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Horst Claassen · Willm Uwe Kampen
Thorsten Kirsch

Localization of collagens and alkaline phosphatase activity during mineralization and ossification of human first rib cartilage

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Abstract The localization of type X collagen and alkaline phosphatase activity was examined in order to gain a better understanding of tissue remodelling during development of human first rib cartilage. First rib cartilages from children and adolescents showed no staining for type X collagen and alkaline phosphatase activity. After onset of mineralization in the late second decade, a peripheral ossification process preceded by mineralized fibrocartilage could be distinguished from a more central one preceded by mineralized hyaline cartilage. No immunostaining for type X collagen was found in either type of cartilage. However, strong staining for alkaline phosphatase activity was detected around chondrocyte-like cells within fibrocartilage adjacent to the peripheral mineralization front, while a weaker staining pattern was observed around chondrocytes of hyaline cartilage near the central mineralization front. In addition, the territorial matrix of some chondrocytes within the hyaline cartilage revealed staining for type I collagen, suggesting that these cells undergo a dedifferentiation process, which leads to a switch from type II to type I collagen synthesis. The study provides evidence that mineralization of the hyaline cartilage areas in human first rib cartilage occurs in the absence of type X collagen synthesis but in the presence of alkaline phosphatase. Thus, mineralization of first rib cartilage seems to follow a different pattern from endochondral ossification in epiphyseal discs.

Introduction

Human first rib cartilage, i.e., the structural cartilage between first rib and manubrium sterni, usually remains cartilaginous until the end of adolescence. In contrast to other cartilages, such as growth plate cartilage of the first rib and sternum, ossification of first rib cartilage only begins when the growth of the trunk and limb skeleton has finished and lasts until an advanced age. The mechanism of ossification, mainly investigated by X-rays (Heinrich 1941; Werner 1978; Koebke and Saternus 1985), is not yet established.

Although much information is available on tissue remodelling that occurs during cartilage-bone metamorphosis in the growth plate of fetal chicken and mammalian long bones, very little is known about matrix changes and mineralization in human first rib cartilage. During development of long bones, chondrocytes undergo a series of differentiation events, which are characterized by a unique pattern of synthesized collagens. While mesenchymal precursor cells produce type I collagen, cells switch to the synthesis of type II, IX, and XI collagen during chondrogenesis (von der Mark 1986) and further to type X collagen when becoming hypertrophic (Schmid and Conrad 1982; Gibson et al. 1983; Castagnola et al. 1986; Schmid and Linsenmayer 1987). Finally, hypertrophic chondrocytes are replaced by bone cells at the chondro-osseous junction.

Mineralization of hypertrophic cartilage during endochondral ossification is accompanied by the synthesis of type X collagen (Schmid and Linsenmayer 1985, 1990; Poole and Pidoux 1989; Kirsch and von der Mark 1991; Reichenberger et al. 1991; Nerlich et al. 1992) and alkaline phosphatase (Bonucci et al. 1992). Both proteins are used as markers for hypertrophic chondrocytes and are thought to be involved in the calcification process.

The purpose of this study was to analyze the distribution of type X collagen and alkaline phosphatase activity in unmineralized and mineralized fibrocartilage and hyaline cartilage during development and ossification of human first rib cartilage. Therefore, the localization of type X collagen and alkaline phosphatase activity was examined in human first rib cartilages of various ages by immuno- and enzymatic staining. In addition, the distribution of these proteins was compared with the localization of type I and II collagens.

H. Claassen (✉) · W.U. Kampen
Anatomisches Institut der Universität Kiel,
Olshausenstrasse 40, D-24098 Kiel, Germany
(0431) 880-1557

T. Kirsch
Department of Biochemistry, School of Dental Medicine,
4001 Spruce Street, University of Pennsylvania,
Philadelphia, PA 19104, USA

Materials and methods

Tissue specimens

First rib cartilages were obtained from autopsies. Rib cartilages from one male child (1 year of age), two male adolescents (12 and 17 years of age), one female adolescent (16 years of age), eight male adults (19–64 years of age) and four female adults (25–58 years of age) were investigated. In all cases right and left first (Fig. 1a) and second rib cartilages were removed from the thoracic shield with adjacent parts of the body of the rib and sternum and X-rayed to determine the stage of mineralization and ossification (Fig. 1b). The caudal half of each first rib cartilage (Fig. 1b) was dissected in sagittal segments to evaluate the site of first bone formation. Segments from the right side were snap-frozen in liquid nitrogen and used for the present immunohistochemical study, those from the left side were embedded in methylmethacrylate for light microscopic investigations (Kampen et al. 1995). Sagitally oriented sections (Fig. 2) of 5–10 μm thickness were cut on a cryostat at −21° C and mounted on poly-L-lysine-coated slides.

Antibodies

The preparation and specificity of a polyclonal rabbit antibody against human type X collagen were described elsewhere (Kirsch and von der Mark 1991). The polyclonal antibody against human type I collagen was a gift from Professor Dr. P.K. Müller (Department of Medical Molecular Biology, Lübeck University). The monoclonal antibody CIID3 is an IgG2a, kappa-isotype antibody cloned after immunization of DBA/1 mouse with native chicken type II collagen. The antibody was shown to react with native type II collagen from chick, mouse, rat, bovine, and human cartilage (Holmdahl et al. 1986), but did not cross-react with other collagens.

Immunohistochemistry

Sections were fixed for 5 min with acetone at 5° C and rinsed 3 times in TRIS-buffered saline (TBS). Before immunostaining, the sections were pretreated with 5 mg/ml sheep testicular hyaluronidase (Boehringer) in TBS, pH 7.3, for 30 min at 37° C. This enzymatic digestion was needed to unmask the antigenic epitopes (von der Mark et al. 1976). The pretreated sections were washed 3 times with TBS and air-dried. After treatment with goat serum for 1 h at room temperature, sections were incubated with the primary antibodies for 3 h at room temperature, followed by washing with TBS buffer, and by incubation for 90 min at room temperature with the respective second fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit or anti-mouse IgG (Medac). Negative controls were performed by omitting the primary antibodies. Sections of human growth plates served as positive controls. Sections were photographed using a Zeiss Axiophot microscope equipped for epifluorescence and phase-contrast microscopy.

Staining for alkaline phosphatase activity

Alkaline phosphatase activity was localized in sections of various first rib cartilages using Naphthol-AS-BI as substrate and Fast Red TR for precipitation, as described by Burstone (1962). Incubation medium was kept at pH 9.2–9.8. Negative controls were performed by inhibiting alkaline phosphatase with tetramisol. For positive controls, sections of human growth plates were used.

Movat's Pentachrome and Azan staining

Serial sections were stained by a modified Movat's Pentachrome technique (Olah et al. 1977) to distinguish between mineralized cartilage and bone areas. In addition, Azan staining was performed to differentiate between fibrocartilage and hyaline cartilage (Romeis 1989).

Results

Control experiments, where the primary antibodies were omitted or alkaline phosphatase activity was inhibited, yielded negative results. In sections of growth plate cartilage, immunostaining for type X collagen was restricted to hypertrophic cartilage. Alkaline phosphatase activity was present around maturing and hypertrophic chondrocytes as well as around osteoblasts of the primary spongiosa of the metaphysis (data not shown).

While the epiphyseal disc of the human first rib shows the organization of a growth plate and undergoes endochondral ossification during childhood, human first rib cartilage shows a different organization (Fig. 2b, c) and remains cartilaginous until the end of the second decade. After onset of ossification in first rib cartilage, a peripheral ossification center can be distinguished from a more central one. While the peripheral mineralization front is preceded by mineralized fibrocartilage, the central one is preceded by mineralized hyaline cartilage.

Fig. 1a, b First rib cartilage. **a** Location of first rib cartilage. **b** X-ray of first and second rib cartilages with sternum and adjacent parts of first and second ribs from a 47-year-old female adult. Ossification started at the lower margin near the lateral end of the first rib cartilage and spread out in a cone-shaped pattern towards manubrium sterni. The *white rectangle* shows the investigated area

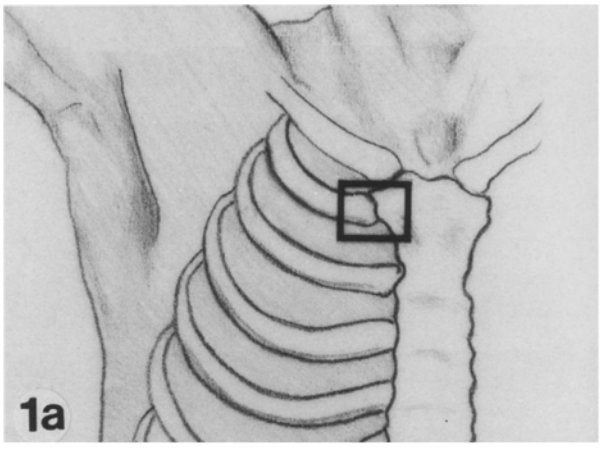
Fig. 2a–c First rib cartilage. **a** Diagram of the section plane. **b** Schematic drawing of first rib cartilage (cross section) after onset of bone formation. **c** Azan staining of first rib cartilage (cross section) from a 26-year-old male adult with the peripheral (*black rectangle*) and the central (*white rectangle*) mineralization front shown in Figs. 7 and 8. (*P* Perichondrium, *1* subperichondrial chondrocytes, *2* intermediate chondrocytes, *3* central chondrocytes, *FC* fibrocartilage, *HC* hyaline cartilage, *MC* mineralized cartilage, *B* bone, *IIM* internal intercostal muscle) **c** ×4

Fig. 3a, b Immunolocalization of type X collagen in the epiphyseal disc of the first rib from a 5-month-old boy. **a** Phase contrast microscopy. **b** Only the matrix of hypertrophic chondrocytes showed immunostaining for type X collagen, while the cells in the proliferative zone were negative for type X collagen. (*P* Perichondrium, *PZ* proliferative zone, *HZ* hypertrophic zone) ×50

Fig. 4a, b Immunolocalization of type X collagen in first rib cartilage from a 1-year-old male child. **a** Phase contrast microscopy. **b** Neither perichondrium nor hyaline cartilage showed immunostaining for type X collagen. (*P* Perichondrium, *HC* hyaline cartilage) ×50

Fig. 5a, b Immunolocalization of type X collagen in first rib cartilage from a 16-year-old female adolescent. **a** Phase contrast microscopy. **b** Hyaline cartilage was negative for type X collagen. (*P* Perichondrium, *HC* hyaline cartilage) ×50

Fig. 6a, b Immunolocalization of type X collagen in a section of first rib cartilage from a 58-year-old female adult. **a** Phase contrast microscopy. **b** The matrix near or in mineralized hyaline cartilage showed no immunostaining for type X collagen. Note that chondrocytes close to mineralized cartilage seem to be small and flattened. (*HC* hyaline cartilage, *MHC* mineralized hyaline cartilage, *B* bone) ×50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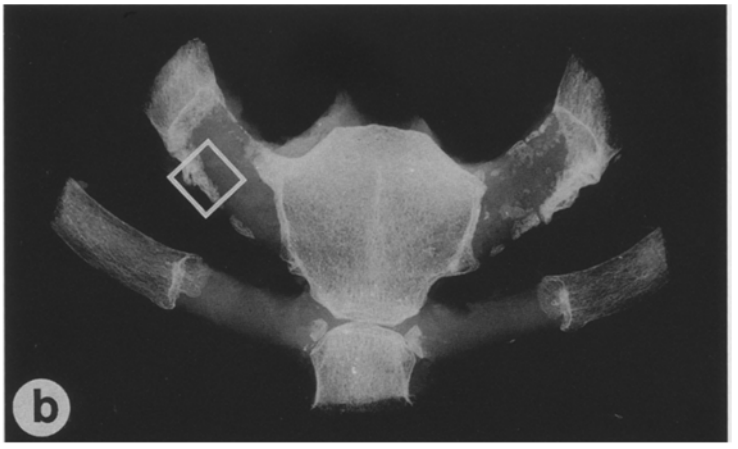
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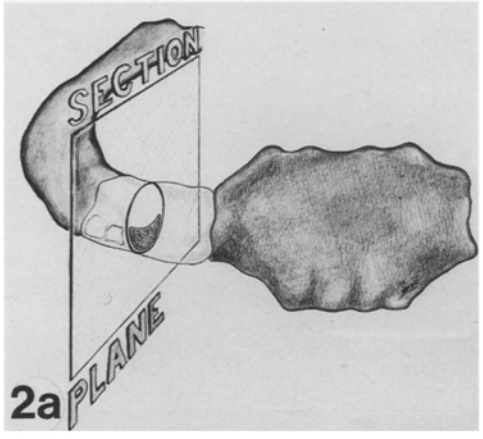
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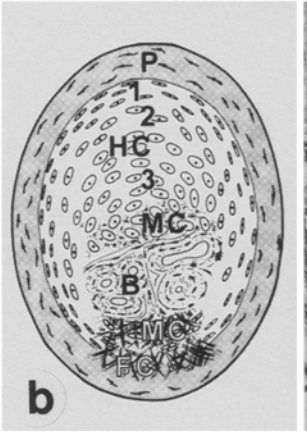
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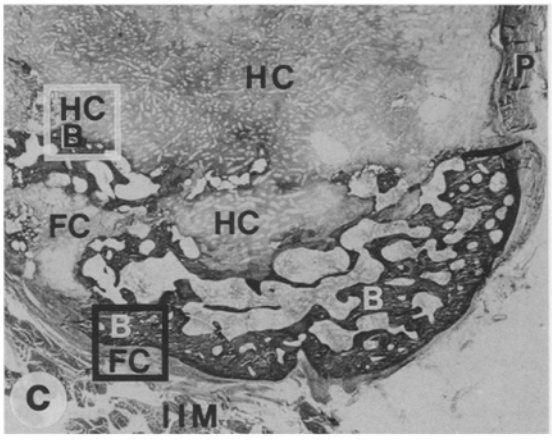
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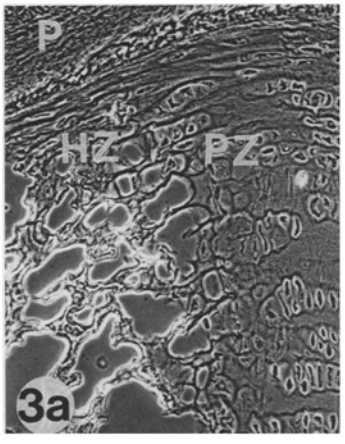
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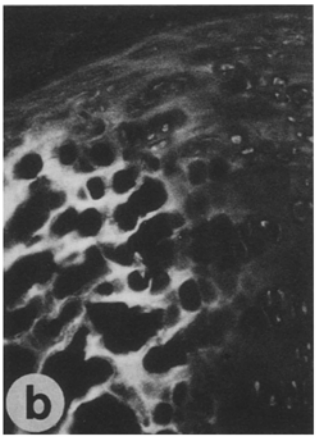
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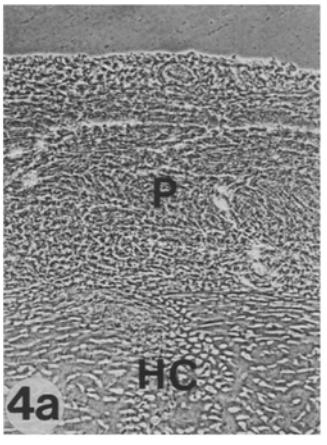
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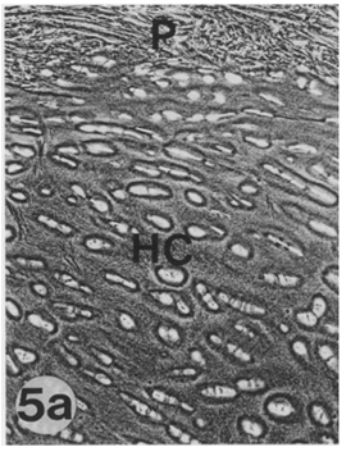
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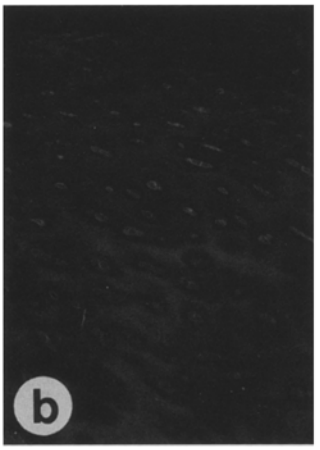
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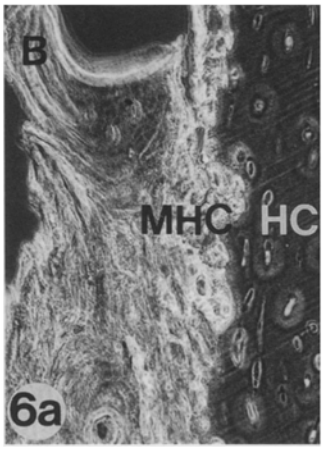
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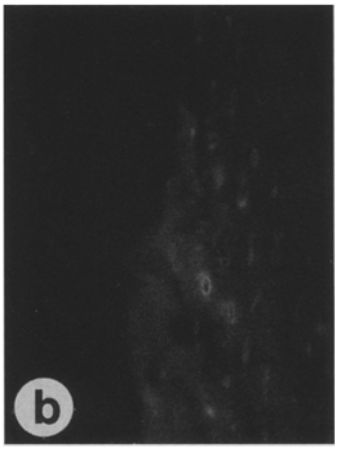
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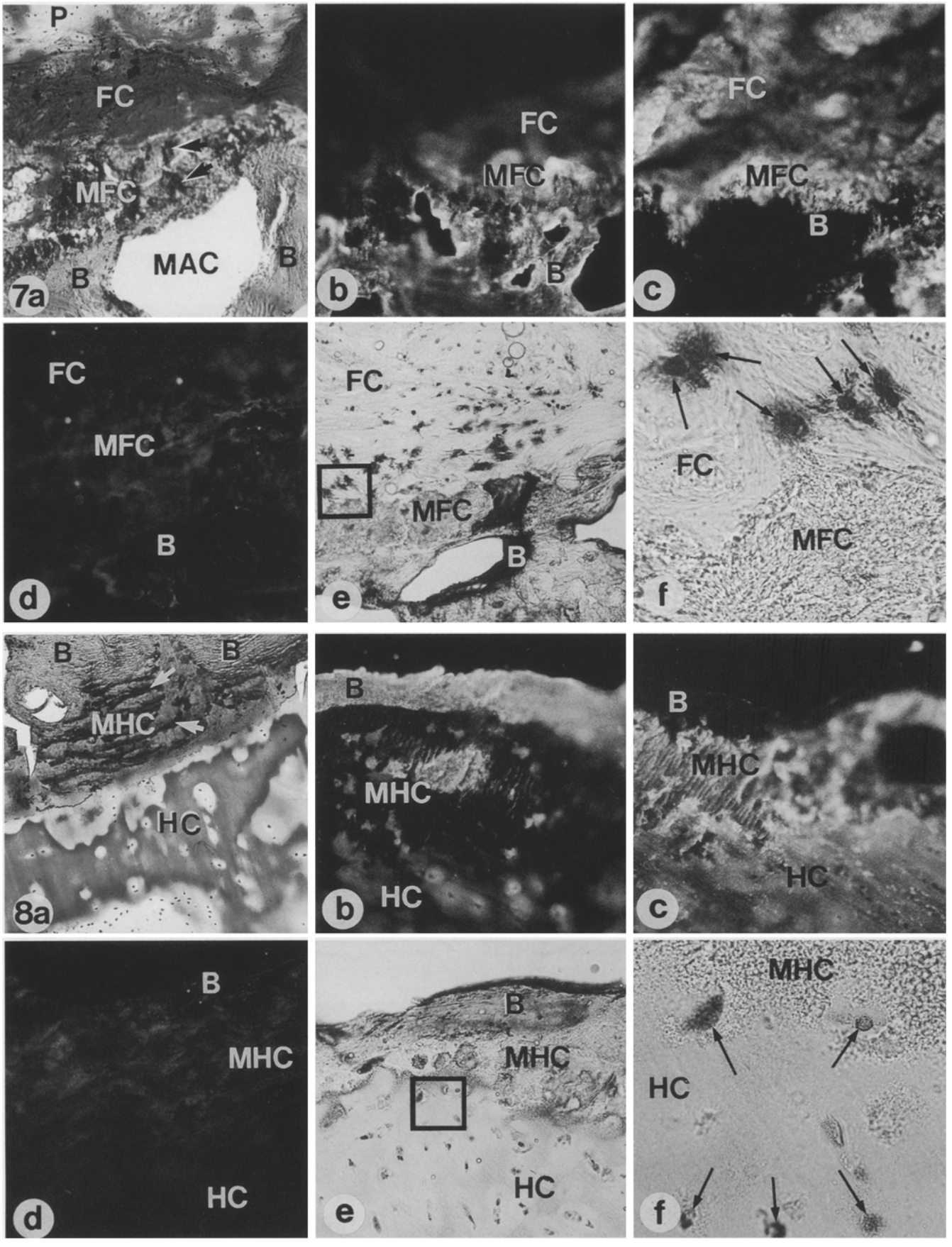
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However, chondrocytes within hyaline cartilage close to or in mineralized areas show a small, flattened morphology, indicating that these cells are not hypertrophic (Fig. 2c).

Figure 3 shows a section of the epiphyseal disc of the first rib from a 5-month-old boy stained with antibodies against type X collagen. While the cells in the hypertrophic and calcifying zone showed a strong immunostaining for type X collagen, the smaller, more flattened cells in the proliferative zone were negative (Fig. 3b). In contrast, first rib cartilage from a 1-year-old male child contained only small, flattened cells, while no large, hypertrophic cells and mineralization were detectable (Fig. 4a). Immunostaining for type X collagen was negative in this cartilage (Fig. 4b). In addition, a peripheral section of first rib cartilage from a 16-year-old female adolescent showed no ossification and was also negative for type X collagen (Fig. 5a, b). No alkaline phosphatase activity was found in either cartilage (data not shown).

After onset of ossification in first rib cartilage, immunostaining for type X collagen still remained negative in all investigated samples. Figure 6a shows one representative, more centrally (see Fig. 2b, c) located section of first rib cartilage from a 58-year-old female adult. The extracellular matrix of centrally localized chondrocytes close to mineralized hyaline cartilage did not react with the type X collagen antibody (Fig. 6b), nor did mineralized hyaline cartilage show immunostaining for type X collagen (Fig. 6b).

Two kinds of mineralizing cartilages could be distinguished in first rib cartilage: a small zone of peripheral fibrocartilage and a large zone of more centrally localized hyaline cartilage (see Fig. 2b, c). These two regions were investigated in detail in first rib cartilage from a 26-year-old male adult (Figs. 7, 8). Serial sections of the peripheral (Fig. 7) and central (Fig. 8) mineralization front were stained with Movat's Pentachrome, antibodies against type I, II and X collagens, or for alkaline phosphatase activity.

At the peripheral mineralization front, fibrocartilage developed near the innermost layer of perichondrium. As demonstrated by Movat's Pentachrome staining, areas of mineralized fibrocartilage preceded bone formation (Fig. 7a). Bone adjacent to areas of fibrocartilage stained intensively with antibodies against type I collagen, while fibrocartilage showed only very weak staining for type I collagen (Fig. 7b). The matrix of peripheral fibrocartilage showed immunoreactivity for type II collagen, which was absent in the osteoid matrix (Fig. 7c). Immunostaining for type X collagen was detected neither in the matrix of unmineralized and mineralized fibrocartilage nor in the bone matrix (Fig. 7d). However, cells in mineralized fibrocartilage as well as cells in unmineralized fibrocartilage close to the mineralization front showed strong enzyme staining for alkaline phosphatase (Fig. 7e, f). In addition, the osteoid rims adjacent to fibrocartilage revealed a strong staining for this enzyme (Fig. 7e).

At the central mineralization front, the hyaline cartilage matrix close to the newly formed bone was mineralized, as demonstrated by Movat's Pentachrome staining (Fig. 8a). The osteoid matrix showed strong immunostaining for type I collagen (Fig. 8b). In addition, the pericellular matrix of some chondrocytes in unmineralized and mineralized hyaline cartilage revealed immunoreactivity for type I collagen (Fig. 8b). Type II collagen was evenly distributed throughout the matrix of unmineralized and mineralized hyaline cartilage (Fig. 8c). No immunostaining for type X collagen was detected in unmineralized and mineralized hyaline cartilage or bone (Fig. 8d). However, some chondrocytes close to or in mineralized cartilage areas showed staining for alkaline phosphatase activity, which was weaker than the staining in fibrocartilage at the peripheral side (Fig. 8e, f).

Fig. 7 Movat's Pentachrome staining (**a**), immunolocalization of type I (**b**), II (**c**), and X collagens (**d**) or staining for alkaline phosphatase enzyme activity (**e, f**) in sections derived from similar areas of the peripheral mineralization front of first rib cartilage from a 26-year-old male adult. **a** Spotted areas (*arrows*) in mineralized fibrocartilage indicate mineralization, while homogenously dark-stained areas indicate unmineralized fibrocartilage. Bone showed a gray staining. Note that mineralized fibrocartilage areas preceded bone formation. **b** The bony matrix reacted with antibodies against type I collagen. Only a faint immunostaining for type I collagen was observed in fibrocartilage. **c** Non-mineralized and mineralized areas of fibrocartilage showed strong immunostaining for type II collagen. **d** No immunostaining for type X collagen was observed in fibrocartilage close to or in mineralized areas. **e** Staining for alkaline phosphatase enzyme activity was detected in and around cells in fibrocartilage and in osteoid matrix. **f** Sector from **e** (*inset*) at higher magnification; note the strong staining for alkaline phosphatase activity in and around chondrocyte-like cells (*arrows*) in fibrocartilage. (*P* Perichondrium, *FC* fibrocartilage, *MFC* mineralized fibrocartilage, *B* bone, *MAC* marrow cavity) **a–e** ×70; **f** ×280

Fig. 8 Movat's Pentachrome staining (**a**), immunolocalization of type I (**b**), II (**c**), and X collagens (**d**) or staining for alkaline phosphatase enzyme activity (**e, f**) in sections derived from similar areas of the central mineralization front of first rib cartilage from a 26-year-old male adult. **a** Bone areas were stained gray. Striped areas (*arrows*) in mineralized hyaline cartilage indicate mineralization, while unmineralized hyaline cartilage areas were stained light gray to white. Note that mineralized cartilage areas preceded bone formation. **b** The osteoid matrix reacted strongly with the antibodies against type I collagen. In addition, the territorial matrix of some chondrocytes in hyaline cartilage showed immunostaining for type I collagen. **c** Type II collagen was evenly distributed in the matrix of hyaline and mineralized cartilage. **d** Matrix close to or in mineralized areas of hyaline cartilage showed no immunostaining for type X collagen. **e** Staining for alkaline phosphatase activity was observed in and around chondrocytes close to the mineralization front, in mineralized cartilage, and in the bone matrix. **f** Sector from **e** (*inset*) at higher magnification; note the positive staining for alkaline phosphatase activity in and around chondrocytes (*arrows*) close to the mineralization front. (*HC* Hyaline cartilage, *MHC* mineralized hyaline cartilage, *B* bone) **a–e** ×70; **f** ×280

Discussion

In this study, the localization of type X collagen and alkaline phosphatase activity was investigated in fibrocartilage and hyaline cartilage during development and ossification of human first rib cartilage. In contrast to the X-

ray studies of Koebke and Saternus (1985), which suggested that a pure ossification without cartilage calcification occurs in first rib cartilage, mineralized cartilage areas preceding bone formation were detected in this study. After onset of ossification, a peripheral mineralization front can be distinguished from a central one. Peripheral bone formation is preceded by mineralization of fibrocartilage (Fig. 7a), while central bone formation is preceded by mineralization of hyaline cartilage (Fig. 8a). Alkaline phosphatase activity is detected in and around cells close to or in both mineralization fronts. Type X collagen is detected neither in the extracellular matrix of fibrocartilage nor in the hyaline cartilage.

Grant and coworkers (1987) performed immunostaining of chicken fracture callus with antibodies against type X collagen. Type X collagen was only localized in areas of fracture callus containing hypertrophic chondrocytes and undergoing endochondral ossification. In contrast, the fibrocartilaginous component of the fracture callus was negative for type X collagen. These findings are consistent with the present results, showing that fibrocartilage close to the peripheral mineralization front of human first rib cartilage is negative for type X collagen.

Many studies have shown that type X collagen is restricted to the hypertrophic and calcifying zones of growth plate cartilage (Schmid and Linsenmayer 1985, 1990; Poole and Pidoux 1989; Kirsch and von der Mark 1991; Reichenberger et al. 1991; Nerlich et al. 1992), sternal cartilage (Gibson and Flint 1985; Schmid and Linsenmayer 1985), and vertebral cartilage (Iyama et al. 1991). A sequence of events was observed in calcifying chicken vertebral bodies (Iyama et al. 1991) and in serial sections of human growth plate and sternal cartilage (Kirsch and von der Mark 1991): first chondrocytes increase in size and become hypertrophic, followed by type X collagen synthesis, and finally, matrix mineralization. However, as shown in this study, chondrocytes in hyaline first rib cartilage close to the central mineralization front show a small and flattened morphology and no immunostaining for type X collagen, suggesting that these cells are not hypertrophic. Some of these cells stain for alkaline phosphatase activity and eventually mineralize their matrix. Thus, mineralization of hyaline cartilage in human first rib cartilage seems to occur independently of cellular hypertrophy and type X collagen synthesis.

Even though the role of type X collagen in cartilage mineralization is not yet clear, it has been suggested that type X collagen is required in the matrix of hypertrophic and calcifying cartilage. A previous study showed that mice carrying a mutated collagen type X transgene develop skeletal deformities, including compression of the hypertrophic zone and a decrease in newly formed bone (Jacenko et al. 1993). However, no immunostaining for type X collagen was detected in the various first rib cartilages investigated in this study. In contrast to growth plate cartilage, first rib cartilage is fully grown at the onset of mineralization. Perhaps this type of hyaline cartilage does not require cellular hypertrophy and type X collagen for mineralization. However, it seems that these chondrocytes need alkaline phosphatase activity to calcify their matrix.

The onset of alkaline phosphatase activity, therefore, does not seem to require the hypertrophic phenotype, but is regulated by other mechanisms. It is possible that the extracellular environment of these chondrocytes, which are adjacent to newly formed osteoid, affects their matrix synthesis, and either osteoblast-derived matrix molecules or cellular communication could lead to an increased synthesis of alkaline phosphatase. A previous study demonstrated only chondrocytes in subperiosteal and perivascular regions of growth plate cartilage undergo modulation to osteoblast-like cells (Galotto et al. 1994). Thus, the extracellular environment of chondrocytes seems to have a crucial influence on their fate (Galotto et al. 1994).

Dearden and coworkers (1974) performed an electron microscopical investigation of human rib cartilages, indicating that chondrocytes become more and more degenerative after onset of mineralization. They are surrounded by halos which contain amorphous material and osmiophilic electron-dense bodies. The fact that chondrocytes in the central part of first rib cartilage show immunostaining for type I collagen (Fig. 8b) strengthens the concept that chondrocytes close to the mineralization front undergo a dedifferentiation process. Whether these changes are the first steps toward cell death in first rib cartilage remains to be established.

In conclusion, the results presented here provide evidence that bone formation in first rib cartilage is accompanied by the mineralization of fibrocartilage and hyaline cartilage. Mineralization of hyaline cartilage seems to occur in the absence of type X collagen but in the presence of alkaline phosphatase. Thus, ossification of human first rib cartilage seems to follow a different pattern from that described for endochondral ossification.

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