SECRETION OF A LATENT NEUTRAL PROTEINASE THAT DEGRADES CARTILAGE PROTEOGLYCANS BY SKIN AND SYNOVIAL FIBROBLASTS IN CULTURE

Ghislaine HUYBRECHTS-GODIN and Gilbert VAES

Laboratoire de Chimie Physiologique, Université de Louvain and *International Institute of Cellular and Molecular Pathology, Avenue Hippocrate, 75, B - 1200 Bruxelles, Belgium*

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1. Introduction

The degradation of cartilage proteoglycans may occur as part of a normal physiological turnover [1,2] or as a consequence of pathological processes, e.g., inflammatory arthritis. To understand and control these destructive processes, the nature of the enzymes responsible for them as well as the types of cell that can secrete such enzymes should be elucidated. Although several lysosomal acid proteinases are able to degrade proteoglycans [3], evidence for their participation to extracellular degradations [3–5] is a matter of debate as their activity depends on an acid pH. We report here that rabbit skin or synovial fibroblasts in culture secrete a metal-dependent neutral proteinase that degrades the protein core of cartilage proteoglycans. The enzyme was found in a latent form in the culture media and was activated by a limited proteolysis with trypsin.

2. Materials and methods

Fragments of skin or synovium (1 mm^3) were explanted from male rabbits (Termonde White strain, aged 1–2 months) in plastic Petri dishes and cultured in a water-saturated atmosphere of air/CO_2 (9:1) in Dulbecco's modified Eagle medium (DMEM) containing glucose (1 g/litre), penicillin (10^5 units/ml) and streptomycin (100 mg/ml) supplemented with 10% (v/v) of heat inactivated (30 min at 56°C) foetal calf serum. The medium was renewed every week. After 3 weeks, the explants were removed and the adherent outgrowing cells were rinsed with phosphate-buffered saline (PBS) [8] and dispersed by incubation for 15 min at 37°C with trypsin (1 mg/ml in PBS). Trypsin was then neutralized by addition of an amount of calf serum sufficient to inhibit completely its activity. The cells were sedimented, washed twice by repeated resuspensions and sedimentations in DMEM and counted in a haemocytometer. They were then plated at a cell density corresponding to half-confluence (1.2×10^5 cells/cm^2) and subcultured in serum-containing DMEM. Cell confluence was obtained after 4 days. Three days later, the cells were washed extensively and further cultured for 7 days in serum-free (unless otherwise indicated) DMEM. The conditioned culture media were then collected and activated with trypsin as in [6,7]. The proteoglycan-degrading neutral proteinase present in serum-free media was fully activated by preincubation for 10 min at 25°C with 8 μg trypsin/ml followed by the addition of a 4-fold excess of soya-bean trypsin inhibitor to block the action of trypsin. It was assayed [7,8] either by measuring the amount of ^{35}S-labelled fragments released at pH 7 from ^{35}S-labelled rabbit ear cartilage or by following, at pH 7.4, the decrease in viscosity of a solution of proteoglycan subunits purified [9] from bovine nasal cartilage. One unit of activity refers to the amount of enzyme that releases 1% initial cartilage ^{35}S radioactivity/h. Gel filtration of the degradation products of ^{35}S-labelled cartilage was done on Sepharose 6B as in [7]. All materials and chemicals were from the same suppliers as in [7,8].

3. Results

The proteoglycan-degrading activity present in either skin or synovial fibroblasts-conditioned serum-free culture media was mostly latent. Only low levels of activity were observed when the assays were done directly on the media but considerably more activity became evident when the media had been submitted to a limited proteolysis by trypsin (fig.1 and fig.4). The latent enzyme was also found in serum-containing conditioned media but larger concentrations of trypsin were then required for its activation (fig.1) as expected from the presence of trypsin inhibitors in serum. After trypsin-activation, the enzyme was found in the culture fluid but not in the cell lysate. This was apparently not due to the presence of inhibitors in the lysate, as the addition of lysate to trypsin-activated medium did not modify the enzyme activity present in the medium.

The proteoglycan-degrading enzyme present in the activated media exerts its optimum activity at about pH 6.5 (fig.2). It is almost completely inhibited by EDTA (1 mM), *o*-phenanthroline (0.1 mM), cysteine (10 mM) and foetal calf serum (5%, v/v) and 60%

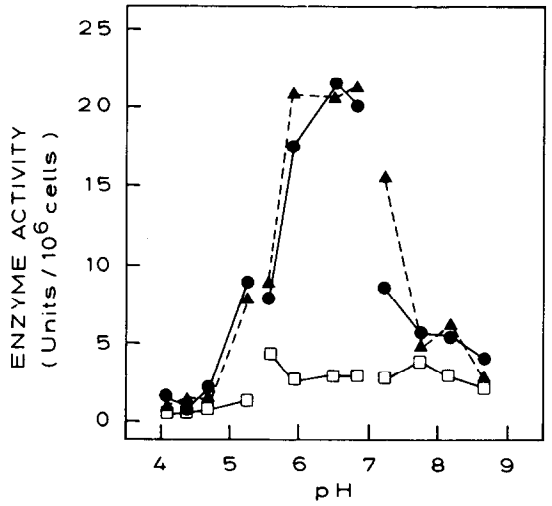

Fig.2. Influence of pH on the proteoglycan-degrading activity of activated culture medium. Incubation of either synovial (●) or skin (▲) fibroblasts-conditioned medium or of fresh non-cultivated medium ((□) as a control for autolysis) was done in the presence of heated ^{35}S-labelled cartilage [8] in acetate (pH 4–5.2), cacodylate (pH 5.6–6.8) or Tris (pH 7.5–8.7) buffers (0.25 M).

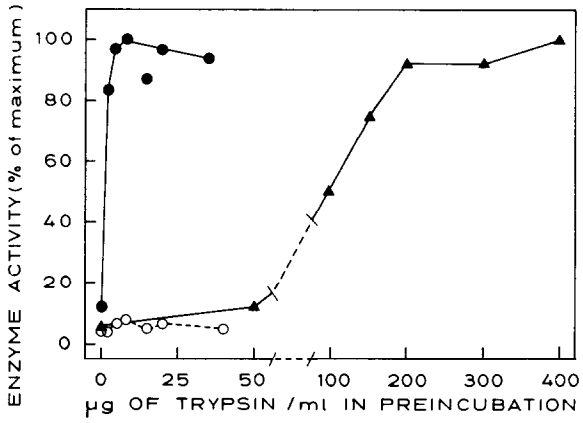

Fig.1. Activation of the proteoglycan-degrading enzyme by trypsin. The activation observed in (a) serum-free synovial fibroblasts-conditioned media (●, ○) is compared to that of (b) skin fibroblasts-conditioned media containing 10% foetal calf-serum (▲). The media were preincubated for 10 min at 25°C with trypsin either before (●, ▲) or (as a control) after (○) the addition of soya-bean trypsin inhibitor. 100% activity (measured on ^{35}S-labelled cartilage) corresponds to 27 units/10^6 cells for (a) and to 38 units/10^6 cells for (b).

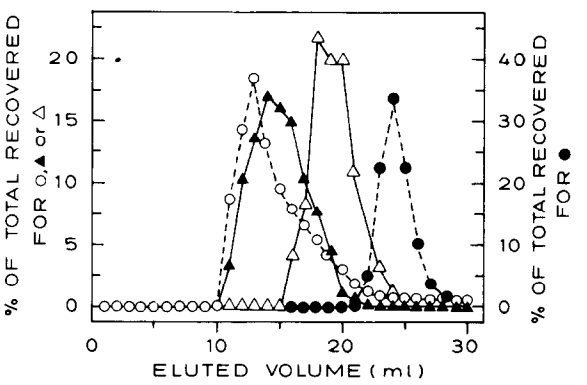

Fig.3. Gel filtration of the ^{35}S-labelled degradation products of cartilage on Sepharose 6B. The chromatographic fractionation of a 1 mg solution of blue dextran (○) and of $Na_2{}^{35}SO_4$ (100 000 dpm) (●) in the eluent was followed by the chromatography of the soluble degradation products obtained after digestion of ^{35}S-labelled cartilage by activated skin fibroblasts-conditioned culture medium for 72 h (corresponding to the release of 54% of the total ^{35}S-label initially present in the cartilage). The digest was chromatographed either as such (▲) or after its further degradation by papain (△).

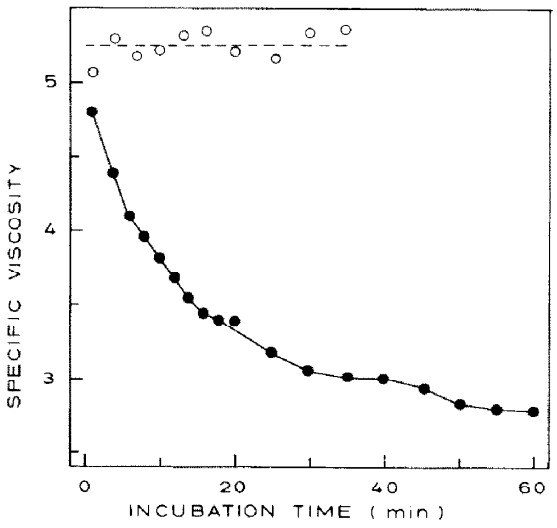

Fig.4. Effect of non-activated (○) and activated (●) synovial fibroblasts-conditioned culture medium on the viscosity of a solution of proteoglycan subunits. The non-activated medium had been supplemented with a preformed mixture of trypsin and of soya bean trypsin inhibitor so as to contain the same final concentrations of both as the activated medium.

inhibited by casein (5 mg/ml). Trasylol (50 μg/ml), 7-amino-l-chloro-3-L-tosylamidoheptan-2-one (TLCK; 2.5 mM), phenylmethanesulphonylfluoride (1 mM) and 4-hydroxymercuribenzoate (1 mM) have no effects. The soluble ^{35}S-labelled cartilage degradation products chromatograph on Sepharose 6B at a position intermediate between blue dextran and the ^{35}S-labelled material obtained by a subsequent papain digestion of these products, well ahead of free $Na_2{}^{35}SO_4$ (fig.3). Moreover, trypsin-activated media decrease the specific viscosity of solutions of purified proteoglycan subunits from bovine nasal cartilage (fig.4).

4. Discussion

This work establishes that rabbit fibroblasts of either skin or synovial origin are able to secrete under culture conditions a proteoglycan-degrading enzyme that is apparently not stored in the cells. The enzyme produces from ^{35}S-labelled cartilage soluble ^{35}S-labelled material of high molecular weight that can be further degraded into smaller fragments by papain. It also lowers the viscosity of solutions of purified proteoglycan subunits. Its activity is optimal around neutrality and inhibited by metal-binding agents but not by inhibitors of serine-proteinase nor by thiol-blocking agents. It can thus be identified as a neutral metal-dependent proteinase that degrades the protein-core of the cartilage proteoglycan subunits. It appears similar to enzymes secreted by mouse bone explants [7] and rabbit macrophages [8] or extracted from human cartilage [10] but it differs from the proteoglycan-degrading neutral serine-proteinases present in human polymorphonuclear leucocytes [3,11].

While the enzymes from rabbit macrophages or human cartilage were detected in a directly active form, the neutral proteinase from rabbit fibroblasts was mostly latent and activatable by trypsin as is the enzyme from mouse bone cultures [7]. The molecular basis for this latency (zymogen or enzyme–inhibitor complex) as well as its physiological significance (precursor or residue of active enzyme) are still unknown. However, it is now evident that not only collagenase [6,12] but also other tissue neutral proteinases may exist in a latent form and thus escape detection unless properly activated by trypsin or by other treatments [13–15].

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