

Terminal Differentiation of Chick Embryo Chondrocytes Requires Shedding of a Cell Surface Protein That Binds 1,25-Dihydroxyvitamin D3^{*[5]}

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Rita Dreier[‡], Birgit Kathrin Günther[‡], Tom Mainz[‡], Ilka Nemere[§], and Peter Bruckner^{‡1}

From the [‡]Institute for Physiological Chemistry and Pathobiochemistry, University Hospital of Münster, 48149 Münster, Germany and [§]Nutrition and Food Sciences, Utah State University, Logan, Utah 84322

Endochondral ossification comprises a cascade of cell differentiation culminating in chondrocyte hypertrophy and is negatively controlled by soluble environmental mediators at several checkpoints. Proteinases modulate this control by processing protein signals and/or their receptors. Here, we show that insulin-like growth factor I can trigger hypertrophic development by stimulating production and/or activation of proteinases in some populations of chick embryo chondrocytes. Cell surface targets of the enzymes include 1,25-dihydroxyvitamin D3 membrane-associated rapid response steroid receptor (1,25 D3 MARRS receptor), also known as ERp57/GRp58/ERp60. This protein is anchored to the outer surface of plasma membranes and inhibits late chondrocyte differentiation after binding of 1,25-dihydroxyvitamin D3. Upon treatment with insulin-like growth factor I, 1,25 D3 MARRS receptor is cleaved into two fragments of ~30 and 22 kDa. This process is abrogated along with hypertrophic development by E-64 or cystatin C, inhibitors of cysteine proteinases. Cell differentiation is enhanced by treatment with antibodies to 1,25 D3 MARRS receptor that either block binding of the inhibitory ligand 1,25-dihydroxyvitamin D3 or inactivate 1,25 D3 MARRS receptor left intact after treatment with proteinase inhibitors. Therefore, proteolytic shedding of 1,25 D3 MARRS receptor constitutes a molecular mechanism eliminating the 1,25-dihydroxyvitamin D3-induced barrier against late cartilage differentiation and is a potentially important step during endochondral ossification or cartilage degeneration in osteoarthritis.

Endochondral ossification is one of two mechanisms of bone formation in vertebrates and is particularly important for development, growth, and repair of long bones. During this process, differentiated cartilage cells transit through a cascade of late differentiation events that sequentially include cell proliferation and several steps of chondrocyte maturation culminating

in hypertrophy. After invasion of blood vessels into hypertrophic cartilage from subchondral bone, the majority of hypertrophic cells undergo apoptosis and the cartilage template is remodeled into trabecular bone. Each chondrocyte differentiation phase is accompanied by a change in cell shape and the expression of stage-specific markers. The cells produce collagens II, IX, and XI at all stages, albeit at different steady-state levels. In addition, the expression repertoire includes collagen VI and matrilin 1 at early proliferative stages and Indian hedgehog during pre-hypertrophy. Collagen X and alkaline phosphatase are well established surrogate markers for the overtly hypertrophic stage of late chondrocyte differentiation. Hypertrophic chondrocytes also reduce, or even terminate, their production of collagen II (1–6). Collagen X is not made in the superficial or intermediate layers of normal articular cartilage but is strongly up-regulated in osteoarthritic cartilage, particularly near surface fissures. For this reason, it has been speculated that osteoarthritis is associated with illegitimate induction of late differentiation in articular chondrocytes (7). Not least for this reason, there has been a considerable interest in the molecular mechanisms of the environmental control of this process.

Late chondrocyte differentiation *in vivo* is controlled by systemic hormones as well as by locally acting autocrine signals derived from chondrocytes themselves or by paracrine signals derived from cells of surrounding tissues, *e.g.* the perichondrium or subchondral blood vessels. When cultured in suspension under several conditions (for review see Ref. 1), chondrocytes *in vitro* can recapitulate late differentiation, and the order of events and their control elements closely resemble those occurring *in vivo*. Late chondrocyte differentiation is subject to positive and negative control elements that interact to regulate the rate and progression of the process (3). Locally produced factors, such as bone morphogenetic proteins, *Wnts*, fibroblast growth factors, *hedgehogs*, insulin-like growth factors (IGFs)², and retinoids, are known so far to influence endochondral ossification (for reviews see Refs. 2, 4, and 5). Likewise, systemic hormones, including growth hormone, thyroid hormone, estrogen, androgen, vitamin D, and glucocorticoids, control the

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¹ To whom correspondence should be addressed: Münster University Hospital, Physiological Chemistry and Pathobiochemistry, Waldeyerstr. 15, 48149 Münster, Germany. Tel.: 49-251-8355591; Fax: 49-251-8355596; E-mail: peter.bruckner@uni-muenster.de.

² The abbreviations used are: IGF, insulin-like growth factor; 1,25-((OH)₂) Vit D3, 1,25-dihydroxyvitamin D3; MMP, matrix metalloproteinase; MARRS receptor, membrane-associated rapid response steroid receptor; ERK, extracellular signal-regulated kinase; MS, mass spectrometry; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; RT-PCR, reverse transcription PCR.

rate and extent of the process at several points. Further, signals may erect barriers against late differentiation at earlier stages but may boost it later. For example, transforming growth factor β 2 and fibroblast growth factor-2 in synergy prevent differentiation at early proliferative stages but fibroblast growth factor-2 alone supports it later (8, 9). In a feedback loop of paracrine control, perichondrial cells, induced by chondrocyte-derived Indian Hedgehog, produce parathyroid hormone-related peptide that delays progression of late differentiation at late proliferative stages (10). However, parathyroid hormone can promote differentiation at other stages (11). Finally, extracellular signals also can stem from suprastructures of the extracellular matrix. For example, fibrils containing cartilage collagens II, IX, and XI interact with chondrocytes through matrix receptors and thereby stabilize the differentiated cartilage phenotype, whereas collagen I-containing fibrils cause the cells to undergo dedifferentiation (12).

At day 17 of *in ovo* development, the sternum of chick embryos still is entirely cartilaginous and contains chondrocytes at different stages of late differentiation. In cells derived from the caudal third, hereafter called caudal cells, hypertrophy is not easily achieved *in vitro*. Cells derived from the cranial third, hereafter called cranial cells, proceed to become overtly hypertrophic and produce large amounts of collagen X when appropriately stimulated *in vitro*, e.g. with insulin-like growth factor I or thyroid hormone. It has been thought that the anabolic response elicited by the hormones was tantamount to the induction of late differentiation. However, this notion does not explain easily the elimination of autocrine barriers against hypertrophy, such as those mentioned above.

Many stationary and diffusible regulators as well as their cell surface receptors are proteins. Therefore, proteinases are not merely destructive effectors of extracellular matrix degradation but also intervene in regulatory networks, both by eliminating control elements and by converting precursors into active agents. For instance, matrix metalloproteinases (MMPs) modify the extracellular matrix microenvironment, resulting in alteration of cellular behavior. They also regulate cell attachment and, thereby, differentiation and apoptosis. In addition, they modulate mediator activities by direct cleavage or by release from extracellular matrix stores. Furthermore, they control the activity of other proteinases by activating their zymogens or by their elimination through proteolysis (13). We have shown that proteinases of endothelial cells abrogate the arrest of late differentiation *in vitro*, most likely by acting in an activation cascade targeting chondrocyte surface components or their soluble ligands (8, 14). Additionally, cysteine proteinases, especially the cathepsins, have been implicated in several proteolytic scenarios during development, growth, remodeling, and aging, as well as in a variety of pathological processes. During endochondral ossification, cathepsins B, H, K, L, and S were detected immunohistochemically in growth plates of rats and humans (15, 16) and are thought to be involved in the proteolysis of several extracellular matrix components.

The seco-steroid 1,25-dihydroxyvitamin D3 (1,25-(OH)₂ Vit D3) is an essential regulator of bone development, growth, and remodeling (17). Vitamin D hormone modulates proliferation and differentiation of growth plate chondrocytes both by mech-

anisms involving the classical nuclear vitamin D receptor and by rapid actions through membrane-associated receptors (18). In chondrocytes of the growth zone, binding of 1,25-(OH)₂ Vit D3 to a membrane-associated vitamin D receptor molecule modulates gene expression through the ERK1/2 pathway activated by phospholipase C. In addition, protein kinase C turns on the synthesis of prostaglandins that also increase ERK1/2 activities. In chondrocytes of the resting zone, however, rapid actions are not mediated through 1,25-(OH)₂ Vit D3 but, rather, via 24,25-dihydroxyvitamin D3. Membrane binding of this vitamin D metabolite induces mitogen-activated protein kinase activity via phospholipase D and decreased prostaglandin production (19). Rapid, non-genomic effects of 1 α ,25-(OH)₂ Vit D3 have been attributed in several tissues and species to a 1,25-dihydroxyvitamin D-binding protein at the cell surface, which is called 1,25-dihydroxyvitamin D3 membrane-associated rapid response steroid receptor (1,25 D3 MARRS receptor) and which is also known as ERp57, GRp58, or ERp60 (GenBankTM accession code 373899) (20–24).

Here, we found that cysteine proteinase activity is essential for progression toward hypertrophy not only in caudal but also in cranial cells. In addition, we found that these cysteine proteinases target membrane-associated signaling by vitamin D hormones, the last barrier against late differentiation of cranial cells.

EXPERIMENTAL PROCEDURES

Antibodies—AB099 is a polyclonal rabbit antibody directed against 1,25 D3 MARRS receptor (20), produced by means of the multiple antigenic peptide system using the first 20 N-terminal amino acids of the putative membrane vitamin D receptor. Horseradish peroxidase-conjugated anti-rabbit IgG from donkey (Amersham Biosciences) served as secondary antibody for immunoblotting. Binding of AB099 in immunohistochemistry was detected with the alkaline phosphatase-anti-alkaline phosphatase protocol with a mouse anti-rabbit IgG-bridging antibody (both DAKO, Glostrup, Denmark).

Chondrocyte Cultures—Chondrocytes were isolated from the cranial third of 17-day-old chick embryo sterna by overnight digestion with collagenase. The cells were cultured in agarose suspension cultures under serum-free conditions as described (25). Briefly, cells were suspended in 0.5% low melting agarose in Dulbecco's modified Eagle's medium (Biochrom) and allowed to sediment on the culture dishes pre-coated with 1% high melting agarose in water. Cells were grown at densities of 2×10^6 cells/ml in Dulbecco's modified Eagle's medium (Biochrom) containing 60 μ g/ml β -aminopropionitrile fumarate, 25 μ g/ml sodium ascorbate, 1 mM cysteine, 1 mM pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin (complete medium) for 14 days at 37 °C and 5% CO₂. For two-dimensional electrophoresis or one-step RT-PCR the cells were cultured on top of agarose layers at high density (8×10^6 cells/ml) in complete medium for 48 h. Where indicated, 100 ng/ml IGF-I, 10^{-6} – 10^{-7} M 1 α ,25-dihydroxyvitamin D3 or 24(R5),25-dihydroxyvitamin D3 (both Biomol), AB099 in a dilution of 1:1000, and/or the proteinase inhibitors E-64 (0.14–14 μ M; Roche Applied Science) or cystatin C (7.5–750 pM; Calbiochem) were added to the medium during the whole culture duration.

Medium was changed every 2–3 days. Micrographs were recorded on day 12 with an inverse microscope (Axiovert 100 equipped with Axiovision 2.0; Zeiss).

Expression of Differentiation Markers—After 14 days in culture, newly synthesized chondrocyte proteins were metabolically labeled for 24 h with 1 μ Ci/ml of [14 C]proline (uniformly labeled, 250 Ci/mmol; PerkinElmer Life Science products). Collagens were isolated after limited digestion with pepsin from the culture medium or the agarose layers and were analyzed by SDS-PAGE on 4.5–15% gradient gels, followed by fluorography as described (26). Alkaline phosphatase activity was monitored in culture medium with *p*-nitrophenyl phosphate as a substrate (27).

One-step RT-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen) as recommended by the manufacturer from chondrocytes cultured in suspension on top of agarose for 7 days. Prior to the one-step PCR protocol (Qiagen), RNA was treated with RNase-free DNase I (Ambion) for 30 min at 37 °C. The following primer sequences were used: β actin forward, ggatgtgcaaggccggtt, β actin reverse, atgctggggtgtgaaggt, amplicon size: 353 bp; collagen X forward, acctgcagatccctgtctat, collagen X reverse, atcaatgacagcactgcctgagg, amplicon size, 161 bp. RT-PCR products were separated on a 2% agarose gel.

Immunohistochemical Analysis—Tibiae of 17-day-old chick embryos were fixed for 24 h in 3% paraformaldehyde in 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS). After decalcification in 10% EDTA in 3% Tris-HCl, pH 7.4, the samples were paraffin-embedded. 3- μ m sections were deparaffinized and subjected to digestion for 90 min at 37 °C with 1 mg/ml of bovine testicular hyaluronidase (Serva) in 20 mM sodium phosphate buffer, pH 5.3, containing 150 mM NaCl (28). After washing with PBS, unspecific antibody binding sites were blocked at 4 °C overnight with PBS containing 10% normal goat serum (DAKO) and 50 mg/ml of bovine serum albumin. Sections were then exposed for 1 h at 37 °C to AB099 (1:1000) or rabbit immunoglobulin fraction (Dako), diluted in blocking solution. Immune complexes were stained with the APAAP detection kit, as recommended by the manufacturer with mouse anti-rabbit IgG as a bridging antibody (Dako) and NBT/BCIP® solution (Sigma) as chromogenic substrate.

Immunoblotting—Proteins in 1 ml of conditioned medium from chondrocytes cultured in agarose were precipitated with 3% (w/v) trichloroacetic acid and were subjected to 4.5–15% SDS-polyacrylamide gel electrophoresis under reducing conditions. Pre-stained protein standards within a range of 207 to 7.5 kDa (Bio-Rad) were used as molecular mass markers. Proteins were electro-transferred onto nitrocellulose membranes (Schleicher and Schuell) for 3 h at 4 °C. Filters were blocked for 1 h in blocking buffer (5% dry skim milk + 1% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20) and incubated overnight with a rabbit antiserum (AB099, diluted 1:5000) directed against 1,25 D3 MARRS receptor or RP1MMP13 (Triple Point Biologics, Inc.; Forest Grove, OR, 1:1000) directed against MMP-13, washed with Tris-buffered saline, and incubated for 2 h with horseradish peroxidase-coupled anti-rabbit IgG antibody from donkey (Amersham Bio-

sciences). Signals were visualized with ECL reagent by exposure on blue light-sensitive autoradiography films (Pierce).

Analysis of Chondrocyte Membrane Fragments by Two-dimensional Gel Electrophoresis or Immunoblotting—In order to enrich for plasma membrane-associated proteins, chondrocytes were harvested after 2 days of culture and cell surface proteins were biotinylated with sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's protocol. Briefly, the cells were washed two times in PBS to remove amine-containing culture medium and proteins. To 2.5×10^7 cells/ml in PBS 2 mM biotin reagent was added and incubated for 30 min at 4 °C. To quench and remove excess biotin reagent, 5 ml of PBS + 100 mM glycine was added and the cells were washed twice in PBS. For isolation of membrane fragments the cells were resuspended in 5 mM Tris-HCl, pH 7.8, 250 mM saccharose containing a proteinase inhibitor mixture (Complete; Roche Applied Science), lysed by three cycles of freeze/thawing, followed by sonification. After removal of nuclei, mitochondria, and the rough microsomes by centrifugation ($1000 \times g$, 30 min, 4 °C), the membrane fragments were pelleted by centrifugation ($150,000 \times g$, 30 min, 4 °C) and resuspended in PBS. Biotinylated membrane fragments were enriched by applying a magnetic separation technique with Magprep™ streptavidin beads (Novagen) as recommended by the manufacturer. Proteins were subjected to immunoblotting with subsequent image analysis (ImageQuant; GE Healthcare) or isoelectric focusing (pH 3–10) using the Zoom IPG Runner on zoom stripes pH 3–10 NL (both Invitrogen) in the following sample buffer: 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 20 mM dithiothreitol, 0.5% 3/10 ampholyte, 0.001% bromphenol blue. The isoelectric focusing run was conducted at 4 °C at 200 V for 20 min, at 450 V for 15 min, at 750 V for 15 min, and at 2000 V for 45 min. Electrophoresis in the second dimension was carried out on a 4.5–15% gradient SDS-PAGE under reducing conditions. Protein standards within a range of 200 to 6.5 kDa (Bio-Rad) were used as molecular mass markers. The gel was stained overnight with Coomassie Brilliant Blue G-250 and destained in HPLC grade A bidest.

Mass Spectrometric Analysis—1,25 D3 MARRS receptor was identified by peptide mass fingerprint (matrix-assisted laser desorption ionization time-of-flight MS analysis) and liquid chromatography MS/MS (nano-HPLC and subsequent quantitative time-of-flight electrospray MS/MS analysis) at the Central Bioanalytics Department (ZBA), Center for Molecular Medicine (ZMMK) Cologne, Germany.

RESULTS

IGF-I-stimulated Late Differentiation of Cranial Cells in Vitro Requires Extracellular Proteinase Activity—In serum-free culture in agarose gels, cranial cells become hypertrophic within 14 days in the presence of 100 ng/ml IGF-I or 25 ng/ml thyroxine. They change their morphology and initiate synthesis of alkaline phosphatase, collagen type X (29), and MMP-13. Newly synthesized radiolabeled collagens II and X after pepsin digestion were identified as characteristic bands migrating with mobilities corresponding to apparent molecular masses of 120 and 58 kDa, respectively, as routinely carried out in several laboratories (1). In developing bone, chondrocytes synthesize this

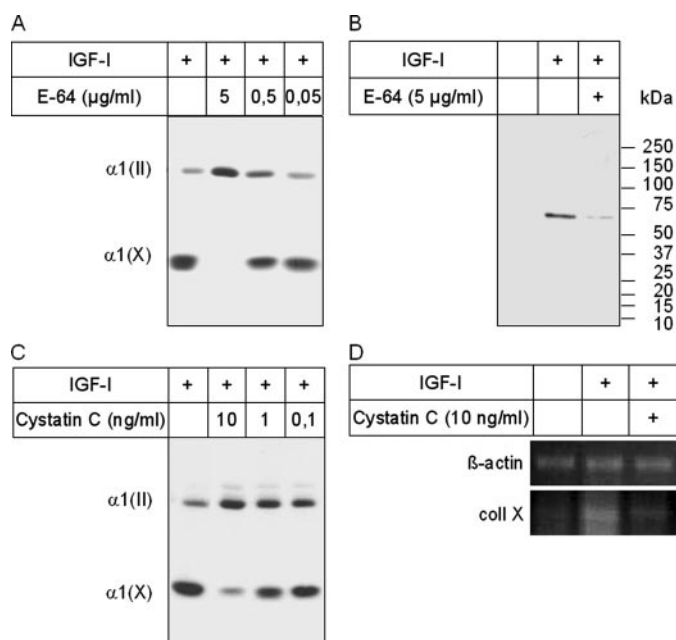


FIGURE 1. Addition of cysteine proteinase inhibitors to cranial chondrocytes in culture inhibits IGF-I-stimulated differentiation in a dose-dependent manner. Chondrocytes isolated from the cranial part of 17-day-old chick embryo sterna were cultured in serum-free suspension cultures for 14 days. 100 ng/ml IGF-I and/or the cysteine proteinase inhibitors E-64 (A and B) or cystatin C (C and D) were added in the indicated concentrations to the medium during the whole culture duration. A and C, fluorographs of SDS-PAGE gels separating newly synthesized, [14 C]proline-labeled, and pepsin-treated collagens are shown. B, immunoblot analysis to detect the hypertrophy marker MMP-13. The signal represents the 60-kDa proenzyme in IGF-I-stimulated cranial chondrocytes. Note: The signal intensity is decreased in cultures treated with E-64. D, agarose gel electrophoresis of one-step RT-PCR products. Note: Cystatin C blocks collagen X expression increased by treatment with IGF-I. Loading control: β actin gene expression.

matrix molecule after they have become hypertrophic and before mineralization of the extracellular matrix occurs (1, 2, 30–33). To determine the role of cysteine proteinases during IGF-I-stimulated chondrocyte differentiation, E-64, a specific inhibitor of papain and of other cysteine proteinases such as cathepsin B or L, or cystatin C, a low molecular mass protein of the cystatin superfamily, were added to the cells during the whole culture period. After 14 days the cultures were metabolically labeled with [14 C]proline and pepsin-treated collagens were isolated from the cell culture medium and analyzed by SDS-PAGE and subsequent fluorography (Fig. 1). In cranial cells stimulated by IGF-I, addition of E-64 dose dependently reduced the production of collagen X, the marker of hypertrophic cells. ~ 5 μ g/ml of E-64 was sufficient to fully block the secretion of the protein. In parallel, collagen II synthesis was increased (Fig. 1A). Inhibition of cysteine proteinases by cystatin C dose dependently produced similar results (Fig. 1C). To confirm that changes in collagen II and X protein are not due to altered proteolysis or processing but rather are associated with terminal chondrocyte differentiation, gene expression of collagen X and β actin (loading control) was analyzed by one-step RT-PCR (Fig. 1D). Collagen X expression is strongly induced after IGF-I stimulation. In the presence of 10 ng/ml cystatin C and IGF-I, however, collagen X expression was strongly diminished. Finally, MMP-13, a metalloproteinase produced by late hypertrophic cells, is another late-stage differentiation marker.

The enzyme is essential for degradation of the extracellular matrix of ossifying hypertrophic cartilage prior to mineralization and remodeling into bone. After treatment with IGF-I, MMP-13 was detected in immunoblots as the 60-kDa proenzyme (34, Fig. 1B). In the presence of 5 μ g/ml of the cysteine proteinase inhibitor E-64, however, MMP-13 secretion is barely visible on the immunoblots. Taken together, these results showed that cysteine proteinases were required for the production of markers of late differentiation by IGF-I-stimulated chondrocyte and indicated that, in the presence of cysteine proteinase inhibitors, the cells remained at earlier stages of late differentiation.

Treatment with E-64 Elevates the Abundance of 1,25 D3 MARRS-binding Protein on the Surface of IGF-I-stimulated Cranial Cells—Because E-64 does not penetrate membranes, cysteine proteinases target components of the culture medium or of the cell surfaces. In a preliminary screen for candidate substrates, proteins on membrane fragments of IGF-I-stimulated cranial cells cultured with or without E-64 were analyzed. In this type of experiments, it was necessary to culture the cells in liquid suspension on top of agarose, a good alternative to suspension culture within agarose gels (1). This allowed easy recovery of the cells after culture. Membrane fragments were prepared from the cells after culture and were subjected to two-dimensional electrophoresis (isoelectric focusing pH 3–10, SDS-PAGE). Gels stained with colloidal Coomassie Blue revealed a number of differences in the protein patterns (Fig. 2A). A 64-kDa protein with an isoelectric point of ~ 5.8 was more abundant in cells cultivated with E-64 (Fig. 2A, arrow). This was in contrast to other proteins on the same gels that produced spots with similar intensities (Fig. 2A, asterisks). The 64-kDa protein was analyzed by peptide mass fingerprinting and liquid chromatography MS/MS and was identified as 1,25 D3 MARRS receptor, also known as ERp57/GRp58/ERp60. Alternatively, biotinylated plasma membrane proteins of IGF-I-stimulated cranial cells cultured with or without E-64 were isolated via MagprepTM streptavidin beads and subjected to immunoblotting using AB099 to detect 1,25 D3 MARRS receptor (Fig. 2B). As quantified by image analysis, there was $\sim 60\%$ more 1,25 D3 MARRS receptor detectable on chondrocyte cell surfaces in the presence of E-64. These results indicated that this membrane-associated vitamin D receptor was a cell surface target of cysteine proteinases and therefore qualified as a candidate attenuator of late differentiation. The protein is eliminated or inactivated by chondrocyte-derived cysteine proteinases upon stimulation with IGF-I.

Cysteine Proteinase Inhibitors Reduce the Proteolytic Processing of 1,25 D3 MARRS Receptor—AB099 is a polyclonal antibody to the N terminus of 1,25 D3 MARRS receptor where the 1,25-dihydroxyvitamin D3 binding site presumably is located.³ To characterize the proteolytic processing of 1,25 D3 MARRS receptor during differentiation, immunoblotting with AB099 was carried out on proteins in spent culture medium of cranial cells, stimulated or not with IGF-I, and cultivated in agarose gels in the presence or absence of E-64. Under all conditions

³ M. C. Farach-Carson, unpublished results.

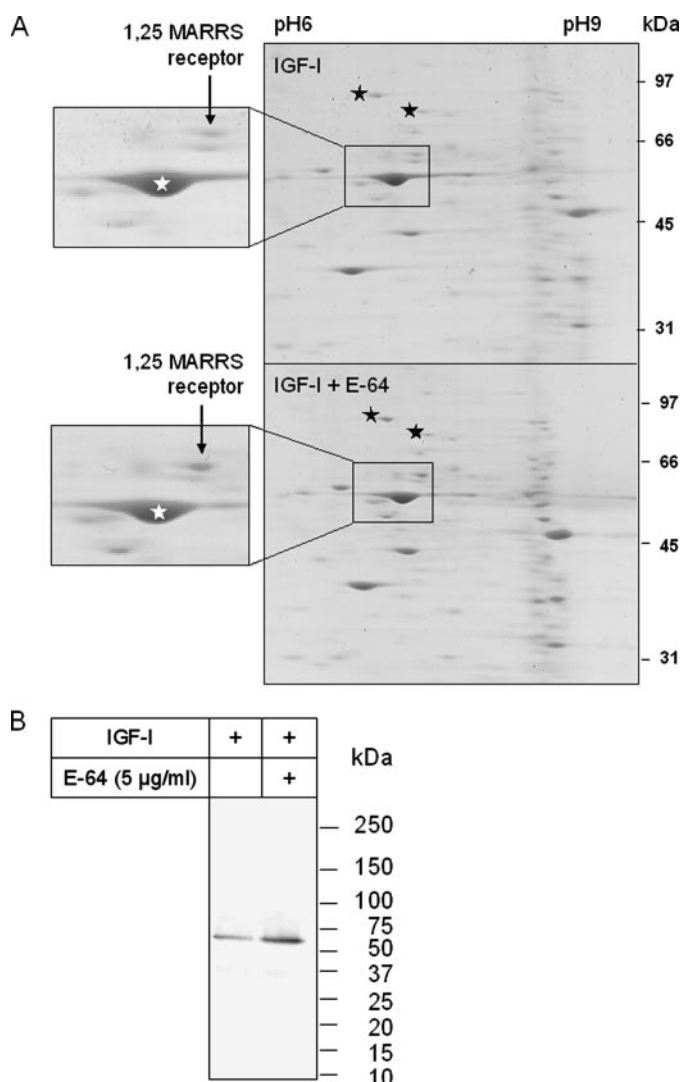


FIGURE 2. Treatment of IGF-I-stimulated cranial chondrocytes with E-64 elevates the abundance of 1,25 D3 MARRS receptor on the cell surface. Chondrocytes were cultivated for 2 days in high density suspension culture in the presence or absence of 5 µg/ml E-64. Isolated membrane-associated cell surface proteins were applied to comparative two-dimensional gel electrophoresis (A) or immunoblotting with AB099 (B). 1,25 D3 MARRS receptor was identified by peptide mass fingerprint and liquid chromatography MS/MS.

applied, 1,25 D3 MARRS receptor was recovered from the culture medium in quantities well detectable by immunoblotting as a set of bands corresponding to polypeptides with apparent molecular masses between 58 and 70 kDa (Fig. 3, *asterisks*). Because 1,25 D3 MARRS receptor is subject to extensive post-translational modification, including complex glycosylation, phosphorylation, and *N*-myristoylation (21), these bands presumably originate from distinct, full-length isoforms of the protein that retain the AB099 epitopes at the N-terminal end and that were not investigated here in further detail. Importantly, however, two proteolytic fragments, F1 and F2, of ~30 and 22 kDa, respectively, appeared upon stimulation with IGF-I for up to 9 days (Fig. 3, *lanes 2 and 5*). These fragments occurred much less in unstimulated (Fig. 3, *lanes 1 and 4*) or in E-64-treated, IGF-I-stimulated cultures (Fig. 3, *lanes 3 and 6*). From these results, we conclude that proteolytic fragmentation of 1,25 D3

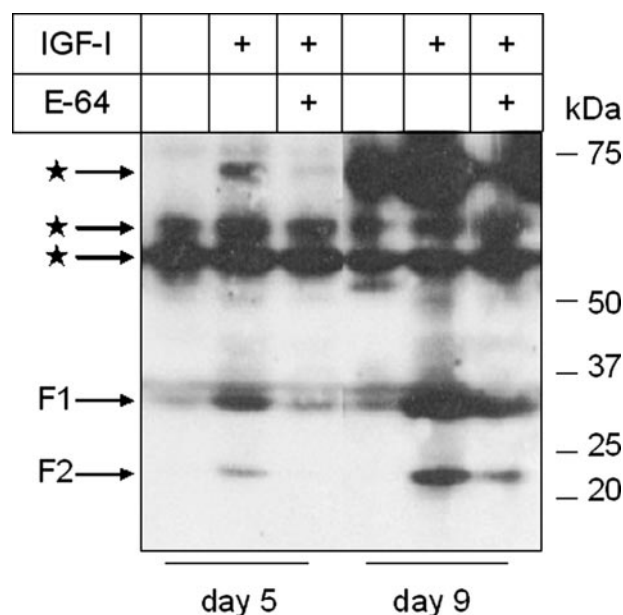


FIGURE 3. E-64 reduces proteolytic processing of 1,25 D3 MARRS receptor in IGF-I-stimulated chondrocyte cultures. Medium aliquots (days 5 and 9) were subjected to SDS-PAGE on 4.5–15% gradient gels under reducing conditions. Proteins were transferred to nitrocellulose and analyzed with AB099. Arrows indicate proteolytic fragments F1 and F2 of 1,25 D3 MARRS receptor. Asterisks denote several post-translational modification isoforms of 1,25 D3 MARRS receptor (21).

MARRS receptor by chondrocyte-derived cysteine proteinases depends on IGF-I signaling.

AB099 (Anti-1,25 D3 MARRS Receptor) Accelerates Chondrocyte Differentiation in Vitro—To characterize the role of the cell surface 1,25 D3 MARRS receptor during differentiation at a functional level, blocking of the function of this membrane-associated vitamin D receptor by the antibody AB099 was assessed in cell cultures of cranial cells, stimulated or not with IGF-I, in the presence or absence of E-64. Irrespective of IGF-I stimulation or proteinase inhibition, the addition of AB099 led to changes in cell morphology. In all cultures in which AB099 was present, more cells showed a hypertrophic appearance at day 12 (Fig. 4A, *arrow*). The cells increased in size and were more granular. More impressive, however, was the detection of large amounts of collagen X in all cultures containing AB099 (Fig. 4B). Even in the absence of IGF-I, blocking by the antibody of vitamin D binding to 1,25 D3 MARRS receptor resulted in hypertrophic differentiation and secretion of collagen X into the medium. Addition of E-64 did not alter this observation. In the agarose layers of these cultures, the fibril-forming collagens are preferentially retained. Therefore, collagen II and only small amounts of collagen X are detectable (Fig. 4C). The more soluble collagen X, however, is retained less in the pericellular matrix and hence is recovered predominantly from the medium (for example, compare *lanes 4* in Fig. 4, B and C). Furthermore, inhibition of chondrocyte differentiation by the cysteine proteinase inhibitor E-64 (Fig. 4, B and C, *lanes 3*) was abrogated by addition of AB099 (Fig. 4, B and C, *lanes 6*), but not by irrelevant rabbit sera (not shown).

1,25 D3 MARRS Receptor Is Present throughout the Whole Chick Tibia Growth Plate—In further experiments, we addressed the question whether the tissue distribution of 1,25

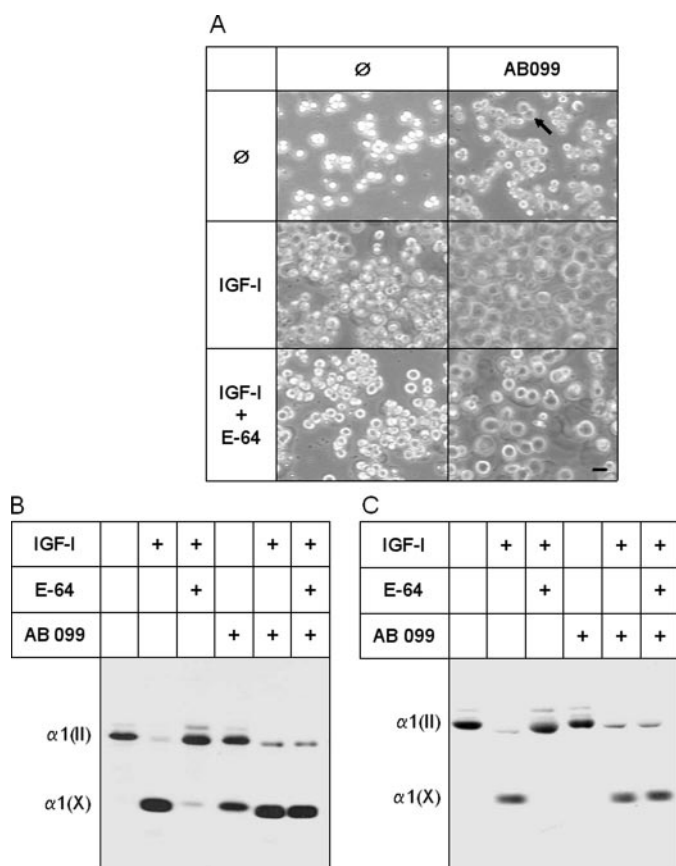


FIGURE 4. Function-blocking antibody AB099 to MARRS receptor accelerates chondrocyte differentiation. Chondrocytes isolated from the cranial part of 17-day-old chick embryo sterna were cultured in serum-free agarose suspension cultures for 14 days. Where indicated, 100 ng/ml IGF-I, 5 $\mu\text{g}/\text{ml}$ E-64, and/or AB099 in a dilution of 1:1000 was added. *A*, microphotographs taken on day 12 of culture; *arrow* points to hypertrophic cells. *Bar*, 20 μm . *B* and *C*, fluorographs of SDS-PAGE gels separating newly synthesized, [^{14}C]proline-labeled, and pepsin-treated collagens isolated from medium (*B*) or agarose layers (*C*).

D3 MARRS receptor was compatible with its possible role *in vivo* as a component of the regulation machinery of late differentiation. Therefore, we analyzed by immunohistochemistry tibial growth plates of 17-day-old chick embryos employing antibody AB099 against 1,25 D3 MARRS receptor. The protein was detectable throughout the whole chick tibia growth plate. Staining was especially prominent within and around chondrocytes of the hypertrophic zone (Fig. 5, *C* and *D*) but was also present at lower intensity in the resting and proliferative zones (Fig. 5, *A* and *B*). Interestingly, strong staining for 1,25 D3 MARRS receptor was observed not only in intracellular compartments (Fig. 5*C*, *arrow*) but also in the extracellular matrix at sites remote from cell surfaces (Fig. 5*D*, *arrowhead*). This was particularly evident in interterritorial regions of the hypertrophic zone (Fig. 5*C*). Closely comparable observations were made in sections of sterna of 17-day-old chick embryos (not shown). In negative controls, treatment with antibody AB099 was either omitted or substituted by addition of normal rabbit immunoglobulins, and very low background labeling was seen in all regions of the growth plates. The presence of 1,25 D3 MARRS receptor in the extracellular matrix is consistent with its initial occurrence on cell surfaces from where it is released,

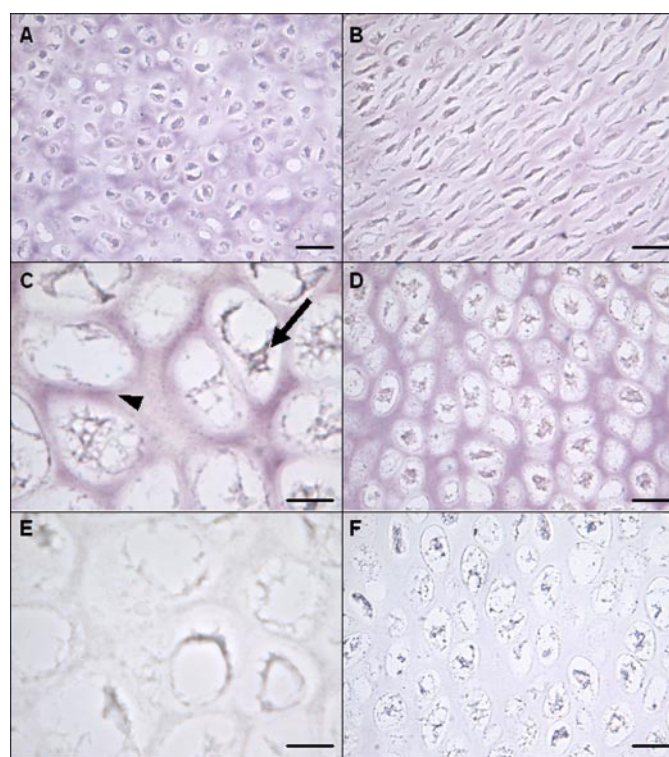


FIGURE 5. AB099 staining of 1,25 D3 MARRS receptor on the cell surface and within the intercellular matrix of hypertrophic zones of chick tibial growth plates. Photographs are shown of sections from paraffin-embedded tibia of 17-day-old chick embryos incubated with AB099, directed against the N terminus of 1,25 D3 MARRS receptor. Immune complexes were detected with the alkaline phosphatase-anti-alkaline phosphatase protocol using NBT/BCIP® solution as a chromogenic substrate. *A*, resting zone, *B*, proliferative zone, *C*, hypertrophic zone. Note intracellular (*arrow*) and pericellular matrix staining near cell surfaces (*arrowhead*). *D*, overview of hypertrophic zone. Note intense matrix staining. *E*, negative control, hypertrophic zone, omission of primary antibody. *F*, negative control, hypertrophic zone, primary antibody was replaced by normal rabbit immunoglobulin fraction (Dako). *Bars*, 20 μm in panels *A*, *B*, *D*, and *F* and 10 μm in panels *C* and *E*.

thus allowing the cells to progress toward later stages of differentiation, including overt hypertrophy.

1,25-Dihydroxyvitamin D3, but Not 24,25-Dihydroxyvitamin D3, Inhibits Chondrocyte Differentiation—Because 1,25 D3 MARRS receptor is a vitamin D-binding protein at the cell surfaces, the influence of 1,25- and 24,25-dihydroxyvitamin D3 on late differentiation was investigated in chondrocytes *in vitro*. Typical physiological concentrations in human plasma of 1,25-dihydroxyvitamin D3 are in the range of 10^{-7} to 10^{-6} M. The vitamin suppressed IGF-I-stimulated late differentiation entirely at 10^{-6} M and partially at 10^{-7} M. This was judged by the increase in collagen II production (Fig. 6*A*) as well as the absence in the cell culture medium of markers of the hypertrophic stage, including collagen X (Fig. 6*A*), alkaline phosphatase activity (Fig. 6*B*), and MMP-13 secretion (Fig. 6*C*). In addition to collagen X production at the protein level, collagen X mRNA expression was investigated by one-step RT-PCR analysis (Fig. 6*D*). Therefore, the absence of collagen X protein in the presence of 10^{-6} M 1,25-dihydroxyvitamin D3 is due to a strong reduction of gene expression that, in turn, is a genuine result of suppression of late differentiation. Consistent with earlier observations by Boyan *et al.* (35), however, addition of another vitamin D variant, *i.e.* 24,25-dihydroxyvitamin D3, led

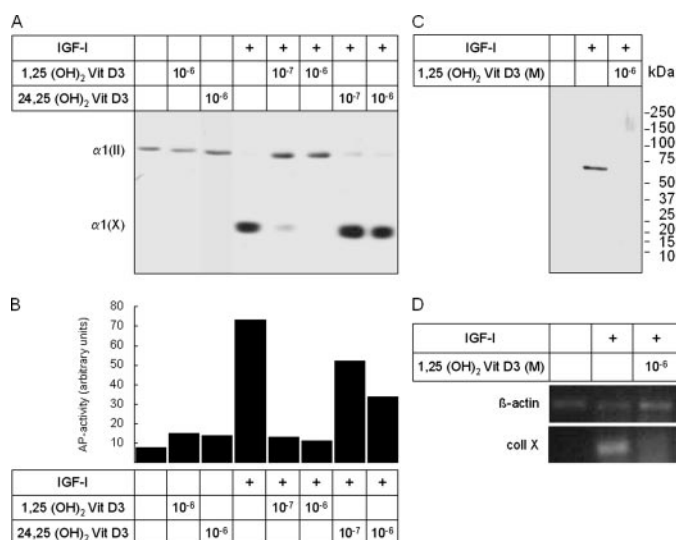


FIGURE 6. 1,25-(OH)₂ Vit D3, but not 24,25-(OH)₂ Vit D3, inhibits chondrocyte differentiation. Chondrocytes isolated from the cranial part of 17-day-old chick embryo sterna were cultured in serum-free suspension cultures for 14 days. 100 ng/ml IGF-I and 1,25-(OH)₂ Vit D3 or 24,25-(OH)₂ Vit D3 in the indicated concentrations were added to the medium during the whole culture duration. *A*, fluorograph of an SDS-PAGE gel separating newly synthesized, [¹⁴C]proline-labeled, and pepsin-treated collagens. *B*, alkaline phosphatase activities were determined in culture medium at day 14 by hydrolysis of *p*-nitrophenyl phosphate. *C*, immunoblot analysis to detect MMP-13. The 60-kDa proenzyme of the collagenase 3 is detectable in IGF-I-stimulated cranial chondrocytes in large amounts. In the presence of IGF-I and 10⁻⁶ M 1,25-(OH)₂ Vit D3, no MMP-13 is detectable. *D*, gel electrophoresis of one-step RT-PCR products. Note: 1,25-(OH)₂ Vit D3 blocks collagen X expression increased by treatment with IGF-I. Loading control: β actin gene expression.

to only minor changes of marker expression. Thus, 24,25-dihydroxyvitamin D3 either does not bind to 1,25 D3 MARRS receptor or cannot signal through this receptor in cranial cells.

AB099 (Anti-1,25 D3 MARRS Receptor) Abrogated the Inhibition of Chondrocyte Differentiation by 1,25-Dihydroxyvitamin D3—To confirm that the inhibition of chondrocyte differentiation by 1,25-dihydroxyvitamin D3 is mediated through 1,25 D3 MARRS receptor, the antibody AB099 was assessed in cell cultures of cranial cells stimulated with IGF-I in the presence or absence of 1,25-dihydroxyvitamin D3. The inhibition of chondrocyte differentiation by 10⁻⁶ M of this vitamin D metabolite, as judged by the absence of collagen X from the culture medium, was completely abrogated by addition of AB099. Under these conditions, cells resumed intensive collagen X production (Fig. 7).

DISCUSSION

To achieve an orderly sequence of late differentiation steps in cartilage destined for ossification, strong barriers set up by environmental signals prevent premature transition from one stage to the next. This is also borne out by chondrocytes from different regions of the chicken sternum. Ossification begins shortly before hatching, progresses in rostro-caudal direction, and is complete in adult birds only. At day 17 of *in ovo* development, the tissue is already committed to ossification but still is entirely cartilaginous. The cranial third contains heterogeneous populations of chondrocytes that easily reach hypertrophy *in vitro* under the influence of anabolic signals. By contrast, the barriers against hypertrophy still are strongly developed in cau-

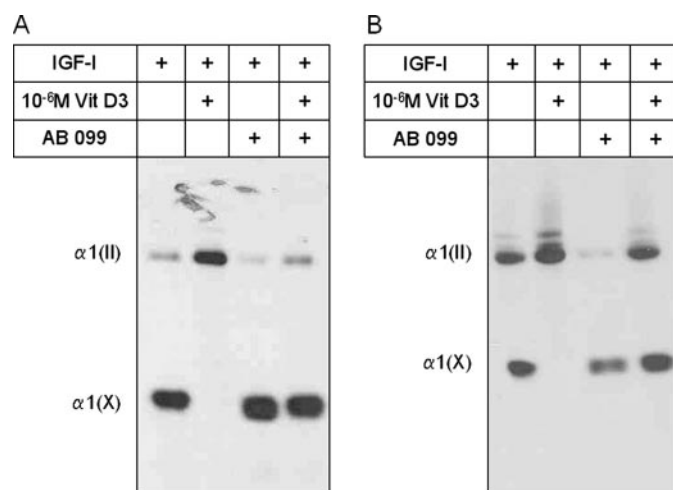


FIGURE 7. Addition of AB099 to chondrocyte cultures compensates the inhibition of chondrocyte differentiation by vitamin D. Chondrocytes isolated from the cranial part of 17-day-old chick embryo sterna were cultured in serum-free agarose suspension cultures for 14 days. Where indicated, 100 ng/ml IGF-I, 10⁻⁶ M Vit D3, and/or AB099 in a dilution of 1:1000 was added. Fluorographs are shown of SDS-PAGE gels separating newly synthesized, [¹⁴C]proline-labeled, and pepsin-treated collagens isolated from cell culture medium (*A*) or agarose layers (*B*).

dal cells, and humoral factors released by such cells can even prevent IGF-I-triggered hypertrophy of cranial cells in co-culture (29).

The autocrine barriers disappear upon exposure of caudal cells to proteinases specifically produced and/or activated by blood vascular endothelial cells. The typical shape changes and marker expression of hypertrophic chondrocytes are achieved also by caudal cells under these conditions. Several proteinase types are involved that act in a cascade-like manner (14). The cascade could consist of sequential steps of proteolytic zymogen activation resembling the stepwise factor conversions in the blood clotting or complement systems. It is also possible that the endothelial proteinases target in an orderly fashion several substrates on or around cartilage cells, including chondrocyte-derived regulatory proteinase zymogens. This proteolytic action directed against the barrier signals or their receptors is sufficient to break down all elements negatively controlling late differentiation in caudal cells *in vitro* (8).

We have shown here that, similarly to rat growth plate chondrocytes at proliferative stages (36), late differentiation of cranial cells still can be restrained by 1,25-dihydroxyvitamin D3. It has been shown previously (37) that rat costal chondrocytes are capable of producing this vitamin D metabolite, which eliminates the necessity of availability of the systemic vitamin D hormone. The concentration of the vitamin at which alkaline phosphatase activity as well as collagen X and MMP-13 production was effectively suppressed corresponds to the upper physiological range in human plasma. However, the effective concentrations of the water-insoluble vitamin in our cultures may well have been lower because the media do not contain sera potentially mediating solubility in aqueous environments. We have shown here that the vitamin D-binding protein at the cell surface, 1,25 D3 MARRS receptor, mediated the vitamin D effect. It remains to be seen whether signaling through the intracellular vitamin D receptor is also involved. We have also shown that

not only caudal, but also cranial, cells depend on proteinases to reach the hypertrophic, collagen X-producing state. However, unlike caudal cells, which depend on proteinases produced by endothelial cells for derepression of late differentiation (14), cranial cells stimulated with IGF-I can produce and activate all enzymes required, and cysteine proteinases are essential in this context. Thus, in view of the fact that ossification of the chicken sternum and, hence, chondrocyte differentiation proceed in rostro-caudal direction, the accumulated evidence from our *in vitro* experiments is consistent with our hypothesis that proteinases are essential positive control elements in the regulatory network of endochondral ossification and their action is required at several distinct stages.

In growth plates of chick tibiae, 1,25 D3 MARRS receptor was detected on chondrocytes throughout all zones, which is consistent with its reported occurrence in rats on resting, post-proliferative, prehypertrophic, and upper hypertrophic zone chondrocytes (growth zone cells) (35). We also found that, particularly in the hypertrophic zone in chick tibiae, this membrane-associated protein also is accumulated far away from cell surfaces within interterritorial regions of the extracellular matrix. It is tempting to speculate that 1,25 D3 MARRS receptor, as a full-length protein, is attached to plasma membranes or to matrix vesicles budding from cells into the extracellular matrix (38). In addition, however, soluble 1,25 D3 MARRS receptor fragments may occur in the extracellular matrix in a vitamin D binding form and thereby sequester the vitamin from the cells. This may contribute to the inactivation of the negative control of chondrocyte differentiation. This concept was also proposed after a detailed analysis by immunoelectron microscopy of 1,25 D3 MARRS receptor distribution in the extracellular matrix in dental tissues and in an odontoblast cell line. 1,25 D3 MARRS receptor-positive membrane vesicle-like structures were localized to the extracellular matrix of MO6-G3 cells, but a proteolytic cleavage and/or the production of a soluble form of 1,25 D3 MARRS receptor was also taken into account (39). Both states, the association of full-length 1,25 D3 MARRS receptor to matrix vesicles budding into the extracellular matrix (40) and the occurrence of shed fragments far off the cells, however, lead to a decline of protein content on the chondrocyte surface, where the vitamin D signaling is mediated.

Finally, we found here that 1,25 D3 MARRS receptor constitutes a crucial restriction checkpoint of late differentiation that is targeted by IGF-I-induced cysteine proteinases of cranial chondrocytes. Proteolytic shedding of 1,25 D3 MARRS receptor or its inactivation by antibodies blocking vitamin D binding triggers chondrocyte size expansion and expression of collagen X, alkaline phosphatase, and MMP-13 as markers for overt hypertrophy. Candidates for the sheddases include the cysteine proteinases cathepsin D and L because both are expressed by growth plate chondrocytes and are thought to be associated with endochondral ossification. Cathepsin D immunostaining was limited to the hypertrophic zone adjacent to the osteochondral junction where the enzyme potentially is involved in cartilage remodeling into bone (16). However, a skeletal phenotype has not been described in cathepsin D-deficient mice, which renders cathepsin D activity less likely to be important in this context (41). Instead, cathepsin L, which also occurs in

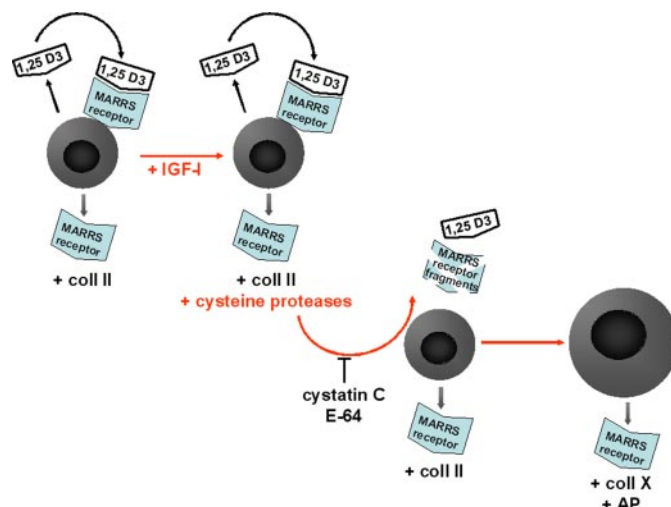


FIGURE 8. Schematic representation of events leading to hypertrophic differentiation in IGF-I-stimulated chondrocytes. 1,25-Dihydroxyvitamin D3 inhibits differentiation via 1,25 D3 MARRS receptor. IGF-I stimulation leads to secretion of cysteine proteinases. These enzymes shed 1,25 D3 MARRS receptor from the cell surface, and differentiation is triggered by fragmentation of the receptor. Cells become hypertrophic and produce collagen X. At any maturation stage the chondrocytes release full-length 1,25 D3 MARRS receptor into the medium.

hypertrophic cartilage, may be the primary catabolic enzyme necessary for cartilage-to-bone remodeling, at least in the absence of cathepsin D. Interestingly, however, cathepsin L is also found in proliferating and early hypertrophic zones, *i.e.* in regions where shedding of 1,25 D3 MARRS receptor is expected to be important for the progression of late chondrocyte differentiation. It is particularly intriguing that cathepsin L-deficient mice have widened and irregular growth plates, which, in turn, results in a reduction of trabecular, but not cortical, bone mass (42). This phenotype is entirely consistent with the notion that cathepsin L is essential for both systematic progression of cartilage differentiation at earlier stages and for remodeling of cartilage into bone at late stages of endochondral bone formation. Cathepsin L, which normally occurs in lysosomes, may well be secreted also by chondrocytes. In another context, secreted cathepsin L was shown to generate the angiogenesis inhibitor endostatin from its precursor, *i.e.* basement membrane-associated collagen XVIII (43).

In summary, we have shown that 1,25-dihydroxyvitamin D3 inhibits the development of overt hypertrophy of chicken chondrocytes *in vitro*, mediated by the cell surface receptor 1,25 D3 MARRS receptor. Cysteine proteinases, such as cathepsin L, are produced by IGF-I-stimulated cranial chondrocytes and are effectors of proteolytic shedding of this protein as an essential step of late chondrocyte differentiation (Fig. 8). It will be interesting to explore whether compromised vitamin D signaling through 1,25 D3 MARRS receptor is an early part of the multi-step etiology of degenerative joint diseases.

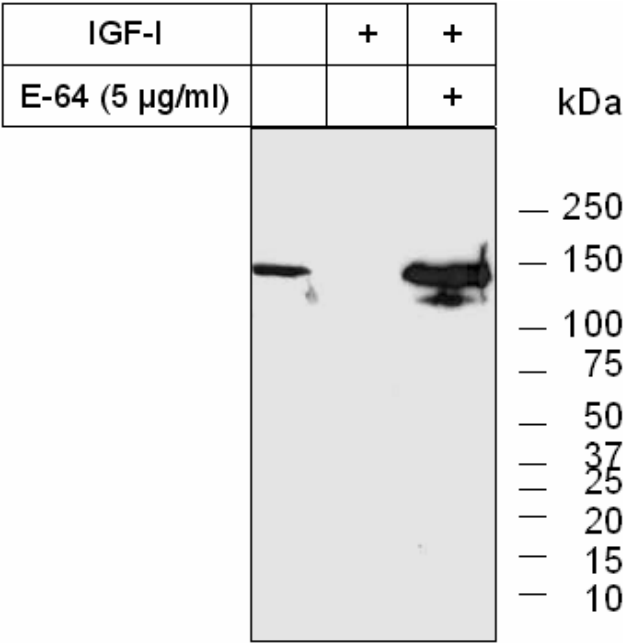
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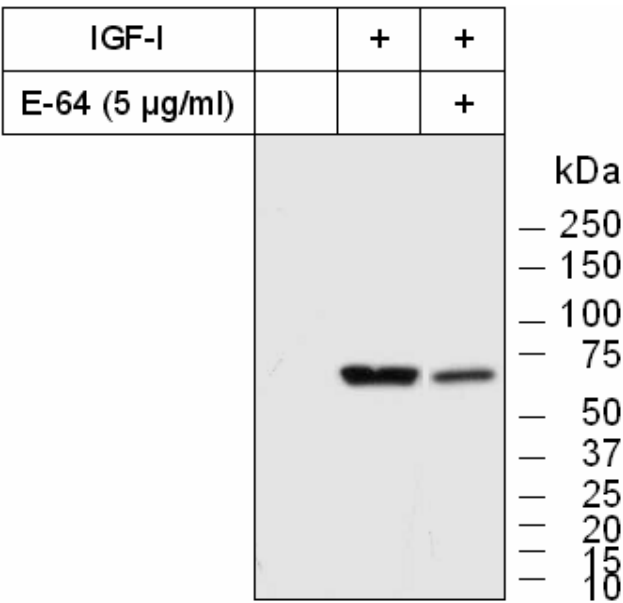
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Immunoblot against collagen II, MAB8887 (chemicon, 1:2000), medium TCA precipitated



Immunoblot against collagen X, (rabbit polyclonal, 1:1000), medium TCA precipitated



Terminal Differentiation of Chick Embryo Chondrocytes Requires Shedding of a Cell Surface Protein That Binds 1,25-Dihydroxyvitamin D3

Rita Dreier, Birgit Kathrin Günther, Tom Mainz, Ilka Nemere and Peter Bruckner

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