Mechanical Stretching of Mouse Calvarial Osteoblasts In Vitro Models Changes in MMP-2 and MMP-9 Expression at the Bone-Implant Interface

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Bone to mechanical loading elicits a biological response that has clinical significance for several areas in dental medicine, including orthodontic tooth movement, tempromandibular joint disease, and endosseous dental implant osseointegration. Human orthopedic studies of failed hip implant sites have identified increased mRNA expression of several collagen-degrading matrix metalloproteinases (MMPs), while in vitro experiments have shown increases in MMP secretion after exposure to inflammatory mediators. This investigation evaluates the effects of mechanical deformation on in vitro osteoblasts by assessing changes in MMP gene expression and enzyme activity. We seeded mouse neonatal calvarial osteoblasts onto flexible 6-well plates and subjected to continuous cyclic mechanical stretching. The expression and activity of mRNA for several MMPs (2, 3, 9, and 10) was assessed. When subjected to mechanical stress in culture, only mRNA specific for MMP-9 was significantly increased compared to nonstretched controls ($P < .005$). Measurement of MMP activity by gelatin zymography demonstrated that none of the MMPs showed increased activity with stretching; however, MMP-2 activity decreased. Our results suggest that in response to stretch, MMP-2 responds rapidly by inhibiting conversion of a MMP-2 to the active form, while a slower up-regulation of MMP-9 may play a role in the long-term remodeling of extracellular matrix in response to continuous mechanical loading. This study suggests that the regulation of metalloproteinases at both the mRNA and protein level are important in the response of bone to mechanical stress.

Key Words: bone, osteoblast, cyclic mechanical stretch, RT-PCR, zymography, metalloproteinase, MMP, TIMP

Introduction

Proteolytic activity and collagenolytic degradation of extracellular matrix at the bone-implant interface have been reported in conjunction with increased mRNA expression of metalloproteinases (MMPs) in otherwise healthy tissues. For example, Sanz and colleagues identified a population of failing titanium endosseous implants that presented clinically without evidence of infection or inflammation, yet displayed considerable mobility and radiographic loss of osseointegration.[1]

MMPs play an important role in skeletal connective tissue degradation and remodeling. These enzymes constitute a family of zinc-dependent endopeptidases, expressed at low levels in normal adult tissues, which regulate the turnover of the extracellular matrix in connective tissues. Levels of MMPs are determined by a balance between their expression and inhibition in tissues by specific proteins, known as tissue inhibitors of metalloproteinases.[2] The cooperative effects of collagenases, gelatinases, and stromelysins—in combination with mechanical forces—suggest a role in MMP-dependent degradation of the extracellular matrix as an important determinant of implant failure due to excess occlusal forces.

Previous studies have demonstrated changes in the RNA expression in cyclically stressed osteoblasts obtained from mouse calvaria.[3] Because this bone cell culture model demonstrates a capacity to respond to mechanical changes, we hypothesize that this model may also show differences in expression of metalloproteinases in response to cyclic mechanical stretch that can be used to model changes at the bone-implant interface.

Materials and Methods

Cell culture

Primary osteoblast cultures were derived from neonatal calvaria from 1- to 2-day-old mouse pups (*Mus musculus* Hsd: ICR [CD 1R], Harlan Spraque Dawley Co, Indianapolis, Ind). The animal-

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use experimental protocol was reviewed and approved by the Animal Care and Use Committee, Dwight David Eisenhower Army Medical Center, Fort Gordon, Ga. All animal husbandry and handling was in accordance with pertinent laws and regulations concerning the use of animals in biomedical research within the Department of Defense. Mouse pups were killed by cervical dislocation, with 140 calvaria aseptically collected, cleansed of soft tissue and periosteum, and subjected to four sequential digests using 0.05% collagenase (Worthington Biochemical Corp, Freehold, NJ). Detached calvarial cells were decanted and placed in a medium for separation from debris on a Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient. Cell counts were established by trypan blue exclusion using a hemocytometer, which generally yielded approximately 2×10^7 total cells. The cell culture medium consisted of phenol-red free Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Equitech-Bio, Inc, Ingram, Texas), 5000 units of penicillin G, 5 mg/mL of streptomycin, 0.5 mg/mL gentamicin, 0.0125 mg/mL fungizone, and 10^{-8} M 1,25-dihydroxyvitamin D_3 (ICN Biochemical, Aurora, Ohio). T-150 cm^2 tissue culture flasks were seeded with 2.5×10^6 cells/flask, and placed in an incubator at 37°C in an atmosphere of 5% carbon dioxide and 95% air. Medium was changed in the primary culture at day 4. At day 6, the cultures had grown to confluence, and the first passage was conducted. When the second passage was grown to confluence, the cells were prepared for freezing. After detachment of cells by collagenase treatment and centrifuging, cell pellets were resuspended in 25% FBS and 75% cell freezing medium (composed of the above medium with 8.7% dimethyl sulfoxide and methylcellulose). Cell suspensions were frozen at −80°C over the course of 24 hours, and then placed in long-term storage in liquid nitrogen at −150°C. For each cell-stretching experiment, frozen cells taken from the second passage were thawed and grown to the fourth passage.

Application of cyclic mechanical stretching

Cyclic mechanical deformation (cell stretching) was applied to the osteoblast cells utilizing the FlexerCell Device (FlexCell International Inc, Chapel Hill, NC). Briefly, this device consists of a vacuum unit connected to a regulator solenoid valve, which is controlled by a computer with a timer program. Thus, the magnitude, duration, and frequency of the cyclic applied force can be varied and controlled. In this study, the deformation regimen used throughout was 3 cycles per minute (0.05 Hz), or 10 seconds of a maximum stretch of 24% elongation followed by 10 seconds of relaxation, according to the regimen used by Buckley and colleagues.[4] This method was selected for mechanical stimulation because it provides a reproducible and well-defined deformation regimen on bone cells in culture. In addition, the intermittent stretch provided by this technique was shown to increase the rate of DNA synthesis and cell division within the first 3 days. Cells were seeded at a density of 1×10^5 cells per 25 mm^2 well in flexible-bottom plates (6 wells per plate) (FlexWell, FlexCell International, Chapel Hill, NC). Four plates were utilized per experiment, with 2 as control and 2 that were subjected to cyclic mechanical stretching. For each condition, an additional 2 mm-thick collagen gel (rat tail collagen) (Becton-Dickinson, Bedford, Mass) at a concentration of 3.9 mg/mL was used as a substrate in 2 of the plates prior to cell seeding. Plates without the addition of native collagen gels contained a thin, dried type I collagen coating that covered the silicone surface, as supplied by the manufacturer.

After confluence was achieved, the medium was changed, and the plates were placed in the 8-plate base manifold unit of the FlexerCell device, which was housed inside the CO_2 incubator. The control plates were also placed in the same incubator but not subjected to mechanical stretching. The specific programmed regimen remained constant and was applied continuously to the cells for periods of 0.5 hr to 7 days. The medium was not changed in any individual experiment until day 4. At the conclusion of each cyclic stretch experiment, the conditioned cell culture media were collected, centrifuged at $500 \times g$, and the supernatants aliquoted for storage at −80°C. Additionally, at several time points (from selected individual wells) conditioned media and RNA were analyzed with termination at 30 minutes, 2 hours, 24 hours, 2 days, and 5 days.

RNA isolation

At the culture time points previously specified, conditioned medium was removed as described, and total RNA from both control and stretched cultures were isolated using acid-guanidium thiocyanate-phenol-chloroform extraction. Cells were lysed directly in each culture well by adding 1.0 mL of TRIzol reagent (Life Technologies, Grand Island, NY) per well. Lysates from sample wells for each plate were pooled into 1 sample for cells that had been mechanically stretched for continuous periods. Individual wells that represented specific time points were separately extracted as a single sample. All samples were incubated for 5 minutes at 30°C to permit the complete dissociation of nucleoprotein complexes. Phase separation was performed with the addition of 0.2 mL of chloroform, vortexing, and centrifugation at $12\,000 \times g$ for 15 minutes at 2°C. Precipitation of the RNA was performed by mixing the RNA-containing aqueous phase with 0.5 mL of isopropyl alcohol, incubating for 10 minutes at 30°C, and subsequent centrifuging at $10\,000 \times g$ at 2°C for 10 minutes. The residual RNA pellet was washed once with 1 mL of 75% ethanol, and after vortexing was centrifuged at $7500 \times g$ for 5 minutes at 2°C. The ethanol was decanted; the pellet was air-dried for 5 minutes and then dissolved in 100 μL of RNase-free Tris buffer (Tris-HCl, pH 7.6). Aliquots were taken for RNA quantification and the samples frozen and stored at −80°C until required for further use. RNA in each sample was quantified using RiboGreen (Molecular Probes Inc, Eugene, Ore).

Reverse transcriptase-polymerase chain reaction protocol

Reverse transcription was performed under the following conditions: 2.5 mM $MgCl_2$, 0.5X polymerase chain reaction (PCR) buffer II, diethylpyrocarbonate (DEPC) treated water, 0.5 mM dNTPs, 0.5 U/μL RNase inhibitor, 1.75 U/μL MuLV reverse transcriptase, and random hexamers at a final concentration of 1.75 μM (GeneAmp RNA PCR Kit, Perkin Elmer/Roche Molecular Systems, Branchburg, NJ). Reverse transcription was facilitated by a thermocycler (GeneAmp PCR System 2400, Perkin Elmer),

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TABLE

Primer sequences used for PCR amplification

Gene	GeneBank Accession	Sequence 5-3′ (Forward) 5-3′ (Reverse)	Product Size (bp)
GADPH	M32599	CCGGTGCTGAGTATGTCGTG TGCTGTTGAAGTCGCAGGAG	597
H4Histone	J00422	ATGTCTGGCAGAGGAAAG TACACCACATCCATAGCG	266
MMP-2	M84324	CTGGGAGAAGGACAAGTG CCTTGGTCAGGACAGAAG	539
MMP-3	X66402	GTGTGGTTGTGTGCTCATC TTCAGAGATCCTGGAGAAAG	420
MMP-9	Z27231	CGGACATTGTCATCCAGTTTG GGGTAGGGCAGAAGCCATAC	504
MMP-10	X76537	ACAAGCCCAGCTAACTTC TCTTGGGAAGCCTTTATC	540
TIMP-2	X62622	GATCAGAGCCAAAGCAGTGAG ACGCGCAAGAACCATCAC	540
TIMP-3	LI9622	GTGGTGGGAAAGAAGCTG AGGCGTAGTGTTTGGACTG	492

programmed with a single cycle consisting of 15 minutes at 42°C (for cDNA template generation), 5 minutes at 99°C (to terminate the reaction) ending with 5 minutes at 5°C. Polymerase chain reactions were then conducted to amplify the cDNA products resulting from the reverse transcription. For each one μL volume of cDNA product, a PCR master mix consisting of the following reagents was added: 1 mM $MgCl_2$, 0.5X PCR buffer II, DEPC treated water, and 1.25 U/100 μL AmpliTaq DNA polymerase. Primers for use in this experiment were designed from GeneBank accessions utilizing the software program Gene Runner (Hastings Software, Hastings, NY), and were synthesized by a commercial laboratory (Genosys Biotechnologies, Inc, The Woodlands, Texas (see the Table). Forward and reverse primer solutions were prepared from 100 μM stocks by adding 5 μL of primer to 45 μL of DEPC water. The PCR master mix contained 0.5 μL of each primer. Thermocycling for polymerase chain reactions began with 2 minutes of incubation at 95°C as the initial melting step, followed by a touchdown cycling program of 30 cycles of 5 seconds each, an annealing step starting at 65°C and decreasing by 0.5°C each cycle, and 72°C for 5 seconds; then 20 cycles of 94°C for 5 seconds, 55°C for 5 seconds, 72°C for 5 seconds, ending with 30 seconds at 72°C.

PCR product evaluation

Two percent agarose gels for electrophoretic separations were prepared using TBE Buffer (0.089 M Tris-borate, 0.089 M Boric acid, and 0.002 M EDTA) and agarose in 15cm^2 geltrays. For electrophoresis, gels were placed horizontally in a bath of 0.5X TBE buffer. Sample buffer (6X) consisted of 0.25% Bromophenol Blue and 30% glycerol in H_2O. One μl of loading buffer and 5 μL of each sample were mixed and pipetted into each well. Gels were electrophoresed at an 80 milliampere (mA) constant current for approximately 3 hours. After electrophoretic separation, DNA was stained using SYBR Green 1 nucleic acid gel stain (Molecular Probes, Inc), for 45 minutes at room temperature. Product bands were visualized with an optical scanner recording fluorescence emission at 520 nm with an excitation wavelength of 497 nm (Storm 860 Optical Scanner, Molecular Dynamics, Sunnyvale, Calif). Densitometry and image analysis of band intensity was subsequently performed with the same apparatus.

Zymography

Zymography or gelatin substrate SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed to ascertain changes in gelatinase activity of conditioned culture medium of cells subjected to mechanical stretching. Precast, commercially available SDS (10%) polyacrylamide gels (0.5 M Tris-Glycine, pH 6.8, NOVEX, San Diego, Calif) with 1 mg/mL gelatin incorporated as a substrate were loaded with 10 μL of conditioned medium from the following sources: unconditioned culture medium (DMEM with 10% FBS), control samples from cells cultured under control conditions, and experimental samples from cells cultured under the application of mechanical forces. Human gelatinase standards MMP-2 (72 kDa) and MMP-9 (92 kDa) were applied with each gel run (Chemicon International, Temecula, Calif). All samples were loaded with an equivalent volume of 2X sample loading buffer (0.5M Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 0.1% Bromophenol Blue) for a final volume of 20 μL per sample. The samples were loaded onto the gels under nonreducing conditions and run at a constant 30–40 mA per gel for 2 hours in a Tris-glycine SDS buffer. Gels were washed twice for 30 minutes in 100 mL of 2.5% Triton X-100 and then placed into 100 mL of 50 mM Tris-HCl, pH 8 for incubation at 37°C for time periods ranging from 4 hours to 24 hours. The gels were stained for 30 minutes with 0.5% Coomassie Brilliant Blue R-250 dye (Pharmacia Biotech) in 20% acetic acid. Gels were then destained in 20% acetic acid for 1 hour until gelatin substrate degradation was clearly visible, appearing as clear bands on a blue background.

RESULTS

Expression of mRNA

The results demonstrated that the mRNA of many MMPs in this mouse calvarial-derived osteoblast culture model is constitutively produced. Expression of mRNA was consistently demonstrated for MMP-2, MMP-3, MMP-9, MMP-10, TIMP-2, and TIMP-3 with or without application of mechanical stress (Figure 1). However, there was a noticeable increase in expression of mRNA for MMP-9 (Gelatinase-B), when compared to nonstretched controls (Figure 1, boxes). The internal controls GAPDH and H4 were consistently expressed with all conditions when subjected to mechanical stress. GAPDH serves as a "housekeeping" gene and is expressed in all vital cells, while H4 histone is more representative of cells undergoing proliferation rather than differentiation. Although many cells in this calvarial osteoblast model have shown varying stages of differentiation, a significant population continues to undergo proliferation as well, as has been observed by other investigators in this laboratory. To

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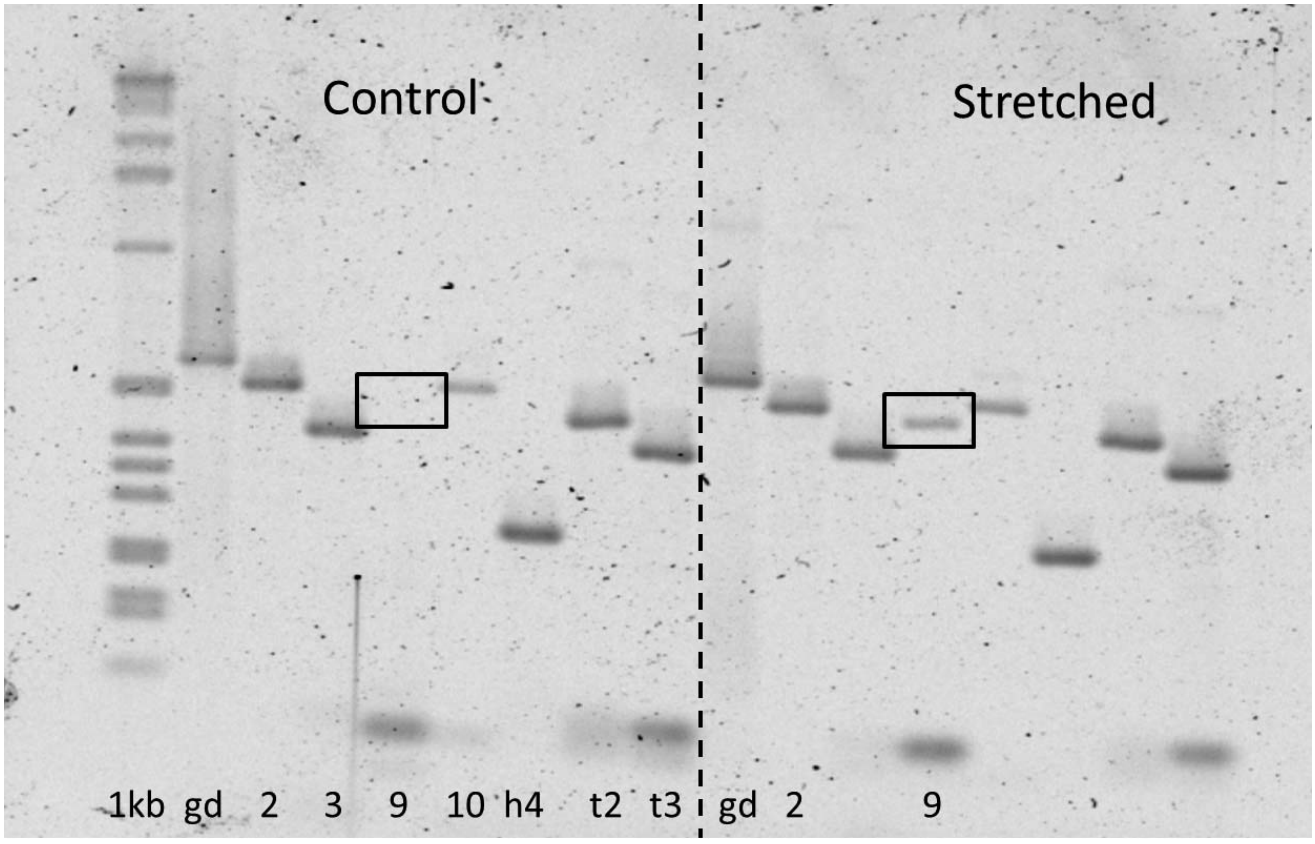

Figure 1. Reverse transcription-polymerase chain reaction agarose gel electrophoresis of MMP expression in control vs stretched cells after 48 hrs showing upregulation of MMP-9 expression with stretching (box). 1kb (1 kilobase DNA ladder standard); gd (GDPH, internal cellular RNA control); 2 (MMP-2, gelatinase A); 3 (MMP-3, stromelysin 1); 9 (MMP-9, gelatinase B); 10 (MMP-10, stromelysin 2); h4 (H4 Histone, internal proliferation control); t2 (TIMP-2, tissue inhibitor of MMP-2); t3 (TIMP-3, tissue inhibitor of MMP-3).

further elucidate the increase of expression of MMP-9, mRNA was analyzed after application of mechanical force for specific time intervals of 0.5, 2, 6, 24, 48, and 96 hours, as with controls. The increase was variable over time but was observed at the first time point—as early as 30 minutes (Figure 2). This general trend of enhanced expression of mRNA for MMP-9 was observed for up to 96 hours. Digitized densitometry of PCR product compared control groups to stretched groups. Statistical analysis of data for the control and stretched groups using the 1-way analysis of variance (ANOVA) demonstrated a statistically significant difference of $P < .005$, for all time points (with the exception of 24 hrs, which showed no statistical difference between stretched and control cells), with a mean relative increase of approximately 60% in MMP-9 mRNA expression (Figure 3).

MMP—gelatinase activity

MMP-2 and MMP-9 gelatinase activity was assayed from collected conditioned cell culture medium after 96 hrs. Constituent activity for both gelatinases was present in DMEM unconditioned medium since it contained 10% FBS. Many different proteases are known to exist in fetal serum; however, serum-free medium was not used in these experiments in order to enable the cells to withstand the regimen of mechanical cycling for prolonged periods. Previous attempts to culture these cells in serum-free medium in this laboratory were generally not successful. Differences in activity of conditioned medium became readily apparent, however, enabling normalization of activity relative to DMEM. Results indicate that MMP-2 (gelatinase-A) activity was strongly expressed in conditioned medium from osteoblasts grown with or without collagen gel, with an observable decrease noted in stretched cells under both conditions (Figure 4, bands near 72 kDa). This activity consistently appeared as the reduction in the doublet band in the zymogram. This difference in molecular weight can be attributed to the preprocessed form of the MMP-2 prior to release from the cells and was therefore not seen in the unconditioned media. However, a decrease in this activity was observed for cells subjected to mechanical force, possibly due to interaction with TIMP-2. When force was applied, there was a noticeable decrease in MMP-2 activity represented in the lightening of the higher pro-MMP-2 band. Expression of MMP-9 gelatinase-B activity was very weakly expressed at this time point. (Figure 4, 92 kDa band). However, a discernible decrease in MMP-9 activity is seen in the stretched cells, especially when grown on the collagen containing media.

Discussion

Many different in vitro osteoblast models have been shown to secrete MMPs in culture.[5–8] These MMPs include the collage-

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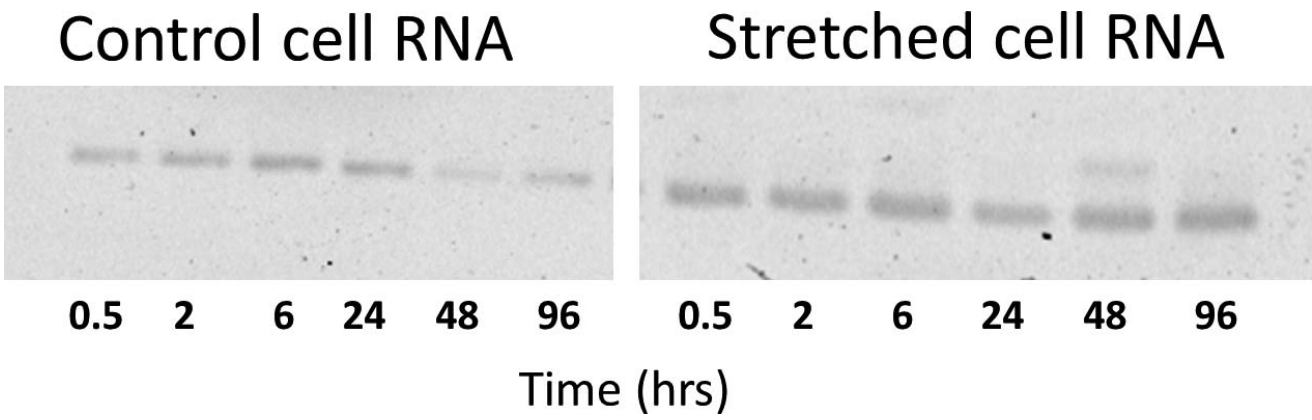

Figure 2. Increased expression of MMP-9, mRNA after application of mechanical force for specific time intervals of 0.5, 2, 6, 24, 48, and 96 hours vs controls. The increase was variable over time but was observed at the first time point, as early as 30 minutes.

nases, stromelysins, and the gelatinases, as well as others, which comprise the 20+ different mammalian MMPs identified over the past several years. Lorenzo and colleagues reported on the ability of newborn mouse calvarial cells to produce both 72 kDa and 92 kDa gelatinases (MMP-2 and MMP-9, respectively) by secreting them into the medium, with increases in activity upon stimulation by known agents such as cytokines, interleukins, and chemical mediators of resorption, such as phorbol esters.[9] Another study by Meikle and colleagues using cultured human osteoblasts found that—in response to parathyroid hormone, vitamin D_3, or mononuclear cell conditioned medium—these osteoblasts were stimulated to synthesize collagenase (MMP-1), gelatinase-B (MMP-9), and stromelysin (MMP-3). Constitutively, these osteoblast cells produced gelatinase-A (MMP-2) and TIMP-1, but only when cultured on a type I collagen substrate.[10] This study for human bone cells correlates well with other data from experiments with rodent-derived osteoblast cultures. The working hypothesis validates the idea that osteoblast-derived MMPs play a role in bone resorption.[11,12]

Our results also showed a similar profile of constitutive MMP-2 and MMP-9 activity in conditioned medium from our osteoblast culture model, where MMP-2 activity was expressed at a much higher level than MMP-9. This difference in activity is consistent with the much higher production in osteoblasts of TIMP-1, the inhibitor of MMP-9, than TIMP-2, inhibitor of MMP-2. This could also explain why, despite the upregulation of MMP-9 mRNA seen in our data, the overall activity of MMP-9 enzyme was negligible. In addition, these findings are also consistent with the higher levels of MMP-2 found in osteoblasts in general. Our results suggest that in response to stretch, MMP-2 responds rapidly by an increase in the conversion of the existing pro-form of MMP-2 to the active form, while a slower up-regulation of MMP-9 may play a role in the long-term remodeling of extracellular matrix in response to continuous mechanical loading. In a study of primary human bone-derived osteoblasts on type I collagen-coated BioFlex plates, mechanical deformation resulted in a reduction of TIMP-2 RNA expression, as compared with nondeformed control cultures.[13]

Others have previously shown that mechanical stimulation of calvarial cell cultures has also resulted in changing phenotypic responses, as measured by proteins such as osteocalcin, osteonectin, alkaline phosphatase, and type I collagen.[14–16] One group found that parathyroid hormone or

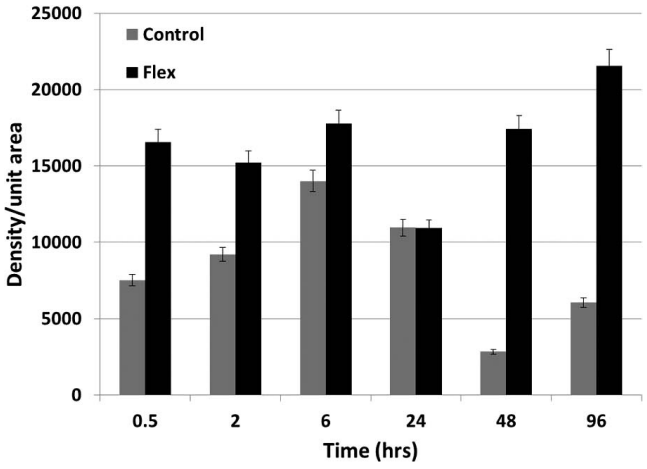

Figure 3. Increased MMP-9 RNA expression in response to mechanical stretching. Digitized densitometry of polymerase chain reaction product comparing control groups to stretched groups at each time point (means of three replicates).

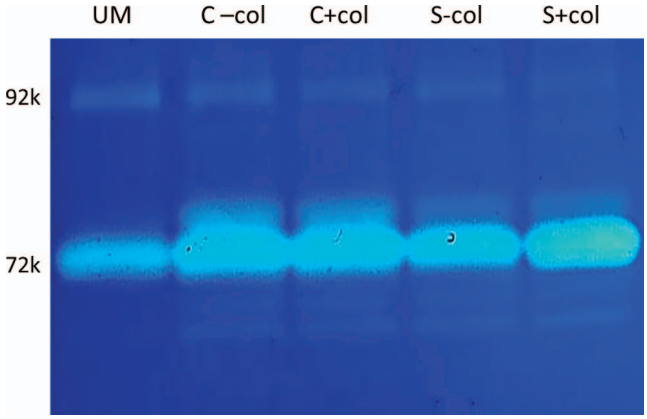

Figure 4. Change in MMP-2 (72k) and MMP-9 (92k) activity with cell stretching. MMP-2 pro-form and MMP-9 band decrease when cells are stretched.

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cyclic application of tension forces (17% elongation, 3 cycles/min) induced collagenase (MMP-1) production in cultured osteoblast-like cells.[17] The effects of mechanical stress on bone cells in vitro have been diverse and difficult to generalize because of variable responses, which may be due to the source of the cells used and the characteristics of the strain applied. Some studies have reported that osteoblastic cells can respond differently to high and low strain.[18,19] With high stress levels, cells respond by increasing proliferation after upregulating prostaglandin synthesis and resorption-stimulating factors (such as MMPs), whereas alkaline phosphatase and collagen synthesis are down-regulated. If low cyclic stress of a more physiologic magnitude is applied, however, cell proliferation becomes inhibited, while alkaline phosphatase and protein synthesis are increased.[18] It has become well established that even within the same osteoblast culture models, there are individual cell populations that are more strain sensitive than others.[20] Liu and co-workers have shown that there can be tremendous variability of mRNA expression among osteoblasts in calvarial cultures. It is speculated that this may reflect differences in stage of cell cycle among individual cells. This observable heterogeneity of the mature osteoblastic phenotype may represent a more flexible pattern of genetic expression rather than a single or unique phenotype.[21]

In our study, 1- to 2-day-old mouse pups were used, which perhaps yielded an enhanced population of cells expressing a more differentiated phenotype. A more recent study of mouse calvaria-derived osteoblasts permitted the seeded cells to produce their own 1–2 mm thick extracellular matrix prior to application of mechanical compression.[7] They found that 6–10% compression at 1 Hz resulted in increased MMP-2 RNA expression.

Other similar studies published since the completion of this work have been used the established mouse MC3T3-E1 preosteoblastic cell line. In the study by Tanaka et al, MMP-9 RNA in MC3T3-E1 cells seeded into type I collagen gels was found to be elevated in response to prolonged broad frequency vibration.[17]

Although expression for MMP-9 mRNA in this culture model was found to be significantly increased when compared to nonstretched controls, there appeared to be no significant differences in expression of MMP-2 mRNA. This was a consistent finding for all conditions studied. Genetic regulation of the 2 gelatinases is known to be distinctly different. Studies of the genomic structure of MMP-2 has revealed that this gene does not have an AP-1 site or TATA box in the promoter region, as do MMP-9 and other MMPs.[22]

Interestingly, the data from the zymograms showed no apparent difference in MMP-9 activity for the control cells grown with or without collagen but was significantly decreased with stretching only when collagen was present (Figure 4, lane S+col).

In contrast, the opposite response was observed for MMP-2. It has been consistently reported as being secreted into the medium of osteoblast cell cultures at a constitutive level. Since the cells from our study exhibited changes of being more fully differentiated when cultured on collagen, this may have rendered them less responsive to the higher stress levels but more sensitive to physiologic loading. The doublet band of activity for MMP-2 was consistently noted in the zymograms. The band of activity of higher molecular mass that was decreased with mechanical loading may represent a latent (pro-enzyme) form of the enzyme.[23,24] Both gelatinases are secreted in latent form, and require cleavage of propeptides for subsequent activation in the extracellular matrix. Constitutive activity of the latent forms of these enzymes is observed in nonreduced gels due to the dissociation from inhibitor-enzyme complexes by SDS. In tissues, MMP-2 is inhibited by TIMP-2 and MMP-9 by TIMP-1, as well as serum proteins such as α-macroglobulin.[25] Activated forms of MMP-2 and MMP-9 migrate to positions of 62 and 82 kDa, respectively, in nonreduced SDS gels, after chemical activation with amino-phenylmercuric acetate.[19] However, this agent was not used in this study, and activated forms of MMPs were not observed on the zymogram gels.

In this study, the results where each secreted gelatinase responded differently to collagen substrate and mechanical stress emphasize earlier findings by others that these two MMPs are regulated very differently. Overall, this could suggest changes in secretion of the MMP molecule. In his study of cultured calvarial osteoblasts, Buckley found the cytoskeletal protein vinculin to be increased 22-fold with mechanical stretch.[11] This protein is intimately associated with integrins of the cell membrane. Cell surface integrins help mediate responses to the extracellular matrix, as well outside mechanical forces, and might play a role in changing secretion of MMPs. MMP-2—which in latent form is a pro-gelatinase—can be activated by another MMP called MT-MMP (MMP-14), which is a membrane associated metalloproteinase. Strongin and colleagues have found that MT-MMP acts as a cell surface receptor for tissue inhibitor of metalloproteinase (TIMP-2 receptor) when activated.[26] It is apparent that changes in the extracellular matrix can modulate both inhibition and activation of metalloproteases, with mechanical stress also playing a role.

Although MMP-9 is found secreted by bone cells in culture, it has not been found in bone tissue localized to osteoblasts. MMP-9 has also been shown to be secreted by various human osteoblast-like tumor cell lines, however, which may indicate incomplete differentiation.[21] Further, immunohistochemical studies using monoclonal antibodies have identified MMP-9 localized in human bone within osteoclasts and mononuclear cells, found to be more highly expressed in pathologic specimens of arthritic bone tissue.[23] MMP-9 has also been shown to be highly expressed during mouse development in cells of osteoclast lineage.[24] Calvarial bone organ cultures have been shown histologically to contain osteoclast precursors, but one immunocytocemical study demonstrated that this activity could be eliminated by stripping the endochondral membranes from the calvaria after harvesting or by subjecting them to enzymatic digest.[28] The mechanism that has been proposed is that MMP-9 acts in concert with MMP-1 and other cysteine or acidic proteases to degrade the organic matrix of bone, allowing access by osteoclasts to the remaining mineralized portion.[23]

Studies using human osteoblastic cells derived as explants from trabecular bone found that cells cultured in a floating collagen lattice were triggered to overexpress MMP-2, MMP-13, and MT1-MMP in response to mechanical contraction, while

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cells on an attached collagen lattice were not triggered.[8] Zymography revealed that enzyme expression for those MMPs were also activated in that study.

In summary, the findings provide significant evidence that in response to cyclic mechanical stretching, cultured osteoblast-like cells derived from neonatal mouse calvaria demonstrate independent changes in MMP-2 and MMP-9 metalloproteinases at transcriptional and post-translational levels. These data are indicative of an increase in mRNA for MMP-9 (gelatinase B), but also a decrease in enzymatic activity and secretion with application of mechanical forces. In contrast, no observable increase was seen for MMP-2 mRNA expression, but an increase in some processed form of MMP-2 (gelatinase A) protein was observed. The differences observed between MMP-2 and MMP-9 suggest that these 2 metalloproteinases are regulated very differently at the transcriptional level and perhaps also at the secretory level; the regulated degradation of the extracellular matrix plays a crucial role in cellular responses to mechanical stress.

Abbreviations

DMEM: Dulbecco's Modified Eagle's Medium

MMP: matrix metalloproteinase

PCR: polymerase chain reaction

Acknowledgment

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