

# Collagenase-3 (MMP-13) Expression in Chondrosarcoma Cells and Its Regulation by Basic Fibroblast Growth Factor

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**Human collagenase-3 (MMP-13) is a member of the matrix metalloproteinase family of enzymes that was originally identified in breast carcinomas and subsequently detected during fetal ossification and in arthritic processes. In this work, we have found that collagenase-3 is produced by HCS-2/8 human chondrosarcoma cells. An analysis of the ability of different cytokines and growth factors to induce the expression of collagenase-3 in these cells revealed that basic fibroblast growth factor (bFGF or FGF-2) strongly up-regulated the expression of this gene. By contrast, other factors, including interleukin-1 $\beta$  and transforming growth factor- $\beta$ , previously found to induce collagenase-3 expression in other cell types, did not exhibit any effect on the expression of this gene in chondrosarcoma cells. Further analysis of the bFGF-induced expression of collagenase-3 in human chondrosarcoma cells revealed that its effect was time and dose dependent, but independent of the *de novo* synthesis of proteins. Western blot analysis revealed that the up-regulatory effect of bFGF on collagenase-3 was also reflected at the protein level as demonstrated by the increase of immunoreactive protein in the conditioned medium of HCS-2/8 cells treated with bFGF. Immunohistochemical analysis of the presence of collagenase-3 in a series of 8 benign and 16 malignant cartilage-forming neoplasms revealed that all analyzed malignant chondrosarcomas stained positively for collagenase-3, whereas only 2 of 8 benign lesions produced this protease. In addition, the finding that bFGF was detected in all analyzed chondrosarcomas, together with the above *in vitro* studies on HCS-2/8 cells, suggest that this growth factor may be an *in vivo* modulator of collagenase-3 expression in these malignant tumors. These results extend the pattern of tumor types with ability to produce this matrix**

**metalloproteinase and suggest that collagenase-3 up-regulation may contribute to the progression of human chondrosarcomas. (*Am J Pathol* 1998, 153:91–101)**

Human collagenase-3 (MMP-13) is a recently identified member of the matrix metalloproteinase (MMP) family that was originally isolated from breast carcinomas.<sup>1</sup> Biochemical characterization of collagenase-3 has revealed that it is a very potent enzyme that, after activation through a proteolytic cascade mechanism, displays a broad spectrum of activity against connective tissue components.<sup>2,3</sup> Thus, collagenase-3 degrades very efficiently the native helix of all fibrillar collagens, with preferential activity on type II collagen, which has led to the suggestion that this enzyme may be of special relevance in the turnover of articular cartilage that is particularly rich in this type of collagen.<sup>3</sup> Consistent with this proposal, collagenase-3 is expressed by human chondrocytes during fetal ossification<sup>4,5</sup> and in inflammatory and degenerative joint diseases, including osteoarthritis and rheumatoid arthritis.<sup>6–10</sup> In addition to its proteolytic activity on fibrillar collagens, collagenase-3 is also a powerful gelatinase and may thus contribute to further degrade the initial cleavage products of collagenolysis to small fragments suitable for further metabolism.<sup>3</sup> Finally, very recent studies have provided evidence that collagenase-3 may also degrade the large cartilage proteoglycan aggregate<sup>11</sup> and other components of the extracellular matrix and basement membranes, including type IV collagen, fibronectin, and tenascin.<sup>12</sup>

The wide spectrum of substrates susceptible to proteolytic degradation by collagenase-3, together with the presence of this enzyme in breast carcinomas but not in normal mammary gland nor in benign mammary lesions, has suggested that it could play a critical role in the uncontrolled lytic processes occurring during malignant tumor progression.<sup>1</sup> In agreement with this proposal, pre-

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**Table 1.** Clinical, Histopathological, and Immunohistochemical Characteristics of Benign and Malignant Cartilage Tumors

Tumor sample	Sex	Age	Location of tumor	Grade	Collagenase-3 (percentage/intensity)	bFGF (percentage/intensity)
Chondrosarcoma	F	82	Hand	I	9.2%/+	10.6%/+
Chondrosarcoma	F	57	Hand	I	20.3%/+++	15.9%/++
Chondrosarcoma	F	39	Humerus	I	10.0%/+	12.8%/+
Chondrosarcoma	F	82	Foot	I	4.3%/+	6.3%/+
Chondrosarcoma	M	41	Calcaneum	I	21.9%/++	18.9%/++
Chondrosarcoma	F	42	Hand	I	18.3%/+	12.5%/+
Chondrosarcoma	M	52	Humerus	I	15.4%/+	12.0%/+
Chondrosarcoma	M	75	Rib	I	15.3%/+	26.3%/+
Chondrosarcoma	M	50	Pubis	I	18.0%/++	14.1%/++
Chondrosarcoma	M	60	Ileum	I	15.4%/++	12.1%/+
Chondrosarcoma	M	55	Rib	II	40.4%/+++	23.2%/++
Chondrosarcoma	M	54	Cricoid	I	8.4%/++	19.6%/+
Chondrosarcoma	F	42	Tibia	I	8.2%/+	11.5%/+
Chondrosarcoma	M	16	Femur	III	24.7%/++	18.5%/++
Chondrosarcoma	M	48	Scapula	I	7.7%/++	6.8%/+
Chondrosarcoma	M	16	Femur	II	30.6%/+++	22.8%/+
Chondroma	M	40	Hand		7.9%/+	0%
Chondroma	M	25	Rib		0%	0%
Chondroma	M	24	Foot		0%	0%
Chondroma	M	68	Femur		0%	0%
Chondroma	M	54	Hand		0%	0%
Chondroma	F	51	Rib		0%	0%
Osteochondroma	F	42	Hand		0%	0%
Chondroblastoma	M	14	Tibia		12.4%/++	15.6%/+

F, female; M, male. Intensity was scored as follows: +, weakly positive tumor; ++, positive tumor; +++, strongly positive tumor.

liminary studies on the prognostic value of collagenase-3 in breast cancer have revealed that this enzyme is a marker of poor clinical outcome in breast cancer patients (F. Vizoso, unpublished data). In addition, recent studies have shown that collagenase-3 is also overexpressed by a subset of laryngeal squamous cell carcinomas with extensive local invasion.<sup>13</sup> However, at present, there is no information on the possible production of this enzyme by tumors other than breast carcinomas and squamous cell carcinomas.<sup>1,13-15</sup> In this regard, the finding that collagenase-3 is produced by chondrocytes during human fetal development and in joint-destructive processes prompted us to examine the possibility that this enzyme could be also associated with tumor processes involving these cells. The malignant cartilage-forming tumors or chondrosarcomas account for approximately 25% of all malignant tumors arising from the skeletal system and are the most frequently diagnosed bone tumors in patients older than 50 years.<sup>16-21</sup> In this work, we provide evidence that collagenase-3 is produced in significant amounts by human chondrosarcoma cells. In addition, analysis of factors potentially responsible for this effect has revealed that bFGF is a strong inducer of collagenase-3 expression in chondrosarcoma cells. Finally, we have evaluated by immunohistochemistry the presence of collagenase-3 in a series of benign and malignant cartilage-forming tumors.

## Materials and Methods

### Materials

All media and supplements for cell culture were obtained from Sigma Chemical Co. (St. Louis, MO) except for fetal

calf serum, which was from Boehringer Mannheim (Mannheim, Germany). Basic fibroblast growth factor (bFGF), acidic FGF (aFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)- $\beta$ 1, interleukin (IL)-1 $\beta$ , 12-O-tetradecanoylphorbol-13-acetate (TPA), dexamethasone, estradiol, and dihydrotestosterone were from Sigma. Rabbit polyclonal IgG anti-bFGF was from Santa Cruz Biotechnology (Santa Cruz, CA). Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim. Double-stranded DNA probes were radiolabeled with [<sup>32</sup>P- $\alpha$ ]dCTP (3000 Ci/mmol) from Amersham International (Little Chalfont, UK) using a commercial random-priming kit purchased from Amersham International.

### Patients and Tumor Specimens

A total of 24 cartilage-forming tumor specimens obtained from patients diagnosed and treated at Hospital de Jove-Gijón, Hospital de Cabueñes-Gijón, and Hospital Central de Asturias-Oviedo (Spain) were analyzed. Sixteen cases corresponded to ten male and six female patients diagnosed of conventional chondrosarcoma (Table 1). None of the patients had received chemo- or radiotherapy before the operation. The age range was 16 to 82 years (mean, 50.7 years). The most frequent site of involvement was the hand (three cases) followed in frequency by the femur and humerus (two cases at each site). Chondrosarcomas were graded according to standard criteria such as cellularity, mitotic activity, and nuclear atypia.<sup>21</sup> In the overall group of sixteen malignant tumors, thirteen were grade I, two were grade II, and only one was a mitotically active grade III tumor. The study population

also included eight cases of benign cartilage-forming neoplasms: six chondromas, one chondroblastoma, and one osteochondroma. The age range of these patients (six males and two females) was 14 to 68 years (mean, 39.7 years). Benign and malignant tumor specimens were routinely fixed and decalcified in 0.3 mol/L EDTA (pH 7.5) before embedding in paraffin. Five-micron sections were cut and stored at room temperature until use for histological diagnosis or immunohistochemical analysis.

### *Cell Culture*

Human chondrosarcoma cells HCS-2/8 were derived from a tumor of the proximal part of the humerus of a 72-year-old Japanese man who had received no surgical treatment or chemical or radiation therapy.<sup>22,23</sup> The tumor was identified as a well differentiated chondrosarcoma as previously described.<sup>22</sup> SW1353 cells were obtained from the American Type Culture Collection (Rockville, MD) and were derived from a primary grade II chondrosarcoma of the right humerus from a 72-year-old Caucasian woman. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were subcultured every 2 weeks by incubation at 37°C for 2 minutes with 0.0125% trypsin in 0.02% EDTA, followed by addition of complete medium, washing, and resuspension in fresh medium. For most experiments, approximately  $5 \times 10^5$  cells were plated out in 100-mm dishes and transferred to serum-free DMEM for 24 hours and then exposed to the different growth factors, cytokines, and tumor promoters at the concentrations and for the times indicated.

### *Northern Blot Analysis*

Total RNA was isolated from HCS-2/8 cells by the guanidium isothiocyanate procedure according to Chomczynski and Sacchi,<sup>24</sup> separated by electrophoresis in 1% agarose-formaldehyde gels, and blotted onto Hybond N nylon filters (Amersham International). Filters containing 20  $\mu$ g of total RNA were prehybridized at 42°C for 3 hours in 50% formamide, 5X SSPE (1X SSPE contains 150 mmol/L NaCl, 10 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L EDTA, pH 7.4), 10X Denhardt's, 2% SDS, and 100  $\mu$ g/ml denatured herring sperm DNA and then hybridized with radio-labeled collagenase-3 full-length cDNA for 20 hours under the same conditions. Filters were washed with 0.1X SSC, 0.1% SDS for 2 hours at 50°C and exposed to autoradiography. RNA integrity and equal loading were assessed by hybridization with a  $\beta$ -actin probe.

### *Reverse Transcription and PCR Amplification of RNA from Human Chondrosarcoma Cells*

Total RNA from HCS-2/8 or SW1353 chondrosarcoma cells, or from a primary chondrosarcoma was used for cDNA synthesis with the RNA polymerase chain reaction

(PCR) kit from Perkin-Elmer/Cetus (Norwalk, CT). The primary chondrosarcoma used in this experiment corresponded to a grade II tumor of the scapula of a 35-year-old man. After RNA isolation and reverse transcription (RT) using 1  $\mu$ g of total RNA and random hexamers as primer, the whole mixture was used for PCR with two oligonucleotides (5'-CCTCCTGGGCCAAATTATGAG-3' and 5'-CAGCTCCGCATCAACCTGCTG-3') specific for collagenase-3. As a positive control, we performed RT-PCR of the different RNA samples with two primers (5'-GTGGGGCCGCTCTAGGCAC-3' and 5'-TTTGATGTCACGCACGATTT-3') specific for human actin. The PCR amplifications were carried out in a GeneAmp 2400 PCR system from Perkin-Elmer for 40 cycles of denaturation (95°C for 2 minutes), annealing (64°C for 30 seconds), and extension (72°C for 30 seconds). The PCR products were analyzed in 2% agarose gels, cloned in pUC18, and sequenced by the dideoxy terminator using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

### *Western Blot Analysis*

Conditioned media were obtained after incubation of HCS-2/8 in serum-free DMEM for 48 hours or supplemented with bFGF, filtered, and dialyzed in an Amicon Centricon-10 microconcentrator (Amicon, Beverly, MA). Proteins from conditioned medium were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing and reducing conditions and transferred to nitrocellulose membranes (Amersham International). After blocking in PBS containing 0.1% Tween-20 (PT buffer) and 5% low-fat dried milk, the membranes were incubated with 0.1  $\mu$ g/ml monoclonal antibody 181-15A12 raised against recombinant human collagenase-3 (kindly provided by K. Iwata, Fuji Chemical Industries, Takaoka, Japan). After extensive washing of the membranes with PT buffer, they were incubated with a horseradish-peroxidase-conjugated goat antiserum against mouse IgGs diluted 1:20,000 in PT containing 3% low-fat dried milk. The membranes were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (ECL system, Amersham International).

### *Immunohistochemical Analysis*

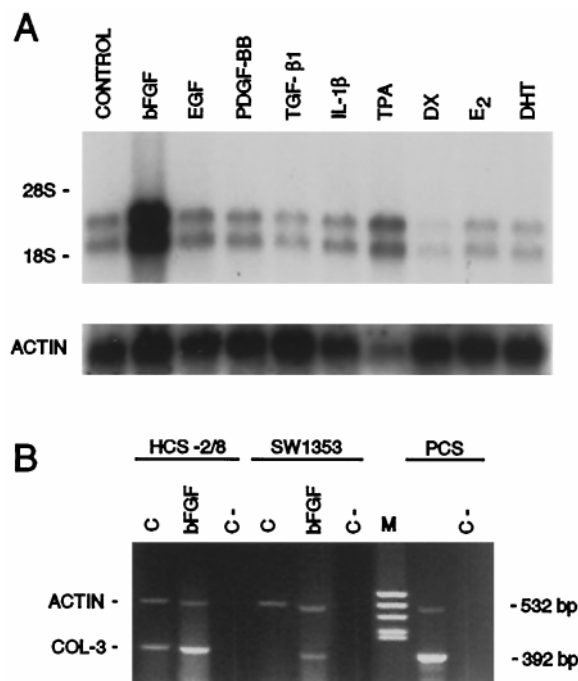
Immunohistochemical assays were performed on 5- $\mu$ m, formalin-fixed, paraffin-embedded tissue sections using the streptavidin-biotin method. After routine deparaffinization, sections were treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes to block endogenous peroxidase activity and then washed with three changes of 0.01 mol/L PBS for 5 minutes each and treated with 1% normal serum for 30 minutes. Sections were incubated in a moist chamber for 12 hours at 4°C with monoclonal antibody 181-14G11 against collagenase-3 (2.5  $\mu$ g/ml). They were then washed with several baths of PBS and incubated sequentially with biotinylated goat anti-mouse antibody (Biomedica Corp., Foster City, CA) and peroxidase-conjugated streptavidin (Biomedica Corp.) for 30 minutes at

room temperature in a moist chamber, washing with PBS between incubations. After washes, sections were treated with a solution containing 0.66 mol/L 3,3'-diaminobenzidine and 2 mmol/L H<sub>2</sub>O<sub>2</sub> in 50 mmol/L Tris/HCl, pH 7.6. Sections were finally counterstained for nuclei with hematoxylin, dehydrated, and mounted with Eukitt. The purity and identity of the antigen used for developing monoclonal antibodies was assessed by automatic Edman degradation of the recombinant human collagenase-3. Specificity of staining was determined using controls that involved incubation of tissues with buffer alone or with an equal amount of IgG from nonimmunized animals. In both cases, there was no significant staining. Furthermore, immunostaining was completely abolished by antiserum preincubation with purified collagenase-3, produced in a vaccinia virus expression system as previously described.<sup>1</sup> Immunohistochemical analysis using rabbit polyclonal anti-bFGF IgGs (0.5 µg/ml; Santa Cruz Biotechnology) was performed in the same way, except that the secondary antibody was a biotinylated goat anti-rabbit antibody (Biomedica Corp). The rabbit anti-bFGF was raised against a synthetic peptide corresponding to positions 40 to 63 in the amino acid sequence of human bFGF. This antiserum did not show cross-reactivity with aFGF (FGF-1). Quantitation of immunoreactivity was performed using an Olympus light microscope interfaced via a Sony camera to an image analysis system (Qwin Pro, Leica, Wetzlar, Germany). The percentages of collagenase-3- and bFGF-immunopositive cells were obtained from 20 random fields per case/section using a 10× objective lens. Semiquantitative estimation of both collagenase-3 and bFGF immunostaining intensities was made in immunopositive cells by arbitrarily assigning +++ when cytoplasmic immunostaining appeared dark brown, ++ when it appeared reddish, and + when it appeared yellowish. Statistical analysis was performed with an SPSS statistical package.

## Results

### Collagenase-3 Is Expressed by Human Chondrosarcoma Cells

To evaluate the possibility that human collagenase-3 is produced by chondrosarcoma cells, HCS-2/8 cells derived from a well differentiated chondrosarcoma were analyzed by Northern blot using as a probe the complete cDNA encoding this enzyme. As illustrated in Figure 1A, two transcripts of approximately 3.0 and 2.5 kb were detected, albeit at low levels, in total RNA extracted from untreated HCS-2/8 cells. The size of these mRNA bands agrees perfectly with that of the two major collagenase-3 transcripts identified in breast carcinomas,<sup>1</sup> squamous cell carcinomas of the head and neck,<sup>13,14</sup> and articular cartilage from arthritic patients.<sup>6-8</sup> Then, and to study the putative factors that could stimulate the expression of collagenase-3 in human chondrosarcoma cells, HCS-2/8 cells were incubated for 24 hours in the presence of a variety of growth factors, cytokines, steroid hormones, and tumor promoters, and total RNA was subjected to



**Figure 1.** Effect of bFGF and other factors on collagenase-3 expression in human chondrosarcoma cells. **A:** Northern blot analysis was performed using 10 µg of total RNA from HCS-2/8 cells incubated for 24 hours in the presence of 10 ng/ml bFGF, 10 ng/ml EGF, 10 ng/ml PDGF-BB, 10 ng/ml TGF-β1, 5 ng/ml IL-1β, 10<sup>-7</sup> mol/L TPA, 10<sup>-7</sup> mol/L dexamethasone (DX), 10<sup>-7</sup> mol/L estradiol (E<sub>2</sub>), and 10<sup>-7</sup> mol/L dihydrotestosterone (DHT). The filter was hybridized with a collagenase-3 cDNA probe, stripped, and subsequently hybridized with a β-actin probe to ascertain equal RNA loading for the different samples. **B:** RT-PCR analysis of collagenase-3 expression in HCS-2/8 and SW1353 chondrosarcoma cells as well as in a primary human chondrosarcoma (PCS). One microgram of total RNA isolated from untreated cells (lanes C), primary chondrosarcomas (PCS), or cells treated with bFGF (10 ng/ml) was reverse transcribed and PCR amplified with oligonucleotides specific for collagenase-3 (392-bp band) and for human actin (532-bp band). Lanes C-, negative controls in which template was omitted. PCR products were separated on a 2% agarose gel run in Tris, borate, and EDTA. pBR322 digested with *Hae*III (Marker V, Boehringer Mannheim) was used as a size marker (lane M).

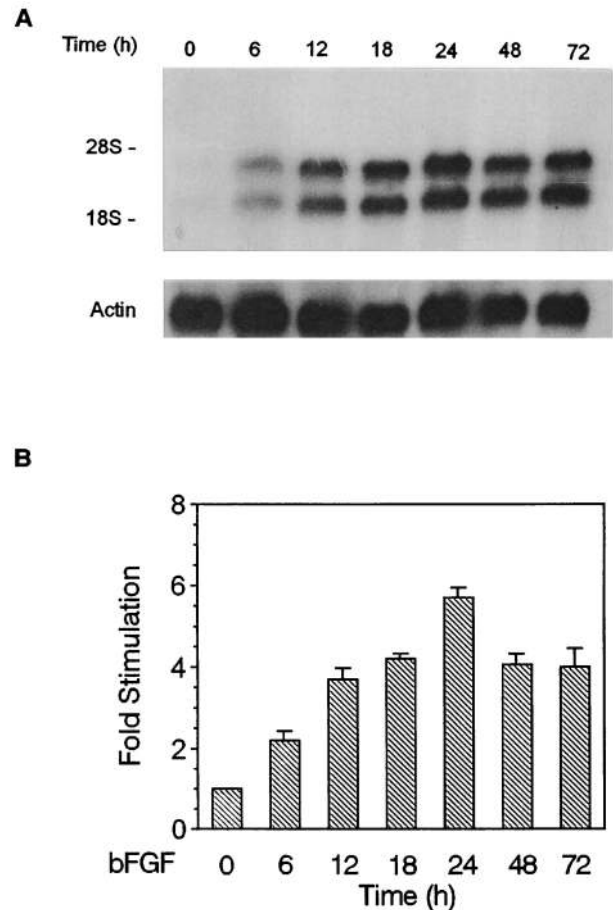
Northern blot analysis using the same collagenase-3 cDNA probe as above. As shown in Figure 1A, bFGF strongly induced the accumulation of the two collagenase-3 mRNA transcripts, resulting in a sevenfold increase over the basal levels observed in the unstimulated cells. The bFGF-mediated induction of collagenase-3 expression in chondrosarcoma cells was also observed in SW1353 cells, a commercially available human chondrosarcoma cell line. However, the level of expression of collagenase-3 in these cells was lower than in HCS-2/8 cells, and RT-PCR was required to increase the sensitivity of detection (Figure 1B). A band of the expected size (392 bp), the identity of which was confirmed by nucleotide sequencing, was amplified from RNA obtained of HCS-2/8 and SW1353 cells treated with bFGF as well as from RNA of a primary chondrosarcoma. Nevertheless, the amount of collagenase-3 RNA amplified from SW1353 cells was consistently lower than that detected in HCS-2/8 cells. These variations could be due to the fact that SW1353 have partially lost their differentiated chondrocyte phenotype, displaying some characteristics typical of fibroblastic cells. Consequently, all subsequent stud-

ies described in the present work were performed with HCS-2/8 cells.

Analysis of the ability of other factors and compounds to up-regulate collagenase-3 expression in chondrosarcoma cells (Figure 1A) revealed that the tumor promoter TPA, a potent inducer of most MMPs in a wide variety of cell types, also induced collagenase-3 expression in HCS-2/8 cells. However, IL-1 $\beta$  did not exhibit any stimulating effect on collagenase-3 expression by HCS-2/8 cells, despite that this pro-inflammatory cytokine has been found to induce collagenase-3 production by normal articular chondrocytes as well as by chondrocytes from osteoarthritic patients.<sup>5-7</sup> The reason for this observation is unclear as IL-1 receptor (type I) is normally expressed in these cells. Nevertheless, the finding that HCS-2/8 cells produce truncated forms of IL-1 could suggest that endogenous abnormal IL-1 may occupy the receptor, thus blocking the effect of exogenously added cytokines (M. Takigawa, unpublished results). Figure 1A also shows that dexamethasone displayed a down-regulatory effect on collagenase-3 expression in HCS-2/8 cells, whereas the remaining analyzed factors including EGF, PDGF-BB, TGF- $\beta$ , estradiol, and dihydrotestosterone did not show any effect on the expression of this metalloproteinase by chondrosarcoma cells.

#### *bFGF Induces Collagenase-3 Expression in Chondrosarcoma Cells through a Mechanism Independent of the de Novo Protein Synthesis*

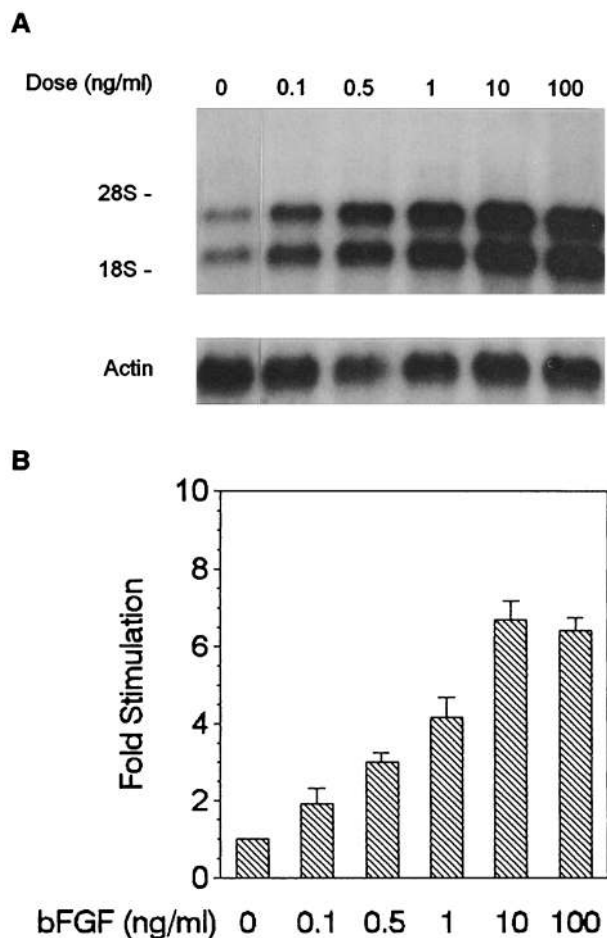
As the above results identified bFGF as a potent inducer of collagenase-3 expression in human chondrosarcoma cells, we undertook a preliminary analysis of the molecular mechanisms and signal transduction pathways mediating this effect. To this purpose, we first examined by Northern blot the time course of the bFGF-induced up-regulation of collagenase-3 expression in HCS-2/8 cells. As can be seen in Figure 2, there was a consistent increase with time in the steady-state collagenase-3 mRNA levels from cells treated with 10 ng/ml bFGF. The up-regulatory effect was already detectable after 6 hours, being maximal 24 hours after bFGF treatment (sixfold over the control cells), and remaining constant for at least 72 hours. The possibility that the induction of collagenase-3 mRNA levels by bFGF was dose dependent was also examined by Northern blot hybridization, and the results obtained are shown in Figure 3. As can be seen, incubation of the cells with 10 ng/ml bFGF resulted in a maximal induction of approximately sevenfold over the control cells, whereas concentrations as low as 0.1 ng/ml still produced an accumulation of collagenase-3 mRNA of approximately twofold over the control. To determine whether the up-regulating effect of bFGF on collagenase-3 mRNA levels was also reflected at the protein level in HCS-2/8 cells, we performed Western blot analysis with conditioned medium from cells treated with 10 ng/ml bFGF for 48 hours. As shown in Figure 4, a clear immunoreactive band of the expected molecular mass (approximately 60 kd) was detected in the 25-fold con-



**Figure 2.** Time course of the effect of bFGF on collagenase-3 expression in human chondrosarcoma cells. **A:** HCS-2/8 cells were cultured for 24 hours in the presence of 10 ng/ml bFGF for the indicated times, and total RNA from each culture was isolated and analyzed by Northern blot, as described in the legend to Figure 1. Filters were hybridized consecutively with labeled probes for collagenase-3 and  $\beta$ -actin. **B:** The hybridization signals were scanned by densitometry, and the values of collagenase-3 mRNA in each sample were corrected for differences of total RNA/lane. The results are expressed as corrected by the control values for each time point.

centrated conditioned medium of HCS-2/8 cells, but not in the medium from untreated cells.

To further analyze the mechanism of induction of the collagenase-3 gene by bFGF, we next cultured HCS-2/8 cells in the presence of cycloheximide, a powerful protein synthesis inhibitor. As can be seen in Figure 5, incubation of these cells with 10  $\mu$ g/ml cycloheximide (added 1 hour before bFGF) did not prevent the bFGF-induced accumulation of collagenase-3 mRNA, despite the fact that this treatment caused an extensive inhibition in overall protein synthesis. We therefore conclude that the induction of collagenase-3 expression by bFGF was independent of the synthesis of proteins *de novo*. By contrast, it is remarkable that a superinduction effect on collagenase-3 mRNA was observed as a consequence of the cycloheximide treatment, suggesting the occurrence in HCS-2/8 cells of a protein factor that acts as a repressor of collagenase-3 gene transcription or an increase in the stability of mRNA transcripts. A similar explanation has been pointed out for the rat collagenase-3 superinduction ob-

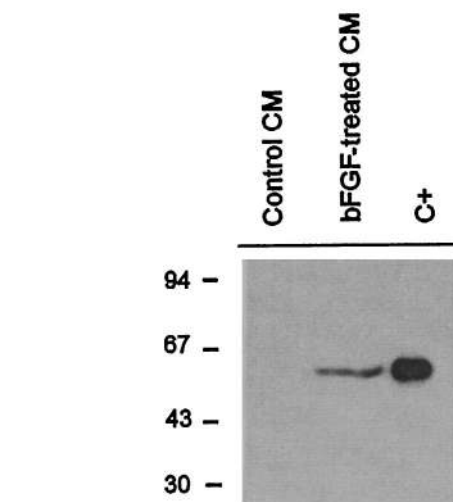


**Figure 3.** Dose-dependence analysis of the effect of bFGF on collagenase-3 mRNA levels in human chondrosarcoma cells. **A:** HCS-2/8 cells were cultured for 24 hours in the presence of the indicated concentrations of bFGF, and total RNA was analyzed by Northern blot, as described in the legend to Figure 1. Filters were hybridized consecutively with labeled probes for collagenase-3 and  $\beta$ -actin. **B:** Autoradiograms were scanned by densitometry, and the values of collagenase-3 mRNA in each sample were corrected for differences of total RNA/lane. Values are represented as relative to the values of control cells.

served after addition of cycloheximide to osteoblastic cells treated with PDGF-BB or retinoic acid.<sup>25,26</sup>

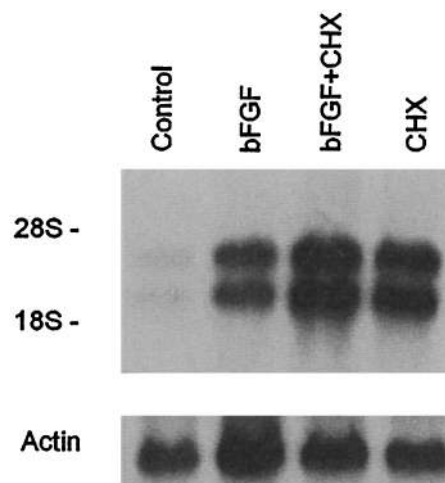
### Immunohistochemical Analysis of Collagenase-3 and bFGF in Benign and Malignant Cartilage Tumors

In an attempt to extend the above *in vitro* observations derived from studies with a chondrosarcoma cell line, we decided to investigate the *in vivo* presence of collagenase-3 in a series of the most frequent benign and malignant cartilage-forming neoplasms. A total of 8 benign lesions and 16 malignant chondrosarcomas were analyzed by immunohistochemical staining with a monoclonal antibody (181-14G11) raised against recombinant human collagenase-3. The monoclonal antibody specificity had been previously confirmed by Western blot analysis of collagenase-3 and a series of different MMPs, including collagenase-1, collagenase-2, stromelysin-1,

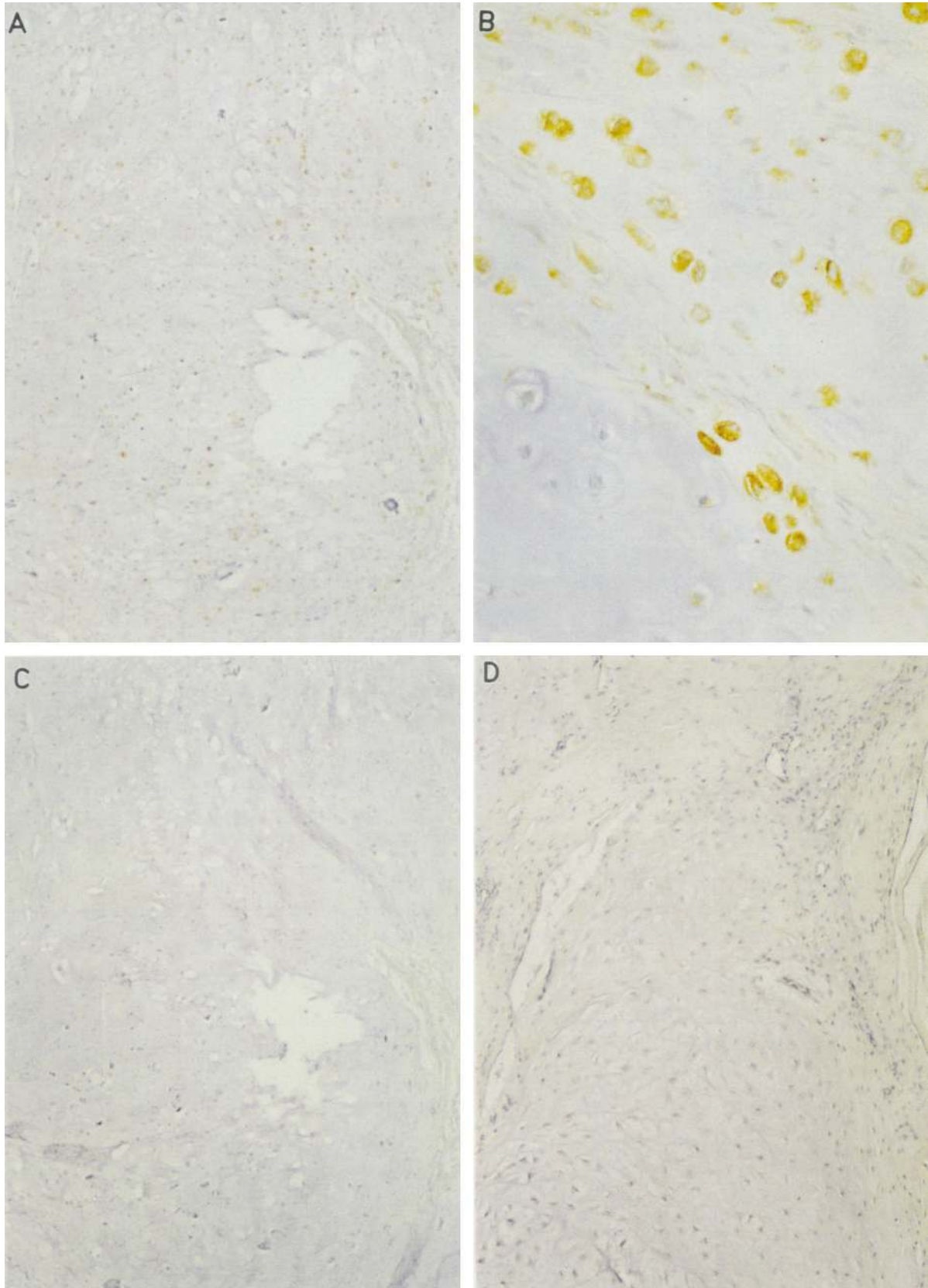


**Figure 4.** Immunoblot analysis of collagenase-3 production by human chondrosarcoma cells treated with bFGF. Cells were cultured in the absence (control CM) or the presence (bFGF-treated CM) of bFGF (10 ng/ml) for 48 hours under serum-free conditions. After incubation, conditioned media were concentrated 25-fold and analyzed by SDS-PAGE along with recombinant purified procollagenase-3 (300 ng; lane C+). After electrophoresis, proteins were transferred to nitrocellulose membranes and the filters were treated with monoclonal antibody 181-15A12 against collagenase-3 (1:5,000). Finally, immunoblots were developed with a chemiluminescence detection reagent.

stromelysin-2, gelatinase-A, gelatinase-B, matrilysin, and MT1-MMP. A single band was detected with collagenase-3, but no immunoreactive band with the 181-14G1 antibody was detected with any of the remaining MMPs (H. Tamei, I. Azumano, K. Iwata, Y. Yoshihara, C. López-Otín, F. Vizoso, V. Knäuper, and G. Murphy, submitted for publication). Immunohistochemical analysis of malignant chondrosarcomas with this monoclonal antibody revealed that all of them were positive for collagenase-3 expression (Figure 6, A and B, and Table 1). Specificity of immunostaining in all cases was determined by using



**Figure 5.** Analysis of the effect of cycloheximide on bFGF-mediated induction of collagenase-3 expression in human chondrosarcoma cells. Effect of bFGF at 10 ng/ml on collagenase-3 expression in HCS-2/8 cells treated for 24 hours in the presence or absence of cycloheximide (CHX; 10  $\mu$ g/ml). After treatments, total RNA was collected and 20  $\mu$ g was loaded in each lane and analyzed by Northern blot. The same blot was stripped and reprobed with a  $\beta$ -actin-encoding probe to confirm equal lane loading.



**Figure 6.** Immunohistochemical analysis of collagenase-3 in benign and malignant cartilage tumors. Immunohistochemical staining of a malignant chondrosarcoma tumor section (**A** and **B**) and a benign chondroma tumor section (**D**) with the monoclonal antibody 181-14G11 raised against human collagenase-3. Immunoreactivity is mainly found in the cytoplasm of small-sized chondrocytes that surround big-sized chondrocytes. No specific immunoreactivity is demonstrable in the benign chondroma tumor (**D**). **C** corresponds to a parallel section of chondrosarcoma that was incubated with antiserum preincubated with purified recombinant collagenase-3. Magnification,  $\times 45$  (**A** and **C**),  $\times 340$  (**B**), and  $\times 110$  (**D**).

controls that involved incubation of tissue sections with buffer alone or with an equal amount of IgG from nonimmunized animals. In addition, immunostaining was completely abolished by antiserum preincubation with purified recombinant collagenase-3 (Figure 6C and data not shown). The immunoreactive pattern of collagenase-3 on chondrosarcoma sections was somewhat variable in both the intensity and percentage of positive cells (Table 1). However, it was common to find strong immunoreactivity in the small-sized and individually arranged chondrocytes located near blood vessels (Figure 6, A and B). Immunoreactivity for collagenase-3 was also present, but at lesser extent and intensity, in chondrocytes of larger size arranged in groups or cell nests (Figure 6, A and B). By contrast, immunohistochemical analysis of benign cartilage-forming tumors using the same monoclonal antibody as above revealed that most of them were negative for collagenase-3 expression (Figure 6D). In fact, only two of eight analyzed lesions (one chondroma and one chondroblastoma) showed some collagenase-3 immunoreactivity. Representative examples of positively stained malignant chondrosarcomas and negative benign tumors and controls are shown in Figure 6, A–D.

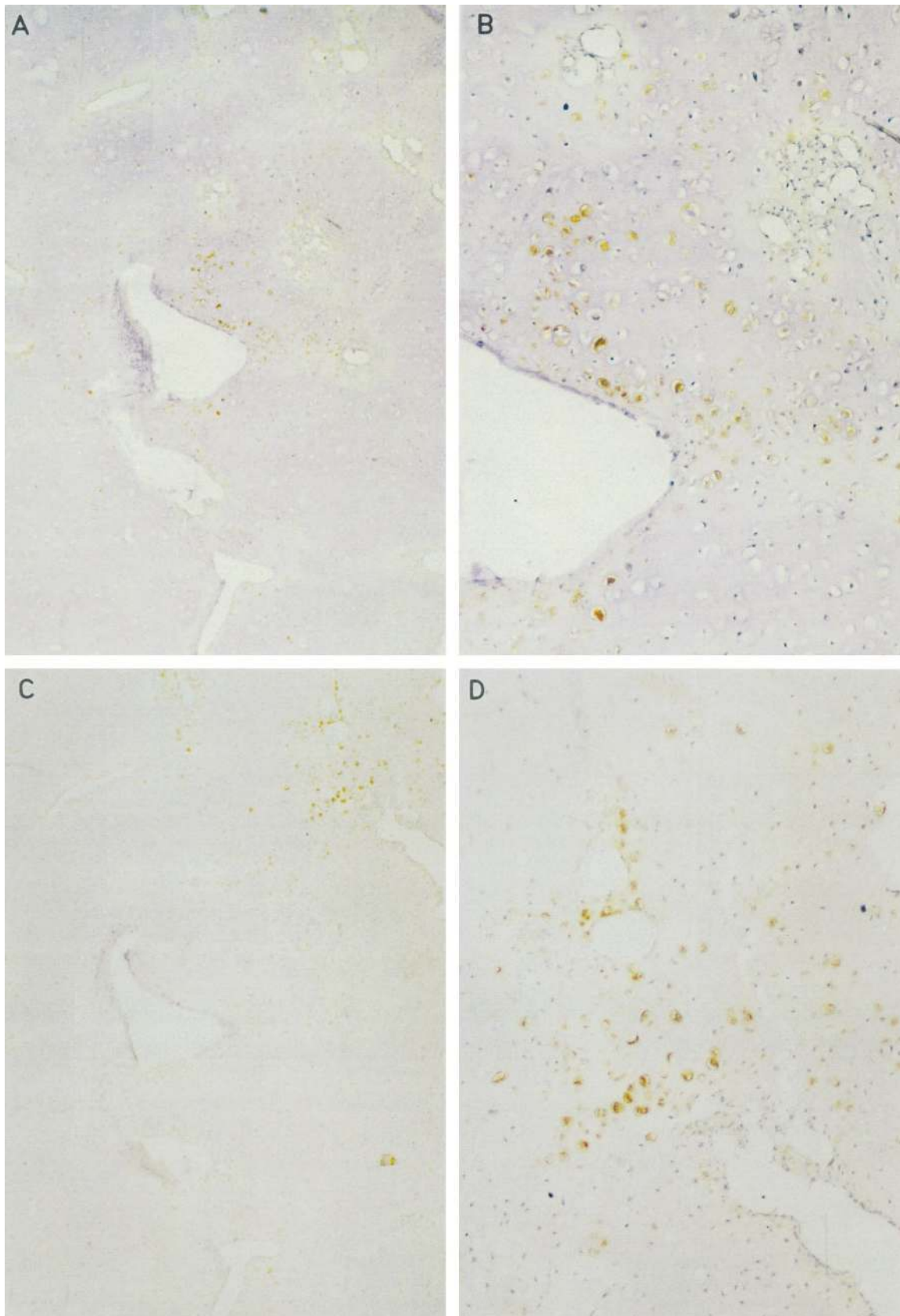
Finally, as the above results had suggested that bFGF could play a major stimulatory effect on collagenase-3 expression in human chondrosarcoma cells, we examined the presence of this growth factor in primary chondrosarcomas by using a commercially available polyclonal antibody raised against human bFGF. As shown in Table 1, and in the representative example depicted in Figure 7, C and D, a clear bFGF immunoreactivity was detected in all analyzed chondrosarcomas as well as in the chondroblastoma case positive for collagenase-3. bFGF immunoreactivity could be also detected in normal non-tumor-involved cartilage, but its expression was very low when compared with that observed in chondrosarcomas (data not shown). Statistical analysis confirmed a significant positive correlation between collagenase-3 and bFGF expression values in chondrosarcomas ( $r = 0.81$ ;  $P < 0.0001$ ). It is also worthwhile mentioning that the pattern of bFGF localization in these chondrosarcoma sections was similar to that of collagenase-3 (Figure 7, A and B), as both proteins were usually found in small-sized chondrocytes located near blood vessels. However, immunoreactive cells for bFGF were not coincidental with those positive for collagenase-3, suggesting that the putative up-regulatory effect of bFGF on collagenase-3 might be mediated through a paracrine mechanism.

## Discussion

In the present study we have provided evidence that collagenase-3, a proteolytic enzyme produced by breast carcinomas, squamous cell carcinomas, and arthritic cartilage, is expressed at high levels by human chondrosarcoma cells. In addition, we have identified bFGF as a potential factor with ability to induce collagenase-3 expression in these cells. Finally, we show that all analyzed human chondrosarcomas produce both collagenase-3 and bFGF, suggesting that this growth factor may be

involved in the *in vivo* production of collagenase-3 by these malignant cartilage-forming tumors.

The finding that collagenase-3 is produced by human chondrosarcomas extends the pattern of tumor types with ability to produce this MMP. In fact, the enzyme was originally cloned from breast carcinomas on the basis of its differential expression in tumors *versus* normal mammary tissues.<sup>1</sup> Subsequently, Heppner et al<sup>27</sup> confirmed that collagenase-3 was produced by approximately 30% of human breast carcinomas. More recently, it has been reported that collagenase-3 is overexpressed in a significant number of squamous cell carcinomas of the head and neck, and this expression is associated with increased local invasion of the tumors.<sup>13,14</sup> The results obtained in the present study demonstrating the production of this enzyme by human chondrosarcomas suggest that collagenase-3 may be associated with a higher number of malignancies than originally thought. In addition, the observation that this enzyme is produced by all chondrosarcomas analyzed in the present work, but not by a significant number of benign lesions or by normal cartilage in adult tissues,<sup>4–9</sup> suggests that this up-regulation may be somewhat linked to the malignant transformation and contribute to the progression of these tumors. This possibility is consistent with previous clinical data showing the production of collagenase-3 by aggressive breast and laryngeal carcinomas as well as with the biochemical properties of this enzyme, which has been characterized as a potent proteinase with a wide substrate specificity, including a preferential degrading activity on type II collagen.<sup>2,6,10</sup> As this collagen type is the most abundant in articular cartilage, the widespread occurrence of a degrading enzyme as collagenase-3 in malignant tumors involving these cells may be a major determinant for facilitating cartilage destruction, thus allowing tumor progression in a similar fashion to that proposed for other MMPs in different tumor types.<sup>27–30</sup> In this regard, it is of interest that, despite the fact that all analyzed chondrosarcomas were positive for collagenase-3 expression, there were clear variations in the percentage of positive cells and the intensity of staining. These variations could be associated with the occurrence of tumors with different clinical outcome. Preliminary analysis aimed at looking for a putative relationship between collagenase-3 levels and a series of biological and clinico-pathological characteristics did not reveal any apparent association, although the limited number of samples analyzed in this study precludes the ability to obtain definitive conclusions. Additional studies with a large number of samples will be required to clarify whether expression levels of collagenase-3 in human chondrosarcomas may help to identify tumors differing in their biological and/or clinical characteristics. Similarly, it will be of interest to examine whether the small percentage of benign lesions producing collagenase-3 could be indicative of an increased risk of malignant transformation in these patients. In fact, it has been previously reported that malignant change can occur in a benign cartilage-forming tumor and that occasional chondroblastomas lacking any unusual histological features have developed distant metastases and have even proved fatal.<sup>19,20</sup>



**Figure 7.** Immunohistochemical analysis of collagenase-3 and bFGF in a malignant chondrosarcoma tumor. Immunohistochemical staining with the monoclonal antibody 181-14G11 (**A** and **B**) or the rabbit IgG anti-bFGF (**C** and **D**) in parallel sections of a malignant chondrosarcoma tumor was performed as described. Note that the pattern of collagenase-3 and bFGF immunoreactive cells are similar but not coincidental. **B** and **D** are higher magnifications of a portion of **A** and **C**, respectively. Magnification,  $\times 45$  (**A** and **C**) and  $\times 110$  (**B** and **D**).

In this work, and as a previous step to identify potential factors that could be responsible for the observed up-regulation of collagenase-3 expression in human chondrosarcomas, we have also examined the ability of different cytokines and growth factors to induce this MMP in chondrosarcoma cells. This analysis revealed that bFGF, a member of the heparin-binding growth factor family with multiple biological functions,<sup>31</sup> was a strong collagenase-3 inducer in these cells. By contrast, a series of factors, such as IL-1 $\beta$ , TGF- $\beta$ , or TNF- $\alpha$ , which have been previously found to play important roles in the induction of collagenase-3 in fibroblasts, keratinocytes, or primary chondrocytes,<sup>32–35</sup> did not show any significant up-regulatory effect on collagenase-3 production by chondrosarcoma cells. These data appear to indicate that the positive effect of bFGF on collagenase-3 expression in these cells is rather specific. In fact, bFGF is unable to induce collagenase-3 expression in a number of analyzed cell lines, including fibroblasts, epithelial breast cancer cells, or keratinocytes,<sup>14,34,35</sup> although this growth factor does induce rat interstitial collagenase, the murine homologue of human collagenase-3, in bone cells.<sup>36,37</sup> It is also worthwhile mentioning that, despite the fact that bFGF has been previously shown to induce or to inhibit the expression of different human MMPs in various cell types, including fibroblasts,<sup>38</sup> endothelial cells,<sup>39</sup> smooth muscle cells,<sup>40</sup> monocytes,<sup>41</sup> and keratinocytes,<sup>42</sup> our findings represent the first report of induction of a human MMP family member by this growth factor in chondrosarcoma cells. Additional analysis of the bFGF-induced expression of human collagenase-3 in these cells revealed that its effect was time and dose dependent, but independent of the *de novo* synthesis of proteins. Nevertheless, it was unclear whether these *in vitro* effects of this growth factor on collagenase-3 expression could reflect its activity *in vivo*, thus making bFGF a potential candidate for inducing expression of this MMP in human chondrosarcomas. In this regard, it is of interest that the presence of bFGF has been described in a variety of human cancers, including breast, endometrial, esophageal, ovarian, renal, uterine cervical, colorectal, pancreatic, or prostatic adenocarcinomas.<sup>43–51</sup> In addition, bFGF levels are usually higher in highly metastatic tumors than in those less aggressive.<sup>48–51</sup> However, although bFGF was originally purified from rat chondrosarcoma cells,<sup>52</sup> no data were available on the occurrence of this growth factor in human chondrosarcomas. The finding that all chondrosarcomas analyzed in the present work are bFGF immunoreactive provides additional support to the proposal that this growth factor may be a modulator of collagenase-3 expression in these tumors. Interestingly, the patterns of intratumor localization of collagenase-3- and bFGF-positive cells are not superimposable. Thus, collagenase-3 was detected in the vicinity of bFGF-producing chondrocytes, but very few cells showed concomitant production of both proteins. These observations suggest that the stimulatory effect of bFGF on the expression of collagenase-3 could be exerted through a paracrine signaling pathway rather than through an autocrine mechanism within the same cell. Finally, it should be mentioned that the precise molecular

mechanisms responsible for collagenase-3 up-regulation in chondrosarcoma cells in response to bFGF have not yet been elucidated, although it is likely that members of the FGF receptor family may be involved.<sup>31,53</sup> The availability of a phenotypically stable chondrosarcoma cell line with ability to produce collagenase-3 after bFGF treatment will be very helpful to further evaluate the precise mechanisms mediating the bFGF-elicited induction of this MMP gene in human chondrosarcomas. These studies may also contribute to a better understanding of the pathogenesis of these tumors that constitute a significant percentage of all malignant tumors arising from the skeletal system, but the biological properties of which remain largely uncharacterized.

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