Critical Reviews in Oral Biology & Medicine

http://cro.sagepub.com/

Proteoglycans At the Bone-Implant Interface

M.M. Klinger, F. Rahemtulla, C.W. Prince, L.C. Lucas and J.E. Lemons

CROBM 1998 9: 449

DOI: 10.1177/10454411980090040401

The online version of this article can be found at:
http://cro.sagepub.com/content/9/4/449

Published by:

http://www.sagepublications.com

On behalf of:

International and American Associations for Dental Research

Additional services and information for *Critical Reviews in Oral Biology & Medicine* can be found at:

Email Alerts: http://cro.sagepub.com/cgi/alerts

Subscriptions: http://cro.sagepub.com/subscriptions

Reprints: http://www.sagepub.com/journalsReprints.nav

Permissions: http://www.sagepub.com/journalsPermissions.nav

>> Version of Record - Jan 1, 1998

What is This?

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

PROTEOGLYCANS AT THE BONE-IMPLANT INTERFACE

M.M. Klinger[1]*
F. Rahemtulla[2]
C.W. Prince[3]
L.C. Lucas[1]
J.E. Lemons[4]

Departments of [1]Biomedical Engineering, Hoehn Building, 1075 13th Street S., [2]Oral Biology, [3]Nutrition Sciences, and [4]Biomaterials, University of Alabama at Birmingham, Birmingham, Alabama 35294;*To whom correspondence should be addressed

ABSTRACT: The widespread success of clinical implantology stems from bone's ability to form rigid, load-bearing connections to titanium and certain bioactive coatings. Adhesive biomolecules in the extracellular matrix are presumably responsible for much of the strength and stability of these junctures. Histochemical and spectroscopic analyses of retrievals have been supplemented by studies of osteoblastic cells cultured on implant materials and of the adsorption of biomolecules to titanium powder. These data have often been interpreted to suggest that proteoglycans permeate a thin, collagen-free zone at the most intimate contact points with implant surfaces. This conclusion has important implications for the development of surface modifications to enhance osseointegration. The evidence for proteoglycans at the interface, however, is somewhat less than compelling due to the lack of specificity of certain histochemical techniques and to possible sectioning artifacts. With this caveat in mind, we have devised a working model to explain certain observations of implant interfaces in light of the known physical and biological properties of bone proteoglycans. This model proposes that titanium surfaces accelerate osseointegration by causing the rapid degradation of a hyaluronan meshwork formed as part of the wound-healing response. It further suggests that the adhesive strength of the thin, collagen-free zone is provided by a bilayer of decorin proteoglycans held in tight association by their overlapping glycosaminoglycan chains.

Key words. Osseointegration, proteoglycans, implant, interface.

(I) *Introduction*

The forcible insertion of a metallic implant into freshly wounded bone is an insult that could produce either chronic inflammation or fibrous encapsulation of the intruder. These results were indeed typical during much of the early history of implantology, when stainless steel and cobalt alloys were the primary implant materials. Some unstable attachments could not adequately support the forces of functional loading and eventually led to failure of the implant (Galante *et al.*, 1991). In the 1960s and '70s, results from the Brånemark group radically modified expectations for root-form (cylindrical screw-type) dental implants by pioneering the use of non-alloyed titanium ("commercially pure" or cpTi) together with techniques that minimized surgical trauma and a passive (non-loaded) phase for healing of the bone (Albrektsson *et al.*, 1983). On-going implant modifications have included the use of porous or bioactive coatings in attempts to form better bone-bonding surfaces (Ducheyne, 1988; Lucas *et al.*, 1993).

In current practice, bone tissue tolerates the presence of titanium implants and usually forms a relatively rigid, load-bearing interface, an outcome termed "osseointegration" by Brånemark *et al.* (1982). These conditions have clearly enhanced the quality of life for large numbers of edentulous and partially edentulous patients. Yet significant challenges remain. Premature loading can disrupt the healing process and may shorten the effective life expectancy of the implant. Most endosteal implants therefore must be securely interfaced with the adjacent bone before the functional prostheses can be attached. The months of waiting and of limited function may be a period of hardship for the patient. With over 300,000 dental implants used annually in the United States alone, a delayed time for integration represents a sizable economic issue.

We have yet to learn all of the reasons for titanium's biocompatibility with bone or, for that matter, the most basic molecular interactions which occur at the implant interface. It is most interesting that several investigators (Linder *et al.*, 1983; Johansson *et al.*, 1989; Steflik *et al.*, 1992; Murai *et al.*, 1996) have observed a thin layer of "proteoglycan-rich" ground substance at intimate contact points between retrieved specimens of implant surfaces and the adjacent bone. This finding makes sense physiologically, *i.e.*, proteoglycans are key components of the extracellular matrix in a variety of tissues where they play significant roles in cell adhesion, proliferation, migration, and differentiation. In bone, they may act to stabilize certain growth factors and have been implicated in the control of mineralization (Gehron Robey, 1989).

A previous article in this journal discussed how sev-

Downloaded from cro.sagepub.com by guest on January 1, 2016 For personal use only. No other uses without permission.

eral components of the extracellular matrix may relate to osseointegration (Stanford and Keller, 1991). By focusing specifically on proteoglycans, this current review has three objectives: (1) to describe briefly the general structure and theorized functions for proteoglycans, particularly in bone; (2) to review critically the evidence for proteoglycans at the bone-implant interface; and (3) to meld this information into a model regarding the role of proteoglycans (PGs) in the interaction of bone with biomaterial surfaces.

(II) Proteoglycans: Structure and Function

Several excellent reviews, including at least three in this journal (Uitto and Larjava, 1991; Rahemtulla, 1992; Bertolami and Messadi, 1994), have treated in various degrees the subject of proteoglycan structure and function (see also Hardingham and Fosang, 1992; Iozzo and Murdoch, 1996). Comprehensive discussions of the PGs of bone and cartilage have been published (Gehron Robey, 1989; Roughley and Lee, 1994). This critique will briefly survey those proteoglycans most likely to be relevant to bone-biomaterial interactions.

The primary structure of proteoglycans consists of a protein core bearing one or more covalently attached sulfated polysaccharide chains, or glycosaminoglycans (GAGs). GAGs are generally classed into five major categories, depending on the nature of the repeating disaccharide units. Chondroitin sulfate (CS) consists of alternating residues of glucuronic acid and N-acetyl-galactosamine; the latter may be sulfated on either the 4- or the 6-carbon. Dermatan sulfate (DS) differs from CS in that most of the glucuronic acid residues have undergone epimerization to iduronic acid. Heparin and heparan sulfate (HS) consist of alternating N-acetyl-glucosamine and either glucuronic or iduronic residues; the monosaccharides are sulfated to a greater degree in heparin than in HS. The keratan sulfate (KS) disaccharide contains N-acetyl-galactosamine and galactose, either of which may be sulfated. Hyaluronic acid (HA), now generally referred to as hyaluronan, is a polymer which consists of repeating, *unsulfated*, disaccharides of glucuronic acid and N-acetyl-glucosamine. HA is usually grouped within the family of proteoglycans, even though it is not found covalently attached to a protein core. It is included in this review because it is often found complexed with true PGs and may also play a significant role in the events leading to osseointegration.

The prototype for proteoglycan structure is the large, chondroitin sulfate PG of bovine nasal cartilage, which bears a polypeptide portion of approximately 220,000 Da. As many as 100 chains of chondroitin sulfate, each consisting of about 40 disaccharide units, are linked *via* xylose-containing linkage regions to serine residues in the center portion of the protein core (Rodén, 1980). These multiple GAG chains, closely spaced and extending outward from the polypeptide, give the molecule its characteristic "bottlebrush" appearance. Several smaller chains of keratan sulfate are attached to the core, in most cases closer to the protein's amino terminus than the CS chains. Several non-GAG oligosaccharides are linked *via* both N- and O-glycosidic linkages to the protein; these carbohydrate chains are structurally similar to those found on many serum and cell-associated glycoproteins. The large cartilage PG is now referred to as aggrecan, due to its ability to form extremely large aggregates with hyaluronan polymers. Several aggrecan PGs bind *via* globular domains at their amino termini to short stretches of glucuronic acid-glucosamine disaccharides in the hyaluronan molecule. The PG:HA interactions are stabilized by non-PG link glycoproteins, resulting in a highly anionic complex with a molecular mass of several million. The high concentration of these huge aggregates in cartilage matrix resists the displacement of water and provides the tissue with much of its resilience (Roughley and Lee, 1994).

Conflicting reports indicate that large chondroitin sulfate PGs (CSPGs) may either inhibit (Dziewiatkowski and Majznerski, 1985; Chen and Boskey, 1986) or stimulate (Linde *et al.*, 1989; Hunter and Szigety, 1992) mineralization, depending to some extent on whether the studies were conducted with soluble or immobilized proteoglycans. Another large CSPG, dubbed versican, is found in developing and healing bone and is gradually replaced by smaller PGs as calcification proceeds. Versican (12 CS chains; mol wt ~1 million) is structurally distinct from aggrecan but shares its ability to bind hyaluronan. A complex of this PG with hyaluronan may provide a short-lived, spongy framework needed for the construction of mature bone (Gehron Robey, 1996).

Heparan sulfate PGs (HSPGs) are synthesized by several osteoblast-like cell lines *in vitro* (Fedarko *et al.*, 1990; Takeuchi *et al.*, 1990; McQuillan *et al.*, 1991). There is some evidence for low levels of HS in mineralized tissues including incisors (Prince *et al.*, 1984) and in human dentin (Branford White, 1978); in these locales, heparan sulfate may be associated with the cell membrane rather than with the extracellular matrix. HSPGs have been implicated in cell attachment as part of focal adhesions which anchor fibroblasts to underlying substrates; these structures contain elevated levels of the syndecan 4 HSPG (Woods and Couchman, 1994). Fibroblast growth factors bind to specific structural regions of HS polysaccharide chains rather than to the protein cores of the proteoglycans (Ruoslahti and Yamaguchi, 1991). HSPGs in growth plate matrix may act as a reservoir for these factors (Chintala *et al.*, 1994).

Two small proteoglycans, decorin and biglycan, are at the opposite end of the size spectrum from aggrecan. Decorin (also known as PG-II) and biglycan (PG-I) both have core polypeptides of approximately 37,000 Da. (Gehron Robey, 1989). Decorin has a single GAG chain

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

near its amino terminus, while biglycan has two such chains, yielding total M_rs of approximately 80,000 and 120,000, respectively. Both PGs are found in a wide variety of tissues in addition to bone and cartilage. Interestingly, decorin in most connective tissues bears a dermatan sulfate GAG chain, while in bone the polysaccharide is chondroitin sulfate. The functional significance of this difference is unknown. While there is about 55% homology in their amino acid sequences, the two proteins are distinct gene products which vary not only in primary structure but also in the regulation of their synthesis (Gehron Robey, 1989). Both decorin and biglycan contain several leucine-rich repeat sequences (LRRs); the possible importance of this feature for biomaterial interactions will be discussed later.

When corneal or tendon tissue preparations are stained with cationic probes such as Cupromeronic Blue, an array of short filaments appears to ornament the surfaces of the collagen fibrils (Scott, 1988, 1990, 1992a,b). These decorative filaments consist of small dermatan sulfate PGs (hence the name decorin) which may restrain the growth or fusion of the fibrils. *In vitro* studies demonstrate that decorin's protein core, rather than its GAG chain, interacts with collagen types I and II (Brown and Vogel, 1989). Decorin and biglycan appear to react with identical binding sites on the collagen molecule, though with considerably different affinities (Schönherr *et al.*, 1995). This difference is underscored by the sensitivity of the interactions to anion concentration. While both PGs bind to collagen in low (3-5 mM) phosphate or sulfate buffers, only decorin binds to the fibrils in higher (30 mM) concentrations of phosphate (Pogány *et al.*, 1994). The core proteins of both decorin and biglycan bind with high affinity to transforming growth factor-β (TGF-β) (Hildebrand *et al.*, 1994). TGF-β 's biological activities may be either neutralized or enhanced when it is complexed with decorin, suggesting that a decorin-rich matrix may serve as a repository for the growth factor (Ruoslahti and Yamaguchi, 1991; Takeuchi *et al.*, 1994).

The mineralized matrix of fetal porcine calvarial bone contains not only decorin but also another, structurally distinct, small CSPG ($M_r \approx 110{,}000$) that appears to be unique to hard tissues (Goldberg *et al.*, 1988). This novel PG (HAPG3 or CSPG III) is secreted by osteoblastic cells and rapidly associates with hydroxylapatite crystals; together with osteopontin, it may be involved in regulating crystal growth (Nagata *et al.*, 1991). This function may be shared by a pair of small keratan sulfate PGs, termed lumican and fibromodulin, which are the most abundant PGs in unmineralized cementum (Cheng *et al.*, 1996).

(III) *Evidence for* PGs *at the* Interface

(A) Retrievals

The presence of proteoglycan-like material at the implant interface was implied nearly 25 years ago when James conducted histochemical studies on Vitallium® implants retrieved from dog maxillae (1973). When the specimens were stained with Periodic Acid Schiff-alcian blue and examined by light microscopy, each revealed an acid mucopolysaccharide (glycosaminoglycan) layer adjacent to the metal surfaces. The most intensely stained areas had a density similar to that of epithelial basement membrane.

Albrektsson *et al.* (1982) and Linder *et al.* (1983) confirmed and extended these findings at the ultrastructural level. These workers sought to avoid the artifacts which can arise when a bulk metal sample is thin-sectioned; this processing can easily disrupt the fine structure of the tissue in the first 10 to 100 nm from the metal surface. As an alternative, they coated polycarbonate rods with thin (120-250 nm) layers of pure titanium or gold and implanted them into adult rabbit tibiae for 12 wks. Examination of the retrieved and sectioned rods by TEM revealed a 20-50-nm zone of collagen-free ground substance closest to the metal surface. This was bordered by a second layer, 100-500 nm in thickness, which contained randomly distributed collagen fibers and occasional osteocytes; a third layer consisted of collagen fibers oriented in orderly bundles. Each of the layers showed some evidence of calcification, with fewer deposits in the zones closest to the metal. Both ruthenium red (RR) and lanthanum intensely stained the 20-50-nm layer, an effect that was greatly diminished if the sections were pre-treated with chondroitinase or hyaluronidase. The authors concluded that the "amorphous" interface zone contains a mixture of CSPGs and hyaluronic acid in unknown proportions. In contrast to the Ti-coated implants, the golden retrievals had a broader RR-positive zone with a minimum thickness of 50 nm.

These investigators subsequently repeated the cpTi studies and compared the tibial responses to polycarbonate plugs coated with zirconium, 316L stainless steel, or titanium-6 aluminum-4 vanadium alloy (Albrektsson *et al.*, 1985; Albrektsson and Hansson, 1986; Johansson *et al.*, 1989). In each case, the interface formed on the pure titanium coatings was consistent with the earlier observations (Linder *et al.*, 1983). However, the other materials yielded quite different results. For zirconium, the PG-rich layer at the metal surface was 40-50 nm thick, followed by a random collagen filament layer up to 300 nm from the surface where collagen bundles could first be detected. At the stainless steel interface, a layer populated by inflammatory cells was separated from the metal by a collagen-free PG coat of several hundred nm thickness. In the case of the titanium alloy, the PG layer ranged in width from 50 to 100 nm, followed by a collagen network up to 1000 nm (1 μm) thick; collagen bundles appeared no closer than 500 to 1000 nm from the metal surface. The bone tissues were more easily dislodged from the

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

gold or stainless steel surfaces than from the pure titanium implant. The PG layers were significantly thicker on the former two materials than on the latter, suggesting "an inverse relationship between the thickness of the ground substance layer and the force of attachment" (Linder *et al.*, 1983). The implications of this observation will be discussed later.

Some years later, Linder and co-workers (1989) analyzed the tissue reactions to implants of bulk metal retrieved from rabbit tibiae after 11 months. On each of the four metals tested (pure titanium, titanium alloy, stainless steel, and Vitallium [CoCrMo]), the thickness of the ground substance layer was less than 50 nm, but no characterization of this layer, i.e., by staining with ruthenium red, was reported. Considerable variation was seen in the organization of collagen fibrils in the adjacent zones and in their distance from the implant surface. Unlike Albrektsson, Linder found no consistent pattern relating interface morphology to implant surface chemistry.

This disagreement illustrates some of the difficulties inherent in studies of bone-metal interfaces. The amorphous zone lacks easily recognizable features and is susceptible to fixation artifact. This technical problem, together with several inventive solutions and their drawbacks, has been reviewed by Linder (1992). For example, metal-sprayed polycarbonate plugs are relatively easy to section, yet questions persist as to whether the chemistry of the surface is truly representative of that of bulk materials; moreover, the mechanical properties of an implant, especially in a load-bearing situation, would be far different for a polymeric core than for a solid metal. Freeze-fracturing bulk metal implants, however, unavoidably removes up to 10 nm of the interface tissue. Interpretation of electron micrographs of the interface is also problematic. Chehroudi *et al.* (1992) coated titanium onto micromachined epoxy substrates and implanted them subcutaneously near rat parietal bones. From their perspective, collagen fibrils abutted directly onto the titanium coating with <u>no</u> intervening amorphous layer. When another investigator viewed the same micrographs, he observed a collagen-free interface zone at the metal surface (D. Steflik, in discussion following Chehroudi *et al.*, p. 102).

Sennerby *et al.* bolstered the case for the amorphous zone with their analysis of solid cpTi screws retrieved from rabbit tibiae (1992). Electropolishing the specimens removed much of the bulk metal near the implant surface and facilitated sectioning of the interface. The fracture technique was applied to a parallel set of samples for separation of the embedded tissue from the metal surface. When they combined the results from these two complementary approaches and took into account the pitfalls of each, the authors concluded that collagen fibrils closely approached the titanium surface but did not penetrate the 100-200-nm-thick layer of amorphous material appearing along the osseointegrated implants.

The thickness and fine structure of the implant interface are likely to be a reflection of several factors, including surface chemistry, time of implantation, and species and age of the host. Pure titanium rods were implanted for 28 days into the tibiae of young and mature rats (Murai *et al.*, 1996). TEM analysis of the demineralized retrievals revealed a 20-50-nm-thick, ruthenium red-positive, amorphous zone between the surface of the titanium and an adjacent layer of collagen fibrils. Interestingly, this morphology is more similar to that seen by Albrektsson *et al.* (1982; Linder *et al.*, 1983) with the titanium-coated polycarbonate plugs than to the observations by Sennerby *et al.* (1992) using the solid titanium implants. The interface zone in the mature, but not the young, rats showed evidence of connective tissue containing blood vessels.

Ultrastructural studies of retrieved dental implants have generally indicated the presence of a PG-rich layer at the interface. Steflik and co-workers (1992, 1993) placed commercial root- and plate-form titanium implants into the mandibles of adult dogs and allowed a five-month unloaded healing period. One group of animals was then killed, while a second group was prosthodontically loaded for an additional six months. The metals were detached from the embedded tissues by cryofracture without decalcification and the interfaces then analyzed by either TEM or high-voltage electron microscopy (HVEM). By examining serial sections along the entire length of the unloaded implants, the authors determined that the thickness of the electron-dense deposit at the interface varied from 50 to 500 nm, and that the zone appeared thinnest where the matrix was most densely mineralized. During a six-month loading period, the zone further constricted to 20-50 nm at sites where mature, densely mineralized bone approached the implant surface. The authors speculated that the zone was glycosaminoglycan in nature, although it was not characterized by histochemical means. In later studies, Steflik *et al.* (1994a,b, 1997) observed that retrieved ceramic (alumina oxide) implants were coated with 20-50 nm of electron-dense, ruthenium-positive deposits similar to those seen on titanium. Osteocytes near the interface extended cellular processes which came into direct contact with the thin amorphous layer at the implant surface.

The PG-rich layer is conspicuously absent from the electron micrographs made by Listgarten *et al.* (1992) of titanium-coated epoxy resin plugs implanted into dog mandibles. Despite the apparent osseointegration of the implants as judged by light microscopy, TEM of the sections indicated that collagen fibrils made direct contact with the titanium surface with no evidence of an inter-

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

vening PG layer. The authors suggested that the relatively rough surface of the experimental implant may have been at least partly responsible for the missing electron-dense layer.

A positive reaction to ruthenium red may well signify an abundance of proteoglycans. But this dye also reacts with carboxyl groups on sialic acid residues (Hayat, 1989) and could thus stain other matrix glycoproteins such as osteopontin (OPN). More specific detection is now possible with commercially available monoclonal antibodies able to distinguish among dermatan sulfate, chondroitin 4-, and 6-sulfate proteoglycans. Digestion of these PGs with chondroitinases ABC or ACII removes the bulk of the GAG chains, while leaving unsaturated oligosaccharide stubs attached to the core proteins (Caterson *et al.*, 1987). Antibodies specific for these stubs were used to analyze GAGs on Ti screws inserted into the thigh bones of normal and diabetic rats (Higuchi *et al.*, 1995). It is unclear from their report how the structure of the interface was preserved during sectioning or the level of detail revealed by the immunochemical staining. Nevertheless, this approach has considerable promise for studies of the interface. These antibodies have been used at the light microscopic level to identify GAGs in rat predentin and dentin (Takagi *et al.*, 1990). Kinne *et al.* (1988) reported the use of anti-decorin antibodies to stain retrieved glass-ceramic implants, but their analysis did not include an examination of the ultrastructure of the interface zone. Immunohistochemical staining of titanium retrievals with anti-OPN antibodies has prompted some to suggest that the amorphous zone consists primarily of OPN and not proteoglycans (Nanci *et al.*, 1994). The presence of one should not exclude the presence of the other, since both are major non-collagenous proteins of bone.

Spectroscopic analysis of retrievals has provided indirect evidence for the presence of PGs at the implant surface. Auger electron spectroscopy (AES) can be used to produce elemental depth profiles of the outermost atomic layers of metal surfaces and can thus determine the thickness and composition of oxide layers on implants. McQueen *et al.* (1982) and Sundgren *et al.* (1986) applied this technique to unimplanted titanium screws and detected a surface oxide layer consisting primarily of TiO_2 with traces of carbon. (Carbon is a persistent contaminant of implant surfaces due to adsorption of airborne dust and hydrocarbons.) After implantation in patients' jaws for up to eight years, the oxide thickness increased from about 3.5 nm to as much as 100 nm. The authors detected sulfur in the outer surface of most of the titanium retrievals but failed to detect this element in the surface oxides of stainless steel implants (pins and wires) retrieved from patients' hands or facial bones. AES is relatively insensitive to sulfur-containing amino acids and disulfide bonds, therefore ruling out most serum and matrix proteins as the source of this signal. Instead, the authors attributed the sulfur peak to CSPGs, each of which may contain hundreds or thousands of ester-linked sulfate groups. Sulfate esters are also found in bone sialoprotein, but at a far lower density (up to 17 sulfates *per* molecule) than in PGs (Midura *et al.*, 1990).

(B) Cell culture studies

Cell culture studies of the bone-biomaterial interface have generally tended to support the presence of the PG-rich layer, but with some notable differences. Davies and co-workers (1990) seeded suspensions of rat bone marrow cells onto discs of commercially pure titanium that had been polished to 600 grit and then sterilized in a steam autoclave. After three wks, the cultures were fixed and stained with ruthenium red, mounted in epon resin, and briefly immersed in liquid nitrogen. This latter step freeze-fractured the interface and separated the metal disc from the fixed tissue. The authors concluded that the interface was faithfully preserved in the resin, since it showed the same pattern of scratches as those visible in the titanium substrate. Cross-sections of the samples revealed two sublayers between the cell bodies and the metal surface. The layer closest to the cells stained more deeply with ruthenium red and was richer in collagen fibers; the layer immediately adjacent to the titanium was more lightly stained and had fewer fibers. Since this PG-poor "bonding zone" had not been observed *in vivo*, it may occur as a special adaptation of cells in culture. Alternatively, this zone may be present in the earlier, but not the later, stages of osseointegration.

Osteoblasts appear to secrete a proteoglycan-rich layer on calcium phosphate surfaces as well as on pure metals. Rat bone marrow cells were cultured for 18 days on plastic coverslips which had been plasma-sprayed with hydroxylapatite (De Bruijn *et al.*, 1993). By varying the spray parameters and heat treatments, they obtained coatings of three average crystallinities (15%, 43%, and 69%). The specimens were thin-sectioned nearly parallel to the surface, rather than at a transverse angle; this orientation made it possible to cut the tissue at the interface before the hydroxylapatite substrate. A 20-60-nm-thick ruthenium-red-positive layer was visible at the interface of the extracellular matrix on the 69% crystalline hydroxylapatite surface. This layer was separated from the substrate by a narrow gap, which the authors ascribed either to tissue shrinkage during fixation or to the decalcification step. The electron-dense layer was less distinct on the hydroxylapatite surfaces of lower crystallinity.

An alternative approach was used by Garvey and Bizios (1994) to examine the interface of rat calvarial osteoblasts cultured on dense hydroxylapatite. The authors used 3-M HCl and 10-M hydrofluoric acid to dissolve the hydroxylapatite coating and underlying glass coverslip carefully away from the fixed and embedded

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

tissue. The thickness of the ruthenium-red-positive zone was greatest (50-120 nm) for cells cultured on rougher HA surfaces than on polished hydroxylapatite or on glass alone. This electron-dense layer was detected after only 7 days in culture, approximately half the time required for the onset of mineralization.

The biosynthesis of proteoglycans on implant materials can be analyzed by metabolic labeling with radioactive precursors. Martin *et al.* (1995) grew MG63 cells, derived from a human osteosarcoma, on commercially pure titanium surfaces of various degrees of roughness. On the basis of radioactivity *per* mg protein, the cells on each of the metal substrates incorporated less [^{35}S]-sulfate into non-dialyzable material than did the controls grown on tissue culture plastic.

In ongoing studies in our laboratory (see abstract, Klinger *et al.*, 1995), we examined PG synthesis on implant metals by MC3T3-E1 cells, a clonal, osteoblast-like cell line derived from newborn mouse calvaria. Analysis of the ^{35}S sulfate-labeled macromolecules by ion exchange on DEAE-Sephacel and gel filtration chromatography on Sepharose CL-4B yielded similar profiles for the total cell extracts on pure titanium and on plastic. Incubation with chondroitinase ABC degraded 45% and 55% of the label from cells on plastic and cpTi, respectively. The remainder of the macromolecular label in each case was degraded by heparitinase, suggesting that both extracts contain a roughly equal mixture of chondroitin sulfate and heparan sulfate proteoglycans.

A subsequent study (see abstract, Klinger *et al.*, 1996a) compared the biosynthesis of ^{35}S-labeled PGs by MC3T3-E1 cells on polished discs of pure titanium, Ti-6Al-4V alloy, and 316L stainless steel. A two-step extraction process was used to separate the radiolabeled PGs in the cell-associated fraction from the PGs in the extracellular matrix. An average of 46% more radioactivity was incorporated (*per* cell) into the cell-associated PGs on each of the three metals than by the same cells grown on plastic. The difference was even more striking for proteoglycans in the extracellular matrix fraction, which presumably would correspond to the ruthenium-red-staining layer seen in the ultrastructural studies. On each of the implant metals, an average of 3.5-fold more label was found in the matrix PGs than in the corresponding fraction from the cells on plastic. Differences between these results and those obtained by Martin *et al.* (1995) could be due to cell type or variations in analytical techniques.

Sulfur has been detected spectroscopically not only on implant retrievals but also in extracellular matrices produced *in vitro*. Davies *et al.* (1991), using imaging electron energy loss spectroscopy, found evidence for sulfated molecules in the matrix secreted by rat bone marrow cells cultured on sputtered cpTi thin films. Osteoblast-like cells from newborn rat calvaria cultured on discs of porous bioactive glass produce an extracellular matrix containing Ca and P in proportions typical of hydroxylapatite (El-Ghannam *et al.*, 1995). Analysis of this material by scanning electron microscopy-dispersive x-ray microanalysis (SEM-EDAX) also detects a small sulfur peak. This signal is absent from the control discs incubated in culture medium but without cells. Siding with Sundgren *et al.*'s conclusions (1986), the authors attribute this peak to the biosynthesis of PGs.

(C) Adsorption studies

The negative electrical charge of the titanium oxide coating at the implant surface might be expected to repel proteoglycans due to the high density of sulfate and carboxylate groups in the GAG chains. Parsegian (1983), who likened this interaction to that observed between negatively charged cell membranes, suggested that small amounts of calcium ions could bridge the negative surface groups and thereby overcome electrostatic repulsion. He also predicted that the surface mixture of titanium oxides contains certain "charge constellations" which match up with corresponding charged groups in the ground substance. If these particular charge configurations are absent from surface oxides on stainless steel or CoCr alloy, then this model would provide valuable clues for understanding titanium's special ability to support osseointegration.

Collis and Embery (1992) came to similar conclusions based on their studies of the adsorption of various glycosaminoglycans to titanium powder. Commercial preparations of heparan sulfate, hyaluronic acid, and chondroitin-6-sulfate bound poorly if at all to the powder. Significantly higher levels of chondroitin-4-sulfate adsorbed to the particles, provided that they had been pre-treated with calcium acetate. This finding is of particular interest, since chondroitin-4-sulfate is the major GAG detected in bone PGs. The authors pictured an electrostatic bridge (TiO_2^-– –Ca^{++}– –$C4S^-$) between the metal surface and proteoglycans in the adjacent tissue.

Preliminary studies from our laboratory indicate that this model may be overly simplistic. We examined the adsorption of radioactively labeled proteoglycans from MC3T3-E1 cells to titanium powder in Tris buffer/0.1% Triton X-100 (Klinger *et al.*, 1996b). The ^{35}S-PGs which adsorbed to the powder were eluted with a mixture of 4 M guanidine HCl and 0.5 M EDTA. Pre-treatment of the titanium powder with $CaCl_2$ stimulated the binding by about 60% compared with the water-treated control; pre-treatment with $LaCl_3$ stimulated the adsorption more than five-fold. The differences in adsorption behavior produced by the Ca and La ions were qualitative as well as quantitative. The average molecular size of the Ca-bound PGs was significantly larger than that of the La-bound PGs as determined by gel filtration on Sepharose CL-4B. Further studies (Klinger *et al.*, 1997) used *sequential* elution with guanidine and EDTA buffers to examine the

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

mechanism of PG adsorption. Surprisingly, EDTA was not effective at eluting ^{35}S-PGs from the $CaCl_2$-treated titanium powder, while 4 M guanidine HCl worked quite well in this regard. The reverse pattern was observed for PGs adsorbed to La-pre-treated titanium. If proteoglycans adsorb to titanium by a simple electrostatic interaction ($GAG^- - -Ca^{++} - -TiO_2^-$), then chelation of the calcium ions by EDTA should efficiently release the bound radioactivity. The observation that guanidine HCl, but not EDTA, achieves this result suggests that the protein portion of the molecules, rather than the polysaccharide chains, mediates the interaction of the PGs with the metal. This conclusion is supported by Ellingsen's finding (1991) that calcium pre-treatment of Ti surfaces enhances the adsorption of non-proteoglycan serum proteins, presumably *via* interactions of the polypeptides with the metal oxide.

(IV) *Model*: Proteoglycans, Hyaluronan, *and the* Bone-Implant *Interface*

It should be clear from the previous discussion that there is no consensus on the molecular structure of the bone-implant interface. At best, we can point to a few general conclusions which are consistent with most, but not all, of the available data. These principles can in turn be used as a basis for theorizing on the role of proteoglycans in the events leading to osseointegration. The following three observations were used as a starting point for developing a working model to describe these interactions:

(I) A thin layer of collagen-free material is present at intimate sites of bone contact with titanium implants. The thickness of this zone appears to decrease as osseointegration is achieved.

(II) Collagen fibrils closest to the interface zone are relatively disorganized, while those at a greater distance appear in oriented bundles.

(III) The thickness of the ruthenium-red-staining interface zone varies with the surface chemistry of the implant, from 20-50 nm for titanium to 1000 nm for stainless steel. The staining intensity is decreased by pre-treatment with chondroitinase or hyaluronidase.

Observation I

Our current model suggests that the relatively thick (> 500 nm) layer observed at immature sites on titanium consists primarily of a hyaluronan meshwork reinforced with chondroitin sulfate proteoglycans. There are ample reasons to suspect the presence of a hyaluronan matrix near the implant surface. The importance of hyaluronan in wound repair (reviewed previously in this journal by Bertolami and Messadi, 1994) is indicated by elevations in hyaluronan levels which occur in the early phases of tissue repair. Weigel *et al.* (1986) have suggested that this molecule, together with fibrin, forms a temporary three-dimensional scaffold at wound sites that influences the motility and inflammatory responses of blood cells. As cell migration proceeds, the matrix is degraded and is replaced by collagen fibers.

Hyaluronan may fulfill a similar role at the bone-implant interface. During bone matrix formation *in vitro*, levels of hyaluronan decrease with time in culture, while those of sulfated proteoglycans either remain constant or increase (Fedarko *et al.*, 1990). A hyaluronan framework may sequester the extracellular space required for subsequent assembly of the mature bone matrix. Hyaluronan influences other cellular behaviors, including proliferation, migration, adhesion, and differentiation, which are undoubtedly critical for achieving osseointegration. These interactions appear to be mediated by hyaladherins, a diverse family of hyaluronan receptors at the cell surface, together with hyaluronan-binding proteins in the matrix (Knudson and Knudson, 1993). One of the major hyaladherins in cartilage matrix is the chondroitin sulfate PG aggrecan which forms large aggregates with hyaluronan; collapse of these aggregates may precede or accompany nucleation and mineral growth (Poole *et al.*, 1989).

Our model asserts that a thick hyaluronan matrix may be formed on virtually any implant surface as a response to surgical trauma and/or as a product of stimulated osteoblasts. The porous hyaluronan matrix would be beneficial during the healing process by contributing to wound hydration and facilitating cell migration (Bertolami and Messadi, 1994). But the matrix would lack structural integrity and be too flimsy to resist micromotion of the implant within its socket. This could result in formation of a soft connective tissue capsule rather than a rigid junction (Brunski *et al.*, 1989). Moreover, high-molecular-weight hyaluronan inhibits the proliferation of certain cell types in a concentration-dependent manner (Goldberg and Toole, 1987). The hyaluronan meshwork must therefore be dismantled fairly quickly and be replaced by a more rigid attachment structure. Our current model presumes that the hyaluronan matrix is broken down *more rapidly* on a Ti or Ti alloy surface than on other implant metals. This enhanced degradation rate would be largely responsible for titanium's special capacity to support osseointegration.

How might different biomaterials affect the pace of hyaluronan depolymerization? Two degradative mechanisms come to mind: enzymatic and free-radical-induced. In wound repair or differentiation, the catabolic process is achieved by a variety of tissue hyaluronidases. Inhibiting the activity of these enzymes theoretically could hinder removal of a hyaluronan meshwork. Chromium corrosion products leached from stainless steel or cobalt alloy implants are significantly more toxic than leached titanium ions (Merritt and Brown, 1993;

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

Messer and Lucas, 1996) and might be expected to have a greater inhibitory effect on hyaluronidases. One might therefore expect the amorphous layer on an inert and non-toxic material to be extremely thin; yet the observed layer on gold-coated implants is significantly thicker than that on titanium (Albrektsson *et al.*, 1982).

An alternative explanation is based on hyaluronan's susceptibility to degradation by free radicals. Research spanning 30 years has established that physiological concentrations of reactive oxygen species can fragment high-molecular-weight polymers of hyaluronan. For example, superoxide generated by a xanthine oxidase/hypoxanthine system markedly reduces the viscosity of hyaluronan solutions within minutes; similar effects are observed when hyaluronan is incubated with activated human neutrophils (Greenwald and Moak, 1986). A small number of scissions in the macromolecule can profoundly decrease the viscosity of a hyaluronan gel and can disrupt aggregates of hyaluronan and chondroitin sulfate proteoglycans (Roberts *et al.*, 1989).

These findings have significance for implant biology, because hydrogen peroxide can be formed by inflammatory cells *in vivo* as part of the response to foreign materials; H_2O_2 is also produced by cultured gingival fibroblasts (Rahemtulla and Rahemtulla, 1994). Tengvall and co-workers (1989b) have examined the aqueous peroxy-gel that forms on titanium surfaces when the bulk metal is incubated with nonphysiological (~10 M) concentrations of H_2O_2; much more dilute H_2O_2 (0.3 M) can produce peroxy-gels on powdered Ti (Tengvall *et al.*, 1989a). Spectrophotometric analysis with a spin trap label indicates that the gel contains superoxide or perhydroxy radicals which may be relatively stable at neutral pH. It has not been determined whether the levels of H_2O_2 produced *in vivo* are of sufficient strength or duration to form peroxy-gels on titanium implant surfaces. But it is intriguing that the dark pigmentation observed frequently on titanium retrievals is similar to the blue/violet-blue color produced *in vitro* when titanium in buffered saline is exposed to hydrogen peroxide (Pan *et al.*, 1996).

The formation of such a gel on implant surfaces may have beneficial biological effects, either by influencing the local inflammatory response or by enhancing the bactericidal effects of tissue peroxidases (Tengvall *et al.*, 1990). Taylor *et al.* (1996) give the Ti-peroxy-gel mixed reviews. It might promote osseointegration by enhancing the growth of the Ti oxide layer. On the other hand, free radicals trapped in the gels may damage extracellular matrix molecules and thus impair the implant's ability to osseointegrate. The authors based this latter conclusion on their finding that Ti-peroxy-gels cleave hyaluronan polymers *in vitro*.

Our current model suggests that the Ti-peroxy-gel may be more of a blessing than an affliction. Accelerating the degradation of the flimsy hyaluronan meshwork would shorten the period when the interface is most vulnerable to micromotion and would lessen the chances for fibrous encapsulation of the implant. Ideally, the majority of the reactive oxygen species in the Ti-peroxy-gel would be consumed as the thick hyaluronan matrix is depolymerized; formation of the rigid attachment zone could then proceed. This outcome would be far more likely on titanium and Ti alloy because H_2O_2 does not cause the formation of peroxy-gels on several other metals, including Zr, Fe, Cu, Cr, Ni, Au, and Al (Tengvall *et al.*, 1989b).

OBSERVATION II

A dense hyaluronan gel near the implant surface might interfere with collagen organization; a more ordered arrangement of bundles would appear at greater distances from the surface, where the matrix is thinner. This may be analogous to the situation in the human vitreous humor, where collagen fibrils are randomly oriented within a thick hyaluronan gel; the fibrils are able to aggregate into parallel bundles in regions that appear to be more segregated from the hyaluronan (Sebag and Balazs, 1989). These effects would likely be caused by spatial exclusion and not by any covalent hyaluronan:collagen interactions (Ren *et al.*, 1991).

OBSERVATION III

This brings us to the third fundamental observation, that osseointegrated sites on titanium implants are characterized by 20-50-nm-thick zones which stain intensely with ruthenium red. Again, it must be stressed that there is little hard information on the composition and orientation of proteoglycans in the amorphous zone observed on titanium surfaces. Nevertheless, we can speculate on these features based on known properties of proteoglycans and collagen proteins. The model suggests that two parallel layers of decorin proteoglycans oppose each other across a gap of 20-50 nm. One layer is bound through its protein cores to a network of collagen fibrils. The second layer is adsorbed, again *via* the protein backbones, to the titanium oxide at the implant surface. The space between the two protein sheets consists of overlapping polysaccharide chains interacting tightly with each other; such a GAG-filled region would be expected to stain intensely with ruthenium red.

This model is based on Scott's vision of proteoglycan:collagen interactions in the corneal stroma (1992a,b). By staining tissue preparations with Cupromeronic blue and then uranyl acetate, Scott obtained high-resolution images of individual glycosaminoglycan chains and could measure their distances to collagen fibrils in the matrix. The resulting scheme is both simple and elegant. The protein cores of decorin and biglycan proteoglycans bind to collagen molecules on the surfaces of adjacent fibrils. Dermatan

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

or chondroitin sulfate chains extend out from the protein cores and overlap with each other, thus bridging and preserving the gap between the collagen fibrils. The distances between the collagen fibrils in the micrographs agree quite well with calculated lengths of extended carbohydrate chains (Thyberg *et al.*, 1975).

The interaction of decorin with collagen appears to be highly complex, perhaps involving multiple protein-protein bonds. Binding occurs in the presence of up to 1% detergent (Triton X-100) and is not reversed by washing with 2 M NaCl (Brown and Vogel, 1989). Decorin's structure suggests that it may also be capable of adsorbing securely to a non-biological material surface. Decorin belongs to a select family of structurally related molecules known collectively as Small Leucine Rich Proteoglycans, or SLRPs (Iozzo and Murdoch, 1996). These SLRPs in turn are members of a larger superfamily of proteins which all contain leucine-rich repeat sequences (LRRs) which vary in length from 20 to 29 amino acid residues. Multiple copies of these sequences are present in tandem in each protein of the superfamily. Decorin and biglycan have ten and eight copies, respectively, of a 24-residue LRR. The functions of this structural feature are uncertain, but all proteins containing LRRs appear to be involved in protein-protein interactions.

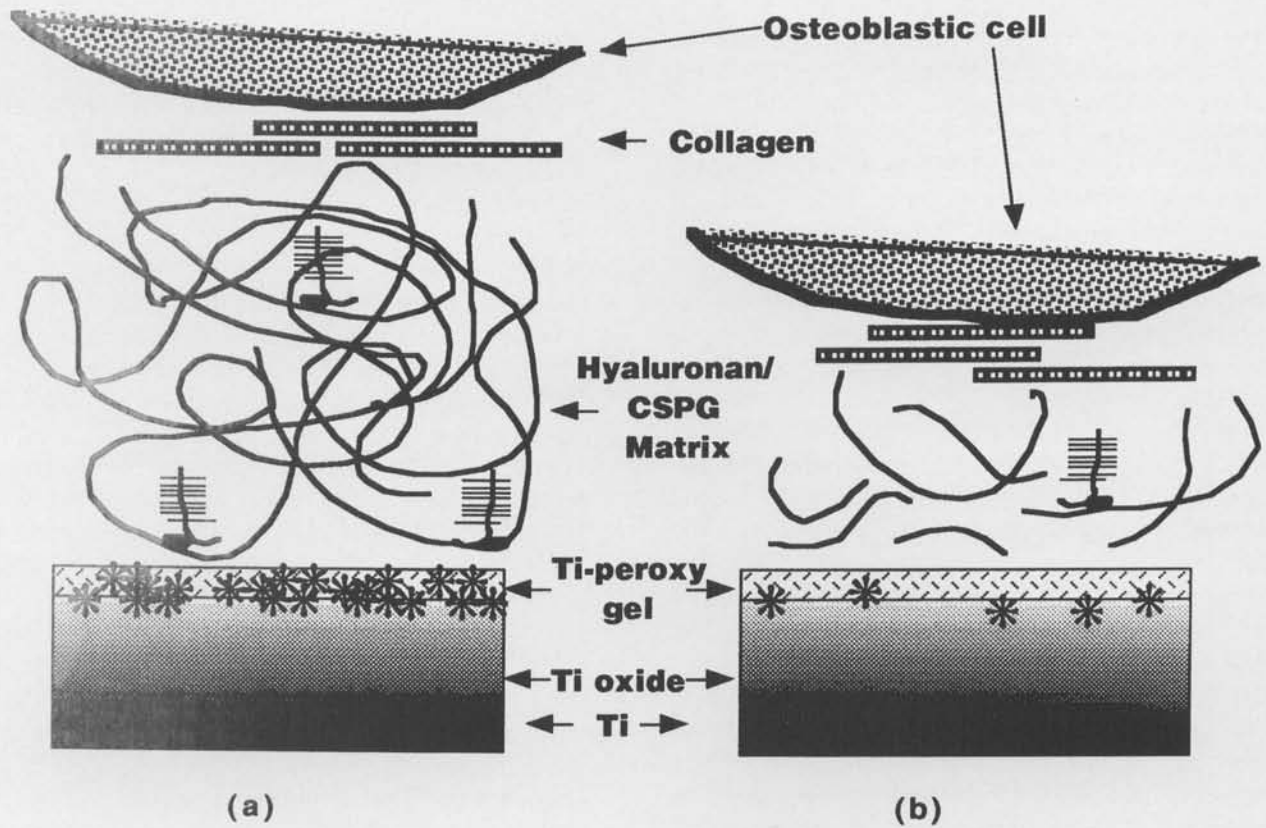
The crystal structure of one such protein, porcine ribonuclease inhibitor protein (RI), indicates a novel three-dimensional configuration (Kobe and Deisenhofer, 1994). RI consists of 15 LRRs of 28-29 residues each. The LRRs appear to be arranged such that all the β-strands and the α-helices are oriented parallel to a single axis. The result is an unusual non-globular, horseshoe-shaped molecule able to bind tightly to large areas on the surfaces of ribonuclease proteins. It is likely that the three-dimensional architecture of RI differs in important respects from that of decorin. However, decorin's ability to bind tightly to a number of proteins such as collagen and TGF-β suggests that its overall structure might be comparable with RI's non-globular, extended shape. Decorin was once thought to have a tadpole shape consisting of a roughly globular protein with a dermatan sulfate tail. When Scott (1996) magnified and re-examined the original electron micrographs, he found that this proteoglycan actually has a horseshoe shape much like RI. Such a structure would have the proper shape and dimensions to stabilize a pair of adjacent collagen triple helices. This same structure might also be ideally suited for binding with high affinity to a metal oxide surface. One may envision a decorin polypeptide in extended conformation, with its ten tandem LRRs forming multiple attachment sites to the surface oxide. The GAG chain at the amino terminal would stretch away from the substrate. When two parallel sheets of these proteoglycans are separated by less than 100 nm, the extended dermatan sulfate chains would overlap and interact with each other.

Such an arrangement could provide substantial adhesive strength, as illustrated by the PGs of the marine sponge *Microciona prolifera*. When placed in calcium- and magnesium-free seawater, these organisms dissociate into cell suspensions. These dispersed cells slowly re-aggregate into cohesive, spherical masses, a process which is enhanced by a large, species-specific complex of cell adhesion proteoglycans. As determined by atomic force microscopic measurements, the binding strength between a single pair of such molecules could theoretically support the weight of 1600 cells (Dammer *et al.*, 1995). The adhesion mechanism appears to consist of carbohydrate-carbohydrate interactions in which polysaccharides extend outward from their respective core proteins and bind to each other in a calcium-dependent manner (Misevic and Burger, 1993).

The ability to self-aggregate has been observed in certain GAGs, including heparan sulfate and dermatan sulfate (Fransson *et al.*, 1983). Overlapping DS chains from neighboring decorin (and biglycan) PGs might thus provide the adhesive force necessary to maintain a rigid attachment to the implant surface. This stabilizing effect would not be expected from CS-decorin, since chondroitin sulfate chains do not appear to self-aggregate (Turley and Roth, 1980). This assertion poses something of a problem. DS-decorin is produced in most connective tissues, but CS-decorin predominates in bone (Gehron Robey, 1989). How then can one account for DS-decorin in new bone matrix at implant interfaces? One possibility is that these DSPGs are secreted by the numerous fibroblastic cells which appear in the collagen matrix near the implant surface (Steflik *et al.*, 1994a). Alternatively, the foreign substrate might somehow influence osteoblastic cell metabolism and alter the biosynthesis of GAGs; indeed, when human bone cells are cultured on plastic, their decorin PGs bear DS, not CS, chains (Beresford *et al.*, 1987).

Granted, this model has limitations. It does not address the role of proteoglycans in the mineralization process at the interface (see Davies, 1996). Nor does it explain the bonding of bone tissue to ceramic surfaces. Blocks of dense sintered hydroxylapatite retrieved from dog mandibles reveal an extremely thin (2-10 nm) electron-dense zone (DeLange *et al.*, 1990); this is significantly narrower than the corresponding material seen on metallic implants. The current model does not account for the presence of other non-collagenous matrix proteins, such as osteopontin or α_2HS-glycoprotein, both of which have been localized to the interface (Nanci *et al.*, 1994). It also does not seek to explain the well-documented effects of such factors as surface energy, topography, and roughness on matrix formation and osseo integration (Kieswetter *et al.*, 1996). Nevertheless, this

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.



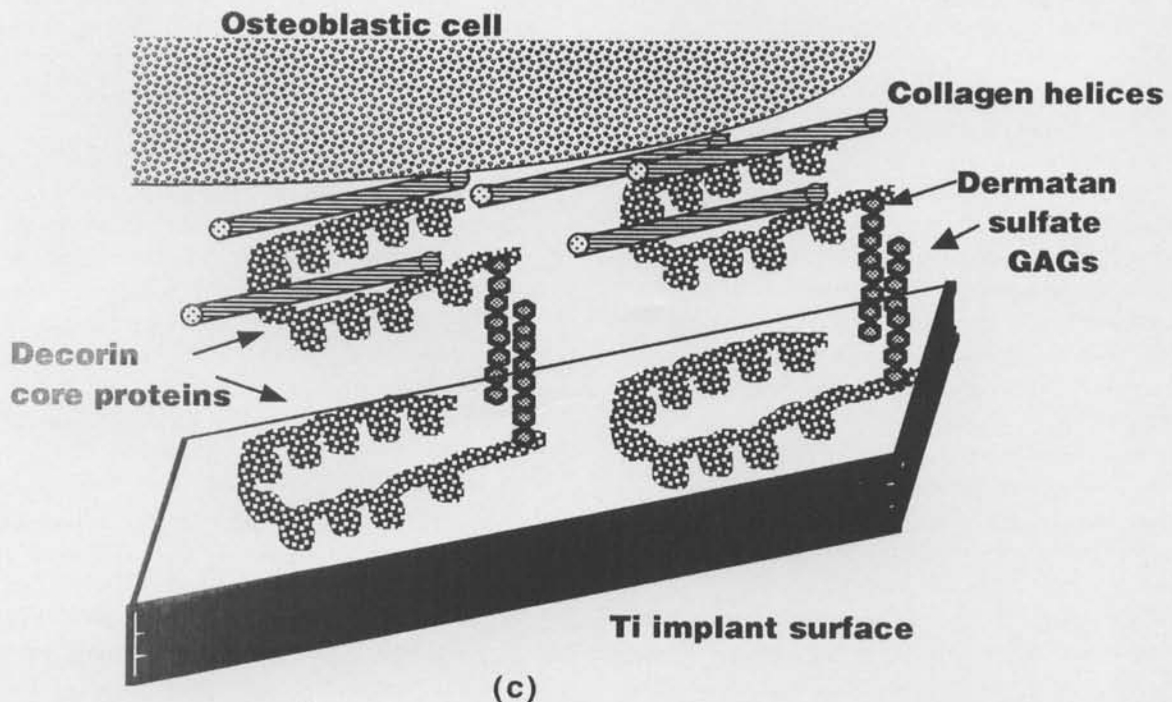


Figure. Model for hyaluronan and proteoglycan interactions at the implant interface. (a) Early phase of healing process. Loose meshwork of hyaluronan polymers and CSPGs fill a gap of approx. 500 nm between the implant surface and nearest collagen fibrils. The titanium oxide surface is coated with a thin Ti-peroxy-gel containing reactive oxygen species (large asterisks). (b) Later stage of healing process. Reactive oxygen species have depolymerized the hyaluronan macromolecules, thus narrowing the gap to 100-500 nm. Most of the free radicals have been consumed in the process. (c) Molecular model of the 20-50-nm "amorphous" zone at the titanium-bone interface. Horseshoe-shaped decorin PGs, with their 10 leucine-rich repeat sequences, form two parallel sheets: one on the implant surface and the second attached to nearby collagen polymers. Dermatan sulfate GAG chains extend outward from the amino termini of the PGs. Association between these overlapping GAG chains forms an adhesive zone that stains intensely with ruthenium red. Not to scale.

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

model may still be useful as a working framework to arrange a growing mass of often-conflicting data and interpretations.

(V) Conclusions and Future Directions

A sizable body of histochemical, spectroscopic, and biochemical data has repeatedly been interpreted to suggest that proteoglycans, as a major component of the extracellular matrix, play a special role in events leading to osseointegration. From our perspective, the evidence for a "proteoglycan-rich" zone at implant interfaces is highly suggestive but not conclusive. Uncertainties arise from sectioning artifacts and from the lack of specificity of certain histochemical techniques. Ultimately, the location and identity of proteoglycans in the collagen-free zone will be determined by means of a combination of antibodies specific for the various core proteins (decorin, biglycan, lumican, CSPG III) as well as by selective histochemical probes such as Cupromeronic blue. These future studies should also focus on hyaluronan, which can now be identified at the electron microscopic level by means of a specific probe derived from the hyaluronan-binding region of a chondrosarcoma proteoglycan (Ripellino *et al.*, 1985). These retrieval studies should be supplemented by *in vitro* determinations of the sequence of PG appearance at the interface and the nature of their interactions with surface oxides.

In the meantime, we have used certain well-established properties of proteoglycans to develop a working model for their appearance and function at implant surfaces. This model begins with the notion that freshly-placed implants induce inflammatory responses which form peroxy-gels on titanium surfaces (Tengvall *et al.*, 1989b). It suggests further that thick hyaluronan meshworks at wound sites must be speedily dismantled if osseointegration is to occur; free radicals in the Ti-peroxy-gels would accelerate this degradative process. The model also proposes that the frequently observed 20-50-nm layer of "amorphous" material at the titanium interface consists of a bilayer of decorin proteoglycans, held tightly together by associations between their overlapping glycosaminoglycan chains. We hope that these suggestions provide plausible explanations for some, but certainly not all, of the most consistently made observations.

Insights into the functions of these molecules in the formation and maintenance of the interface zone will no doubt suggest new surface modifications. It is already commonplace in clinical practice to see long-lasting, load-bearing connections form between oral implants and adjacent bone. The stability of these junctures is presumably dependent on interactions between extracellular matrix biomolecules and outermost layers at implant surfaces. Treatments which enhance these interactions will hasten the achievement of osseointegration and thus result in better patient care.

Acknowledgments

Part of the research described here was supported by the NSF-EPSCoR program. We also thank the two anonymous reviewers for helpful suggestions which improved the clarity and completeness of this manuscript.

References

Albrektsson T, Hansson H-A (1986). An ultrastructural characterization of the interface between bone and sputtered titanium or stainless steel surfaces. *Biomaterials* 7:201-205.

Albrektsson T, Brånemark P-I, Hansson H-A, Ivarsson B, Jönsson U (1982). Ultrastructural analysis of the interface zone of titanium and gold implants. In: Clinical applications of biomaterials. Lee AJC, Albrektsson T, Brånemark P-I, editors. New York: John Wiley & Sons, pp. 167-177.

Albrektsson T, Brånemark P-I, Hansson H-A, Kasemo B, Larsson K, Lundstrom I, *et al.* (1983). The interface zone of inorganic implants *in vivo*: titanium implants in bone. *Ann Biomed Eng* 11:1-27.

Albrektsson T, Hansson H-A, Ivarsson B (1985). Interface analysis of titanium and zirconium bone implants. *Biomaterials* 6:97-101.

Beresford J, Fedarko N, Fisher L, Midura R, Yanagashita M, Termine J, *et al.* (1987). Analysis of the proteoglycans synthesized by human bone cells *in vitro*. *J Biol Chem* 262:17164-17172.

Bertolami CN, Messadi DV (1994). The role of proteoglycans in hard and soft tissue repair. *Crit Rev Oral Biol Med* 5:311-337.

Brånemark P-I, Adell R, Albrektsson T, Carlsson G, Haraldson T, Lekholm U, *et al.* (1982). Osseointegrated titanium implants in the rehabilitation of the edentulous patient. In: Clinical applications of biomaterials. Lee AJC, Albrektsson T, Brånemark P-I, editors. New York: John Wiley & Sons, pp. 133-141.

Branford-White CJ (1978). Molecular organization of heparan sulfate proteoglycans from human dentine. *Arch Oral Biol* 23:1141-1144.

Brown DC, Vogel KG (1989). Characteristics of the *in vitro* interaction of a small proteoglycan (PG II) of bovine tendon with type I collagen. *Matrix* 9:468-478.

Brunski J, Hipp J, Cochran G (1989). The influence of biomechanical factors at the tissue-biomaterial interface. *Mater Res Soc Symp Proc* 110:505-515.

Caterson B, Calabro T, Hampton A (1987). Monoclonal antibodies as probes for elucidating proteoglycan structure and function. In: Biology of proteoglycans. Wight TN, Mecham RP, editors. Orlando, FL: Academic Press, pp. 1-26.

Chehroudi B, Ratkay J, Brunette DM (1992). The role of implant surface geometry on mineralization *in vivo*

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

and *in vitro*: A transmission and scanning electron microscopic study. *Cells Mater* 2:89-104.

Chen C, Boskey A (1986). The effects of proteoglycans from different cartilage types on *in vitro* hydroxyapatite proliferation. *Calcif Tissue Int* 39:324-327.

Cheng H, Caterson B, Neame P, Lester G, Yamauchi M (1996). Differential distribution of lumican and fibromodulin in tooth cementum. *Connect Tissue Res* 34:87-96.

Chintala SK, Miller RR, McDevitt CA (1994). Basic fibroblast growth factor binds to heparan sulfate in the extracellular matrix of rat growth plate chondrocytes. *Arch Biochem Biophys* 310:180-186.

Collis JJ, Embery G (1992). Adsorption of glycosaminoglycans to commercially pure titanium. *Biomaterials* 13:548-552.

Dammer U, Popescu O, Wagner P, Anselmetti D, Güntherodt H-J, Misevic GN (1995). Binding strength between cell adhesion proteoglycans measured by atomic force microscopy. *Science* 267:1173-1175.

Davies JE (1996). *In vitro* modeling of the bone/implant interface. *Anat Rec* 245:426-445.

Davies JE, Lowenberg B, Shiga A (1990). The bone-titanium interface *in vitro*. *J Biomed Mater Res* 24:1289-1306.

Davies JE, Ottensmeyer P, Shen X, Hashimoto M, Peel SAF (1991). Early extracellular matrix synthesis. In: The bone-biomaterial interface. Davies JE, editor. Toronto, Canada: University of Toronto Press, pp. 214-228.

De Bruijn JD, Flach FS, De Groot K, Van Blitterswijk CA, Davies JE (1993). Analysis of the bony interface with various types of hydroxyapatite *in vitro*. *Cells Mater* 3:115-127.

De Lange GL, De Putter C, De Wijs FLJA (1990). Histological and ultrastructural appearance of the hydroxyapatite-bone interface. *J Biomed Mater Res* 24:829-845.

Ducheyne P (1988). Titanium and calcium phosphate ceramic dental implants, surfaces, coatings and interfaces. *J Oral Implantol* 14:325-340.

Dziewiatkowski D, Majznerski L (1985). Role of proteoglycans in endochondral ossification: inhibition of calcification. *Calcif Tissue Int* 37:560-564.

El-Ghannam A, Ducheyne P, Shapiro IM (1995). Bioactive material template for *in vitro* synthesis of bone. *J Biomed Mater Res* 29:359-370.

Ellingsen JE (1991). A study on the mechanism of protein adsorption to TiO_2. *Biomaterials* 12:593-596.

Fedarko NS, Termine JD, Young MF, Gehron Robey P (1990). Temporal regulation of hyaluronan and proteoglycan metabolism by human bone cells *in vitro*. *J Biol Chem* 265:12200-12209.

Fransson L-Å, Cöster L, Nieduszynski IA, Phelps CF, Sheehan JK (1983). Self-association of copolymeric glycosaminoglycans (proteoglycans). In: Molecular biophysics of the extracellular matrix. Arnott S, Rees DA, Morris ER, editors. Clifton, NJ: Humana Press, pp. 95-118.

Galante J, Lemons J, Spector M, Wilson P, Wright T (1991). The biologic effects of implant materials. *J Ortho Res* 9:760-775.

Garvey B, Bizios R (1994). A method for transmission electron microscopy examination of the osteoblast/hydroxyapatite interface. *J Appl Biomater* 5:39-45.

Gehron Robey P (1989). The biochemistry of bone. *Endo Metab Clin N Am* 18:833-902.

Gehron Robey P (1996). Vertebrate mineralized matrix proteins: structure and function. *Connect Tissue Res* 35:131-136.

Goldberg HA, Domenicucci C, Pringle G, Sodek J (1988). Mineral-binding proteoglycans of fetal porcine calvarial bone. *J Biol Chem* 263:12092-12101.

Goldberg R, Toole B (1987). Hyaluronate inhibition of cell proliferation. *Arthr Rheum* 30:769-778.

Greenwald RA, Moak SA (1986). Degradation of hyaluronic acid by polymorphonuclear leukocytes. *Inflammation* 10:15-30.

Hardingham TE, Fosang AJ (1992). Proteoglycans: many forms and many functions. FASEB J 6:861-870.

Hayat MA (1989). Principles and techniques of electron microscopy. Biological applications. 3rd ed. Boca Raton, FL: CRC Press, pp. 285-289.

Higuchi Y, Matukawa F, Ashida T, Gonda Y (1995). Studies of glycosaminoglycans to titanium implants in the diabetic rat (abstract). *J Dent Res* 74:435.

Hildebrand A, Romarís M, Rasmussen L, Heinegård D, Twardzik D, Border W, *et al.* (1994). Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor β. *Biochem J* 302:527-534.

Hunter GK, Szigety SK (1992). Effects of proteoglycan on hydroxyapatite formation under non-steady-state and pseudo-steady-state conditions. *Matrix* 12:362-368.

Iozzo RV, Murdoch AD (1996). Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J 10:598-614.

James R (1973). A histopathological study of the nature of the epithelium surrounding implant posts. *Oral Implantol* 3:137-159.

Johansson C, Lausmaa J, Ask M, Hansson H-A, Albrektsson T (1989). Ultrastructural differences of the interface zone between bone and Ti6Al4V or commercially pure titanium. *J Biomed Eng* 11:3-8.

Kieswetter K, Schwartz Z, Dean D, Boyan B (1996). The role of implant surface characteristics in the healing of bone. *Crit Rev Oral Biol Med* 7:329-345.

Kinne RW, Schmitz HJ, Gross U (1988). Collagenous and non-collagenous bone proteins in rats and rabbits at the bone-implant interface of bone-bonding and non-

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

bonding glass-ceramic. In: Implant materials in biofunction. De Putter C, De Lange GL, De Groot K, Lee AJC, editors. Amsterdam: Elsevier, pp. 73-78.

Klinger MM, Rahemtulla F, Prince CW, Lucas LC (1995). Proteoglycans synthesized by osteoblast-like MC3T3-E1 cells on cp-Ti (abstract). *Ann Biomed Eng* 23(Suppl 1):S-100.

Klinger MM, Rahemtulla F, Prince CW, Lucas LC (1996a). Proteoglycans at the bone-biomaterial interface *in vitro* (abstract). In: Abstracts, Fifth World Biomaterials Congress. Vol. II, p. 427.

Klinger MM, Rahemtulla F, Prince CW, Lucas LC (1996b). Adsorption of proteoglycans to titanium powder (abstract). *Ann Biomed Eng* 24(Suppl. 1):S-76.

Klinger MM, Rahemtulla F, Prince CW, Lucas LC (1997). Effect of cations on proteoglycan adsorption to titanium oxides (abstract). In: Abstracts, Society for Biomaterials Annual Meeting, p. 450.

Knudson CB, Knudson W (1993). Hyaluronan-binding proteins in development, tissue homeostasis, and disease. FASEB J 7:1233-1241.

Kobe B, Deisenhofer J (1994). The leucine-rich repeat: a versatile binding motif. *Trends Biochem Sci* 19:415-421.

Linde A, Lussi A, Crenshaw M (1989). Mineral induction by immobilized polyanionic proteins. *Calcif Tissue Int* 44:286-295.

Linder L (1992). Ultrastructure of the bone-cement and the bone-metal interface. *Clin Orthop Rel Res* 276:147-156.

Linder L, Albrektsson T, Brånemark P-I, Hansson H-A, Ivarsson B, Jonsson U, *et al.* (1983). Electron microscopic analysis of the bone-titanium interface. *Acta Orthop Scand* 54:45-52.

Linder L, Obrant K, Boivin G (1989). Osseointegration of metallic implants. II. Transmission electron microscopy in the rabbit. *Acta Orthop Scand* 60:235-239.

Listgarten MA, Buser D, Steinemann SG, Donath K, Lang NP, Weber HP (1992). Light and transmission electron microscopy of the intact interfaces between non-submerged titanium-coated epoxy resin implants and bone or gingiva. *J Dent Res* 71:364-371.

Lucas LC, Lacefield WR, Ong JL, Whitehead RY (1993). Calcium phosphate coatings for medical and dental implants. *Colloids Surf* (A) *Physicochem Eng Asp* 77:141-147.

Martin JY, Schwartz Z, Hummert TW, Schraub DM, Simpson J, Lankford J, *et al.* (1995). Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (MG63). *J Biomed Mater Res* 29:389-401.

McQueen D, Sundgren J-E, Ivarsson B, Lundström I, af Ekenstam B, Svensson A, *et al.* (1982). Auger electron spectroscopic studies of titanium implants. In: Clinical applications of biomaterials. Lee AJC, Albrektsson T, Brånemark PI, editors. New York: John Wiley & Sons, pp. 179-185.

McQuillan DJ, Findlay DM, Hocking AM, Yanagashita M, Midura RJ, Hascall VC (1991). Proteoglycans synthesized by an osteoblast-like cell line (UMR 106-01). *Biochem J* 277:199-206.

Merritt K, Brown S (1993). Release of hexavalent chromium from corrosion of stainless steel and cobalt-chromium alloys. *J Biomed Mater Res* 29:627-633.

Messer RLW, Lucas LC (1996). Metabolic effects of $Cr^{(+3)}$ and $Cr^{(+6)}$ salt solutions (abstract). *Ann Biomed Eng* 24(Suppl. 1):S-76.

Midura RJ, McQuillan DJ, Benham KJ, Fisher LW, Hascall VC (1990). A rat osteogenic cell line (UMR 106-01) synthesizes a highly sulfated form of bone sialoprotein. *J Biol Chem* 265:5285-5291.

Misevic GN, Burger MM (1993). Carbohydrate-carbohydrate interactions of a novel acidic glycan can mediate sponge cell adhesion. *J Biol Chem* 268:4922-4929.

Murai K, Takeshita F, Ayukawa Y, Kiyoshima T, Suetsugu T, Tanaka T (1996). Light and electron microscopic studies of bone-titanium interface in the tibiae of young and mature rats. *J Biomed Mater Res* 30:523-533.

Nagata T, Goldberg HA, Zhang Q, Domenicucci C, Sodek J (1991). Biosynthesis of bone proteins by fetal porcine calvariae *in vitro*. Rapid association of sulfated sialoproteins (secreted phosphoprotein-1 and bone sialoprotein) and chondroitin sulfate proteoglycan (CS-PGIII) with bone mineral. *Matrix* 11:86-100.

Nanci A, McCarthy GF, Zalzal S, Clokie CML, Warshawsky H, McKee MD (1994). Tissue response to titanium implants in the rat tibia: ultrastructural, immunocytochemical and lectin-cytochemical characterization of the bone-titanium interface. *Cells Mater* 4:1-30.

Pan J, Thierry D, Leygraf C (1996). Hydrogen peroxide toward enhanced oxide growth on titanium in PBS solution: blue coloration and clinical relevance. *J Biomed Mater Res* 30:393-402.

Parsegian VA (1983). Molecular forces governing tight contact between cellular surfaces and substrates. *J Prosthet Dent* 49:838-842.

Pogány G, Hernandez DJ, Vogel KG (1994). The *in vitro* interaction of proteoglycans with type I collagen is modulated by phosphate. *Arch Biochem Biophys* 313:102-111.

Poole AR, Matsui Y, Hinek A, Lee ER (1989). Cartilage macromolecules and the calcification of cartilage matrix. *Anat Rec* 224:167-179.

Prince CW, Rahemtulla F, Butler WT (1984). Incorporation of $[^{35}S]$sulphate into glycosaminoglycans by mineralized tissues *in vivo*. *Biochem J* 224:941-945.

Rahemtulla F (1992). Proteoglycans of oral tissues. *Crit Rev Oral Biol Med* 3:135-162.

Rahemtulla F, Rahemtulla B (1994). Human gingival fibroblasts (HGF) in primary culture release reactive oxygen species (abstract). *J Dent Res* 73:278.

Ren Z, Brewton R, Mayne R (1991). An analysis by rotary

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

shadowing of the structure of the mammalian vitreous humor and zonular apparatus. *J Struct Biol* 106:57-63.

Ripellino JA, Klinger MM, Margolis RU, Margolis RK (1985). The hyaluronic acid binding region as a specific probe for the localization of hyaluronic acid in tissue sections. *J Histochem Cytochem* 10:1060-1066.

Roberts CR, Roughley PJ, Mort JS (1989). Degradation of human proteoglycan aggregate induced by hydrogen peroxide. Protein fragmentation, amino acid modification and hyaluronic acid cleavage. *Biochem J* 259:805-811.

Rodén L (1980). Structure and metabolism of connective tissue proteoglycans. In: The biochemistry of glycoproteins and proteoglycans. Lennarz W, editor. New York: Plenum Press, pp. 267-371.

Roughley PJ, Lee ER (1994). Cartilage proteoglycans: structure and potential functions. *Microsc Res Technique* 28:385-397.

Ruoslahti E, Yamaguchi Y (1991). Proteoglycans as modulators of growth factor activities. *Cell* 64:867-869.

Schönherr E, Witsch-Prehm P, Harrach B, Robenek H, Rauterberg J, Kresse H (1995). Interaction of biglycan with type I collagen. *J Biol Chem* 270:2776-2783.

Scott JE (1988). Proteoglycan-fibrillar collagen interactions. *Biochem J* 252:313-323.

Scott JE (1990). Proteoglycan:collagen interactions and subfibrillar structure in collagen fibrils. Implications in the development and ageing of connective tissues. *J Anat* 169:23-35.

Scott JE (1992a). Supramolecular organization of extracellular matrix glycosaminoglycans, *in vitro* and in the tissues. FASEB J 6:2639-2645.

Scott JE (1992b). Morphometry of Cupromeronic blue-stained proteoglycan molecules in animal corneas, versus that of purified proteoglycans stained *in vitro*, implies that tertiary structures contribute to corneal ultrastructure. *J Anat* 180:155-164.

Scott JE (1996). Proteodermatan and proteokeratan sulfate (decorin, lumican/fibromodulin) proteins are horseshoe shaped. Implications for their interactions with collagen. *Biochemistry* 35:8795-8799.

Sebag J, Balazs EA (1989). Morphology and ultrastructure of human vitreous fibers. *Invest Ophthalmol Vis Sci* 30:1867-1871.

Sennerby L, Thomsen P, Ericson LE (1992). Ultrastructure of the bone-titanium interface in rabbits. *J Mater Sci:Mater Med* 3:262-271.

Stanford CM, Keller JC (1991). The concept of osseointegration and bone matrix expression. *Crit Rev Oral Biol Med* 2:83-101.

Steflik DE, Sisk AL, Parr GR, Gardner LK, Hanes PJ, Lake FT, *et al.* (1992). High-voltage electron microscopy and conventional transmission electron microscopy of the interface zone between bone and endosteal dental implants. *J Biomed Mater Res* 26:529-545.

Steflik DE, Sisk AL, Parr GR, Gardner LK, Hanes PJ, Lake FT, *et al.* (1993). Osteogenesis at the dental implant interface: high-voltage electron microscopic and conventional transmission electron microscopic observations. *J Biomed Mater Res* 27:791-800.

Steflik DE, Sisk AL, Parr GR, Lake FT, Hanes PJ, Berkery DJ, *et al.* (1994a). Transmission electron and high-voltage electron microscopy of osteocyte cellular processes extending to the dental implant surface. *J Biomed Mater Res* 28:1095-1107.

Steflik DE, Parr GR, Sisk AL, Lake FT, Hanes PJ, Berkery DJ, *et al.* (1994b). Osteoblast activity at the dental implant-bone interface: Transmission electron microscopic and high voltage electron microscopic observations. *J Periodontol* 65:404-413.

Steflik DE, Corpe RS, Lake FT, Sisk AL, Parr GR, Hanes PJ, *et al.* (1997). Composite morphology of the bone and associated support-tissue interfaces to osseointegrated dental implants: TEM and HVEM analyses. *Int J Oral Maxillofac Implants* 12:443-453.

Sundgren J-E, Bodo P, Lundstrom I (1986). Auger electron spectroscopic studies of the interface between human tissue and implants of titanium and stainless steel. *J Colloid Interf Sci* 110:9-20.

Takagi M, Hishikawa H, Hosokawa Y, Kagami A, Rahemtulla F (1990). Immunohistochemical localization of glycosaminoglycans and proteoglycans in predentin and dentin of rat incisors. *J Histochem Cytochem* 38:319-324.

Takeuchi Y, Matsumoto T, Ogata E, Shishiba Y (1990). Isolation and characterization of proteoglycans synthesized by mouse osteoblastic cells in culture during the mineralization process. *Biochem J* 266:15-24.

Takeuchi Y, Kodama Y, Matsumoto T (1994). Bone matrix decorin binds transforming growth factor-β and enhances its bioactivity. *J Biol Chem* 269:32634-32638.

Taylor GC, Waddington RJ, Moseley R, Williams KR, Embery G (1996). Influence of titanium oxide and titanium peroxy gel on the breakdown of hyaluronan by reactive oxygen species. *Biomaterials* 17:1313-1319.

Tengvall P, Elwing H, Sjöqvist L, Lundström I, Bjursten L (1989a). Interaction between hydrogen peroxide and titanium: a possible role in the biocompatibility of titanium. *Biomaterials* 10:118-120.

Tengvall P, Lundström I, Sjöqvist L, Elwing H, Bjursten L (1989b). Titanium-hydrogen peroxide interaction: model studies of the influence of the inflammatory response on titanium implants. *Biomaterials* 10:166-175.

Tengvall P, Hörnsten EG, Elwing H, Lundstrom I (1990). Bactericidal properties of a titanium-peroxy gel obtained from metallic titanium and hydrogen peroxide. *J Biomed Mater Res* 24:319-330.

Thyberg J, Lohmander S, Heinegård D (1975). Proteoglycans of hyaline cartilage. Electron-micro-

Downloaded from cro.sagepub.com by guest on January 1, 2013 For personal use only. No other uses without permission.

scopic studies on isolated molecules. *Biochem J* 151:157-166.

Turley EA, Roth S (1980). Interactions between the carbohydrate chains of hyaluronate and chondroitin sulfate. *Nature* 283:268-271.

Uitto V-J, Larjava H (1991). Extracellular matrix molecules and their receptors: an overview with special emphasis on periodontal tissues. *Crit Rev Oral Biol Med* 2:323-354.

Weigel P, Fuller GM, LeBoeuf RD (1986). A model for the role of hyaluronic acid and fibrin in the early events during the inflammatory response and wound healing. *J Theoret Biol* 119:219-234.

Woods A, Couchman JR (1994). Syndecan 4 heparan sulfate proteoglycan is a selectively enriched and widespread focal adhesion component. *Molec Biol Cell* 5:183-192.

Downloaded from cro.sagepub.com by guest on January 1, 2015 For personal use only. No other uses without permission.