tube formation of BCEs in the fibrin plus collagen gel. The addition of either RGDS or GRGDS peptides (100 μg/ml) in the culture medium 12 h after the beginning of cultivation significantly suppressed the formation of tubular structures by BCEs in the fibrin plus collagen gel, but the RGES peptides (100 μg/ml) did not inhibit the tube formation of BCEs in the fibrin plus collagen gel. The inhibitory effect of RGDS peptides on tube formation was not observed on the collagen gel (Fig. 7).

When 5 ng/ml of bFGF was added to the culture medium, tube formation by BCEs in collagen gel was enhanced by about 2 times as compared with that in culture medium without the addition of bFGF, and this enhancing effect of exogenous bFGF on tube formation of BCEs was not inhibited by 100 μg/ml of RGDS peptides added to the culture medium (Fig. 8).

DISCUSSION

The central finding of this study is that the angiogenic effect of fibrin *in vitro* is mediated by bFGF and uPA released by BCEs. One

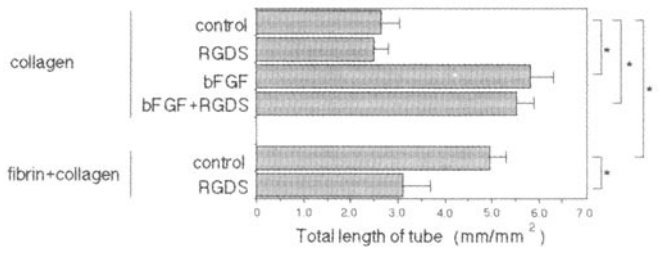

FIG. 8. Effect of RGD-containing peptides on the angiogenic effect of bFGF in the collagen gel. Five nanograms per milliliter of bFGF added to the culture medium increased the length of the tubular structures formed by BCEs in the collagen gel by about 200%, and this effect was not inhibited by 100 μg/ml of RGDS added to the culture medium. Tube formation of BCEs in the fibrin plus collagen gel was significantly inhibited by the same dose of RGDS. Mean values (± SD) of three experiments are shown. . $*P < 0.01$.

milligram per milliliter of fibrin added to the collagen gel significantly increased the length of the tubular structures formed by BCEs by about 1.8 times. Fibrin has also been reported to induce angiogenesis *in vitro* and *in vivo*. Olander et al. (29) reported that fibrin enhances endothelial cells to organize tubular structures *in vivo*, and Dvorak et al. (9) reported that fibrin-containing gel induced angiogenesis *in vitro*. Nicosia et al. (25) performed a quantitative assay for the angiogenic effect of fibrin *in vitro*, and demonstrated that fibrin gel (3 mg/ml) stimulated angiogenesis by 1.7 times compared with collagen.

The intimal neovascularization has been demonstrated in human atherosclerotic lesions (2,15,39,41) and Bini et al. (3) reported that the deposition of fibrin II (des-A, B fibrin) was increased in the advanced atherosclerotic aorta, although fibrinogen and fibrin I (des-A fibrin) were contained in normal or early atherosclerotic aorta. These findings suggest the possibility that fibrinogen, des-A fibrin, and des-A, B fibrin affect the angiogenetic activity in atherosclerotic lesions, so we also examined the angiogenic effect of fibrinogen and des-A fibrin. In fact, fibrinogen, des-A fibrin, and fibrin enhanced the angiogenesis *in vitro* and no apparent difference in the extent of these enhancing effects was recognized among them.

The mechanism of the angiogenic effect of fibrin is not yet fully clarified. Dejana et al. reported that fibrinogen induced endothelial cell adhesion, microfilament reorganization (7) and migration (8). Angiogenesis is thought to be regulated by several growth factors (5,20,42), proteases (19,43), and extracellular matrices (16,21,26), and thus we examined which growth factor(s) and/or protease(s) play an important role in the angiogenic effect of fibrin *in vitro*. At first we examined the participation of bFGF activity, which has been reported to be an angiogenic factor released by BCEs in an autocrine or paracrine manner, or both (36,38). Anti-bFGF IgG added in culture media and gels significantly suppressed the facilitating effect of fibrin on the tube formation of BCEs whereas the same dose of anti-bFGF IgG did not affect the tube formation of BCEs in type I collagen gel. These results indicate that the angiogenic effect of fibrin *in vitro* is mainly mediated by bFGF released from the BCEs probably in an autocrine manner. bFGF enhances endothelial cells to synthesize proteases such as plasminogen activators and collagenase (37), which have been shown to participate in angiogenesis both *in vitro* (43) and *in vivo* (19). Thus we next examined whether plasminogen activator activity relates to the angiogenic effect of fibrin *in vitro*. Tube formation by the BCEs in fibrin plus collagen gel was significantly suppressed by anti-uPA IgG, but the suppression by anti-tPA IgG was not significant. These results confirm that uPA synthesized and released by BCEs also participates in the angiogenic effect of fibrin *in vitro*. bFGF is also known to enhance proliferation of BCEs in two-dimensional culture system (37), so an increased number of BCEs might be concerned with the angiogenic effect of fibrin *in vitro*. However, in our experiments, BCEs grew to confluency 24 h after they were seeded both on the collagen gel and on the fibrin plus collagen gel. In addition, the angiogenic effect of fibrin was almost completely suppressed by anti-uPA IgG. So we suggest that, in these experiments, angiogenic effect of bFGF is mediated mainly by the increased secretion of uPA from BCEs, but not by enhanced proliferation of BCEs.

Basic fibroblast growth factor lacks a signal peptide in its amino acid sequence (1) and the mechanism of its release is still obscure. Some investigators have reported that bFGF is released from cells when the cells are damaged by mechanical injury (12,18) or radiation injury (13). Interleukin 1 also lacks a signal peptide, but it is confirmed that interleukin 1 is released from endothelial cells and regulates their growth (6). bFGF is reported to be deposited in extracellular matrices (33) and exists in exocytotic vesicles of endothelial cells (44), so bFGF might be released by an unknown mechanism.

RGD is a peptide found in the amino acid sequence of several extracellular matrices such as fibronectin, laminin, fibrinogen, and others (34), and has been well known to be recognized by integrin receptors. Cherech (4) reported that endothelial cells expressed RGD-directed adhesion receptor, and Languino et al. (17) revealed the possibility that the interaction between fibrinogen and endothelial cells was mediated by the RGD sequence. In our study, RGD-containing peptides inhibited angiogenic effect of fibrin *in vitro*, but showed no effect on angiogenesis in collagen gel. This result indicates that RGD sequence in fibrin is concerned with its angiogenic effect *in vitro*, whereas the RGD sequence in type I collagen is not concerned with its angiogenic effect *in vitro*. Nicosia and Bonanno (27), however, reported that the RGD-containing peptides inhibited the angiogenesis in rat tail collagen gel using an experimental model of embedding rat aorta ring in collagen gel. They applied RGD-containing peptide from the beginning of induction of angiogenesis, but we added RGD-containing peptide into the culture media 12 h after the beginning of cultivation to achieve an attachment of BCEs on the surface of the gels. This difference of experimental design might be one of the causes of this discrepancy.

Presta et al. (30) reported that DGR in the amino acid sequence of bFGF participated in the mitogenic activity of bFGF, and exogenous RGD-containing peptides partially inhibited the mitogenic activity of bFGF. So the possibility that RGD-containing peptides inhibited not only the interaction between fibrin and BCEs but also the angiogenic effect of bFGF was considered. But the inhibitory effect of RGD-containing peptides on bFGF itself is thought to be relatively little, because RGD-containing peptides did not suppress the angiogenic effect of exogenous bFGF.

Migration of endothelial cells is an important step in angiogenesis, and uPA plays an important role in migration of endothelial cells (19,43). bFGF is known to stimulate endothelial cell proliferation, migration, and protease production (22,31). Fibrinogen is also reported to induce endothelial cell migration, and this effect is mediated by the interaction between RGD sequence in fibrinogen and integrin receptor on endothelial cells (17). So, in this study, migration

of BCEs were thought to be stimulated by fibrin via two different pathways, autocrinological bFGF and RGD-integrin interaction. Werb et al. (40) reported that signal transduction through the fibronectin receptor induces the expression of collagenase and stromelysin by rabbit synovial fibroblasts, but the correlation between the signal transduction via integrin and the expression of growth factors or proteases in endothelial cells has yet to be clarified. Therefore, further investigations on this point are necessary.

In summary, we demonstrated that fibrin stimulated the tube formation of BECs in type I collagen gel, and the enhanced release of bFGF and uPA by BCEs participated in the angiogenic effect of fibrin *in vitro*. In addition, the anchoring of BCEs to the fibrin via RGD sequence might be related to the acceleration of tube formation of BCEs. By these processes, fibrin is considered to play an important role in tissue repair and tumor growth.

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