Kinin-Forming Activity of Cultured Mouse Fibroblasts L-929[1] (37410)

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Components of a vasopeptide kinin-forming system previously were found in the Ehrlich ascites tumor (1) and the transplanted rat Murphy-Sturm lymphosarcoma (MSLS) (2). Crude extracts of the MSLS tissue contained kininogen substrate, a kinin-destroying enzyme, and a prekallikrein capable of being activated by acetone at pH 7.8 This kallikrein utilized human kininogen substrate but did not form kinin when incubated with the tumor kininogen. Subsequent studies revealed that the tumor extracts also contained a kinin-forming system, active at acid pH (pH 4–5) and capable of digesting the tumor kininogen substrate (3).

The MSLS acid kinin-forming system was similar to that found previously by Greenbaum *et al.* in polymorphonuclear (PMN) leukocytes (4–7) and malignant cells (6, 7), with respect to pH optimum, effect of inhibitors, and nature of the active form. However, whereas the PMN kinin-forming activity was found localized in the nuclear membrane debris fraction, the MSLS acid kinin-forming activity was shown to be present in the soluble protoplasmic protein fraction (3). Acid kinin-forming activity also was present in extracts of the rat Walker carcinoma and rabbit adenocarcinoma (unpublished data).

Since the Murphy-Sturm lymphosarcoma had a considerable network of fibroblasts, the possibility was suggested that cellular elements other than tumor cells may have contributed to the kinin-forming activity observed in the tumor. Thus, a study was undertaken to explore the kinin-forming potential of a pure line of rodent fibroblasts grown in stationary cell culture.

Methods. Cell culture. Mouse fibroblasts L-929, originating from Microbiological Associates (Bethesda, MD), was obtained through the courtesy of Dr. J. Kite (Dept. of Microbiology, State University of New York, Buffalo). The cells were grown for 4 days either in roller bottles or flasks in minimum Eagle's medium containing 10% fetal calf serum. At the time of study, the cells were scraped free from the flask wall, suspended in minimum Eagle's medium, the cell number determined by microscopic count, and centrifuged at 3500 rpm. The cells then were resuspended in physiological saline at a concentration of 6×10^6/ml saline and disrupted by freeze-thaw technique repeated 10 times. Aliquot quantities of 0.5 ml of this suspension approximating 3×10^6 cells were used for the assays.

Kininogen substrates. Both rat plasma and MSLS tissue kininogen substrate were used throughout the study. Tumor substrate was prepared by homogenizing 1 g of tumor per ml saline for 5 min in a Servall homogenizer placed in an ice bath. The homogenate was centrifuged at 3500 rpm for 30 min in a refrigerated centrifuge, the supernatant collected and heated up to 80° in a water bath. The preparation was centrifuged once again at 3500 rpm for 30 min and lyophilized in a Vortis lyophilizer. No attempt was made to purify the preparation further. For use, 30 mg of this substrate preparation was suspended in 0.5 ml of a 1 *M* acetic acid/acetate buffer, pH 4.0. Citrated rat plasma, obtained by cardiac puncture from an anesthetized rat, was used as a source of plasma kininogen. At pH 4.0, neither the rat plasma nor tumor substrate preparation alone generated any kinin activity in control tubes.

Kinin-forming activity of fibroblasts. 0.5 ml of L-cell suspension was incubated with 0.5 ml of tumor substrate (30 mg protein) for

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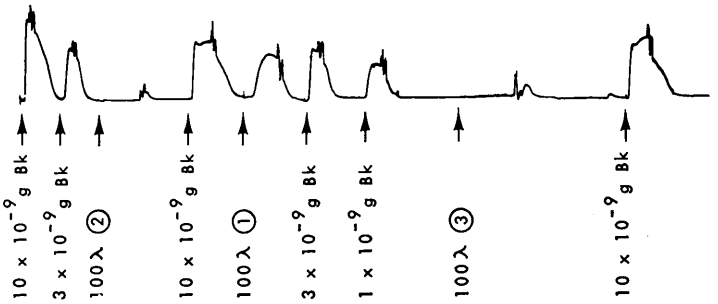

FIG. 1. Smooth muscle stimulating activity (rat uterus) formed during 60 min incubation of fibroblasts L-cells with substrate from Murphy-Sturm lymphosarcoma. Bk is bradykinin control. 1 = L-cells + substrate; 2 = L-cells + buffer; 3 = saline + substrate.

60 min at pH 4.0, 37°. (Initially, 1 mg of 1,10-phenanthroline was added to inhibit any kininase activity. However, this addition was unnecessary since it was found that incubation of the L-cells for 30–60 min at pH 4.0 destroyed all kininase activity when tested against synthetic bradykinin (Sandoz). Furthermore, the kininase activity was not regenerated when the suspensions were readjusted to neutral or alkaline pH following incubation at acid pH). Following incubation, 0.5 ml of 1 *M* Tris buffer was added to bring the pH up to 7.8. One-hundred microliter aliquots were studied for smooth muscle stimulating activity on the isolated rat uterus preparation perfused with modified Tyrodes solution at room temperature (8). Similar assays were conducted with the L-cell suspension using 0.2 ml rat plasma, to which was added 0.3 ml acetic acid/acetate buffer. Throughout the studies synthetic bradykinin (Sandoz) was used as a reference standard. The polypeptide character of the smooth muscle stimulating activity was ascertained by incubation of the samples showing kinin activity with 10 μg of carboxypeptidase B (Worthington Labs, Freehold, NJ).

pH Profile. The pH profile of the kinin-forming activity was determined by incubating 0.5 ml of the L-cell suspensions with 0.5 ml of tumor substrate at a pH ranging from 2 to 8 by individual adjustment of the pH with 0.1 *N* NaOH or 0.1 *N* HCl. One milligram of 1,10-phenanthroline was added to all tubes for uniformity, since the L-cell kininase was active at neutral and alkaline pH. Control studies showed 1,10-phenanthroline to be an effective inhibitor of the L-cell kininase activity.

Time and concentration study. The time course for kinin formation at pH 4.0 and 37° was studied by incubating the L-cell-tumor substrate mixture for 30, 60, 90, 120, and 180 min. The effect of increasing concentrations of L-cells on kinin formation also was studied following 120 min incubation at pH 4.0, 37°. L-cell concentrations used were 0.375×10^6, 0.75×10^6, 1.5×10^6, and 3×10^6.

Inhibitor study. Various natural and synthetic inhibitors were studied for their effect on the kinin-forming activity of the L-cell suspensions. Maximum concentrations of the inhibitors contained in 0.1 ml volume were incubated for 120 min together with 0.5 ml L-cell suspension and 0.5 ml tumor substrate at pH 4.0 and 37°, and then 100-μl aliquots assayed for kinin activity. For comparison, the effect of the same inhibitors on the kinin-forming activity of MSLS extracts (at kinin-forming activities equivalent to the L-cells) was studied. Inhibitors studied included soya bean trypsin inhibitor (SBTI),[2] lima bean trypsin inhibitor (LBTI),[2] ovomucoid trypsin inhibitor,[2] TRASYLOL,[3] heparin,[4] epsilon aminocaproic acid (EACA),[5] 4-aminoethylcyclohexane-1-carboxylic acid (AMCHA),[5] and phenylpyruvic acid[6]. A dose-response study with phenylpyruvic acid also was conducted.

Cell fractionation. Ultracentrifugal cell

[2] Worthington Labs, Freehold, NJ.

[3] Farbenfabriken Bayer AG, Leverkusen, Germany.

[4] Pan-Heparin, Abbott Labs, Chicago, IL.

[5] Lederle Labs, Pearl River, NY.

[6] Cal Biochem, Los Angeles, CA.

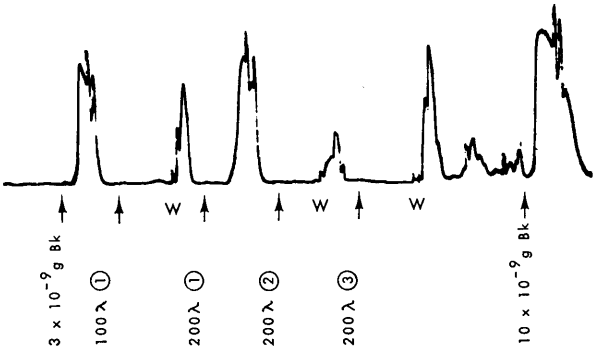

FIG. 2. Smooth muscle stimulating activity (rat uterus) formed during 120 min incubation of fibroblast L-cells with substrate from rat plasma. Bk is bradykinin control. 1 = L-cells + plasma substrate; 2 = buffer + plasma substrate; 3 = L-cells + buffer; w = wash.

fractionation studies were carried out to identify the cellular localization of the kinin-forming activity. Sixty-million L-cells were spun down at 3500 rpm, the cell pellet retained and resuspended in 10 ml of 0.25 *M* sucrose containing 0.00018 *M* $CaCl_2$. This cell suspension was frozen and thawed 10 times, and respun at speeds of 600*g* for 10 min (nuclear fraction), 20,000*g* for 30 min (mitochondrial fraction), and 50,000*g* for for 120 min (microsomal fraction). The remainder constituted the soluble protoplasmic protein fraction. Aliquots of these fractions were assayed with tumor substrate for kinin-forming activity as described above.

Results. Kinin-forming activity of L-cells. Figure 1 shows the smooth muscle stimulating activity formed during the 60-min incubation of L-cells with the tumor substrate. Approximately 1.0 ng of kinin activity per mg of substrate was generated based on a comparison with synthetic bradykinin. Thus, a total of 30 ng of kinin was formed from the 30 mg substrate present in the tube. It will be seen (Fig. 5) that 150 ng of kinin is formed from that amount of substrate when the incubation time is increased to 180 min under the same conditions. Appropriate cell and substrate controls did not have any kinin activity. The same concentration of L-cells released 112.5 ng kinin per ml rat plasma after 120 min incubation period (Fig. 2). Incubation of the L-cell-substrate mixture with 10 μg of carboxypeptidase B destroyed the smooth muscle stimulating activity (Fig. 3). The fibroblast suspension did not contain kininogen substrate but did have kinin-destroying activity that was inactivated at acid pH as well as by 1,10-phenanthroline.

pH Profile. The cell-tumor substrate mixture formed kinin only at pH 4.0 following 120 min incubation at 37° (Fig. 4). At this incubation time period, a total of 90 ng activity was generated in the incubation tube (3 ng/mg substrate). The possibility that the kinin-forming enzyme destroyed kinin was excluded by control studies, where-

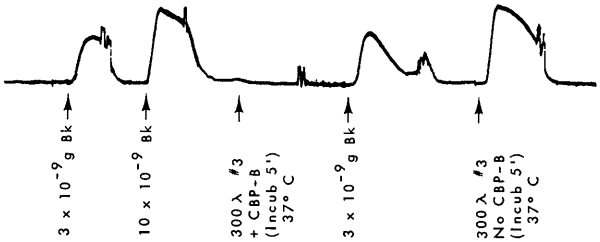

FIG. 3. Effect of carboxypeptidase B (CBP-B) on smooth muscle stimulating activity formed following 60 min L-cell incubation with Murphy-Sturm lymphosarcoma substrate. Bk is bradykinin control.

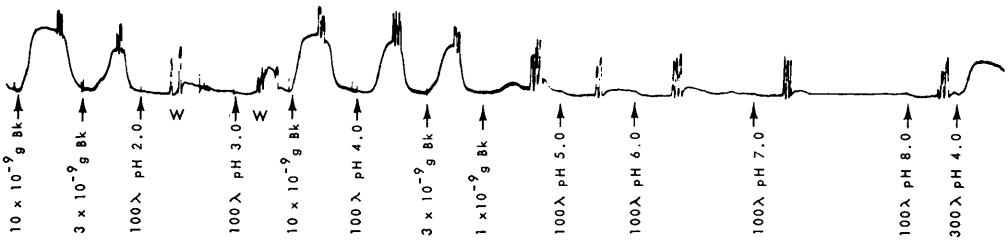

FIG. 4. pH Profile of kinin-forming activity in L-cell suspension incubated with Murphy-Sturm lymphosarcoma substrated for 60 min at 37°. Activity was noted only at pH 4.0. Bk = bradykinin; w = wash.

by full recovery of synthetic bradykinin was achieved after incubation with the L-cell suspension for 120 min at pH 4.0.

Time and concentration study. Incubation of the L-cells with tumor substrate at pH 4.0 for increasing time periods yielded increasing amounts of kinin (Fig. 5). A linear relationship between kinin formed and time of incubation was obtained within the 180-min time period studied. At the highest incubation time period, maximum kinin formation had not been achieved. An amount of activity equivalent to 150 ng of kinin per incubation mixture was generated. A linear relationship also obtained with respect to the concentration of L-cells used in the incubate with tumor substrate. Increasing concentrations of L-cell yielded increased amounts of kinin when incubated for 120 min (Fig. 6). Total amount of kinin formed in the incubate was 120 ng (4 ng/mg substrate).

Inhibitor study. None of the classical natural or synthetic protease inhibitors at the concentrations studied affected signifi-

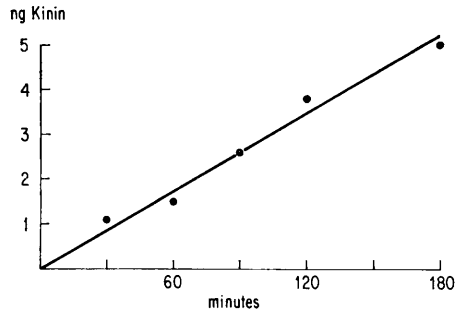

FIG. 5. Time course for kinin formation following incubation of L-cell suspension with Murphy-Sturm lymphosarcoma substrate at pH 4.0 and 37°. (Nanograms kinin released per milligram substrate.)

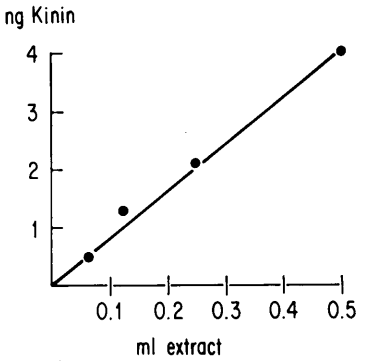

FIG. 6. Effect of varying concentrations of L-cells on kinin formation following 120-min incubation with Murphy-Sturm lymphosarcoma at pH 4.0, 37°. Each milliliter L-cell extract contained 6 × 10^6 cells. (Nanograms kinin released per milligram substrate.)

cantly the kinin-forming activity of the L-cells at pH 4.0 (Fig. 7). The results were were comparable to those obtained with the extracts of the MSLS. However, phenylpyruvic acid, at a concentration of 0.006 *M*, did inhibit the kinin-forming activity of both the L-cells and MSLS. This inhibitory effect was dose related (Fig. 8) and was obtained also with the MSLS (2).

Cell fractionation. Ultracentrifugal studies show the major portion of the kinin-forming activity (76.6%) in the soluble protoplasmic protein fraction of the L-cell (Fig. 9). Minor activity was seen in the nuclear fraction (6.1%), while the mitochondrial and microsomal fractions contained less than 1%. A total of 84.1% of the activity was recovered from these fractions.

Discussion. This line of cultured fibroblasts was shown to have kinin-forming activity at an acid pH, resembling the

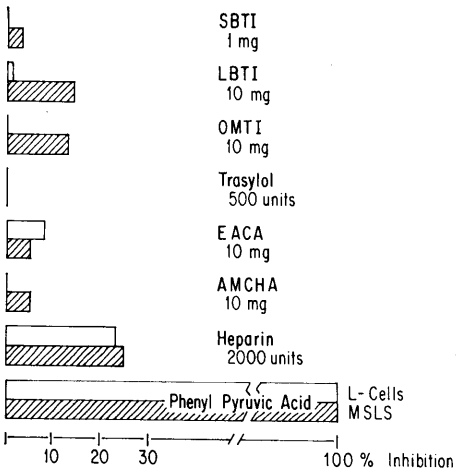

FIG. 7. Effect of protease inhibitors on kinin-forming activity of L-cells and Murphy-Sturm lymphosarcoma (MSLS). Substrate was from MSLS. SBTI = soya bean trypsin inhibitor; LBTI = lima bean trypsin inhibitor; OMTI = ovomucoid trypsin inhibitor; EACA = epsilon amino caproic acid; AM CHA = 4-aminoethylcyclohexane-1-carboxylic acid.

activity present in the MSLS extracts with regard to optimum pH, kinetics of kinin formation, nature of the active enzyme form, action of protease inhibitors, and site of cell localization. Acid proteinases acting on other substrates as hemoglobin and artificial esters have been reported in tumor interstitial fluid (9), human gastric carcinoma (10), renal carcinoma (11), and Jensen sarcoma (12).

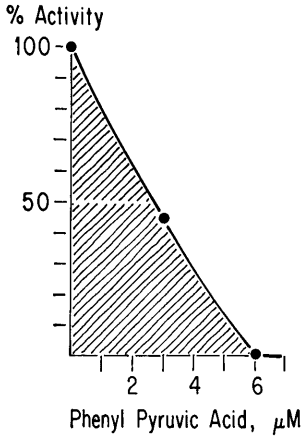

FIG. 8. Dose-response relationship of the inhibitory activity of phenyl pyruvic acid on kinin-forming activity of L-cells. 100% activity represents no inhibition.

While acid proteases in PMN cells were described previously (13–15), Greenbaum and Yamafuji were the first to report on the kinin-forming activity of acid proteases from PMN cells (4). They provided a comparison of the kinin-forming activity from PMN cells and mouse leukemia L-1210 cells (6). A summary of their studies has been published recently (7). Studies with the acid kinin-forming system in the MSLS tissue and mouse fibroblasts reported here show the system to be comparable in nearly all aspects with that reported by Greenbaum. The proteinase activity is the same with regard to pH optimum, nature of the active form, and action of inhibitors. Like the PMN leuko-

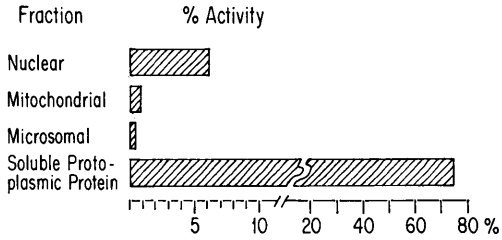

FIG. 9. Cellular localization of kinin-forming activity of L-cells.

kininogenase, the MSLS acid proteinase utilizes kininogen substrates from human plasma only when the substrate is made available by warming the plasma to 57° for 15 min (7). However, while the main cellular location of the leukokininogenase is in the nuclear membrane debris fraction (low-speed fraction), the activity of both the fibroblasts and MSLS tissue was found in the soluble protoplasmic protein fraction.

It is known that transplanted tumor cells, during the initial stage of growth, induce fibroblast proliferation at the transplant subcutaneous site of the host (16). The continued growth of solid tumors would not appear to depend upon the presence of fibroblasts since fibroblast growth is suppressed as tumor cell growth becomes more luxuriant. However, Yoshida tumor cells of the rat can be maintained *in vitro* only in the presence of normal rat fibroblasts (17). Bovine fibroblasts contain a number of neutral peptide

hydrolases (18).

It has been postulated previously that acid proteinases in tumor tissue might utilize appropriate tumor or plasma substrates to provide a continued source of smaller peptides for tumor growth (3). Some formed peptides with vasoactivity could influence the vasculature during tumor growth or alter cell membrane permeability when intracellular conditions dictate, as in the production of an unfavorable tissue acid environment. Thus, a mechanism may be provided for the correction of intracellular conditions that may influence tumor growth.

The ability of fibroblasts to generate kinin under controlled conditions has been demonstrated. Further study is required to purify this kinin-forming enzyme, and elucidate its biochemical profile. It may be suggested that in a heterogenous cell population, as in a solid tumor, acid proteinases from several types of cells may utilize available substrates for smaller peptide and kinin formation.

Summary. The kinin-forming potential of a cultured cell line of mouse fibroblasts, L-929, has been studied. The fibroblasts were found to form kinin at acid pH from both rat plasma and Murphy-Sturm lymphosarcoma kininogen substrate preparations. The kinin formed was destroyed following incubation with carboxypeptidase B. The kinetics of kinin formation relative to incubation time and concentration of fibroblasts also were studied. None of the conventional plant, mammalian, or synthetic protease inhibitors had any significant effect on the kinin-forming activity of the fibroblasts. Phenylpyruvic acid, however, did inhibit this activity. The activity appeared localized in the soluble protoplasmic portion of the cell, as determined by ultracentrifugal technique.

1. LeBlanc, P. P., and Back, N., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 807 (1970).

2. Back, N., and Steger, R., *in* "Bradykinin and Related Peptides" (F. Sicuteri, M. Rocha e Silva, and N. Back, eds.), Vol. 8, p. 225. Plenum Press, New York (1970).

3. Back, N., and Steger, R., *in* "Vasopeptides: Chemistry, Pharmacology and Pathophysiology" (N. Back, and F. Sicuteri, eds.), p. 417. Plenum Press, New York (1972).

4. Greenbaum, L. M., and Yamafuji, K., *in* "Hypotensive Peptides" (E. G. Erdos, N. Back, and F. Sicuteri, eds.), p. 252. Springer Verlag, New York (1966).

5. Greenbaum, L. M., and Kim, K. S., Brit. J. Pharmacol. **29**, 238 (1967).

6. Greenbaum, L. M., Freer, R., Chang, J., Semento, G., and Yamafuji, K., Brit. J. Pharmacol. **36**, 623 (1969).

7. Greenbaum, L., Amer. J. Pathol. **68**, 119 (1972).

8. Back, N., and Steger, R., Proc. Soc. Exp. Biol. Med. **133**, 740 (1970).

9. Sylven, B., and Bois-Svensson, I., Cancer Res. **25**, 458 (1965).

10. Etherington, D. J., and Taylor, W. H., Biochem. J. **115**, 43 (1969).

11. Schersten, T., Wahlquist, L., and Johansson, L. G., Cancer **23**, 608 (1969).

12. Kampschmidt, R. F., and Wells, D., Cancer Res. **28**, 1938 (1968).

13. Cohn, Z., and Hirsch, J., J. Exp. Med. **112**, 983 (1960).

14. Lapresle, C., and Webb, T., Biochem. J. **84**, 455 (1962).

15. Wasi, S., Murray, R., Macmorine, D., and Movat, H., Brit. J. Exp. Pathol. **47**, 411 (1966).

16. Grossfeld, H., Crossley, M. L., and Allison, J. B., Cancer Res. **23**, 770 (1963).

17. Lettre, R., *in* "Cancer Cells in Culture" (H. Katsuto, ed.), p. 103. Univ. of Tokyo Press, Tokyo (1968).

18. Schwabe, C., Biochemistry **8**, 771 (1969).

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