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Okadaic Acid Is a Potent Angiogenesis Inducer

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Okadaic acid, which is a non-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoter and an inhibitor of protein phosphatases 1 and 2A, induced angiogenesis in the chorioallantoic membrane of the chick embryo. Its potent angiogenic activity was dose-dependent. The minimum effective dose was 5 fmol/egg and the effective dose for 50% induction was 90 fmol/egg. These results indicated that okadaic acid exhibits angiogenic activity one order of magnitude stronger than that of TPA (reported previously). Moreover, the time-course of angiogenesis induction by okadaic acid was much slower than that by TPA. The difference is consistent with the time-courses of other biochemical and biological activities and also various gene expressions induced by okadaic acid and TPA, indicating that the difference in the time-course is associated with their mechanisms of action. We conclude that okadaic acid induces angiogenesis through a different pathway than does TPA, indicating the existence of a new mechanism of angiogenesis induction.

Key words: Okadaic acid — TPA — Angiogenesis — Tumor promotion

Angiogenesis seems to play a critical role in the progression of solid tumors,[1] based on the evidence that various angiogenesis inhibitors, such as angiostatic steroids, microbial products and sulfated chitin derivatives, suppress the growth of primary tumor or lung tumor metastasis.[2–6] Most tumors are able to induce angiogenesis,[1,7] and various substances show angiogenic activity.[8] Morris *et al.* reported that 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces angiogenesis.[9] Recently Fujiki's group in Tokyo reported that okadaic acid is a non-TPA type tumor promoter on mouse skin.[10] Okadaic acid is a polyether compound of a C_{38} fatty acid, isolated from the black sponge, *Halichondria okadai.*[11] Okadaic acid is as strong a tumor promoter as TPA, and acts differently on the cells than TPA.[12] Namely, okadaic acid specifically inhibits the activities of protein phosphatases 1 and 2A, resulting in an increase of phosphoproteins in the cell,[13,14] whereas TPA is a potent activator of protein kinase C.[15] Thus, we studied induction of angiogenesis by okadaic acid, and compared it with that by TPA.

Angiogenic activity was determined by the chorioallantoic membrane (CAM) assay as described previously.[16] Methylcellulose (4000 cps; Tokyo Kasei Kogyo Co. Ltd., Tokyo) disks were prepared by air-drying 1% methylcellulose solution containing a test sample and 2.5% ethanol on Teflon rods according to the method of Crum *et al.*[17] Okadaic acid was isolated from a black sponge, *Halichondria okadai,* as described previously.[11] TPA was purchased from LC Services Corp., Woburn, MA. Fertilized chick eggs (Ohmiya Kakin Lab., Ohmiya) were incubated in a humidified egg incubator at 37°C. After a 3-day incubation, a 1-cm square window on the egg shell was made and eggs were incubated for an additional 7 days. A methylcellulose disk impregnated with a test sample was carefully implanted on the surface of the 10-day-old CAM. Angiogenic response on the treated CAM was evaluated under an Olympus stereoscope at the indicated time, being graded as negative or positive on the basis of infiltration of blood vessels into the area of the implanted methylcellulose disk, as described previously.[16]

Figure 1 shows angiogenic activity on the third day after implantation of a methylcellulose disk impregnated with okadaic acid at a dose of 50 pmol/egg and TPA at a dose of 200 pmol/egg. Figure 2 shows the dose-response curve of angiogenic activity by okadaic acid, which was assessed 3 days after implantation. Okadaic acid at a dose of 5 fmol/egg significantly induced angiogenesis and its effective dose for 50% induction was 90 fmol/egg. Five pmol of okadaic acid/egg induced angiogenesis in all treated CAMs. As reported previously, TPA at a concentration of 8 μM, which corresponds to 80 pmol/egg, induces angiogenesis in 93% of CAMs.[9] Therefore, we think that okadaic acid is at least one order of magnitude more potent than TPA in induction of angiogenesis. Angiogenin, which is a peptide isolated from a human colon adenocarcinoma cell line, is a strong angiogenesis inducer. Angiogenin at a dose of 35 fmol/egg starts to induce angiogenesis, as reported previously.[18] From these data, okadaic acid is thought to

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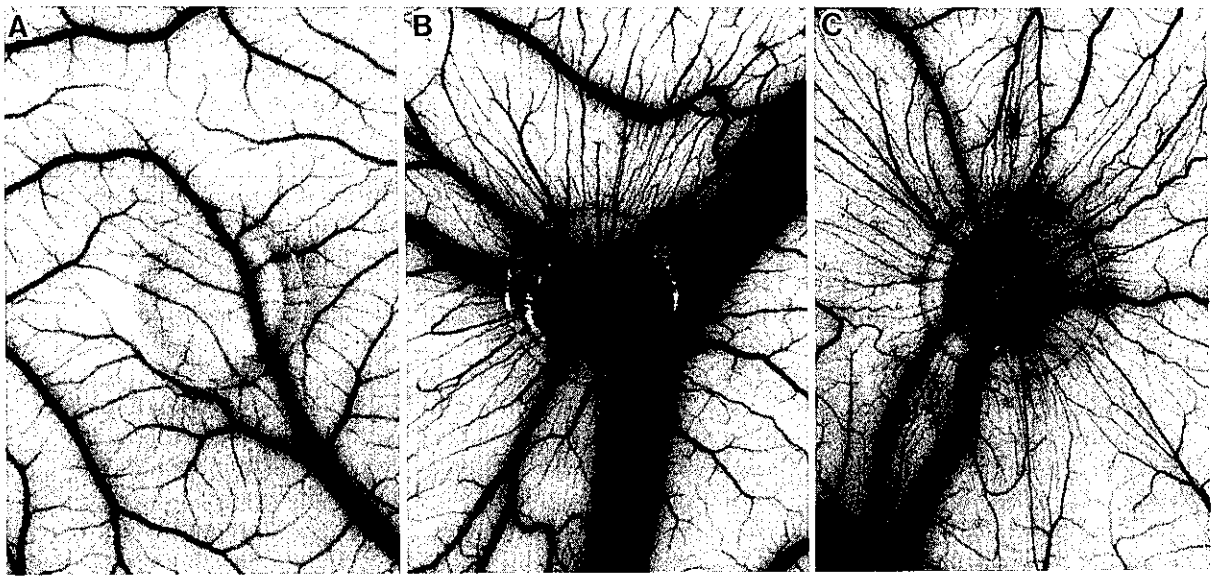

Fig. 1. Angiogenic activity on the third day after implantation of a methylcellulose disk impregnated with the vehicle (A, control), okadaic acid (B, 50 pmol/egg) or TPA (C, 200 pmol/egg) into CAM of chick embryo. The 10-day-old CAM was treated with the vehicle, okadaic acid or TPA for 3 days. ×8.

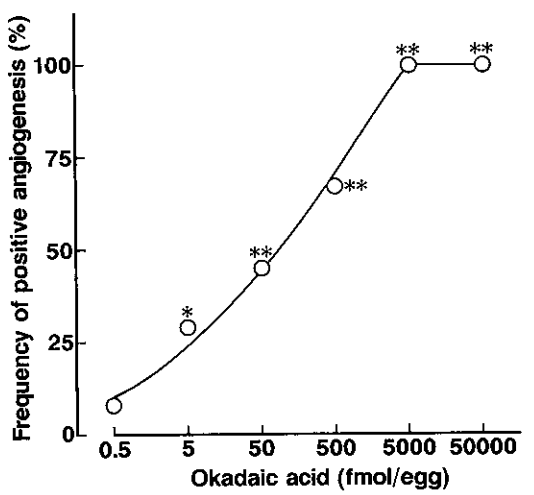

Fig. 2.. Dose-response curve of angiogenesis induction by okadaic acid. Angiogenic activity was assessed 3 days after the implantation and 12–21 eggs were employed for each dose. * $P<0.02$ compared to the control, whose positive angiogenic activity value was 4% (2/51); ** $P<0.001$ compared to the control (Fisher's exact probability test).

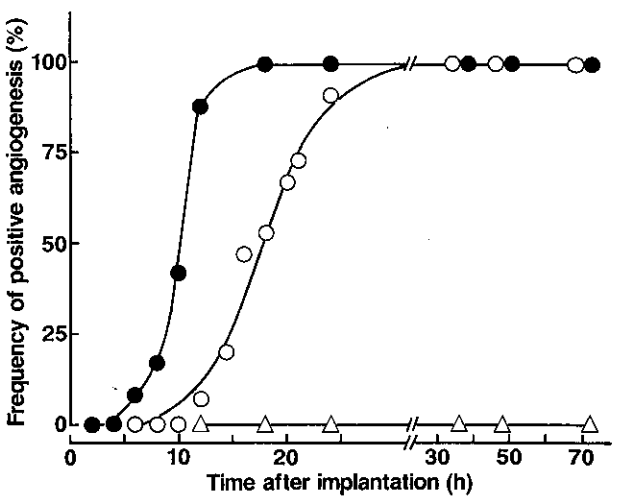

Fig. 3. Time-course of induction of angiogenesis by okadaic acid (○) and TPA (●). The numbers of eggs used were 15, 15 and 12 for the vehicle (△; control), okadaic acid (50 pmol/egg) and TPA (200 pmol/egg), respectively.

be one of the most potent angiogenesis inducers known at present.

Figure 3 shows the time-courses of angiogenesis induction by okadaic acid at a dose of 50 pmol/egg and by TPA at a dose of 200 pmol/egg. The respective doses of okadaic acid and TPA were thought to be functionally comparable to each other with respect to their abilities to induce angiogenesis, based on the results for okadaic acid shown in Fig. 2 and the previous results for TPA mentioned above.[9] It was remarkable that okadaic acid

induces angiogenesis much more slowly than TPA, namely, angiogenic activity by okadaic acid was not detected until 12 h after implantation and then reached a maximum at 24 h after treatment. In contrast, TPA induced angiogenesis much more rapidly; most of the treated CAMs showed a positive angiogenic response at 12 h after treatment. There was an 8-h difference in treatment times with okadaic acid and TPA to achieve the 50% induction.

It has recently been reported that okadaic acid shows a delayed time-course in induction of other biological and biochemical effects and also gene expressions compared with TPA, although both okadaic acid and TPA induce various common responses. Okadaic acid, unlike TPA, stimulated prostaglandin E_2 production with a 6-h lag phase in rat peritoneal macrophages.[19] Expressions of c-*fos*, transin and urokinase genes induced by okadaic acid in mouse keratinocytes were delayed about 3, 14 and 6 h, respectively, when compared to the induction by TPA.[20] In addition, okadaic acid-induced NF-κB in Jurkat cells was delayed about 5 h compared to the induction by TPA.[21] We also know now that these gene expressions are activated by various enhancer elements such as TPA responsive element (TRE) and serum responsive element (SRE).[22,23] In particular, the okadaic acid responsive element (ORE), which was first found at -137 in the human collagenase promoter, is also involved in gene expression of okadaic acid.[23] We assume that angiogenesis induction by okadaic acid is mediated through a signal transduction pathway involving ORE as well as TRE and SRE. Alternatively, it might be possible that some proteases, such as urokinase and collagenase, are involved in angiogenesis induction by okadaic acid, because the expressions of these two protease activities were induced by okadaic acid or TPA,[20,22-24] and their inhibitors affected angiogenesis induction.[25,26]

Based on the evidence that two potent tumor promoters commonly induce angiogenesis, we should discuss how angiogenesis is related to tumor promotion. It was reported that retinoids and vitamin D_3 analogues, which are potent inhibitors of tumor promotion of TPA on mouse skin, also inhibit angiogenesis induction in CAMs.[27-30] Moreover, we have recently demonstrated that an angiogenesis inhibitor, medroxyprogesterone acetate (MPA), inhibits tumor promotion by okadaic acid on mouse skin. Cotreatment with MPA and sarcophytol A, another inhibitor of carcinogenesis, resulted in enhanced inhibitory effects on tumor promotion.[31] These results strongly indicated that the angiogenic response by a tumor promoter is involved in one stage of tumor development, that is, tumor promotion. Previous studies also showed that there was an intimate relationship between angiogenesis and neoplastic progression,[32] and that induction of angiogenesis occurred during the transition from hyperplasia to neoplasia, indicating that angiogenesis induction is one of the important events in carcinogenesis.[33] Further study is required to elucidate the role of angiogenesis inhibitors in prevention of tumor growth.

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REFERENCES

1) Folkman, J. Tumor angiogenesis. *Adv. Cancer Res.*, **43**, 175–203 (1985).
2) Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C. and Taylor, S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science*, **221**, 719–725 (1983).
3) Sakamoto, N. and Tanaka, N. G. Effect of angiostatic steroid with or without glucocorticoid activity on metastasis. *Invasion Metastasis*, **7**, 208–216 (1987).
4) Oikawa, T., Hiragun, A., Yoshida, Y., Ashino-Fuse, H., Tominaga, T. and Iwaguchi, T. Angiogenic activity of rat mammary carcinomas 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).
5) Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H. and Folkman, J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. *Nature*, **348**, 555–557 (1990).
6) Murata, J., Saiki, I., Makabe, T., Tsuta, Y., Tokura, S. and Azuma, I. Inhibition of tumor-induced angiogenesis by sulfated chitin derivatives. *Cancer Res.*, **51**, 22–26 (1991).
7) Gullino, P. M. Angiogenic factor(s). *Handb. Exp. Pharmacol.*, **57**, 427–449 (1981).
8) Blood, C. H. and Zetter, B. R. Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim. Biophys. Acta*, **1032**, 89–118 (1990).
9) Morris, P. B., Hida, T., Blackshear, P. J., Klintworth, G. K. and Swain, J. L. Tumor-promoting phorbol esters induce angiogenesis *in vivo*. *Am. J. Physiol.*, **254**, C318–C322 (1988).
10) Suganuma, M., Fujiki, H., Suguri, H., Yoshizawa, S., Hirota, M., Nakayasu, M., Ojika, M., Wakamatsu, K.,

Yamada, K. and Sugimura, T. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc. Natl. Acad. Sci. USA,* **85,** 1768–1771 (1988).

11) Tachibana, K., Schener, P. J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y. and Schmitz, F. J. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria. J. Am. Chem. Soc.,* **103,** 2469–2471 (1981).
12) Fujiki, H., Suganuma, M., Nishiwaki, S., Yoshizawa, S., Yatsunami, J., Matsushima, R., Furuya, H., Okabe, S., Matsunaga, S. and Sugimura, T. Specific mechanistic aspects of animal tumor promoters: The okadaic acid pathway. *In* "Relevance of Animal Studies to Evaluate Human Cancer Risk," ed. R. D'Amato, T. J. Slaga, W. Farland and C. Henry (1991). John Wiley & Sons, Inc., New York, in press.
13) Sassa, T., Richter, W. W., Uda, N., Suganuma, M., Suguri, H., Yoshizawa, S., Hirota, M. and Fujiki, H. Apparent "activation" of protein kinases by okadaic acid class tumor promoters. *Biochem. Biophys. Res. Commun.,* **159,** 939–944 (1989).
14) Yatsunami, J., Fujiki, H., Suganuma, M., Yoshizawa, S., Erikson, J. E., Olson, M. O. and Goldman, R. D. Vimentin is hyperphosphorylated in primary human fibroblasts treated with okadaic acid. *Biochem. Biophys. Res. Commun.,* **177,** 1165–1170 (1991).
15) Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature,* **308,** 693–698 (1984).
16) Oikawa, T., Hirotani, K., Yamaoka, K., Katayama, T., Iwaguchi, T., Ito, H., Morita, I., Murota, S. and Hiragun, A. 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).
17) Crum, R., Szabo, S. and Folkman, J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science,* **230,** 1375–1378 (1985).
18) Fett, J. W., Strydom, D. J., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F. and Vallee, B. L. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry,* **24,** 5480–5486 (1985).
19) Ohuchi, K., Tamura, T., Ohashi, M., Watanabe, M., Hirasawa, N., Tsurufuji, S. and Fujiki, H. Okadaic acid and dinophysistoxin-1, non-TPA-type tumor promoters, stimulate prostaglandin E_2 production in rat peritoneal macrophages. *Biochim. Biophys. Acta,* **1013,** 86–91 (1989).
20) Hollady, K., Fujiki, H. and Bowden, T. Okadaic acid induced expression of both early and secondary response genes in mouse keratinocytes. *Mol. Carcinog.* (1991), in press.
21) Thévenin, C., Kim, S. J., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., Fauci, A. S. and Kehrl, J. H. Induction of nuclear factor-κB and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific inhibitor of phosphatases 1 and 2A. *New Biologist,* **2,** 793–800 (1990).
22) Kim, S. J., Lafyatis, R., Kim, K. Y., Angel, P., Fujiki, H., Karin, M., Sporn, M. B. and Roberts, A. B. Regulation of collagenase gene expression by okadaic acid, an inhibitor of protein phosphatases. *Cell Regul.,* **1,** 269–278 (1990).
23) Levy, J. P., Fujiki, H., Angel, P. and Bowden, G. T. An inactive mutant TPA responsive element acts as an active okadaic acid response element in mouse keratinocytes. *Proc. Am. Assoc. Cancer Res.,* **32,** 291 (1991).
24) Whitham, S. E., Murphy, G., Angel, P., Rahmsdorf, H. J., Smith, B. J., Lyons, A., Harris, T. J. R., Reynolds, J. J., Herrlich, P. and Docherty, A. J. P. Comparison of human stromelysin and collagenase by cloning and sequence analysis. *Biochem. J.,* **240,** 913–916 (1986).
25) Ashino-Fuse, H., Takano, Y., Oikawa, T., Shimamura, M. and Iwaguchi, T. Medroxyprogesterone acetate, an anticancer and anti-angiogenic steroid, inhibits the plasminogen activator in bovine endothelial cells. *Int. J. Cancer,* **44,** 859–864 (1989).
26) Tamargo, R. J., Bok, R. A. and Brem, H. Angiogenesis inhibition by minocycline. *Cancer Res.,* **51,** 672–675 (1991).
27) Verma, A. K., Shapas, B. G., Rice, H. M. and Boutwell, R. K. Correlation of the inhibition by retinoids of tumor promoter-induced mouse epidermal ornithine decarboxylase activity and of skin tumor promotion. *Cancer Res.,* **39,** 419–425 (1979).
28) Chida, K., Hashiba, H., Fukushima, M., Suda, T. and Kuroki, T. Inhibition of tumor promotion in mouse skin by 1α,25-dihydroxyvitamin D_3. *Cancer Res.,* **45,** 5426–5430 (1985).
29) Oikawa, T., Hirotani, K., Nakamura, O., Shudo, K., Hiragun, A. and Iwaguchi, T. A highly potent anti-angiogenic activity of retinoids. *Cancer Lett.,* **48,** 157–162 (1989).
30) Oikawa, T., Hirotani, K., Ogasawara, H., Katayama, T., Nakamura, O., Iwaguchi, T. and Hiragun, A. Inhibition of angiogenesis by vitamin D_3 analogues. *Eur. J. Pharmacol.,* **178,** 247–250 (1990).
31) Suganuma, M., Yoshizawa, S., Yatsunami, J., Nishiwaki, S., Furuya, H., Okabe, S., Nishiwaki-Matsushima, R., Frenkel, K., Troll, W., Verma, A. K. and Fujiki, H. Mechanisms of action of new antitumor promoters. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms III," ed. G. Bronzetti, S. De Flora, S. M. Shankel and M. D. Waters (1991). Plenum Press, New York, in press.
32) Ziche, M. and Gullino, P. M. Angiogenesis and neoplastic progression *in vitro. J. Natl. Cancer Inst.,* **69,** 483–487 (1982).
33) Folkman, J., Watson, K., Ingber, D. and Hanahan, D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature,* **339,** 58–61 (1989).