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Evaluation of Angiogenic Inhibitors with an *in vivo* Quantitative Angiogenesis Method Using Agarose Microencapsulation and Mouse Hemoglobin Enzyme-linked Immunosorbent Assay

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In the present work, using a previously reported *in vivo* quantitative tumor-angiogenesis model, we attempted to ascertain whether this animal model is suitable for practical use in monitoring inhibitors of tumor angiogenesis. Mouse sarcoma-180 cells, human A431 cells or rat C6 cells microencapsulated in agarose beads were implanted s.c. into C57BL/6 mice. The level of blood vessel induction at the agarose pellet site was evaluated using mouse hemoglobin enzyme-linked immunosorbent assay on day 10 after implantation. Hydrocortisone, tetrahydro-S, medroxyprogesterone acetate, pentosan polysulfate and suramin inhibited blood vessel growth in our *in vivo* tumor-angiogenesis assay system, and heparin enhanced the antiangiogenic effects of hydrocortisone and tetrahydro-S. These results are almost entirely consistent with those observed in common assay systems, and suggest that this method may be useful for the identification and quantitative evaluation of inhibitors of tumor angiogenesis.

Key words: Tumor angiogenesis — Angiogenic inhibitor — Agarose-microencapsulation — Mouse hemoglobin ELISA

Angiogenesis, the process of generation of new capillary blood vessels, is associated with the primary initiation and/or progression of various pathological conditions, such as tumor growth and metastasis, diabetic retinopathy, arteriosclerosis and rheumatoid arthritis.[1–3] In contrast, angiogenesis is not usually active in the normal adult except during wound repair, ovulation, menstruation, and the formation of the placenta.[4, 5] Therefore, the identification of reliable and effective angiogenesis inhibitors has potential as a curative approach to angiogenic diseases without side effects.

In 1983, Folkman *et al.* reported that the combination of cortisone and heparin prevented angiogenesis and caused tumor regression.[6] On the basis of this finding, an extensive search was carried out for combinations of various steroids and heparin with antiangiogenic effect. These studies led to the identification of a new biological activity of steroids, which is distinguished from glucocorticoid and mineralocorticoid activity, termed the angiostatic activity.[2, 7, 8] Thereafter, the understanding of antiangiogenic substances has expanded rapidly, with the development of an *in vitro* angiogenesis assay system using cultured cells. However, little progress has been made in the identification of novel antiangiogenic drugs, because a reproducible and quantitative *in vivo* angiogenesis assay system has not yet been established.

A reliable *in vivo* antiangiogenic activity screening system is necessary for the comprehensive evaluation of angiogenic inhibition. We have developed a new *in vivo* system of monitoring blood vessel induction by agarose gel-microencapsulated tumor cells with mouse hemoglobin enzyme-linked immunosorbent assay (mHb-ELISA).[9, 10] This *in vivo* angiogenesis model has enabled us to assess the tumor angiogenesis quantitatively, rapidly and easily. Additionally, we found that agarose hydrogel-microencapsulated tumor cells were isolated from the host's immune system but could acquire sufficient nutrition to support cell proliferation, and our established system enabled us to evaluate the angiogenic potential of various tumor types across histocompatibility and species barriers.[9] We confirmed that the newly formed capillaries in our *in vivo* angiogenesis assay system have the specific characteristics of the tumor vessel.[10]

The purpose of the present study was to determine whether our *in vivo* angiogenesis model could be applied to identify potential inhibitors of tumor-induced blood vessel growth. We tested the inhibitory effects on angiogenesis induced in our *in vivo* system of five known angiogenic inhibitors (hydrocortisone,[6, 7, 11–13] tetrahydro-S,[6, 7, 12, 13] medroxyprogesterone,[13–18] pentosan polysulfate[19–21] and suramin[8, 13, 22, 23]).

MATERIALS AND METHODS

Animals Female ddY mice (5 weeks old) and male C57BL/6 mice (6 weeks old) were purchased from Shimizu Experimental Animal Co., Ltd., Kyoto.

Tumor cells A431, a human epidermoid carcinoma cell line, was purchased from RIKEN Cell Bank (Tsukuba).

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A431 was cultured on porous microcarriers (Asahi Kasei, Tokyo) in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS, Filtron Pty. Ltd., Brooklyn, Australia). Rat C6 glioma cells were also cultured on porous microcarriers in DMEM supplemented with 10% FCS. The culture of tumor cells on microcarriers was carried out as previously described.[9] Sarcoma-180 (S-180) cells were maintained intraperitoneally by serial passage in ddY mice.

Microencapsulation of tumor cells or microcarrier-attached tumor cells in agarose hydrogel S-180 cells, microcarrier-attached A431 cells (A431-MC) or microcarrier-attached C6 cells (C6-MC) were microencapsulated in agarose microbeads following the method originally developed by Nilsson *et al.*[24, 25] with our slight modification.[9, 10] Briefly, a 4% (wt%) AGAROSE-LGT (Nacalai Tesque Inc., Kyoto) solution (1 ml) in Eagle's minimum essential medium (MEM, Nissui Seiyaku Co., Ltd.) was autoclaved in a 50 ml glass centrifuge tube, then cooled and kept at 37°C in a water bath. S-180 cells (1 ml of 2.5×10^7 cells/ml) or A431-MC or C6-MC (1 ml, containing 0.5 ml of A431-MC or C6-MC) in DMEM were incubated at 37°C and mixed with the above agarose solution. Then liquid paraffin (20 ml), which had been autoclaved and cooled to 37°C, was poured into the centrifuge tube. The liquids were emulsified with manual shaking to obtain the desired bead size. The centrifuge tube was immersed in an ice bath for 10 min to gel the agarose solution. The agarose beads were washed with Hanks' balanced salt solution 3 times to remove the liquid paraffin.

Implantation of agarose microbeads in mice and measurement of angiogenesis with mHb-ELISA A 300 μl portion of agarose microbeads with S-180 cells, A431-MC or C6-MC, or cell-free agarose beads was implanted subcutaneously near the dorsal midline of 6-week-old male C57BL/6 mice. On day 10, the agarose bead pellets were resected and homogenized, and the mHb level was evaluated with mHb-ELISA as a measure of angiogenesis induced on the pellet (Fig. 1).[9] The protein level of the homogenized solution was also measured by use of a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The percentage inhibition of angiogenesis was determined by comparing the mHb (μg)/protein (mg) ratio of drug treatment groups to that of the untreated group (control).

Administration of angiogenic inhibitors Hydrocortisone 21-phosphate (HC, Sigma Chemical Co., St. Louis, MO) was administered orally from day 1 at tapering doses (250, 100 or 50 mg/kg/day for 3 days, followed by 100, 50 or 25 mg/kg/day for 3 days, and then 50, 25 or 12.5 mg/kg/day for 3 days) with or without administration of 30 mg/kg/day of heparin sodium (Daiichi Pure Chemicals Co., Ltd., Tokyo), p.o. Similarly, tetrahydro S (THS, 5β-pregnane-3α, 17α, 21-triol-20-one, Sigma Chemical Co.) was administered orally from day 1 at tapering doses (100 mg/kg/day, 50 mg/kg/day, and 25 mg/kg/day for 3 days each) with or without administration of 30 mg/kg/day of heparin sodium, p.o. Medroxyprogesterone acetate (MPA, Sigma Chemical Co.) was administered i.m. at a constant dose (4.8, 2.4 or 1.2 mg/kg/day for 9 days) from day 1. Suramin (donated by Dr. Noriko Tanaka, Daiichi Pharmaceutical Co., Ltd., Tokyo) was

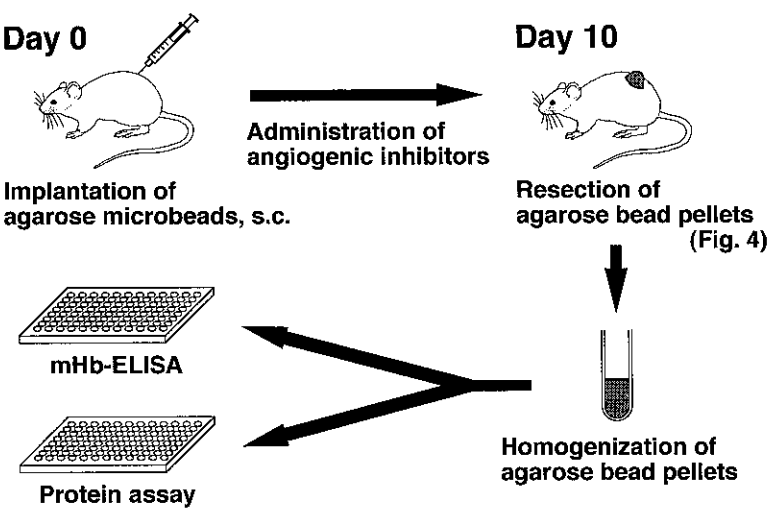

Fig. 1. Protocol outlining the analysis of *in vivo* tumor angiogenesis.

administered i.p. also at a constant dose (200, 100 or 50 mg/kg/day) for 4 days from day 4. Pentosan polysulfate (PPS, Sigma Chemical Co.) was administered i.p. at either 25 or 12.5 mg/kg/day for 9 days from day 1.

Statistical methods The significance of differences in the percentage inhibition of angiogenesis was evaluated by using Student's *t* test.

RESULTS

Inhibitory effects of angiogenic inhibitors on angiogenesis induced by S-180 cells entrapped in agarose beads Quantitative analysis of the hemoglobin concentration at the site implanted with S-180 cells entrapped in agarose beads revealed that HC inhibited 65% of angiogenesis at the lower dose and 80% at the higher dose. Moreover, the combination of HC and heparin markedly diminished the angiogenesis at the site implanted with agarose beads containing S-180 cells to only 5% of that in the untreated control. THS alone reduced angiogenesis to 10% of the untreated control, and the inhibitory effect was almost total with coadministration of heparin (Fig. 2).

Both MPA and suramin dose-dependently inhibited angiogenesis, the maximum response of $<70\%$ inhibition being obtained at dose levels of 4.8 mg/kg/day for MPA and 200 mg/kg/day for suramin. Although dose dependency was not observed in the group administered PPS, PPS showed a 50–60% inhibitory effect in our assay system (Fig. 3).

Inhibitory effects of angiogenic inhibitors on angiogenesis induced by A431 cells entrapped in agarose beads HC alone, THS alone or the combination of HC and heparin had only a slight inhibitory effect (about 30%) on blood vessel growth in the experiment using A431 cells entrapped in agarose beads. However, when THS was administered with heparin, formation of new blood vessels was inhibited by 80% (Figs. 4 and 5).

Inhibitory effects of angiogenic inhibitors on angiogenesis induced by C6 cells entrapped in agarose beads HC alone caused a 75% reduction in hemoglobin level at the site implanted with C6 cells entrapped in agarose beads, and even greater inhibition (85%) of angiogenesis occurred when HC was combined with heparin. PPS and suramin demonstrated 75% and 45% antiangiogenic activity, respectively (Fig. 6).

DISCUSSION

In the present study, we attempted to determine whether our *in vivo* angiogenesis model could be applied to identify potential inhibitors of tumor-induced angiogenesis. We accordingly tested the inhibitory effects of five known angiogenic inhibitors (HC, THS, MPA, PPS and suramin) on the angiogenesis induced in our *in vivo* system.

HC dose-dependently inhibited the angiogenesis induced by S-180 cells or A431 cells entrapped in agarose beads, and THS also inhibited the angiogenesis induced

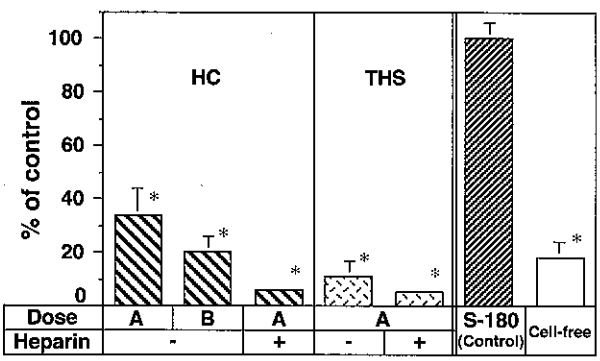

Fig. 2. Inhibitory effects of angiostatic steroids with or without heparin on the angiogenesis induced by S-180 cells entrapped in agarose beads. C57BL/6 mice were implanted with S-180 cells entrapped in agarose beads at day 0, and were treated with HC or THS for 9 days. Tapering doses of steroids were administered p.o. from day 1: (A) 100 mg/kg/day for 3 days, followed by 50 mg/kg/day for 3 days and 25 mg/kg/day for 3 days with (+) or without (−) administration of 30 mg/kg/day of heparin sodium, p.o.; and (B) 250 mg/kg/day for 3 days, followed by 100 mg/kg/day for 3 days and 50 mg/kg/day for 3 days. The mHb levels at the area implanted with agarose beads were determined by mHb-ELISA on day 10. Each value represents the mean±SEM for 3–5 mice. * $P<0.001$ significantly different from the control.

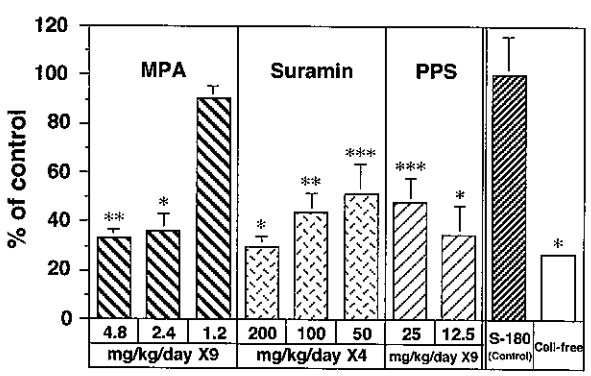

Fig. 3. Inhibitory effects of MPA, suramin and PPS on the angiogenesis induced by S-180 cells entrapped in agarose beads. C57BL/6 mice were implanted with S-180 cells entrapped in agarose beads at day 0, and were treated with MPA or PPS for 9 days. Treatment with suramin was given from day 4 to day 7. The mHb levels at the area implanted with agarose beads were determined by mHb-ELISA on day 10. Each value represents the mean±SEM for 4–9 mice. * $P<0.005$, ** $P<0.01$, *** $P<0.05$ compared to control.

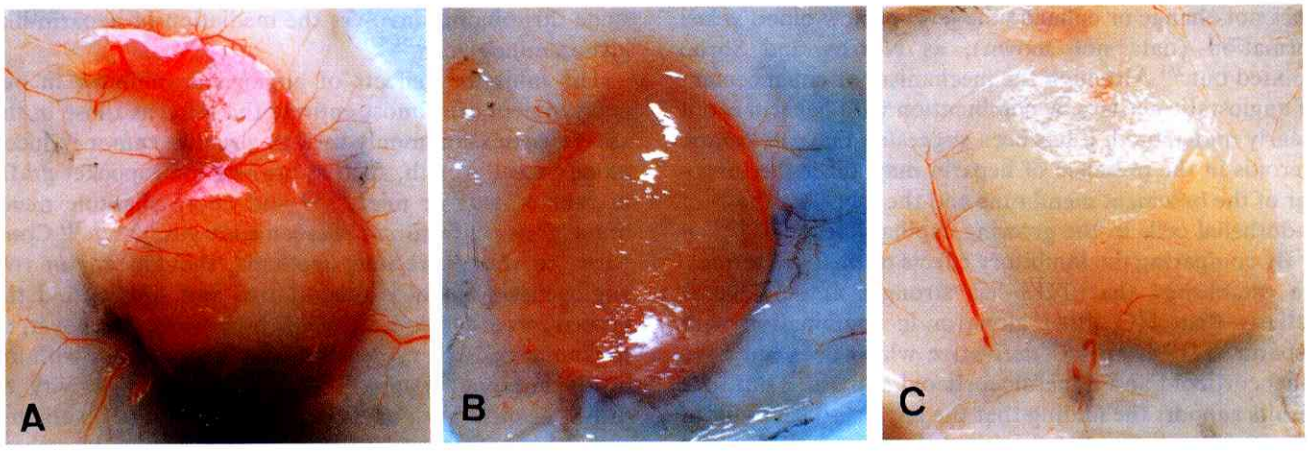

Fig. 4. Inhibitory effect of THS with heparin on the angiogenesis induced by A431 cells entrapped in agarose microbeads in C57BL/6 mice. Agarose microbeads with (A) or without (C) A431 cells were subcutaneously implanted near the dorsal midline of C57BL/6 mice. THS was administered orally from day 1 at tapering doses (100 mg/kg/day, 50 mg/kg/day, and 25 mg/kg/day for 3 days each) with administration of 30 mg/kg/day of heparin sodium, p.o., to the mice implanted with agarose microbeads containing A431 cells (B). Ten days later, agarose gel pellets were observed under a stereoscopic microscope.

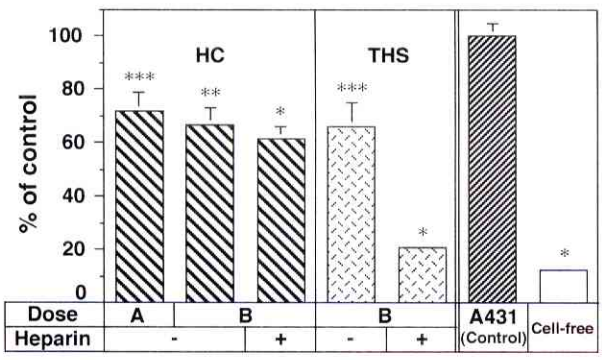

Fig. 5. Inhibitory effects of angiostatic steroids with or without heparin on the angiogenesis induced by A431 cells entrapped in agarose beads. C57BL/6 mice were implanted with A431 cells entrapped in agarose beads at day 0, and were treated with HC or THS for 9 days. Tapering doses of steroids were administered p.o. from day 1: (A) 50 mg/kg/day for 3 days, followed by 25 mg/kg/day for 3 days and 12.5 mg/kg/day for 3 days; and (B) 100 mg/kg/day for 3 days, followed by 50 mg/kg/day for 3 days and 25 mg/kg/day for 3 days from day 1 with (+) or without (−) administration of 30 mg/kg/day of heparin sodium, p.o. The mHb levels at the area implanted with agarose beads were determined by mHb-ELISA on day 10. Each value represents the mean± SEM for 3–5 mice. * $P<0.001$, ** $P<0.01$, *** $P<0.05$ compared to control.

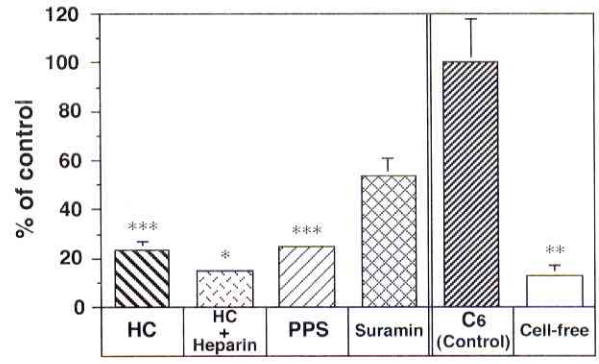

Fig. 6. Inhibitory effects of HC, PPS and suramin on the angiogenesis induced by C6 cells entrapped in agarose beads. C57BL/6 mice were implanted with C6 cells entrapped in agarose beads at day 0, and were treated with HC or PPS for 9 days from day 1. HC was administered p.o. at tapering doses (100 mg/kg/day for 3 days, followed by 50 mg/kg/day for 3 days and 25 mg/kg/day for 3 days) with or without administration of 30 mg/kg/day of heparin sodium, p.o. PPS was administered p.o. at 25 mg/kg/day for 9 days. Suramin was administered i.p. at 100 mg/kg/day from day 4 to day 7. The mHb levels at the area implanted with agarose beads were determined by mHb-ELISA on day 10. Each value represents the mean±SEM for 5–8 mice. * $P<0.001$, ** $P<0.005$, *** $P<0.01$ compared to control.

by both types of cells. Moreover, these inhibitory effects were increased by the combination with steroid or with heparin. The combination of HC and heparin also showed greater effect than HC alone in the system using C6 cells. These results suggest that heparin promotes the antiangiogenic activity of steroids, because heparin alone

does not change or enhance the level of new blood vessel formation (data not shown), as Norrby and Sorbo pointed out.[26] Although the mechanism of enhancement of angiostatic activity by combination with heparin is not clearly understood, it has been suggested that angiostatic steroids in the presence of heparin may affect the turnover of the basement membrane and the DNA synthesis of endothelial cells in the growing capillary vessel.[7, 27, 28]

By comparing the inhibitory effects of the two steroids, we established that THS has stronger antiangiogenic activity than HC either alone or in combination with heparin. THS is an HC derivative which does not have glucocorticoid and mineralocorticoid activity. Thus, our results support the finding that the angiostatic activity of steroids is not correlated with their glucocorticoid and mineralocorticoid activity.[2, 6]

Antiangiogenic activity of MPA, PPS and suramin was also detected in our *in vivo* angiogenesis assay system. The mechanism of angiogenesis inhibition by MPA is thought to be inhibition of collagenase activity and plasminogen activator activity, which is necessary for degradation of the basement membrane of the parent blood vessels.[14, 18] On the other hand, PPS and suramin, which are polysulfated polyanions, are thought to inhibit the mitogenic effect of fibroblast growth factor (FGF), which is a potent angiogenic factor in endothelial cells, by interfering with FGF binding to the FGF receptor, since this binding involves the mediation of heparin-like glycosaminoglycan.[21–23]

The inhibitory effects of angiogenic inhibitor in our assay system are almost entirely the same as those in the assay systems commonly used to visualize tumor-induced blood vessel growth, such as corneal micropocket grafts in rabbits, rats or mice,[12, 14] and chorioallantoic membrane grafts in fertilized chicken embryos.[2, 6–8, 13, 29] Compared to these screening methods, though, our *in vivo* angiogenesis model is quantitative and simple, and the procedures are technically easy to perform. Moreover, as the tumor cells are protected from direct contact with the host's immune system, the antiangiogenic activity of drugs against various tumor types may be evaluated across histocompatibility or species barriers. Given these encouraging results, we hope that our *in vivo* angiogenesis assay system will be generally useful in research on inhibitors of tumor-induced angiogenesis.

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REFERENCES

1) Algire, G. H., Legallais, F. Y. and Park, H. D. Vascular reactions of normal and malignant tissues *in vivo*. II. The vascular reactions of normal and neoplastic tissues of mice to a bacterial polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*) culture filtrates. *J. Natl. Cancer Inst.*, **8**, 53–62 (1947).
2) Folkman, J. and Ingber, D. E. Angiostatic steroids: method of discovery and mechanism of action. *Ann. Surg.*, **206**, 374–383 (1987).
3) Goldie, I. The synovial microvascular derangement in rheumatoid arthritis and osthoarthritis. *Acta Orthop. Scand.*, **40**, 751–764 (1970).
4) Kirsner, R. S. and Eaglstein W. H. The wound healing process. *Dermatol. Clin.*, **11**, 629–640 (1993).
5) Heldin, C.-H., Usuki, K. and Miyazono, K. Platelet-derived endothelial cell growth factor. *J. Cell. Biochem.*, **47**, 208–210 (1991).
6) Crum, R., Szabo, S. and Folkman, J. A new class of steroids inhibits angiogenesis in the presence of heparin or heparin fragment. *Science*, **230**, 1375–1378 (1985).
7) Ingber, D. E., Madri, J. A. and Folkman, J. A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. *Endocrinology*, **119**, 1768–1775 (1986).
8) Wilks, J. W., Scott, P. S., Vrba, L. K. and Cocuzza, J. M. Inhibition of angiogenesis with combination treatments of angiostatic steroids and suramin. *Int. J. Radiat. Biol.*, **60**, 73–77 (1991).
9) Okada, N., Fushimi, M., Nagata, Y., Fukunaga, T., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. A quantitative *in vivo* method of analyzing human tumor-induced angiogenesis in mice using agarose microencapsulation and hemoglobin enzyme-linked immunosorbent assay. *Jpn. J. Cancer Res.*, **86**, 1182–1188 (1995).
10) Okada, N., Kaneda, Y., Miyamoto, H., Yamamoto, Y., Mizuguchi, H., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. Selective enhancement by tumor necrosis factor-α of vascular permeability of new blood vessels induced with agarose hydrogel-entrapped Meth-A fibrosarcoma cells. *Jpn. J. Cancer Res.*, **87**, 831–836 (1996).
11) Lee, J. K., Choi, B., Sobel, R. A., Chiocca, E. A. and Martuza, R. L. Inhibition of growth and angiogenesis of human neorofibrosarcoma by heparin and hydrocortisone. *J. Neurosurg.*, **73**, 429–435 (1990).
12) Li, W. W., Casey, R., Gonzalez, E. M. and Folkman, J. Angiostatic steroids potentiated by sulfated cyclodextrins inhibit corneal neovascularization. *Invest. Ophthalmol. Visual Sci.*, **32**, 2898–2905 (1991).

13) Gagliardi, A., Hadd, H. and Collins, D. C. Inhibition of angiogenesis by suramin. *Cancer Res.*, **52**, 5073–5075 (1992).
14) Gross, J., Azizkhan, R. G., Biswas, C., Bruns, R. R., Hsieh, D. S. T. and Folkman, J. Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. *Proc. Natl. Acad. Sci. USA*, **78**, 1176–1180 (1981).
15) 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).
16) Fujimoto, J., Hosoda, S., Fujita, H. and Okada, H. Inhibition of tumor angiogenesis activity by medroxyprogesterone acetate in gynecologic malignant tumors. *Invasion Metastasis*, **9**, 269–277 (1989).
17) Fujimoto, J., Hosoda, S., Fujita, H. and Okada, H. Inhibition of tumor angiogenesis activity in C3H mouse mammary tumor by medroxyprogesterone acetate. *Acta Obstet. Gynaecol. Jpn.*, **41**, 77–82 (1989).
18) 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).
19) Zugmaier, G., Lippman, M. E. and Wellstein, A. Inhibition by pentosan polysulfate (PPS) of heparin-binding growth factors released from tumor cells and blockage by PPS of tumor growth in animals. *J. Natl. Cancer Inst.*, **84**, 1716–1724 (1992).
20) Pienta, K. J., Murphy, B. C., Isaacs, W. B., Isaacs, J. T. and Coffey, D. S. Effect of pentosan, a novel cancer chemotherapeutic agent, on prostate cancer cell growth and motility. *Prostate*, **20**, 233–241 (1992).
21) Wellstein, A., Zugmaier, G., Califano III, J. A., Kern, F., Paik, S. and Lippman, M. E. Tumor growth dependent on Kaposi's sarcoma-derived fibroblast growth factor inhibited by pentosan polysulfate. *J. Natl. Cancer Inst.*, **83**, 716–720 (1991).
22) Pesenti, E., Sola, F., Mongelli, N., Grandi, M. and Spreafico, F. Suramin prevents neovascularisation and tumor growth through blocking of basic fibroblast growth factor activity. *Br. J. Cancer*, **66**, 367–372 (1992).
23) Takano, S., Gately, S., Neville, M. E., Herblin, W. F., Gross, J. L., Engelhard, H., Perricone, M., Eidsvoog, K. and Brem, S. Suramin, an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. *Cancer Res.*, **54**, 2654–2660 (1994).
24) Nilsson, K., Scheirer, W., Merten, O. W., Ostberg, L., Liehl, E., Katinger, H. W. D. and Mosbach, K. Entrapment of animal cells for production of monoclonal antibodies and other biomolecules. *Nature*, **302**, 629–630 (1983).
25) Nilsson, K., Scheirer, W., Katinger, H. W. D. and Mosbach, K. Entrapment of animal cells. *Methods Enzymol.*, **135**, 399–410 (1987).
26) Norrby, K. and Sorbo, J. Heparin enhances angiogenesis by a systemic mode of action. *Int. J. Exp. Pathol.*, **73**, 147–155 (1992).
27) Cariou, R., Harousseau, J. L. and Tobelem, G. Inhibition of human endothelial cell proliferation by heparin and steroids. *Cell Biol. Int. Rep.*, **12**, 1037–1047 (1988).
28) Sakamoto, N. and Tanaka, N. G. Mechanism of the synergistic effect of heparin and cortisone against angiogenesis and tumor growth. *Cancer J.*, **2**, 9–13 (1988).
29) Maragoudakis, M. E., Sarmonika, M. and Panoutsacopoulou, M. Antiangiogenic action of heparin plus cortisone is associated with decreased collagenous protein synthesis in the chick chorioallantoic membrane system. *J. Pharmacol. Exp. Ther.*, **251**, 679–682 (1989).