

Cellular Heterogeneity in Cultured Human Chondrocytes Identified by Antibodies Specific for $\alpha 2(XI)$ Collagen Chains

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Abstract. Collagen type XI is a component of hyaline cartilage consisting of $\alpha 1(XI)$, $\alpha 2(XI)$, and $\alpha 3(XI)$ chains; with 5–10% of the total collagen content, it is a minor but significant component next to type II collagen, but its function and precise localization in cartilaginous tissues is still unclear. Owing to the homology of the $\alpha 3(XI)$ and $\alpha 1(II)$ collagen chains, attempts to prepare specific antibodies to native type XI collagen have been unsuccessful in the past.

In this study, we report on the preparation and use for immunohistochemistry of a polyclonal antibody specific for $\alpha 2(XI)$ denatured collagen chains. The antibody was prepared by immunization with the isolated $\alpha 2(XI)$ chain and reacts neither with native type XI collagen nor type I, II, V, or IX by ELISA or immunoblotting, nor with $\alpha 1(XI)$ or $\alpha 3(XI)$, but with $\alpha 2(XI)$ chains. Using this antibody, it was possible to specifically localize $\alpha 2(XI)$ in cartilage by pretreating tissue sections with 6 M urea. In double immuno-

fluorescence staining experiments, the distribution of $\alpha 2(XI)$ as indicative for type XI collagen in fetal bovine and human cartilage was compared with that of type II collagen, using a monoclonal antibody to $\alpha 1(II)$. Type XI collagen was found throughout the matrix of hyaline cartilage. However, owing to cross-reactivity of the monoclonal anti- $\alpha 1(II)$ with $\alpha 3(XI)$, both antibodies produced the same staining pattern. Cellular heterogeneity was, however, detected in monolayer cultures of human chondrocytes. Numerous cells stained intracellularly for $\alpha 2(XI)$ but not for $\alpha 1(II)/\alpha 3(XI)$ and vice versa, besides the majority of the cells that stained with both antibodies. These findings suggest that (a) chondrocytes may not always simultaneously synthesize $\alpha 1(II)$ and $\alpha 2(XI)$ collagen chains in cell culture; and (b) that a subpopulation of chondrocytes may exist in vitro that synthesize type XI collagen molecules containing $\alpha 2(XI)$ but not $\alpha 3(XI)$ subunits.

THE assembly of the highly hydrated, space-filling extracellular matrix of hyaline cartilage involves the interaction of specific proteoglycans and the collagen types II, IX, and XI that are unique for this tissue (for review, see Mayne and von der Mark, 1983; van der Rest and Mayne, 1987; Eyre and Wu, 1987), with the exception of vitreous humor (Swann and Scotsman, 1980) and certain transient embryonic epithelia (Linsenmayer et al., 1977; von der Mark et al., 1977; Thorogood et al., 1986). Type VI collagen recently described in cartilage (Ayad et al., 1984; Keene et al., 1988) also exists in skin and other connective tissues (Timpl and Engel, 1987; Keene et al., 1988), and type X only occurs in hypertrophic cartilage (Schmid and Linsenmayer, 1985; Capasso et al., 1984; Gibson and Flint, 1985). The size, degree of cross-linking, and the orientation of collagen fibres determines to a large extent the biomechanical properties of hyaline cartilage. Thus, the role of the individual collagen types in the assembly of cartilage collagen fibrils and the three-dimensional collagenous meshwork is the subject of intense investigation.

Using electron microscopical technique, Vaughan and coauthors (1987) were able to show that type IX collagen molecules, which are covalently cross-linked to type II collagen (Eyre et al., 1987), decorate the periphery of type II collagen fibrils in a D-periodic pattern. Type VI collagen forms a fine fibrous meshwork interwoven with the type II collagen fibrils (Keene et al., 1988). Type XI collagen apparently does not assemble into separate supramolecular structures in cartilage, but is integrated into type II collagen fibrils; it may control the fibril diameter (Mendler et al., 1989).

Type XI collagen is thought to be a 1:1:1 heterotrimer consisting of $\alpha 1(XI)$, $\alpha 2(XI)$, and $\alpha 3(XI)$ chains (Burgeson and Hollister, 1979; Morris and Bächinger, 1987). Parent type XI collagen molecules consisting of either $[\alpha 1(XI)_n \alpha 2(XI)_m]$ or $[\alpha 3(XI)]_3$ have never been observed. Type XI collagen has the character of a "hybrid" collagen: $\alpha 1(XI)$ and $\alpha 2(XI)$ are similar to $\alpha 1(V)$ and $\alpha 2(V)$ in terms of solubility and migration on SDS-PAGE (Burgeson and Hollister, 1979; Reese and Mayne, 1981; von der Mark et al., 1982); $\alpha 3(XI)$ is probably coded by a common type II collagen gene and differs

from $\alpha 1(\text{II})$ by posttranslational modifications (Furuto and Miller, 1983; Eyre and Wu, 1987), possibly also by amino acid substitutions that give rise to a different CNBr-peptide pattern (von der Mark et al., 1982).

Collagen types IX and XI always coextract together with type II collagen; e.g., from human articular cartilage (Burgesson and Hollister, 1979), chick sternal cartilage (Reese and Mayne, 1981; von der Mark et al., 1982), and from bovine and porcine cartilage (Eyre and Wu, 1987). In chondrocyte cultures modulated by bromodesoxyuridine or grown to senescence, the transition from type II synthesis to type I collagen is accompanied by a change from type XI to type V collagen (Mayne et al., 1984), suggesting coordinate regulation of types II and XI. In this study, however, we provide evidence by immunofluorescence double staining that in cultured chondrocytes the $\alpha 2$ subunit of type XI collagen may not always be simultaneously synthesized with $\alpha 1(\text{II})$. Using polyclonal antibodies specific for $\alpha 2(\text{XI})$ and monoclonal antibodies recognizing $\alpha 1(\text{II})$ and $\alpha 3(\text{XI})$ chains (Holmdahl et al., 1986), we show that in monolayer cultures of human chondrocytes numerous cells stain for $\alpha 2(\text{XI})$ but not for $\alpha 1(\text{II})/\alpha 3(\text{XI})$ collagen chains and vice versa, although a majority of cells stain with both antibodies. The rabbit antibody specific for denatured $\alpha 2(\text{XI})$ collagen chains was obtained by immunization with purified $\alpha 2(\text{XI})$ and can also be used to identify type XI collagen in cartilage after denaturing tissue sections with urea.

Materials and Methods

Collagen Preparation

Epiphyseal cartilage from long bones of 3–6 mo calf fetuses was dissected free of adhering connective tissue, cut in pieces, weighed, and homogenized at 4°C with an Ultraturrax (Janke and Kunkel, FRG) in 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, containing protease inhibitors (1 mM EDTA, 1 mM *N*-ethylmaleimide and 1 mM phenyl methane sulfonyl fluoride) and 50 $\mu\text{g}/\text{ml}$ β -aminopropionitrile. The extract was centrifuged for 30 min at 50,000 g, and the residual pellet was subsequently extracted with 4 M guanidinium hydrochloride 0.05 M Tris-HCl, pH 7.4, then with 0.5 M acetic acid, and finally with 5 mg pepsin/g wet weight cartilage in 0.5 M HOAc for 24 h at 4°C as described previously (von der Mark et al., 1982). The pepsin extract was neutralized, and the collagens were separated into 3 fractions by subsequent precipitation with 0.7, 1.2, and 2 M NaCl in 0.5 M acetic acid as described elsewhere (Shimokomaki et al., 1980). The 1.2 M NaCl fraction containing type XI collagen was dissolved in 0.5 M acetic acid and reprecipitated with 0.86 and 1.2 M NaCl.

Collagens type II and IX from bovine fetal cartilage were obtained in the same preparation and purified according to von der Mark et al. (1982). Collagen types I and V were extracted and purified from human placenta as described by Benz et al. (1978).

Isolation of $\alpha 2(\text{XI})$

$\alpha 1$, $\alpha 2$, and $\alpha 3(\text{XI})$ collagen chains were separated by chromatography on carboxymethyl cellulose at 42°C in 0.04 M sodium acetate, pH 4.8, containing 6 M urea as described previously (von der Mark et al., 1982). The purity of the α -chains was checked by SDS-gel electrophoresis in 7% acrylamide minigels using the buffer system of Laemmli (1970).

Antibodies

Rabbits were immunized by subcutaneous injection with 1 mg purified $\alpha 2(\text{XI})$ in 1 ml 1 M NaCl/0.05 M Tris-HCl, pH 7.5, emulsified in 1 ml complete Freund's adjuvant. Blood was collected from the ear vein after 2 mo following two booster injections, 1 mg $\alpha 2(\text{XI})$, in incomplete Freund's adjuvant 4 and 6 wk after the first immunization. The antibody titer was checked by ELISA (see below). The antiserum was absorbed with collagen types I, II, V, and IX, all coupled to Sepharose CL6B, and with fibronectin. From

the preabsorbed sera, anti $\alpha 2(\text{XI})$ was purified by affinity chromatography on $\alpha 2(\text{XI})$ chains (1 mg coupled to 5 ml Sepharose CL6B) as described previously (von der Mark et al., 1976).

Rabbit antibodies against human type II collagen were obtained by immunization with native type II collagen extracted from human fetal epiphyseal cartilage. Specific antibodies were isolated after cross absorption with types I, V, and IX collagen followed by affinity chromatography on type II collagen as described previously (von der Mark et al., 1976).

The monoclonal antibody CII D3 is a Ig G2a, kappa isotype antibody cloned after immunization of a DBA/1 mouse with native chick type II collagen (Holmdahl et al., 1986). The antibody has been shown to react with native type II collagen from chick, mouse, rat, bovine, and human cartilages, but not with type I collagen (Holmdahl et al., 1986).

ELISA

Antibody titers were determined by ELISA using collagen-coated microtiter plates (coating solutions: 50 μg collagen/ml; Engvall and Perlmann, 1971). The second antibody was peroxidase-conjugated goat anti-rabbit-Ig (Bio-Rad Laboratories, Richmond, CA) diluted 1:5,000. Color substrate was 3,3'-5,5'-tetramethylbenzidine.

Immunoblotting

Collagen type I, II, V, and IX were Western blotted onto nitrocellulose filters (Bio-Rad Laboratories) after electrophoresis in 7% polyacrylamide-SDS gels (Towbin et al., 1979). After blocking with low fat milk protein, blotted proteins were immunolabeled with rabbit anti $\alpha 2(\text{XI})$ or monoclonal anti type II collagen (CII D3) both diluted 1:100 with PBS, using peroxidase-labeled goat anti-rabbit-Ig (Bio-Rad Laboratories) as second antibodies, or rabbit anti-mouse-Ig (Bio-Rad Laboratories), and chloronaphthol as color substrate.

Cell Culture

Human chondrocytes were prepared from femoral head cartilage of an autopsied 22-wk-old fetus. The cartilage was dissected free of adhering connective tissue, and digested with trypsin (2 mg/ml; Serva, Heidelberg, FRG) in PBS for 45 min at 37°C followed by clostridium collagenase (2 mg/ml; CLS II; Worthington, Freehold, NJ) in Ham's F12 medium containing 10% FCS at 37°C. When the cartilage was digested (usually after 8–12 h), chondrocytes were filtered through 2 layers of a 20 mesh nylon net and washed by centrifugation. Cells were plated at 2×10^5 cells/60 mm tissue culture dish and grown in F12 medium containing 10% FCS, Penicillin/Streptomycin (100 U/ml), glutamine (200 μM), and sodium ascorbate (50 $\mu\text{g}/\text{ml}$) (Dessau et al., 1978).

Collagen Biosynthesis

First passage chondrocytes monolayer cultures were labeled for 8 h with 10 $\mu\text{Ci}/\text{ml}$ of [1-2,3]- ^3H proline (30 Ci/mmol; Amersham Buchler GmbH, Braunschweig, FRG) in DME without serum, containing sodium ascorbate (50 $\mu\text{g}/\text{ml}$), β -aminopropionitrile (50 $\mu\text{l}/\text{ml}$), and glutamine (180 $\mu\text{g}/\text{ml}$). The newly synthesized collagens secreted into the medium and retained in the cell layer were digested with pepsin at 4°C for 12 h and analyzed by SDS-gel electrophoresis and fluorography as described (Hermann et al., 1980).

Immunofluorescence

8- μm -cryostat sections were cut from fetal bovine and human cartilage and mounted on polylysine-coated glass slides. To reveal $\alpha 2(\text{XI})$ -staining, sections were treated with testicular hyaluronidase (2 mg/ml) for 20 min at 20°C and then with 6 M urea, 0.05 M sodium acetate, pH 4.8, for 20 min in a humidified atmosphere at 50°C. Sections were washed in PBS and stained with affinity-purified rabbit anti $\alpha 2(\text{XI})$ (10 $\mu\text{g}/\text{ml}$) followed by fluorescein-conjugated goat anti-rabbit-Ig. For double staining with monoclonal antibody CII D3, fluorescein-conjugated goat anti-mouse-Ig (1:40 dilution; Jackson Laboratory, Bar Harbor, ME) and rhodamin-conjugated goat anti-rabbit (1:40 dilution; Bio Yeda, Israel) was used. Sections were mounted in 10% mowiol 4–88, (Hoechst AG, FRG), 25% glycerol, 0.1 M Tris-HCl, pH 8.5, and viewed under a Diaplan microscope (E. Leitz, Wetzlar, FRG) equipped for epifluorescence.

Chondrocyte monolayer cultures were fixed with 70% ethanol for 10 min and stained with antibodies as described previously (von der Mark and von der Mark, 1977).

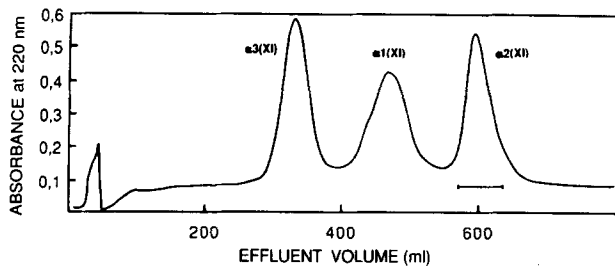


Figure 1. Separation of the α chains of bovine type XI collagen by carboxymethylcellulose chromatography. Pepsin-extracted type XI collagen from fetal bovine epiphyseal cartilage was purified by fractional salt precipitation (Shimokomaki et al., 1980) (see Fig. 2 a), denatured and applied to a 2.5×15 -cm column of carboxymethylcellulose, equilibrated in 0.04 M sodium acetate, 6 M urea, pH 4.8. Elution was achieved with a linear salt gradient (0–0.12 M NaCl) at 42°C as described previously (von der Mark et al., 1982).

Results

Preparation of Antibodies Specific for Type XI Collagen

Owing to the high degree of homology of $\alpha 3(XI)$ with $\alpha 1(II)$, previous attempts in our laboratory have failed to obtain antibodies against native type XI collagen without cross reaction with type II collagen. For this reason, $\alpha 1(XI)$ and $\alpha 2(XI)$ chains from fetal bovine cartilage were isolated and purified by carboxymethyl cellulose chromatography as described previously for chicken cartilage (von der Mark et al., 1982) (Fig. 1). The homogeneity of the α chains was checked by SDS-PAGE (Fig. 2). Rabbits were immunized with purified $\alpha 2(XI)$ chains; the antiserum was absorbed with collagen types I, II, IX, and V collagen and purified by affinity chromatography on $\alpha 2(XI)$ coupled to Sepharose. The specificity of the antibody for $\alpha 2(XI)$ and the absence of cross-reactivity with native collagen types I, II, and IX was determined by ELISA (Fig. 3). Interestingly, the purified $\alpha 2(XI)$ antibodies failed to react with native type XI collagen (Fig. 3). They were also specific for $\alpha 2(XI)$ in immunoblotting and stained neither $\alpha 1(XI)$ nor $\alpha 3(XI)$, nor $\alpha 1(II)$, nor human $\alpha 2(V)$ chains (Fig. 4 B). The monoclonal antibody CII D3 prepared against chick type II collagen reacted with bovine (not shown) and human type II collagen in the immunoblot, as well as with bovine $\alpha 3(XI)$, but not with $\alpha 1(XI)$ or $\alpha 2(XI)$ (Fig. 4 A).

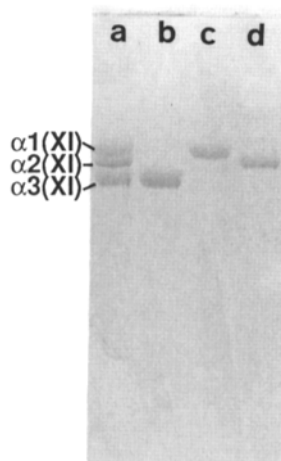


Figure 2. SDS-PAGE showing pepsin-extracted bovine type XI collagen, purified by precipitation with 1.2 M NaCl in 0.5 M acetic acid (a), and α chains, separated by carboxymethylcellulose chromatography (Fig. 1). b, $\alpha 3(XI)$; c, $\alpha 1(XI)$; d, $\alpha 2(XI)$. 7% polyacrylamide gel, Coomassie blue stained.

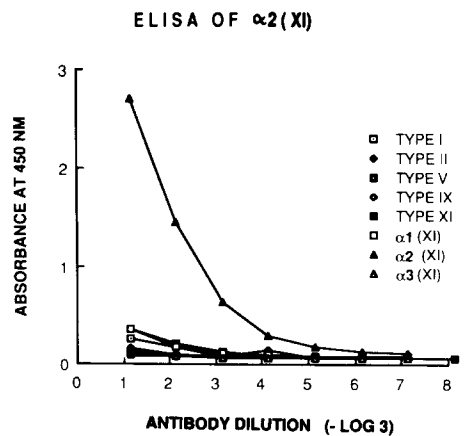


Figure 3. Titer of affinity-purified rabbit anti $\alpha 2(XI)$ (0.1 mg/ml) against various native collagen types and denatured $\alpha 1$, $\alpha 2$, and $\alpha 3(XI)$ chains, determined by ELISA. 96 microtiter dishes were coated with 50 μ g/ml of native collagen or α chains.

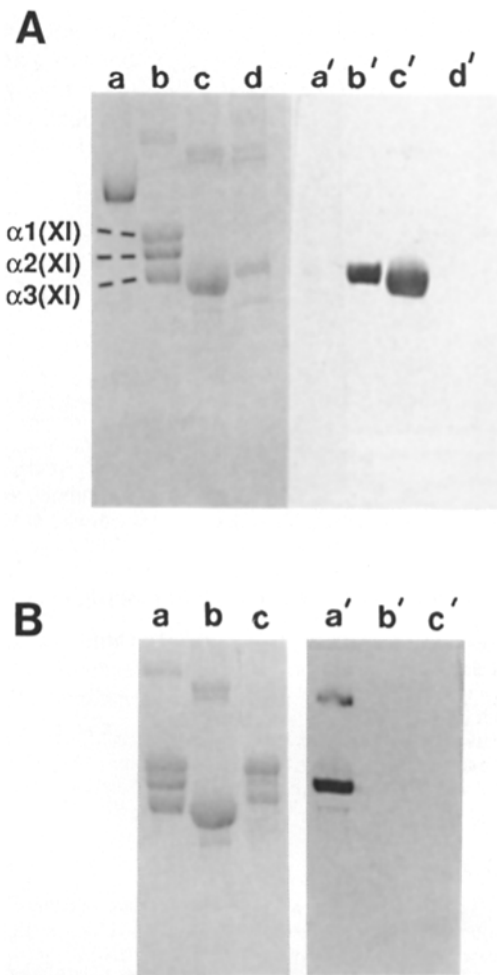


Figure 4. Immunoblotting of collagen types I, II, V, IX, and XI with monoclonal anti-type II collagen (A) and purified rabbit anti- $\alpha 2(XI)$ (B); A, a and a': bovine type IX collagen; b and b': bovine type XI collagen; c and c': human type II collagen; and d and d': human type I collagen. a–d, Coomassie blue-stained SDS-gel after blotting; a'–d', immunostaining with monoclonal antibody CII D3. The monoclonal antibody binds only to $\alpha 1(II)$ (c) and $\alpha 3(XI)$ (b). B, a and a': bovine: type XI collagen; b and b': human type II collagen; c and c': human type V collagen. a, b, and c, Coomassie blue staining; a', b', c': immunostaining with anti- $\alpha 2(XI)$.

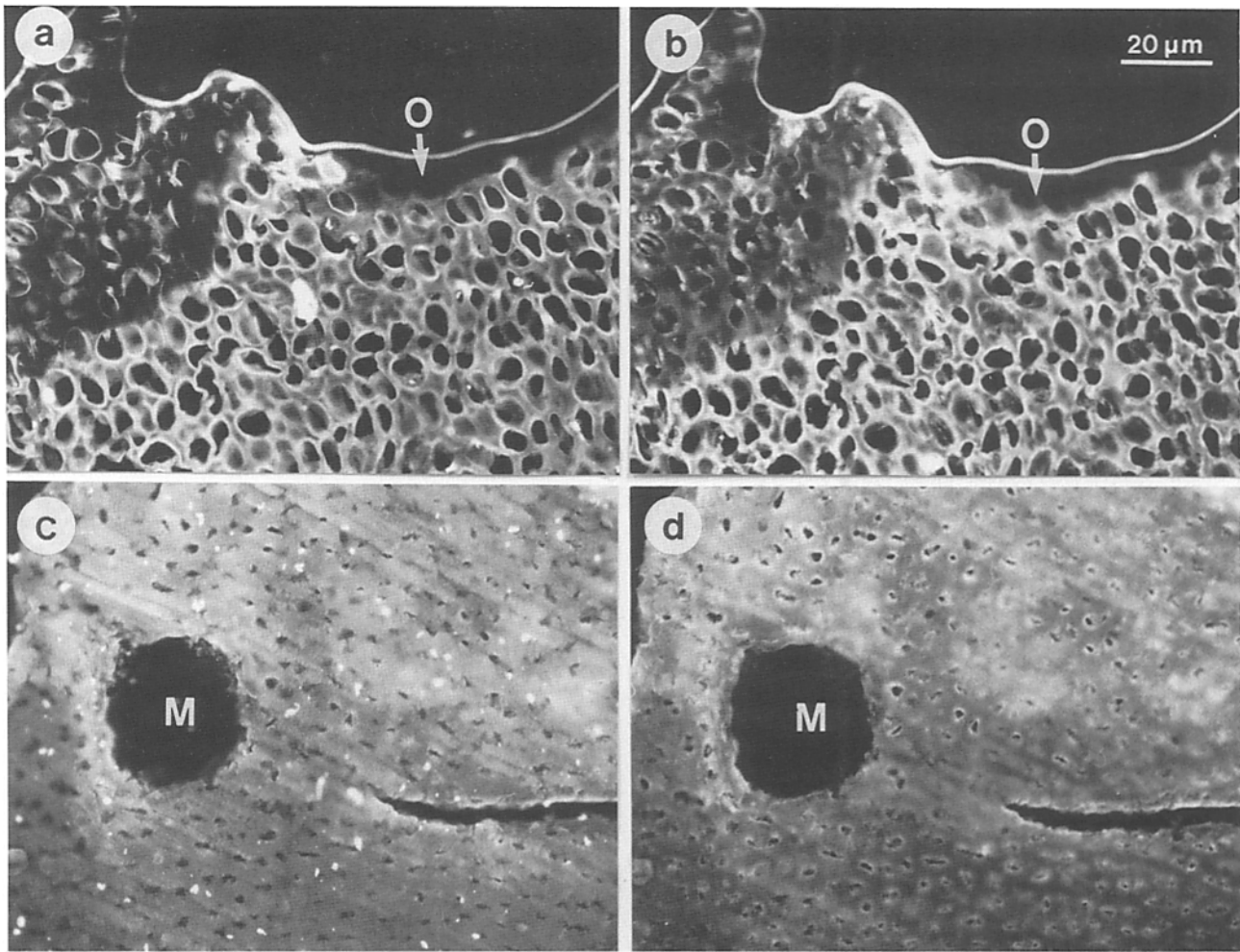


Figure 5. Immunofluorescence localization of types II (*a* and *c*) and XI (*b* and *d*) collagen in fetal bovine epiphyseal cartilage. Frozen sections were denatured for 20 min with 6 M urea at 50°C, treated with testicular hyaluronidase and double stained with rabbit anti $\alpha 2(\text{XI})$ (*b* and *d*), followed by rhodamine-conjugated goat anti-rabbit, and with monoclonal anti type II collagen CII D3 (*a* and *c*), followed by FITC-labeled goat anti-mouse-IG. *M*, bone marrow sprout; *O*, osteoid.

Localization of Type XI Collagen in Fetal Cartilage

In cryostat sections of bovine fetal cartilage, the antibody to $\alpha 2(\text{XI})$ did not reveal any immunofluorescence reaction even after hyaluronidase treatment. However, after denaturing the tissue collagen with 6 M urea, pH 4.8, for 20 min at 50°C,

the entire cartilaginous area was labeled (Fig. 5, *b* and *d*). Perichondral tissue and endochondral bone were unlabeled. Double immunofluorescence staining with the monoclonal antibody to type II collagen revealed extensive colocalization of both collagen types in hypertrophic (Fig. 5, *a* and *b*) as well as in resting (Fig. 5, *c* and *d*) fetal bovine cartilage. Similar results were obtained after staining of fetal human cartilage (not shown).

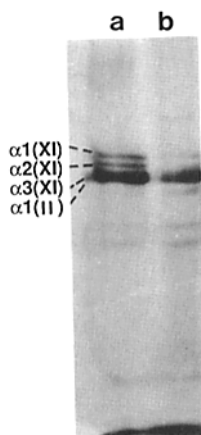


Figure 6. Biosynthesis of type XI collagen in cultures of human fetal chondrocytes. 3-wk monolayer cultures of chondrocytes derived from femoral head cartilage were labeled with [^3H]proline for 8 h, and the collagens from cell layer (*a*) and culture medium (*b*) were pepsin-digested and analyzed by SDS-gel electrophoresis on 7% polyacrylamide gels, followed by fluorography. The majority of type XI collagen is retained in the cell layer (*a*).

Identification of Intracellular Type XI Collagen in Chondrocyte Cultures

Chondrocytes from human fetal femoral heads were grown in monolayer cultures for 1 wk and labeled metabolically with [^3H]proline to assess type XI collagen synthesis. Both secreted and cell bound collagens were digested with pepsin at 4°C, and analyzed by SDS-PAGE (Fig. 6). Besides a majority of $\alpha 1(\text{II})$ and some $\alpha 2(\text{I})$ chains, $\alpha 1(\text{XI})$ and $\alpha 2(\text{XI})$ as well as fragments of type IX collagen were identified. The band of $\alpha 3(\text{XI})$ is probably covered by $\alpha 1(\text{II})$.

When subcultures were fixed and stained with antibodies to human type II collagen, only ~50% of the cells showed intracellular reactions (Fig. 7 *b*), indicating the start of cellu-

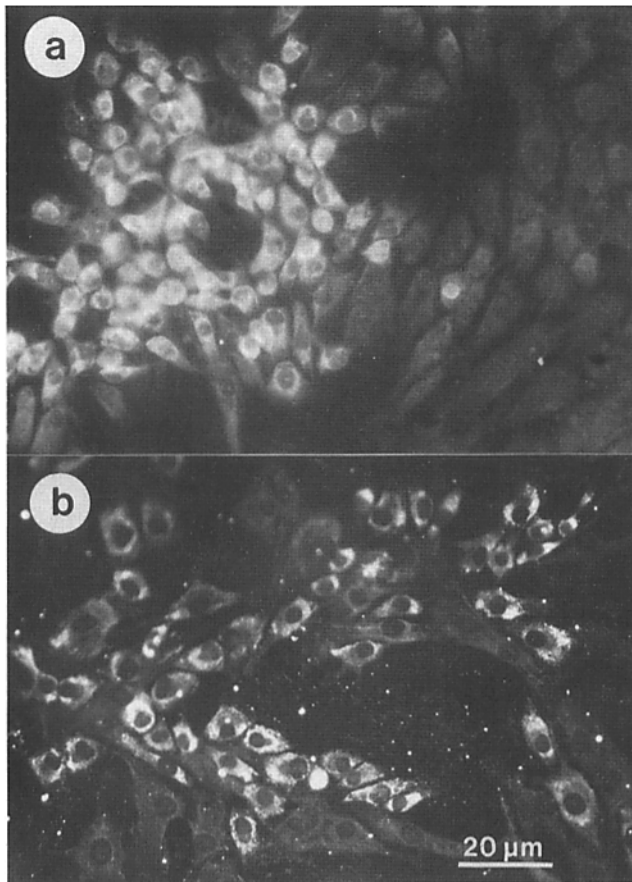


Figure 7. Immunofluorescence staining of human fetal chondrocytes with rabbit anti- $\alpha 2(\text{XI})$ (a) and rabbit anti-human type II collagen (b) followed by FITC-conjugated goat anti-rabbit Ig. First passage monolayer cultures of epiphyseal chondrocytes were fixed 4 d after plating with 70% ethanol to reveal intracellular immunoreaction.

lar dedifferentiation. Staining with anti $\alpha 2(\text{XI})$ revealed intracellular reaction in ~ 20 – 30% of the cells (Fig. 7 a). Only cells with polygonal, chondrocyte-like morphology were stained; stellate, fibroblast-like cells did not stain with $\alpha 2(\text{XI})$ antibodies.

Double immunofluorescence staining with rabbit anti $\alpha 2(\text{XI})$ and the monoclonal antibody CII D3 specific for $\alpha 2(\text{II})$ and $\alpha 3(\text{XI})$, however, demonstrated that such chondrocyte cultures contained cells that had synthesized $\alpha 2(\text{XI})$ chains but not $\alpha 1(\text{II})$ or $\alpha 3(\text{XI})$ and vice versa (Fig. 8), although the majority of positive cells reacted with both antibodies. Frequently, $\alpha 2(\text{XI})$ and $\alpha(\text{XI})$ and vice versa, (Fig. 8), although the majority of positive cells reacted with both antibodies. Frequently, $\alpha 2(\text{XI})$ and $\alpha 1(\text{II})$, or $\alpha 3(\text{XI})$, were located in different intracellular domains. Fig. 8 a, for example, shows a perinuclear cluster of $\alpha 1(\text{II})$ and/or $\alpha 3(\text{XI})$ (probably the Golgi apparatus), while, in the same cell, $\alpha 2(\text{XI})$ staining is more pronounced in the peripheral cytoplasm, but absent from the $\alpha 1(\text{II})$ -positive, perinuclear domain.

Discussion

In this study, we report on the preparation of an antibody specific for $\alpha 2(\text{XI})$ collagen that can be used for the im-

munolocalization of type XI collagen in hyaline bovine and human fetal cartilages. While previous attempts to obtain antibodies specific for type XI collagen by immunization with native type XI collagen molecules have failed as a result of cross-reactivity with types II and V collagen, immunization with purified $\alpha 2(\text{XI})$ chains led to the production of antibodies specific for this polypeptide.

The availability of an antibody recognizing only denatured $\alpha 2(\text{XI})$ chains but not native type XI collagen might be useful for the detection of collagen degradation in cartilage. Recently, Dodge and Poole (1989) have reported on the immunohistochemical detection and quantitative analysis of type II collagen chains and fragments in rheumatoid cartilage or bovine cartilage explants treated with Interleukin 1.

The anti $\alpha 2(\text{XI})$ antibody can, however, also be used for the identification of intact type XI collagen in tissue sections. Similar to immunofluorescence localizations of type V collagen using monoclonal antibodies that required acid or pepsin pretreatment of tissue sections (von der Mark and Öcalan, 1982; Linsenmayer et al., 1983), reaction with type XI collagen in tissue sections could be achieved after treatment of the section with urea. In double staining experiments with a monoclonal antibody against type II collagen, we found the same distribution for type XI collagen and type II collagen in the extracellular matrix of fetal bovine and human cartilages. In accordance with this finding, Mendler et al. (1989) have shown by immunogold-labeling using anti $\alpha 2(\text{XI})$ that type XI collagen is incorporated into type II collagen fibrils; also in their study the antigenic epitopes of type XI collagen were exposed only after partial disruption of the fibrils.

Similarly, by immunofluorescent investigations (von der Mark and Öcalan, 1982; Linsenmayer et al., 1983) and fibril reconstitution experiments (Adachi and Hayashi, 1985), it has become evident that type V collagen is integrated into type I collagen fibrils (Birk et al., 1988) and may be involved in the control of fibril growth. Thus, type XI collagen is frequently considered as the type V collagen-analogue of hyaline cartilage (Burgeson and Hollister, 1979; Reese and Mayne, 1981; Eyre and Wu, 1987).

Several lines of evidence indicate coordinate synthesis of type II and type XI collagen in hyaline cartilage: (a) $\alpha 1(\text{II})$ and $\alpha 3(\text{XI})$ have a common genetic origin; (b) type II and type XI collagen are consistently found together in cartilage; (c) a concomitant change from type II and XI collagen synthesis to type I and V collagen synthesis follows the modulation of chondrocytes by BUdR or growth to senescence (Mayne et al., 1984). Here we present data, however, which indicate that, at the level of an individual chondrocyte, the subunits of type II and XI collagen are not necessarily simultaneously synthesized. Using antibodies specific for $\alpha 2(\text{XI})$ and $\alpha 1(\text{II})$, we have identified cells staining intracellularly for $\alpha 2(\text{XI})$ but not for $\alpha 1(\text{II})$ collagen, and vice versa. The fact that in the same culture $\alpha 2(\text{XI})$ -positive and $\alpha 1(\text{II})/\alpha 3(\text{XI})$ -negative, as well as $\alpha 1(\text{II})/\alpha 3(\text{XI})$ -positive and $\alpha 2(\text{XI})$ -negative cells were observed excluded the possibility of antigen masking, cross-reactivity of antibodies, or lack of species cross-reactivity. Antigen masking in individual cells is also unlikely in view of the observation that even chondrocytes that have developed a pericellular matrix in cell culture still take up antibody after alcohol fixation and reveal intracellular fluorescence reactions (Dessau et al., 1981).

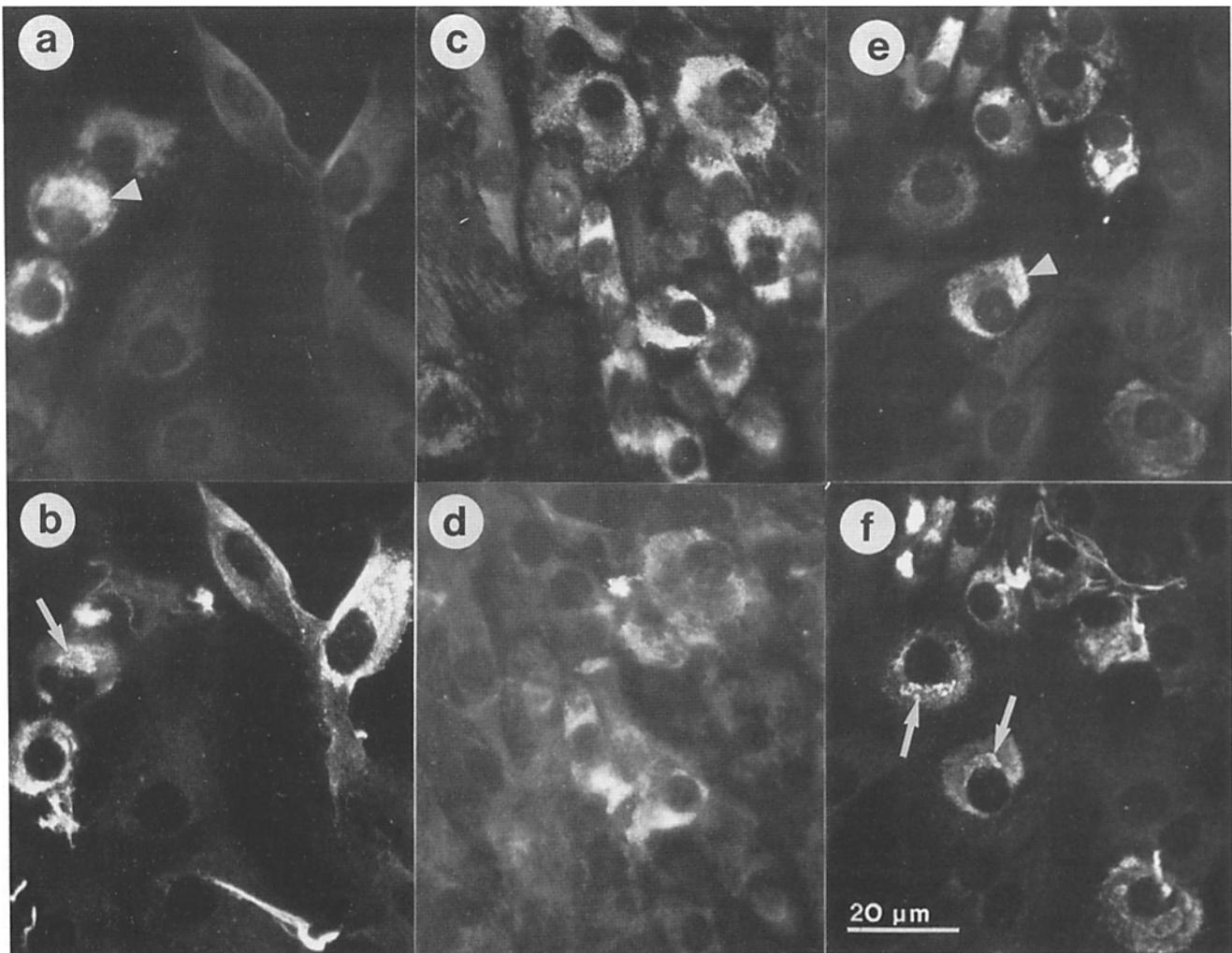


Figure 8. Immunofluorescence double staining of human fetal chondrocytes (as in Fig. 7) with rabbit anti- $\alpha 2(\text{XI})$ (a, c, and e), followed with rhodamine-conjugated goat anti-rabbit-Ig, and monoclonal CII D3 (b, d, and f), followed by FITC-conjugated goat anti-mouse-Ig. Arrows point to perinuclear locations of type II collagen synthesis; arrow heads mark cytoplasmic locations of $\alpha 2(\text{XI})$ in the same cell.

These observations suggest that either different populations of chondrocytes emerge in monolayer culture synthesizing either $\alpha 1(\text{II})$ or $\alpha 2(\text{XI})$ (or, as many cells, both simultaneously), or that the synthesis of the subunits of type II and type XI does not occur synchronously in all cells. The latter hypothesis is supported by the observation that in some cells staining with both antibodies, the reaction for $\alpha 1(\text{II})/\alpha 3(\text{XI})$ seems to be more intense in the Golgi region, while the $\alpha 2(\text{XI})$ antibody stained preferentially the peripheral cytoplasmic domains, probably the region of the RER. However, this differential staining pattern could also be a result of the inability of the anti $\alpha 2(\text{XI})$ antibody to react with the native type XI collagen molecules in the Golgi region, and a higher affinity of the monoclonal antibody to native, densely packed type II collagen molecules in the Golgi region as compared to nascent $\alpha 1(\text{II})$ and $\alpha 3(\text{XI})$ chains in the RER. Studies at the electronmicroscopical level would be required to answer this question.

Since fibril assembly occurs extracellularly, this finding is not in conflict with the idea that after secretion of complete

type II and XI collagen molecules seem habitually to be incorporated into the same fibrils (Mendler et al., 1989).

The observation that some cells stained for $\alpha 2(\text{XI})$ but not with the monoclonal antibody specific for $\alpha 1(\text{II})/\alpha 3(\text{XI})$, suggests that even the synthesis of the subunits of type XI collagen may not occur simultaneously in some cells. The question remains open, whether in such cells the $\alpha 2(\text{XI})$ chains assemble into triplehelical molecules together with $\alpha 1(\text{XI})$ but not $\alpha 3(\text{XI})$, or whether the $\alpha 2(\text{XI})$ chains are degraded intracellularly. The possibility that molecules are synthesized and secreted containing only $\alpha 1(\text{XI})$ and $\alpha 2(\text{XI})$ but not $\alpha 3(\text{XI})$ subunits is not unlikely in view of the fact that also type V collagen molecules have been identified containing only $\alpha 2(\text{V})$ but not $\alpha 1$ or $\alpha 3(\text{V})$ subunits (Haralson et al., 1980).

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