Europ. J. Cancer Vol. 16, pp. 1257–1261.

0014-2964/80/0901-1257 $02.00/0

Proteoglycan- and Fibrin-Degrading Neutral Proteinase Activities of Lewis Lung Carcinoma Cells*

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Abstract—*Lewis lung carcinoma cells in culture, spontaneously degrade cartilage proteoglycan and fibrin but not collagen. These processes are due, at least in part, to the secretion by the cells of a proteoglycan-degrading neutral serine proteinase and of plasminogen activator.*

INTRODUCTION

THE ABILITY of tumor cells to metastasize to distant sites may be dependent on a multitude of factors, among which is their capacity to secrete proteinases affecting components of the surrounding extracellular matrices [1]. To investigate this possibility, we undertook experiments with a spontaneously metastazing tumor, the Lewis lung carcinoma (3LL). We report here that 3LL cells spontaneously degrade fibrin and proteoglycan but not collagen when they are cultured either at the surface of plates coated with these macromolecules or in contact with dead cartilage.

MATERIALS AND METHODS

Cells

3LL cells and their L20 variants (obtained from Dr. T. Boon, ICP, Brussels) were cultured at 37°C under air/CO_2 (9:1) in Dulbecco's medium (DMEM, Gibco) containing streptomycin (100 mg/l) and penicillin (10^5 U/l) and supplemented with 10% heat-inactivated (30 min at 56°C) foetal calf serum (FCS, Gibco). At confluence, they were dispersed with phosphate-buffered saline [2] containing 2 mM EDTA, sedimented and washed twice in DMEM.

Accepted 13 March 1980.
*This work was supported by grants from the Cancer Research Funds of the Caisse Générale d'Épargne et de Retraite and from the Belgian Fonds de la Recherche Scientifique Médicale.

Degradation of proteoglycan and collagen by cells in culture

Co-cultures of 3LL cells with pieces of heat-inactivated rabbit ear cartilage biosynthetically labelled with ^{35}S in its proteoglycan were done according to Hauser and Vaes [2]. The cells (2×10^5) were distributed in the wells of Falcon Microtest plates and cultured together with the cartilage in 0.2 ml DMEM supplemented with 15% acid-treated [3] FCS. Controls involved cartilage cultured without cells either in the same medium (to evaluate the autolysis of cartilage) or in 0.2 ml of DMEM containing 25 μg trypsin [2]. Degradation of native collagen was considered to occur only when the amount of hydroxyproline lost from the cartilage during the culture was significantly larger than the maximum amount solubilized by trypsin.

The cells were also cultured on proteoglycan–collagen plates according to a procedure that will be published in detail elsewhere (Ch. Peeters-Joris, X. Emonds-Alt and G. Vaes, in preparation). The principle of that assay consists in monitoring the release of soluble radioactive degradation products from a dish (Costar Multiwell plate) coated with a dried, reconstituted mixed gel made of ^3H-labelled [4] bovine nasal proteoglycan-aggregates [5] and of ^{14}C-labelled collagen [6]. The coating of the plates was done as in our previous work [7], by using however a mixture of [^{14}C] collagen and buffer supplemented by 580 μg of [^3H] proteoglycan aggregates/ml. Cells ($2 \times 10^4 - 4 \times 10^5$) (or blank medium without cells) were distributed

in the coated wells and cultured for up to 7 days in 1 ml DMEM supplemented with 1% acid-treated and, for some experiments, plasminogen-depleted [8] FCS.

Degradation of fibrin by cells in culture

[^{3}H] Fibrin coated Multiwell plates were prepared from ^{3}H-labelled [3] fibrinogen (Miles Laboratories). Cells (or blank medium without cells) were incubated on these plates in the same way as on the proteoglycan–collagen plates and the release of [^{3}H] labelled soluble products was monitored [3]. To evaluate whether fibrinolysis was plasminogen-dependent or not, cultures were done in DMEM supplemented with 1% acid-treated FCS depleted [8] or not of plasminogen. The efficiency of the plasminogen-depletion was verified by incubating fibrin plates under the conditions of the cell cultures with 5 U of urokinase (Leo Pharmaceuticals)/ml in DMEM supplemented with plasminogen-depleted FCS: only 10–15% of the ^{3}H was then released after 3 days in contrast with a 60% release after 2 hr of culture in DMEM containing non-depleted FCS.

Assays of enzyme activities in cell lysates and in conditioned media from cultures of 3LL cells

3LL cells were cultured up to confluence (5 days) in DMEM supplemented with 10% FCS, depleted or not of plasminogen. The conditioned culture media were filtered on 0.22 μm Millipore® filters and concentrated (20×) by $(NH_3)_2SO_4$ precipitation at 50% saturation, the precipitate being collected by centrifugation, dissolved in phosphate-buffered saline containing NaN_3 (0.1 mg/ml) and dialyzed extensively against the same buffer. The cell lysates were prepared by resuspending the cells in phosphate-buffered saline containing 0.1% (w/v) Triton X-100 (4×10^5 cells/ml). The enzyme activities were assayed on proteoglycan/collagen- or on fibrin-coated plates by incubating the plates for 24 hr at 37°C with 0.5 ml of cell lysate or of 20 x-concentrated conditioned media per well; heat-inactivated (10 min at 100°C) preparations were used for the blanks. Assays of neutral proteinases were also done on ^{3}H-acetylated casein as previously described [6].

Gel filtration of the products of degradation of ^{3}H-labelled proteoglycan on Sepharose 6B

Proteoglycan–collagen plates were digested for 5 days by 2×10^5 cells in culture/well, as described above; the medium was then filtered on 0.22 μm Millipore® filters. Alternatively, plates were digested for 48 hr at 37°C by concentrated (20×) conditioned medium from 3LL cell cultures (0.25 ml/well) or by trypsin (25 μg/well). Samples (0.5 ml) of these digests were chromatographed on a Sepharose 6B column (1.6 cm × 10 cm; 0.32 ml/min; 1 ml fractions) equilibrated and eluted at 4°C with phosphate-buffered saline. The ^{3}H-radioactivity was then measured in the various fractions. The void volume and the total volume of the column were measured by the chromatography of 0.5 ml of buffer containing 0.5 mg of Blue Dextran and 0.05 μCi of ^{3}H-proline.

RESULTS

Degradation of proteoglycan by cells in culture

When cultivated together with [^{35}S] cartilage, both the 3LL cells and their L20 variants degraded almost completely within a few days the ^{35}S-labelled proteoglycan of the cartilage. However they did not degrade native cartilage collagen as they were unable to release more hydroxyproline in soluble form than did trypsin (Table 1).

When cultivated on [^{3}H] proteoglycan-[^{14}C] collagen plates, the 3LL cells caused also the release of soluble ^{3}H-labelled degradation products from proteoglycan (Fig. 1a). They did however not degrade native collagen (data not shown). The action on proteoglycan was related to the number of cells in culture (Fig. 1b). It was abolished when the cells were cultured with cycloheximide or when they were killed prior to the cultures (Fig. 1a). The soluble ^{3}H-labelled degradation products that accumulated in the culture fluid chromatographed on a Sepharose 6B column at a position near that of the soluble products released from the plates by trypsin, well below that of intact proteoglycan aggregates (Fig. 2).

Degradation of fibrin by cells in culture

3LL cells cultivated on ^{3}H-labelled-fibrin plates caused a rapid release of soluble ^{3}H-labelled degradation products that was dependent on the number of cells put in culture and abolished when the cells were cultured with cycloheximide or after their killing. It was lowered but not abolished when the cultures were done in the presence of plasminogen-depleted serum (Fig. 3).

Table 1. *Degradation of* ^{35}S*-labelled cartilage by 3LL cells and their L20 variants in culture*

Experimental group	No. of cultures	Cumulative release (% of total) during culture					
		day 0–3		day 0–5		day 0–7	
		^{35}S	Hydroxyproline	^{35}S	Hydroxyproline	^{35}S	Hydroxyproline
1. Controls for autolysis	3	14.5±2.5	14.5±5.6	28.5±2.4	18.6±7.1	49.1±0.4	23.9±8.8
2. Trypsin digestion	4	84.6±12.3	19.8±8.3	90.1±13.2	25.2±8.5	91.6±13.3	29.4±8.7
3. 3LL cells	3	51.9±22.4*	25.7±7.0	75.5±19.2†	29.7±8.8	87.5±12.5†	33.9±9.9
4. 3LL-L20 cells	3	44.8±25.6	19.5±11.5	80.1±16.3†	24.6±12.0	93.0±5.9‡	31.3±14.2

The cell cultures, the controls for autolysis and the trypsin digestions were run as described in Materials and Methods. The cartilage discs were transferred to new culture wells, containing fresh medium and (for groups 3 and 4) cells, after 3 days culture and again 2 days later. The cumulative release of soluble ^{35}S-labelled material and hydroxyproline is presented as means±S.D. The statistical significance of the differences observed between the cell cultures (groups 3 and 4) and the controls (group 1) was evaluated with Student's *t*-test (*, $P<0.05$; †, $P<0.01$; ‡, $P<0.001$).

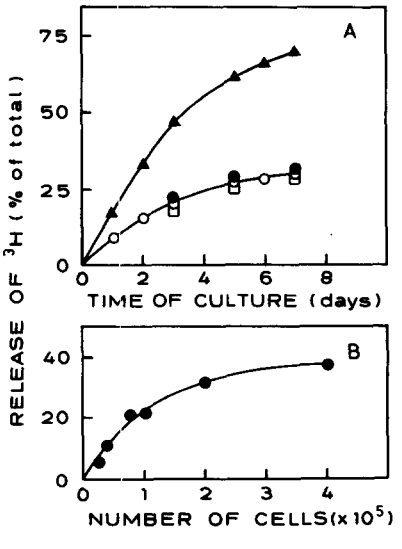

Fig. 1. *Proteoglycan degradation by 3LL cells in culture.* *3LL cells were cultured in the wells of* [^{3}H] *proteoglycan/*[^{14}C] *collagen-coated plates. The release of* ^{3}H*-labelled material from proteoglycan is expressed as a percentage of the total amount of proteoglycan* (^{3}H) *initially present in the wells. Each point is the mean of* 3 *cultures.* (*A*) *Effect of the time of culture. Cells were cultured either as such* (▲) *or with* 2 μg *of cycloheximide/*ml (●) *or after their killing by repeated freezing and thawing* (□). *Blanks* (○), *determined by incubation of the plates with medium but without cells, have here not been substracted from the other experimental data.* (*B*) *Effect of cell number. The indicated number of cells were cultured for* 3 days. *Blanks have been substracted from the results.*

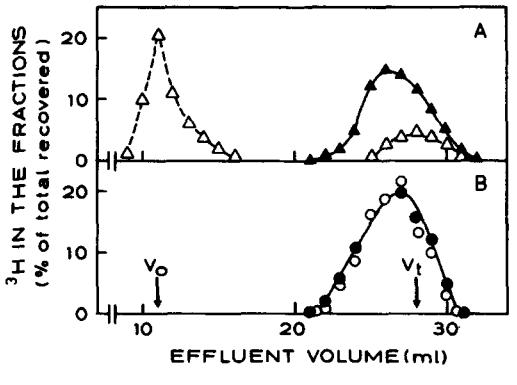

Fig. 2. *Gel filtration of the* ^{3}H*-labelled degradation products of proteoglycan on Sepharose* 6*B.* (*A*) *Chromatography of the preparation of* ^{3}H*-labelled proteoglycan aggregates used to prepare the plates* (△) *and of the products of degradation of the plates by trypsin* (▲). *The labelled material that chromatographs around* V_t *represents dialysable degradation products generated from the proteoglycans during their storage.* (*B*) *Chromatography of the products of degradation of the plates by either cells in culture* (●) *or conditioned medium* (○). V_o, *void volume,* V_t, *total volume.*

Enzymes secreted by cells in culture

A search was made for neutral proteinases active on fibrin, proteoglycan, collagen or casein in the cell lysates and in the conditioned media surrounding 3LL cells in culture. No significant activity was found in the

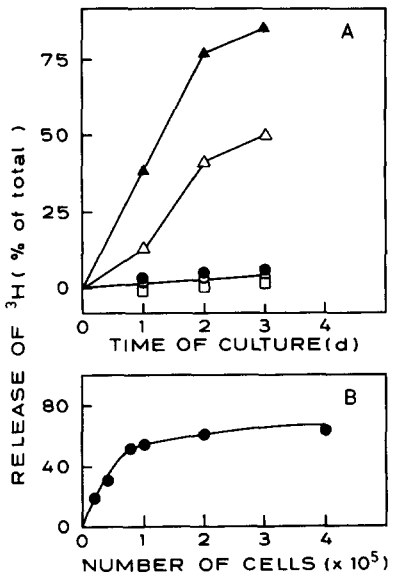

Fig. 3. Fibrin degradation by 3LL cells in culture. The release of ^{3}H-labelled material from [^{3}H] fibrin-coated culture plates is expressed as a percentage of the total amount of fibrin (^{3}H) initially present in the wells. Each point is the mean of 3 cultures. (A) Effect of the time of culture and of plasminogen depletion. Cells (2×10^5) were cultured with 1% acid-treated FCS either as such (▲) or with 2 μg of cycloheximide/ml (●) or after their killing by repeated freezing and thawing (□). They were also incubated with 1% acid-treated, plasminogen-depleted FCS (△). Blanks (○) (see Fig. 1) have here not been substracted from the other experimental data. (B) Effect of cell number. The indicated number of cells were cultured for 2 days. Blanks have been substracted from the results.

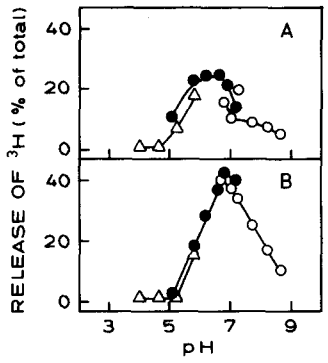

Fig. 4. Influence of pH on the proteoglycan-degrading and on the fibrinolytic activity of conditioned media of 3LL cell cultures. The incubations were done in 0.1 M buffers: sodium acetate/acetic acid (△), sodium cacodylate/HCl (●) or Tris/HCl (○). Blanks have been substracted.

lysates. Over 24 hr, concentrated (20 ×) cell free media (0.5 ml/well) degraded 30% of the [^{3}H] fibrin and 24% of the [^{3}H] proteoglycan from the proteoglycan–collagen plates. They degraded also casein (61 U/ml) but they had no effect on collagen, even after trypsin-treatments (5–500 μg/ml; 10 min at 25°C) aimed at the activation of potential latent neutral collagenase [6]. Their activity on fibrin was totally dependent on the presence of plasminogen while that on proteoglycan was almost unchanged (22% of degradation) with plasminogen-depleted culture media. About half of the activity exerted on casein (28 U/ml) was still manifested in the absence of plasminogen.

The proteoglycan-degrading activity of the conditioned media was optimal around pH 6.5 and their fibrinolytic activity around pH 7 (Fig. 4). Both activities, as well as the caseinolytic activity of the conditioned media, were almost completely inhibited by soya bean trypsin inhibitor (0.8 mg/ml), aprotinin (Trasylol, 2.5–5 mg/ml) and phenylmethane sulfonyl fluoride (2–5 mM) but they were not affected by EDTA (2.5–10 mM), 1,10-phenanthroline (2.5 mM), cysteine (2.5 mM) or 4-hydroxy-mercuribenzoate (1 mM). The fibrinolytic activity was abolished by ε-aminocaproic acid at a concentration (50 mM) that had only an insignificant inhibitory effect (−9%) on the proteoglycan-degrading activity.

DISCUSSION

Our studies demonstrate that 3LL cells in culture spontaneously degrade cartilage proteoglycan into soluble fragments but have no effect on interstitial collagen. These cells also degrade fibrin by an action that appears due, in part, to a plasminogen-activator as it is reduced in the absence of plasminogen. However, part of the fibrinolysis is plasminogen-independent and might thus involve another proteinase.

Enzymes responsible for proteoglycan or fibrin degradation could not be detected in non-concentrated conditioned culture media. After concentration (20 ×) of the media, their activity was low compared to that directly exerted by the cells in culture on the substrates. This could be explained by a lack of stability of the enzymes in the media, or by the presence of inhibitors, or by an association of the greatest part of the enzymes with insoluble cellular structures. It is therefore uncertain whether the enzymes found in conditioned media represent validly those that were active in the direct digestion of the substrates by the cells during the cultures and whether the properties observed for the soluble, presumably secreted enzymes may be

extended to the whole of the enzyme capacity of the living cells. The whole of the fibrinolytic activity recovered in the conditioned media was plasminogen-dependent and inhibited, as would be expected for either plasminogen activator or plasmin, by inhibitors of serine proteinases and by ε-aminocaproic acid. On the contrary, the proteoglycan-degrading activity of the media was almost completely plasminogen-independent and not affected by ε-aminocaproic acid. It was optimal around neutral pH and inhibited by inhibitors of serine proteinases but not of metalloproteinases nor of thiol-dependent proteinases. It is thus distinct from the proteoglycan-degrading metal-dependent neutral proteinase secreted in culture by mouse bone explants [6], rabbit macrophages [2] or rabbit fibroblasts [9] and more similar to the two proteoglycan-degrading neutral serine proteinases (cathepsin G and elastase) present in human polymorphonuclear leucocytes [10]. The plasminogen-independent fraction of the caseinolytic activity detected in the conditioned media may possibly be due to the same proteinase.

The lack of collagenolytic effect exerted by 3LL cells on interstitial (types 1 and 2) collagen may indicate that a direct production of 'interstitial' collagenase does not condition the metastatic behaviour of the 3LL cells. It appears however important to search for factors that may regulate the *in vivo* secretion of collagenase by these cells. Also, cancer cells could stimulate surrounding cell types from the host to secrete collagenase. Finally 3LL cells could produce a collagenase active only on basement membrane collagen [11].

Acknowledgement—We thank Mrs. Th. Cogi-Vinckx for her excellent technical assistance.

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