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Inhibition of Tumorigenesis in Mouse Skin by Leupeptin, a Protease Inhibitor from *Actinomycetes*

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SUMMARY

Leupeptin, with the structure *N*-acetyl(*N*-propionyl)-L-leucyl-L-leucyl-DL-argininal, was isolated from *Actinomycetes* and is a potent and specific inhibitor of proteases. In this work, leupeptin was found to inhibit tumorigenesis in mouse skin induced by a single, noncarcinogenic dose of 7,12-dimethylbenz(a)anthracene followed by repeated application of croton oil. Tumors that had already been induced were scarcely affected by leupeptin.

The activity of *p*-toluene-sulfonyl-L-arginine methyl ester esterase in mouse skin was markedly increased by treatment with croton oil and was inhibited by leupeptin both *in vitro* and *in vivo*. The effect of leupeptin in repressing tumorigenesis seems to be due to its inhibition of *p*-toluene-sulfonyl-L-arginine methyl ester esterase in the skin and not to a direct effect on tumors.

INTRODUCTION

In the recent works of Klassen *et al.* (16) and Troll *et al.* (26), it was reported that synthetic inhibitors of proteases markedly depressed tumorigenesis in mouse skin induced by painting the skin with DMBA[1] and croton oil. Therefore, they suggested that certain proteases might be involved in tumorigenesis.

Aoyagi *et al.* (2, 3) isolated a group of compounds, which were named leupeptin and which were potent, specific inhibitors of proteases, from the culture media of various species of *Actinomycetes*. The structure of leupeptin was determined to be *N*-acetyl(*N*-propionyl)-L-leucyl-L-leucyl-DL-argininal (15, 17). It is a strong, competitive inhibitor of proteolysis by plasmin, trypsin, or papain (2). Furthermore, leupeptin inhibits thrombokinase and kallikrein, markedly inhibits blood coagulation, and produces a curative effect on inflammation with edema (2).

This work concerns the effect of leupeptin on tumorigenesis in mouse skin induced by a single, noncarcinogenic dose of DMBA and then by repeated painting of the skin with croton oil. Leupeptin markedly inhibited tumorigenesis induced in this way. It was also found that leupeptin inhibited the activity of TAME esterase in the skin of animals treated with croton oil.

MATERIALS AND METHODS

Chemicals. DMBA was obtained from Distillation Products Industries, Rochester, N. Y. Croton oil and TAME were obtained from Sigma Chemical Co., St. Louis, Mo. DMSO was obtained from Daiichi Pure Chemicals Co., Tokyo, Japan, and was distilled before use. Leupeptin was a mixture of *N*-acetyl- and *N*-propionyl-L-leucyl-L-leucyl-DL-argininals that was prepared as described previously (3, 15, 17). The structure of leupeptin is shown in Chart 1.

Animals. Female ICR, Swiss strain mice about 60 days old were used. At this age, the dorsal hair follicles were usually quiescent. The mice were kept on laboratory chow (Central Laboratories for Experimental Animals, Tokyo, Japan) and tap water *ad libitum*.

Tumorigenesis Experiment. For testing of the effect of leupeptin on tumorigenesis in mouse skin, the skins on the backs of 4 groups of 30 female ICR mice were shaved and painted with 125 μg of DMBA in 0.25 ml of acetone. Starting 3 weeks later, the mice in 3 groups were painted 3 times a week with 0.25 ml of 0.03% croton oil in acetone. Two hours after the croton oil treatment, 2 of these groups were painted with 0.25 and 1.25 mg, respectively, of leupeptin in 0.25 ml of DMSO. The 3rd group was painted with 0.25 ml of DMSO alone and served as a control.

In these experiments, the amount of solution applied was measured in a 0.25-ml syringe, and the incidence of tumors was recorded at weekly intervals.

Determination of TAME Esterase Activity. The hair on the backs of female ICR mice was shaved with an animal hair clipper. The skin was removed and chopped up finely with scissors. It was then homogenized 3 times at the maximum speed in a VirTis homogenizer for 2-min periods with deionized water to give a 10% homogenate. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was used to measure enzyme activity. All procedures used for preparation of this solution were carried out in ice water.

TAME esterase activity in the solution was determined as follows. The reaction mixture contained 0.4 ml of the test solution, 0.2 ml of 40 mM TAME, 0.2 ml of 0.5 M phosphate buffer (pH 7.0), and 0.2 ml of deionized water or leupeptin in a total volume of 1.0 ml. The reaction mixture was incubated at 37° for 4 hr, unless stated otherwise.

[1] The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; DMSO, dimethyl sulfoxide.

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After incubation, the amount of TAME remaining was determined by a slight modification of the method of Hestrin (11), as follows. To the whole reaction mixture were added 1.5 ml of alkaline hydroxylamine solution (2 M hydroxylamine hydrochloride mixed with an equal volume of 3.5 N sodium hydroxide) and, after standing for 15 min at room temperature, 1.0 ml of 8 N hydrochloric acid and 1.0 ml of 18% trichloroacetic acid were added. Then, 1.0 ml of 10% ferric chloride in 0.1 N hydrochloric acid was added. The turbidity, which was due mainly to the test solution, was removed by centrifugation at 18,000 X *g* for 20 min. The absorbance of the clear supernatant was read at 530 nm. The specific activity of TAME esterase was calculated after the amount of protein in the solution was determined by the method of Lowry *et al.* (19).

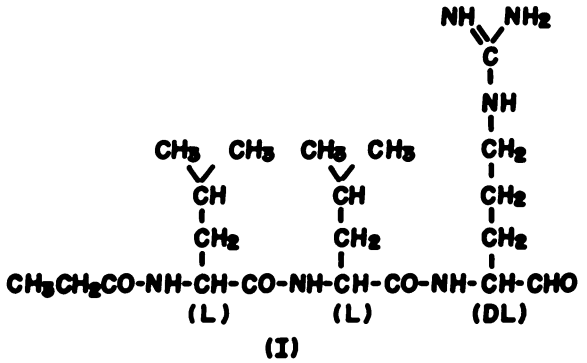

Chart 1. Structure of leupeptins. *I*, *N*-propionyl-L-leucyl-L-leucyl-DL-argininal; *II*, *N*-acetyl-L-leucyl-L-leucyl-DL-argininal.

RESULTS

Effect of Leupeptin on Tumorigenesis of Mouse Skin. Tumors developed in the skin of control mice from 6 weeks after the beginning of croton oil treatment, and from this time the incidence of the tumors increased progressively, as shown in Table 1. On the other hand, tumors developed only after 8 weeks in the group that had been treated with 0.25 mg of leupeptin. In this group, 3 tumors appeared in 2 mice after 10 weeks, but the incidence of tumors was much less than in the control group, as shown in Table 1. Treatment of the mice with 1.25 mg of leupeptin did not result in significantly more inhibition of tumorigenesis, and the incidence of tumors in the groups receiving 0.25 and 1.25 mg of leupeptin was similar, as shown in Table 1.

Thirty weeks after croton oil treatment, most of the tumors were benign tumors (papillomas) of various sizes. There was no consistent difference between the sizes of tumors in mice of the control group and in those treated with leupeptin. However, the average number of tumors in the latter was much less than in control mice, as shown in Table 1. The group of 30 mice that were painted with 125 μg of DMBA in 0.25 ml of acetone but that were not treated with croton oil did not develop tumors. The incidence of tumors in another group of 30 mice that were painted with 0.03% croton oil after DMBA treatment but that were not painted with DMSO was similar to that in mice painted with DMSO after croton oil. Therefore, tumors seem to be induced by croton oil treatment, and the inhibition of the tumorigenesis by leupeptin seems to be due to counteraction of the stimulus by croton oil.

Survival of mice treated with leupeptin was slightly less than that of the mice not treated with leupeptin; however, no

Table 1

Inhibition of tumorigenesis in mouse skin by leupeptin

Mice were painted with 0.25 ml of 0.03% croton oil in acetone 3 times a week, beginning 3 weeks after a single application of 125 μg of DMBA in 0.25 ml of acetone. DMSO solution, 0.25 ml, with or without leupeptin was applied 2 hr after treatment with croton oil.

	Tumor incidence					
	Control (DMSO alone)		Leupeptin (0.25 mg)		Leupeptin (1.25 mg)	
Croton oil treatment (wk)	No. of mice with tumors/ survivors	Total no. of tumors/ survivors	No. of mice with tumors/ survivors	Total no. of tumors/ survivors	No. of mice with tumors/ survivors	Total no. of tumors/ survivors
6	2/30	2/30	0/30	0/30	1/28	2/28
8	7/30	37/30	0/30	0/30	1/28	2/28
10	11/30	78/30	2/30	3/30	4/28	11/28
15	18/30	115/30	7/29	15/29	5/26	34/26
20	19/29	130/29	7/27	25/27	8/26	34/26
25	21/28	179/28	9/26	48/26	11/26	44/26
30	24/28	194/28	9/26	48/26	11/26	44/26

Table 2

In vitro effect of leupeptin on protease activity of mouse skin

Skins from 10 normal and croton oil-treated mice, respectively, were pooled. Protease activity was determined as described in the text.

Enzyme source	Leupeptin (mg/ml)	Activity (μmoles TAME/4 hr/mg protein)
Normal skin	0	0.63
Skin after croton oil treatment	0	1.60
	0.1	1.28
	0.5	0.72
	2.5	0.14

Table 3

In vivo effect of leupeptin on protease activity of mouse skin treated with croton oil

Skins from 5 mice with or without treatment with leupeptin were pooled. Protease was assayed as described in the text.

Leupeptin (mg/mouse)	Activity (μmoles TAME/4 hr/mg protein)
0	1.95
1	1.24
5	0.86

significant toxic effect of leupeptin was observed, and the general conditions of treated and untreated mice were similar.

Effect of Leupeptin on Mouse Skin TAME Esterase. Leupeptin is a potent inhibitor of various proteases; therefore its marked inhibition of tumorigenesis in mouse skin induced by croton oil is probably somehow related to its specific inhibition of skin TAME esterase induced by croton oil treatment. Accordingly, the effects of treatment with croton oil and of subsequent treatment with leupeptin on the activities of skin TAME esterase were observed. In preliminary work, TAME esterase was extracted from the skin with 0.1% Triton X-100 instead of deionized water because it was thought that this detergent would effectively extract proteases from the lysosomal fraction (10, 21). However, the specific activity of TAME esterase in the extract was scarcely different from that in a solution extracted with deionized water, as described before. Therefore, in this work, enzyme was extracted with deionized water. The enzyme activities of the normal skins were low, but the incubation for 4 hr enabled us to determine the activity precisely. The reactions of the enzymes from the normal and the 0.3% croton oil-treated skins proceeded linearly up to 4 hr. Data in Table 2 show that the activity of the skin TAME esterase had increased almost 2.5 times at 24 hr after 0.3% croton oil treatment. The TAME esterase activity was significantly inhibited by addition of leupeptin, 0.1 mg/ml, and it was progressively inhibited by higher concentrations of leupeptin. The activity was less than 10% of the control in the presence of leupeptin, 2.5 mg/ml.

For confirmation of the inhibitory effect of leupeptin on TAME esterase in skin after croton oil treatment *in vivo*, mice were treated with croton oil, and 2 hr later some of them were painted with leupeptin. Skins from 5 animals in each group were combined. Skin extracts were prepared 22 hr after leupeptin treatment for measurement of TAME esterase activity. As shown in Table 3, the TAME esterase activity was inhibited almost 40 and 60% by painting mice with 1 and 5 mg, respectively, of leupeptin.

DISCUSSION

Leupeptin strongly inhibited tumorigenesis in mouse skin in the initial phase of tumor development. However, later tumors appeared in the mice treated with leupeptin, and their incidence was almost one-half that of control mice 30 weeks after treatment with croton oil. The latent periods in induction of tumors in mice treated with leupeptin were definitely longer than those in mice that were not treated with leupeptin. While the delay in induction of tumors attributable to leupeptin treatment and the general features of development of tumor are similar to those observed in mice treated with the synthetic inhibitor of protease, tosyl lysine chloromethyl ketone, by Troll *et al.* (26), the quantities of leupeptin used were at least 10 times larger than that of tosyl lysine chloromethyl ketone.

Doses of 0.25 and 1.25 mg, respectively, of leupeptin per mouse caused similar inhibition of tumorigenesis. Thus, the extent of inhibition caused by a dose of 0.25 mg/mouse may be the upper limit of its effect, so that a larger dose did not cause further inhibition.

Tumorigenesis was markedly inhibited by leupeptin, but growth of tumors that had already formed was scarcely affected by leupeptin. That is, the tumors in mice painted with leupeptin grew in a manner similar to that in mice without leupeptin. Therefore, the inhibition of tumorigenesis was not due to a direct action of leupeptin on tumor cells but probably to its effect on the process of tumorigenesis, in which the elevated TAME esterase activity induced by croton oil may be involved.

As expected, leupeptin definitely inhibited TAME esterase from mouse skin *in vitro*. Leupeptin also inhibited TAME esterase in mouse skin *in vivo* but less than *in vitro*. This lower inhibition *in vivo* may be because leupeptin did not penetrate well into the deeper layers of the skin, which contained significant amounts of TAME esterase. Another possible explanation is that the turnover of TAME esterase in the skin was so rapid that active TAME esterase was produced within 24 hr after a single administration of leupeptin, and so the inhibition of TAME esterase by leupeptin was masked by this further formation of active TAME esterase.

The discrepancy between the marked inhibitory effect of leupeptin on the tumorigenesis of skins and the relatively weak inhibition of TAME esterase of skins *in vivo* is difficult to explain from the present experimental data, and detailed investigations on this point are under way.

It is unknown how croton oil stimulates proteases in the skin, but there is evidence that croton oil accumulates in membranes and interacts with certain substances in membranes (6, 18, 23–25, 28). Furthermore, proteases, together with other lysosomal enzymes, may increase in the skin after treatment with croton oil (16, 22, 26, 27). Proteases from plasma may pass through the vascular membrane because of an increase in permeability and may cause this increase in

proteases and inflammation in the skin (4, 5, 9, 14, 16, 20, 26, 27).

The mechanisms underlying enhanced tumorigenesis attributable to increase in TAME esterase activity induced by croton oil may be related to gene activation. This activation may occur by removal of repressor substances, possibly nuclear proteins in chromatin, regulating gene expression, as suggested by Troll *et al.* (26). Proteases may also change the structure of the cell membrane by digesting protein components in the membrane, and they may produce cells of a nature similar to that of transformed cells. On trypsin treatment, contact-inhibited, normal 3T3 cells were found to be converted to cells with a surface membrane structure similar to that of cells transformed with oncogenic viruses (8). Furthermore, proteases were found to increase in the membranes of cells infected with oncogenic viruses (7). The importance of such proteolytic activity in alteration of the surface membrane in malignant cell transformation was also suggested recently by Inbar *et al.* (13). Moreover, the finding that the changes in surface membranes are associated not only with malignant cell transformation but also with reversion of tumor cells to nonmalignant cells (12) emphasizes the role of proteases in tumorigenesis in modification of the surface membranes. Lysosomal enzymes other than proteases (1), released as a result of labilization of lysosomal membranes by the actions of proteases, may also be involved in the mechanisms of tumorigenesis.

Details of the contributions of proteases to the process of tumorigenesis remain to be elucidated.

REFERENCES

1. Allison, A. C. Lysosomes and Cancer. *In:* J. T. Dingle and H. B. Fell (eds.), Lysosomes in Biology and Pathology, Vol. 2, pp. 178–204. Amsterdam: North Holland Publishing Co., 1969.
2. Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishizuka, M., Takeuchi, T., and Umezawa, H. Biological Activities of Leupeptins. J. Antibiotics Tokyo Ser. A, *22:* 558–568, 1969.
3. Aoyagi, T., Takeuchi, T., Matsuzaki, A., Kawamura, K., Kondo, S., Hamada, M., Maeda, K., and Umezawa, H. Leupeptins, New Protease Inhibitors from *Actinomycetes.* J. Antibiotics Tokyo Ser. A, *22:* 283–286, 1969.
4. Barnhart, M. I., Quintana, C., Lenon, H. L., Blyhm, G. B., and Riddle, J. M. Proteases in Inflammation. Ann. N. Y. Acad. Sci., *146:* 527–539, 1968.
5. Belman, S., and Troll, W. The Inhibition of Croton Oil-promoted Mouse Skin Tumorigenesis by Steroid Hormones. Cancer Res., *32:* 450–454, 1972.
6. Bogart, B., Prutkin, L., and Ocken, P. R. The Localization of Phorbol Ester ^{14}C-Acetate in Papillomas That Were Initiated with 7,12-DMBA and Promoted with Phorbol Ester. J. Invest. Dermatol., *56:* 140–146, 1971.
7. Bosmann, H. B., and Pike, G. Z. Glycoprotein Synthesis and Degradation: Glycoprotein: *N*-Acetylglucosamine Transferase, Proteolytic and Glycosidase Activity in Normal and Polyoma Virus Transformed BHK Cells. Life Sci., *9:* 1433–1440, 1970.
8. Burger, M. M. A Difference in the Architecture of the Surface Membrane of Normal and Virally Transformed Cells. Proc. Natl. Acad. Sci. U. S., *62:* 994–1001, 1969.
9. Davies, P., Rita, G. A., Krakauer, K., and Weissmann, G. Characterization of a Neutral Protease from Lysosomes of Rabbit Polymorphonuclear Leucocytes. Biochem. J., *123:* 559–569, 1971.
10. Dicken, C. H., and Decker, R. H. Biochemical Evidence for the Presence of Lysosomes in the Epidermis. J. Invest. Dermatol., *47:* 426–431, 1966.
11. Hestrin, S. The Reaction of Acetylcholine and Other Carboxylic Acid Derivatives with Hydroxylamine, and Its Analytical Application. J. Biol. Chem., *180:* 249–261, 1949.
12. Hozumi, M., Miyake, S., Mizunoe, F., Sugimura, T., Irie, F. R., Koyama, K., Tomita, M., and Ukita, T. Surface Properties of Non-Tumorigenic Variants of Mouse Mammary Carcinoma Cells in Culture. Intern. J. Cancer, *9:* 393–401, 1972.
13. Inbar, M., Ben-Bassat, H., and Sachs, L. A Specific Metabolic Activity on the Surface Membrane in Malignant Cell-Transformation. Proc. Natl. Acad. Sci. U.S., *68:* 2748–2751, 1971.
14. Janoff, A., Klassen, A., and Troll, W. Local Vascular Changes Induced by the Cocarcinogen, Phorbol Myristate Acetate. Cancer Res., *30:* 2568–2571, 1970.
15. Kawamura, K., Kondo, S., Maeda, K., and Umezawa, H. Structure and Synthesis of Leupeptins Pr-LL and Ac-LL. Chem. Pharm. Bull. Tokyo, *17:* 1902–1909, 1969.
16. Klassen, A., Janoff, A., and Troll, W. Possible Role of a Neutral Protease in Tumor-Promotion by Phorbol Ester. Proc. Am. Assoc. Cancer Res., *11:* 44, 1970.
17. Kondo, S., Kawamura, K., Iwanaga, J., Hamada, M., Aoyagi, T., Maeda, K., Takeuchi, T., and Umezawa, H. Isolation and Characterization of Leupeptins Produced by *Actinomycetes.* Chem. Pharm. Bull. Tokyo, *17:* 1896–1901, 1969.
18. Kreibich, G., Witte, I., and Hecker, E. On the Biochemical Mechanism of Tumorigenesis in Mouse Skin. IV. Methods for Determination of Fate and Distribution of Phorbol Ester TPA. Z. Krebsforsch., *76:* 113–123, 1971.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., *193:* 265–275, 1951.
20. Salaman, M. H., and Roe, F. J. C. Cocarcinogenesis. Brit. Med. Bull., *20:* 139–144, 1964.
21. Shamberger, R. J. Increase of Lysosomal Enzymes in Skin Cancer. Nature, *213:* 617–618, 1967.
22. Shamberger, R. J. Inhibitory Effect of Vitamin A on Carcinogenesis. J. Natl. Cancer Inst., *47:* 667–673, 1971.
23. Sivak, A., Ray, F., and Van Duuren, B. L. Phorbol Ester Tumor-Promoting Agents and Membrane Stability. Cancer Res., *29:* 624–630, 1969.
24. Sivak, A., and Van Duuren, B. L. Phenotypic Expression of Transformation: Induction in Cell Culture by a Phorbol Ester. Science, *157:* 1443–1444, 1967.
25. Sivak, A., and Van Duuren, B. L. Cellular Interactions of Phorbol Myristate Acetate in Tumor Promotion. Chem.-Biol. Interactions, *3:* 401–411, 1971.
26. Troll, W., Klassen, A., and Janoff, A. Tumorigenesis in Mouse Skin: Inhibition by Synthetic Inhibitors of Proteases. Science, *169:* 1211–1213, 1971.
27. Van Duuren, B. L. Tumor-Promoting Agents in Two-Stage Carcinogenesis. Progr. Exptl. Tumor Res., *11:* 31–68, 1969.
28. Weissmann, G., Troll, W., Van Duuren, B. L., and Sessa, G. A New Action of Tumor-Promoting Agents: Lysis of Cell and Intracellular Membranes. J. Clin. Invest., *46:* 1131, 1967.