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Collagen Fibril Growth During Chicken Tendon Development: Matrix Metalloproteinase-2 and Its Activation

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Abstract

The role of matrix metalloproteinases (MMPs) in collagen fibrillogenesis during development was studied in the well-characterized chicken metatarsal tendon. Collagen fibrils are initially assembled as intermediates and the mature fibrils assemble by linear and lateral growth from intermediates. We hypothesize that this involves the turnover of fibril-associated molecules mediated by expression and activation of matrix metalloproteinase-2 (MMP-2). We demonstrate changes in the ratio of full-length to truncated MMP-2 during tendon development, consistent with enzyme activation. The level of full length proMMP-2 remains relatively unchanged, however, the truncated form of MMP-2 is highest prior to and during fibril growth. Membrane type matrix metalloproteinase-3 (MT3-MMP, MMP-16) is fibroblast-associated and involved in the regulation of MMP-2 as well as in direct matrix turnover. The ratio of full-length proMT3-MMP/truncated (active) MT3-MMP has a pattern similar to that of full-length proMMP-2/truncated (active) MMP-2 during tendon development. Regulation of proMMP-2 activation involves complex formation with active MT3-MMP and TIMP-2. The constant low TIMP-2 expression seen in tendon development is consistent with this role. Isolation of collagen fibrils from pre-fibril growth tendons (14 day) in the presence of activated MMP-2 is associated with premature fibril growth seen as increased fibril diameters compared to controls. These data implicate MMP-2/MT3-MMP in the initiation of and progression of fibril growth, matrix assembly and tendon development. This may involve turnover of fibril-associated molecules involved in regulating linear and lateral growth, such as small leucine-rich proteoglycans and fibril-associated collagens. Activation of proMMP-2 dependent on MT3-MMP would allow a focal control of turnover.

Keywords

Matrix Metalloproteinases; MMP-2; Collagen Fibril Growth; Tendon; Development

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INTRODUCTION

Matrix metalloproteinases (MMPs) are important during development, normal matrix turnover and repair as well as processes such as chronic inflammation, metastasis and embryonic implantation (Fairbairn et al. 1985; Mignatti et al. 1986; Woessner, Jr. 1994; Birkedal-Hansen 1995; Brinckerhoff and Matrisian 2002). The MMPs are a family of matrix degrading proteinases with broad substrate specificity and are capable of degrading virtually all matrix components (Nagase and Woessner, Jr. 1999; McCawley and Matrisian 2001; Brinckerhoff and Matrisian 2002). Extracellular matrix turnover and remodeling are slow processes in mature tissues, but matrix remodeling is essential during development and growth. Elucidating the roles of both assembly and turnover during collagen fibrillogenesis and matrix assembly is essential to developing an understanding of how continuously functional connective tissues, such as tendon, grow and remodel.

MMP-2 and MMP-9 (Gelatinases A and B), have activity against a host of traditional matrix components, including denatured collagen molecules (gelatin) and native fibrillar and non-fibrillar collagens, laminin, aggrecan and vitronectin (Aimes and Quigley 1995; Woessner, Jr. and Nagase 2000; McCawley and Matrisian 2001). MMP-2 (72-KDa gelatinase, gelatinase A) also can degrade decorin (Imai et al. 1997) and activate other MMPs as well as growth factors (McCawley and Matrisian 2001). Mice deficient in MMP-2 do not display significant deficiencies during development and growth (Itoh et al. 1997). However, increased MMP-2 levels observed associated with decreased uptake in the absence of thrombospondin 2 were associated with altered collagen fibril structure and tendon organization during development (Kyriakides et al. 1998; Bornstein et al. 2000; Yang et al. 2000; Bornstein et al. 2004).

MMPs with a transmembrane domain (MT-MMPs), are activators of MMP-2 (Takino et al. 1995; Shofuda et al. 1998; Shimada et al. 1999). MT3-MMP (MMP-16) is widely expressed and is capable of degrading many extracellular matrix components including type III collagen (Matsumoto et al. 1997; Shimada et al. 1999). MT3-MMP is localized at the cell surface. Unlike other MMPs, MT-MMPs are rapidly degraded by autolysis after maturation to prevent excess activity because unlike other MMPs, these enzymes are intracellularly activated by furin or a similar serine proteinase (Sato et al. 1996). It has been shown by in situ hybridization that chicken MT3-MMP is expressed at high levels in a number of developing tissues (Yang et al. 1996; Huh et al. 2007). The different tissue distributions for individual MT-MMPs suggest tissue-specific roles in extracellular matrix turnover (Shimada et al. 1999). The MT-MMPs are likely to play important roles in localized hydrolysis and controlled site-specific activation of other MMPs.

Activated MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs). TIMP-2, but not TIMP-1, has been shown to stimulate MT-MMP-mediated activation of MMP-2 on the cell surface. Activation occurs at low TIMP concentrations because binding of MMP-2 to MT-MMP increases, but inhibits the same processes at higher concentrations where MT1-MMP is saturated by TIMP-2 leaving no free MT-MMP available for MMP-2 activation (Strongin et al. 1995; Butler et al. 1998). Therefore, cellular activation of MMP-2 is dependent on concentration, and the balance between free MT-MMP and MT-MMP/TIMP-2 complex (Butler et al. 1998).

During tendon development, MMPs have a potential role in the turnover of fibril-associated macromolecules involved in the regulation of collagen fibril assembly. Fibrils are initially assembled as short, small diameter intermediates. These immature intermediates are the building blocks of the mature fibril (Birk and Trelstad 1986; Birk et al. 1989; 1990; 1991; Birk and Zycband 1994; Kadler et al. 1996; Graham et al. 2000). Fibril intermediates were characterized in tendon and other connective tissues, including cornea and dermis (Birk et al.

1989;1995;1996; Holmes et al. 1994). During tendon development, intermediates are incorporated into fibers and maintained. However, during a narrow period of chicken tendon development (17–18 days) the intermediates mature to form long fibrils of indeterminate length characteristic of the mature, mechanically functional tendon (Birk et al. 1995; 1997). The assembly of intermediates into longer, continuous fibrils with larger diameters allows for growth and dramatically increases the tensile strength of tissues such as the tendon. The controlled expression, activation or inhibition of the MMPs during development may alter the surface of fibril intermediates and/or the inter-fibrillar matrix thereby regulating their growth. Decorin, other closely related leucine-rich repeat proteoglycans, fibril-associated collagens types XII and XIV and amino-terminal domains of fibrillar collagens all interact with the fibril surface and have been implicated with the regulation of tendon fibrillogenesis (Birk and Mayne 1997; Iozzo 1999; Ezura et al. 2000; Zhang et al. 2003; 2005). These regulatory matrix macromolecules are all substrates for MMP-2/MT3-MMP, implicating these enzymes in the focal control of matrix turnover required during tendon development.

The present study examined the levels of MMP-2 in developing chicken metatarsal tendons. The levels were analyzed at different periods in the fibril growth phase; i.e., prior to, during and after. Our hypothesis is that tightly regulated, focal MMP activity is required for linear and lateral growth of fibrils from immature intermediates. This involves MMPs, such as MMP-2 and MT3-MMP as well as TIMPs. Our results indicate that the beginning of tendon fibril growth (14 day) is associated with the presence of both full-length and truncated forms of MMP-2 and MT3-MMP, consistent with enzyme activation. The amount of the proforms remains the same, but the truncated forms disappear towards the end of fibril growth (19 day). Ex vivo studies indicate that MMP activity may be involved in the removal of a component(s) from the immature fibril surface thus allowing adjacent fibril intermediates to associate and the fibril growth phase to proceed.

MATERIALS AND METHODS

Extraction of MMPs from metatarsal tendons

White Leghorn chicken embryos were obtained from Spafas (Preston, CT). For extraction of MMPs, chicken metatarsal tendons were dissected from staged embryos (Hamburger and Hamilton 1951) and washed 3 times with PBS. Soluble proteins were extracted from tendon by homogenization with an extraction buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1mM CaCl₂, 1 μM ZnCl₂, 0.1% Triton X-100, and 0.05% sodium azide. Homogenates were centrifuged at 13,000 rpm for 30 min at 4°C and the supernatant was collected. Extracts were analyzed using zymography and immuno-blots for MMPs. Samples were normalized for total protein or constant wet tendon weight. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, USA). Normalization for constant wet tendon weight or protein concentration yielded comparable, virtually identical results. During this period of development tendon cells are non-proliferative (Nurminskaya and Birk 1998).

Zymography

Tendon extracts were analyzed using zymography as reported (Hibbs et al. 1987). Briefly, SDS-polyacrylamide gels (7.5 or 10%) were prepared and gelatin (from bovine skin; Sigma; St. Louis, MO) or casein (Sigma) was included in the gels at a concentration of 1%. Equal amounts of extract normalized for protein (30μg) or wet weight were separated by SDS-PAGE under non-reducing conditions. After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 30 min, washed in dH₂O and then incubated in 50 mM Tris, pH 7.6, 10 mM CaCl₂ overnight at 37°C. Enzymatic activity was identified, after the gel was stained with Coomassie Brilliant Blue (Sigma) and destained.

Collagenase activity for type I collagen

Collagenase assays were done as described previously (Levine et al. 1984). Samples of tendon extracts normalized for constant wet weight, were activated with 2 µg trypsin for 5 min at 37°C, followed by addition of 2 µg of soybean trypsin inhibitor (Sigma). The assays were performed in a final volume of 300 µl containing 30 µg of [³H]-collagen, 50 mM Tris-HCl (pH 7.8), 20 mM NaCl, 10 mM CaCl₂, 20 µg/ml BSA, and 35 µl of activated or unactivated tissue samples. The reaction was carried out for 2 h in a 37°C shaking water bath and terminated by the addition of Na₂EDTA. To further degrade the partially digested collagen, 100 µg of chymotrypsin (Sigma) and 100 µg of trypsin (Sigma) were added and incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.4 mg/ml BSA and 1 volume of cold 20% trichloroacetic acid on ice. The undigested collagen in the precipitate was collected on glass fiber filters, washed and radioactivity was determined. In each assay the control samples, unactivated and activated samples were run in triplicate and processed in parallel. Collagenase activity was expressed as the difference between the control and experimental sample.

Immuno-blot analysis

Analyses were performed using standard techniques (Huh et al. 2007). Equal amounts of protein lysates (30 µg) were separated using SDS-PAGE under non-reducing conditions and the proteins were transferred electrophoretically onto nitrocellulose membranes (0.2 µm, Schleicher and Schuell). The membranes were blocked in the presence of 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h to minimize nonspecific binding. Antibodies against MMP-2 were purchased from Chemicon (Temecula, CA), MT3-MMP from Calbiochem (San Diego, CA), and TIMP-2 from Sigma (St. Louis, MO). Primary antibodies were diluted in TBS-T containing 5% dry milk and then incubated for 1 h and detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or goat anti-mouse IgG for 1 h. Specific antibody binding was visualized using an enhanced chemiluminescence detection kit (Amersham, Biosciences) with X-ray film exposure.

Ex vivo studies of fibril growth

Metatarsal tendons were dissected from 14 day White Leghorn embryonic chickens and cultures established as previously described (Doane and Birk 1991). Briefly, tendons were incubated with 200 units/ml bacterial collagenase (Sigma) in Minimal Essential Medium (Gibco Laboratories) with sodium bicarbonate buffer, 50 µg/ml gentamicin and 2.5 µg/ml fungizone (CMEM) for 2 h at 37°C with trypsin added to a final concentration of 0.25% after 1 h. Tendons were filtered through a Swinex filter with Spectropor mesh (105 µm). The cell suspension was centrifuged at 1,000 rpm for 5 min, the pellets resuspended in MEM with 20% fetal bovine serum, and plated in tissue culture flasks with MEM with 10% fetal bovine serum, with 0.05 mg/ml ascorbate added to the medium after 48 h. The cultures were washed 3 times with serum free MEM followed by culture for 24 h in serum free media. The conditioned media was collected, centrifuged to remove cell debris and stored frozen. Prior to use, the conditioned media was activated with 1mM aminophenylmercuric acetate (APMA).

The effects of MMP-2 on fibril growth were analyzed after isolation of tendon fibrils with conditioned or control media. Activated media was treated with APMA and contained predominately MMP-2 activity. Controls included activated media with EDTA, a non-specific MMP inhibitor and MEM. Fibril intermediates were isolated from 14 day chicken embryo tendons essentially as described (Birk et al. 1995; 1996). Briefly, White Leghorn chicken embryos were obtained from Spafas (Preston, CT) and staged (Hamburger and Hamilton 1951). Metatarsal tendons at 14 days of development were dissected, washed in PBS (pH 7.2) at 4°C followed by homogenization in conditioned or control culture media with a Dounce homogenizer. The tendon homogenate was incubated in media for a total of 4 h at 37°C, centrifuged at 16,000 × g for 20 sec and the supernatant containing extracted fibrils was

removed and analyzed for these experiments. The fibrils present at this stage of tendon development are homogeneous, with small diameters (Birk et al. 1990; 1995; Birk and Mayne 1997; Ezura et al. 2000).

Fibrils treated with conditioned media or control preparations were analyzed by transmission electron microscopy. Fibrils were absorbed onto butvar/carbon coated grids. The grids were washed with PBS and negatively stained with 1% phosphotungstic acid (pH 7.0). Specimens were then examined and photographed using a Philips CM10 transmission electron microscope operating at 80 kV. Calibrated micrographs were randomly selected from each experimental group in a masked manner. Fibril diameters were measured from 6 independent groups. The differences between the experimental and each control group were analyzed using a t test. Fibril diameters also were determined from day 14 and day 20 chicken embryo tendons. Tendons were processed for electron microscopy as previously described (Birk and Trelstad 1986). Fibril diameters were measured from transverse sections as previously described (Ezura et al., 2000).

RESULTS

MMP-2 expression in developing tendons

The MMPs present in tendon extracts and the developmental patterns of appearance were analyzed using gelatin zymograms. Extracts from both 14- and 19-day tendons contained a band of zymogram activity with a mobility of 70 kDa consistent with proMMP-2. In addition, a second gelatinolytic band was present with a mobility about 8 kDa less in apparent molecular weight, consistent with the truncated active form of MMP-2 (Fig. 1a). In the 14- and 19-day tendon extracts, proMMP-2 was observed in comparable amounts. In contrast, the truncated form of MMP-2 predominated in 14-day tendon extracts. The 14-day tendon extracts contained approximately five fold more activated form than the 19-day extracts. In addition, no 92-kDa gelatinase activity, consistent with MMP-9, was detected in either stage of tendon development. Positive controls of rabbit corneal fibroblast conditioned medium containing MMP-9 were included on the zymograms (data not shown) (Matsubara et al. 1991).

The identities of the gelatinolytic activities with a relative mobility of ~70 and 62 kDa, observed by zymography, was confirmed using immuno-blot analysis and an antibody against MMP-2 (Fig. 1b). The immuno-blots showed reactivity of the putative proMMP-2 and MMP-2 bands with the anti-MMP-2 antibodies, thus confirming their identity. Higher molecular weight bands reactive with anti-MMP-2 antibody were present with approximately equal densities in both 14 and 19 day tendon extracts. These bands may represent proMMP-2 complexes with other molecules. In addition, lower molecular weight MMP-2 reactive bands were observed. These probably represent degradation products with the faster migrating bands representing the MMP-2 pro-domain. Consistent with the zymogram, no reactivity was observed with antibodies against MMP-9 (data not shown).

To define the developmental expression pattern of MMP-2, tendon extracts were analyzed between embryonic days 14 and 20 using zymography. As shown in Fig. 2, there was a significant decrease in the ratio of proMMP-2/truncated MMP-2 activity from 14 to 19 days of tendon development, consistent with enzyme activation. The ratio of MMP-2 forms was further defined relative to stages in tendon fibril growth. A ratio consistent with enzyme activity was highest in tendons from 14-day embryos. At day 15 there was a consistent decrease in the activity ratio. The activity ratio reached a plateau and remained high between day 15 and day 18 of tendon development (pre fibril growth and growth phase). The activity ratio decreased significantly from 18 to 20 days of development. There was a plateau at a low enzyme level in 19 and 20 day tendons. Across all developmental stages studied (day 14 to 20) the proMMP-2

activity band was low and comparable. The observed differences in band ratio were primarily due to changes in activated MMP-2.

Type I Collagenolytic/MMP-3 activity in developing tendons

Type I collagenolytic activity was determined at 14- and 19-days of tendon development. Both 14- and 19-day tendon extracts exhibited type I collagenolytic activity, the activity from 14-day tendons was greater than that from 19-day both in tendon extracts that were untreated or treated with soybean trypsin inhibitor to activate latent enzyme. The differences were approximately five fold in unactivated and more than two fold in activated extracts, respectively (Fig. 3). The expression pattern was similar to that of MMP-2. To analyze MMP-3 (stromelysin) in tendons during different stages of development, extracts underwent electrophoresis on casein zymograms. Both 14- and 19-day tendons contained easily detectable levels of caseinolytic activity. However, this corresponded to MMP-2, no activity was seen in the ~45-55-kDa range expected from the mammalian MMP-1 (collagenase)/MMP-3 (stromelysin) enzymes (data not shown). In addition, zymograms were prepared using fibronectin, laminin and a mixture of decorin and lumican. These matrix components are all substrates for mammalian MMP-3, but chicken tendon extracts demonstrated no activity in the ~45-55-kDa range. This was in contrast to the mammalian enzyme, included as a positive control, that demonstrated activity with an apparent molecular weight of 51-kDa (data not shown).

MT3-MMP (MMP-16) and TIMP expression correlates with MMP-2 activation in developing tendon

High activity levels of MMP-2 identified on zymograms and confirmed by immuno-blot analysis (Fig. 1) were associated with the period of rapid fibril growth at 14 to 18 days of development. During development, activation of MMPs must be a tightly controlled process and focal control of activation involves cell surface associated MT-MMPs and TIMPs. MT3-MMP protein expression was analyzed using immuno-blots to address its role in regulation of MMP-2 activation (Fig. 4a). Both proMT3-MMP and MT3-MMP were detected in 14- and 19-day tendon extracts. Increased levels of the active form, intermediate forms as well as the pro-form of MT3-MMP were consistently observed in 14-day compared to 19-day tendon extracts. In addition, strong reactivity with high molecular weight forms of MT3-MMPs were only detected in 14-day tendon extracts. These forms remain unidentified, but may represent complexes of MT3-MMP with proMMP-2 and/or TIMP-2. Moreover, relatively weak reactivity with low molecular weight forms (presumably the result of autolysis) also were detected only in extracts from 14-day tendon. Based on high levels of truncated MT3-MMP, we postulate that MT3-MMP is involved in converting proMMP-2 to active MMP-2 during the transition to fibril growth in the developing tendon.

TIMP-2 expression was investigated at 14 and 19 days of tendon development using immuno-blot analysis. TIMP-2, but not TIMP-1, was shown to stimulate MT-MMP-mediated activation of proMMP-2 on the cell surface at low concentration (Strongin et al. 1995; Butler et al. 1998). The level of TIMP-2 expression was comparable in both day 14 and 19 tendon extracts (Fig 4b). This is consistent with a potential role of TIMP-2 in proMMP-2 activation. At low concentrations of TIMP-2, it is likely that proMMP-2 activation is positively regulated by TIMP-2 in the presence of high levels of MT3-MMP expression at day 14 compared to at day 19.

MMPs initiate lateral fibril growth ex vivo

To address the role of MMP-2 in tendon fibrillogenesis, fibrils from 14-day chicken embryos were isolated in MMP-2 containing conditioned medium. Like the tendon extracts, the conditioned media from 14-day tendon fibroblasts contained predominantly MMP-2 activity

(Fig. 5a,b). No MMP-9 or other activity was observed using gelatin and casein zymography (Fig. 5b). Chicken tendons at 14 days of development are composed of a homogeneous population of small diameter fibrils. In contrast, 19–21 day tendons contain a heterogeneous population of larger diameter fibrils undergoing lateral growth associated with maturation of the tendon (Fig. 5c,d). The presence of MMP-2 during fibril isolation yielded fibrils that were in the lateral fibril growth stage. In contrast, fibrils isolated in the absence of MMPs or with MMPs and EDTA had not started fibril growth (Fig. 5e,f). The isolated fibrils under all conditions were intact with a characteristic 67nm banding pattern. The fibrils isolated in control media, with no MMP-2 or inactive MMPs, had mean diameters of $35.8 \pm 7.9\text{nm}$ (24 to 58 nm) and $39.4 \pm 6.9\text{nm}$ (21 to 66 nm), respectively (Fig. 5f). In addition, the fibril diameter distribution was symmetric and characteristic of that seen in 14-day tendons. In contrast, fibrils isolated in the presence of active MMP-2 had significantly larger diameters than when no MMP-2 was present, i.e., MEM ($p < 0.001$) or activated media with EDTA ($p < 0.01$). These fibrils had mean diameters of $46.3 \pm 9.5\text{nm}$ (26 to 75 nm). The fibril diameter distribution was not symmetric, was skewed to the right, i.e., larger diameters and had a broader range. These data are comparable with that seen in 20-day tendons where fibril growth has occurred (Fig. 5h).

DISCUSSION

The role of MMPs in collagen fibrillogenesis during tendon development was studied using the well-characterized chicken metatarsal tendon model. Our studies demonstrated significant changes in the ratio of MMP-2 full-length, pro-forms to truncated, active-forms during tendon development consistent with changes in enzyme activity; this is correlated with entrance into and progression through fibril growth stages. Fibril growth stages are periods of rapid matrix assembly and tendon growth. The ratio of truncated to full-length MMP-2 and MT3-MMP was higher prior to and during fibril growth than at the later stages characteristic of mature fibrils. This correlation implicates MMP-2/MT3-MMP in the initiation of and progression of normal collagen fibril growth, matrix assembly and tissue development.

Our data demonstrate high levels of MMP-2 activity associated with tendon fibril growth suggesting a key role for MMP-2 in this important developmental event. In the thrombospondin 2-deficient mouse there is an abnormal increase in MMP-2 activity. This altered expression pattern is associated with changes in cellular and matrix organization as well as abnormal collagen fibril structure in the developing tendon (Bornstein et al. 2000; 2004; Yang et al. 2001). The fibrils have larger diameters and irregular contours (Kyriakides et al. 1998) consistent with altered lateral fibril growth. This dysfunctional regulation is consistent with our data indicating abnormal lateral growth when 14 day fibrils are isolated in the presence of MMP-2. These data support an important role for regulated MMP-2 activation during tendon development. These conclusions are not supported by findings from the mouse MMP-2-deficient model, where growth was not significantly impacted (Itoh et al. 1997). However, the potential compensation by other MMPs in the absence of MMP-2 can not be excluded in this model.

The ratio of MT3-MMP forms had a similar pattern to that observed for MMP-2, i.e., high truncated form at 14 days and low at 19 days of tendon development. The observed developmental changes correlate with the amount of MMP-2 truncated form detected in tendon extracts. Since the proMMP-2 levels were relatively constant, this suggests that activation of MMP-2 may be the regulatory step. Our data suggest a role for MT3-MMP in activation of proMMP-2 during tendon development. proMMP-2 binds to TIMP-2 bound to MT-MMP by forming a complex at the cell surface where the uninhibited free MT-MMP then cleaves and partially activates the tethered proMMP-2 (Strongin et al. 1995; Deryugina et al. 1997; Nagase 1998). A residual portion of the MMP-2 propeptide is removed by another MMP-2 molecule

at the cell surface to yield a fully active, mature form of MMP-2 which can then be released from the cell surface. Active MMP-2 also can be inhibited by another TIMP-2 molecule or left in an uninhibited active state depending on local MMP-2:TIMP-2 molar ratios (Strongin et al. 1995; Deryugina et al. 1997; Nagase 1998). In normal developing tendon in vivo, we assume that TIMP-2 expression levels at day 14 are low enough to activate MMP-2 because high levels of both active MMP-2 and MT3-MMP were detected. Our data also demonstrate relatively low TIMP-2 concentrations, presumably to levels that favor MMP-2 activation, but constant expression of TIMP-2 across the developmental period studied. During tendon development, this correlation between MMP-2 activity and MT3-MMP expression level and the constant low expression of TIMP-2 indicated that there may be focal regulation of matrix turnover involved in the regulation of fibrillogenesis.

MT-MMPs are capable of degrading many extracellular matrix components including native collagen, adhesive glycoproteins and growth factors (d'Ortho et al. 1997). Degradation of collagen type III was very efficient with MT3-MMP (Matsumoto et al. 1997; Shimada et al. 1999). Type III collagen was expressed early in chicken tendon development, during initial assembly of fibril intermediates, but prior to fibril growth steps (Birk and Mayne 1997) and removal of type III collagen was proposed to be required prior to linear and lateral fibril growth. Taken together, our studies suggest that MT3-MMP play an important role in extracellular matrix turnover not only by activating MMP-2, but also by acting directly on extracellular matrix macromolecules, including type III collagen. Our results demonstrate a collagenase activity with an expression pattern similar to the MMP-2. Collagenase (MMP-1) can degrade the fibrillar collagens as well as others and promote the activation of MMP-2 (Crabbe et al. 1994). Potentially these MMPs may work together to facilitate the turnover of type III collagen and other matrix components. Alternatively the reported collagenase activity could be the result of the known collagenolytic activity of both MMP-2 (Aimes and Quigley 1995) and MT3-MMP (Shimada et al. 1999). Our model of fibrillogenesis, from preformed intermediates, does not include the turnover of entire collagen fibrils, only associated molecules. Therefore, we favor the later explanation where collagenase activity present is secondary to the activity required for turnover of fibril-associated molecules.

The data suggest that MT3-MMP has crucial roles in tissue remodeling during tendon development, both as an activator of proMMP-2 and as a membrane-associated enzyme capable of degrading a spectrum of extracellular matrix molecules. In addition, its presence at the fibroblast surface provides a mechanism to localize MMP-2 activity. Therefore, MT3-MMP provides turnover directly, local activation of other enzymes and the compartmentalization of these processes at the fibroblast surface. These important functions suggest that MT3-MMP is a key regulator of matrix turnover during development. The MT1-MMP-deficient mouse supported this suggestion. In this model, where MMP-2 and TIMP-2 were normal, there were severe developmental defects in a spectrum of connective tissues, including bone, cartilage, teeth and soft connective tissues (Holmbeck et al. 1999; 2004; Zhou et al. 2000). Postnatal growth was severely affected in the MT1-MMP-deficient mice. In the mouse, the stage of linear and lateral fibril growth begins around postnatal day 10 (Ezura et al. 2000; Zhang et al. 2005) and presumably the coordinate activity of MMP-2 and MT3-MMP is involved in the regulation of this transition.

Our previous studies of tendon fibril growth have demonstrated that the fibril intermediate was the precursor to the mature, continuous collagen fibril. Day 14 embryonic tendons are composed of small diameter, short (~10µm) fibril intermediates (Birk et al. 1989). This is a rapidly growing stage with a high rate of collagen synthesis and fibril assembly. From day 14 to 19, the fibril intermediates increase in length and diameter, forming mature fibrils (Birk et al. 1995; 1996; 1997). We hypothesized that fibril growth involves lateral associations and fusion of intermediates associated with an alteration in the fibril surface and/or interfibrillar

matrix (Birk et al. 1995). In the chicken, from day 14 to day 20, there are changes in a number of fibril-associated molecules including type III collagen (Birk and Mayne 1997), decorin (Birk et al. 1995), fibromodulin (Nurminskaya and Birk 1996) and type XIV collagen (Young et al. 2000). We were unable to detect stromelysin-1 activity during this period of tendon development. This enzyme has activity toward numerous fibril-associated and interfibrillar molecules, including proteoglycans and fibril-associated collagens (McCawley and Matrisian 2001). MMP-2 and MT3-MMP have broad substrate specificity, including the fibril-associated molecules or members of these classes found in developing tendon (Imai et al. 1997; Woessner, Jr. and Nagase 2000; McCawley and Matrisian 2001). This substrate specificity of MMP2/MT3-MMP and lack of stromelysin further support its role in matrix turnover during tendon fibrillogenesis.

Overall, the present data indicate that collagen fibril growth during chicken tendon development both, in vivo and in vitro, involves MMP-2 dependent mechanisms. These data implicate MMP-2/MT3-MMP in the initiation and progression of collagen fibril growth, matrix assembly and tendon development. This could involve the turnover of fibril-associated molecules involved in regulating linear and lateral fibril growth, such as small leucine-rich proteoglycans and fibril-associated collagens. Turnover dependent on the activation of proMMP-2 by fibroblast-associated MT3-MMP would allow for a regulated, focal control of turnover.

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Abbreviations Used

MMP	matrix metalloproteinase
MT-MMP	membrane type-matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinases
EDTA	ethylenediaminetetraacetic acid
APMA	aminophenylmercuric acetate

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Figure 1. Alteration in the ratio of full-length to truncated MMP-2 forms in developing tendons
(a) Gelatinolytic activities from chicken embryo tendon extracts from 14-day and 19-day tendons were analyzed by zymography on gelatin-containing 10% SDS-PAGE gels. Activity with a relative mobility consistent with proMMP-2 and activated MMP-2 was present in extracts from both 14- and 19-day tendons. There was no activity corresponding to MMP-9 (92-kDa gelatinase) at either developmental stage. **(b)** Immuno-blot analysis of extracts from developing tendon were done to confirm the identity of the activities consistent with proMMP-2 and MMP-2 on gelatin zymograms. The immuno-blot analysis using 7.5% SDS-PAGE with an antibody against MMP-2 demonstrated that these activities were proMMP-2 and MMP-2. Higher molecular weight bands that may represent complexes with MT3-MMP and TIMP-2

(*) were observed as well as lower molecular weight, MMP-2 reactive bands, that may represent proMMP-2 degradation products (**). MW standards (kDa) are included.

Figure 2. MMP-2 activity as a function of tendon development

Tendon extracts between 14- and 20-days of development were analyzed on gelatin zymograms. All tendon extracts contained both proMMP-2 and MMP-2 activity. There was an approximately 30% decrease in proMMP-2 activity from day 14 to day 15 of development. Between day-15 and day-20 proMMP-2 levels were comparable. MMP-2 activity decreased with development. The MMP-2 activity was highest preceding and during the fibril growth phase (14–18 days), but decreased significantly after the period of rapid fibril growth (19–20 days). The amount of extract loaded was normalized to constant wet tendon weight. When normalized to constant DNA or protein nearly identical results were obtained. The figure represents 3 to 5 independent experiments for each developmental stage ($\pm$ SEM).

Figure 3. Characterization and developmental expression of collagenase

Type I collagenolytic activity from tissue extracts of 14- and 19-day tendons. Both the activated and unactivated samples contained increased type I collagenase activity at 14 relative to 19 days of development. Equal amounts of sample relative to tendon wet weight were activated by treatment with trypsin. Reaction mixtures containing enzyme from the tissue extracts and [^3H] labeled type I collagen were incubated for 2 h at 37°C, followed by digestion of the partially degraded collagen substrate with trypsin and chymotrypsin. The undigested collagen was precipitated with TCA and the percent digestion was determined. Collagenase activity was expressed as the percent of control samples. The figure represents at least three different experiments for each stage ($\pm$ SEM).

Figure 4. Immuno-blot analysis of MT3-MMP and TIMP-2 in tendon development

MT3-MMP and TIMP-2 expression at 14 and 19 days of development using immuno-blot analysis of tendon extracts. **(a)** Both ProMT3-MMP (63-KDa) and MT3-MMP (54-KDa) were expressed at both developmental stages. However, there was significantly higher expression of MT3-MMP at 14 days compared to 19 days of tendon development. Both higher molecular weight bands, that may represent complexes of MT3-MMP with MMP-2 and/or TIMP-2 (}) and barely detectable low molecular weight reactivity, presumably autolytic products (arrowheads) were observed only in extracts from 14-day tendons. **(b)** The expression of TIMP-2 in the 14- and 19-day tendon extracts also was analyzed. A band migrating at approximately 20 kDa consistent with TIMP-2 was observed at both developmental stages.

TIMP-2 was expressed in relatively comparable amounts at both developmental stages. The data suggest that MT3-MMP and TIMP-2 at the cell surface might provide a focal regulation of MMP-2 activation, providing a mechanism for focal degradation directly influenced by the cell in developing tissues. MW standards (kDa) are included.

Figure 5. MMP-2 initiates fibril growth ex vivo

To determine if MMP-2 was sufficient to initiate fibril growth, fibrils were isolated and analyzed from 14-day tendons in the presence of activated conditioned media, activated media with a non-specific MMP inhibitor EDTA and control media. The conditioned medium contained primarily MMP-2 and its precursor form by gelatin (a) and casein (b) zymography. Electron micrographs of fibrils in transverse sections from 14 day (c) and 20 day (d) tendons are presented. The pre-fibril growth tendons contained a homogeneous population of small diameter fibrils (c) whereas the post-fibril growth tendon had a heterogeneous distribution of larger diameter fibrils (d). The fibrils were analyzed by transmission electron microscopy and the diameters determined from transverse sections as in (c,d) and the distributions are presented

in **(f,h)** (blue). Fibrils isolated in control **(e)** and activated **(g)** media all were intact with the characteristic 67nm banding pattern **(e,g)**. However, the fibrils isolated in the presence of MMP activity were consistently larger in diameter. The mean diameters of fibrils isolated in the control media and activated media with EDTA **(f)** were significantly smaller than fibrils isolated in media containing activated MMP-2 **(h)** ($p<0.001$ and $p<0.01$ respectively). The fibrils isolated in control media where MMP activity was low or absent were smaller than the fibrils isolated in the presence of MMPs. In addition to the shift to larger diameters, the fibril diameter distribution from tendons isolated with activated MMPs developed a significant right hand shoulder relative to the control distribution. This shoulder contains larger diameter fibrils. The right hand shift of the distribution and development of a shoulder with large diameter fibrils is characteristic of tendons during fibril growth. These distributions are comparable to those seen in normal tendon development at 14 and 20 days of development (blue). Activated Media, conditioned media from 14 day tendon fibroblasts treated with 1mM APMA; Control, medium essential medium (MEM) not incubated with fibroblasts; Activated media + EDTA, conditioned media with 10 mM EDTA as a non-specific inhibitor of MMP activity.