INHIBITION OF ANGIOGENESIS BY STAUROSPORINE, A POTENT PROTEIN KINASE INHIBITOR

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The effect of staurosporine, a potent inhibitor of protein kinases, on embryonic angiogenesis was studied in an *in vivo* assay system involving chorioallantoic membranes of growing chick embryo. Staurosporine inhibited embryonic angiogenesis in a dose-related manner, the ID_{50} value being 71 pmol/egg. Staurosporine dose-dependently suppressed the proliferation of vascular endothelial cells, an important event involved in the angiogenesis process. The IC_{50} value was 0.88 nM. In contrast, staurosporine did not affect the migration of vascular endothelial cells. These results suggest that staurosporine affected embryonic angiogenesis probably by inhibiting endothelial cell proliferation. In addition, these results might support the notion that certain protein kinase(s) could be implicated in induction of angiogenesis and also that staurosporine would be a useful compound for studying a mode of action of angiogenesis occurring in various diseases, including tumor development.

Angiogenesis occurs in normal physiological and pathophysiological conditions, such as in embryonic development, in wound healing, in diabetic retinopathy, in psoriasis and in tumor development[1]. In particular, the important role of angiogenesis in the progressive growth of solid tumors is indicated by previous observations that various angiogenesis inhibitors, including angiostatic steroids, microbial products and angiostatic vitamins, have a tumor growth-inhibitory effect[1~3]. Angiogenesis is a result of concerted sequence of events which contains several steps, including migration and proliferation of vascular endothelial cells[1,2]. In addition to various growth factor peptides, several chemicals, such as prostaglandins, 1-butyryl-glycerol and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cause an angiogenic response[1,4,5]. TPA, a potent tumor promoter, is well-known to induce its tumor promotion activity by binding to and activating protein kinase C which is a key enzyme involved in both signal transduction on cell membrane and cell proliferation[6]. TPA also stimulates the growth of vascular endothelial cells and this stimulation is inhibited by staurosporine which is a potent inhibitor of protein kinases, such as protein kinase C and protein tyrosine kinase, isolated from a strain of *Streptomyces*[7~9]. We previously found that herbimycin A, a microbial inhibitor of certain protein tyrosine kinase, exhibit angiogenesis-inhibitory activity in the chorioallantoic membrane (CAM) assay system[10]. These findings prompted us to speculate that certain protein kinase(s) might play an important role during angiogenesis. To verify

this speculation, we examined the effect of staurosporine on angiogenesis in the CAM.

In this study we demonstrate that staurosporine inhibits embryonic angiogenesis in a dose-dependent manner, and also affect the proliferation of vascular endothelial cells *in vitro*. These results show that the antiangiogenic activity of staurosporine might involve the inhibition of certain protein kinase(s) responsible for angiogenic endothelial cell growth.

Materials and Methods

Chemicals

Staurosporine was generously supplied by Dr. T. TAMAOKI, Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. Ethylene-vinyl acetate copolymer 40 (EV 40) was a generous gift from Mitsui-DuPont Polychemical Co., Ltd., Tokyo, Japan. DULBECCO's modified EAGLE's medium (DMEM) was purchased from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum (FBS) was obtained from Biocell Lab., Carson, CA. Fisetin was obtained from Aldrich Chem. Co., Milwaukee, WI and genistein from Extrasynthese Co., Genay, France.

Assay of Antiangiogenic Activity in CAM

Antiangiogenic activity was assessed by placing an EV pellet containing a test sample on the 4.5-day-old CAM, as described previously[10,11]. In brief, fertilized eggs (Ohmiya Kakin Lab., Ohmiya, Japan) were incubated in a humidified egg incubator at 37°C. After a 4.5-day incubation, an EV pellet with or without the sample examined was placed on the 4.5-day-old CAM, where embryos with chorioallantois of 3~5 mm diameter were used in this experiment. Following additional 2-day incubation, an appropriate volume of 20% fat emulsion was injected into the chorioallantois, so that the vascular network stood out against the white background of lipid. The antiangiogenic response was evaluated by measuring the avascular zone in the CAM. The response was scored as effective when the avascular zone exceeded 3 mm according to our method[10].

Vascular Endothelial Cells

Endothelial cells were prepared from bovine carotid artery and maintained in DMEM supplemented with 10% FBS, as described previously[12,13]. The cells at passages 7~15 were used for subsequent experiments.

Vascular Endothelial Cell Proliferation Assay

The proliferation of endothelial cells was determined in triplicate, as described[13]. After endothelial cells (1×10^4 cells/well) were plated onto the wells of a 24-multiwell dishes (Falcon; Becton Dickinson Co., Lincoln, NJ) containing 1 ml of DMEM with 5% FBS and allowed to be attached to the substratum of the dish for 5 hours at 37°C under an atmosphere of 5% CO_2 in air, various concentrations of samples (20 μl) were added into the medium and incubated in the same manner. Following incubation at 37°C for 3 days, the cells were trypsinized and then counted in a Coulter counter ZBI (Coulter Electronics Inc., Hialeah, FL).

Vascular Endothelial Cell Migration Assay

The endothelial cell migration was determined in a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD) involving nucleopore membrane filter with 10-μm thickness and 8-μm pore size as described[12]. The stimulation of cell migration was induced by serum; the concentrations of FBS in the lower and upper wells were 10 and 2%, respectively. Staurosporine were added to both lower and upper wells. Cell suspensions (50 μl) in 2% FBS-DMEM were added to the wells at cell density of 2×10^4/well. After migration assays were run at 37°C for 3 hours in 5% CO_2 in the incubator, the cells at the upper surface of the nucleopore membrane were removed. The cells at the lower surface of the membrane were fixed in 90% ethanol and stained with hematoxyline solution, and then the total cell number was counted.

Statistical Analysis

Results as to the incidence of antiangiogenic activity were analyzed by FISHER's exact probability test with $P<0.05$ as the level of significance.

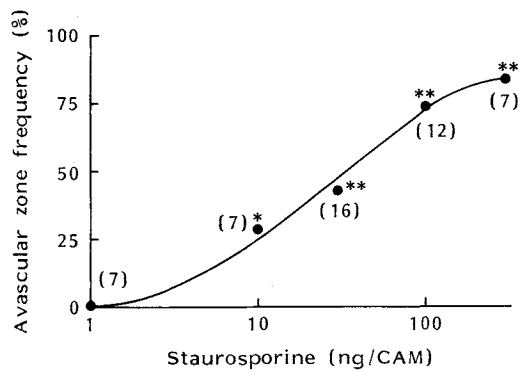
Results

Antiangiogenic Activity of Staurosporine

The effect of staurosporine on embryonic angiogenesis was examined by placing an EV pellet containing various doses of the agent on the surface of growing 4.5-day-old CAM. Fig. 1 shows the dose-response relationship for the appearance of an avascular zone. Staurosporine markedly inhibited embryonic angiogenesis. This potent inhibition was dose-dependent. The minimum effective dose required for causing an avascular zone in CAM was 10 ng (21 pmol) per egg, when compared with the effects of empty pellets (control) which did not produce an avascular zone in any of 29 CAMs tested. The ID_{50} value was 33 ng/egg. Representatives of these experiments are illustrated in Fig. 2. Staurosporine produced significant avascular zone in the CAM, while an empty pellet did not exert such an effect in any of the treated CAMs.

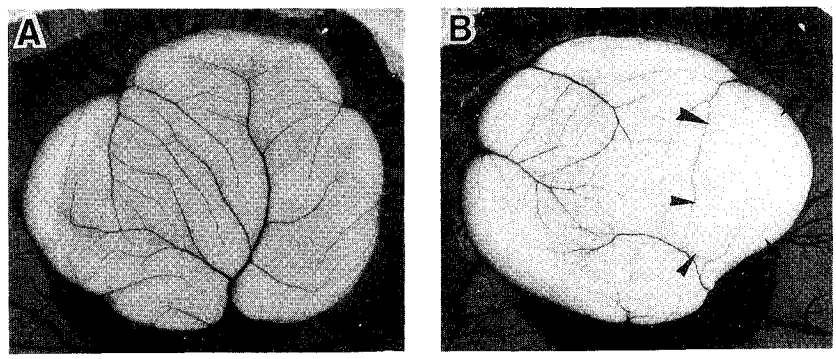
To further determine possible involvement of protein kinase in angiogenesis induction, two other protein kinase inhibitors, *i.e.*, fisetin[14], a plant flavonoid, and genistein[15], a microbial isoflavone compound, were also examined as to their angiogenesis-inhibitory effects at the dose of 100 ng/egg. The value of antiangiogenic activity by this dose of staurosporine was 75% (9/12). Fisetin, although

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



After EV pellets containing various doses of staurosporine 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=29) which did not cause antiangiogenic activity; ** $P<0.001$ compared to the control.

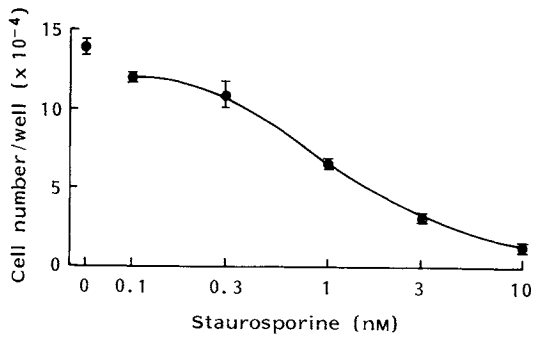
Fig. 2. Effect of staurosporine on angiogenesis in CAM 2 days after placement of EV pellets with staurosporine (A, 0 ng/pellet; B, 100 ng/pellet).



EV pellets with staurosporine produced an avascular zone (surrounded with arrows) showing antiangiogenic activity, while empty pellets without the agent did not. Magnification, ×2.2.

showed less effect than staurosporine, significantly inhibited angiogenesis, a value for antiangiogenic activity being 43% (3/7). There was a trend for genistein to inhibit angiogenesis, although this did not reach statistical significance; a value for antiangiogenic activity was 22% (2/9). This might be because the dose of genistein used was not sufficient to inhibit angiogenesis, that is, higher doses of genistein than that used in this study could affect angiogenesis with significant levels.

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



Endothelial cells were incubated in the presence of various concentrations of staurosporine for 72 hours and then were counted.

Effect of Staurosporine on Proliferation of Vascular Endothelial Cells

To determine whether or not the inhibition of angiogenesis by staurosporine was due to its inhibition of vascular endothelial cell proliferation, endothelial cells were cultured for 72 hours in the presence of various concentrations of staurosporine. Staurosporine inhibited the proliferation of endothelial cell in a concentration-dependent manner. The IC_{50} value was 0.88 nM.

To determine whether or not staurosporine affected another event in the angiogenesis process, its effect on the migration of endothelial cells was also examined. Staurosporine, at concentrations of $0.1 \sim 10$ nM, did not inhibit the endothelial cell migration.

Discussion

Recent studies have shown that TPA causes angiogenesis in both *in vivo* and *in vitro* assay systems[5,16]. TPA induces its various biological and biochemical activities by binding to and activating protein kinase C[6]. Thus it is expected that an inhibitor of protein kinase C could inhibit angiogenesis, but a direct evidence for this has not yet been obtained. In this study we demonstrate that staurosporine, a potent protein kinase C inhibitor, exhibits angiogenesis-inhibitory activity. The inhibition occurred in the picomolar range. This finding suggests that protein kinase C is implicated in induction of angiogenesis. It might also imply possible involvement of other protein kinase in angiogenesis induction, because staurosporine also inhibit several protein kinases in addition to protein kinase C[9]. This might be supported by the present observation that fisetin and probably genistein, these two agents known to be inhibitors of protein kinase, affected angiogenesis and also by our previous finding that herbimycin A, a protein tyrosine kinase inhibitor, inhibits angiogenesis[10]. Alternatively, it might be possible that staurosporine modifies the differentiation of angiogenic endothelial cells, resulting in angiogenesis inhibition, since staurosporine modulates cell differentiation induced by various agents, retinoic acid and 1α,25-dihydroxyvitamin D_3[17] and since our previous observations that retinoids and vitamin D_3 analogs, both of which are well-known to modify cell differentiation, are potent angiogenesis inhibitors[11,18].

The antiangiogenic potency of staurosporine with an ID_{50} value of 71 pmol/egg was stronger than those of herbimycin A with an ID_{50} value of 260 pmol/egg, 1α,25-dihydroxyvitamin D_3 with an ID_{50} value of 340 pmol/egg and retinoic acid with an ID_{50} value of 330 pmol/egg[10,11,18]. Staurosporine appears to exhibit similar antiangiogenic activity as 22-oxa-1α,25$(OH)_2D_3$ with an ID_{50} value of 96 pmol/egg[18] and has somewhat less an angiogenesis-inhibitory effect than the most potent angiogenesis inhibitor, a synthetic retinoid Ch 55 with an ID_{50} value of 24 pmol/egg[11].

Proliferation of vascular endothelial cells is thought to be prerequisite for angiogenesis. Indeed, recent studies have shown that several angiogenesis inhibitors, such as platelet factor-4, a cartilage-derived collagenase inhibitor and fumagillin analogs, exhibit a growth-inhibitory effect on vascular

endothelial cells *in vitro*[2,4]. Our *in vitro* experiments revealed that staurosporine inhibits vascular endothelial cell proliferation with an ID_{50} value of 0.9 nM. DAVIET *et al.* also found that staurosporine affected the proliferation of vascular endothelial cells[7]. The ID_{50} value was 1.3 nM, which was comparable to the ID_{50} value obtained in our study. These findings indicate that staurosporine expresses antiangiogenic activity by inhibiting certain protein kinase(s) associated with the proliferation of angiogenic endothelial cells.

In this study staurosporine did not inhibit the migration of vascular endothelial cells. On the other hand ROSEN *et al.* reported that staurosporine inhibited vascular endothelial cell migration[19]. This discrepancy might due to the difference in stimulators used for inducing cell migration; serum in our study and scatter factor (or TPA) in their experiment as a stimulator was used, respectively. Alternatively, it might result from the difference in the source of vascular endothelial cells used; our cells were derived from carotid and their cells from brain.

On the basis of the previous findings that staurosporine exhibits different biological and biochemical activities in various *in vitro* and *in vivo* systems[20~27] and the present observation that it is also able to inhibit strongly angiogenesis in an *in vivo* system, we should discuss the specificity of staurosporine as an angiogenesis inhibitor. Taking all the findings together, it seems that staurosporine is a potent angiogenesis inhibitor although it dose not selectively affect angiogenesis. When estimated on an IC_{50} value available as to cell proliferation inhibition of staurosporine, although our vascular endothelial cells (IC_{50}=0.9 nM) is more sensitive to the antibiotic than other cell types[22,28,29], including Walker carcinoma cells (IC_{50}=40 nM), T-24 human bladder carcinoma cells (IC_{50}=29 nM), HL-60 promyelocytic leukemia cells (IC_{50}=130 nM), bovine corneal endothelial cells (IC_{50}=22 nM) and mouse megakaryoblastic cells (IC_{50}=130 nM), their values for IC_{50} are all within a nM range of concentration and roughly similar each other. However, we should emphasize that the present observation is interesting that both the inhibition of endothelial cell proliferation and blood vessel formation in the CAM caused by staurosporine apparently occur at similar concentrations, although not conclusive, because local staurosporine concentration in CAM could not be determined at present.

In summary, staurosporine, a potent inhibitor of protein kinases, has the ability to inhibit within a picomolar range of dose. This indicates possible role of certain protein kinase(s) during angiogenesis. In addition, the present study indicate that staurosporine is useful in elucidating a molecular mechanism of angiogenesis. Further study is in progress to determine which protein kinase(s) is a key role in angiogenesis induction.

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References

1) FOLKMAN, J. & M. KLAGSBRUN: Angiogenic factors. Science 235: 442~447, 1987
2) MOSES, M. A. & R. LANGER: Inhibitors of angiogenesis. Biotechnology 9: 630~634, 1991
3) 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
4) KLAGSBRUN, M. & P. A. D'AMORE: Regulators of angiogenesis. Ann. Rev. Physiol. 53: 217~239, 1991
5) MORRIS, P. B.; T. HIDA, P. J. BLACKSHEAR, G. K. KLINTWORTH, & J. L. SWAIN: Tumor-promoting phorbol esters induce angiogenesis *in vivo*. Am. J. Physiol. 254 (Cell Physiol. 23): C318~C322, 1988
6) NISHIZUKA, Y.: The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308: 693~698, 1984
7) DAVIET, I.; J. M. HERBERT & J. P. MAFFRAND: Tumor-promoting phorbol esters stimulate bovine cerebral cortex capillary endothelial cell growth *in vitro*. Biochem. Biophys. Res. Commun. 158: 584~589, 1989
8) 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
9) NAKANO, H.; E. KOBAYASHI, I. TAKAHASHI, T. TAMAOKI, Y. KUZUU & H. IBA: Staurosporine inhibits

tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiotics 40: 706～708, 1987

10) 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
11) 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
12) 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
13) OIKAWA, T.; K. HIROTANI, K. YAMAOKA, T. KATAYAMA, T. IWAGUCHI, H. ITO, I. MORITA, S. MUROTA, T. TOMINAGA & A. HIRAGUN: Angiogenic factor of a rat mammary tumor cell line (RMT-1) (I). Secretion of two distinct angiogenic factors into serum-free conditioned medium by RMT-1 cells. Cancer Lett. 59: 57～66, 1991
14) FERRIOLA, P.; V. CODY & E. MIDDLETON, Jr.: Protein kinase C inhibition by plant flavonoids: Kinetic mechanisms and structure-activity relationships. Biochem. Pharmacol. 38: 1617～1624, 1989
15) AKIYAMA, T.; J. ISHIDA, S. NAKAGAWA, H. OGAWARA, S. WATANABE, N. ITOH, M. SHIBUYA & Y. FUKAMI: Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262: 5592～5595, 1987
16) MONTESANA, R. & L. ORCI: Phorbol esters induce angiogenesis *in vitro* from large-vessel endothelial cells. J. Cell. Physiol. 130: 284～291, 1987
17) OKAZAKI, T.; Y. KATA, T. MOCHIZUKI, M. TASHIMA, H. SAWADA & H. UCHINO: Staurosporine, a novel protein kinase inhibitor, enhances HL-60-cell differentiation induced by various compounds. Exp. Hematol. 16: 42～48, 1988
18) 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
19) ROSEN, E. M.; S. JAKEN, W. CARLEY, P. M. LUCKETT, E. SETTER, M. BHARGAVA & I. D. GOLDBERG: Regulation of motility in bovine brain endothelial cells. J. Cell. Physiol. 146: 325～335, 1991
20) YAMADA, S.; K. HIROTA, K. CHIDA & T. KUROKI: Inhibition of phorbol ester-caused induction of ornithine decarboxylase and tumor promotion in mouse skin by staurosporine, a potent inhibitor of protein kinase C. Biochem. Biophys. Res. Commun. 157: 9～15, 1988
21) YAMAMOTO, S.; I. KIYOTO, E. AIZU, T. NAKADATE, Y. HOSODA & R. KATO: Differential inhibition by staurosporine, a potent protein kinase C inhibitor, of 12-*O*-tetradecanoylphorbol-13-acetate-caused skin tumor promotion, epidermal ornithine decarboxylase induction, hyperplasia and inflammation. Carcinogenesis 10: 1315～1322, 1989
22) GRUNICKE, H.; J. HOFMANN, K. MALY, F. UBERALL, L. POSCH, H. OBERHUBER & H. FIEBIG: The phospholipid- and calcium-dependent protein kinase as a target in tumor chemotherapy. Adv. Enzyme Regul. 28: 201～216, 1989
23) YOSHIZAWA, S.; H. FUJIKI, H. SUGURI, M. SUGANUMA, M. NAKAYASU, R. MATSUSHIMA & T. SUGIMURA: Tumor-promoting activity of staurosporine, a protein kinase inhibitor on mouse skin. Cancer Res. 50: 4974～4978, 1990
24) NABESHIMA, T.; S. OGAWA, H. NISHIMURA, K. FUJI, T. KAMEYAMA & Y. SASAKI: Staurosporine facilitates recovery from the basal forebrain-lesion-induced impairment of learning and deficit of cholinergic neuron in rats. J. Pharmacol. Exp. Ther. 257: 562～566, 1991
25) ABE, K.; M. YOSHIDA, T. USUI, S. HORINOUCHI & T. BEPPU: Highly synchronous culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp. Cell Res. 192: 122～127, 1991
26) KULYK, W. M.: Promotion of embryonic limb cartilage differentiation *in vitro* by staurosporine, a protein kinase C inhibitor. Dev. Biol. 146: 38～48, 1991
27) 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
28) MEYER, T.; U. REGENASS, D. FABBRO, E. ALTERI, J. ROSEL, M. MULLER, G. Caravatti & A. Matter: A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. Int. J. Cancer 43: 851～856, 1989
29) HONMA, Y.; J. OKABE-KADO, T. KASUKABE, M. HOZUMI, S. KAJIGAYA, T. SUDA & Y. MIURA: Induction by some protein kinase inhibitors of differentiation of a mouse megakaryoblastic cell line established by coinfection with abelson murine leukemia virus and recombinant SV40 retrovirus. Cancer Res. 51: 4649～4655, 1991