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Kinin-Destroying (Kininase) Activity of Cultured Rodent Fibroblasts L-929[1, 2] (39505)

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Extracts of a cultured cell line of rodent fibroblasts, L-929, were reported to have kinin-forming activity when incubated at acid pH with kininogen substrate from rat plasma or Murphy-Sturm lymphosarcoma tissue (1). The kinin-forming acid protease was purified and further characterized with regard to pH optimum, cellular localization, and molecular weight (2). While the fibroblast preparation did not contain kininogen substrate, it did have kinin-destroying activity that was inactivated at acid pH as well as by 1,10-phenanthroline (1). In view of the possible involvement of the vasopeptide kinin system in the growth and development of transplanted tumors containing considerable fibroblast networks (3), the nature of this kinin-destroying activity of fibroblasts was studied further and forms the basis of this report.

Methods. Cell culture and cell extract preparation. Mouse fibroblasts L-929, obtained from Microbiological Associates (Bethesda, Maryland), were grown for 4 days in roller bottles in minimum Eagle's medium containing 10% fetal calf serum as described previously (1). The cells were scraped from the bottle wall at the time of study, suspended in minimum Eagle's medium, the cell number was determined by microscopic examination, and the cell suspension was centrifuged in a refrigerated centrifuge at 3500 rpm. The cells then were resuspended in physiologic saline and disrupted by freeze-thaw technique repeated 10 times. Aliquot volumes of 0.2-0.5 ml of the cell extract, approximating 6×10^6 cells, were used for the assays.

Kinin-destroying activity. Fibroblast suspensions (0.2-0.5 ml) were incubated at 37° with 0.5 ml of synthetic bradykinin (1×10^{-6} g, Sandoz, Hanover, New Jersey) dissolved in 0.05 *M* phosphate buffer, pH 7.4. Additional buffer was added to 1 ml when indicated, and at 1-, 3-, and 5-min intervals, 1 mg of 1,10-phenanthroline was added to individual incubation mixtures to terminate the reaction. One hundred-microliter aliquots were studied for residual bradykinin activity on the isolated rat uterus muscle preparation perfused at room temperature with modified Tyrodes solution (4). Control samples contained only buffer and synthetic bradykinin. The 10% fetal calf serum was prepared commercially by heating for 1 hr at 56°. Heating of a carboxypeptidase *N* preparation for 5 min at 56° was reported to decrease the kinin-destroying activity by 80% (5). Incubation of 0.5 ml of the 10% fetal calf serum with 1×10^{-6} g of bradykinin confirmed the fact that the serum per se did not have any kinin-destroying activity. Throughout the study, synthetic bradykinin was used as the reference standard.

pH Profile. The pH profile of the kinin-destroying activity was determined by incubating 0.5 ml of the cell extract with 1×10^{-6} g of bradykinin in 0.5 ml of various buffers ranging in pH from 4.0 to 10.0. The mixtures were incubated for 5 min at 37°, and the reaction was terminated by the addition of 1 mg of 1,10-phenanthroline. The residual kinin activity was assayed on the isolated rat uterus, and the percentage loss of smooth muscle stimulating (kinin) activity was calculated by the following formula:

$$\frac{\text{Activity}_{\text{standard}} - \text{Activity}_{\text{experimental}}}{\text{Activity}_{\text{standard}}} \times 100.$$

Time and concentration study. The time course for kinin destruction at pH 7.0 and 37° was studied by incubating the cell extract-bradykinin mixture for 1-min time intervals up to 5 min. The effect of increasing

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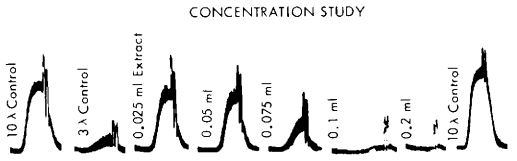

FIG. 1. Smooth muscle-stimulating activity of incubates containing 1×10^{-6} g of synthetic bradykinin and increasing volumes of fibroblast cell extracts (0.025–0.2 ml). Control activity of 10λ and 3λ bradykinin (following incubation for 5 min with phosphate buffer) is shown.

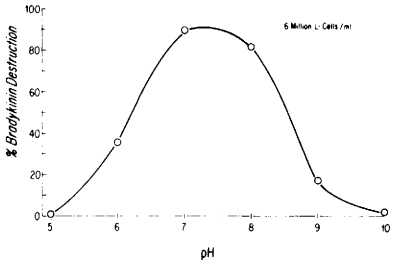

FIG. 2. pH Profile of kinin-destroying (kininase) activity of fibroblast cell extract (0.5 ml) incubated with 1×10^{-6} g of synthetic bradykinin for 5 min at 37°. The reaction was terminated by the addition of 1 mg of 1,10-phenanthroline.

volumes of cell extract on kinin destruction (from 0.02 to 0.20 ml) also was studied following 5-min incubation with synthetic bradykinin (1×10^{-6} g) at pH 7.0 and 37°.

Inhibitor study. The following natural and synthetic inhibitors were studied for their effect on the kinin-destroying activity of the fibroblast cell extract: aprotinin (Trasylol, Farbenfabriken Bayer AG, Leverkusen, Germany), soya bean trypsin inhibitor (SBTI, Worthington Labs, Freehold, New Jersey), lima bean trypsin inhibitor (LBTI, Worthington), heparin (Pan-Heparin, Abbott Labs, Chicago, Illinois), epsilon aminocaproic acid (EACA, Lederle Labs, Pearl River, New York), 4-aminoethylcyclohexane-1-carboxylic acid (AMCHA, Lederle Labs), 2,3-dimercaptopropanol (BAL, Cal Biochem, Los Angeles, California), and 1,10-phenanthroline (Cal Biochem). Increasing concentrations of inhibitor in 0.1 ml of volume were added to 0.2 ml of cell extract and 0.2 ml of phosphate buffer, pH 7.0. The cell extract–inhibitor preparations were allowed to stand for 5 min at room temperature, and then 0.5 ml of bradykinin (1×10^{-6} g in a phosphate buffer) was added. The mixture was agitated and the tubes were placed in a 37° water bath for an additional 5 min. The reaction was stopped by placing the tubes on ice, and the residual kinin in 100-μl aliquots was assayed on the isolated rat uterus. Control tubes contained the cell extract, buffer, and synthetic bradykinin. The extent of inhibition was recorded as percentage of residual kinin activity compared to the standard control tubes.

Cell fractionation. Ultracentrifugal cell fractionation studies were carried out to identify the subcellar site of the kininase activity (6, 7). Fibroblast cells (60×10^6) were suspended in 10 ml of 0.25 *M* sucrose–1.8×10^{-4} *M* $CaCl_2$ solution. The suspension was frozen and thawed 10 times to disrupt the cells and spun at 600*g* for 10 min to obtain the sedimented nuclear fraction. The supernatant yielded a mitochondria pellet fraction when centrifuged at 10,000*g* for

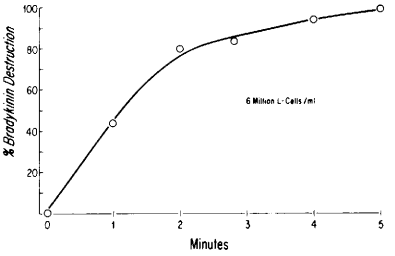

FIG. 3. Time course of kinin-destroying activity following incubation of 0.2 ml of fibroblast cell extract (1.2×10^6 cell equivalent) with 1×10^{-6} g of synthetic bradykinin at 1- to 5-min time intervals at 37° and pH 7.0.

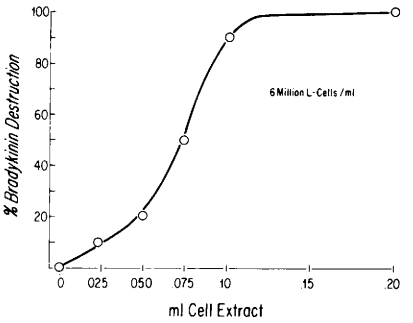

FIG. 4. Effect of increasing concentrations of fibroblast cell extract on the destruction of synthetic bradykinin (1×10^{-6} g) following incubation for 5 min at 37° and pH 7.0.

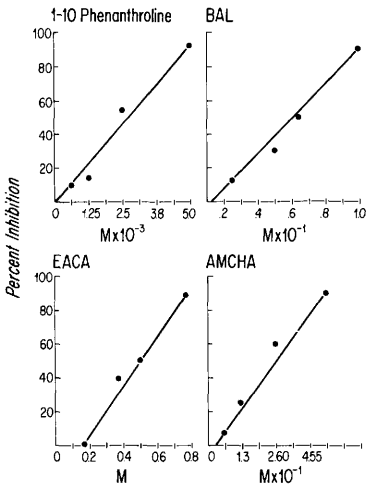

FIG. 5. Effect of 1, 10-phenanthroline, 2,3-dimercaptopropanol (BAL), epsilon amino caproic acid (EACA), and 4-aminoethylcyclohexane-1-carboxylic acid (AMCHA) on the kinin-destroying activity of fibroblast cell extracts. Two-tenths of a milliliter of extract was incubated with increasing concentrations of inhibitors for 5 min, to which was added thereafter 1×10^{-6} g of bradykinin for an additional 5 min.

TABLE 1. CELLULAR LOCALIZATION OF KININ-DESTROYING ACTIVITY OF L-929 FIBROBLAST CELLS.

Cell fraction	Total protein (%)	Kininase activity (%)
Whole cell homogenate	100	100
Nuclear (600*g*)	12.3	20.0
Mitochondrial (20,000*g*)	4.9	5.0
Microsomal (100,000*g*)	1.9	0.0
Soluble Protoplasmic (100,000*g*)	52.0	64.2

30 min and a microsome fraction after centrifugation at 100,000*g* for 60 min. The remaining supernatant constituted the soluble protoplasmic protein fraction. Cell fraction aliquots of 0.2–0.4 ml were incubated with 1×10^{-6} g of bradykinin to determine the kininase activity as described above.

Results. Kinin-destroying activity of fibroblast extracts. Figure 1 shows the smooth muscle stimulating responses of the incubation mixtures containing 1×10^{-6} g of bradykinin with increasing volume aliquots of fibroblast extracts. The incubations were carried out at pH 7.4 for 5 min at 37°. The incubation mixtures demonstrated increased kinin-destroying activity, while control tubes containing only bradykinin and buffer retained undiminished kinin activity after the 5-min incubation period.

pH Profile. Optimum kinin-destroying activity occured at a neutral pH of 7.0–7.4, Fig. 2, while no kinin-destroying activity was observed at a pH of 5 or lower and at pH 10.0. Thus, activity ranged over a pH of 6.0–9.0. Residual kinin activity in the bradykinin–extract incubation mixtures was compared with control tubes containing bradykinin and buffer and incubated under the same time and pH conditions.

Time and concentration study. Incubation of 0.2 ml of the fibroblast cell extract with 1×10^{-6} g of bradykinin at 37° and pH 7.0 for increasing time intervals resulted in total destruction of the kinin activity within 5 min, Fig. 3. Fifty percent of the kinin activity was destroyed following 1.2 min of incubation whereas almost 80% was destroyed after 2 min. Increasing concentrations of fibroblast extract (0.02–0.2 ml) resulted in increased kinin destruction when incubated with 1×10^{-6} g of bradykinin for 5 min at 37° and pH 7.0, Fig. 4. An aliquot of 0.07 ml of extract caused 50% destruction, while 0.1 ml destroyed 90% of the kinin.

Inhibitor study. The inhibitory profiles of four agents that inhibited the kinin-destroying activity are represented in Fig. 5. The agents included the classical kininase inhibitor 1,10-phenanthroline (ID_{50}, 4.375×10^{-3} *M*); BAL (ID_{50}, 0.64×10^{-1} *M*); and two weak inhibitors, EACA (ID_{50}, 0.5 *M*) and AMCHA (ID_{50}, 0.238 *M*). At the highest doses studied (indicated in the parentheses), the following agents had no effect on the fibroblast kinase activity: aprotinin (1000 units), soya bean trypsin inhibitor (10 mg), lima bean trypsin inhibitor (10 mg), and heparin (2000 units).

Cell fractionation. Ultracentrifugal cell fractionation showed the soluble protoplasmic protein fraction to contain 64.2% of the kininase activity, while the nuclear portion had 20% and the mitochondria fraction had only 5%, Table 1. The microsome fraction did not contain any activity. Approximately 90% of the total kinin-destroying activity was recovered from these cell fractions.

Discussion. This mouse fibroblast cell line was shown previously to have an acid pro-

tease capable of forming kinins from suitable substrates (1). Purification and further characterization of this enzyme have been achieved (2). The present study has focused on the kinin-destroying activity of this fibroblast cell line (1). This activity was shown to have a pH optimum at neutrality, similar to the activity reported in extracts of rabbit and human leukocytes (8, 9), kidney (10, 11), rat liver (12), and rabbit brain (13).

Studies with the inhibitor suggest that the fibroblast kinin-destroying activity is metal dependent. Both 1,10-phenanthroline, an effective metal-dependent kininase inhibitor (11), and 2,3-dimercaptopropanol (BAL) blocked the fibroblast kinin-destroying activity in a dose-dependent fashion. BAL has been reported to inhibit rat plasma kininase activity *in vitro* (14). The proteinase inhibitor aprotinin and the soya and lima bean trypsin inhibitors were not effective against the fibroblast kinin-destroying activity similar to previously published reports with activities from other tissue sources (15). Aprotinin did inhibit the kininase activity of rabbit brain (16) and rabbit granulocyte extracts (17). The synthetic agents EACA and AMCHA were found to be very weak inhibitors of the fibroblast kinin-destroying activity. EACA, at concentrations of 3×10^{-3} M, did inhibit the kinin-destroying activity of human plasma but not that of hemolyzed human red blood cells or guinea pig serum (15).

The major portion of the kinin-destroying activity resided in the soluble protoplasmic protein fraction of the fibroblast cell (obtained after centrifugation at 100,000g for 1 hr). High kinin-destroying activity has been reported in the soluble final supernatant fraction of homogenized rat liver (18), human granulocytes (9), rabbit brain (16, 19), and rat brain (20). Activity also has been found among particulate fractions of rabbit brain cell homogenates (19) and in a cell fraction of rat kidney homogenate that sedimented with the microsomal fraction (11).

Thus, this fibroblast cell line has both kinin-forming (1, 2) and kinin-destroying potential. These studies were undertaken following the observation that transplanted rodent tumors, with a mixed tumor cell-fibroblast cell population, have both an alkaline and acid kinin-forming enzyme and kininase activity (3, 21). Present data suggest that the fibroblast cell contributes to the overall kinin-enzyme system activity associated with the tumor tissue. While the role of fibroblasts in tumor growth and development awaits clarification, their proliferation at tumor transplant sites (22) and localization between the growing edges of necrotic areas in solid tumor transplants (23) would suggest some relationship with tumor growth mechanisms. Further study is required to establish whether these mechanisms are related to the vasopeptide kinin protease system present in both the fibroblasts and tumor tissue. Reports of tumor inhibition by protease inhibitors (24, 25) provides indirect evidence for a possible role of proteases in malignancy.

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