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Cyclooxygenase-2 Expression in Chondrosarcoma

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Key Words
Chondrosarcoma · Cyclooxygenase · Enchondroma

Abstract
Objective: In recent years it has become evident that tissue cyclooxygenase-2 (COX-2) may play a role in carcinogenesis and tumor malignancy. There is now a mounting body of information that strongly implies that COX-2 inhibitors may be of some value in the management of patients with carcinomas, and most recently several similar reports have appeared relating to sarcomas. ***Methods:*** The authors studied 32 samples of cartilage tumors from our tumor tissue bank for the presence of COX-2 by a Western blot technique. There were 29 patients from whom the samples were obtained, including 8 with enchondromas and 21 with chondrosarcomas. ***Results:*** Thirteen of the 24 chondrosarcoma samples and none of the 8 enchondromas were positive for COX-2. An attempt was made to correlate these results with clinical data including age, gender, staging according to the Musculoskeletal Tumor Society, anatomical site, ploidic pattern, presence of metastases and death rate but no statistically valid correlation could be found. ***Conclusion:*** It is evident that COX-2 may play some role in chondrosarcoma but not in the benign enchondroma and that further studies with COX-2 inhibitors are warranted.

Introduction

In the recent past, cyclooxygenase-2 (COX-2) inhibitors have shown promise in terms of a chemotherapeutic effect on cancers. Studies have shown some positive actions on colorectal [1–5], breast [6, 7], prostate [8, 9], skin [10–12], hepatocellular [13], gastric [14–16], pancreatic [17], uterine [18], and lung cancer [19, 20]. To date, however, little is known of the effect of these agents on sarcomas. Although some of the trials of COX-2 inhibitors have involved sarcomas in animal species [21, 22], only one recent report has defined the presence of COX-2 in a high percentage of rhabdomyosarcomas, osteosarcomas and Ewing's sarcomas [23]. There have as yet been no reported trials of the COX-2 inhibitors in patients with sarcomatous tumors.

The Massachusetts General Hospital Orthopedic Oncology Service maintains a tumor bank containing almost 1,000 specimens of connective tissue tumors and has, in addition, considerable information regarding the nature of the tumors and the outcome for the patients whose tumors were preserved in the tissue bank [24]. Because none of the samples of chondrosarcoma received adjunctive radiation or chemotherapy treatment prior to resection, and are varied in histological grade and staging according to the Musculoskeletal Tumor Society (MSTS) [25], the specimens were selected to be analyzed for COX-

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Table 1. Data for enchondromas

Sample	Age years	Gender	Stage	Location	Ploidy	COX-2
1	58	female	0	proximal humerus	diploid	none
2	44	female	0	distal femur	diploid	none
3	41	female	0	proximal humerus	diploid	none
4	48	female	0	proximal tibia	diploid	none
13	71	female	0	proximal tibia	diploid	none
14	49	female	0	distal femur	diploid	none
15	33	male	0	finger	diploid	none
16	42	female	0	distal femur	diploid	none

2 protein. In all there were 24 specimens from 21 patients; in addition 8 enchondromas were selected to serve as controls.

Chondrosarcomas are malignant cartilaginous tumors most commonly arising within or adjacent to a bone. They represent 25% of all bone sarcomas and are the most frequent primary malignant bone tumor in patients over 50 years of age [26]. Computerized data regarding 450 of these lesions treated by our oncology group between 1972 and 2002 showed the average age of the patients to be 27.6 ± 17.2 years (range 7–82), and males were more frequently affected than females [26]. Survival statistics were available for 388 of these patients, and the 5-year survival is 75% and the 10-year survival 71%. The usual method of treatment for chondrosarcomas is resection, and there is little documentation to suggest that malignant cartilaginous tumors respond to adjuvant or neoadjuvant chemotherapy or radiation as some other sarcomas do [27, 28]. Local recurrence of a chondrosarcoma, regardless of how it is treated, increases the likelihood of eventual metastasis and death [26, 27].

Materials and Methods

Tumor Specimens

Tumor specimens obtained at the time of surgery were immediately stored at –80°C in the tumor tissue bank of the Massachusetts General Hospital Department of Orthopedic Oncology. Thirty-two specimens were obtained from the tumor bank. These were from 8 patients with enchondromas and 21 with chondrosarcomas. Two of the latter patients had more than one specimen taken at different times during their course (biopsy, resection and in 1 case, local recurrence), raising the total number of samples for the malignant lesions to 24. The age range for the enchondroma group was 33–71, and none of the patients has died. In the chondrosarcoma group with 21 patients, the age range was from 31 to 80, and 5 patients have died of disease. Tables 1 and 2 provide descriptions of the patients and the tumors.

Ploidic Studies. Segments of tissues weighing 0.2 g are minced and treated with collagenase in phosphate-buffered saline. The suspension is incubated at 37°C and agitated and passed through a 100-μm nylon mesh filter and introduced into a flow cytometer using a blue-green light from a CR6 argon ion laser. The pattern is assessed by analysis of a computer-generated X-Y plot in the form of a histogram. The tissue is studied for apoptotic rates and also assessed for concentrations of diploid, S-phase and aneuploid cells and compared against a control from normal blood cells [29].

Preparation of Tumor Extracts

Tumor samples weighing 100–210 mg were obtained from the tissue bank and washed once with 500 μl of phosphate-buffered saline to remove residual media. The tissue fragments were suspended in 250 μl of extraction buffer (100 m*M* NaCl, 10 m*M* Tris HCl, pH 7.8, 10 m*M* EDTA, 1%Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, and protease inhibitor cocktail as recommended by the supplier (Complete Protease Inhibitor Tablet, Roche Molecular Biochemicals, Mannheim, Germany). The tumor specimens were extracted at 4°C overnight on a shaker, and extracts were clarified by centrifugation for 20 min at 12,000 *g* at 4°C. Supernatants were recovered and stored at –80°C. Protein concentrations of the tumor extracts were determined using the BioRad Protein Assay kit (BioRad Laboratories, Hercules, Calif., USA), using bovine serum albumin as a standard.

Detection of COX-2 Protein by Western Blotting

Equal quantities of protein (80 μg) from each tumor extract were run on a SDS-polyacrylamide (7.5%) gel. The resolved proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 20 m*M* Tris HCl, pH 7.8, 500 m*M* NaCl, 0.1% Tween-20 (TBST) with 5% dry milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, Mich., USA) at 0.5 μg/ml in TBST with 5% dry milk. The membrane was washed with TBST and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Cell Signaling Technology, Beverly, Mass., USA) at a dilution of 1:2,000 for 2 h at room temperature. After washing, White Lightning Chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, Mass., USA) was used for signal development. After detection of COX-2, blots were stripped and re-probed with monoclonal anti-human β-actin (Sigma, St. Louis, Mo., USA) as a control for loading of intracellular protein. Images were scanned using Kodak Image Station 400.

Table 2. Data for chondrosarcomas

Sample	Age years	Gender	Death	MSTS stage	Location	Metastases	Ploidy	COX-2
1	44	male	false	3	hemipelvis	viscera	aneuploid	no
2	39	male	false	1B	sacrum	none	aneuploid	no
3	42	male	false	1B	sacrum	none	aneuploid	yes
4	42	male	false	1B	sacrum	none	aneuploid	no
5	59	female	false	1B	hemipelvis	none	diploid	yes
6	51	male	true	3	mid-femur	lung	diploid	no
7	59	male	false	2B	distal femur	none	aneuploid	no
8	58	male	false	2B	distal humerus	none	diploid	no
9	53	male	false	2B	distal femur	lung	aneuploid	yes
10	33	female	false	1A	finger	none	diploid	no
11	64	male	true	3	clavicle	bone	aneuploid	no
12	39	male	false	1A	scapula	none	aneuploid	no
13	31	female	false	2B	femur	none	aneuploid	yes
14	53	male	false	1A	femur	none	diploid	yes
15	80	male	true	3	femur	bone	aneuploid	yes
16	80	male	true	3	femur	bone	diploid	yes
17	63	male	true	3	scapula	none	aneuploid	no
18	38	male	false	2A	humerus	none	aneuploid	yes
19	63	male	false	1B	hemipelvis	none	diploid	yes
20	31	female	false	1A	scapula	none	diploid	yes
21	65	female	false	3	distal femur	lung	aneuploid	no
22	77	male	false	1B	scapula	none	diploid	yes
23	33	male	false	1B	lumbar spine	none	diploid	yes
24	51	male	true	2B	mid-tibia	none	aneuploid	yes

As can be noted, samples 2, 3 and 4 came from the sacral area of the same patient at different times. Samples 15 and 16 came from the femur of the same patient.

To confirm the specificity of COX-2 immunoreactivity, blots were incubated in parallel with anti-COX-2 antibody that had been preincubated with the immunizing peptide (Cayman Chemical) to block protein-antibody complex formation specific for COX-2. Prior to use, the peptide was dissolved in water at a concentration of 10 mg/ml. The blocking peptide was added to the antibody in TBST with 5% bovine serum albumin at a final concentration of 10 μg/ml, and the mixture was incubated for 1 h at room temperature prior to application of the antibody to the membrane.

Results

In order to determine whether COX-2 is expressed in cartilage tumors, proteins extracted from specimens of benign and malignant cartilage tumors were analyzed by Western blot. COX-2 protein was not detectable in any of the 8 enchondroma samples. In contrast, 13 of 24 chondrosarcoma extracts had detectable COX-2 protein (see fig. 1 for representative data). To determine whether the tumor extracts contained comparable amounts of intracellular proteins, blots were reprobed for β-actin. All tumor samples displayed a 42-kD signal when the antibody for actin was used, although the intensity of the signal was somewhat variable (fig. 1).

Control experiments were performed in order to confirm the authenticity of the COX-2 signals on Western blot (fig. 2). The immunoreactive protein in chondrosarcoma extracts migrated between 60 and 80 kD, comigrating with the 72-kD purified ovine COX-2 electrophoresis standard from Cayman Chemical. Preincubation of the anti-COX-2 antibody with a blocking peptide corresponding to the sequence of the immunizing peptide eradicated the signals (fig. 3). In five chondrosarcoma extracts that were positive, two bands appeared on the blot, and both signals were abrogated if the antibody was blocked (for example fig. 3, lane 8). The nature of the additional immunoreactive band, corresponding to a protein of approximately 90 kD, is unknown.

The analysis of the relation of COX-2 to other variables in the chondrosarcoma specimens is shown in table 3. COX-2 was detected in 11 of the 21 samples (excluding the three samples from 2 patients which had the

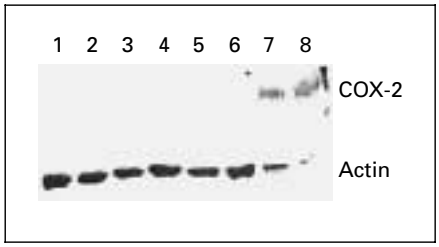

Fig. 1. Western blots for COX-2 and actin proteins in enchondroma and chondrosarcoma. Lanes 1–4 are enchondroma protein extracts, and lanes 5–8 are chondrosarcoma protein extracts. Two chondrosarcoma extracts (lanes 7 and 8) have COX-2 immunoreactive bands. All lanes displayed a band for β-actin.

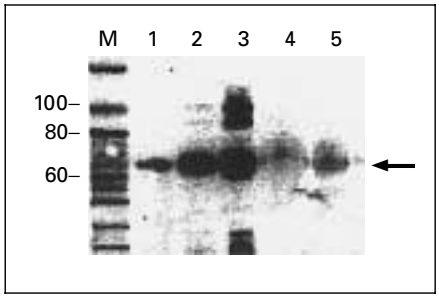

Fig. 2. Electrophoretic mobility of COX-2. Lane M = Biotinylated protein standard (Cell Signaling Technologies). Lanes 1–3 show COX-2 electrophoresis standard of 72 kD at increasing concentrations (0.2, 1 and 2 μg, lanes 1–3, respectively). Lanes 4 and 5 contain protein extracted from 2 chondrosarcomas, each displaying a band for COX-2 protein that comigrates with the COX-2 standard.

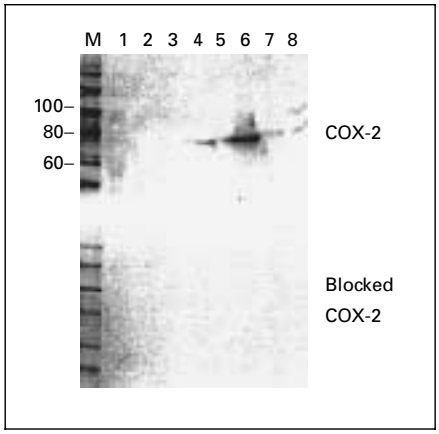

Fig. 3. Western blots for COX-2 protein (top) and antibody blocking for COX-2 (bottom). Lane M = Standard protein size ladder. Lanes 1–8 are chondrosarcoma protein extracts. Lanes 5–8 show COX-2 immunoreactive bands, and the signals are abrogated if the antibody is blocked with peptide specific for COX-2 (bottom panel). Note that the immunoreactivity at ~90 kD in lane 8 is also blocked.

Table 3. Correlation of COX-2 results with demographic data

		n	COX-2	PCT
Gender	Male	16	8	50.0
	Female	5	3	60.0
Site	Upper	8	3	37.5
	Spine	5	3	60.0
	Lower	8	5	62.5
MSTS stage	1	9	6	66.7
	2	6	4	66.7
	3	6	1	16.7
Metastasis	Yes	7	2	28.6
	No	14	9	64.3
Ploidy	Aneuploid	12	5	41.7
	Diploid	9	6	66.7
Outcome	Dead	5	2	40.0
	Alive	16	9	56.3
Age	<40 years	7	4	57.1
	39–60 years	8	4	50.0
	>59 years	6	3	50.0

None of these data show a statistical difference by χ^2 test or ANOVA. The closest correlations are stage of metastasis and ploidy, all of which are less than 0.3.

identical response pattern). As can be noted, 8 of 16 males and 3 of 5 females were COX-2 positive. Six of 9 tumors that showed a diploid DNA pattern and 5 of 12 that were aneuploid were positive for Cox-2. The MSTS stage 3 patients (4) had fewer numbers of COX-2 positive samples and more of the 16 patients who remained alive were positive for COX-2 than of the 5 who died. None of these comparative data achieved statistical significance.

Discussion

This study demonstrated that detectable levels of COX-2 protein were expressed in 54% of malignant chondrosarcomas and none of the benign enchondromas. These data strongly support the concept that COX-2 protein is upregulated in chondrosarcoma of the bone.

Several different mechanisms could provide an important link between COX-2 and the biologic behavior of chondrosarcoma. Enhanced synthesis of prostaglandins, a consequence of upregulation of COX-2, can act to increase cell proliferation [3], promote angiogenesis [30], and inhibit immune surveillance [3]. All of these effects could conceivably lead to growth of malignant cells. Other studies have shown that the overexpression of COX-2

inhibits apoptosis [1] and enhances invasiveness [9], both of which may also play a role.

As noted in the Introduction, the presence of COX-2 increases some of the key factors in cancer development [1, 3, 8–11, 15–17, 19, 30], and COX-2 inhibitors have shown promise in terms of adjuvant chemotherapy for malignant carcinomas [2, 4, 7, 12, 19, 31], as well as more recently in increasing radiation sensitivity of sarcomatous lesions [21, 22]. The study reported here as well as a recent one in other sarcomatous tissues [23] support the possibility that these tumors might respond favorably to treatment with Cox-2 inhibitors. Our data show that at least half of the chondrosarcomas studied have a sufficient amount of COX-2 present to theoretically respond as well as the carcinomas to this form of therapy. Further, it is certainly possible that greater than 54% of chondrosarcomas express COX-2, since the specimens studied had varying COX-2 concentrations and some might have had COX-2 below the level of detection in the Western blot system utilized.

In view of these findings, it is of some interest to assess the role of COX-2 and COX-2 inhibitors in normal articular cartilage and other connective tissue tumors. In 1995, Geng et al. [32] performed a study demonstrating that COX-2 mRNA could be induced by exposure of cartilage cells in culture to IL-1, TNF-α, IL-6 and other inflammatory agents. Similarly, in a study by Mastbergen et al. [33] published in 2002, the authors demonstrated that COX-2 inhibitors had no effect on normal cartilage but showed a remarkable action on cartilage that was inflamed. Some recent preliminary studies from our laboratory showed that osteoarthritic cartilage could be stimulated to demonstrate increased apoptotic activity with in vitro exposure to COX-2 inhibitors. As a result of these observations and the study reported here, we have analyzed the effect of COX-2 inhibitors on specimens of connective tissue tumors including especially chondrosarcomas and chordomas. The major findings from this beginning experiment suggest a significant increase in apoptotic activity in all of the tissues analyzed and a modest decrease in DNA synthesis in some of the specimens. These data support the findings in this study in relation to the possible treatment of patients with chondrosarcoma and other connective tissue tumors with COX-2 inhibitors.

Conclusion

Malignant cartilaginous tumors do not respond as well to adjuvant or neoadjuvant chemotherapy and radiation as many other sarcomas. Local recurrence of a chondrosarcoma, regardless of how it is treated, is likely to result in eventual metastasis and death. COX-2 inhibitors have shown promise in terms of an addition to the current chemotherapy and radiation utilized for malignant tumors. The results of this study show that over half of a series of chondrosarcomas displayed COX-2 protein, which was not present in benign cartilage tumors. Based on these results, it seems important to further define the biologic role that COX-2 may play in malignant sarcomas, and to determine whether selective inhibitors of COX-2 may be useful in altering or ameliorating some of the malignant attributes of chondrosarcomas or other connective tissue tumors.

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