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Ca^{2+} binding properties of type X collagen

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Type X collagen is a developmentally regulated collagen that is only synthesized by chondrocytes of the hypertrophic and calcifying zone in fetal cartilage. There is evidence in the literature that type X collagen may be involved in cartilage calcification. Here we show that type X collagen synthesis precedes calcium deposition in nodules of fetal human chondrocytes forming in cell culture and present evidence that type X collagen binds calcium in a specific and dose dependent manner. In an assay using bovine type X collagen coupled to beads and $^{45}Ca^{2+}$ we determined a total of about 15 binding sites per α1(X) chain with a dissociation constant of 32 μM.

Type X collagen; Calcium binding; Cartilage calcification; Chondrocyte hypertrophy

1. INTRODUCTION

During the process of endochondral bone formation epiphyseal cartilage undergoes a series of biochemical and cellular changes involving chondrocyte proliferation, hypertrophy and matrix calcification which prepare the cartilage for replacement by bone. Chondrocytes mature during this process in discrete differentiation steps as manifested by a change in the collagen type expression pattern. In the resting and proliferative zone chondrocytes synthesize type II, IX and XI collagen, but turn on type X collagen synthesis in the hypertrophic zone [1–6]. Type X collagen, a short, network forming collagen [7,8] was originally found in cultures of fetal chondrocytes in monolayer [9], in organ cultures [10], on agar [11] and in collagen gels [12].

There is evidence from several studies that type X collagen is involved in cartilage calcification: (1) immunolocalization studies on samples of calcifying chick cartilage revealed type X collagen at sites of calcification [4,13]; (2) comparative studies of normal and rachitic chick epiphyseal cartilage showed an altered type X collagen synthesis in rachitic cartilage [14,15]; (3) in cultures of chick chondrocytes Ca^{2+} accumulation was determined during the onset of type X collagen synthesis [16–18]; (4) type X collagen has been shown to bind to matrix vesicles [16,19]. These cell-derived membrane-inverted particles have been reported to initate calcification [20,21]. However, the role of type X collagen in calcification remains obscure. Here we present evidence for direct binding of Ca^{2+} to bovine and human type X collagen and on the occurrence of calcium deposits within nodules of type X collagen producing human chondrocytes cultured on agarose.

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2. MATERIALS AND METHODS

2.1. *Chondrocyte culture*

Culture medium was Ham's modified F12 (Seromed), supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland); 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Agarose was obtained from Bio-Rad, ascorbic acid from Sigma Chemical Co. (St. Louis, MO, USA). Cells were cultured at 37°C in 5% CO_2, 95% air.

2.2. *Isolation of chondrocytes and cell culture conditions*

Femoral head cartilage (35-week-old autopsied human fetus, 24 h postmortem) was dissected free from adhering connective tissue, cleaned, washed in phosphate-buffered saline pH 7.2 and digested for 30 min at 37°C with 2 mg/ml trypsin (Gibco) followed by digestion with collagenase (2 mg/ml Worthington) for 8 h at 37°C. The released chondrocytes were centrifuged and resuspended in culture medium (Ham's F12 medium without ascorbic acid). 2 ml chondrocyte suspension (3×10^6 cells/ml) was plated in 10-cm dishes previously coated with a thin layer of 1.5% agarose. Cells were fed by substituting half of the medium every 2–3 days.

For immunofluorescence staining cells were transferred on plastic culture dishes (2×10^6 cells/60-mm dish) and cultured for 24 h in Ham's F12 medium supplemented with 50 μg/ml ascorbic acid and 4 U/ml hyaluronidase (Boehringer, Mannheim, Germany). Then cells were washed with phosphate-buffered saline, fixed with 70% ethanol and air-dried. Immunofluorescence staining was performed by incubation with rabbit anti collagen type X IgG for 3 h. Then after washing the cells were incubated with Texas red conjugated donkey anti rabbit IgG (Amersham) for 1 h. For demonstrating the localization of calcium deposits, cells were stained with 0.5% Alizarine red S solution pH 4.0 for 5 min at room temperature. Control cells were decalcified with a solution of 300 mM EDTA/ 50 mM Tris pH 7.5 for 1 h at room temperature prior staining. Stained cells were washed three times with water and ethanol. Cells were viewed under a Leitz Diaplan microscope equipped for epifluorescence.

2.3. *Isolation of type X collagen*

Type X collagen was isolated from bovine and human fetal growth plate cartilage and purified as described previously [5,22].

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2.4. *Purification of chondrocalcin*

Chondrocalcin was isolated from fetal bovine growth plate cartilage and purified by anion-exchange chromatography on a Fast Q column and gel-filtration chromatography on a Superose 12 prep column (Pharmacia, Sweden) as described previously. Fraction C of the gel-filtration chromatography contained pure chondrocalcin [22].

Pepsin-extracted type II, IX and XI collagens from fetal human or bovine epiphyseal cartilage were purified by fractional salt precipitation as described elsewhere [23].

2.5. *Ca^{2+} binding assays*

2.5.1. Slot blot assay.

Different concentrations of the purified proteins were applied onto nitrocellulose filters (Schleicher and Schuell) using a slot blot machinery. The labeling of proteins with $^{45}Ca^{2+}$ was carried out as described by Maryane et al. [24]. The nitrocellulose sheets were incubated in a solution containing 5 mM $MgCl_2$, 60 mM KCl and 10 mM imidazole HCl pH 6.9 for 2 h with three changes of buffer. The nitrocellulose filters were then transferred to a new buffer containing 2 mCi of $^{45}CaCl_2$/liter (1.7 mCi/mol) and incubated for 15 min at room temperature. The filters were washed with 50% ethanol, tried and subjected to autoradiography. The proteins providing $^{45}Ca^{2+}$ were visualized by exposure to a Kodak XAR-5 film at −80°C. Band intensity was measured by scanning the nitrocellulose filters.

2.5.2. Solid phase $^{45}Ca^{2+}$ binding assay.

To quantitate the Ca^{2+} binding known concentrations of purified bovine type X collagen (60 μg) and chondrocalcin (60 μg) were coupled to Affigel 10 beads (1 ml suspension, Bio-Rad). Proteins were coupled in a solution containing 0.1 M MOPS/80 mM $CaCl_2$ pH 7.5 at 4°C overnight to the activated beads. After centrifugation the protein concentration in the supernates was determined to calculate the coupling efficiency. Aliquots of 100 μl beads containing 4.7 μg of type X collagen were incubated with different concentrations of $^{45}CaCl_2$ in a solution of 5 mM $MgCl_2$, 60 mM KCl and 10 mM imidazole HCl pH 6.8 for 1 h at room temperature. The beads were washed twice with the same buffer, and after centrifugation aliquots of the supernates were taken for scintillation counting. To determine the amount of bound $^{45}Ca^{2+}$ the beads were incubated with 300 mM EDTA/50 mM Tris pH 7.5 for 30 min and centrifuged; aliquots of the supernates were taken for scintillation counting. The amount of $^{45}Ca^{2+}$ bound is expressed as mol of Ca^{2+} bound/mol protein, assuming a molecular mass of 65 500 for bovine α1(X) chain [25] and 35 000 for the 35-kDa subunit of chondrocalcin [26].

2.6. *Protein determination*

Protein concentrations of samples were determined according to Bradford (1976) [27] with the microassay procedure described by Bio-Rad.

3. RESULTS

3.1. *Mineral deposition in cultured hypertrophic chondrocytes*

When fetal human chondrocytes were grown on agarose in Ham's F12 medium lacking ascorbic acid, most of the cells arranged into nodules. After transfer of these nodules to monolayer immunostaining with a polyclonal rabbit antibody against human type X collagen at the following day revealed that after 28 days over 90% of the cells in the nodules showed a positive reaction (Fig. 1A). When aggregates of type X collagen synthesizing, hypertrophic chondrocytes were stained with Alizarine red S, calcium deposits were localized in the center of the nodules (Fig. 1B). Staining was absent in nodules decalcified with EDTA prior staining (Kirsch et al., in preparation). The correlation between the staining for type X collagen and for calcium deposits in the center of chondrocyte nodules confirmed previous observations made in calcifying chick chondrocyte cultures [16–18], that type X collagen is involved in the calcification process.

3.2. *Ca^{2+} binding properties of type X collagen*

To determine whether type X collagen has an affinity for calcium, various amounts of bovine and human type X collagen dotted onto nitrocellulose filters were incubated with $^{45}Ca^{2+}$. Densitometric evaluation of the amount of bound radioactive calcium indicated that both bovine and human type X collagen bound calcium in a dose dependent manner. Chondrocalcin (C-pro-

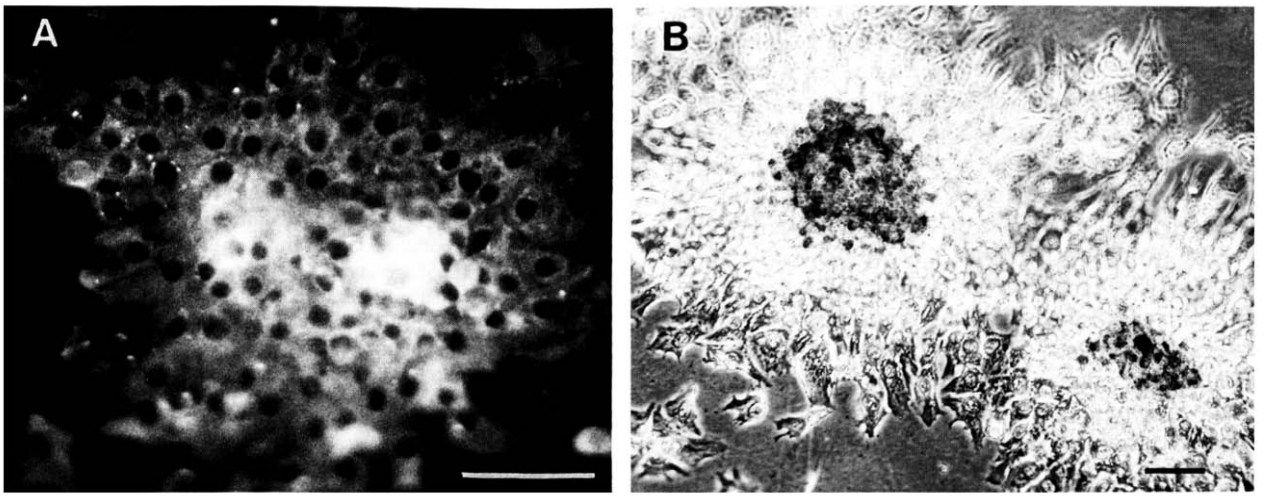

Fig. 1. Localization of type X collagen (A) and calcium deposits (B) in nodules of human fetal chondrocytes cultured for 28 days on agar, and then transfered to monolayer for one day for immunostaining. Fixed cells were immunostained with an antibody against human type X collagen followed by Texas red conjugated donkey anti rabbit IgG (A). Calcium deposits were detected by Alizarine red S staining (B). Note the correlation between type X collagen staining and Alizarine red S staining in the center of nodules. Bar, 50 μm.

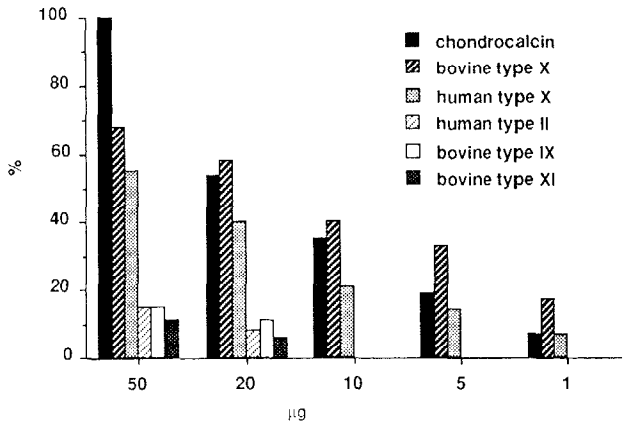

Fig. 2. Ca^{2+} binding properties of bovine and human type X collagen. Different concentrations (50,20,10,5,1 μg) of chondrocalcin, bovine type II, IX, XI collagens and bovine and human type X collagen were applied onto nitrocellulose filters and incubated with $^{45}CaCl_2$ (see section 2). Chondrocalcin, which is known to bind calcium, was used as positive control. Autoradiographs were analyzed by densitometry; the data are expressed as integrated optical density for each band. The optical density obtained for the Ca^{2+} binding of 50 μg chondrocalcin was set as 100%. In contrast to type II, IX and XI collagen, bovine and human type X collagen showed a dose dependent binding of calcium.

peptide of type II collagen) that is known to be a calcium binding protein [28] was used as positive control. Other cartilage collagens, like type II, type IX and type XI collagen did not show any significant calcium binding (Fig. 2).

To quantitate the Ca^{2+} binding to type X collagen, purified bovine type X was coupled to Affigel beads with a coupling efficiency of 70%. Aliquots of type X collagen beads were incubated with different concentrations of $^{45}CaCl_2$. After centrifugation and washing, free calcium was determined by scintillation counting of the supernatants; bound calcium was released with EDTA from the beads and counted. As shown in Fig. 3A Ca^{2+} bound to bovine type X collagen in a saturable manner, similar to Ca^{2+} binding to chondrocalcin (Fig. 3B). Analysis of the binding data by the method of Scatchard [29] (inserts of Fig. 3A,B) revealed that bovine type X collagen bound 15 calcium molecules per α chain with a K_d of ~32 μM. Chondrocalcin bound 6 calcium molecules per 35-kDa subunit with a K_d of ~8 μM.

4. DISCUSSION

Immunohistological and in situ hybridization studies of different calcifying cartilages from chick and mammalian species have documented the restriction of type X collagen to the hypertrophic and calcifying zone of cartilage [1,4–6,13,22,30]. In vitro it has been demonstrated by several investigators that hypertrophic, type X collagen synthesizing chondrocytes have the ability to calcify [16–18]. Here we show that also in fetal human chondrocytes after four weeks culture on agarose calcium deposits can be detected by Alizarine red S staining in the center of the nodules. Mineral deposition

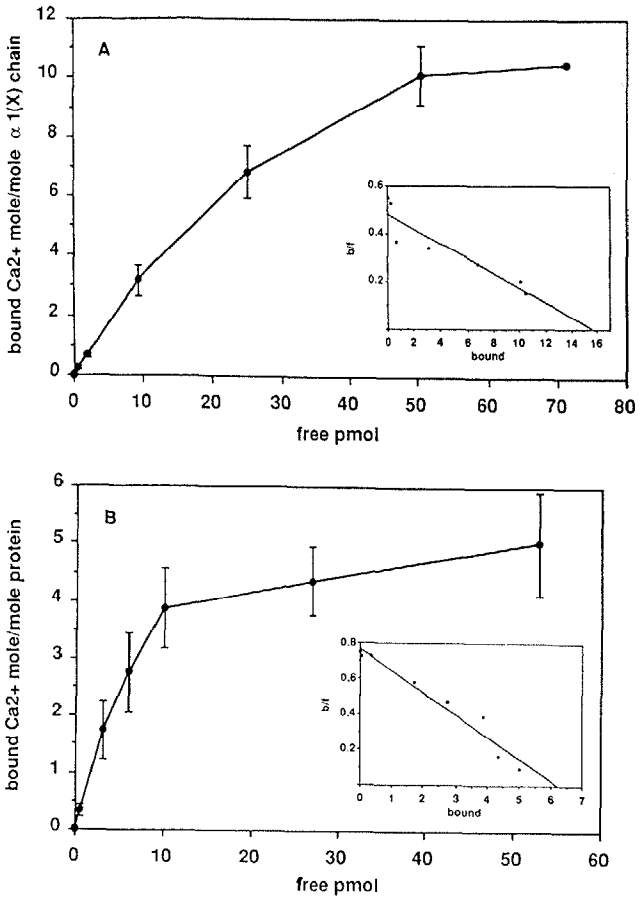

Fig. 3. High-affinity binding of Ca^{2+} to bovine type X collagen (A) and to chondrocalcin (C-propeptide of type II collagen, B). Experimental values for bound Ca^{2+} at different concentrations of free Ca^{2+} were obtained as described in section 2 and were fitted to a binding curve. All values were determined three times, except the value for type X collagen at the highest $^{45}Ca^{2+}$ concentration. The insets show the same data plotted according to Scatchard [28]. The amount bound (mol of Ca^{2+} bound per mol of bovine α1(X) chain or the 35-kDa subunit of chondrocalcin) is plotted against bound/free where bound is again in mol/mol protein and free is the Ca^{2+} concentration (molar) in the supernates as determined by scintillation counting. Bovine type X collagen binds 15 mol of Ca^{2+} per mole of α1(X) chain with a K_d of approximately 32×10^{-6} μM, while the 35-kDa subunit of chondrocalcin binds 6 calcium molecules with a K_d of approximately 8×10^{-6} μM.

is preceded by type X collagen synthesis, suggesting that type X collagen plays a role in the mineralization. However, the function of type X collagen in cartilage calcification remains unclear. A direct binding of calcium to type X collagen has never been shown. Here we present evidence for the calcium binding properties of bovine and human type X collagen. In contrast to type II, IX and XI collagens, type X collagen binds calcium with high affinity. Quantitative binding studies revealed that bovine type X collagen has 15 binding sites for Ca^{2+} per α1(X) chain; these sites have values for K_d of about 32 μM. The type X collagen sequence does not contain calcium binding motifs [7,25,31]. But especially the C-terminal globular domains of the α1(X) chains are rich in acidic amino acids that may bind calcium [25].

The mechanism of cartilage mineralization during endochondral ossification is a complicated process and far from being completely understood. Several extracellular matrix proteins have been claimed to be involved in the calcification, including proteoglycans [32,33], the C-propeptide of type II collagen (chondrocalcin) [34], type X collagen [1], the matrix Gla protein [35] and osteonectin [36]. Matrix vesicles which have been reported to initiate mineralization [20,21] bind to type II and type X collagen [16], as well as to the link protein and hyaluronan-binding region of aggrecan [37]. Matrix vesicle proteins, belonging to the family of Ca-phospholipid binding annexins, are involved in these interactions [38–40]. Thus the data obtained in this study showing the Ca^{2+} binding properties of type X collagen suggest, that calcium may act as a mediator of the interaction of matrix vesicles with type X collagen. Further experiments have to confirm this hypothesis.

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