Volume 306, number 2,3, 169–172 FEBS 11297 July 1992

Expression of c-*fos*, c-*jun*, *jun*-B, metallothionein and metalloproteinase genes in human chondrocyte

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Received 5 May 1992

Normal and osteoarthritic (OA) human articular cartilage chondrocytes, released enzymatically in the presence of 0.5% fetal calf serum, display constitutive expression of early response activating protein (AP-1) genes; c-*fos*, c-*jun* and *jun*-B. Among the late AP-1 responsive genes, total metallothionein (MT) and stromelysin mRNAs were expressed at high levels in both normal and OA chondrocytes, while collagenase and hMT-II_A mRNA levels were elevated only in OA individuals. Despite the common AP-1 sequences present in their promoter regions, the three late genes were differentially expressed.

Osteoarthritis; Chondrocyte; fos/jun; Collagenase; Stromelysin; Metallothionein; mRNA

1. INTRODUCTION

Progressive degradation of articular cartilage is one of the most important manifestations of osteoarthritis (OA) [1]. The cartilage chondrocytes could be triggered by certain stimuli to destroy their own extracellular matrix components, such as collagen type II and proteoglycans [2]. OA cartilage has been shown to contain elevated levels of zinc-containing metalloproteinases [3,4], such as collagenase and stromelysin, compared to their specific inhibitor, tissue inhibitor of metalloproteinases [5]. Expression of metalloenzyme genes can be induced by cytokines and tumor promoters [3,4]. Another group of 6–7 kDa proteins, metallothioneins (MT), not only bind but are also inducible by heavy metal (Cd^{2+}, Cu^{2+}, Zn^{2+}) salts [6]. They are involved in trace-metal nutrient (Cu^{2+} and Zn^{2+}) metabolism, possibly donating these essential metals to various metalloenzymes and transcription factors [6,7]. One of several MT genes, hMT-II_A, like metalloproteinases, is inducible by tumor promoter [8] and interleukin-1 [9]. Analysis of the promoter regions of the human collagenase, stromelysin and MT-II_A genes revealed that they contain a 9 bp sequence (TRE, tumor promoter-responsive element) which is recognized by the activating protein-1 (AP-1) complex of transcription factors [8,10] encoded by the fos/jun family of protooncogenes. This site is implicated in the transcriptional regulation of the collagenase gene by phorbol esters [8,10,11], interleukin-1 [12] and tumor necrosis factor α [13]. Other studies suggest that the AP-1 site in wild-type stromelysin promoter is needed for basal but not tumor promoter tetradecanoyl phorbol acetate (TPA) induction of the stromelysin gene [14]. Involvement of additional factors has been suggested [15] and it has been shown that the presence of AP-1 proteins is not always followed by expression of their responsive target genes [16].

The fos/jun nuclear protooncogenes belong to a group of 'early response' genes which are rapidly and transiently transcribed when quiescent cells are stimulated with serum to re-enter in cell cycle leading to DNA replication and cell division [17]. The transcription of early genes in the absence of protein synthesis is followed by the expression of various late target genes, such as collagenase [12], stromelysin and MT.

Human cartilage is a very resilient tissue and contains very few chondrocytes, making direct RNA extraction very difficult and giving a very poor yield. This study was aimed at investigating if enzymatically released normal and OA chondrocytes could be useful for studying differences in expression of the aforementioned early and late genes under minimal serum [0.5%] conditions. Our data demonstrate, for the first time, that chondrocytes differential express various early and late genes showing some differences between normal and diseased cells.

Abbreviations: Ap-1, activating protein-1; hMT-II_A, human metallothionein II_A; OA, osteoarthritic.

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2. MATERIALS AND METHODS

2.1. *Specimen selection and RNA extraction*

Cartilage specimens were obtained either from OA patients (2 males/3 females, mean age 70.8 years) after total knee replacement surgery or from normal individuals (3 males, mean age 78.6 years) at postmortem during autopsy at Notre-Dame and Saint-Luc Hospitals, Montreal, Qué. The diagnostic criteria for OA patients were based on clinical and radiological evaluations [18]. For normal controls, only patients with no history of joint disease and a macroscopic normal knee cartilage were used.

Cartilage obtained from tibial plateaus and femoral condyles were washed with sterile phosphate-buffered saline solution (PBS), and the chondrocytes released under sterile conditions by sequential enzymatic digestion; 2 h with pronase (2.0 mg/ml; Sigma, St-Louis, MO), followed by 18 h with collagenase (1 mg/ml; Sigma) at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratory, Grand Island, NY) containing 25 mM HEPES (Gibco), 0.5% heat-inactivated fetal calf serum (FCS, Gibco), 100 U/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). The digested tissue was centrifuged and the pellet washed three times with cold PBS. The cells were lysed with guanidinium isothiocyanate, and total RNA isolated by the CsCl–ultracentrifugation technique [19]. The RNA pellet was resuspended in sterile water, extracted with phenol and chloroform, recovered by ethanol precipitation and quantified by spectrophotometry [19].

2.2. *Northern blotting and hybridization analyses*

Total RNA (5 μg) was fractionated in several identical copies of 1.2% formaldehyde-agarose gels [19], 28 S and 18 S ribosomal RNA bands visualized by ethidium bromide staining and then electroblotted (Transblot, Bio-Rad, Mississauga, Ont.) onto zetaprobe nylon membranes (Bio-Rad) using 30 mM sodium phosphate buffer, pH 6.8, at a current of 500 mA for 12 h. Following complete transfer, wet membranes were wrapped in plastic and placed for 10 min on an ultraviolet box for crosslinking. Pre-washing was performed in a buffer containing 3 × SET (1 × SET = 0.15 M NaCl, 0.03 M Tris-HCl, 2 mM EDTA, pH 8.0), 10 × Denhardt's solution and 1% sodium dodecyl sulfate at 68°C for 3 h. The prehybridization buffer consisted of 3 × SET, 10 × Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate, 10 μg/ml poly(A), 250 μg per/ml yeast RNA, and 50 μg/ml of sheared and denatured salmon sperm DNA. The probes were as follows; for human c-*fos* [20] a 9 kb *Eco*RI fragment (American Type Culture Collection (ATCC) #41042, Rockville, MD) cloned into pBR322; for c-*jun* a mouse cDNA (2.6 kb *Eco*RI fragment in pGEM-2 from ATCC #63026) [21]; and for human *jun*-B (provided by Dr. Shaun McColl, Université Laval, Ste-Foy, Qué.), a 1 kb *Eco*RI–*Pst*I cDNA fragment cloned in pUC12, were utilized. The respective fragments were excised from the gels and labelled to a high specific activity (10^8 cpm/μg) with the Promega Biotech (Madison, WI) random primer labelling (Prime-a-Gene, Promega) kit. Metallothionein (MT) probes have been previously described [22]. The MT-IIpg (processed gene) detects mRNAs encoded by all human MT genes. The human metallothionein-II_A (provided by Dr. Dean Hamer, National Institute of Health), MT-IF and MT-IG specific fragments were from the 3′ ends of these genes and have been shown to be specific for the respective genes [22]. cRNA probes from these fragments previously cloned in pGEM-2 vector were synthesized utilizing SP6 (hMT-II_A) or T7 (hMT-IIpg, MT-IF and MT-IG) polymerases (Promega Biotech) following linearization of the plasmids.

The antisense radiolabelled RNA (cRNA) probes were synthesized from cloned cDNA fragments for collagenase (provided by Dr. D. Edwards, University of Calgary, Calgary, Canada), stromelysin-1 (provided by Dr. G. Goldberg, Washington University, St. Louis, MO) and glyceraldehyde phosphate dehydrogenase (GAPDH) (ATCC #57090, cloned in pGEM-3Z vector) employing [α-^{32}P]CTP (New England Nuclear, Madison, WI). The former two probes were synthesized from linearized bluescript plasmids by T3 polymerase promoter and GAPDH by T7 polymerase according to the protocols provided by Promega Biotech. Following 12 h of prehybridization at 68°C in 15 ml of buffer, hybridization was performed in 4 ml of the buffer for 36 h at 68°C (except mouse c-*jun* where 60°C was used) in the presence of 6×10^6 cpm of each probe. Each filter was hybridized in separate bags with the respective probes. Stringent serial post-hybridization washes were conducted at 68°C, for which the final wash was 0.1% SET, 0.1% SDS and 0.1% sodium pyrophosphate. For GAPDH, and hMT-II_A, MT-IF and MT-IG probes, filters were treated with RNAse A and RNAse T1 for 30 min at 37°C in 2 × SET to remove any non-specific hybridization [22]. The membranes were rinsed for 15 min at room temperature in 3 × SET and subjected to autoradiography using Kodak XAR5 films and Cronex intensifying screen (Dupont Canada, Mississauga, Ont.) for various periods of time at −80°C.

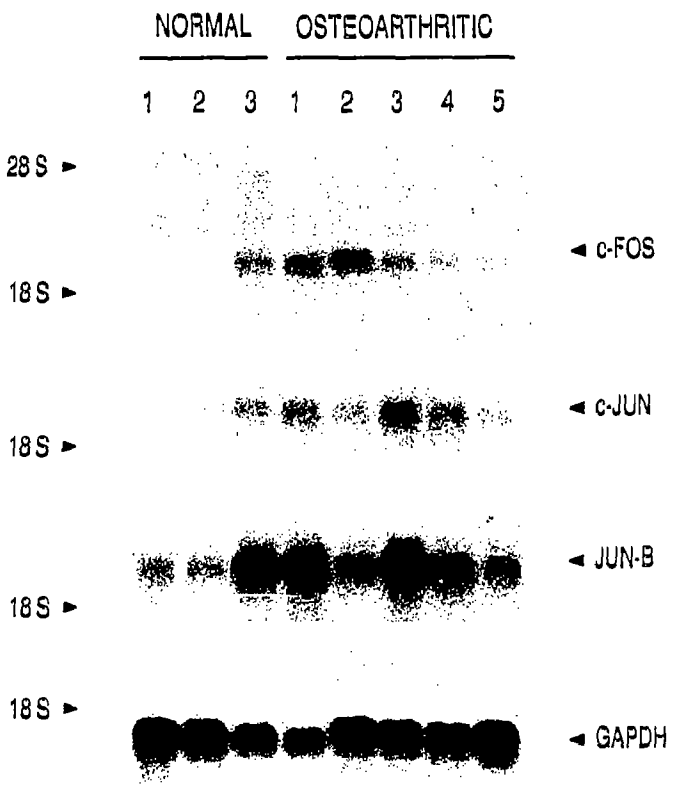

Fig. 1. Expression of 'early response' mRNAs in normal and osteoarthritic chondrocytes. Identical Northern blots of total RNA (5 μg/lane) from different individuals (denoted by numbers) were hybridized with the indicated probes and autoradiographed for 96 h (c-*fos*), 7 days (c-*jun* and *jun*-B) and 32 h (GAPDH). The position of 28 S and 18 S ribosomal RNAs are indicated. All the bands are at the expected positions.

3. RESULTS AND DISCUSSION

To evaluate whether enzymatically liberated chondrocytes from human articular cartilage expressed early c-*fos*, c-*jun* and *jun*-B genes under very low (0.5%) serum conditions, identical RNA blots were hybridized to the respective probes. The results shown in Fig. 1 suggest that the chondrocytes from normal individuals produced very low (lanes 1,2) to moderate (lane 3) levels of c-*fos*, c-*jun* and *jun*-B mRNAs. All 5 OA chondrocytes expressed the three genes with slight elevation of c-*fos*

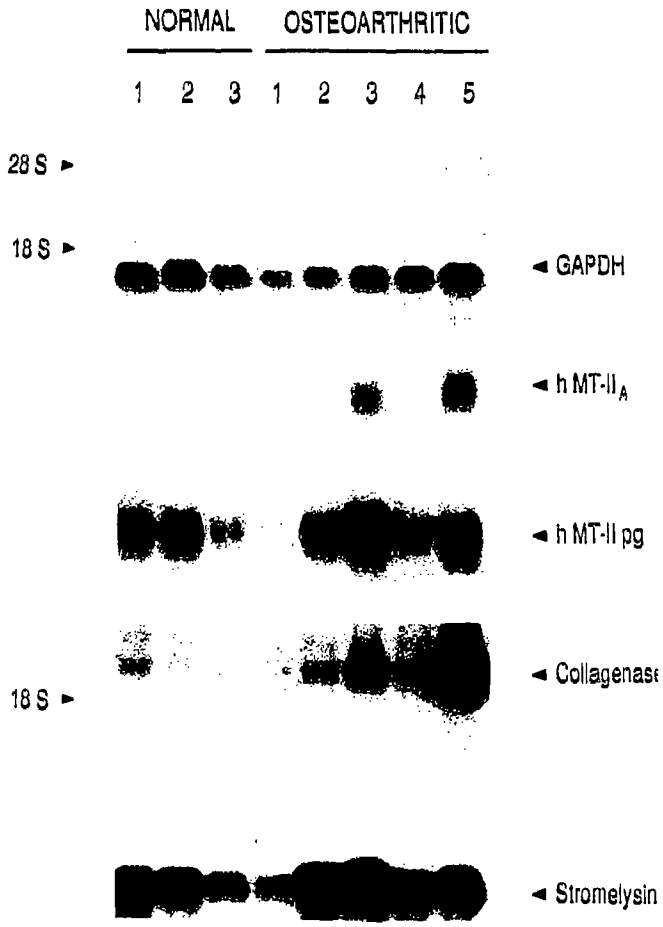

Fig. 2. Expression of 'late response' mRNAs in normal and osteoarthritic chondrocytes. Four identical Northern blots from the individuals in Fig. 1 were hybridized with the respective probes and film-exposed for 24 h (GAPDH and hMT-II$_A$), 18 h (hMT-II pg), 20 h (collagenase) and 6 h (stromelysin). The filters hybridized with GAPDH and hMT-II$_A$ probes were treated with RNAse A and T1 to detect hMT-II$_A$-specific RNA. The hMT-IIpg detects total MT mRNAs.

mRNA in two individuals (OA lanes 1,2). The c-*jun* and *jun*-B appeared to be coordinately expressed. The weak signal in c-*jun* is due to weaker hybridization with the heterologous mouse probe. The ubiquitously expressed glyceraldehyde phosphate dehydrogenase mRNA levels showed very little variation. These results demonstrate that human articular chondrocytes express the early-response mRNAs. However, one must analyze these data with caution; the slightest differences in normal and OA may not reflect an in vivo phenomenon, as these mRNAs may be induced either by mechanical disturbance of extracellular matrix or by serum factors during digestion of cartilage with pronase and collagenase. The early mRNAs have been shown to be induced in other cell types in response to injury and stress [23]. This issue will be resolved only when direct extraction of RNA from cartilage is possible.

Following detection of early-response mRNAs, we wished to determine if the AP-1-responsive late genes were expressed in human chondrocytes. The promoter regions of human metallothionein II$_A$, collagenase and stromelysin genes have previously been shown to contain an AP-1 recognition sequence [8,10]. Fig. 2 illustrates the Northern hybridization analysis, and revealed that both normal and OA chondrocytes contained a very high constitutive level of stromelysin and total metallothionein (MT) mRNAs, with some variation among different individuals. Stromelysin RNA levels were greater (6 h exposure) than total MT (18 h exposure). In OA cells, a slight increase was noted in all but one case. When three metallothionein gene-specific probes [22] were employed, only AP-1 responsive, hMT-II$_A$- specific RNAse A and T1-resistant transcripts could be detected with slight elevation in two OA individuals. No transcript for hMT-I$_F$ and hMT-I$_G$ genes could be detected under these conditions (data not shown). It is possible that MT genes other than MT-II$_A$ also contribute to the total MT mRNA. In contrast with very high stromelysin mRNA levels in both normal and OA, collagenase mRNA was elevated in 4 cases of OA chondrocytes compared to barely detectable levels in normal cells. It is quite intriguing that closely related metalloproteinases, collagenase and stromelysin are expressed differentially in identical released chondrocytes. We have repeatedly observed preferential expresssion of stromelysin over collagenase in directly extracted RNA from OA synovial membranes (Zafarullah et al., unpublished results). Discoordinate expression of the two genes has also been observed in cultured rheumatoid synovial fibroblasts [24]. Furthermore, cycloheximide, an inhibitor of protein synthesis, selectively induced the stromelysin gene and prevented collagenase mRNA induction by IL-1 [25]. Despite the similarity of the regulatory mechanisms via AP-1, these late genes may have additional gene-specific mechanisms. It is possible that stromelysin and total MT transcripts were induced either by small amounts of serum factors or by mechanical stress during enzymatic digestion of cartilage matrix. Co-induction of both early and late genes might indicate a relationship between the regulatory and the AP-1-responsive genes. Others have shown that expression of early genes is not always followed by the expression of late genes [16], questioning the regulatory role of AP-1 encoding genes.

In summary, this study demonstrates that several early and late genes are expressed simultaneously in chondrocytes released from the human cartilage in the presence of 0.5% serum. With the exception of collagenase, only minor differences between normal and OA could be detected, thus suggesting that approaches such as in situ hybridization could be more useful to evaluate the in vivo expression of these genes.

Acknowledgements: The generous gifts of cDNAs for collagenase from Dr. Dylan Edwards (University of Calgary, Calgary, Alta), stromely-

sin from Dr. Greg Goldberg (Washington University, St. Louis, MO), *jun*-B from Dr. Shaun McColl, Université de Laval, Qué., and human MT-II$_A$ from Dr. Dean Hamer (National Institute of Health, USA) were profoundly appreciated. The authors are grateful to Ms. Nathalie Gendron and Mr. Gilles Viau for their expert technical assitance. We thank Ms. Santa Fiori for processing this manuscript. The authors acknowledge the support of the Arthritis Society (Canada), the University of Montréal's Faculty of Medicine (FIRFM) and the Fonds de la recherche en santé du Québec (FRSQ).

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