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Inhibition of Angiogenesis by Rhizoxin, a Microbial Metabolite Containing Two Epoxide Groups

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Previous studies by our and other groups have shown that microbial products containing more than one epoxide group, including eponemycin, radicicol, depudecin and AGM-1470, exhibit anti-angiogenic activity in an *in vivo* assay system involving chorioallantoic membranes (CAMs) of growing chick embryos. Based on these findings, rhizoxin, a microbial metabolite that contains two epoxide groups and exhibits anti-tubulin activity, was tested for anti-angiogenic activity in a CAM assay system. Rhizoxin caused dose-dependent inhibition of embryonic angiogenesis, the ID_{50} value being 2 ng (3.2 pmol) per egg. In addition, this compound (2 mg/kg i.p.) significantly suppressed neovascularization induced by M5076 mouse tumor cells in a mouse dorsal air sac assay system, compared to the vehicle alone ($P<0.05$). These results indicate that rhizoxin is a novel inhibitor of angiogenesis, and that it has potential as a new therapeutic agent for cancer.

Key words: Angiogenesis — Angiogenesis inhibitor — Rhizoxin — Epoxide group — Angiostatic therapy

Recent studies have revealed that angiogenesis is an essential event in the growth and metastasis of cancer cells.[1–5] Namely, the network of newly formed blood vessels provides malignant tumor cells with nutrients and oxygen necessary for their growth and also affords a pathway for metastasis to remote tissues or organs. Therefore, compounds able to suppress angiogenesis are potential therapeutic agents for cancer. On the basis of this concept, the development of angiogenesis inhibitors has attracted much attention.[1–5]

We previously showed that eponemycin, an epoxide group-containing microbial metabolite, has potent anti-angiogenic activity in a chorioallantoic membrane (CAM) assay system.[6] Radicicol and depudecin, which contain one and two epoxide groups, respectively, also exert inhibitory effects on embryonic angiogenesis under the same assay conditions.[7,8] Likewise, other groups showed that fumagillin and its analogs containing two epoxide groups, such as AGM-1470 (or TNP-470) and FR-118487, are potent inhibitors of angiogenesis in a CAM, cornea or mouse dorsal air sac assay system.[9,10] Furthermore, a method was established for the large-scale synthesis of (−)-ovalicin, a microbial product, which contains two epoxide groups, is structurally similar to fumagillin and exhibits almost as potent anti-angiogenic activity as AGM-1470.[11] Ovalicin was supposed to be superior to AGM-1470 in physicochemical stability. These findings led us to speculate that the epoxide group might be a useful marker in the search for novel inhibitors of angiogenesis. As a first step to substantiate this speculation we assessed the effect of rhizoxin, a microbial metabolite containing two epoxide groups, on neovascularization both in the CAM and mouse dorsal assay systems. The reason for selecting this compound from among epoxide group-containing agents is that it has anti-tubulin activity,[12] because recent studies have shown that compounds with anti-tubulin activity, including 2-methoxyestradiol, taxol and nocodazole, influence angiogenic responses in *in vitro* and/or *in vivo* model systems.[13–15]

Rhizoxin was isolated as described previously.[16] Ethylene-vinyl acetate copolymer 40 (EV) was kindly donated by Mitsui-DuPont Polychemicals, Tokyo. M5076 mouse tumor cells were kindly provided by Dr. Tashiro, Cancer Chemotherapy Center, Tokyo. Female ICR mice (5-week-old) were purchased from Charles River Japan Inc., Atsugi.

To assess the effect of rhizoxin on embryonic angiogenesis, the CAM assay was performed as described previously.[17] EV pellets impregnated with various doses of rhizoxin were placed on the surface of CAMs of 5-day-old chick embryos, followed by incubation at 37°C for 2 days in a humidified egg incubator. The anti-angio-

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genic activity was evaluated, the response being graded as either non-effective or effective as previously described.[17] When the diameter of the avascular zone on a treated CAM exceeded 3 mm in diameter, the anti-angiogenic response was taken as effective.

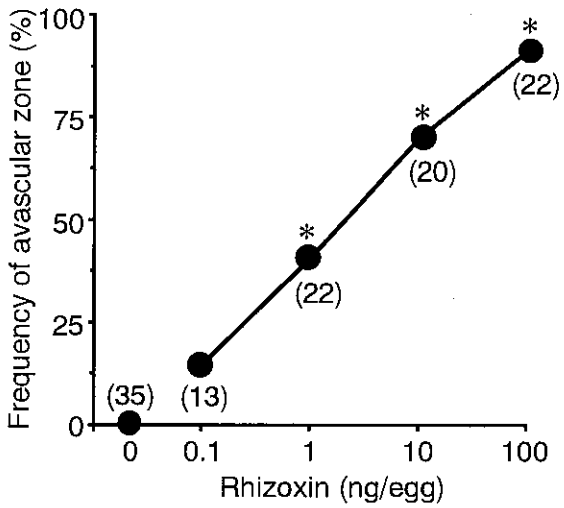

Fig. 1. Inhibitory effect of rhizoxin on embryonic angiogenesis. After 5-day-old CAMs had been treated with EV pellets impregnated with various doses of rhizoxin for 2 days, the anti-angiogenic effects were assessed by measuring the avascular zones. The points show the frequency (%) of avascular zones exhibiting a significant anti-angiogenic response. The values in parentheses are the numbers of CAMs used. * $P<$ 0.001, compared to the control (Fisher's exact probability test).

The effect of rhizoxin on tumor-induced angiogenesis was assessed in the mouse dorsal air sac assay system, as described previously.[18] A chamber was prepared by covering both sides of a Millipore ring (inner diameter, 10 mm; thickness, 2 mm) with Millipore filters (pore size, 0.45 μm), and filled with M5076 tumor cells (2.5×10^7 cells) suspended in phosphate-buffered saline (PBS). The chamber was implanted into a subcutaneous dorsal air sac formed in a female ICR mouse (8- to 10-week-old) by injecting an appropriate volume of air. Four mice per group received 0 or 2 mg/kg of rhizoxin i.p. on days 0 and 4 after implantation of the chambers. On day 5 the implanted chambers were removed from the subcutaneous fascia of the treated mice, then a black ring having the same inner diameter as the Millipore ring was placed on the same site. The angiogenic response was evaluated under a dissecting microscope by counting newly formed blood vessels longer than 3 mm in length within the area encircled by the black ring. Angiogenesis indexes 0, 1, 2 and 3 indicate that the numbers of neovessels were 0, 1, 2 and 3 or more, respectively. The newly formed blood vessels were morphologically distinguishable from the preexisting background vessels by their tortuous nature, as described previously.[18–21]

First, rhizoxin was examined for inhibitory effect on embryonic angiogenesis in the CAM assay system, which is one of the most widely used assay systems for determining anti-angiogenic activity. The CAMs of growing chick embryos were treated for 2 days with EV pellets impregnated with various doses of rhizoxin, ranging

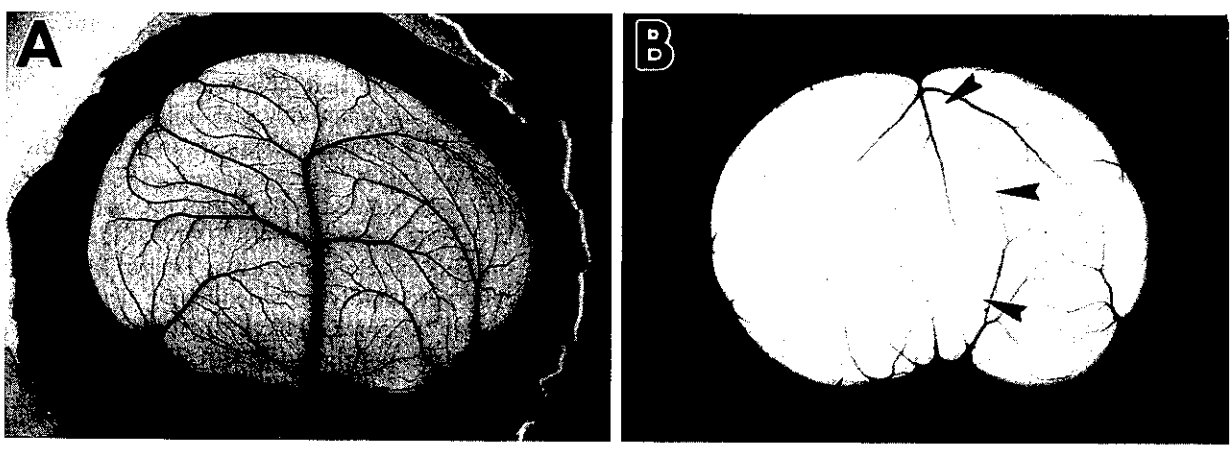

Fig. 2. Effect of rhizoxin on angiogenesis in CAMs 2 days after placement of EV pellets impregnated with rhizoxin (A, 0 ng/egg; B, 100 ng/egg). An appropriate volume of a fat emulsion was injected into the chorioallantois so that the vascular network in the CAM stood out against the white background of the lipid. Rhizoxin-containing EV pellets led to the formation of a significant avascular zone (indicated by arrowheads), showing anti-angiogenic activity, while control EV pellets did not have such an effect. ×2.6.

from 0.1 to 100 ng/egg. Rhizoxin, at the lowest dose (0.1 ng/egg), did not exhibit significant inhibitory activity against neovascularization, but inhibited embryonic angiogenesis in a dose-dependent manner (Fig. 1). The ID_{50} value was 2 ng (3.2 pmol) per egg. Typical results are shown in Fig. 2. Rhizoxin potently inhibited embryonic angiogenesis, producing a significant avascular zone (Fig. 2B), while an empty EV pellet without rhizoxin (Fig. 2A) did not exert such an effect on any of the CAMs treated ($n=35$).

Table I shows a comparison of the anti-angiogenic ability of rhizoxin with those of other angiogenesis inhibitors we have examined so far in our CAM assay system, on a molar basis. Rhizoxin exhibits weaker anti-angiogenic activity than eponemycin,[6] the most powerful known inhibitor, but appears to have a more potent angiogenesis-inhibitory effect than other angiogenesis inhibitors, including radicicol,[7] depudecin,[8] 15-deoxyspergualin,[24] 9α-fluoromedroxyprogesterone acetate (FMPA),[25] a synthetic retinoid Re 80,[22] 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$),[23] 22-oxa-$1,25(OH)_2D_3$,[23] and wortmannin.[26]

Table I. Comparison of Anti-angiogenic Potency between Rhizoxin and Other Compounds in Our CAM Assay

Compounds	ID_{50} (pmol/egg)	Reference no.
15-Deoxyspergualin	960	24
Depudecin	1500	8
$1,25(OH)_2D_3$	340	23
Eponemycin	0.25	6
FMPA	2500	25
Herbimycin A	260	5
22-Oxa-$1,25(OH)_2D_3$	96	23
Radicicol	540	7
Re 80	6.3	22
Retinoic acid	330	17
Rhizoxin	3.2	
Staurosporine	71	5
Wortmannin	70	26

We next examined whether or not rhizoxin, when administered systemically, affects tumor-induced angiogenesis, using the mouse dorsal air sac assay model. We used M5076 mouse tumor cells as an inducer of tumor angiogenesis because these mouse tumor cells, which exhibit a high metastatic ability,[27] were found to induce neovascularization and to be sensitive to angiogenesis inhibitors such as fumagillin[9] and tecogalan (or DS-4152).[28] The results of representative experiments are shown in Fig. 3. The control chamber containing PBS produced little or no angiogenic response (Fig. 3A). The chamber containing M5076 tumor cells strongly induced angiogenesis (Fig. 3B). The M5076-induced angiogenesis was dramatically suppressed by i.p. injection of rhizoxin at 2 mg/kg on days 0 and 4 after the implantation of the chambers (Fig. 3C). These angiogenic responses were evaluated under a dissecting microscope by counting the newly formed blood vessels showing the characteristic zigzagging of tumor cell-induced new vasculature (Fig. 4). The angiogenesis index was 0.25 ± 0.5 (mean$\pm$SD; $n=4$) in the control group receiving a PBS-containing chamber, followed by treatment with the vehicle (i.e., experimental group A). The experimental group B receiving an M5076 tumor cell-containing chamber, when

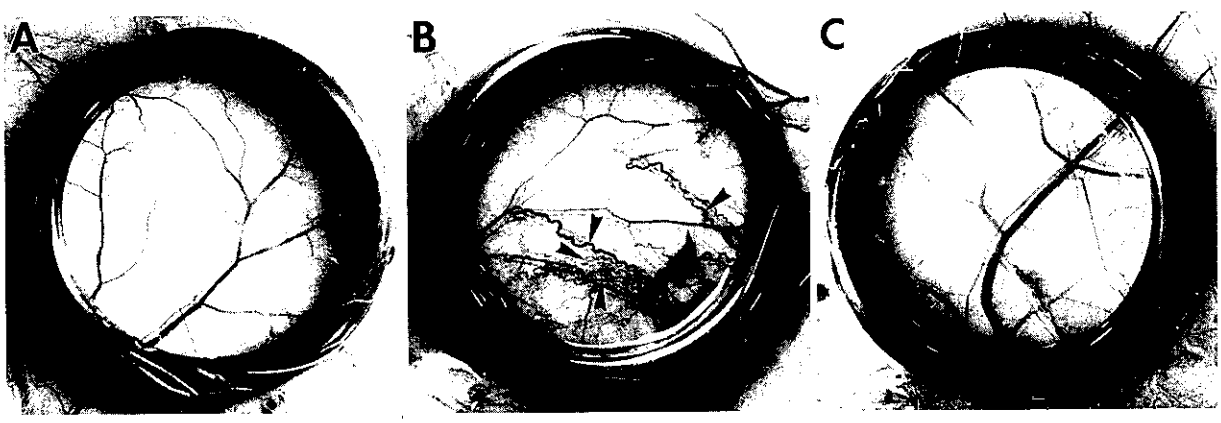

Fig. 3. Effect of rhizoxin on the angiogenic response 5 days after implantation of a Millipore chamber containing M5076 tumor cells. Rhizoxin or the vehicle was administered i.p. at days 0 and 4. A, Mice implanted with a chamber containing PBS were treated with the vehicle (i.e. control group); mice implanted with a chamber containing M5076 cells (2.5×10^7 cells) were treated with the vehicle (B) or 2 mg/kg rhizoxin (C). Note that rhizoxin (C) strongly inhibited M5076-induced formation of new tortuous blood vessels (indicated by arrowheads), compared to the vehicle (B). $\times4.4$.

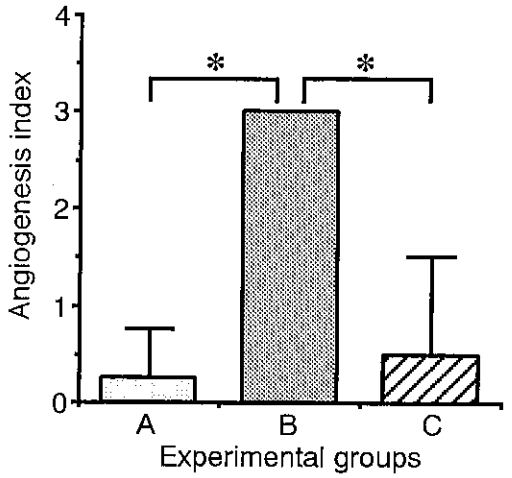

Fig. 4. Inhibitory effect of rhizoxin on M5076 tumor cell-induced angiogenesis. Groups A and B received chambers containing PBS and M5076 tumor cells, respectively, and each group was also treated with the vehicle. Group C received M5076 tumor cell-containing chambers, followed by the administration of rhizoxin (2 mg/kg i.p.) twice. Values are mean±SD ($n=4$). * $P<0.05$ between the indicated groups (Mann-Whitney U test).

treated with the vehicle, exhibited a significant angiogenic response, the angiogenesis index being 3 ± 0 ($n=4$), compared to the experimental group A ($P<0.05$). Compared with the vehicle-treated group (i.e., experimental group B), significant suppression of the angiogenic response triggered by M5076 cells occurred in the experimental group C treated with rhizoxin (2 mg/kg, twice), the angiogenesis index being 0.5 ± 1 ($n=4$) ($P<0.05$). There was no difference in the angiogenic response between the experimental groups A and C ($P>0.05$).

These results identified rhizoxin as an *in vivo* inhibitor of angiogenesis, which tends to support our hypothesis that the epoxide group is a useful indicator in the search for new inhibitors of angiogenesis. This finding was confirmed by means of two bioassays, i.e., the CAM and mouse dorsal air sac assay systems. So far, only three agents have been found to exhibit anti-angiogenic effects in both of these assays, as far as we know.[9, 24, 28, 29] They include AGM-1470 and tecogalan, which are currently under clinical trial in patients with cancer or AIDS.[1-5] Rhizoxin seems to have almost the same anti-angiogenic activity as these two angiogenesis inhibitors.

Previous *in vitro* experiments involving eponemycin have indicated the involvement of its inhibitory effect on vascular endothelial cell migration in its anti-angiogenic action.[6] The anti-angiogenic effect of radicicol probably involves its suppressive action against the secretion of plasminogen activator activity by vascular endothelial cells.[7] Preliminary experiments involving cultured vascular endothelial cells showed that rhizoxin at picomolar concentrations influenced the proliferation of, and plasminogen activator production and tube formation by endothelial cells. Thus, further *in vitro* experiments are necessary to confirm these preliminary results suggesting that rhizoxin, like other epoxide group-containing angiogenesis inhibitors, can suppress functions of vascular endothelial cells related to *in vivo* angiogenesis. Furthermore, the anti-tubulin activity of rhizoxin[12] might be relevant to the mechanism of its anti-angiogenic action, since it has been suggested that the anti-angiogenic effects of 2-methoxyestradiol and taxol might involve their anti-tubulin actions,[13, 14] and since nocodazole, a microtubule inhibitor, affected Matrigel-dependent tube formation by human umbilical vein endothelial cells.[15]

In conclusion, the results of the present investigation suggest that rhizoxin is a novel inhibitor of angiogenesis, though the mechanism underlying its anti-angiogenic action remains to be established.

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