# Differential expression of mRNAs for endopeptidases in phenotypically modulated ('dedifferentiated') human articular chondrocytes

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**Abstract Human articular chondrocytes modulated away from their original phenotype by serial subcultures in monolayer differentially express mRNAs for endopeptidases. The mRNAs for the cathepsins B and L are extremely low in differentiated cells, but are soon expressed in parallel with the loss of the differentiated state. In contrast, the mRNA for collagenase-1 is strongly expressed by differentiated chondrocytes and declines rapidly following phenotypic modulation. The mRNA for stromelysin-1 and the tissue inhibitor of metalloproteinases-2 is high and does not appreciably change after modulation. Chondrocyte activation induced by alteration of its original phenotype leads to the expression of endopeptidases in a way that markedly differs from that induced by cytokines. The results are relevant to cartilage catabolism in osteoarthritis and suggest a prominent role of fibroblastic metaplasia on the part of the chondrocytes as a mechanism of expressing catabolic endopeptidases.**

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*Key words:* Cathepsin B; Cathepsin L; Collagenase-1; Stromelysin-1; TIMP-2; Osteoarthritis

## 1. Introduction

Most of the research on chondrocyte-mediated breakdown of articular cartilage has essentially focused on the action of metalloproteinases released after cell stimulation by cytokines [1,2], while other endogenous enzymes have received less attention [3,4]. Studies from this laboratory have shown that phenotypic modulation of articular chondrocytes results in the biosynthesis and release of high amounts of cathepsin B. This enzyme has been proposed to be a marker of the 'dedifferentiated' chondrocyte phenotype [5] and its secretion was shown to depend directly on protein synthesis [6]. Phenotypic modulation of chondrocytes dramatically stimulated the secretion and intracellular accumulation of active cathepsin B, while the extracellular activity of collagenase-1 was even suppressed [7]. On the other hand, cell stimulation by interleukin-1β resulted in a large increase of extracellular collagenase activity, while the secretion of cathepsin B was not affected and its intracellular pool was only moderately increased [7]. The role of cathepsin B in sustaining the chronicity of osteoarthritis, by acting as an antagonist of cartilage regeneration, has been documented in a study with human osteoarthritic articular cartilage [8,9]. These ex vivo results also pointed to the similarity between osteoarthritic and artificially modulated chondrocytes and suggested fibroblastic metaplasia as a plausible mechanism for cathepsin B induction [9].

Since our previous studies were based on protein (enzyme activity) assays, the question arose whether the gene expression of the involved enzymes correlated with the protein findings. In the present study we analyzed the mRNA levels for the cathepsins B and L, collagenase-1, stromelysin-1 and TIMP-2 in human articular chondrocytes phenotypically modulated by serial subcultures. As an internal control, results were compared to those of type I collagen mRNA expression, for which data are known from the literature.

## 2. Materials and methods

### 2.1. Cell isolation and culture

Chondrocytes were prepared 8 h postmortem from cartilage pooled from both femoral heads and condyli of a healthy male individual aged 25 years according to methods detailed elsewhere [9]. Monolayer cultures were established at a density of $2 \cdot 10^6$ cells/25 $cm^2$ culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Subculturing was performed by replating in monolayer after trypsinization.

### 2.2. RNA extraction, probes and Northern blot analysis

Total RNA was extracted with commercial kits provided by QIAGEN. Cells were harvested by trypsinization, pelleted by centrifugation, resuspended in lysis buffer, homogenized with the QIAshredder method and RNA was extracted using the RNeasy midi RNA system. After heat-denaturation, equal amounts of total RNA (10.6 μg/lane) from primary culture and subcultures were separated electrophoretically in 2.6 M formaldehyde, 1% agarose gels and transferred to nylon membranes using a vacuum blotting system (Vacu Gene TMXL, Pharmacia) followed by exposure to ultraviolet light for crosslinking. cDNA clones for human cathepsin B (250 bp), human collagenase-1 (210 bp), human tissue inhibitor of metalloproteinases-2 (300 bp) and human type I collagen α1(I) chain (230 bp) had previously been obtained by a subtractive cDNA cloning approach [10]. cDNA clones for human prostromelysin-1 (780 bp) and human cathepsin L (1345 bp) were kindly provided by Prof. H. Nagase and Prof. B. Wiederanders, respectively. A cDNA fragment of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to compensate for differences in sample loading. cDNAs were labeled with [$^{32}$P]deoxycytidine-5′-triphosphate using a random primer labeling kit (Promega). After 5–6 h of prehybridization the membranes were hybridized with $^{32}$P-labeled DNA probes for 14–16 h, then washed 3 times each with 2× sodium chloride sodium citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS) for 30 min at room temperature and with 1×SSC/0.1% SDS for 30 min at 55°C. Autoradiography was carried out at −70°C using an Agfa Curix Ortho HTA film and an intensifying screen for 1 or 4 days. Nylon membranes were reprobed with the appropriate cDNAs after stripping by boiling in 0.5% SDS/0.1×SSC buffer for 20 min. Images were quantified using an electrophoresis data software system (ONE-Dscan, Billerica, MA, USA).

## 3. Results

Human articular chondrocytes were phenotypically modu-

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*Abbreviations:* GAPDH, glyceraldehyde 3-phosphate dehydrogenase

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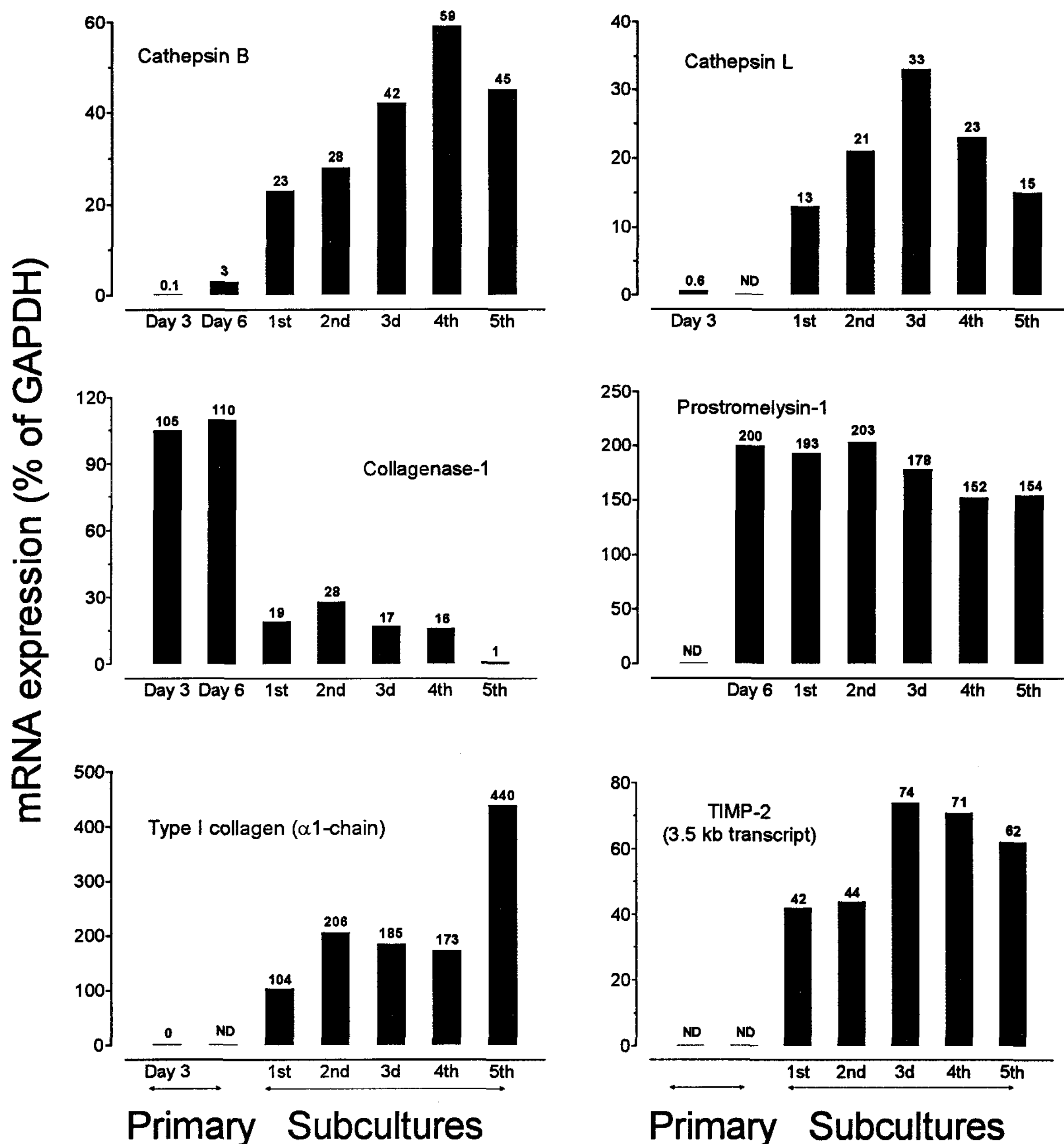


Fig. 1. Northern blot analysis of mRNA expression by human articular chondrocytes phenotypically modulated by subcultures. The intensity of the bands was determined by scanning densitometry of autoradiography films. To compensate for small variations in sample loading, results were normalized to the GAPDH mRNA of the same lane after exactly the same exposure and are shown as per cent of the band intensity ratio. Numbers on the top of the bars show the actual values. ND = not determined.

lated by serial monolayer subcultures as detailed previously for rabbit chondrocytes [5]. By measuring the enzymatic activity (data not shown), we confirmed that cathepsin B is a typical marker of the 'dedifferentiated' phenotype also in human articular chondrocytes. Namely, the intracellular pool of cathepsin B as well as its secretion increased dramatically parallel with the loss of the differentiated state of the cells and with a gradual morphological change from rounded-polygonal to a fibroblast-like type. mRNA was analyzed by the Northern blot method in primary cultures, up to the fifth subculture, and probed with cDNAs for cathepsin B, cathepsin L, collagenase-1, stromelysin-1, type I collagen and TIMP-2 (Fig. 1). Day 3 in Fig. 1 means the third day after the beginning of the cell isolation procedure from cartilage. This represents the first time-point at which the chondrocytes were free of debris and formed a uniform cell population with rounded morphology while still retaining the original biochemical phenotype. At Day 6 all cells were adherent and had begun to develop a fibroblast-like morphology. Freshly isolated chondrocytes hardly expressed detectable levels of cathepsin B and L mRNA. Expression of the two cathepsins became apparent after subculturing the cells and reached considerable levels in later subcultures. As an important internal control, type I collagen, previously shown to parallel the loss of chondrocytic differentiated functions [11,12], showed a similar pattern of expression. In fact, the mRNA level for the

α1(I) collagen chain was zero in differentiated chondrocytes and increased to a maximum response in the fifth subculture. Similar results have been reported for human fetal epiphyseal chondrocytes [13] and for bovine articular chondrocytes [14,15]. Collagenase-1 showed an opposite trend with respect to the two cathepsins and type I collagen: its mRNA decreased rapidly when the cells were passaged into the first subculture and was very low in the fifth subculture. Stromelysin-1 was strongly expressed, but did not show appreciable variations of its mRNA from the primary culture up to the fifth subculture. As previously noted by Stetler-Stevenson et al. [16] and Zafarullah et al. [17], TIMP-2 showed two transcripts (3.5 and 1.0 kb, respectively), and data in Fig. 1 refer to the major (3.5 kb) transcript. The mRNA for TIMP-2 was highly expressed in subcultures 1–5 showing a consistent trend to be slightly higher in subcultures 3–5 with respect to subcultures 1–2. Only a few cells were available for experiments with primary cultures, therefore some measurements could not be performed (ND in Fig. 1).

## 4. Discussion

In this paper we use the term 'phenotypic modulation' [18], others prefer 'dedifferentiation' [13,19], to describe the property of articular chondrocytes to synthesize type I collagen and cathepsin B under monolayer culture conditions, and to revert to the normal state with the production of type II collagen but no cathepsin B after transfer to a 3-dimensional culture system. We emphasize that in monolayer culture chondrocytes are not transformed into or overgrown by fibroblasts. Rather, the cells assume morphological and biochemical aspects resembling those of fibroblasts, a property also referred to as 'fibroblastic metaplasia'. Previous studies from this laboratory [9] demonstrated that osteoarthritic chondrocytes share with chondrocytes that are artificially modulated away from their original phenotype [5] the common property of expressing high levels of cathepsin B. On the basis of enzyme activity measurements, we pointed out that chondrocyte activation through the action of cytokines or by phenotypic modulation results in a completely different expression pattern for key enzymes such as collagenase-1 and cathepsin B [7]. Moreover, the differentiated chondrocyte did not secrete appreciable amounts of cathepsin B, but secreted high levels of collagenase-1, while the phenotypically modulated chondrocyte showed the opposite trend [5,7]. The results of this study confirmed, at the transcriptional level, the trends observed for enzymatic activity. In addition, cathepsin L, hitherto not reported from human articular chondrocytes, was shown to be expressed in a way similar to cathepsin B, being also a product of cells modulated away from their original phenotype. Conversely, stromelysin-1 did not show macroscopic changes and can be considered to be a constitutive, housekeeping enzyme, not responsive to alterations due to phenotypic chondrocyte modulation, although being sensitive to cytokine stimulation [20]. The high, persistent expression of TIMP-2 during several subcultures of the chondrocytes fully agrees with the result by Zafarullah et al. that this inhibitor is constitutively expressed in normal as well as in osteoarthritic chondrocytes [17].

Information pooled from this and our previous contributions [5–9], suggests that chondrocyte activation in osteoarthritis cannot only occur through cytokine stimulation, but also through a cytokine-independent mechanism based on fibroblastic metaplasia. We proposed earlier that the two routes of chondrocyte activation can either coexist or represent temporarily distinct episodes [7]. This concept was reinforced by ex vivo studies on osteoarthritic human cartilage [8,9] that highlighted the role of cathepsin B as a decisive factor in maintaining the chronic character of osteoarthritis. Further support for the role of cathepsin B proposed by us comes from a recent investigation in a rabbit model of osteoarthritis [21]. On the basis of the evidence accumulated to date, it seems reasonable to assume that destructive processes in osteoarthritis are dominated by cytokine-induced metalloendopeptidases during episodes of joint inflammation. On the other hand, repair phases are soon initiated to compensate for the matrix loss, but are unsuccessful through the action of cathepsin B expressed by phenotypically modulated chondrocytes, an event that can occur in the absence of cytokines. The fact that the two routes of chondrocyte activation lead to transcriptional and translational expression of cysteine and matrix metalloproteinases relevant to cartilage catabolism in opposite ways, may have not only pathogenetic, but also pharmacological implications.

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