Isolation and differential secretion of metalloproteinase by superficial chondrocytes in articular cartilage

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Summary. *Chondrocytes from superficial layers of articular cartilage have distinct phenotypic properties which are different from those of cells obtained from the deeper areas. We describe a method that isolates highly purified articular cartilage chondrocytes from the superficial layers. When the superficial cells are stimulated in vitro with a source of cytokines, they secrete greater amounts of metalloproteinase compared to chondrocytes obtained from a deeper area.*

Résumé. *Des études récentes ont démontré que les chondrocytes originaires des couches superficielles du cartilage articulaire possèdent des caractéristiques phénotypiques différentes des caractéristiques des cellules provenant de la région profonde du tissu. Nous décrivons une nouvelle méthode qui permet l'isolement d'une population des chondrocytes superficiels de haute pureté. Nous montrons aussi que quand les chondrocytes superficiels sont stimulés in vitro avec des cytokines, on peut constater une sécrétion plus importante de caséinase par rapport au niveau de sécrétion des cellules profondes.*

Introduction

Although the mechanisms of cartilage damage in inflammatory and noninflammatory arthritis are not well understood, chondrocyte activation and

increased secretion of metalloproteinases by articular chondrocytes play an important part in this process [8, 9]. Recent studies by ourselves [16] and other workers [1–6] have emphasised the phenotypic differences between superficial (SC) and deep chondrocytes (DC) which may be relevant to the process of cartilage damage.

Most previous experiments involved chondrocytes, obtained from superficial slices of cartilage cut by hand with a scalpel, consisting of from 11% to 33% of the total cell population [16], whereas it has been estimated that SC make up no more than 10% of chondrocytes obtained from pieces of full thickness cartilage.

In this study, we describe a new technique which allows the isolation of highly purified SC consisting of 2% to 6% of the total chondrocyte population. We also investigated the secretion by SC of metalloproteinase when stimulated by cytokine compared with cells obtained from the deeper layers of cartilage.

Materials and methods

Chondrocytes

Slices of articular cartilage were obtained from the carpo-metacarpal joints of one year old cows. The slices were cut into 10 mm diameter discs using a metal corkborer (Fisher Scientific Co. #07-845C No. 7). As shown in Fig. 1, they were placed between the lid of U-shaped bottomless microtitre plates with 96 wells (Dynatech) so that the articular surface of the cartilage discs served as the bottom of the wells. The tissues were kept in place by metal clamps compressing the lid and the microtitre plate to establish a tight seal between the wells and the tissues. The wells were then filled with 400 µl of a testicular hyaluronidase (Sigma Biochemicals) 1 mg/ml in Hanks basal salt solution (HBSS) and incubated at room temperature with continuous shaking. After washing the wells

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Fig. 1. Diagram showing the method used for the isolation of chondrocytes from the superficial layers of articular cartilage

Fig. 2A–D. Flow cytometry analysis of the isolated chondrocyte populations. **A** Forward scatter plot showing bimodal distribution with respect to size. **B** Dot plot of the same cell population. **C** Side scatter plot showing cell distribution with respect to complexity. **D** Dot plot of chondrocytes obtained after partial collagenase digestion of the articular surface

Fig. 3. Histological section showing digestion of the outermost layers of cartilage after treatment with collagenase for 5 h

with phosphate buffer saline (PBS), pH 7.2, containing 0.001 M EDTA, the discs were sequentially incubated with 0.25% trypsin for 30 min and then with clostridial collagenase 2 mg/

ml (specific activity: 150–200 U/mg) (Worthington Biochemicals, Freehold, New Jersey) in HBSS-10% foetal calf serum (FCS) for 1 h at 37 °C. After 4 h further incubation with fresh collagenase, the released SC were harvested for further culture. To obtain DC, the cartilage discs were incubated over night with collagenase as already described. Cell viability was >90% as determined by trypan blue exclusion.

Flow cytometry

The chondrocyte suspensions were washed in HBSS, filtered through gauze, and analysed in a FacStar flow cytometer (Becton-Dickinson Systems, Mountain View, California). Data processing was carried out in a Hewlett-Packard computer using the Lysys programme.

Chondrocyte activation

Cell suspensions in RPMI 1640 culture medium (GIBCO)-10% FCS, at $2-4 \times 10^4$ cells/well, were cultured for 3 days at 37 °C in air and 5% CO₂. Cell activation was achieved by adding a 10% dilution of a phytohaemagglutinin-stimulated human mononuclear cell supernatant [7]. At the end of the culture period, the supernatants were harvested and stored at –70 °C until assayed for metalloproteinase activity.

Metalloproteinase assay

This was measured in the supernatants with or without the addition of 1 mM APMA [15]. 100 µl of supernatant was added to an equal volume of [¹²⁵I]-casein containing 100 µg protein and 1.6×10^5 cpm. After incubation for 19 hours at 37 °C, the reaction was stopped by the addition of 200 µg unlabelled casein and 100 µl 25% trichloroacetic acid solution. After centrifugation at 7000 rpm for 20 min, 200 µl of the supernatant was collected and the soluble radioactivity measured in a gamma-scintillation spectrometer. One unit of metalloproteinase activity was defined as the degradation of 1 µg casein per minute at 37 °C.

Results

Collagenase incubation of the cartilage discs for 5 h yielded 9000 to 25000 (16000 ± 3000 ; $n = 5$) cells per disc comprising 2.6% ($4.5\% \pm 0.8\%$, $n = 5$) of the total cell population. Measurement of mean cell diameters in the two populations was performed with a calibrated objective attached to an optical microscope. The mean cell diameter of SC (14.1 ± 1.0 µm) was significantly smaller than that of DC (15.7 ± 1.4 µm; $P < 0.001$; $n = 20$).

Flow cytometry analysis (forward scatter vs side scatter) of the cells obtained from full thickness cartilage pieces showed two populations: one with smaller size and higher complexity of cells, and a second larger population with larger size and lesser complexity. DC obtained from discs previously digested with collagenase for 5 h showed that the former cell population had disappeared (Fig. 2). Histological examination of the partly

digested discs showed absence of the flat and elongated superficial cells (Fig. 3).

The next series of experiments were designed to investigate cytokine-mediated responses in terms of metalloproteinase secretion of the highly purified SC and DC populations. Incubation of SC and DC with a source of cytokines in 3 separate experiments resulted in a significantly higher increase of stimulated metalloproteinase secretion by SC (SC: 625 ± 77 mU/ 10^6 cells. DC: 107 ± 75 mU/ 10^6 cells; $P < 0.01$).

Discussion

The phenotypic characteristics of SC have been studied by several investigators. Flow cytometry analysis has shown that SC are smaller and exhibit a more complex cytoplasm [1] in keeping with the well known flattened appearance of these cells. Metabolic differences between the two cell populations have been reported. SC synthesize less aggrecan [2] and collagen [3] than DC; they appear, however, to produce nonaggregating small proteoglycans in greater quantities [10]. The superficial cells secrete greater amounts of matrix-degrading metalloproteinases when stimulated with cytokines [5]. Moreover, they are more responsive to interleukin-1 [5, 6] as judged by its proteoglycan synthesis inhibitory activity, probably because there are 6 times greater numbers of membrane receptors for this cytokine [6]. We have also shown that rheumatoid synovial fluid IgM antibodies bind to, and kill preferentially, SC, when compared to DC [16]. Some studies are based on cytokine stimulation of cartilage explants in organ culture. The results of these types of experiments may therefore depend on the preferential diffusion of large peptides into the superficial layers of the tissues since it has been shown that this area is much more permeable to large molecules than the deeper areas [11]. Manual dissection of cartilage yields slices of variable thickness containing a mixture of SC and DC. For these reasons, we devised a method that allows for the isolation of highly purified SC populations. Controlled collagenase digestion of cartilage discs allowing contact of the enzyme only with the articular surface of the tissues resulted in the isolation of a homogeneous population of smaller and more complex chondrocytes with characteristics previously described for SC.

We also show that the SC isolated by the method described above show increased secretion of metalloproteinase compared with DC when the cells are stimulated in vitro with a source of cy-

tokines. These studies complement previous observations showing preferential localization of stromelysin protein [3] and mRNA [12] to the cells of the superficial layer of normal articular cartilage.

The articular surface is subjected to direct mechanical forces, and its macromolecular organization is probably partly responsible for its lubrication characteristics, which result in very low friction coefficients, and its peculiar permeability properties. In addition, the intact surface may be conformed in such a way as to prevent adhesion of synovial tissue and synovial fluid cells [9]. This area is in the front line of attack in inflammatory joint diseases so that it would be exposed very early to various noxious factors, including II-1, contained in synovial exudates. Further studies of superficial chondrocytes in health and disease should provide valuable insight into the pathology of cartilage damage in inflammatory and non-inflammatory arthritis. The method described should facilitate studies dealing with the biological properties of this chondrocyte subpopulation in health and disease.

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