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Type X collagen expression and hypertrophic differentiation in chondrogenic neoplasias

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Abstract Little is known about matrix biochemistry and cell differentiation patterns in chondrogenic neoplasms. This is the first description of the focal expression of collagen type X by neoplastic chondrocytes in situ and its incorporation into the extracellular matrix of cartilaginous tumors. This shows that neoplastic chondrocytes have the potential to undergo the full program of cell differentiation, including hypertrophy, comparable to their physiological counterparts in the growth plate. However, only in benign osteochondromas was a zonal expression of type X collagen found similar to that observed in the growth plate, where the cells immediately above the ossification frontier are selectively positive for type X collagen. In enchondromas and chondrosarcomas, the expression was randomly distributed within the tumors. Surprisingly, in less differentiated chondrosarcomas with spindle-shaped cells and non-cartilaginous extracellular matrix, exceptional expression of collagen type X was observed, which indicates potential uncoupling of collagen type X expression from the differentiated chondrocytic phenotype in neoplastic chondrocytes in vivo.

Introduction

Chondroid neoplasms are defined by the presence of neoplastic cartilage formation. Biochemical and immunohistochemical analyses indicated that the matrix composition of chondroid tumors is similar to that of fetal and adult hyaline cartilage (Mankin et al. 1980; Remberger and Gay 1977; Ueda et al. 1990). Little informtion is available to date on cell differentiation in neoplastic cartilage and, in particular, whether it follows the same pattern of differentiation as fetal cartilage. The differentiation processes in fetal growth plate chondrocytes can be monitored by changes in the expression of collagen genes (for review see Cancedda et al. 1995; von der Mark 1986): collagen types I, IIA, and III are expressed by chondroprogenitor cells in the perichondrium (Sandell et al. 1991; Treilleux et al. 1992), collagen types II, IX, and XI, and the chondroitin sulfate proteoglycan, aggrecan, are typical gene products of differentiated chondrocytes (Müller et al. 1977; Sandberg and Vuorio 1987; Vornehm et al. 1996), while hypertrophic chondrocytes are marked by the expression of collagen type X (Marriott et al. 1991; Reichenberger et al. 1991; Schmid and Linsenmayer 1985a, b). Recently, evidence has been accumulating of the further differentiation of hypertrophic chondrocytes to type I collagen expressing osteoblast-like cells (Cancedda et al. 1992; Kirsch et al. 1992; Roach et al. 1995).

The exact function of the short-chain collagen type X within the developing fetal growth plate is still unresolved. It is thought to be involved in the calcification process in the lower hypertrophic zone (Alini et al. 1994; Kirsch and von der Mark 1992; Kwan et al. 1989; Schmid and Linsenmayer 1987), a possibility supported by the restriction of type X collagen to the calcified zone in adult articular cartilage (Gannon et al. 1991; Walker et al. 1995) and its prevalence in the calcified chick egg shell (Arias et al. 1991).

In this study, we analyzed the occurrence and expression pattern of collagen type X as a marker of hypertrophy in neoplastic chondrocytes in order to elucidate the differentiation potency of neoplastic chondrocytes in vivo. For this purpose we have selected the most common benign and malignant chondrogenic tumors (Fechner and Mills 1993): enchondromas, osteochondromas, and (conventional) chondrosarcomas.

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Materials and methods

Tissue preparation and histochemistry

Seven benign osteochondromas, seven benign enchondromas, and 13 malignant (conventional) chondrosarcomas of various grades were analyzed (for exact description of morphological features of the tumor types investigated see Schajowicz 1994; Unni 1996). Additionally, five samples of fetal growth plate cartilage were included in the series for comparison. Specimens were fixed with 10% formalin immediately after removal, decalcified in 0.3 M EDTA, and embedded in paraffin wax. Toluidine blue and safranin O stainings were performed to estimate the proteoglycan content (Rosenberg 1971).

Immunohistochemistry

Deparaffinized sections were incubated with testicular hyaluronidase (2 mg/ml, 60 min at 37° C) and pronase (1 mg/ml, 60 min at 37° C) or protease (0.02 mg/ml, 60 min at 37° C). Primary antibodies were incubated overnight at 4° C and visualized using alkaline phosphatase-labeled secondary antibodies. Nuclei were counterstained with hematoxylin. Monoclonal antibodies to type II collagen were kindly provided by R. Holmdahl (CII-E8; Klareskog et al. 1986). Monoclonal antibodies to type X collagen were prepared as described elsewhere (Girkontaité et al. 1996). Polyclonal antibodies against S-100 protein were purchased from Dakopatts (Glostrup, Denmark).

Preparation of RNA probes

The RNA probes for human collagen chains α1(I), α1(II) (Aigner et al. 1992, 1993), α_1(X) (Reichenberger et al. 1991), and 18 S rRNA (Aigner et al. 1992) were linearized and transcribed as described previously.

In situ hybridization

In situ hybridization was performed as described in detail elsewhere (Aigner et al. 1992). Briefly, deparaffinized sections were digested with proteinase K, postfixed, acetylated, and dehydrated. The sections were hybridized for 12–16 h at 43° C riboprobes. After hybridization, the tissue sections were washed at 40° C in 0.5×SSC/0.5% β-mercaptoethanol, treated with RNAses A and T_1, washed again for 2 h at 45° C in 2×SSC/50% formamide/0.5% β-mercaptoethanol. After three rinses in 0.1×SSC at room temperature the slides were dehydrated.

Autoradiography was performed (Kodak NTB-2 nuclear track emulsion) for 4 days at 4° C and sections counterstained in 5% Giemsa dye.

Control experiments

The specificity of the cDNA probe was ascertained by computerized homology search, northern blotting experiments, and in situ hybridization experiments in the fetal growth plate (Reichenberger et al. 1991). Sense transcripts as well as unrelated cDNA probes were used as negative controls and never showed more than background signals (data not shown).

As negative controls for immunohistochemical stainings, the primary antibody was replaced either by preimmune serum, unspecific mouse or rabbit serum (BioGenex, San Ramon, Calif., USA) or TRIS-buffered saline (pH 7.2).

Results

Fetal growth plate cartilage (data not shown) showed strong staining for cartilage proteoglycans and collagen type II throughout the extracellular matrix. In contrast, type X collagen was restricted to the hypertrophic zone. Correspondingly, collagen type X collagen mRNA expression was found solely in hypertrophic chondrocytes, whereas type II collagen mRNA was seen in all chondrocytes. Bone was negative for collagen type X except for cartilage remnants in freshly formed bone trabecules, which were also positive for collagen type II and cartilage proteoglycans. No other tissue types, such as fibrous, vascular or muscle tissue, showed expression of collagen types II and X at the mRNA or protein level.

Osteochondromas are benign “cartilage-capped bony projections on the external surface of a bone” (World Health Organization definition). They show a highly structured tissue organization, illustrated in Fig. 1a. Mesenchymal cell layers of fibrous appearance overlay cartilaginous tissue. In the deep zone, ossification was observed. Histochemical and immunohistochemical analyses of the cartilaginous layer revealed typical components of cartilage matrix: cartilage proteoglycans and collagen type II (Fig. 1b). Type X collagen expression was restricted to the cartilaginous zone immediately above the ossification frontier in all osteochondromas investigated (Fig. 1c), while the fibrous layer and the upper cartilaginous zone were negative. Bone showed collagen type X in cartilage cores of bone trabecules. These were also positive for collagen type II and cartilage proteoglycans (Fig. 1a, b) and, thus, resembled the cartilage remnants found in freshly formed bone trabecules in the fetal growth plate.

In contrast to osteochondromas, chondromas showed no structured tissue organization, but in most cases a conglomerate of cartilage lobules was observed (Fig. 1d–f), which resembles chondrosarcomas. Histochemically and immunohistochemically, strong collagen type II (Fig. 1e) and cartilage proteoglycan (Fig. 1d) staining could be detected throughout the extracellular tumor matrix. Type X collagen expression was restricted to randomly distributed foci in some tumors (Fig. 1f), but most areas were negative.

Chondrosarcomas are malignant tumors characterized by the formation of cartilage, but not of bone, by the tumor cells. In general, chondrosarcomas showed a rather heterogeneous pattern of tissue formation. It was important for the analysis to distinguish between low and highly differentiated areas.

In highly differentiated areas containing hyaline matrix, strong staining for cartilage proteoglycans (Fig. 1g) and collagen type II (Fig. 1h) was observed. Type X collagen was found in most tumors multifocally (Figs. 1i, 2d, h, i, j, h). In situ hybridization analyses often revealed a very strong expression of type X collagen mRNA, confirming active expression of this collagen type (Fig. 2a, f, h). The expression level was often similar to the high levels found in the hypertrophic fetal

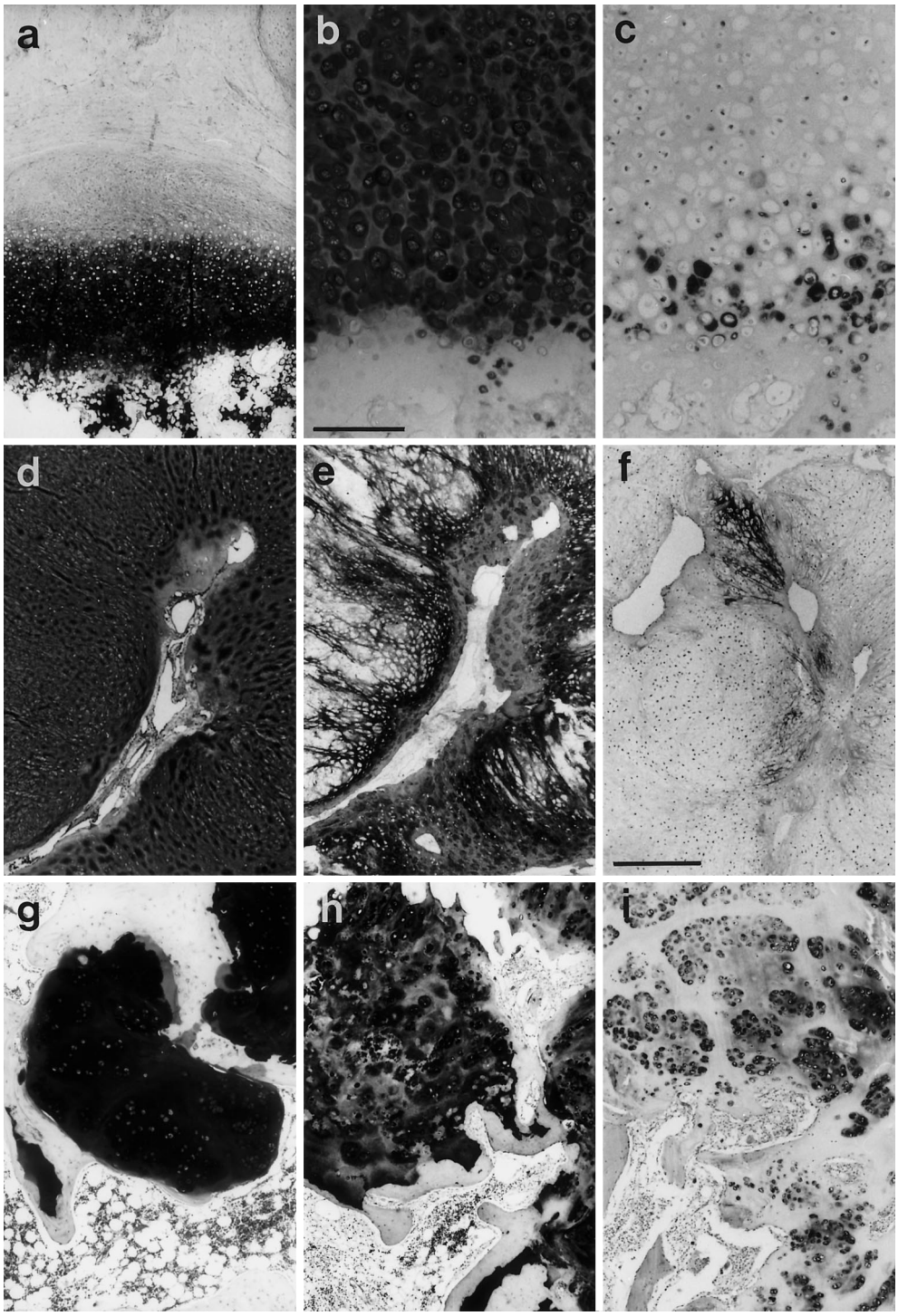

Fig. 1 Histochemical detection of cartilage proteoglycans (**a, d, g**) and immunohistochemical detection of collagen types II (**b, e, h**) and X (**c, f, i**) in benign osteochondroma (**a–c**), benign enchondroma (**d–f**), and malignant highly differentiated chondrosarcoma (**g–i**). *Bars* **a, d–i** 250 µm, **b, c** 100 µm

growth plate chondrocytes (not shown). The collagen type X-expressing cells were mostly large round cells. However, not all large round cells showed type X collagen expresion at the mRNA or protein level (data not shown). Thus, no direct correlation between cell morphology and expression pattern was apparent.

Overall, the distribution of type X collagen was focal. In some tumors, whole lobules expressed type X collagen mRNA (Fig. 2f) and, correspondingly, showed lobular staining for type X collagen protein (Fig. 2g). In other lobules of the same tumors, no mRNA or protein of type X collagen was found at all. Some lobules showed positivity selectively at their margins (Fig. 2h, i). Although in many areas the distribution of mRNA signals correlated with protein staining, in other regions, type X collagen could be observed immunohistochemically without detectable levels of type X collagen mRNA, indicating previous synthetic activity of the now silent cells.

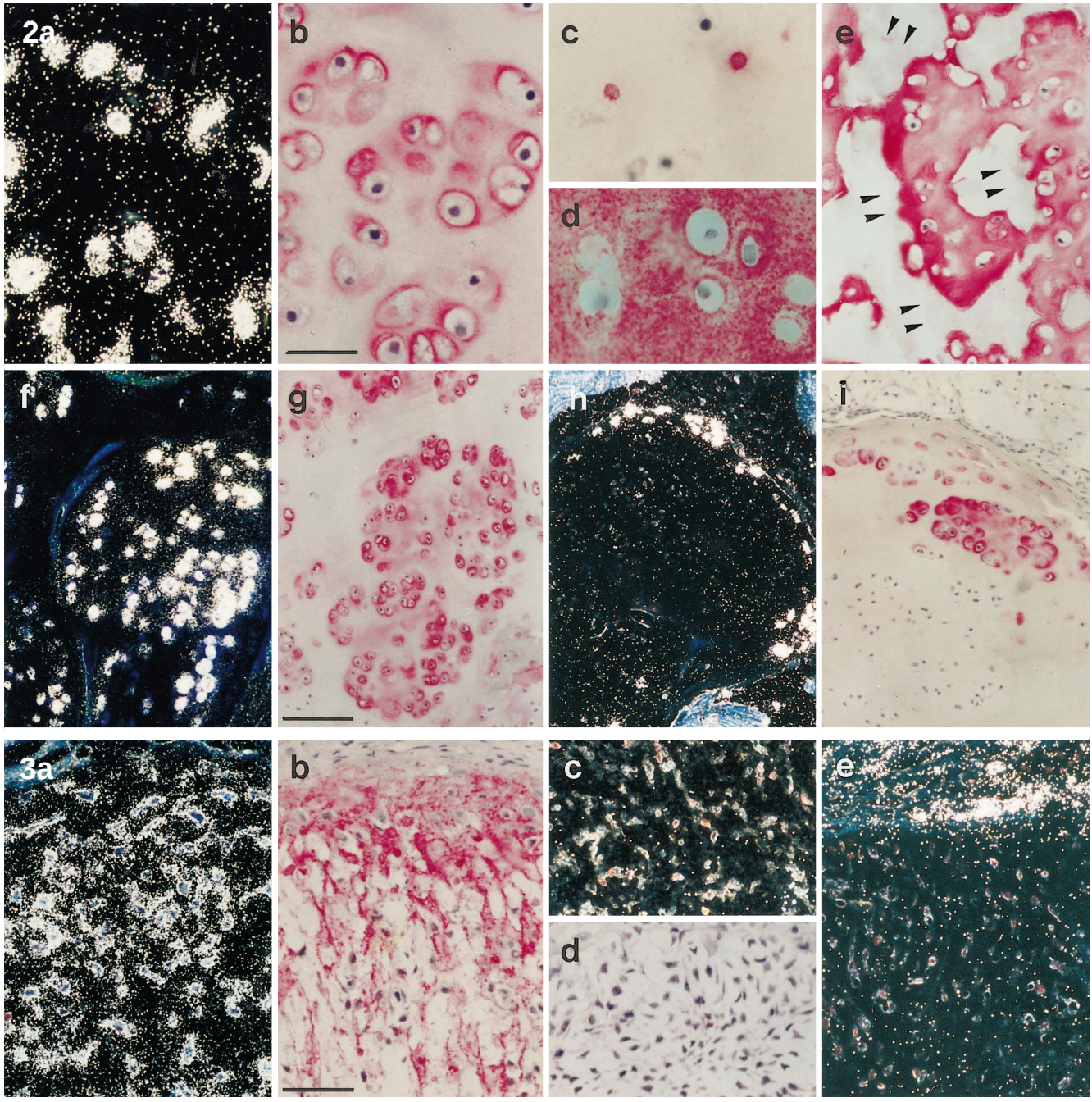

Fig. 2 In situ hybridization (**a, f, h**) and immunohistochemical (**b–e, g, i**) analyses of collagen type X expression in highly differentiated chondrosarcoma tumor areas: collagen type X protein could be detected mostly pericellularly (**b, g**); in some areas, intracellular (**c**) and interterritorial (**d, e**) staining was observed. In many areas of high differentiation, expression of type X collagen was associated with calcification and bone formation (**e** *arrowheads* bone trabecules). **a, f, h** dark field micrographs; *bars* **a–d** 50 µm, **e–i** 100 µm

Fig. 3 In situ hybridization (**a, c, e**) and immunohistochemical (**b, d**) analyses of collagen types X (**a, b**), II (**c, d**) and I (**e**) in low differentiated chondrosarcoma tumor areas with spindle-shaped cells. Although no type II collagen mRNA (**c**) and protein (**d**) was detected in the neoplastic cells, these cells expressed type X collagen (**a, b**). They also did not express type I collagen (**e** the fibroblasts of the tumor capsule seen in the upper part of the figure showed moderate expression levels and served as an internal positive control). *Bar* 50 µm

In many cases, the strongest immunohistochemical staining for collagen type X was seen pericellularly (Fig. 2b, g). However, in some areas, interterritorial staining (Fig. 2d) and, less frequently, selective intracellular staining was also seen (Fig. 2c). At some sites, the expression of type X collagen was associated with calcification and bone formation in the tumors (Fig. 2e) and remnants of calcified, collagen types X- and II-positive cartilage were found in newly formed bone trabecules, similar to those in the fetal growth plate.

Non-chondroid areas of chondrosarcomas showed less abundant extracellular matrix and spindle-shaped or stellate neoplastic cells (Fig. 3d). Diminished staining for cartilage proteoglycans and little or no expression of collagen type II mRNA and protein (Fig. 3c, d) indicated

the lack of full chondrocytic differentiation of the neoplastic cells. Exceptionally, collagen type X mRNA expression and deposition of the protein was detected in these areas (Fig. 3a, b). The type X-positive cells were negative for type I collagen mRNA (Fig. 3e). However, they were positive for S-100 protein (not shown). In non-chondroid tumor areas, no calcification or bone formation was observed, irrespective of the prevalence of type X collagen mRNA and protein.

Discussion

In this study, we were first able to demonstrate chondrocyte hypertrophy in chondrogenic neoplasms using type X collagen as a marker for this particular differentiation status of chondrocytes (Marriott et al. 1991; Reichenberger et al. 1991; Schmid and Linsenmayer 1985a, b). We were able to show the presence of collagen type X as a new component of the extracellular matrix in benign and malignant tumors containing chondroid areas.

Whereas in enchondromas and chondrosarcomas a random expression of type X collagen was found, osteochondromas showed a highly structured tissue organization and a zonal expression pattern of collagen types II and X similar to that which occurs in the mammalian fetal growth plate cartilage (Kirsch and von der Mark 1990; Reichenberger et al. 1991). Thus, osteochondromas resemble osteophytes, which arise next to articular cartilage and show similar morphology and cell differentiation patterns (Aigner et al. 1995). In contrast, the very focal type X collagen expression in enchondromas and conventional chondrosarcomas reflects a very focal cell differentiation pattern in these neoplasms, which explains in part the high morphologic heterogeneity typical for these tumors (Unni 1996).

Central features of hypertrophic differentiation were preserved in many neoplastic chondrocytes of all tumor types investigated: type X collagen expressing cells were mostly large and rounded in appearance, and showed a fully differentiated chondrocytic phenotype, as further demonstrated by the expression of the typical chondrocyte gene products, type II collagen and cartilage proteoglycans. In addition, the association of collagen type X expression and calcification or bone formation (Kirsch and von der Mark 1990, 1992; Reichenberger et al. 1991) was maintained in part of the neoplastic cartilages. It will need further studies to evaluate which portion of the spotty calcification pattern, which is characteristic of chondroid neoplasms, e.g., on X-ray micrographs, is directly related to sites of type X collagen expression.

Remarkable and so far not described in vitro or in vivo, was the exceptional expression of collagen type X in areas which lacked full chondrocytic cell differentiation, as indicated by the absence of a cartilaginous, collagen type II- and proteoglycan-positive extracellular matrix and the rounded chondrocytic shape of the cells. No alkaline phosphatase activity, calcification or ossification is observed in these sites in the tumors (O'Neal and Ackerman 1952; Sanerkin 1980). Thus, the expression of type X collagen seems not to be constitutively associated with the hypertrophic chondrocytic phenotype and does not lead by itself to matrix calcification. A similar situation was recently suggested to exist in the surface zone of normal articular cartilage (Rucklidge et al. 1996). However, this analysis was only done on one sample and we and others were not able to confirm this observation in larger series of normal articular cartilage specimens (Girkontaité et al. 1996; Walker et al. 1995; our own unpublished results).

The collagen type X-expressing cells in non-cartilaginous areas also do not represent posthypertrophic, osteoblast-like cells (Galotto et al. 1994; Kirsch and von der Mark 1992; Roach et al. 1995), as they do not express collagen type I mRNA. However, from our data it cannot be ruled out that the phenomenon observed results from some sort of abortive differentiation of the cells to osteoblast-like cells. In this case one could expect that the cells have already stopped type II, but not type X, collagen synthesis, have undergone morphological changes but have not yet started expression of characteristic bone proteins such as type I collagen.

Overall, our study demonstrates for the first time type X collagen as a new constituent of the extracellular tumor matrix of cartilaginous neoplasms. Our data demonstrate that neoplastic chondrocytes display the full potential of differentiation patterns also found in fetal growth plate chondrocytes. In general, type X collagen expression is linked to the fully differentiated chondrocytic phenotype. However, our results give further evidence that type X collagen expression is not necessarily restricted to hypertrophic chondrocytes (Arias et al. 1991) and thus is not an absolutely reliable marker of hypertrophy in, at least neoplastic, chondrocytes. On the other hand, our own and other data show that condrocyte hypertrophy, defined as cell body enlargement, also occurs without the expression of type X collagen (Chung et al. 1995).

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