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The Proteasome Is Involved in Angiogenesis

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The proteasome is a recently identified intracellular protease whose catalytic active site is a threonine residue and has been shown to play key roles in a variety of important intracellular events, including cell cycle progression, the antigen-presenting pathway, and apoptosis. However, its biological significance in multicellular organisms is still largely unknown because of lack of experimental systems for its study. Here we verified potential involvement of the proteasome in angiogenesis using lactacystin, a specific proteasome inhibitor. Lactacystin treatment resulted in almost complete prevention of *in vivo* neovascularization in the developing chick embryo chorioallantoic membrane. It also inhibited vascular endothelial tube formation on Matrigel, a model for *in vitro* angiogenesis, in a concentration-dependent fashion. Moreover, it prevented production of plasminogen activator, an important protease responsible for induction of angiogenesis, by endothelial cells, which correlated well with its suppression of intracellular proteasome activity. Our studies suggest that the proteasome operates in the process of angiogenesis, a phenomenon essential in important physiological and pathological settings.

Angiogenesis, the formation of new blood vessels, is a fundamental feature in physiological situations like embryogenesis and in refractory disease states such as growth and metastasis of tumors, rheumatoid arthritis, AIDS and diabetic retinopathy (1–3). This is the reason why angiogenesis has attracted recent attention. Angiogenesis involves a sequence of multiple events of vascular endothelial cells, including dedifferentiation, acquisition of migrative and proliferative abilities with high matrix-degrading activity, and tube formation of the cells. Many factors are involved in positive or negative regulations of angiogenesis (1–6). Positive regulators of angiogenesis include mitogens such as fibroblast growth factors and endothelial cell growth factor, matrix-degrading enzymes like plasminogen activator (PA) and integrins $\alpha v\beta 3$ and $\alpha v\beta 5$. Examples of negative angiogenesis regulators are thrombospondin-1, angiostatin and endostatin. However, the mechanism of angiogenesis in cells is not yet fully understood.

The proteasome is a recently identified intracellular protease, and its function(s) in cells has been the focus of recent attention because the protease is being uncovered to play critical roles in a variety of important intracellular events, including cell cycle progression, antigen-presenting pathway and apoptosis (7–9). Cell growth largely depends on cell cycle progression and is an important phase in the process of angiogenesis. In addition, there is increasing evidence suggesting a correlation between apoptosis and angiogenesis. For example, some angiogenesis inhibitors, including angiostatin and endostatin, induce dormancy of primary tumors and metastases by indirectly increasing apoptosis in tumor cells (10–12), and antagonists of $\alpha v\beta 3$ exhibit anti-angiogenic activity through direct induction of apoptosis in proliferative endothelial cells during angiogenesis (13, 14). These findings strongly suggest that the proteasome exerts its functions in the process of angiogenesis, but the evidence to prove the role of this protease in neovascularization is not provided.

To verify this hypothesis, we here examined the effect of lactacystin, a specific proteasome inhibitor (15, 16), on neovascularization in the developing chick embryo chorioallantoic membrane (CAM), a widely used *in vivo* angiogenesis model, and investigated the influence of lactacystin on endothelial cell functions associated with angiogenesis.

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Abbreviations used: PA, plasminogen activator; CAM, chorioallantoic membrane; EV, ethylene vinyl-acetate copolymer 40; HDMECs, human dermal microvascular endothelial cells; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; EGF, epidermal growth factor; BSA, bovine serum albumin; IC_{50}, concentration for half-maximal inhibition.

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MATERIALS AND METHODS

Materials. Ethylene-vinyl acetate copolymer 40 (EV) was a gift from Mitsui-DuPond Polychemicals (Tokyo, Japan). Matrigel was purchased from Collaborative Biochemical Products, Becton-Dickinson Labware (Bedford, MA). MCDB 131, human recombinant epidermal growth factor (EGF), heparin and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Endothelial cell growth supplement (ECGS) was purchased from Upstate Biotechnology (Lake Placid, NY). Antibiotics were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Moregate (Melbourne, Australia). Human dermal microvascular endothelial cells (HDMECs) were purchased from Cell Systems (Kirkland, WA). Lactacystin was isolated as described previously (15).

CAM assay. CAM assay was conducted as described (17). An EV pellet impregnated with increasing doses of lactacystin was placed carefully on the CAM surface of 5-day-old chick embryos, and these embryos were incubated in a humidified incubator at 37°C. After incubation for 2 days, an appropriate volume of 20% fat emulsion was injected into the chorioallantois to improve visualization of the vascular network. Inhibition of angiogenesis was determined by measuring the avascular zone in the CAM. A positive anti-angiogenic response was assessed as an avascular zone of $\geqslant$3 mm in diameter.

Determination of tube formation. Tube formation was determined as described (18). Aliquots (0.2 ml/well) of Matrigel (10 mg/ml) at 4°C were introduced into 24-multiwell dishes (Sumitomo-Bakelite, Tokyo, Japan) and allowed to polymerize at 37°C for 60 min. HDMECs (2×10^4 cells per cm^2) were seeded onto gelatinized dishes (Iwaki Glass, Tokyo, Japan), and incubated at 37°C for 24 hr in a humidified chamber under 5% CO_2 in the presence of the indicated concentrations of lactacystin in complete medium, composed of MCDB 131 with 10% FBS, 1% antibiotics, 10 μg/ml ECGS, 10 ng/ml EGF and 10 μg/ml heparin. The cells (7.5×10^4 cells/well) were plated onto the Matrigel in 1 ml of complete medium containing the corresponding doses of lactacystin, and incubated at 37°C in a humidified chamber under 5% CO_2. After incubation for 18 hr, the formation of tube-like structures of HDMECs was analyzed by phase contrast microscopy (x 100 magnification), and the total lengths of tubular structures in five randomly chosen microscopic fields per well were measured using a Leica Q500MC image analyzer equipped with Qwin image analysis software (Cambridge, UK).

PA activity assay. PA activity was determined as described (19). HDMECs (1.5×10^5 cells/well) in 1 ml of complete medium were seeded onto gelatin-coated 24-multiwell dishes (Sumitomo-Bakelite, Tokyo, Japan), incubated at 37°C for 24 h in a humidified chamber under 5% CO_2, and then incubated in MCDB 131 containing 0.1% BSA with or without ECGS, EGF and heparin in the presence or absence of various concentrations of lactacystin for 18 hr. Then the serum-free conditioned medium was collected by centrifugation for examination of its PA activity. Reactions were performed at 37°C and pH 7.4 in 0.1 M Tris-HCl containing plasminogen (Daiichi Chemicals, Tokyo, Japan) and D-Val-Leu-Lys-*p*-nitroanilide (S-2251; Chromogenix, Mölndal, Sweden), substrates for PA and plasmin, respectively. At the same time, the cell number of cultures was determined with a Coulter counter after trypsinization. Activity was expressed in urokinase units (U) per 10^5 cells. In separate experiments, HDMECs (1.5×10^5 cells/well) in 2 ml of complete medium were seeded onto gelatin-coated 35-mm culture dishes (Iwaki, Tokyo, Japan), incubated at 37°C for 24 h in a humidified chamber under 5% CO_2, and treated with lactacystin in the same conditions as those mentioned above. After 18-hr incubation, cells attached to dishes were washed two times with phosphate-buffered saline (pH 7.4), and extracted with 0.5 ml of 0.5% Triton X-100 in phosphate-buffered saline. PA activity in the cell extracts was determined as described above. Protein concentration was determined with BSA as a standard according to the manufacture's instructions (DC protein assay; Bio-Rad, Hercules, CA).

Proteasome activity assay. In parallel with experiments on PA production, the proteasome activity in the cells was determined using succinyl-Leu-Leu-Val-Tyr-methylcoumarylamide as described (20). After treatment with lactacystin for 18 hr, HDMECs were disrupted by sonication. The extracts were centrifuged at 9200 X *g* for 20 min at 4°C and the supernatants were tested for proteasome activity. One unit of proteasome activity is defined as the amount hydrolyzing 1 nmol of the substrate per min at 37°C.

HDMEC proliferation. In the presence of indicated concentrations of lactacystin HDMECs (1×10^4 cells/well) were cultured in the gelatinized 24-multiwell dish containing 1 ml of complete medium. After 72 hr, the medium containing lactacystin was replaced by fresh medium without lactacystin. After further incubation for 72 hr, the cells were refed with fresh medium without lactacystin. Cell numbers were counted at the indicated times in a Coulter counter after trypsinization.

RESULTS AND DISCUSSION

We used the CAM assay to examine the effect of lactacystin on neovascularization, because if a test sample could block at least one of angiogenic endothelial functions, the CAM assay has the advantage of identifying it as an angiogenesis inhibitor (5, 21). Also, the CAM assay can examine a topical effect of a test sample. Lactacystin inhibited angiogenesis in CAM, causing an avascular zone (Fig. 1b), whereas the vehicle alone, as a control, had no effect (Fig. 1a). The inhibition was dose-dependent (Fig. 1c), a significant avascular zone in half the CAMs examined being observed with 3.6 μg per egg. Addition of 100 μg of lactacystin induced a marked avascular zone in all the CAMs examined, indicating the role of the proteasome in embryonic angiogenesis. No apparently adverse effect on the growth of the embryos were observed, as judged by the size of developing embryos during a 2-day experimental period. Thus the anti-angiogenic effect of lactacystin is apparently not due to toxicity (see below). One might ask why the growth of the embryos was not affected by lactacystin treatment because angiogenesis is important in embryo development. The reason for this is not clear, but it may be due to differences in levels of lactacystin concentrations between the extraembryonic CAMs and intraembryos; lactacystin levels in intraembryonic tissues might be insufficient to inhibit angiogenesis, thereby not suppressing embryo growth.

Next, we examined the *in vitro* effect of lactacystin on tube formation of HDMECs, a process that is a final stage in angiogenesis (22, 23). Exposure to lactacystin (Fig. 2b–d) but not the vehicle alone (0.1% dimethylsulfoxide) (Fig. 2a) resulted in drastic suppression of the formation of capillary-like structures on Matrigel, an extract of basement membrane components. Quantitative measurements with an image-analyzer showed that lactacystin prevented the formation of tube-like structures, its IC_{50} being 1.6 μM. This IC_{50} value is comparable to those for its inhibition in other experiments, such as on the inductions of neuritogenesis (15), cell cycle arrest (24) and apoptosis (25).

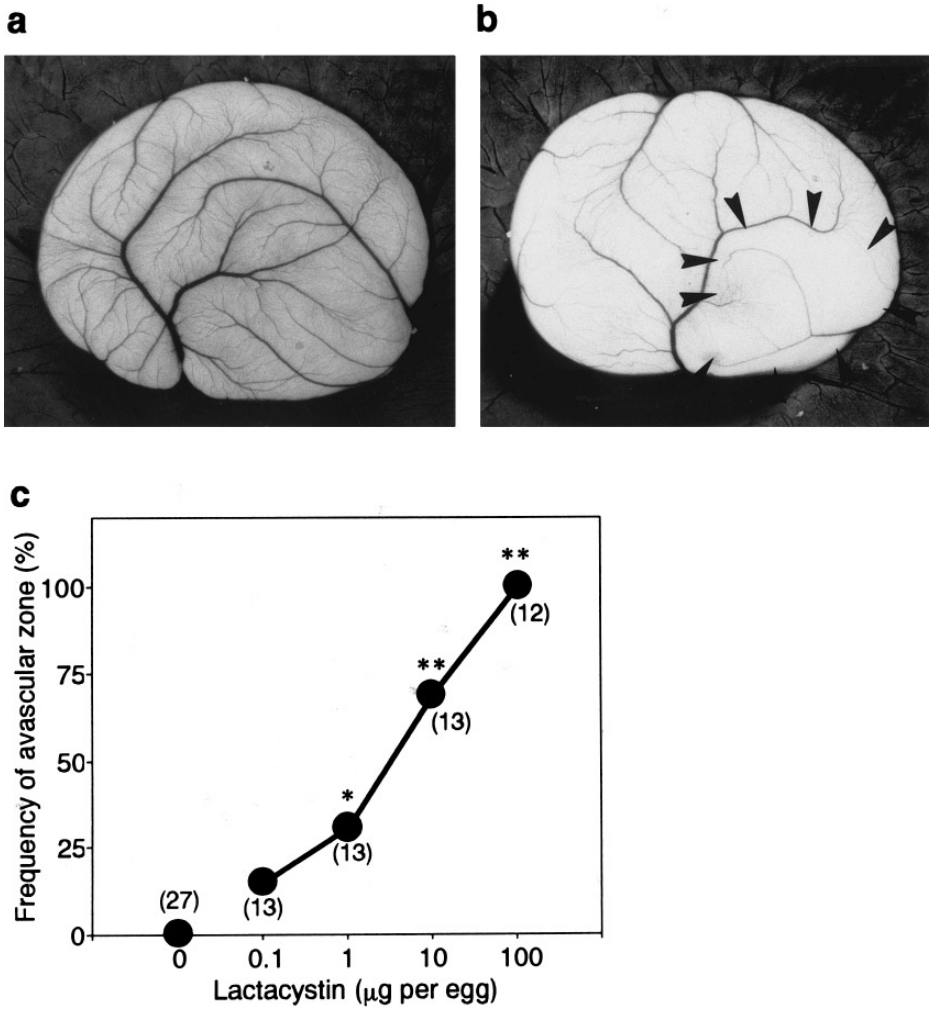

FIG. 1. Effect of lactacystin on *in vivo* angiogenesis. CAM-angiogenesis assay was carried out with (b) and without (a) addition of a lactacystin-containing EV pellet (100 μg per egg). Results show data in a representative experiment. Arrowheads indicate an avascular zone. Magnification, 2.6 X. (c) Dose-dependent inhibition of angiogenesis by lactacystin. Points indicate the frequencies (%) of avascular zones exhibiting an anti-angiogenic response. Values in parentheses are numbers of membranes examined. *, $p < 0.01$; **, $p < 0.001$ compared with the value for control CAMs (n = 34), not showing an avascular zone. Statistical analysis was done by Fisher's exact probability test.

Intriguingly, secretion of PA is known to contribute to destruction of the extracellular matrix by endothelial cells, a process that is essential for angiogenesis. Thus the effects of anti-angiogenic agents probably involve their inhibitory effects on extracellular secretion of PA in vascular endothelial cells (19, 26). Accordingly we have proposed that any agent that can inhibit endothelial PA production may be an inhibitor of angiogenesis (5). Independently, other groups have suggested that PA has an important role in induction of angiogenesis (27–29). Lactacystin markedly inhibited PA secretion from confluent quiescent endothelial cells, HDMECs, into serum-free conditioned medium without affecting cell viability (Fig. 3a, b), and cell-associated PA levels were inhibited after lactacystin administration (Fig. 3c). Such inhibition of extracellular and cell-associated PA levels by lactacystin correlates well with its suppression of intracellular proteasome activity as determined by inhibition of ability to hydrolyze succinyl-Leu-Leu-Val-Tyr-methylcoumarylamide (Fig. 3d), a good substrate of the proteasome (20). Moreover, ECGS upregulated the extracellular and cell-associated PA levels of HDMECs to about 300% of the control levels in untreated HDMECs, and lactacystin at concentrations of[3] 1 μM reduced these upregulated levels to those of untreated cultures (Fig. 3f, g) but had little or no effect on the cell number of cultures (Fig. 3e). This is similar to the inhibition of the proteasomal activity (Fig. 3h). These results imply that net production of extracellular and cell-associated PA by endothelial cells is regulated by proteasome function. The enzymatic activity of PA is determined by a balance of both

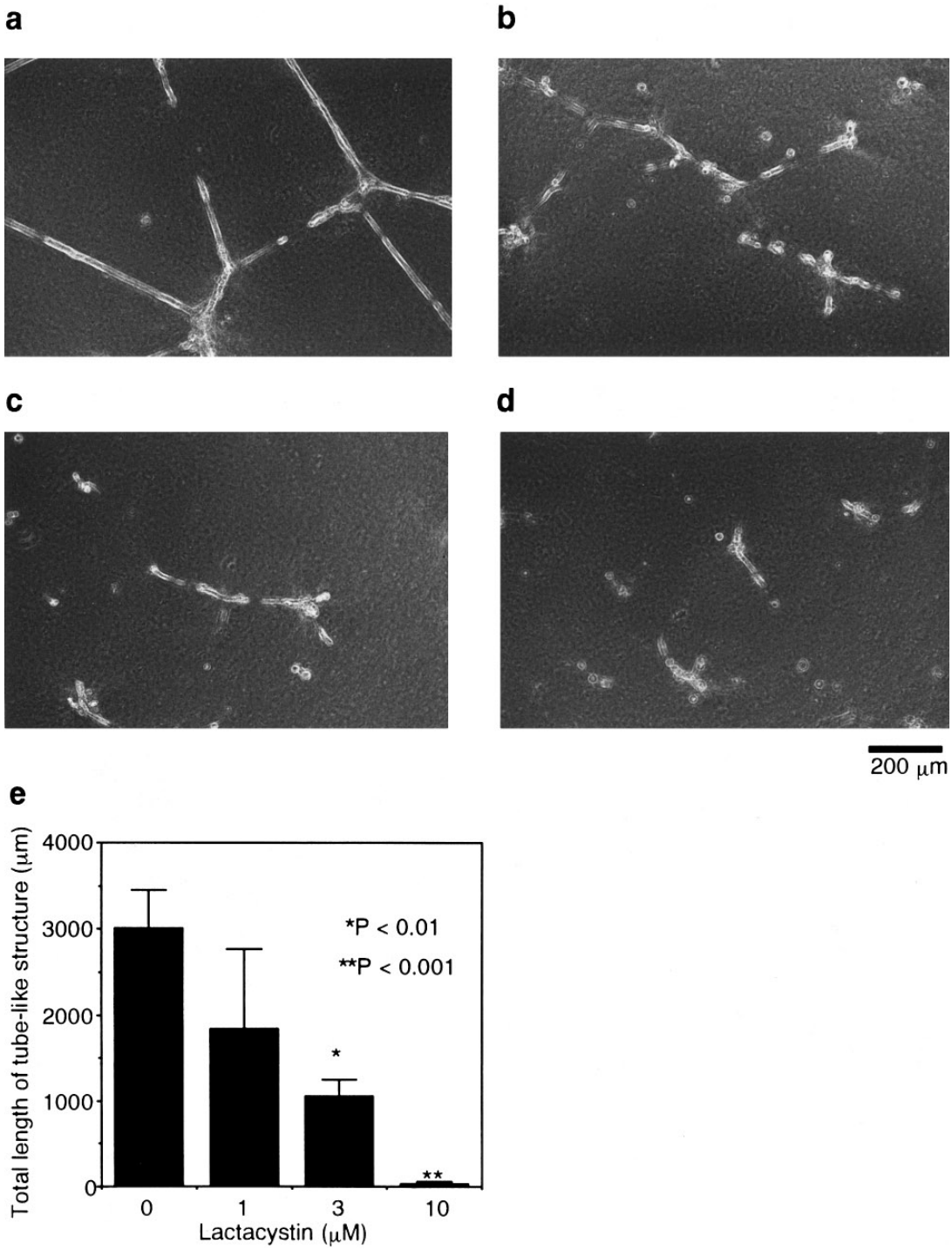

FIG. 2. Inhibition by lactacystin of in vitro tube formation of endothelial cells on Matrigel. The doses of lactacystin used were 0 μM for a, 1 μM for b, 3 μM for c and 10 μM for d. Results show representative data. Bar = 200 μm. e: Length of tubular structures of HDMECs determined with an image analyzer equipped with Qwin image analysis software. Values are means ± S.D. from four separate wells. Significant difference was based on Student's *t* test, *, $p < 0.01$; **, $p < 0.001$ *versus* control (in the absence of lactacystin).

levels of PA and its inhibitors, such as plasminogen activator inhibitor (PAI)-1 (28, 29). Thus it might be possible that the inhibition of endothelial cell PA production by lactacystin results from suppressed PA synthesis, elevated PAI synthesis, or both. Further study will be required for elucidating this mechanism of inhibition of PA production.

Next we examined whether lactacystin affects the

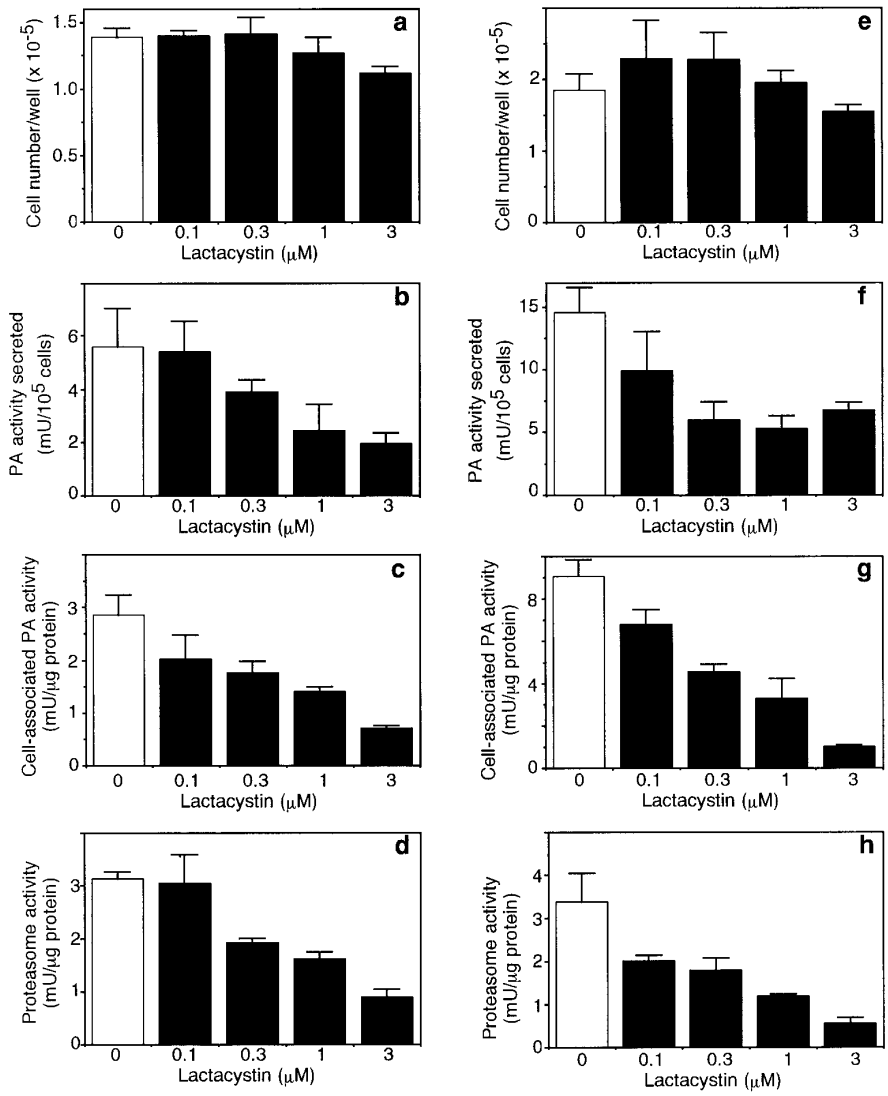

FIG. 3. Effect of lactacystin on extracellular and cell-associated PA levels in HDMECs. Cells were treated with lactacystin as indicated in serum-free cultures in the absence (a–d) and the presence of ECGS, EGF and heparin (e–h). After 18-hr culture, cell numbers (a, e), extracellular PA activity (b, f), cell-associated PA activity (c, g) and proteasome activity (d, h) were measured. Data are means ± S.D. from four separate wells.

growth of endothelial cells, because this cell activity is implicated in angiogenesis (1–6). As shown in Fig. 4, lactacystin concentration-dependently inhibited the division of growing cultures of HDMECs, the IC_{50} value being 3.5 μM. This is in marked contrast to its little or no effect on the viability of quiescent HDMECs (Fig. 3a). This inhibition was reversible, since the suppressed growth of HDMECs incubated in the presence of lactacystin at up to 10 μM at 37°C for 72 h was reversed by removal of lactacystin from the cultures. The antiproliferative activity of lactacystin is not limited to endothelial cells, having been shown with various other types of cells (15, 24, 25). Therefore, growth-arrest by lactacystin may be involved in its inhibition of neovascularization observed by CAM assay (Fig. 1b). However, the blockade of tube formation and PA production of endothelial cells by lactacystin appeared to be independent on its antiproliferative effect, because these experiments were carried out using cells in the quiescent state (Figs. 2, 3). Thus the proteasome presumably plays an essential role in the multiple steps of angiogenesis. It may function in controlling the intracellular stability of as yet unidentified proteins required to induce angiogenesis.

There is no evidence that lactacystine affects activities of any enzymes other than the proteasome; lactacystine inhibits selectively proteasome activity by binding its catalytic active threonine residue, but not inhibit activities of other proteases, including those of serine-, cysteine-, aspartic-, and metallo-proteases (8, 16). This is highly likely to imply that lactacystine is a selective inhibitor of the proteasome. Nothing is known about the role of other cellular proteases in angiogenesis *in vivo and in vitro*, to

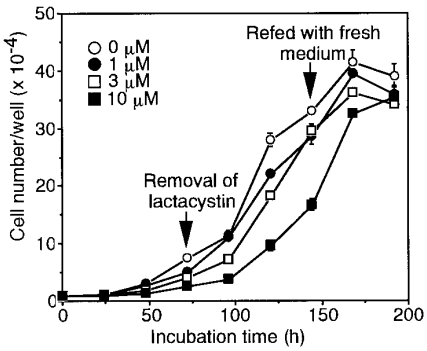

FIG. 4. Inhibitory effect of lactacystin on the proliferation of HDMECs and its reversibility. Cells were treated by lactacystin at the various concentrations indicated. After 72 hr, the medium containing lactacystin was replaced by fresh medium without lactacystin. After further incubation for 72 hr, the cells were refed with fresh medium without lactacystin. Cell numbers were counted at the indicated times in a Coulter counter after trypsinization. Data are means ± S.D. from four separate wells.

our knowledge. On the other hand, some inhibitors of extracellular proteases, which play roles in angiogenesis, have been found to suppress angiogenic responses in different experimental systems, including PA inhibitor and tissue inhibitors of metalloproteases (1, 3–5).

Our results provide evidence that the proteasome acts as a key regulator in *in vivo* and *in vitro* neovascularization, a biologically important response, because lactacystin, has an anti-angiogenic effect. This is the first paper showing the significance of the proteasome in a biological phenomenon in multicellular organisms. It is impractical to treat angiogenic diseases by systemic administration of lactacystin because of the critical functions of the proteasome in many cellular events, but local administration of lactacystin might have an anti-angiogenic effect, and so be therapeutically effective against some angiogenesis-dependent diseases such as cancer, rheumatoid arthritis, AIDS and diabetic retinopathy. The exact molecular mechanism of the anti-angiogenic action of lactacystin remains to be determined. But our results should facilitate understanding of the mechanism of neovascularization and the biological importance of the proteasome in the body, and help in development of useful therapeutic agents for angiogenic disorders.

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REFERENCES

1. Folkman, J. (1995) *Nature Med.* **1,** 27–31.
2. Risau, W. (1997) *Nature* **386,** 671–674.
3. Bouck, N., Stellmach, V., and Hsu, S. C. (1996) *Adv. Cancer Res.* **69,** 135–174.
4. Auerbach, W., and Auerbach, R. (1994) *Pharmacol. Ther.* **63,** 265–311.
5. Oikawa, T. (1995) *Curr. Med. Chem.* **1,** 406–417.
6. Folkman, J. (1996) *Nature Med.* **2,** 167–168.
7. Hochstrasser, M. (1995) *Curr. Opin. Cell Biol.* **7,** 215–223.
8. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Ann. Rev. Biochem.* **65,** 801–847.
9. Hilt, W., and Wolf, D. H. (1996) *Trends Biochem. Sci.* **21,** 96–102.
10. Holmgren, L., O'Reilly, M. S., and Folkman, J. (1995) *Nature Med.* **1,** 149–153.
11. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. (1996) *Nature Med.* **2,** 689–692.
12. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. (1997) *Cell* **88,** 277–285.
13. Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994) *Cell* **79,** 1157–1164.
14. Strömblad, S., Becker, J. C., Yebra, M., Brooks, P. C., and Cheresh, D. A. (1996) *J. Clin. Invest.* **98,** 426–433.
15. Õmura, S., Fujimoto, T., Otoguro, K., Matsuzaki, K., Moriguchi, R., Tanaka, H., and Sasaki, Y. (1991) *J. Antibiot. (Tokyo)* **44,** 113–116.
16. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* **268,** 726–731.
17. Oikawa, T., Hirotani, K., Nakamura, O., Shudo, K., Hiragun, A., and Iwaguchi, T. (1989) *Cancer Lett.* **48,** 157–162.
18. Oikawa, T., Sasaki, M., Inose, M., Shimamura, M., Kuboki, H., Hirano, S., Kumagai, H., Ishizuka, M., and Takeuchi, T. (1997) *Anticancer Res.* **17,** 1881–1886.
19. Ashino-Fuse, H., Takano, Y., Oikawa, T., Shimamura, M., and Iwaguchi, T. (1989) *Int. J. Cancer* **44,** 859–864.
20. Tanaka, K., Yoshimura, T., Kumatori, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K., and Takagi, T. (1988) *J. Biol. Chem.* **263,** 16209–16217.
21. Nguyen, M., Shing, Y., and Folkman, J. (1994) *Microvascular Res.* **47,** 31–40.
22. Kubota, Y., Kleinman, H. K., Martin, G. R., and Lawley, T. J. (1988) *J. Cell Biol.* **107,** 1589–1598.
23. Cockerill, G. W., Gamble, J. R., and Vadas, M. A. (1995) *Int. Rev. Cytol.* **159,** 113–160.
24. Fenteany, G., Standaert, R. F., Reichard, G. A., Corey, E. J., and Schreiber, S. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3358–3362.
25. Imajoh-Ohmi, S., Kawaguchi, T., Sugiyama, S., Tanaka, K., Õmura, S., and Kikuchi, H. (1995) *Biochem. Biophys. Res. Commun.* **217,** 1070–1077.
26. Oikawa, T., Ito, H., Ashino, H., Toi, M., Tominaga, T., Morita, I., and Murota, S. (1993) *Eur. J. Pharmacol.* **241,** 221–227.
27. Mignatti, P., and Rifkin, D. B. (1996) Curr. *Topics Microbiol. Immunol.* **213**(Part 1), 33–50.
28. Pepper, M. S., Vassalli, J.-D., Wilks, J. W., Schweigerer, L., Orci, L., and Montesano, R. (1992) *J. Cell. Biochem.* **55,** 419–434.
29. Ono, M., Izumi, H., Yoshida, S., Goto, D., Jimi, S., Kawahara, N., Shono, T., Ushiro, S., Ryuto, M., Kohno, K., Sato, Y., and Kuwano, M. (1996) *Cancer Chemother. Pharmacol.* **38**(Suppl.), S78–S82.