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Short communication

Potent inhibition of angiogenesis by wortmannin, a fungal metabolite

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Abstract

Wortmannin ([1*S*-(1α,6*b*α,9*a*β,11α,11*b*β)]-11-(acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-3*H*-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione), a fungal metabolite that is as a selective inhibitor of phosphatidylinositol 3-kinase, was evaluated for its potential as an inhibitor of in vivo angiogenesis in a bioassay system involving growing chick embryo chorioallantoic membranes. It showed dose-dependent inhibitory activity against embryonic angiogenesis. This inhibition occurred at a dose as low as 1 ng (2.3 pmol) per egg and the ID_{50} value was 30 ng/egg. These findings suggest that wortmannin is a new angiogenesis inhibitor, and that it may be a lead antibiotic for a novel class of therapeutic agents for angiogenesis-dependent diseases like cancer, diabetic retinopathy and rheumatoid arthritis.

Keywords: Wortmannin; Phosphatidylinositol 3-kinase inhibitor; Anti-angiogenic activity; Angiogenesis, embryonic; Chorioallantoic membrane

1. Introduction

Angiogenesis, the outgrowth of new blood vessels from existing vasculature, is expected to be a promising target for treatment of various diseases, including cancer, diabetic retinopathy and rheumatoid arthritis (Battegay, 1995; Folkman, 1995; Oikawa, 1995). This is based on the fact that the induction and/or maintenance of these diseases are largely dependent on neovascularization, and that the treatment for these so-called angiogenic disorders needs the development of a new therapeutic strategy because of the current lack of satisfactory therapy.

Angiogenesis is a cascade reaction which includes many important cellular events, such as the proliferation and chemotaxis of and tube formation by vascular endothelial cells, and is thought to be controlled by a number of factors such as growth factors and extracellular matrix components (Battegay, 1995; Folkman, 1995; Oikawa, 1995). Growth factors triggering angiogenesis include basic and acidic fibroblast growth factors, vascular endothelial growth factor, hepatocyte growth factor, epidermal growth factor and platelet-derived growth factor (Battegay, 1995; Folkman, 1995). The membrane receptors of these growth factors contain the respective specific functional tyrosine kinase domains, suggesting that various tyrosine phosphorylation pathways might participate in the regulation of angiogenesis (Oikawa, 1995). This suggestion may be supported by the finding that different agents able to inhibit tyrosine kinase activity affect in vivo angiogenic responses, including herbimycin A (Oikawa et al., 1989b), erbstatin (Oikawa et al., 1993a) and lavendustin A (Hu and Fan, 1995). In addition, a GTP-binding (G)-protein-dependent pathway might be involved in the process of angiogenesis, since Matrigel-induced tube formation of cultured vascular endothelial cells was blocked by pertussis toxin (Bauer et al., 1992), which uncouples certain G-proteins, such as G_i or G_o, from the receptors via the ADP-ribosylation of the G-proteins and hence interferes with the response of cells to receptor stimulation (Ui et al., 1995). Considering these findings together with other information showing that the activation of phosphatidylinositol 3-kinase occurs in a number of cellular signaling systems involving receptor tyrosine kinases or G-protein-coupled receptors (Stephens et al., 1993; Ui et al., 1995; Shepherd et al.,

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1996), it seems fascinating to speculate that phosphatidylinositol 3-kinase could be a potential target for finding new angiogenesis inhibitors.

As the first attempt to verify this speculation, we here examined the effect of wortmannin ([1*S*-(1α,6*b*α,9*a*β,11α,11*b*β)]-11-(acetyloxy)-1,6*b*,7,8,9*a*,10,-11,11*b*-octahydro-1-(methoxymethyl)-9*a*,11*b*-dimethyl-3*H*-furo[4,3,2-de]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione), a fungal metabolite originally identified as an antifungal agent, on in vivo angiogenesis using the growing chick embryo chorioallantoic membrane assay. Recent studies have shown that this antibiotic is a potent and selective inhibitor of phosphatidylinositol 3-kinase, the IC_{50} value for this enzyme being in the low nanomolar range (Ui et al., 1995).

2. Materials and methods

2.1. Materials

Wortmannin was purchased from Kyowa Medex (Tokyo, Japan). Its chemical structure is shown in Fig. 1A. Ethylene-vinyl acetate copolymer 40 was kindly donated by Mitsui-DuPont Polychemicals (Tokyo, Japan).

2.2. Anti-angiogenic activity assay

Anti-angiogenic activity was determined by using the growing chick embryo chorioallantoic membrane assay, as described previously (Oikawa et al., 1989a, 1993b). In short, the chorioallantoic membranes of 4.5-day-old chick embryos were treated with ethylene-vinyl acetate copolymer 40 containing, or not, various doses of wortmannin at 37°C for 2 days in a humidified egg incubator, after which an appropriate volume of 20% fat emulsion was injected into the chorioallantois to show the vascular network better. The anti-angiogenic response was assessed as positive when the avascular zone exceeded 3 mm in diameter; only the frequency of such zones was monitored.

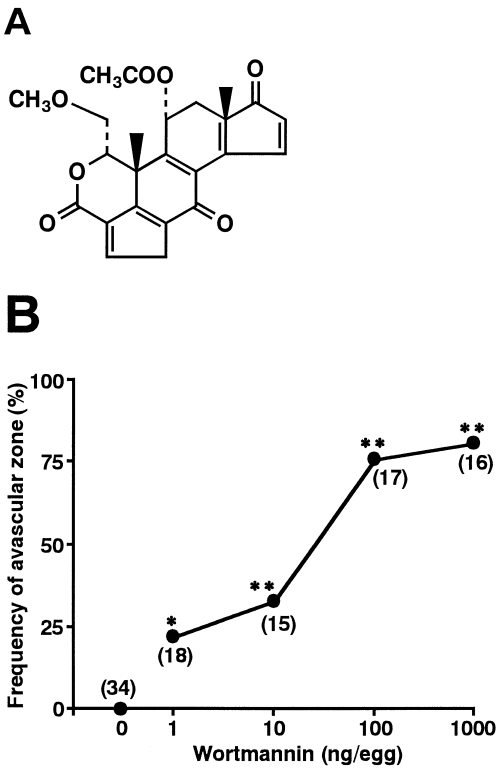

Fig. 1. (A) Chemical structure of wortmannin. (B) Inhibitory activity of wortmannin on embryonic angiogenesis. Ethylene-vinyl acetate copolymer 40 pellets containing various doses of wortmannin were placed on 4.5-day-old chorioallantoic membranes. After 2 days, the anti-angiogenic effect was determined by measuring the avascular zones. The points show the frequency (%) of avascular zones exhibiting an anti-angiogenic response. The values in parentheses are the numbers of membranes examined. * $P < 0.05$ compared to control (i.e., empty) pellet-treated membranes ($n = 34$), which did not show an avascular zone; ** $P <$ 0.001 compared to the control.

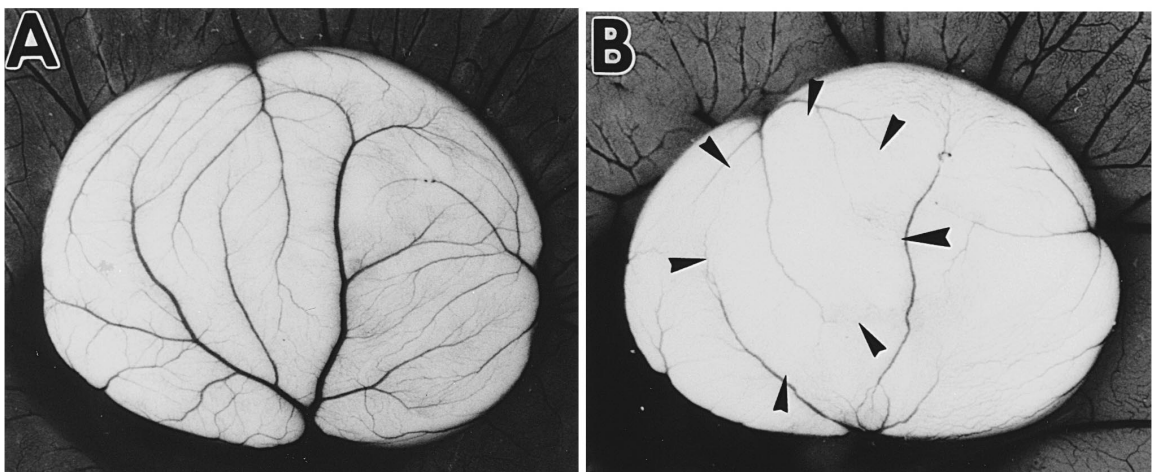

Fig. 2. Effect of wortmannin on angiogenesis in chorioallantoic membranes 2 days after implantation of ethylene-vinyl acetate copolymer 40 pellets. Note that a wortmannin-containing ethylene-vinyl acetate copolymer 40 pellet caused the appearance of a significant avascular zone (indicated by arrowheads), reflecting anti-angiogenic activity (B), while a control pellet (A) did not. Magnification ×2.6.

2.3. Statistics

The data concerning anti-angiogenic activity were analyzed by means of Fisher's exact probability test, $P < 0.05$ being taken as the level of significance.

3. Results

After the 4.5-day-old chorioallantoic membranes were treated with different doses of wortmannin for 2 days, the dose-response relationship for the appearance of an avascular zone was determined. Treatment with wortmannin caused a dose-dependent suppression of embryonic angiogenesis (Fig. 1B). As compared to the effect of control ethylene-vinyl acetate polymer 40 pellets alone, which did not exhibit anti-angiogenic activity in any of the chorioallantoic membranes treated ($n = 34$), the minimum effective dose of wortmannin required for the appearance of an avascular zone was 1 ng (2.3 pmol) per egg ($n = 18$). The ID_{50} value obtained for wortmannin was 30 ng/egg. The results of representative experiments are shown in Fig. 2. Wortmannin (1000 ng/egg) potently suppressed embryonic angiogenesis, causing a significant avascular zone (Fig. 2B), while control, empty ethylene-vinyl acetate copolymer 40 pellets did not produce such an avascular zone in any of the 34 chorioallantoic membranes examined.

4. Discussion

Previous findings have suggested the possibility that different cellular signaling systems are involved in the induction of in vivo angiogenesis, including those involving tyrosine phosphorylation and probably G-protein-coupled-receptor pathways (Oikawa et al., 1989b, 1993a; Oikawa, 1995; Bauer et al., 1992; Battegay, 1995; Folkman, 1995). It is known that activation of phosphatidylinositol 3-kinase is a crucial step downstream of these signaling pathways in various cellular responses (Stephens et al., 1993; Ui et al., 1995; Shepherd et al., 1996). In the present study we have demonstrated that wortmannin, a fungal metabolite, is a potent angiogenesis inhibitor which exerts its effect in the picomolar dose range, as assessed in an in vivo assay system involving growing chick embryo chorioallantoic membranes. Wortmannin was previously found to act as a selective inhibitor of myosin light chain kinase (Ui et al., 1995). However, recent studies have revealed that the fungal metabolite is a potent and selective inhibitor of phosphatidylinositol 3-kinase, with inhibition occurring at low nanomolar concentrations (Ui et al., 1995). The concentration of the fungal metabolite required for inhibition of this enzyme is about 100-fold lower than that required for inhibition of myosin light chain kinase, and at concentrations up to 1 μM it has little or no effect on other kinases, including protein kinases A, C and G and phosphatidylinositol 4-kinase (Ui et al., 1995). Taken together, it seems reasonable to speculate that wortmannin influences in vivo angiogenesis through inhibition of phosphatidylinositol 3-kinase, although the possibility that other molecule(s) may be a target cannot be completely excluded, because there is one report suggesting that this microbial product may affect phospholipase A_2 at low nanomolar concentrations (Cross et al., 1995).

If the above speculation is true, then the question of which step(s) of in vivo angiogenesis requires the activity of phosphatidylinositol 3-kinase arises. Previous studies could give a clue to the answer to this question. Tube formation by cultured vascular endothelial cells on Matrigel was found to be strongly inhibited by pertussis toxin, an inhibitor of G-protein-coupled signaling pathways (Bauer et al., 1992). It has been shown that this toxin can block G-protein-receptor-coupled phosphatidylinositol 3-kinase activation in various cellular responses (Ui et al., 1995). In addition, hepatocyte growth factor, which shows angiogenic activity in vivo, has been shown to induce tubulogenesis in cultured vascular endothelial cells or renal collecting duct epithelial cells, probably through binding to and tyrosine phosphorylation of its high-affinity receptor, c-met, and subsequent association of several intracellular proteins, such as phosphatidylinositol 3-kinase (Cantley et al., 1994; Rosen and Goldberg, 1995). Although the effect of wortmannin on vascular tube formation in vitro has not yet been examined, hepatocyte growth factor-mediated tubulogenesis in cultured renal epithelial cells is inhibited by this fungal metabolite at low nanomolar concentrations, indicating that this inhibition might come from the direct effect of the fungal metabolite on phosphatidylinositol 3-kinase (Derman et al., 1995). Taken together, it is possible that phosphatidylinositol 3-kinase activity is involved in the step of tube formation by vascular endothelial cells during angiogenesis. An alternative possibility is that this enzyme may contribute to mitogenesis and chemotaxis of endothelial cells, since the enzyme was found affect these two responses in other cell types, including renal epithelial cells (Derman et al., 1995; Ui et al., 1995; Shepherd et al., 1996).

In comparison with the potency of other angiogenesis inhibitors we previously identified using the chorioallantoic membrane assay (Oikawa, 1995), wortmannin (ID_{50} = 70 pmol/egg) seems to have almost the same potency as staurosporine (ID_{50} = 71 pmol/egg), a potent protein kinase inhibitor. Wortmannin seems to exhibit stronger anti-angiogenic activity than 22-oxa-1α,25-dihydroxyvitamin D_3 (ID_{50} = 96 pmol/egg), herbimycin A (ID_{50} = 260 pmol/egg), retinoic acid (ID_{50} = 330 pmol/egg), 1α,25-dihydroxyvitamin D_3 (ID_{50} = 340 pmol/egg), erbstatin (ID_{50} = 450 pmol/egg), radicicol (ID_{50} = 540 pmol/egg), 15-deoxyspergualin (ID_{50} = 960 pmol/egg), depudecin (ID_{50} = 1500 pmol/egg) and medroxyprogesterone acetate (ID_{50} = 260 nmol/egg), although it appears to have weaker

anti-angiogenic activity than eponemycin ($ID_{50} = 0.25$ pmol/egg) and four synthetic retinoids, Re 80 ($ID_{50} = 6.3$ pmol/egg), Ch 55 ($ID_{50} = 22$ pmol/egg), Am 580 ($ID_{50} = 23$ pmol/egg) and Am 80 ($ID_{50} = 28$ pmol/egg).

In summary, the present paper is the first to show the possible involvement of phosphatidylinositol 3-kinase signaling in the regulation of in vivo angiogenesis. Wortmannin might be a lead antibiotic for a novel class of therapeutic agents for angiogenic diseases and also a useful probe for understanding the mechanism of angiogenesis.

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