Abstract  It has been shown that transforming growth factor-β (TGF-β) has a potent stimulatory effect on the growth of chondrosarcoma cells in vitro. In order to examine the production of this family of growth factors and their receptors in vivo, we studied the expression of TGF-β isoforms 1, 2, and 3 and of TGF-β receptor types I and II (TGF-βRI and TGF-βRII) in a series of 24 chondrosarcomas of bone using immunohistochemistry and reverse-transcription polymerase chain reaction analysis. For comparison, five enchondromas and five osteochondromas were also analyzed. TGF-β1 was expressed in 3 benign lesions (30%) and 18 chondrosarcomas (75%), with a significantly higher expression in grade-2 and -3 tumors than in grade-1 tumors (P=0.002). TGF-β2 was identified in 8 benign lesions (89%) and 21 chondrosarcomas (87.5%), with increased expression in grade-2 and -3 chondrosarcomas in comparison with grade-1 tumors (P=0.05). TGF-β3 was detected in 6 benign lesions (60%) and 17 chondrosarcomas (70.8%), with no significant differences between chondrosarcomas of different histologic grade (P=0.6). Twenty-three chondrosarcomas (95.8%) expressed both TGF-β receptor types I and II. Reverse-transcription polymerase chain reaction analysis performed on ten chondrosarcomas confirmed the presence of low or absent levels of TGF-β1 and -β2 mRNA in grade-1 chondrosarcomas, while grade-2 chondrosarcomas presented high levels of transcript of both cytokines. High levels of TGF-βRI and RII mRNA were also detected. Chondrosarcomas with TGF-β1 and TGF-β2 overexpression (>20% of tumor cells) had a significantly higher expression of the cell proliferation marker MIB-1 (P=0.006 and P=0.0003, respectively), while no significant correlation was found between TGF-β3 expression and proliferative activity (P=0.5). When TGF-β isoform and receptor expression were examined with respect to disease-free survival, TGF-β1 overexpression was significantly associated with a shorter disease-free survival (P=0.004, log-rank test). Our data indicate that TGF-β isoforms are produced by neoplastic cells of chondrosarcomas and could have a potential role as autocrine growth stimulators in these neoplasms.

Keywords Chondrosarcoma · TGF-β · TGF-β receptors · Immunohistochemistry · mRNA

Introduction  Transforming growth factor β (TGF-β) is a potent cell regulatory peptide that variably affects proliferation, differentiation, and extracellular matrix synthesis. At least three TGF-β isotypes with similar biologic activities have been identified in mammals, which exert their autocrine/paracrine functions through the interaction with specific heterodimeric receptor complexes formed by two different transmembrane proteins (TGF-βRI and TGF-βRII) [26]. High contents of TGF-β1 are accumulated in cartilage tissue, where the growth factor is produced by chondrocytes and released in the extracellular matrix in latent forms, consisting of a 100-kDa complex of TGF-β conjugated with a latency-associated peptide (LAP) and of a large latent complex formed by the small 100-kDa complex covalently bound to the latent TGF-β binding protein 1 (LTBP1) [1, 2, 3, 23]. Activation of even small amounts of latent TGF-β may have dramatic effects on cartilage, since chondrocytes exhibit phenotypic responses to the active growth factor at concentrations 10- to 100-fold below plasma levels of TGF-β (1–50 ng/ml) [1].

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TGF-β has been implicated in the growth of several neoplasms, and recent studies have suggested that dysregulation of TGF-β autocrine control appears to have a role in the growth of bone tumors, such as Ewing’s sarcoma and osteosarcoma [9, 18, 20]. In addition, in vitro studies have demonstrated that TGF-β1 is produced by human chondrosarcoma cells [5], and the cytokine has a potent stimulatory effect on the growth of rat chondrosarcoma [12, 19]. However, no information is currently available concerning the production of this family of growth factors by human chondrosarcomas in vivo. Therefore, we studied the expression of TGF-β1, β2, β3 and of TGF-βRI and -RII in a series of 24 chondrosarcomas of bone and 10 benign cartilaginous lesions of bone using immunohistochemistry and reverse-transcription polymerase chain reaction (RT-PCR) analysis for mRNA determination. Furthermore, we correlated TGF-β isoform expression in chondrosarcomas with the histologic grade of the tumor and with proliferative activity assessed by means of MIB-1 immunostaining.

**Subjects and methods**

**Subjects**

Our study was conducted on 24 consecutive conventional chondrosarcomas of bone from 24 patients. Complete clinical information was available for all patients, and paraffin-embedded tissue blocks not submitted to decalcification procedures were retrieved in each case to perform the immunohistochemical analysis. Fourteen patients were male and ten were female, with ages ranging at the time of diagnosis between 25 years and 71 years (median 52 years). Thirteen tumors were located in the trunk, including the pelvis (8 patients) and the scapula (5 patients), whereas 11 involved the extremities, including the femur (7 patients), the humerus (3 patients), and the tibia (1 patient). One patient who developed a chondrosarcoma of the femur was affected by Ollier’s disease. Six patients had stage-IA chondrosarcoma [7], 4 had stage-IB chondrosarcoma, 1 had stage-IIA chondrosarcoma, 12 had stage-IIB chondrosarcoma, and 1 had a stage-III A chondrosarcoma. All patients underwent surgical treatment consisting of surgical resection in 22 cases, curettage in 1 case, and amputation in 1 case. Hematoxylin and eosin stained sections were employed by one pathologist (A.F.) to grade each lesion according to the criteria of Evans [8]. Ten patients had grade-1 tumors, 10 had grade-2 tumors, and 4 had grade-3 tumors.

Patients were followed for a minimum of 36 months or until death (median follow-up 62 months). There were four patients who presented with local failures and six who presented with distant failures. Of the four patients who developed local recurrence, three also developed distant metastases. Four patients were alive with disease and three died due to metastasis after 14, 24, and 36 months from diagnosis.

In addition, we studied a group of ten benign cartilaginous lesions, including five enchondromas and five osteochondromas.

**Immunohistochemistry**

Immunohistochemical staining was done on formalin-fixed, paraffin-embedded tumor tissue fragments using the avidin–biotin complex (ABC) technique (Vector, Burlingame, Calif.). The primary rabbit polyclonal antibodies against TGF-β1, TGF-β2, TGF-β3, TGF-βRI, and TGF-βRII (Santa Cruz Biotechnology, Calif.) were applied at 1:50 dilution. A semi-quantitative system was employed to evaluate the level of antigen expression: immunoreactivity was scored as either absent (−), low (+, less than 20% of positive tumor cells), moderate (+++, 21–75% of positive tumor cells), or diffuse (+++, more than 75% of positive tumor cells). Proliferative activity was evaluated using the MIB-1 monoclonal antibody (Immunotech, Marseille, France) after microwave pretreatment of dewaxed and rehydrated sections. Scoring of the immunostaining was performed using a standard light microscope equipped with an eyepiece grid of 10×10 squares and a 40× objective. In each case, at least 500 tumor cells were counted from areas with high and low expression of the antigen and the results were expressed as percentage of positive cells.

Negative controls were obtained by substituting the primary antibody with non-immune rabbit or mouse serum.

**Reverse-transcription polymerase chain reaction analysis**

RT-PCR was performed using total RNA obtained from fresh tumor tissue fragments. The sequences of sense and antisense primers employed were as previously described [16]. The RT procedure was performed using the 3′ primers under the following conditions: RT at 42°C for 50 min, inactivation of reverse transcriptase at 70°C for 15 min, and RNase H digestion at 37°C for 20 min. One PCR cycle (30 cycles total) consisted of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and polymerization at 72°C for 1 min. PCR products were electrophoresed on 1–1.5% agarose gel, visualized by ethidium bromide staining, and photographed under ultraviolet light.

**Statistical analysis**

Statistical analysis was performed using Statistica 5.1 software (Statsoft Inc., Tulsa, Okla.). Two-tailed Fisher’s exact test was used to analyze the correlation between TGF-β isoform expression and the histologic features of the lesions. The Mann-Whitney U test and one-way analysis of variance (ANOVA) were used to assess the differences in proliferative activity determined on tumor tissue sections, according to TGF-β isoform expression and histologic features of the lesion. The cumulative probability of disease-free survival was calculated using the Kaplan-Meier method. Logrank analysis was employed to compare curves. P values <0.05 were considered significant.

**Results**

The results of the immunohistochemical studies are summarized in Table 1. Overall, cytoplasmic positivity for TGF-β1 was identified in neoplastic cells of 18 chondrosarcomas (75%) and in 3 benign lesions (30%). TGF-β1

<table>
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<tr>
<th>Table 1 Correlation between transforming growth factor (TGF)-β isoform and receptor (RI and RII) expression, proliferative activity and histologic features in 34 cartilaginous lesions of bone</th>
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<td>Benign lesions (10)</td>
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<td>G1 chondrosarcomas (10)</td>
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<td>G2/G3 chondrosarcomas (14)</td>
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<td>P value (G2/3 vs G1)</td>
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