INHIBITION OF ANGIOGENESIS BY ERBSTATIN, AN INHIBITOR OF TYROSINE KINASE

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Here we describe the inhibitory effect of erbstatin, a specific tyrosine kinase inhibitor, on *in vivo* angiogenesis. Inhibition of angiogenesis was determined in a bioassay system involving chorioallantoic membranes of growing chick embryos. Erbstatin produced a dose-dependent inhibitory action on embryonic angiogenesis. This inhibition occurred at as small a dose as 10 ng/egg and the ID_{50} value was 80 ng/egg. To analyze this inhibition, *in vitro* experiments involving vascular endothelial cells were also performed. Erbstatin affected the proliferation of vascular endothelial cells, one of angiogenic components. This inhibition was dose-dependent, the IC_{50} value being 3.6 μM. These data indicate that erbstatin-sensitive tyrosine kinase(s) is involved in angiogenic endothelial cell proliferation, and that experiments involving erbstatin will provide an important clue to understand a mechanism of angiogenesis.

It is widely accepted that angiogenesis is a key factor in the progression of malignant tumors. Evidence has been accumulating that a variety of growth factors induce an angiogenic response is *in vivo* assay systems involving rabbit cornea or chick embryo chorioallantoic membrane (CAM). These angiogenic mitogens include acidic and basic fibroblast growth factors, epidermal growth factor (EGF), vascular endothelial growth factor and hepatocyte growth factor[1~4]. Interestingly, their respective receptors have been found to show tyrosine kinase activity[4~7], suggesting that tyrosine phosphorylation might be an important signal in the angiogenesis process. In relation to this, it was interesting that phosphotyrosine-modified proteins were concentrated at the cell-to-cell adherens junctions in vascular endothelial cells of various tissues during chick embryo development and that specific proto-oncogenic tyrosine kinases of the *src* family were enriched in the cell-to-cell adherens junctions[8,9]. In addition, vanadate, a specific inhibitor of phosphotyrosine phosphatase, promotes vascular endothelial cells to form vessel-like tubules and also to produce two types of plasminogen activators, proteolytic enzymes known to be involved in an angiogenic response[10].

We found recently that staurosporine, isolated from a strain of *Streptomyces*, inhibits *in vivo* angiogenesis in the picomolar range of dose[11]. Because this microbial product was originally identified as a potent protein kinase C inhibitor[12], it was suggested that it might elicit its antiangiogenic action by inhibiting kinase activity. In addition to this inhibitory activity, staurosporine was also found to inhibit tyrosine kinase and cAMP-dependent protein kinase within a similar range of concentrations to that required for inhibition of protein kinase C[13]. Thus it might be also possible that the antiangiogenic action of staurosporine is associated with the inhibition of tyrosine kinase or cAMP-dependent protein kinase or both. Specific inhibitors of protein kinase C, tyrosine kinase or cAMP-dependent protein kinase should provide a clue to this problem.

Erbstatin was isolated from the culture filtrate of a strain of *Streptomyces* as an inhibitor of tyrosine kinase activity of the EGF receptor[14]. It also inhibits the activity of the *src* tyrosine kinase, but dose not inhibit cAMP-dependent protein kinase or protein kinase C[15], implying that erbstatin, unlike staurosporine, is a specific inhibitor of tyrosine kinase.

Here we show that erbstatin dose-dependently exerts an angiogenesis-inhibitory effect in an *in vivo* assay system involving growing chick embryo CAM. *In vitro* experiments revealed that erbstatin also inhibits the proliferation of vascular endothelial cells in a concentration-dependent manner. These findings indicate the possibility that tyrosine kinase plays a role in vascular endothelial cell growth, a crucial component of the angiogenesis process, and that erbstatin evokes an antiangiogenic action, probably through inhibition of the tyrosine kinase activity.

Materials and Methods

Chemicals

Erbstatin was isolated as described previously[14]. Ethylene-vinyl acetate copolymer 40 (EV 40) was a generous gift from Mitsui-DuPont Polychemical Co., Ltd., Tokyo, Japan. EV 40 was used in the present study, after being fully washed in an excess of 99% ethanol to remove impurities affecting the CAM assay. DULBECCO's modified EAGLE's medium (DMEM) was a product of Sigma Chemical Co., St. Louis, MO. Fetal bovine serum (FBS) was obtained from Biocell Lab., Carson, CA and a mixture of penicillin-streptomycin from Gibco Lab., Grand Island, NY.

Preparation of EV 40 Pellets Containing Erbstatin

Erbstatin was dissolved in ethanol at a concentration of 1 mg/ml, sequentially diluted with ethanol and then mixed with an equal volume of 5% EV 40 dissolved in methylene chloride. Aliquots (20 μl) of a mixture were dropped on sterilized glass plates and then air-dried. Dried films of EV 40 were made into pellets using a microspatula and stored at -40°C until used.

Assay of Antiangiogenic Activity in CAM

Inhibition of angiogenesis was determined in a bioassay system involving CAMs of growing chick embryos as described[16]. An EV 40 pellet containing the indicated dose of erbstatin was placed carefully on the CAM surface of 4.5-day-old chick embryo and then incubated in a humidified egg incubator. After 2 days of incubation, an appropriate volume of 20% fat emulsion was injected into the chorioallantois to improve visualization of the vascular network. Inhibition of angiogenesis was assessed by measuring the avascular zone in the CAM. An antiangiogenic response was assessed as effective when the avascular zone exceeded 3 mm.

Assay of Vascular Endothelial Cell Proliferation

Vascular endothelial cells were prepared and maintained in basal medium (DMEM containing 25 mM

Hepes, 100 units/ml benzylpenicillin and 100 μg/ml streptomycin) supplemented with 10% FBS, as described previously[11,17]. Endothelial cells (5×10^3 cell/well) were plated into the wells of 24-multiwell dishes (Falcon; Becton Dickinson Co., Lincoln, NJ) containing 1 ml of basal medium supplemented with 5% FBS. Five hours after plating of the cells, aliquots (20 μl) of various concentrations of erbstatin dissolved in 5% ethanol-basal medium were added directly into the medium, where the final concentration of ethanol was identical in all wells and was 0.1%. After 72-hours incubation at 37°C under an atmosphere of 5% CO_2 in air, the cells were trypsinized and then counted in a Coulter counter ZBI (Coulter Electronics Inc., Hialeah, FL).

Statistical Analysis

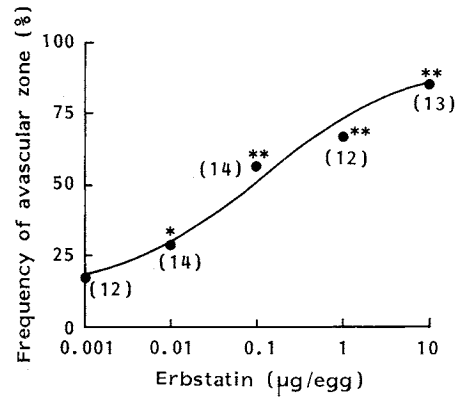
Data as to the incidence of antiangiogenic activity were analyzed by means of FISHER's exact probability test, $p<0.05$ being taken as the level of significance. Results as to the endothelial cell proliferation were analyzed by means of STUDENT's t-test with $p<0.05$ as the level of significance.

Results

Antiangiogenic Effect of Erbstatin

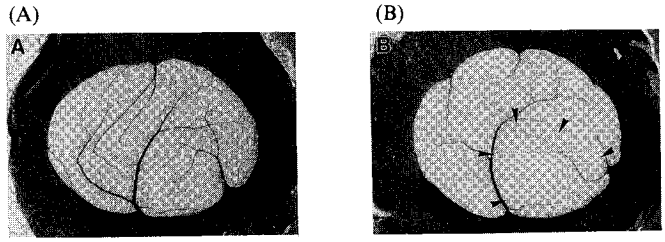
Erbstatin was examined for its antiangiogenic effect in the CAM assay system. Fig. 1 represents the dose-response relationship for the appearance of an avascular zone. Erbstatin inhibited the embryonic angiogenesis dose-dependently. In comparison with the effects of empty EV pellets (control) which did not cause inhibition of embryonic angiogenesis in any of 20 CAMs examined, the minimum effective dose required for causing an avascular zone was 10 ng (56 pmol) per egg, and the ID_{50} value was 80 ng/egg. Representatives of these experiments are shown in Fig. 2. Erbstatin effectively inhibited embryonic angiogenesis to cause a significant avascular zone, while an EV pellet alone did not elicit such an action in any of the CAMs treated.

Fig. 1. Inhibitory effect of erbstatin on embryonic angiogenesis.



After EV pellets containing various doses of erbstatin were placed on the 4.5-day-old CAM, the antiangiogenic activity was assessed as described in Materials and Methods. The values in parentheses show the number of CAMs used. * $P<0.05$ compared to empty pellet-treated (*i.e.*, control) CAMs (n=20) which did not cause antiangiogenic activity; ** $P<$ 0.001 compared to the control.

Fig. 2. Effect of erbstatin on angiogenesis in CAM 2 days after placement of EV pellets with erbstatin (A, 0 ng/pellet; B, 100 ng/pellet).

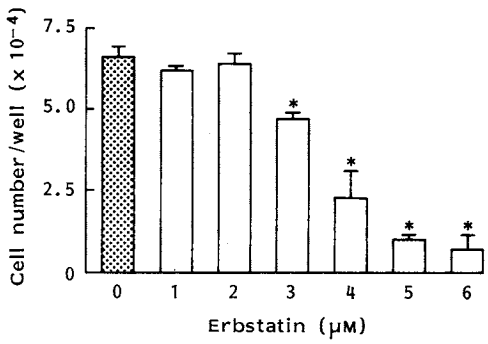


EV pellets with erbstatin caused an avascular zone (surrounded with arrows) showing antiangiogenic activity, while empty pellets without the agent did not. Magnification, × 1.6.

Effect of Erbstatin on Proliferation of Vascular Endothelial Cells

To find a clue to the understanding of a mechanism of angiogenesis inhibition by erbstatin, *in vitro* experiments were carried out to determine whether or not the microbial product affected proliferation of vascular endothelial cells, a key step in the angiogenesis process. It exerted an inhibitory effect on the endothelial cell proliferation in a concentration-dependent manner, the IC_{50} being 3.6 μM.

Fig. 3. Effect of erbstatin on proliferation of vascular endothelial cells.



Endothelial cells were incubated in the presence of various concentrations of erbstatin for 72 hours and then were counted. The bars show the mean values and SD (n=3). * $P<0.01$ compared to the control.

Discussion

Angiogenesis has become an attractive target of studies on cancer treatment, because different types of angiogenesis inhibitors have been proven effective in growth inhibition of solid tumors[18~23]. However, the molecular bases of angiogenesis have not yet been well understood. An angiogenesis inhibitor with an unique mode of action seems to give us useful information to approach this important problem.

Our previous study showed that staurosporine affects *in vivo* angiogenesis, the ID_{50} value being 71 pmol/egg[11]. This indicated that certain protein kinases, including protein kinase C, tyrosine kinase and cAMP-dependent protein kinase, might play a crucial role in induction of angiogenesis, because staurosporine exerts potent inhibitory effects on different protein kinases in a similar concentration range[13]. Thus, which type(s) of protein kinase is associated with angiogenesis induction remains to be clarified. To approach this problem, in this study we examined the effect of erbstatin, a specific inhibitor of tyrosine kinase, on angiogenesis in the CAM assay, and found that it is an effective inhibitor of angiogenesis. Taking the present result together with our previous observation that herbimycin A, another specific tyrosine kinase inhibitor, effectively inhibits angiogenesis[24], it is conceivable to assue that certain tyrosine kinase plays a role in induction of angiogenesis *in vivo*.

When compared on a molar basis, erbstatin appears to be weaker in antiangiogenic ability (ID_{50}= 450 pmol/egg) than a synthetic retinoid Ch 55 (ID_{50}=24 pmol/egg), 22-oxa-1α,25-dihydroxyvitamin D_3 (ID_{50}=96 pmol/egg), staurosporine (ID_{50}=71 pmol/egg) and eponemycin (ID_{50}=250 fmol/egg)[11,16,25,26]. On the other hand, erbstatin seems to exert similar antiangiogenic effect as herbimycin A (ID_{50}= 260 pmol/egg), retinoic acid (ID_{50}=330 pmol/egg) and 1α,25-dihydroxyvitamin D_3 (ID_{50}=340 pmol/egg)[16,24,26]. Taking these findings together, erbstatin seems to be a moderate angiogenesis inhibitor.

We would like to discuss the question that what kind of tyrosine kinase is inhibited by erbstatin. Erbstatin was originally identified as an inhibitor of tyrosine kinase activity of the receptor of EGF, known to be an inducer of angiogenesis, although it exerts an inhibitory effect on neither protein kinase C nor cAMP-dependent protein kinase. It also inhibited the *src* tyrosine kinase[14,27], as did herbimycin A[28]. In addition, there are interesting findings that phosphotyrosine-modified proteins were found to be concentrated at the cell-to-cell adherens junctions in various tissues during development of chick embryo and the members of the *src* family, including c-*src* and c-*yes* tyrosine kinases, were enriched in the cell-to-cell adherens junctions[8,9]. Considering these findings, the *src* tyrosine kinase or its related tyrosine kinase would be a promising candidate of targets of erbstatin. It might also be possible that the tyrosine kinase of the receptors of a variety of putative angiogenic factors, including EGF, fibroblast growth factor, vascular endothelial growth factor and hepatocyte growth factor, might be susceptible to erbstatin, because their growth factors contain a tyrosine kinase domain[4~7].

Previous study showed that erbstatin inhibits the growth of A431 cells with an IC_{50} value of 20 μM or IMC-carcinoma cells with an IC_{50} value of 17 μM[14]. Taking these findings together with the present observation that erbstatin dose-dependently inhibited proliferation of vascular endothelial cells, the IC_{50}

being 3.6 μM, the endothelial cells seem to be more sensitive to the microbial product than these two tumor cell lines although it does not selectively inhibit the endothelial cell growth.

The present *in vitro* experiments involving vascular endothelial cells suggested that the cell proliferation inhibitory effect of erbstatin is probably involved in the antiangiogenic mechanism. However, this inhibitory action on the endothelial cell growth appeared less effective than those of other angiogenesis inhibitors, such as staurosporine and eponemycin[11,25], which show growth-inhibitory activity against endothelial cells. This might be in part due to the previously reported instability of erbstatin in serum[29,30]. Another possibility might be that erbstatin exhibits an antiangiogenic effect through the modification of the differentiation state of angiogenic endothelial cells, taking the previous observation that the microbial product induces the change of transformed cells into morphologically normal phenotype cells[31], together with our previous findings that cell differentiation modifiers such as retinoids and vitamin D_3 analogs are also effective angiogenesis inhibitors[16,26,32]. Further study on elucidation of the mechanism of antiangiogenic activity of erbstatin will undoubtedly provide us an important clue of better understanding of a mechanism of angiogenesis.

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References

1) FOLKMAN, J. & M. KLAGSBRUN: Angiogenic factors. Science 235: 442~447, 1987
2) BLOOD, C. H. & B. R. ZETTER: Tumor interactions with the vasculature and tumor metastasis. Biochim. Biophys. Acta 1032: 89~118, 1990
3) FOLKMAN, J. & Y. SHING: Angiogenesis. J. Biol. Chem. 267: 10931~10934, 1992
4) BUSSOLINO, F.; M. F. DI RENZO, M. ZICHE, E. BOCCHIETTO, M. OLIVERO, L. NALDINI, G. BAUDINO, L. TAMAGNONE, A. COFFER & P. M. COMOGLIO: Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. 119: 629~641, 1992
5) HUNTER, T. & J. A. COOPER: Protein-tyrosine kinases. Annu. Rev. Biochem. 54: 897~930, 1985
6) ULLRICH, A. & J. SCHLESSINGER: Signal transduction by receptors with tyrosine kinase activity. Cell 61: 203~212, 1990
7) DE VARIES, C.; J. A. ESCOBEDO, H. UENO, K. HOUCK, N. FERRARA & L. T. WILLIAMS: The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255: 989~991, 1992
8) TANAKA, K. & S. J. SINGER: Phosphotyrosine-modified proteins are concentrated at the membranes of epithelial and endothelial cells during tissue development in chick embryos. J. Cell Biol. 106: 1757~1764, 1988
9) TSUKITA, S.; K. OISHI, T. AKIYAMA, Y. YAMANASHI, T. YAMAMOTO & S. TSUKITA: Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. J. Cell Biol. 113: 867~879, 1991
10) MONTESANO, R.; M. S. PEPPER, D. BELIN, J.-D. VASSALLI & L. ORCI: Induction of angiogenesis in vitro by vanadate, an inhibitor of phosphotyrosine phosphatases. J. Cell. Physiol. 134: 460~466, 1988
11) OIKAWA, T.; M. SHIMAMURA, H. ASHINO, O. NAKAMURA, T. KANAYASU, I. MORITA & S. MUROTA: Inhibition of angiogenesis by staurosporine, a potent protein kinase inhibitor. J. Antibiotics 45: 1155~1160, 1992
12) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1986
13) TAMAOKI, T.: Use of specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *In* Methods in Enzymology. Vol. 201. *Eds.*, T. HUNTER & B. M. SEFTON, pp. 340~347, Academic Press, 1991
14) UMEZAWA, H.; M. IMOTO, T. SAWA, K. Isshiki, N. MATSUDA, T. UCHIDA, H. IINUMA, M. HAMADA & T. TAKEUCHI: Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. J. Antibiotics 39: 170~173, 1986
15) IMOTO, M.; K. UMEZAWA, T. SAWA, T. TAKEUCHI & H. UMEZAWA: *In situ* inhibition of tyrosine kinase by erbstatin. Biochem. Int. 15: 989~995, 1987
16) OIKAWA, T.; K. HIROTANI, O. NAKAMURA, K. SHUDO, A. HIRAGUN & T. IWAGUCHI: A highly potent antiangiogenic activity of retinoids. Cancer Lett. 48: 157~162, 1989
17) KANAYASU, T.; J. NAKAO-HAYASHI, N. ASUWA, I. MORITA, T. ISHII, H. ITO & S. MUROTA: Leukotriene C_4 stimulates angiogenesis in bovine carotid artery endothelial cells *in vitro*. Biochem. Biophys. Res. Commun. 159: 572~578, 1989

18) FOLKMAN, J.: Tumor angiogenesis. Adv. Cancer Res. 43: 175~203, 1985
19) OIKAWA, T.; A. HIRAGUN, Y. YOSHIDA, H. ASHINO-FUSE, T. TOMINAGA & T. IWAGUCHI: Angiogenic activity of rat mammary carcinoma induced by 7,12-dimethylbenz[a]anthracene and its inhibition by medroxyprogesterone acetate: possible involvement of antiangiogenic action of medroxyprogesterone acetate in its tumor growth inhibition. Cancer Lett. 43: 85~92, 1988
20) MOSES, M. A. & R. LANGER: Inhibitors of angiogenesis. Biotechnology 9: 630~634, 1991
21) KLAGSBRUN, M. & P. A. D'AMORE: Regulators of angiogenesis. Ann. Rev. Physiol. 53: 217~239, 1991
22) OIKAWA, T.; Y. YOSHIDA, M. SHIMAMURA, H. ASHINO-FUSE, T. IWAGUCHI & T. TOMINAGA: Antitumor effect of 22-oxa-1α,25-dihydroxyvitamin D_3, a potent angiogenesis inhibitor, on rat mammary tumors induced by 7,12-dimethylbenz[a]anthracene. Anti-Cancer Drugs 2: 475~480, 1991
23) EDGINGTON, S. M.: Angiogenic and angiostatic drugs: Reshaping biotech's future. Biotechnology 10: 981~985, 1992
24) OIKAWA, T.; K. HIROTANI, M. SHIMAMURA, H. ASHINO-FUSE & T. IWAGUCHI: Powerful antiangiogenic activity of herbimycin A (named angiostatic antibiotic). J. Antibiotics 42: 1202~1204, 1989
25) OIKAWA, T.; M. HASEGAWA, M. SHIMAMURA, H. ASHINO, S. MUROTA & I. MORITA: Eponemycin, a novel antibiotic, is a highly powerful angiogenesis inhibitor. Biochem. Biophys. Res. Commun. 181: 1070~1076, 1991
26) OIKAWA, T.; K. HIROTANI, H. OGASAWARA, T. KATAYAMA, O. NAKAMURA, T. IWAGUCHI & A. HIRAGUN: Inhibition of angiogenesis by vitamin D_3 analogues. Eur. J. Pharmacol. 178: 247~250, 1990
27) IMOTO, M.; K. UMEZAWA, K. ISSHIKI, S. KUNIMOTO, T. SAWA, T. TAKEUCHI & H. UMEZAWA: Kinetic studies of tyrosine kinase inhibition by erbstatin. J. Antibiotics 40: 1471~1473, 1987
28) UEHARA, Y.; Y. MURAKAMI, S. MIZUNO & S. KAWAI: Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A. Virology 164: 294~298, 1988
29) IMOTO, M.; K. UMEZAWA, K. KOMURO, T. SAWA, T. TAKEUCHI & H. UMEZAWA: Antitumor activity of erbstatin, a tyrosine protein kinase inhibitor. Jpn. J. Cancer Res. 78: 329~332, 1987
30) UMEZAWA, K.; T. HORI, H. TAJIMA, M. IMOTO, K. ISSHIKI & T. TAKEUCHI: Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. FEBS Lett. 260: 198~200, 1990
31) HONMA, Y.; J. OKABE-KADO, R. KASUKABE, M. HOZUMI & K. UMEZAWA: Inhibition of *abl* oncogene tyrosine kinase induces erythroid differentiation of human myelogenous leukemia K562 cells. Jpn. J. Cancer Res. 81: 1132~1136, 1990
32) OIKAWA, T.; M. SHIMAMURA, H. ASHINO, I. MORITA, S. MUROTA, J. ABE, Y. NISHII & T. TOMINAGA: A novel vitamin D_3 analogue with an antiangiogenic action. Drug News Perspect, in press