Short Communication

Matrix Gene Expression Analysis and Cellular Phenotyping in Chordoma Reveals Focal Differentiation Pattern of Neoplastic Cells Mimicking Nucleus Pulposus Development

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Chordoma is the fourth most common malignant primary neoplasm of the skeleton and almost the only one showing a real epithelial phenotype. Besides classic chordoma, so-called chondroid chordoma was described as a specific entity showing cartilage-like tissue within chordomatoid structures. However, since its first description, strongly conflicting results have been reported about the existence of chondroid chordoma and several studies suggested chondroid chordomas being in fact low-grade conventional chondrosarcomas. In the present study, we used cytoprotein expression profiling and molecular *in situ* localization techniques of marker gene products indicative of developmental phenotypes of chondrocytes to elucidate origin and biology of chondroid chordoma. We were able to demonstrate the chondrogenic potential of chordomas irrespectively of the appearance of overt cartilage formation by identifying the multifocal expression of type II collagen, the main marker of chondrocytic differentiation. Additionally, the cartilage-typical large aggregating proteoglycan aggrecan was present throughout all chordomas and, thus, a very characteristic gene product and marker of these neoplasms. Biochemical matrix composition and cell differentiation pattern analysis showed a high resemblance of classic chordomas and in chondroid areas of chondroid chordomas to the fetal chorda dorsalis, whereas chondroid areas of chondroid chordomas showed features similar to adult nucleus pulposus. This demonstrates on the cell function level the chon-

drocytic differentiation potential of neoplastic chondroid cells as a characteristic facet of chordomas, mimicking fetal vertebral development, ie, the transition of the chorda dorsalis to the nucleus pulposus. Our study firmly establishes a focal real chondrocytic phenotype of neoplastic cells in chordomas. Chondroid chordoma is neither a low-grade chondrosarcoma nor a misnomer as discussed previously. (*Am J Pathol* 2001, 158:1571–1578)

Chordoma is the fourth most common primary malignant neoplasm of the skeleton and almost the only one showing expression of epithelial cell markers. It was first described by Virchow in 1857¹ and in 1858 by Müller² who suggested it to be of notochordal origin. Besides classic chordoma, so-called chondroid chordoma was described by Heffelfinger and colleagues³ in 1973 as a specific entity, which might have a better prognosis. However, since then conflicting results have been reported on the existence of this cartilaginous tumor variant^{4–7} and several studies suggested chondroid chordomas being in fact low-grade chondrosarcomas rather than a chordoma variant^{4–7} and/or denied chondroid differentiation in chordomas at all.^{4,8} Histological and ultrastructural examination was not able to settle the discussion.⁴ Special ultrastructural features such as desmosomes, simple cell junctions, or peculiar tubular structures seen in chordomas⁹ are also found in nonepithelial tumors including chondrosarcomas.^{10–12}

In the present study, we investigated the biochemical composition of the extracellular tumor matrix as well as the matrix gene expression pattern in classic and chondroid chordomas in comparison to cell and tissue mor-

Supported by a post-doctoral scholarship to D. Gottschalk of the Ernst-und-Berta-Grimmke-Stiftung and the Wilhelm-Sander-Stiftung (München, Germany; grant 96.050.2).

Accepted for publication January 16, 2001.

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Table 1. Primary Antibodies and Enzymatic Pretreatments Used for Immunohistochemical Analyses

	Clone	Species (isotype)	Dilution	Digestion	Source
Pan-epithelial cytokeratins	Lu5	m (IgG1)	1:100	P	Dianova (Germany)
Cytokeratin 19	RCX10 8	m (IgG1)	1:100	P	Dako (Denmark)
Epithelial membrane antigen (EMA)	E29	m (IgG2a)	1:100	—	Dako (Denmark)
Vimentin	V9.1	m (IgG1)	1:1000	Pt	Dako (Denmark)
S-100 protein	—	r	1:1000	P	Dako (Denmark)
Collagen I	—	r	1:100	H, Pt	Synbio (Germany)
Collagen II	C2-E8	m (ns)	1:200	H, P	R. Holmdahl ⁵⁶
Collagen III	—	r	1:2000	H, P	Dr. Günzler (Aventis, Germany)
Collagen VI	—	r	1:2000	H, Pt	Dr. Timpl ⁵⁷
Collagen X	X-34	m (ns)	1:100	H, Pt	Dr. von der Mark ⁵⁸
Aggrecan proteoglycan	5G5A12	m (ns)	1:1000	H, Pt	Dr. Perris (Avioli, Italy)

m, Mouse monoclonal; ns, not specified isotype; r, rabbit polyclonal; H, hyaluronidase (Boehringer Mannheim, Mannheim, Germany; 2 mg/ml in phosphate-buffered saline, pH 5, 60 minutes at 37°C); P, pronase (Boehringer Mannheim, 2 mg/ml in PBS, pH 7.3, 60 minutes at 37°C); Pt, protease XXIV (Sigma, 0.02 mg/ml in PBS, pH 7.3, 60 minutes at room temperature).

phology and the cytoprotein profile of the neoplastic cells. Herein, the analysis of the matrix gene expression pattern allowed us to identify and characterize mesenchymal cell differentiation within the neoplasms that is not unequivocally possible by morphological or cytoprotein analysis.^{13,14} Using this approach, we could identify and trace the cellular differentiation pattern in chordomas including chondroid chordoma and could unequivocally identify focal chondroid differentiation as a characteristic facet of chordomas.

Materials and Methods

Tissue Preparation and Histochemistry

Twenty-two specimens of chordomas (15 classic and 7 chondroid chordomas) diagnosed according to conventional criteria^{15,16} and four samples of fetal vertebral columns with remnants of chorda dorsalis tissue (22 to 36 weeks of gestation) were routinely fixed, embedded in paraffin, and 3- μ m sections cut.

The high molecular weight acid mucopolysaccharides (glycosaminoglycans) that are found abundantly in cartilaginous tissues, were visualized by toluidine blue staining. The presence of collagens in the extracellular tumor matrix was demonstrated by Masson-Goldner's staining.

Immunohistochemistry

Deparaffinized sections were enzymatically pretreated and epitopes detected using mono- and polyclonal antibodies (Table 1) as described previously.¹⁷

As negative control for immunohistochemical stainings, the primary antibody in control sections was replaced by nonimmune mouse or rabbit serum (BioGenex, San Ramon, CA) or Tris-buffered saline (pH 7.2). None of the negative controls showed any signal.

Preparation of RNA Probes—in Situ Hybridization

Suitable fragments of human collagen chains α_1 (II) and α_1 (X), and aggrecan core protein mRNA were selected¹⁸

and transcribed *in vitro* to generate digoxigenin-labeled antisense and sense transcripts as described elsewhere.¹⁹

In situ hybridization was performed as described in detail elsewhere.²⁰ Briefly, deparaffinized sections were pretreated and hybridized for 12 to 16 hours at 44°C (α_1 (II) and α_1 (X)) or 55°C (aggrecan) with riboprobes at a final concentration of ~1 ng/ml. After washing, the immunological detection of the digoxigenin-labeled probes was performed using the digoxigenin-detection-kit (Boehringer-Mannheim, Mannheim, Germany). The exposure time was 3 days for all three probes.

Probe Specificity and Control Experiments

To avoid false-positives because of cross-hybridization, cDNA fragments were checked experimentally on fetal growth-plate specimens in parallel experiments (data not shown; Ref. 18). A probe for 18S rRNA was used as a positive control for preservation of the RNA in the samples during the technical procedures (see Figure 3j). In selected cases sense transcripts served as negative controls and revealed only background signals.

Results

Fetal Chorda Dorsalis

Remnants of the chorda dorsalis present in the fetal vertebral column specimens showed histologically characteristic chords of vacuolated cells surrounded by a myxoid, histochemically collagen-poor and glycosaminoglycan-rich extracellular matrix (Figure 1a). Immunohistochemically, the cells were strongly reactive for epithelial cytokeratins, in particular cytokeratin 19 (Figure 1e), as well as epithelial membrane antigen (EMA) (Figure 1f), S-100 protein (Figure 1d), and vimentin. The extracellular matrix was strongly positive for aggrecan proteoglycan (Figure 1b) and to a lesser extent for type VI collagen, but negative for collagen types II (Figure 1c) and X. Collagen types I and III were observed focally.

Figure 1. Remnants of the fetal chorda dorsalis in the developing nucleus pulposus of the vertebral column showed strand-like cell formations within a glycosaminoglycan-rich (a) and aggrecan-rich (b) and collagen type II-negative (c) extracellular matrix. The surrounding cartilage endplate was positive for aggrecan (b) and type II collagen (c). The chordal cells are positive for S-100 protein (d), cytokeratin 19 (e), and EMA (f). a: Toluidine blue staining for glycosaminoglycans. b–f: Immunostainings for aggrecan (b), type II collagen (c), S-100 protein (d), cytokeratin 19 (e), and EMA (f). Original magnifications: $\times 100$ (a), $\times 50$ (b–f).

Chordomas

Histomorphology and Cytoprotein Profile (Table 2)

Fifteen classic and seven chondroid chordomas were investigated. Histologically, all cases contained areas showing chords or nests of cells with partly vacuolated cytoplasm embedded in a myxoid tumor matrix (see Figure 3a). The cases diagnosed as chondroid chordomas

additionally contained areas showing morphologically chondrocyte-like cells lying in a cartilage-like extracellular tumor matrix (see Figure 3k).

Immunodetection of cytoproteins showed strong staining in neoplastic cells throughout all chordomas for S-100 protein (Figure 2d) and vimentin (Figure 2c). Cytokeratin 19 (Figure 2a) and EMA (Figure 2b) were positive throughout classic chordomas. In chondroid chordomas,

Table 2. Distribution of Cytoproteins in Chorda Dorsalis, Nucleus Pulposus,^{16,26,27} Classic Chordoma, Chondroid and Nonchondroid Areas of Chondroid Chordoma, and Low-Grade Conventional Chondrosarcoma (Primary Central Conventional Chondrosarcoma, G1)^{4,15}

	EMA	pan-CK	CK 19	S-100	Vim
Chorda dorsalis	+	+	+	+	+
Nucleus pulposus	(+)	(+)	(+)	+	+
Classic chordoma	+	+	+	+	+
Chondroid chordoma					
Chondroid area	+	+	+	+	+
Transitional area	+	+	+	+	+
Chondroid area	–/(+)	–/(+)	–/(+)	+	+
Conventional chondrosarcoma	–	–	–	+	+

+, majority of cells positive; (+), very focally positive; –, negative; EMA, pan-CK, CK 19, and vim: cytoplasmic staining; S-100, cytoplasmic and/or nuclear staining; CK, epithelial cytokeratins; CK 19, cytokeratin 19; vim, vimentin.

Figure 2. Cytoprotein profile of neoplastic cells in chordoid (**a–c**) and chondroid (**d–f**) tumor areas. In chordoid areas, immunostaining showed the tumor cells to be positive for epithelial cytokeratins (**a**), EMA (**b**), and vimentin (**c**). In chondroid tumor areas neoplastic chondrocytic cells were positive for S-100 protein (**d**), partly positive (**large arrowheads**) and partly negative (**small arrowheads**) for epithelial cytokeratins (**e**) and in particular cytokeratin 19 (**f**). **a–f:** Immunostainings. Original magnifications: $\times 50$ (**a–c**), $\times 100$ (**d–f**).

both were positive in the chordoid areas and in part of the chondrocyte-like cells (Figure 2, e and f; large arrowheads). The chondrocyte-like cells in the centers of the chondroid areas were, however, negative for the investigated epithelial cell markers (Figure 2, e and f; small arrowheads).

Analysis of Aggrecan Proteoglycan Expression and Distribution (Table 3)

The expression and distribution of aggrecan proteoglycan, the main proteoglycan of cartilaginous tissue, was investigated on the mRNA and protein level by *in situ*

Table 3. Distribution of Major Extracellular Matrix Components in Chorda Dorsalis, (Nondegenerated) Nucleus Pulposus,^{29,40} Classic Chordoma, Chondroid and Nonchondroid Areas of Chondroid Chordoma and (Primary Central) Low-Grade Conventional Chondrosarcoma (G1)⁴¹

	GAG	Agg (m/p)	COL	COL2 (m/p)	COL10 (m/p)
Chorda dorsalis	++	++/++	+	–/–	–/–
Nucleus pulposus	++	++/++	++	+/++	–/–
Classic chordoma	++	++/++	+	(+)/(+)	–/–
Chondroid chordoma					
Chordoid areas	++	++/++	+	–	–/–
Transitional areas	++	++/++	++	++/++	–/–
Chondroid areas	+++	++/++	+++	++/+++	–/–
Conventional chondrosarcoma	+++	++/++	+++	++/+++	(+)/(+)

+++ , strongly positive; ++ , positive; + , weakly positive; (+) , only focally positive; – , negative; all antigens; extracellular matrix staining. Agg, aggrecan; COL2,10, collagen type II, X; GAG, glycosaminoglycans; pc, pericellular; m, mRNA; p, protein.

hybridization and immunohistochemistry. Histochemical analysis was performed to confirm the presence of sulfated glycosaminoglycans that represent the typical component of the sugar side chains of the large aggregating proteoglycan aggrecan. Very similar to the fetal chorda dorsalis, a high GAG/aggrecan content of the extracellular matrix was detected throughout classic and chordoid areas of chordoid chordomas histochemically as well as by immunostaining (Figure 3, g and h). In particular the chordoid areas of chordoid chordomas were positive for aggrecan and glycosaminoglycans (Figure 3, m and n). *In situ* hybridization showed high mRNA expression levels for aggrecan in neoplastic cells of classic and chordoid chordomas (Figure 3, i and l) and, thus, excluded that this aggrecan-rich matrix was produced by surrounding nonneoplastic stromal cells.

Analysis of the Collagenous Tumor Matrix (Table 3)

In classic chordomas, the extracellular tumor matrix was mostly collagen-poor as shown by histochemical analysis. Immunohistochemically, in particular type VI collagen could be demonstrated. Types I and III collagens were found only focally. Type II collagen was absent in most tumor areas (Figure 3c). Correspondingly, no type II collagen mRNA expression was observed in most tumor cells (Figure 3b). However, focal expression and deposition of type II collagen was seen also in classic chordomas without histologically evident chordoid matrix formation (Figure 3d). Histochemically, these areas had a relatively collagen-rich tumor matrix.

Chordoid chordomas showed similar collagen expression and distribution pattern as classic chordomas in the chordoid areas. High type II collagen mRNA (Figure 3o) and protein levels (Figure 3p) were found in the chordoid tumor compartments. Type VI collagen was mainly concentrated in the pericellular matrix compartment. Types I and III collagens were also focally present.

mRNA expression or protein deposition of type X collagen was not observed in the classic or chordoid chordoma (Figure 3; e, f, q, and r).

Discussion

Despite discrepancies in the past, the cytoprotein profile seems now to be well established for classic chordomas and nonchordoid portions of chordoid chordomas. Both show positivity for epithelial cytokeratins, in particular cytokeratin 19,^{14,21} and EMA as well as vimentin and S-100 protein.^{13,22–24} An identical cellular phenotype is seen in cells of the fetal chorda dorsalis^{16,25} and interestingly, also in the nucleus pulposus during childhood.^{26,27} At this stage the cells of the nucleus pulposus have started to express and deposit cartilage matrix.^{28,29} Notably, most cells of the nucleus pulposus in adulthood lose their epithelial markers and show an immunophenotype similar to chondrocytes with positivity for S-100 protein and vimentin, but not for cytokeratin and EMA.^{26,27,30} A similar switch in the cellular phenotype was also seen in cells of chordoid chordomas that confirms and extends

previous investigations.^{7,24} Also morphologically, parallel changes can be observed, both in nucleus pulposus development and chordoid chordomas, where physaliferous cells change to a rounded, chondrocyte-typical cell shape.

Since its first description by Heffelfinger and colleagues,³ chordoid chordoma remained controversial. Morphological and cytoprotein analyses alone were not able to resolve the continuing issue. Jeffrey and co-workers⁸ even doubted that there exists any cartilaginous differentiation at all in chordomas and others considered chordoid chordoma as low-grade chondrosarcomas.^{4,6,8,22} In the present article, using a combination of morphological features, the cytoprotein profile, and the matrix gene expression pattern as three independent and complementary tools to identify cell and tissue differentiation,¹⁷ we could show that all of the described features of classic or chordoid chordomas are within the spectrum of the developmental differentiation pathway from the chorda dorsalis into the nucleus pulposus. This was indicated by morphology and the cytoprotein profile of the cells as outlined above. Matrix gene expression analysis, however, allowed us to firmly establish this concept on the cell function level. In chordoid areas of chordoma, most neoplastic cells produce a collagen type VI and focally I- and III-positive, but type II collagen-negative extracellular tumor matrix similar to the matrix found in the chorda dorsalis.^{25,31} Significantly, both tissues contain high amounts of chondroitin and keratan sulfate-rich proteoglycans,^{32,33} identified in this study as aggrecan. Later in vertebral development, chorda dorsalis cells become part of the nucleus pulposus and start to express collagen type II, the characteristic marker of chondrocytic differentiation.³⁴ An analogous switch of expression pattern is observed more or less focally in most chordomas even before cartilage formation is visible morphologically.²⁵ Thus, in contrast to Taniguchi and colleagues,³⁵ we could clearly show the presence of type II collagen also in classic chordoma, although type II collagen is not found throughout all chordomas as suggested by Wojno and co-workers.⁷ Notably, no expression of type X collagen, which is a marker of hypertrophic differentiation of chondrocytic cells and thought to precede matrix calcification,^{36,37} was found in any chordoma. This might explain why chordoid chordomas do not extensively calcify as other cartilaginous neoplasms or degenerated nucleus pulposus, both of which have been shown to express multifocally type X collagen.^{29,38–40}

None of our chordoid chordoma cases showed pure cartilaginous differentiation without significant tumor portions resembling classic chordoma. Having clearly demonstrated the potential of chondrocytic cell differentiation in chordomas virtually indistinguishable from neoplastic chondrocytes found, eg, in low-grade conventional chondrosarcomas,⁴¹ according to our analysis, pure chordoid chordoma is a theoretical possibility. This could include neoplasms even without any focal expression of epithelial cytokeratins or EMA.^{6,22} However, this seems to the authors as an as unlikely extreme as, eg, a completely cartilaginous-differentiated pleomorphic adenoma: also

Figure 3. a–j: Chordoid chordoma/chordoid areas of chondroid chordoma. Conventional histology revealed the typical morphology of chordoid chordoma (**a**). No mRNA expression (**b**) and no protein (**c**) of type II collagen was found in most tumor areas, whereas others showed focal deposition of type II collagen (**d**). No mRNA expression (**e**) or protein deposition (**f**) was detectable for type X collagen, whereas aggrecan expression was detectable on the histochemical (**g**), protein (**h**), and mRNA (**i**) level throughout the neoplasms. **j:** Positive *in situ* hybridization control using a probe for 18S rRNA. **k–r:** Chondroid areas of chondroid chordoma. Conventional histology showed typical chondrocytic cells lying in cell lacunae and surrounded by an abundant hyaline extracellular matrix (**k**). *In situ* analysis demonstrated the mRNA expression and protein deposition of aggrecan proteoglycan (**l** and **n**) and type II collagen (**o** and **p**). The presence of highly glycosylated proteoglycans was confirmed histochemically (**m**). No mRNA expression (**q**) or protein deposition (**r**) was visible for type X collagen. **a:** H&E staining; **g** and **m:** toluidine blue staining for glycosaminoglycans; **b, c, e, l, o,** and **q:** *in situ* hybridization experiments for type II collagen (**b** and **o**), type X collagen (**e** and **q**), 18S rRNA (**j**), and aggrecan (**i** and **l**); **c, d, f, n, p,** and **r:** immunostainings for type II collagen (**c, d,** and **p**), type X collagen (**f** and **r**), and aggrecan (**h** and **n**). Original magnifications: $\times 100$ (**a, i, k–r**); $\times 50$ (**b–h, j**).

in pleomorphic adenomas extensive cartilaginous differentiation can occur,¹⁷ but still no chondroma-like pleomorphic adenoma of the parotid was described in the literature despite the frequency of this neoplasm. Instead, chondroid differentiation is always focal within these tumors and most likely also in chordoma. More definitive proof of the (non)existence of pure chondroid chordoma and its differentiation from low-grade conventional chondrosarcoma might come from genetic studies using techniques such as recently applied for dedifferentiated or conventional chondrosarcomas and that allow the identification of irreversible genetic alterations markers during tumor development.^{42–44} So far reported cytogenetic alterations in chordomas^{45–48} did not reveal any significant similarities to alterations known from similar analyses in conventional chondrosarcomas.^{49–51} This provides supportive evidence for the basic difference of chondroid chordomas and (conventional) chondrosarcomas.

Our results support the notion that analysis of extracellular matrix gene expression has the potential to become a diagnostic tool in tumor pathology, in particular, because cytoprotein markers are not always unequivocal for detecting or excluding mesenchymal tumor differentiation.^{13,14} Thus, the detection of, eg, type II collagen in the tumor matrix clearly identifies the chondrogenic potential of a neoplasm even without overt cartilage formation^{41,52,53} and therefore excludes, eg, metastatic mucinous carcinoma that might come into differential diagnosis.^{25,53} Irrespectively of chondrocytic differentiation, the detection of aggrecan allows the differentiation of chordoma from clear cell carcinomas and mucinous carcinomas that accumulate mucinous polysaccharides, but not aggrecan (our own unpublished data). Both entities might be a problem in differential diagnosis in particular in limited biopsy specimens.⁵⁴

Altogether, our study firmly establishes a real cartilaginous phenotype of a subset of neoplastic cells in chordomas that appears more or less focally in many chordomas and that justifies also biologically the term chondroid chordoma, depending on the abundance of this phenomenon.³ Our results, however, clearly support the notion that chondroid chordomas are a variant of classic chordoma and do not imply a substantially different biology. This is also reflected clinically by their overall similar prognosis.⁵⁵ The focal chondrocytic differentiation is delineated in development by the transition of the chorda dorsalis to the nucleus pulposus and can, thus, be considered as a focal maturation process within chordomas. Our results refute the suggestion that chondroid chordoma is a low-grade chondrosarcoma or a misnomer.^{4,6,8,11} However, our data support the notion that one has to be reluctant to claim that monodifferentiated chondroid chordomas exist that are virtually indistinguishable from low-grade conventional chondrosarcomas.^{6,7,22}

Acknowledgments

We thank Dr. Günzler (Höchst Co., Frankfurt, Germany), Dr. Holmdahl (Uppsala, Sweden), Dr. Timpl (MPI, Munich, Germany), Dr. R. Perris (Avioli, Italy), and Dr. K. von

der Mark (Erlangen, Germany) for kindly providing us with antibodies to collagen types II, III, VI, and X and aggrecan; Ms. K. Herbig for expert photographic assistance; and Louise A. McKenna for critically reviewing the manuscript.

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