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Titanium implants induce expression of matrix metalloproteinases in bone during osseointegration

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Abstract—Implanted pure titanium fixtures are able to completely integrate with bone, in part because of the formation of a strong extracellular matrix (ECM) bond at the titanium-bone interface. In this study, we used a rodent femur model of intramedullary osseointegration to analyze the changes in immunoreactivity of ECM-controlling matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase-3 (TIMP-3), and tumor necrosis factor alpha (TNF-alpha) during osseointegration. We observed dramatic increases in MMP-2, MMP-9, MMP-7, TIMP-3, and TNF-alpha in osteocytes, osteoclasts, haversian canals, and the interface matrix in bone ipsilateral to the titanium implant. An increase in TIMP-3, MMP-9, and MMP-7 in hypertrophied chondrocytes and the vascular component of the epiphysial growth plate was also observed in experimental bone. These findings were not seen in contralateral or sham-operated bone, where the titanium fixtures were threaded into the femur and immediately removed. Our data link titanium-induced bone remodeling to changes in expression and distribution of MMPs.

Key words: bone, endosteal, histopathology, intramedullary, matrix, MMP, osseointegration, TIMP, titanium, TNF.

INTRODUCTION

The remarkable success of endosteal titanium implants in dental, cranial-maxillary facial reconstruction, and orthopedic applications [1] can be attributed to the capability of pure titanium implants to become permanently integrated with living bone, a phenomenon defined as *osseointegration* [2]. Direct contact between living bone and the surface of a load-carrying titanium implant forms a strong structural and functional extracellular matrix (ECM) bond at the interface that is composed of proteoglycans, glycoproteins, and adhesion molecules [3–12]. This matrix bond increases in strength over time [1,4], promoting reparative osteogenesis at the interface that results in clinical fixation of the implant [6].

Bone matrix turnover is regulated by the extracellular zinc-dependent enzyme family of matrix metalloproteinases (MMPs) comprising collagenases, gelatinases, stromelysins and membrane-type MMPs [13]. Bone development and remodeling requires activity of MMPs for matrix maintenance and repair, bone resorption, and the coupling

Abbreviations: BSA = bovine serum albumin, DAB = diaminobenzidine, ECM = extracellular matrix, MMP = matrix metalloproteinases, NIH = National Institutes of Health, OD = optical density, PBS = phosphate buffered saline, TIMP = tissue inhibitors of metalloproteinases, TNF = tumor necrosis factor, VA = Department of Veterans Affairs.

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to bone formation [12–14]. MMP-9 (gelatinase B) is thought to be important in controlling osteoclast differentiation and recruitment into remodeling bone [14–18]. Both MMP-9 and MMP-2 (gelatinase A) have been implicated in bone resorption that results in the loosening of prostheses [19–20]. MMP-7 (matrilysin) degrades proteoglycans [21], the key structural substrates for adhesion of titanium fixture to bone [3–4,10–11]. This potent proteoglycanase has also been shown to regulate macrophage migration during bone resorption through the release of the immunomodulatory cytokine tumor necrosis factor alpha (TNF-α) [22]. A number of MMPs release soluble TNF-α from its transmembrane precursor form [23], and TNF-α in turn, induces MMP gene expression, including MMP-9 [24]. TNF-α promotes bone resorption [25–26] and governs signaling mechanisms between osteoblasts and osteoclasts through the regulation of endocrine stimulants of bone resorption, such as parathyroid hormone [14].

With the use of a transverse fracture model in TNF-α receptor knockout mice, TNF-α has been shown to be critical for osteoprogenitor cell recruitment and intramembranous bone formation [27]. MMP function is regulated through expression, activation from a proenzyme form, and importantly, interaction with naturally occurring tissue inhibitors of metalloproteinases (TIMPs). Within the TIMP family, TIMP-3 is a multifunctional protein exclusively localized in ECM [28] and is a potent modulator of angiogenesis [29], a process considered fundamental in coupling bone resorption and formation [30]. In vitro studies have shown that titanium particles induced MMP-2 and TNF-α in cultured macrophages [31–33] and TNF-α in osteoblast-like cells [34], while MMP-2 and MMP-9 were stimulated by titanium substrates in fibroblasts [35] and by pure titanium discs in primary human osteoblasts [36].

To better understand the in vivo molecular mechanisms of osseointegration, we previously developed a rat femur model of intramedullary osseointegration and observed dynamic changes in neural-immune activity in bone and protein gene product 9.5 (PGP 9.5) and calcitonin gene-related peptide-positive (CGRP-positive) sensory nerve fibers at the titanium-bone interface [37]. We are now reporting that pure titanium threaded rods implanted in the medullary cavity of rat femurs generate increases in immunoreactive MMP-2, MMP-7, MMP-9, TNF-α, and TIMP-3 that correlate with the structural and functional remodeling in bone, resulting in osseointegration.

MATERIALS AND METHODS

Titanium Fixtures

Experimental implants were manufactured from commercially pure titanium at the Brånemark Institute (Gothenburg, Sweden). The implant screw was Grade 4 titanium with a 11.4 mm-long, smooth middle section, a core diameter of 1.5 mm, and a 4.3 mm-long M2 thread at each end. A 2 mm-deep hexagonal hole (1.2 mm diameter) was manufactured at the distal end of the implant to facilitate insertion. We kept autoclaved fixtures in dry glass containers and handled them with titanium instruments to avoid contamination.

Animals and Surgery

Adult male Sprague-Dawley rats (N = 42; 225–250 g; Harlan Labs, Indianapolis, IN) were handled in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23, Rev. 1985). All procedures were performed in accordance with protocols approved by the University of California, San Diego, and the Department of Veterans Affairs (VA) Healthcare Committee on Animal Research. An anesthetic solution containing sodium pentobarbital (Nembutal, 50 mg/mL; Abbott Labs, North Chicago, IL), diazepam (5 mg/mL, Steris Labs, Phoenix, AZ) and saline (0.9%, Steris Labs) was injected intraperitoneally. Surgery was performed on the front of the lower limb, unilaterally under sterile conditions as previously described [37].

Briefly, we opened the femoral medullary cavity via a flexed knee joint with a 1.7 mm diameter hand drill and threaded with an M2 pretapping device to the appropriate depth. The implant screw was inserted with a hex key and countersunk 5 to 7 mm below the knee joint. After reposition of the patella, we closed the wound with 3-0 absorbable sutures using continuous stitches in joint capsule and skin. The animals were allowed to recover in a sensory-enriched environment without restricting their mobility. Sham surgery consisted of exposing, drilling, and tapping the medullary space, and threading the titanium fixture into the cavity, followed by its immediate removal. We did not operate on the contralateral femur and used it for control.

In a small subset of animals, loading was accomplished by osteotomy of the femur 8 weeks after implanting the titanium fixture. An osteotomy was performed, and a 2 mm length of femur was removed at the midlength of the fixture. This procedure provided clinical relevance

for human amputee applications in which osseointegration of the fixture is allowed to occur in the unloaded state prior to attachment of a peripheral prosthesis.

Tissue Processing

Tissue was harvested at 4, 8, and 12 weeks postsurgery as summarized in **Table 1**. Anesthetized rats were perfused with Tyrodes buffer (134 mM sodium chloride, 0.9 mM potassium chloride, 1.0 mM magnesium chloride, 4.0 mM magnesium sulfate, 0.34 mM monobasic sodium phosphate, 5.0 mM glucose, and 12.0 mM sodium bicarbonate, pH 7.4) at room temperature, then with ice-cold (4 °C) Tyrodes buffer, immediately followed with a picric acid formaldehyde fixative containing 2.0 percent paraformaldehyde and 0.15 percent picric acid in 0.1 M phosphate buffer at room temperature, as suggested [38]. Femur bone was harvested and fixed in a picric acid formaldehyde fixative for 48 hours at 4 °C. Then it was rapidly decalcified in Immunocal Formic Acid Decalcifier (American Master Tech Scientific, Inc., Lodi, CA) at 4 °C for about 72 hours until flexible and cut longitudinally or transversely with a single-edge razor blade. The titanium fixture was carefully removed, and femurs were further rinsed in distilled deionized water for 3 hours. All tissue was embedded in paraffin and cut in 10 μm-thick sections.

Antibodies

The following antibodies were used in our studies:

1. Mouse antihuman MMP-2 (rat-compatible, Chemicon, Temecula, CA; 1:1,500).
2. Goat anti-MMP-7 (Santa Cruz Biotech, Santa Cruz, CA; 1:500).
3. Rabbit antirat MMP-9 (Torrey Pines Biolabs, Houston, TX; 1:1,000).
4. Rabbit antihuman TIMP-3 (rat-compatible, Chemicon; 1:800).
5. Goat anti-TNF-α (R&D Systems, Minneapolis, MN, 1:200).

We used normal mouse, goat, or rabbit serum in place of matching primary antibody to control staining specificity.

Table 1.
Experimental animal groups.

Postimplantation (wk)	Group	Animals
4	Titanium	8
4	Sham	5
8	Titanium	12
8	Sham	5
8 + 4 of Loading	Titanium	11
8 + 4 of Loading	Sham	5
12	Titanium	5
12	Sham	5

Immunohistochemical Analysis

We used an avidin-biotin detection system with 3,3'-diaminobenzidine (DAB) substrate (Vector Labs, Burlingame, CA). Tissue was deparaffinized with xylene, rehydrated in a graded ethanol series to 70 percent, and washed in phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 3 percent H_2O_2, and tissue was permeabilized with 0.5 percent Triton X-100 in PBS for 30 minutes. Nonspecific binding was blocked with 5 percent normal horse serum for MMP-2, rabbit serum for MMP-7 and TNF-α, and goat serum for MMP-9 and TIMP-3 (Vector) for 1 hour at room temperature. Primary antibodies were diluted in 0.1 percent bovine serum albumin (BSA) (Sigma) and 0.5 percent normal serum in PBS and applied overnight at 4 °C. Following extensive PBS rinses, one of the following biotinylated secondary antibodies (Vector; 1:200 dilution) were applied for 1 hour at room temperature:

1. Horse antimouse for MMP-2.
2. Rabbit antigoat for MMP-7 and TNF-α.
3. Goat antirabbit for MMP-9 and TIMP-3.

An avidin-biotin complex (ABC) (Elite, Vector) was applied for 30 minutes at room temperature. Sections were developed with DAB (Vector), counterstained with 0.2 percent methyl green (Fisher Scientific, Pittsburgh, PA), and mounted with Permount media (Fisher).

Image Analysis

The image analysis system consisted of a Leica DMRB microscope (Leica Microsystems, Bannockburn, IL), a 12-bit Leica DFC (dense fibrillary component) 300 digital camera (McBain Instruments, Chatsworth, CA), and an Apple G4 computer. Openlab 3.1.2 image analysis software (Improvision Inc., Lexington, MA) measured integrated optical density (OD) of the immunoreactive area and intensity; profiles are presented as a ratio of immunoreactive counts over total counts. For analysis, at least four animals were used per time point, with five high-power fields per animal. For contralateral and sham control tissues, each tissue was individually assessed and the OD averaged for each animal. The results of image

analysis are reported as the mean ± standard error (SE) of *N* observations. Statistical significance ($p < 0.05$) was calculated by analysis of variance (ANOVA), followed by Tukey's posthoc test.

RESULTS

We reported the changes in the distribution of MMP-2, MMP-7, MMP-9, TIMP-3, and TNF-α that were consistently seen in animals with permanent titanium implants (experimental), but not in the contralateral (unoperated) side, or in femurs into which the implant had been threaded and then immediately removed (sham-operated). Overall, a significant increase in MMP-2, MMP-7, MMP-9, and TIMP-3 staining was observed at 4, 8, and 12 weeks postimplantation relative to controls, while the increase in TNF-α levels was transient in osteocytes only and declined after 4 weeks postimplantation. Given that five antigens were analyzed in four different animals groups and several bone structures were implicated, we quantified and summarized data in **Table 2** and presented representative findings and appropriate controls in **Figures 1** and **2**.

Our histological analysis of the titanium-bone interface at 4, 8, and 12 weeks following transplant surgery indicated successful osseointegration with normal bone adjacent to the fixture without significant inflammatory reaction. New normal bone appeared adjacent to and fully occupying the space between fixture threads. *Interfacial matrix* localized directly between the titanium implant and remodeling bone displayed the increase in MMP-9 and its matrix-specific natural inhibitor TIMP-3 [28] at 4 weeks postimplantation (**Figure 1(a)**). No immunoreactivity was noted for either antigen in contralateral or sham-operated femurs (**Figure 1(b)**).

Osteocytes in proximity to the implant were numerous and slightly enlarged, as described previously [3,8]. In the ipsilateral compact bone, osteocytes were strongly immunoreactive for MMP-2 and MMP-7 at all time points and for TNF-α and MMP-9 only at 4 weeks postimplantation (**Figure 1(c)**; **Table 2**). In control contralateral and sham femurs, osteocytes were only reactive for MMP-2, displaying mild staining of occasional osteocytes (**Figure 1(d)**) and showing a mean 18-fold increase with titanium implant relative to sham controls (**Table 2**). MMP-9 has previously been shown to control osteoclast maturation and migration [13,16–18], and it was markedly induced in osteoclasts of experimental (**Figure 1(e)**), but not sham bone (**Figure 1(f)**) at 4 weeks postimplantation. Other antigens showed no reactivity in osteoclasts at the time points we analyzed.

Table 2.
Summary of immunohistochemical evaluation of implanted bone.

Marker	Structure	4 Weeks	8 Weeks	8 Weeks + Load	12 Weeks
MMP-2	OC	22.7 ± 1.2	18.2 ± 2.1	14.5 ± 0.4	17.01 ± 0.3
	HC	57.3 ± 4.0	71.3 ± 6.0	103.0 ± 14.0	59.7 ± 5.0
MMP-7	OC	24.5 ± 0.8	29.0 ± 1.4	12.0 ± 2.1	22.0 ± 0.8
	HC	69.3 ± 4.0	62.0 ± 8.0	189.0 ± 9.3	41.0 ± 5.0
	GP	44.2 ± 2.0	—	—	—
MMP-9	Ti-B	7.2 ± 0.4	5.3 ± 0.6	4.4 ± 0.8	2.4 ± 0.3
	OC	2.0 ± 0.2	—	—	—
	Ocl	5.2 ± 0.3	—	—	—
	Obl	3.0 ± 0.16	—	—	—
	GP	4.0 ± 0.6	—	—	—
TNF-α	OC	12.0 ± 2.2	—	—	—
TIMP-3	Ti-B	1453.0 ± 23.0*	563.0 ± 21.0	563.0 ± 21.0	153.0 ± 14.0
	GP	6.0 ± 0.4	—	—	—

Note: Mean fold increase in staining intensity ± standard error (SE) for each marker was estimated in experimental bone at 4, 8 (without and with loading), and 12 weeks after titanium implantation, relative to control sham-operated and/or contralateral bone (mean value of all controls is used). Values are significant ($p < 0.05$); *$p < 0.01$.

GP = growth plate, HC = haversian canals, Obl = osteoblasts, OC = osteocytes, Ocl = osteoclasts, Ti-B = titanium-bone interface, TIMP = tissue inhibitors of metalloproteinases, MMP = matrix metalloproteinases, TNF = tumor necrosis factor

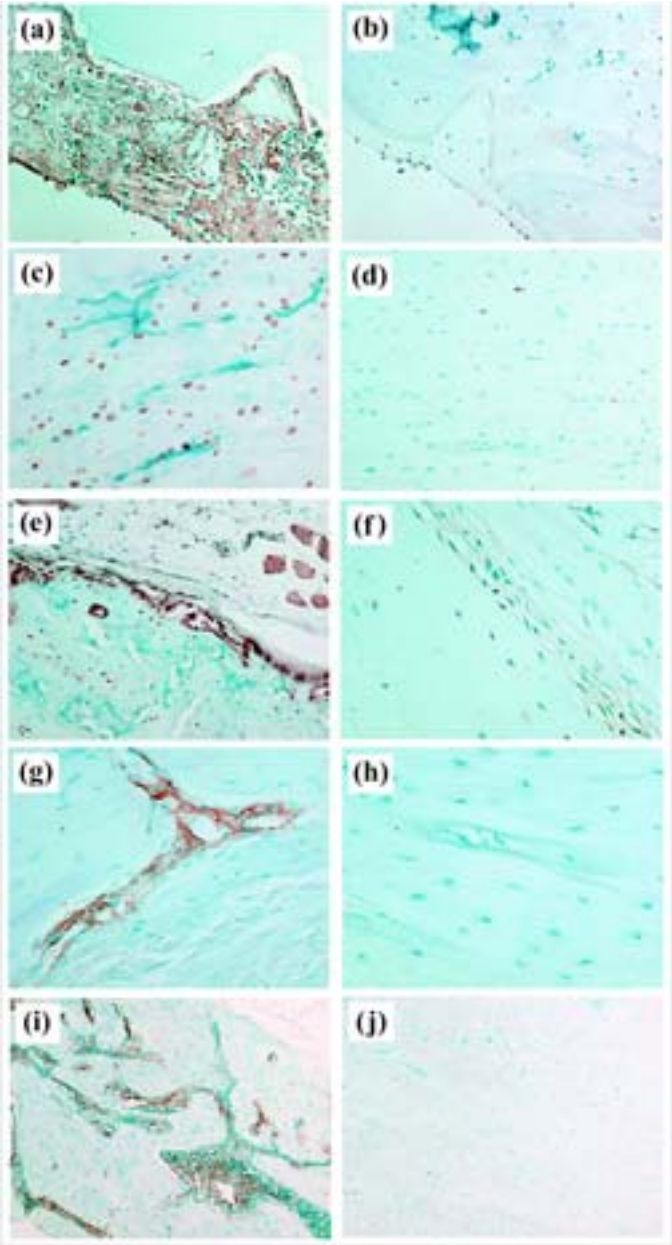

Figure 1.
Matrix metalloproteinase (MMP) distribution in bone undergoing osseointegration. At interface of titanium with bone, MMP-9 and tissue inhibitors of metalloproteinases-3 (TIMP-3) are induced at 4 weeks postimplantation in **(a)** experimental but not in **(b)** sham femur. Osteocytes are enlarged and immunoreactive for MMP-2 and MMP-7 in all experimental bone (not shown), while MMP-9 and tumor necrosis factor-α (TNF-α) at only 4 weeks **(c)** postimplantation, but not in **(d)** sham. MMP-9 is markedly increased in osteoclasts of **(e)** implanted, but not **(f)** sham bone. Haversian canals are prominently stained for MMP-7 and MMP-2 in **(g)** ipsilateral, but not in **(h)** sham femur. At 8 weeks postimplantation, followed by a 4-week loading period, haversian canals were **(i)** strongly proliferated compared to **(j)** sham. Magnification: **(a)–(f)**, 200×; **(g)–(h)**, 500×; **(i)–(j)**, 165×.

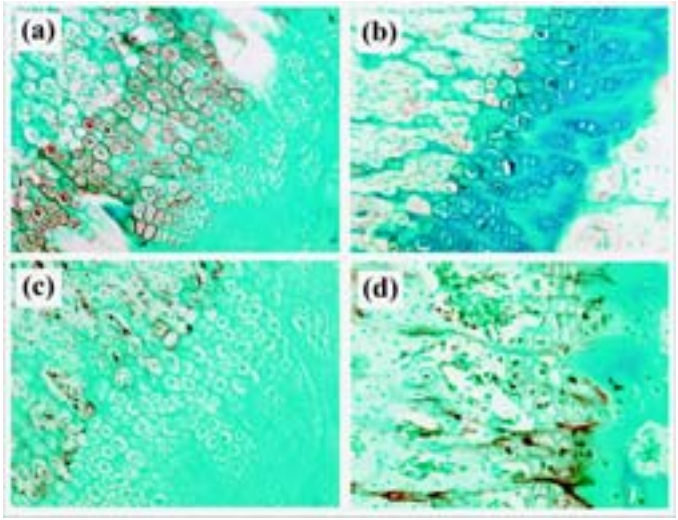

Figure 2.
Matrix metalloproteinase (MMP) distribution at epiphysial growth plate during osseointegration. Hypertrophied zone is vastly intensified showing dramatic changes in MMP-7 staining in hypertrophied chondrocytes of **(a)** experimental compared to **(b)** sham femur. **(c)** Experimental bone was also reactive for MMP-9 in osteogenic cells at maturation zone, while **(d)** tissue inhibitors of metalloproteinases-3 (TIMP-3) stained in endochondral vessels and/or thin bone deposit layer of titanium implants, but not sham femur (not shown). Magnification: **(a)–(d)**, 200×.

Haversian canals containing neurovascular channels were branching and stained prominently for MMP-7 and MMP-2 in ipsilateral (**Figure 1(g)**), but not contralateral or sham femur (**Figure 1(h)**) at 4, 8, and 12 weeks postimplantation. Loading of the implant had a particularly strong effect on proliferation of haversian canals and was associated with intensified staining for both MMP-7 and MMP-2 (**Figure 1(i)**). No staining was seen in contralateral or sham-operated femur (**Figure 1(j)**). A reduced number of osteocytes and osteocyte immunoreactivity was observed in the bone that underwent loading (**Table 2**).

The *epiphysial growth plate*, where the hyaline cartilage is being replaced by bone, was reactive in experimental rats at 4 weeks postimplantation, but not later time points. It showed an increase in MMP-7 in hypertrophied chondrocytes, particularly at the zone of maturation in ipsilateral (**Figure 2(a)**), but not contralateral or sham femur (**Figure 2(b)**). At the osteogenic zone, occasional cells, presumably osteoblasts, were stained for MMP-9 (**Figure 3(c)**) and TIMP-3, which also distributed along the capillaries and/or thin layer of bone deposits (**Figure 4(d)**) in experimental, but not control tissue.

Periosteal vessels were positively stained for MMP-2 and MMP-7 throughout analyzed tissues, with no apparent differences between experimental and control bone (not shown).

DISCUSSION

Integration of external titanium fixtures into living bone (osseointegration) occurs through active bone remodeling [1], resulting in sensory neuronal changes facilitating perception of peripheral mechanical stimuli through an attached prosthesis (osseoperception) [39]. This marks the first demonstration of titanium-induced increase in MMPs, TNF-α, and TIMP-3 that was specific to bone undergoing osseointegration; that is, the changes were associated with permanent pure titanium implants rather than the bone surgery alone.

The formation of a strong ECM bond provides an effective mechanical connection of titanium to bone facilitating intercellular signaling at the interface [3–6,9,11]. Proteoglycans are essential components of the titanium-stimulated matrix [10]. Increased expression of a potent proteoglycanase MMP-7 in osteocytes and collagen bundles of haversian canals during osseointegration may reflect its role in maintaining matrix content. MMPs regulate turnover and function of matrix collagens and adhesion molecules contributing to the solubilization of osteoid [21] that is essential in maintaining bone turnover during implantation. Matrix thickness impacts bone formation during osseointegration, and heparan sulfate-binding molecules have been implicated in this process [5]. TIMP-3, which we find localized at the contact site with titanium, is one such heparan-binding molecule [40].

Coupling of bone resorption and bone formation ensures successful osseointegration of titanium [8–9]. When activated by the presence of a titanium fixture, osteoclasts migrate from the endosteal surface to the titanium implant [6] to control bone resorption [7,12].

New bone formed during implantation promotes endochondral ossification of the growth plate [6]. We demonstrated that osteoclasts stimulated by titanium in vivo are reactive for MMP-9, essential for osteoclast migration [13,16–18]. During osseointegration, chondrocytes of the growth plate express MMP-7, which maintains cartilage proteoglycan turnover [21]. MMP-9 is a regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes [41]. Expressed at the zone of maturation and vascular output in bone undergoing osseointegration, MMP-9 and its inhibitor TIMP-3 may be important in regulating chondrocyte function [42]. The balance between angiogenic MMP-9 [41] and angiostatic TIMP-3 [29] in endochondral vessels may be important in coupling bone resorption to bone formation [30].

Fibrosis and periprosthetic osteolysis are limiting problems of titanium implantology. TNF-α is a potent mediator of inflammation associated with these problems [43]. Brånemark dental implants were found lacking in expression of TNF-α, while showing induction of immunoprotective cytokines, such as transforming growth factor-β (TGF-β) [44]. Our model is characterized by the absence of inflammation and, as seen in the **Table 2**, lack of TNF-α expression, with the exception of osteocytes at 4 weeks postimplantation. Low inflammation associated with pure titanium fixtures relative to metal alloys and cement may in part be related to this pattern of TNF-α expression.

The capability of osseointegrated titanium to transmit mechanical stimuli [39] is of particular clinical importance [45]. Discovering molecular mechanisms in titanium-stimulated bone that promote central mechanosensation is fundamental. TNF-α is known to modulate mechanosensory pathways in peripheral nerves [46,47]. Functional adaptation of bone and its mechanosensation is thought to be a primary function of osteocytes [48,49], which appear to function as early carriers of TNF-α during osseointegration. Its up-regulation in mechanosensory osteocytes may be important in the restoration of the mechanosensory network in bone, leading to osseoperception. Axonal transport of TNF-α [50] may contribute to the central plasticity in response to the integrating implant.

A recent study has shown that MMP-9 messenger ribonucleic acid (mRNA) expression in human osteoblasts in vitro is stimulated with titanium, but was decreased with zirconium and was unreactive to alumina ceramics [36]. While the present data demonstrate that titanium-induced osseointegration is associated with dynamic changes in MMPs, the activating (TNF-α) and inhibiting (TIMP-3) factors of MMPs relative to sham surgery, a detailed in vivo analysis of the effect of alternative materials on the patterns of MMP expression and distribution is forthcoming. Variations between human and rodent response to titanium fixtures may exist because of the differences in bone maturation and turnover. Yet, a rat femur model of intramedullary

osseointegration and osseoperception provides a paradigm for studying molecular mechanisms of these phenomena in connection with their clinical application [37].

CONCLUSION

In conclusion, we hypothesize that MMPs are involved in the formation of properly constituted ECM and of bone remodeling processes that promote integration of pure titanium threads with newly forming bone (summarized in **Table 3**). MMP-dependent molecules may be potential candidates for the coating and other biochemical surface modifications of implants.

Table 3.
Proposed function of MMP-related molecules in osseointegration.

Marker	Proposed Function
MMP-2	Solubilization of osteoid
MMP-7	Solubilization of osteoid Maintaining content of proteoglycan matrix Chondrocyte function at growth plate
MMP-9	Osteoclast migration/function Growth plate angiogenesis Apoptosis of hypertrophic chondrocytes
TNF-α	Sensory (osseoperceptive) transduction via mechanosensory osteocytes
TIMP-3	Matrix thickness maintenance at titanium-bone interface Growth plate angiogenesis via MMP-9 regulation

MMP = matrix metalloproteinases
TNF = tumor necrosis factor
TIMP = tissue inhibitors of metalloproteinases

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