Expression of matrix metalloproteinases during vascularization and ossification of normal and impaired avian growth plate

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ABSTRACT: Enzymes of the matrix metalloproteinase (MMP) family regulate angiogenesis and are involved in the endochondral ossification process. Tibial dyschondroplasia (TD) and rickets are 2 disorders associated with impairments in this process, mainly in the vascularization of the avian growth plate. In this paper, we induced TD and rickets and studied the expression patterns of 4 members of the MMP family known to be important for endochondral ossification, MMP-2, 3, 9, and 13, in normal and impaired avian growth plates. The expression of MMP-3, 9, and 13 was reduced in the lesions and lined up parallel to the expulsion of blood vessels, which was extended up to the border of the lesion, but did not penetrate into it. Matrix metalloproteinase-2 was not expressed in the TD lesion but was overexpressed in the rachitic lesion. We also studied the differentiation stage of the chondrocytes populating the lesions and found that the rachitic lesions were populated with proliferative chondrocytes, whereas the TD lesions were filled with chondrocytes that presented both proliferative and hypertrophic markers. These results suggest that MMP-3, 9, and 13 play a role in the vascularization and ossification processes, whereas MMP-2 is related to chondrocyte differentiation and may be involved in cartilage remodeling in the avian growth plate.

Key words: chondrocyte, rickets, tibial dyschondroplasia

INTRODUCTION

The growth plates, where endochondral ossification takes place (Hunziker, 1994), are organized in several horizontal zones of chondrocytes: reserve, proliferative, prehypertrophic, and hypertrophic (Gerber and Ferrara, 2000). The avian growth plate contains longer columns of chondrocytes than its mammalian counterpart, more cells are found in each zone, and it is highly vascularized (Pines and Hurwitz, 1991; Praul et al., 2000). This vascularization requires matrix degradation (Bittner et al., 1998).

The matrix metalloproteinases (MMP) are a family of proteases whose substrates are the extracellular matrix (ECM) proteins (Massova et al., 1998; Murphy et al., 1999). The MMP family is involved in proteolytic cleavage and remodeling of the ECM (Vu, 2001) and regulate angiogenesis (Rundhaug, 2005). Twenty-four distinct MMP have been cloned in humans, but only MMP-1, 2, 3, 9, 10, 13, and 14 have been shown to be involved in endochondral ossification (Zhou et al., 2000; Malmud, 2006).

Disruptions in ossification can lead to skeletal abnormalities such as tibial dyschondroplasia (TD) and rickets. Tibial dyschondroplasia is characterized by the presence of a nonvascularized, nonmineralized lesion that extends from the epiphysis into the metaphysis of the proximal tibiotarsal growth plates (Leach and Nesheim, 1965, 1972). An efficient and common method for TD induction is the use of dithiocarbamates, such as thiram (Rath et al., 2004). Rickets, a syndrome characterized by expansion of the growth plate, develops when growing bones fail to mineralize. Nutritional rickets results from inadequate sunlight exposure or nutrient intake, in particular vitamin D, but also calcium or phosphorus (Nield et al., 2006).

In this paper, we studied the expression of 4 members of the MMP family in the vascularization of the growth plate.


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Table 1. Primers for collagen (Col) types II and X, osteopontin (OPN), and for matrix metalloproteinase (MMP)-2, MMP-3, MMP-9, and MMP-13

<table>
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<th>Gene</th>
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<th>Reverse, 5′ to 3′</th>
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<td>GTCCCCAGAAC</td>
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<td>CAAACTCCTG</td>
<td>GTCACCTAC</td>
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<td>X56772</td>
<td>CCACCTGGAT</td>
<td>TTCAATCCT</td>
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<td>GGAAGACCTG</td>
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Matrix metalloproteinases in avian growth plate

plate using 2 known avian disorders, thiram-induced TD and vitamin D deficiency-induced rickets.

**MATERIALS AND METHODS**

All procedures were approved by the Animal Care Welfare Committee of the Volcani Center of the Agricultural Research Organization.

Alcian blue, silver nitrate, levamisole, and thiram were purchased from Sigma Chemical (St. Louis, MO); digoxigenin 2′-deoxyuridine 5′-triphosphate was from Enzo (Mannheim, Germany); DIG RNA Labeling Mix, 4-nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were from Roche (Wiesbaden, Germany). The kit for immunohistochemistry of proliferating cell nuclear antigen (PCNA) was purchased from Zymed (San Francisco, CA).

**Animals and Induction of TD and Rickets**

Cobb strain broiler chicks (1 d of age; n = 150) were obtained from a commercial hatchery (Brown Hatcheries, Hod Hasharon, Israel). Chicks were raised for 22 d under the recommended temperature regimen and were fed as follows: the control group received standard diets according to National Research Council recommendations (n = 50); the second group (TD) received the same diet containing 50 mg/kg of thiram (n = 50), and the third group (rickets) received a diet with no vitamin D (n = 50); all groups had free access to food and water. The rickets group was housed in the dark. On d 3, 5, 8, 11, 15, and 22, the birds (n = 4 treatment−1 d−1) were killed by cervical dislocation, and the proximal growth plates of the right and left tibia were shaved longitudinally to determine the incidence and severity of TD and rickets. Sections were collected from the center of the bone for further analysis. Whole growth plates were collected and fixed in 4% paraformaldehyde for further analysis.

**Preparation of Probes**

Probes for in situ hybridization were prepared for chicken collagen (Col) types II and X, osteopontin (OPN), MMP-2, MMP-3, MMP-9, and MMP-13, using PCR amplification from cDNA of both chicken growth plates and primary cultured chondrocytes isolated from the proximal tibial growth plates (Table 1). Probe sizes ranged from 600 to 800 bp. The PCR products were ligated into pGEM constructs using the pGEM T Easy kit (Promega, Madison, WI) according to the protocol of the manufacturer, to be used as probes for in situ hybridization. Restriction enzymes were used to create sense or antisense probes. Antisense sequences for Col types II and X, OPN, MMP-3, and MMP-9 were cut with the restriction enzyme Nde1; sense sequences for those genes were cut with Nco1. Antisense for MMP-2 was cut with Neo1, and sense was cut with Nde1. Antisense for MMP-13 was cut with Apa1, and sense was cut with Sac1. All restriction enzymes were purchased from Fermentas (Glen Burnie, MD).

**Histological Staining, Immunohistochemistry, and In Situ Hybridization of Growth Plate Sections**

Growth plates were fixed overnight in 4% paraformaldehyde (Sigma) in PBS at 4°C. The samples were dehydrated in graded ethanol solutions, cleared in chloroform, embedded in Paraplast, and 5-μm sections were prepared. The mineralized bone was cut with a low-profile blade 819 (Leica, Wetzlar, Germany). Alcian blue and Von Kossa staining were performed with 0.6% Alcian blue 8 GX in 70% ethanol and with 2% silver nitrate exposed to sunlight. Alkaline phosphatase (Gentili et al., 1993) activity was detected with a substrate solution (NBT + BCIP) for the enzyme. For PCNA immunohistochemistry, sections were deparaffinized with xylene, washed twice in 100% ethanol, and treated with 3% H2O2 in distilled water for 30 min to block endoge-
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Figure 1. Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on endochondral ossification of the chicken tibia. Alcian blue and Von Kossa staining on d 8, 15, and 22 (7.5×).

Nons peroxidase activity. Next, the sections were rehydrated through a series (95, 80, 70%) of ethanol solutions, rinsed in 0.05% PBS-Tween, and incubated for 1 h in 3% normal goat serum in PBS-Tween. Sections were then incubated for 1 h with biotinylated mouse anti-PCNA primary antibody (Zymed) and washed 3 times for 10 min each in 0.05% PBS-Tween. After washing, sections were incubated with streptavidine peroxidase for 10 min and washed again with PBS-Tween. Next, sections were incubated with substrate solution for PCNA (5 mg 3,3-diaminobenzidine, 5 μL of H₂O₂ in 10 mL of double-distilled water) for 15 min and washed with double-distilled water.

The in situ hybridizations were performed as described by Reich et al. (2005). The sections were deparaaffinized in 100% xylene, rehydrated through a graded series (95, 80, 70%) of ethanol solutions, rinsed in distilled water (5 min), and incubated in 2× sodium chloride-sodium citrate buffer at 55°C for 30 min. The sections were then rinsed in distilled water and treated with proteinase K (10 μg/mL in 0.2 M Tris-HCl, 5 mM EDTA, pH 7.5) for 10 min. After digestion, the slides were rinsed with distilled water, fixed in 10% formaldehyde in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded (95, 80, 70%) ethanol solutions, and air-dried for several hours. The sections were then hybridized with digoxigenin-labeled antisense probes for Col II, Col X, or OPN or with 35S-labeled probes (10 ng) for MMP-2, 3, 9, or 13 (Reich et al., 2005). Digoxigenin-labeled probes were detected using a polyclonal antidigoxigenin antibody attached to alkaline phosphatase (ALP; Gentili et al., 1993), which, when it reacts with its substrate (NBT and BCIP), produces a color response. Endogenous ALP was inhibited with levamisole (Knopov et al., 1997). The sections were counterstained with Methyl green (Zymed) and photographed under bright field. Radioactive signals of 35S-labeled probes were intensified using a radiographic emulsion (Eastman Kodak Company, Rochester, NY). The sections were incubated with the emulsion solution for 1 mo in the dark at room temperature. After developing them, the sections were stained with hematoxylin for nuclear staining and photographed using bright or
dark field. No signal was observed in any of the hybridizations with sense probes, which were used as controls.

RESULTS

Induction of TD and Rickets

We used thiram to induce TD and vitamin D deficiency to cause rickets. After 22 d, 85% of the birds in the thiram group showed TD lesions in the proximal tibia, and 100% of the birds in the vitamin D-deficient group had rickets. All of the birds in the control group exhibited a normal phenotype.

The Endochondral Ossification Process in TD and Rickets

The effect of TD and rickets on the endochondral ossification process of the chicks was characterized by Alcian blue and Von Kossa staining of the cartilage matrix, for proteoglycans and minerals, respectively, on d 8, 15, and 22. In both syndromes, the bone was less mineralized than in the controls, as reflected by the thickened Alcian blue-stained area representing the cartilaginous growth plate (Figure 1). The extension from the growth plate of the TD lesions exhibited an asymmetric outline, whereas rickets was characterized by symmetrical expansion of the growth plate.

Chondrocyte Differentiation in TD and Rickets

We used several markers to determine chondrocyte differentiation status: the expression levels of Col II, a known marker of proliferative chondrocytes, and Col X, a known marker of hypertrophic chondrocytes, as well as of osteopontin (OPN), a gene expressed by hypertrophic chondrocytes and osteoblasts, were studied in TD and rickets by in situ hybridization analysis. In addition, the activity level of ALP (Gentili et al., 1993), which is a marker of hypertrophic chondrocytes and osteoblasts (Gentili et al., 1993), was examined in the syndromes. Representative slides from d 8 are presented in Figure 2.

Tibial dyschondroplasia had no effect on the proliferative chondrocytes, as can be seen by the expression pattern of Col II, which was similar to that of the control group (Figure 2). In contrast, an expansion of the proliferative zone was observed in rickets, because the Col II-expressing area was wider (Figure 2). The widening of the proliferative zone in rickets was followed by expansion of the hypertrophic zone, as can be seen by the location of Col X, OPN, and ALP in those slides (Figure 2). Although Col X is expressed only by hypertrophic chondrocytes, OPN and ALP are also expressed by osteoblasts; hence, Col X is expressed only in the growth plate, whereas the others are expressed in the trabecular bone as well. In contrast to rickets, TD affected mainly the hypertrophic chondrocytes; the Col X-expressing zone expanded asymmetrically (Figure 2), in accordance with the characteristics of TD lesion. Alkaline phosphatase activity was found in the TD lesion. Interestingly, although it is a hypertrophic marker, OPN was not expressed throughout the lesion but only in the center of it (Figure 2). To completely verify the differentiation status of the cells populating the TD lesion, we stained for PCNA, a marker for proliferating cells. Surprisingly, proliferating cells were found in the middle of the TD lesion, in the prehypertrophic Col X-expressing cells, whereas in the control group, there were no proliferating cells in this zone (Figure 3).

MMP Expression in TD and Rickets

We studied, in TD and rickets, the expression patterns of 4 members of the MMP family, which are known for their importance in the process of endochondral ossification, MMP-2, 3, 9, and 13 (Ortega et al., 2004), and compared them with normal growth plate, using in situ hybridization analysis.

Matrix metalloproteinase-2 (gelatinase A) is expressed in the compact bone, the perichondrium, and the proliferative zone, as well as in cells surrounding the blood vessels of the epiphysis and the bone (Figure 4). Hypertrophic chondrocytes do not express MMP-2. In agreement with this, the TD lesion, which originates from prehypertrophic and hypertrophic chondrocytes, did not express this gene (Figure 4). Note the very wide cartilaginous lesion in TD compared with the control. In contrast, rickets is characterized by an expansion of the proliferative zone; therefore, the MMP-2 expression area was very wide in this syndrome (Figure 4).

Matrix metalloproteinase-3 (stromelysin-1) is expressed in cells surrounding the blood vessels penetrating the hypertrophic and proliferative zones of the growth plate (Figure 5), as well as in hypertrophic chondrocytes, mainly those surrounding the blood vessels. Because vessels do not penetrate the TD and rachitic lesions, less MMP-3 expression was detected there. The distribution of MMP-3 mRNA differed in the 2 lesions: in both TD and rickets, its expression was stronger at the rim of the vessels compared with the control; however, whereas in the rachitic lesion the expression patterns were shaped like arrowheads; in TD they appeared truncated, probably in accordance with vessel morphology (Figure 5).

Matrix metalloproteinase-9 (gelatinase B) is expressed in cells surrounding the blood vessels in the bone and in the proliferative, hypertrophic, and epiphysial zones of the growth plate (Figure 6). In both TD and rickets, MMP-9 expression was less than in the control growth plate (Figure 6), indicating less blood penetration into it. Matrix metalloproteinase-9 expression demonstrated the expansion of blood vessels from the lesions in the 2 syndromes.

Matrix metalloproteinase-13 (collagenase-3) is expressed in cells surrounding the blood vessels of the bone as well as the epiphysis, proliferative, and hypertrophic zones of the growth plate and in hypertrophic
Figure 2. Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on chondrocyte differentiation stage of the chicken tibial growth plate on d 8. Expression of collagen type II (Col II), collagen type X (Col X), and osteopontin (OPN) was studied by in situ hybridization analysis. Alkaline phosphatase (ALP; Gentili et al., 1993) activity was studied with a kit for the detection of this enzyme. All micrographs were taken at 7.5x.

DISCUSSION

In this work, we studied morphology, differentiation stage, and MMP expression in 2 syndromes, TD and rickets, characterized by impaired vascularization of the growth plate. Tibial dyschondroplasia was induced by the addition of thiram in the diet of the chicks. Rickets was induced by the removal of vitamin D from the diet and housing the birds in the dark. Alcian blue and Von Kossa staining of representative growth plates from each group confirmed the induction of the disorders. In the thiram group, the asymmetric nonvascularized, nonmineralized lesion characteristic of TD developed, whereas in the vitamin D-deficient group, sym-

chondrocytes (Figure 7). Although it is a hypertrophic-specific gene, MMP-13 was not expressed in the TD lesion. Its expression stopped at the border of the lesion, together with the blood vessels. A similar expression pattern was seen in rickets (Figure 7).
Figure 3. Effect of tibial dyschondroplasia (TD) vs. control (Ctrl.) on chondrocyte proliferation of the chicken tibial growth plate. Proliferating cell nuclear antigen assay was performed on d 8. In the control group, few proliferating cells (brownish, stained nuclei) were found in the prehypertrophic zone (pre HZ), and no proliferating cells were found in the hypertrophic zone (HZ). In the TD group, proliferating cells were common in the pre HZ zone. The pre HZ is shown at greater magnification in the lower panel. Upper panel, 100×; and lower panel, 200×.

Figure 4. Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on matrix metalloproteinase (MMP)-2 expression. In situ hybridization was performed on d 5 and 8 with 35S-labeled riboprobes for avian MMP-2. All micrographs were taken under dark field illumination at 7.5×.
metrical cartilaginous extension of the growth plate was observed. The changes in the growth plate leading to rickets are very controlled, creating a lesion resulting from prolonged proliferation of the cells, whereas the changes leading to TD appear much more random, resulting in a lesion filled with cells in different stages of differentiation.

Rickets is a skeletal abnormality resulting from decreased bone mineralization, which is largely caused by deficiencies in calcium and vitamin D (Nield et al.,

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**Figure 5.** Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on matrix metalloproteinase (MMP)-3 expression. In situ hybridization was performed on d 8 with $^{35}$S-labeled riboprobes for avian MMP-3 (7.5×). Greater magnification of blood vessels shows the differential distribution of MMP-3 mRNA in these syndromes (40×). The 7.5× micrographs were taken under dark field illumination, whereas those at 40× were taken under bright field.

**Figure 6.** Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on matrix metalloproteinase (MMP)-9 expression. In situ hybridization was performed on d 5 and 8 with $^{35}$S-labeled riboprobes for avian MMP-9. All micrographs were taken under dark field illumination at 7.5×.
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It is characterized by expansion of the growth plate and inhibition of ossification. Vitamin D is critical for skeletal development and cellular function because of its effect on calcium homeostasis by promoting intestinal calcium absorption (Leanne et al., 2007). The TD lesion develops through an unknown mechanism. Visual observation is sufficient to support the concept that TD cartilage is not vascularized, but whether it is a result of defective blood vessels or damaged cartilage has yet to be determined. Two main paradigms are used to explain TD etiology: Rath et al. (2005) suggested that a metabolic dysfunction leads to the destruction of blood capillaries in the transition zone, whereas others suggested that the lesion occurs when prehypertrophic cells are unable to differentiate to hypertrophic chondrocytes (Praul et al., 2000; Webster et al., 2003). Orth and Cook (1994) also suggested that the primary event is chondrocyte-related, suggesting that the chondrocytes secrete an immature cartilage, which becomes highly cross-linked and is resistant to resorption and vascularization by the metaphyseal vessels. We recently suggested that the defective chondrocytes fail to synthesize and secrete MMP, and thus, the matrix is not properly degraded, less blood vessels penetrate into the growth plate, calcification is inhibited, and the nonvascularized, nonmineralized TD lesion is formed (Simsa et al., 2007b). In the current study, we found that the cells in the TD lesion express the hypertrophic markers Col X and ALP and that an island of cells in the center of the lesion expresses OPN. The cells do not express Col II, which is a marker for proliferative chondrocytes, but some cells undergo proliferation, as demonstrated by PCNA staining of the lesion. These results suggest that the cells in the TD lesion are not at a defined stage of differentiation and that they exhibit a pattern of gene expression characteristic of that observed with endoplasmic reticulum stress in growth plate chondrocytes (Tsang et al., 2007). It can be suggested that increased hypoxia due to the lack of metaphyseal blood vessel penetration triggers the endoplasmic reticulum stress (Leach and Monsonego-Ornan, 2007).

In this study, we characterized the expression pattern of 4 members of the MMP family, which are critical for proper endochondral ossification, MMP-2, 3, 9, and 13, in the normal chicken growth plate and in TD and rickets. The expression of MMP-2 described here is unique to avian species (Simsa et al., 2007a) and suggests a role for this gene in proliferative chondrocyte survival or function, or both. Tibial dyschondroplasia did not alter MMP-2 expression, whereas the MMP-2-expressing domain was markedly widened in the rachitic growth plate. Among the MMP examined, MMP-2 can serve as a marker to distinguish between TD and rickets, because the changes in its expression are most pronounced between those 2 syndromes.

Matrix metalloproteinase-3 (stromelysin-1) has broad substrate specificity, including proteoglycans, casein, fibronectin, and Col III, IV, and V. Besides digesting ECM components, MMP-3 activates several pro-MMP, such as pro-MMP-1 and pro-MMP-9 (DeClerck

![Figure 7. Effect of tibial dyschondroplasia (TD) and rickets vs. control (Ctrl.) on matrix metalloproteinase (MMP)-13 expression. In situ hybridization was performed on d 5 and 8 with 35S-labeled riboprobes for avian MMP-13. All micrographs were taken under dark field illumination at 7.5×.](image-url)
and Laug, 1996). The expression of MMP-3 in hypertrophic chondrocytes is probably associated with its ability to degrade proteoglycans (it is the most potent proteoglycanase among all the MMP), and it is therefore important for endochondral ossification (Armstrong et al., 2002; Visse and Nagase, 2003). There is little information on the expression of this gene in the avian growth plate (Simsa et al., 2007a). Our finding regarding its unique expression pattern at the edge of the blood vessels in TD and rickets suggests its involvement in vascularization of the chicken growth plate.

Matrix metalloprotease-9 is expressed in cells surrounding the blood vessels in the bone and in the proliferative, hypertrophic, and epiphyseal zones of the growth plate. This enzyme is a key regulator of growth plate angiogenesis, as described in mouse (Vu et al., 1998) and avian (Tong et al., 2003; Simsa et al., 2007a) growth plates. Its expression in TD and rickets was lower than in the control growth plate, in agreement with the known occurrence of less vascular penetration into the damaged growth plates (Leach and Nesheim, 1965, 1972).

The localization of MMP-13 mRNA suggests that its expression in cells surrounding the blood vessels is associated with a role in vascular penetration into the growth plate. Furthermore, in TD and rickets, its expression stopped at the border of the lesions, together with the blood vessels. The expulsion of MMP-9 and MMP-13 expression, in parallel to the expulsion of the blood vessels, suggests that the MMP play a role in the vascularization impairments in TD and rickets. These results are complementary to our previous findings showing downregulation of MMP after thiram exposure in avian growth plate chondrocytes both in vivo and in vitro (Simsa et al., 2007b) and support our hypothesis that one of the early events in TD is failure of the defective chondrocytes to synthesize and secrete MMP, causing improper degradation of the matrix, less blood vessels penetrating the growth plate, inhibition of calcification, and formation of the nonvascularized, nonmineralized TD lesion (Simsa et al., 2007b). The extreme phenotype in mice lacking both MMP-9 and MMP-13 that exhibits severely impaired endochondral bone, characterized by diminished ECM remodeling and delayed vascular recruitment (Stckens et al., 2004), has characteristics resembling both TD and rickets lesions, supporting our suggestion that MMP are involved in the development of those syndromes.

An important finding of this work was the undefined differentiation stage of the chondrocytes populating the TD lesion. It is possible that because of this intermediate differentiation stage, the chondrocytes fail to send signals for blood penetration into the growth plate. As a result, the endothelial cells surrounding the blood vessels express less MMP. Together, these 2 events could lead to the formation of a nonvascularized lesion.

LITERATURE CITED


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