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Effects of rhizoxin, a microbial angiogenesis inhibitor, on angiogenic endothelial cell functions

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Abstract

Our previous study revealed that rhizoxin ([1*S*-[1*R**,3*R**,5*S**,8*R**(1*R**,2*S**,3*E*,5*E*,7*E*),10*R**,11*S**,13*S**,14*E*,16*S**,17*S**]]-10-hydroxy-8-[2-methoxy-1,3,7-trimethyl-8-(2-methyl-4-oxazolyl)-3,5,7-octatrienyl]-11,16-dimethyl-4,7,12,18-tetraoxatetracyclo[15.3.1.03,5.011,13]heneicos-14-ene-6,19-dione) has a potent inhibitory effect on in vivo angiogenesis. However, little is known regarding the mechanism by which rhizoxin exhibits antiangiogenic activity. In this study, we examined its effects on the functions of endothelial cells associated with neovascular formation in vivo, using cultured vascular endothelial cells. Rhizoxin concentration-dependently inhibited the proliferation of bovine carotid artery endothelial cells, human umbilical vein endothelial cells and human dermal microvascular endothelial cells, the IC_{50} values being 7, 5 and 0.4 nM, respectively. In addition, it reduced the extracellular plasminogen activator level in bovine vascular endothelial cells in the low nM range, and suppressed the migration of human dermal microvascular endothelial cells in the pM range. Furthermore, it blocked the tubular morphogenesis of human umbilical vein endothelial cells and human dermal microvascular endothelial cells on Matrigel in a concentration-dependent manner; the IC_{50} values being 40 and 130 pM, respectively. These results suggest that rhizoxin exhibits antiangiogenic activity through the combined inhibition of some functions of endothelial cells responsible for induction of in vivo angiogenesis.

Keywords: Rhizoxin; Microbial angiogenesis inhibitor; Angiogenic endothelial function; Plasminogen activator; Tube formation

1. Introduction

Pharmacological research faces the critical problem of how effective means of treatment of diseases difficult to cure, including cancer, rheumatoid arthritis, diabetic retinopathy and acquired immunodeficiency syndrome, can be developed. Recent studies have revealed that these refractory diseases depend largely on neovascularization, indicating that the inhibition of angiogenesis could be a new strategy for their treatment (Battegay, 1995; Folkman, 1995; Carmeliet and Jain, 2000; Oikawa, 2001). This is the major reason why angiogenesis has attracted recent attention in the field of pharmacological research. The key to the development of such an angiostatic therapy is to develop useful angiogenesis inhibitors, because they are likely to improve the treatment of these angiogenesis-related diseases. A number of research groups have shown that various substances are effective in inhibition of angiogenesis and/or in the treatment of angiogenic diseases like cancer at the experimental animal model level. Examples are TNP-470 ((3*R*,4*S*,5*S*,6*R*)-5-methoxy-4-[(2*R*,3*R*)-2-methyl-3-(3-methyl-2-butenyl)-oxiranyl]-1-oxaspiro[2,5]oct-6-yl (chloroacetyl) carbamate) (Ingber et al., 1990), angiostatin (a 38-kDa plasminogen fragment) (O'Reilly et al., 1994), endostatin (a 20-kDa C-terminal fragment of collagen XVIII) (O'Reilly et al., 1997), matrix metalloproteinase inhibitors like batimastat (*N*4-[(1*S*)-2,2-dimethyl-1-[(methylamino)carbonyl]propyl]-*N*1,2-dihydroxy-3-(2-methylpropyl)-(2*S*, 3*R*)-butanediamide) (Hidalgo and Eckhardt, 2001), inhibitors of the vascular endothelial growth factor

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signaling pathway (Kim et al., 1993; Brekken et al., 2000), and antagonists of some integrins, such as αvβ3 and αv, (Brooks et al., 1994; Kerr et al., 1999). The use of these antiangiogenic agents for cancer treatment is being evaluated through clinical trials.

Using our chorioallantoic membrane assay, we have attempted to find a low molecular weight compound that exhibits antiangiogenic activity from among microbial products. This is based on the finding that microorganisms are well known to provide a lot of useful substances such as antibiotics and enzyme inhibitors. With such an approach, we have found that different microbial metabolites, such as herbimycin A (17-demethoxy-15-methoxy-11-0-methylgeldanamycin) (Oikawa et al., 1989) and eponemycin ((4*S*)-1,2-epoxy-2-hydromethyl-4-(*N*-isooctanoyl-L-serylamino)-6-methylhept-6-ene-3-one) (Oikawa et al., 1991), have antiangiogenic activity. This might imply that microorganisms constitute a rich repository of natural antiangiogenic agents (Oikawa, 2001). Eponemycin is the most potent inhibitor of angiogenesis with our chorioallantoic membrane assay, the ID_{50} value being 250 fmol (0.1 ng) per egg. Recently, we found that rhizoxin ([1*S*-[1*R**,3*R**,5*S**, 8*R**(1*R**, 2*S**,3*E*,5*E*,7*E*),10*R**,11*S**,13*S**,14*E*,16*S**,17*S**]]-10-hydroxy-8-[2-methoxy-1,3,7-trimethyl-8-(2-methyl-4-oxazolyl)-3,5,7-octatrienyl]-11,16-dimethyl-4,7,12,18-tetra oxatetracyclo[15.3.1.03,5.011,13]heneicos-14-ene-6,19-dione), which was isolated from *Rhizopus chinensis*, suppresses embryonic and tumor cell-induced angiogenesis (Onozawa et al., 1997). On a molar basis, this microbial agent is the second strongest inhibitor of angiogenesis with our chorioallantoic membrane assay system, the ID_{50} value being 3.2 pmol per egg. However, it remains unclear which event(s) in the angiogenesis process is affected by rhizoxin.

In the present study, our aim is to determine the effects of rhizoxin on the vascular endothelial cell functions associated with angiogenesis, including plasminogen activator production, migration and proliferation, and tube formation in cultured vascular endothelial cells.

2. Materials and methods

2.1. Materials

Rhizoxin was a generous gift from Prof. Shigeo Iwasaki (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). Its chemical structure is shown in Fig. 1. Dulbecco's modified Eagle's medium (DMEM), Molecular, Cellular and Developmental Biology (MCDB)-131, epidermal growth factor, heparin and medroxyprogesterone acetate were purchased from Sigma (St. Louis, MO). Fetal bovine serum was obtained from Moregate (Melbourne, Australia). A mixture of penicillin and streptomycin was purchased from Gibco (Grand Island, NY). *H*-D-Val-L-Leu-L-Lys-*p*-nitroanilide (S-2251), a chromogenic substrate of plasmin, and plasminogen were purchased from Chromogenix (Mölndal, Sweden). Matrigel was from Collaborative Biochemical Products, Becton-Dickinson Labware (Bedford, MA); and endothelial cell growth supplement was from Upstate Biotechnology (Lake Placid, NY).

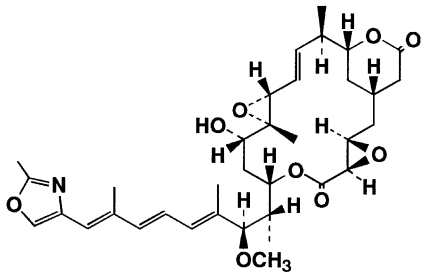

Fig. 1. Chemical structure of rhizoxin.

2.2. Cells

Human umbilical vein endothelial cells and human dermal microvascular endothelial cells were obtained from Cell Systems (Kirkland, WA). Bovine carotid artery endothelial cells were kindly provided by Prof. Ikuo Morita (Tokyo Medical and Dental University, Tokyo, Japan).

2.3. Chromogenic substrate assay for plasminogen activator

Plasminogen activator activity secreted by bovine carotid artery endothelial cells into conditioned medium was determined as described previously (Oikawa et al., 1993). In short, bovine vascular endothelial cells (2×10^5 cells/well) were plated onto and grown in a 24-multiwell dish containing 1 ml of DMEM supplemented with 10% fetal bovine serum (growth medium for bovine carotid artery endothelial cells) at 37 °C for 24 h under humidified 5% CO_2–95% air in an incubator, and then further incubated in 0.5 ml of serum-free growth medium containing various concentrations of rhizoxin or medroxyprogesterone acetate (1 μM) for an additional 24 h. Medroxyprogesterone acetate was included as a positive control because it is a potent inhibitor of angiogenesis and of plasminogen activator secretion by endothelial cells (Ashino-Fuse et al., 1989; Oikawa et al., 1993). Following collection and centrifugation of the serum-free conditioned medium, the resulting supernatant was examined as to its plasminogen activator activity. The assay mixture contained 0.5 CU/ml plasminogen and 0.76 mM S-2251 (the chromogenic substrate for plasmin). At the same time, cells after 24-h incubation were counted with a Coulter counter. The activity was expressed in urokinase units (U) per 10^5 cells.

2.4. Zymography of plasminogen activator

Zymographic analysis of plasminogen activator in serum-free conditioned medium prepared above was performed by a modification of the method of Vassalli et al. (1984). Aliquots

of the serum-free conditioned medium corresponding to the same number of cells were electrophoresed in 10% polyacrylamide gel containing 0.2% sodium dodecyl sulfate (SDS) under nonreducing conditions. The electrophoresed gels were washed in 2.5% Triton X-100 solution and distilled water, after which they were overlayered on the substrate gels containing 8% polyacrylamide, 0.1 CU/ml plasminogen and 1% fat-free milk as a source of casein, and incubated at 37 °C for 16 h under a humidified atmosphere to allow proteolysis. The substrate gels were stained with 0.1% Amide Black 10B in 10% acetic and 10% isopropanol.

2.5. Vascular endothelial cell proliferation

Vascular endothelial cell proliferation was assayed as described previously (Oikawa et al., 1993, 1997, 1998). In the presence of various concentrations of rhizoxin, endothelial cells (1×10^4 cells/well) were incubated in a 24-multiwell dish (non-coated dish for bovine vascular endothelial cells; gelatin-coated dish for human vascular endothelial cells) containing 1 ml of growth medium (DMEM supplemented with 10% fetal bovine serum for bovine vascular endothelial cells; MCDB-131 supplemented with 10% fetal bovine serum, 10 μg/ml endothelial cell growth supplement, 10 ng/ml epidermal growth factor and 10 μg/ml heparin for human vascular endothelial cells) at 37 °C under humidified 5% CO_2–95% air in an incubator. After 72-h incubation, the cells were trypsinized and then counted with a Coulter counter Z1 (Coulter Japan, Tokyo, Japan).

2.6. Cell migration assay

Vascular endothelial cell migration was determined by means of a wound healing migration assay (Sato and Rifkin, 1988; Oikawa et al., 2001). Two scratches of 5 mm in width were made, with a razor blade, in gelatin-coated 35-mm culture dishes containing confluent monolayers of human dermal microvascular endothelial cells, washed twice with MCDB-131 containing 0.1% bovine serum albumin, and then further incubated for 18 h in MCDB-131 containing 0.1% bovine serum albumin, 10 μg/ml endothelial cell growth supplement, 10 ng/ml epidermal growth factor and 10 μg/ml heparin, in the presence of the indicated concentrations of rhizoxin. After being stained with Giemsa, the cells that had migrated across the edge of the wound were recorded at ×100 magnification by a color video camera recorder (TK-1280U; Victor, Tokyo, Japan). The total number of cells that had migrated in 10 randomly chosen microscopic fields per dish was determined with microcomputer-assisted NIH Image (Version 1.58).

2.7. Tube formation on Matrigel

Endothelial tube formation on Matrigel was conducted as described previously (Oikawa et al., 1998, 2001). Matrigel (2 mg/0.2 ml/well) at 4 °C was added to a 24-multiwell dish and then allowed to polymerize at 37 °C for about 1 h. Human umbilical vein endothelial cells or human dermal microvascular endothelial cells (7.5×10^5 cells/well) were plated onto the Matrigel in 1 ml of growth medium and then incubated at 37 °C in a humidified 5% CO_2 chamber. After 18-h incubation, the formation of tube-like structures by endothelial cells was analyzed by phase contrast microscopy at ×100 magnification, and the total length of tubular structures in five randomly chosen microscopic fields per well was determined with a Leica Q500MC image analyzer equipped with QWin image analysis software (Cambridge, UK).

2.8. Statistics

Statistical analysis was performed using StatView 4.51 software (Abacus Concepts, Berkeley, CA). Bonferroni/Dunn's multiple range test was used for planned comparisons among the various treatment groups. The level of significance was set at $*p < 0.05$ and $**p < 0.01$. Values are expressed as the mean ± S.D., and the p value is given for differences with the control group.

3. Results

3.1. Effect of rhizoxin on the extracellular plasminogen activator level in vascular endothelial cells

Fig. 2B shows the plasminogen activator level in serum-free conditioned medium produced by bovine vascular

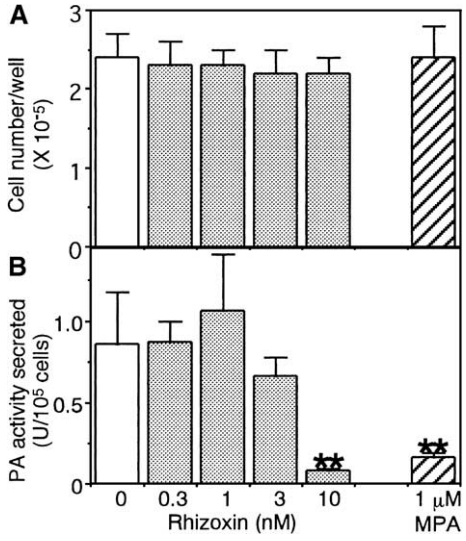

Fig. 2. Effect of rhizoxin on the extracellular level of plasminogen activator (PA) in vascular endothelial cells. Confluent cultures of bovine endothelial cells grown on 24-well plates were incubated in 1 ml of serum-free medium containing various concentrations of rhizoxin or medroxyprogesterone acetate (MPA). After 24-h culture, the extracellular PA activity was determined, and expressed in urokinase units (mU) per 10^5 cells (B). The cell number was determined with a Coulter counter after trypsinization (A). Values represent means ± S.D. for four wells. $**p < 0.01$ vs. the vehicle group.

endothelial cells in the presence of various concentrations of rhizoxin, as measured by means of the chromogenic substrate assay involving S-2251. The hydrolysis of S-2251 by the endothelial cell-conditioned medium occurred in the presence of plasminogen, whereas in the absence of plasminogen, hydrolysis of S-2251 was undetectable (< 1 mU per 10^5 cells). There was a trend for rhizoxin to inhibit the production of plasminogen activator activity by vascular endothelial cells in a concentration-dependent manner. The inhibitory potency of 10 nM rhizoxin seemed to be greater than that of 1 μM medroxyprogesterone acetate, which was included as a positive control in the experiments for comparison. There was no significant difference in the cell number after 24 h between the control and rhizoxin-treated cultures, suggesting that this agent showed no cytotoxicity toward confluent vascular endothelial cells (Fig. 2A).

Fig. 3A shows the results of zymographic analysis of the plasminogen activator activity in the samples (i.e. conditioned medium of endothelial cells treated with rhizoxin) used in Fig. 2. The proteolytic activity, visualized as a white band, depended on the presence of plasminogen in the substrate gel, indicating that the proteolytic activity is due to plasminogen activator. Rhizoxin treatment potently inhibited the production of plasminogen activator in endothelial cells. In addition, the zymographic analysis showed

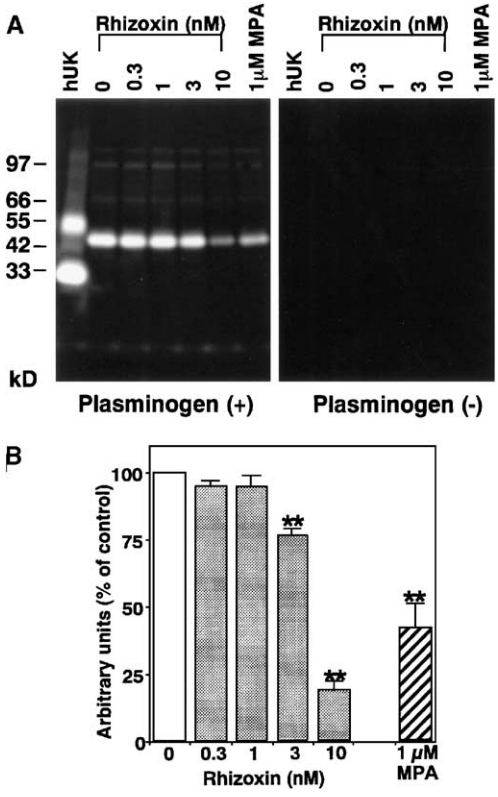

Fig. 3. (A) Zymography of extracellular plasminogen activator in vascular endothelial cells treated with rhizoxin or medroxyprogesterone acetate (MPA). The samples used in Fig. 2 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the extracellular proteolytic activity was examined in the presence and absence of plasminogen in the substrate gel. Human urokinase (hUK) was electrophoresed at the same time. (B) Image analysis of the zymograph of plasminogen activator presented in (A). The intensities of bands were determined with a Leica Q500MC image analyzer equipped with QWin image analysis software, and expressed in arbitrary units. Values represent means ± S.D. ($n = 4$). $^{**}p < 0.01$ vs. the vehicle group.

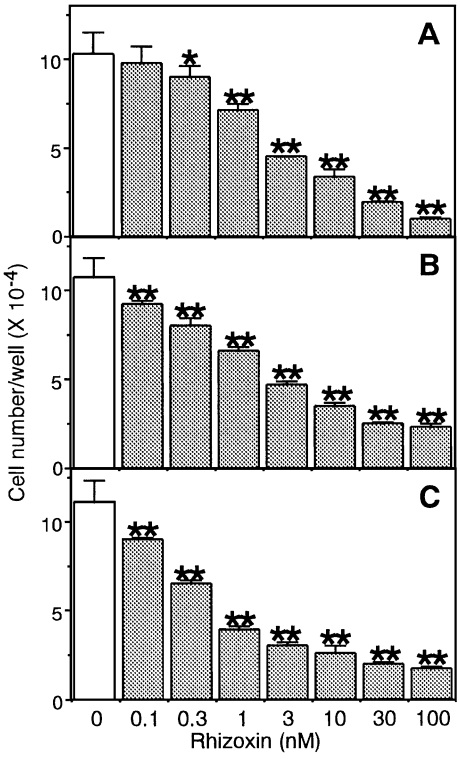

Fig. 4. Effect of rhizoxin on cell proliferation by bovine carotid artery endothelial cells (A), human umbilical vein endothelial cells (B) and human dermal microvascular endothelial cells(C). After 72-h culture in 24-well plates in the presence of rhizoxin, the cell number was determined with a Coulter counter. Values represent means ± S.D. for three wells. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. the vehicle group.

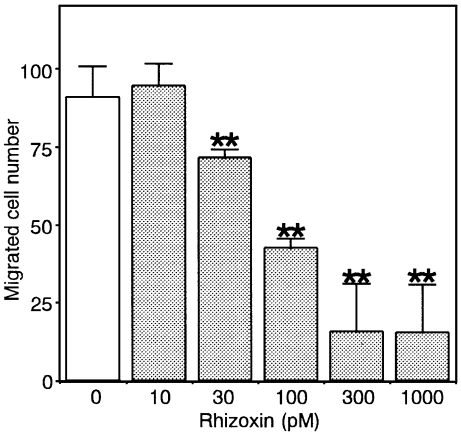

Fig. 5. Effect of rhizoxin on the migration of vascular endothelial cells. The migration of human dermal microvascular endothelial cells was determined with a wound-healing assay. Values are means ± S.D. ($n = 4$). $^{**}p < 0.01$ vs. the vehicle group.

that our bovine vascular endothelial cells predominantly secreted the plasminogen-dependent proteolytic activity seen at the position of 45 kDa, which corresponds to the known molecular weight of bovine urokinase-type plasminogen activator (Pepper et al., 1987; Saksela et al., 1987; Peverali et al., 1994), indicating that the predominant form of plasminogen activator produced by our endothelial cells is the urokinase-type one.

Image analysis of a zymograph of the plasminogen activator in the conditioned medium of endothelial cells treated with rhizoxin showed that the plasminogen activator production by vascular endothelial cells was inhibited by rhizoxin in a concentration-dependent fashion, the ID_{50} value being 5 nM (Fig. 3B).

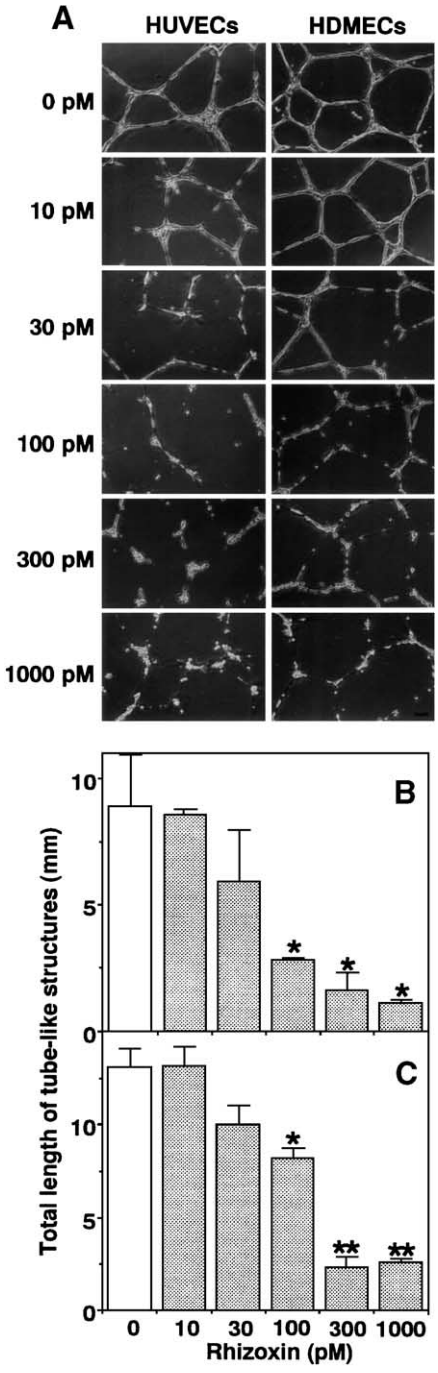

Fig. 6. (A) Effect of rhizoxin on tube formation by human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) on Matrigel. Scale bar, 0.1 mm. Quantitative analysis of endothelial tube formation on Matrigel by human umbilical vein endothelial cells (B) and human dermal microvascular endothelial cells (C) treated with rhizoxin. The total length of tube-like structures per field was determined with a Leica Q500MC image analyzer equipped with QWin image analysis software. Values represent means ± S.D. for two wells. $*p < 0.05$ and $**p < 0.01$ vs. the vehicle group.

3.2. Effect of rhizoxin on the proliferation of vascular endothelial cells

Whether or not rhizoxin affects the proliferation of vascular endothelial cells was examined. It concentration-dependently prevented endothelial cell proliferation (Fig. 4). The IC_{50} values were 7, 2, and 0.4 nM for bovine carotid artery endothelial cells, human umbilical vein endothelial cells, and human dermal microvascular endothelial cells, respectively.

3.3. Effect of rhizoxin on the migration of vascular endothelial cells

The effect of rhizoxin on the migration of human dermal microvascular endothelial cells was examined with a wound healing migration assay. It exhibited concentration-dependent suppression of human microvascular endothelial cell migration, the ID_{50} value being 95 pM (Fig. 5).

3.4. Effect of rhizoxin on tube formation by vascular endothelial cells

Fig. 6A shows the effect of rhizoxin on tube formation by human umbilical vein endothelial cells or human dermal microvascular endothelial cells on Matrigel. It exerted an inhibitory effect on the tube formation by human umbilical vein endothelial cells or human dermal microvascular endothelial cells in the picomolar range. To further analyze the inhibition of tube formation observed in Fig. 6A, the length of the tube-like structures was determined with an image analyzer (Fig. 6B). Rhizoxin concentration-dependently suppressed endothelial tube formation, the IC_{50} values for human umbilical vein endothelial cells and human dermal microvascular endothelial cells being 50 and 130 pM, respectively.

4. Discussion

Our previous study showed that rhizoxin is a microbial inhibitor of in vivo angiogenesis (Onozawa et al., 1997). However, little is known about the mechanism by which rhizoxin exerts antiangiogenic activity. In the present study

we therefore investigated the effects of rhizoxin on angiogenic endothelial cell functions.

Angiogenesis is a cascade reaction that includes various cellular responses, including the production of extracellular matrix-degrading enzymes like plasminogen activator in endothelial cells. In this respect we previously reported a possible mechanism by which the inhibition of plasminogen activator by medroxyprogesterone acetate might contribute to its antiangiogenic activity (Ashino-Fuse et al., 1989). In the present study, rhizoxin treatment resulted in remarkable reduction in the extracellular plasminogen activator activity in vascular endothelial cells, as measured by means of an assay involving chromogenic substrate S-2251. Plasminogen activator activity is controlled by the net balance between plasminogen activators and their inhibitors. Thus, it was likely that the reduction in plasminogen activator activity caused by rhizoxin is due to a decrease in the level of plasminogen activator protein or an increase in the level of its inhibitor protein, or both. Several studies have shown that zymographic analysis of plasminogen activator provides clues for understanding the mechanisms of action of agents that interfere with the plasminogen activator level (Fotsis et al., 1993; Keski-Oja et al., 1988; Pepper et al., 1990; Arakawa et al., 2002). Therefore, the effect of rhizoxin on the plasminogen activator level was further evaluated by zymography. Plasminogen activator activity, visualized as white bands, was inhibited by rhizoxin in a concentration-dependent manner. Overall, these findings indicated that rhizoxin decreases the level of plasminogen activator protein, resulting in reduced activity. However, the possibility cannot be ruled out that rhizoxin increases the protein levels of inhibitors of plasminogen activator because we did not examine it in this study. Thus, further studies on this point are needed.

Another suggestion as to the effect of rhizoxin on the plasminogen activator level is that the plasminogen activator/plasmin system might represent an attractive target for inhibition of angiogenesis. In this regard, different inhibitors of angiogenesis, including angiostatic steroids like medroxyprogesterone acetate (Ashino-Fuse et al., 1989; Blei et al., 1993; Yamamoto et al., 1994), radicicol (Oikawa et al., 1993), TNP-470 (Maier et al., 1997), 16 K prolactin (Lee et al., 1998), and 15-deoxy-delta12,14-prostaglandin J_2 (Xin et al., 1999), have been found to decrease the plasminogen activator level in vascular endothelial cells.

The migration of vascular endothelial cells is one of the initial phases in the angiogenic process. Previous studies have shown that endothelial migration needs the concomitant production of plasminogen activator and plasminogen activator inhibitor-1, because both the activator and inhibitor are induced in migrating endothelial cells (Pepper et al., 1987, 1992). Thus, it is reasonable to assume that inhibition of plasminogen activator interferes with angiogenesis. So, in the present study, we also examined the effect of rhizoxin on endothelial migration because it affected the level of plasminogen activator, as mentioned above, and found that it dose-dependently inhibited the migration of human endothelial cells with a wound healing migration assay. Therefore, this inhibitory effect might be responsible for its antiangiogenic action. Previously, some inhibitors of angiogenesis, including platelet factor 4 (Sharpe et al., 1990), thrombospondin (Good et al., 1990), paclitaxel (Belotti et al., 1996), endostatin (Dhanabal et al., 1999) and nonsteroidal anti-inflammatory drugs like aspirin (Dormond et al., 2001), were found to prevent endothelial cell migration.

The angiogenic response also requires the induction of endothelial cell proliferation. So we examined the effect of rhizoxin on the proliferation of endothelial cells. It exhibited a dose-dependent inhibitory effect on three different types of endothelial cells. Therefore, it is reasonable to assume that this inhibitory effect might be involved in its previously observed antiangiogenic activity in vivo. On the other hand, rhizoxin has been shown to have a growth-inhibitory effect on several cancer cell types, including P388 murine leukemia cells, K562 human myelogenous leukemia cells and L1210 murine leukemia cells (Tsuruo et al., 1986; Bai et al., 1991), indicating that the growth-inhibitory activity of rhizoxin is unlikely to be specific for endothelial cells. In addition, it has been reported that it is unlikely for rhizoxin to be developed as a classical cytotoxic agent for the treatment of cancer (Christian et al., 1997; Roberge et al., 2000). Recent studies have shown the antiangiogenic effects of anti-cancer agents that are cytotoxic to cancer cells. Examples are paclitaxel, vincristin and tegafur (Schirner et al., 1998; Yonekura et al., 1999; Shibata et al., 1998). In addition, an alternative antiangiogenic schedule for the administration of chemotherapeutic agents such as cyclophosphamide and vinblastine has been developed (Browder et al., 2000; Klement et al., 2000). With this antiangiogenic schedule for administration, the chemotherapeutic agents more effectively control tumor growth, regardless of whether or not the tumor cells are drug-resistant. Collectively, it may be possible that rhizoxin can be redeveloped as an antiangiogenic agent, given its potent effects on angiogenic endothelial cell functions observed in this study as well as in vivo angiogenesis in the previous study.

Vascular endothelial cells have the ability to form tube-like structures when cultured on extracellular matrix components like Matrigel, which is composed of basement membrane components (Kubota et al., 1988; Grant et al., 1989). So this reaction is regarded as an in vitro model of angiogenesis that requires reorganization of cytoskeleton components such as microfilaments and microtubules, endothelial cell migration and synthesis of proteins like collagen (Grant et al., 1991). Furthermore, plasminogen activator appears to be involved in endothelial tube formation (Schnaper et al., 1995). On the other hand, however, endothelial cell proliferation is unlikely to contribute to the formation of tube-like structures. As mentioned above, rhizoxin decreased the level of plasminogen activator activity in endothelial cells. Also, it suppressed endothelial cell migration. These observations raised the possibility that

rhizoxin might exhibit inhibitory activity against endothelial tube formation. So we examined this possibility in this study, and demonstrated the inhibitory effect of rhizoxin on tube formation in two types of endothelial cells. However, the molecular mechanism of its action on tube formation is not yet clear. As mentioned above, microtubules play a role in tube formation, and nocodazole, a synthetic anti-tubulin agent that shows antimitotic activity, was found to prevent tube formation (Zimrin et al., 1995). In addition, previous studies have shown that inhibitors of microtubules, including 2-methoxyestradiol (Klauber et al., 1997) and paclitaxel (Belotti et al., 1996), exhibit antiangiogenic activity. Rhizoxin has also been found to act as a tubulin inhibitor (Takahashi et al., 1987). Overall, it is conceivable that the anti-tubulin activity of rhizoxin might be involved in its inhibition of endothelial tube formation.

One might ask why rhizoxin reduced the extracellular plasminogen activator level and cell proliferation in vascular endothelial cells in the nM range while suppressing endothelial migration and tube formation (i.e. in vitro angiogenesis) in the pM range. The reason for the difference in the potency of the effects of rhizoxin on these angiogenic endothelial cell functions is not clear. Thus, further studies on this point are needed.

In conclusion, the present study demonstrated that rhizoxin exhibits antiangiogenic activity through the combined inhibition of multiple functions of angiogenic endothelial cells. Given the potent inhibitory effects of rhizoxin on angiogenesis in vivo and in vitro, rhizoxin is likely to have the potential to be developed as an antiaangiogenic agent.

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