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Adsorption of proteoglycans to metallic implant materials.

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ADSORPTION OF PROTEOGLYCANS TO METALLIC IMPLANT MATERIALS

by

MARTIN M. KLINGER

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Adhesive biomolecules in the extracellular matrix are the likely source of much of the strength and stability of the bone-implant interface. Proteoglycans, as major components of this matrix, appear to play a critical role in the events leading to osseointegration. This notion is supported by a considerable body of both histochemical and spectroscopic data obtained from implant retrievals. The studies described here were designed to clarify some of the relationships between proteoglycans and the surfaces of metallic implant materials. As an *in vitro* model, this work used MC3T3E1 cells, a clonal line of osteoblastic cells derived from newborn mouse calvaria. The studies compared the biosynthesis of proteoglycans on four implant metals: pure titanium (cpTi), titanium-6Al-4V (Ti-6-4), 316L stainless steel (316L SS), and cobalt chrome molybdenum (CoCrMo). In addition, radiolabeled MC3T3 proteoglycans were adsorbed to titanium powders and to bulk samples of the four metals. Titanium surfaces were precoated with proteoglycans in attempts to stimulate cell attachment. The experimental results yield the following conclusions: 1) Differences in the surface oxides of titanium- and chrome-based alloys do not cause significant differences in the biosynthesis of proteoglycans by proliferating osteoblastic cells on these materials. However, these metals as a group are distinguishable from tissue culture plastic in this regard. 2) Pretreatment of cpTi and Ti-6-4 with certain proteoglycans (decorin, biglycan, aggrecan, all derived from bovine cartilage) does not enhance osteoblast attachment to the metal surfaces. 3) Pretreatment of titanium powder with lanthanum chloride or hydrogen peroxide significantly alters its capacity to adsorb proteoglycans. 4) The mechanism of proteoglycan binding to titanium oxides is not a

simple electrostatic interaction between the surface and anionic polysaccharide chains; rather, it also appears to involve hydrophobic interactions between the surface oxide and the polypeptide portion of the molecules. 5) Titanium- and chrome-based alloys differ qualitatively and quantitatively in the adsorption of proteoglycans. The difference is subtle; however, at least one sulfated protein, Mr approx. 120,000, binds detectably to cpTi and to Ti-6-4 and not to 316L SS or to CoCrMo.

DEDICATION

To Karen and Hannah.

And to Reba. There's one thing that won't change.

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This work was possible only because of the support and friendship and gentle prodding of my primary advisor, Dr. Linda C. Lucas. A dozen roses.

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And thank you, Jan, for being there.

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LIST OF ABBREVIATIONS

316L SS	316 low carbon stainless steel
AES	Auger electron spectroscopy
BSA	bovine serum albumin
CoCrMo	cobalt-chrome-molybdenum alloy
cpTi	commercially pure titanium
CS	chondroitin sulfate
CSPG	chondroitin sulfate proteoglycan
DS	dermatan sulfate
EDTA	ethylene diamine tetraacetic acid
GAG	glycosaminoglycan
GuHCl	guanidine hydrochloride
HA	hyaluronic acid
HAPGs	hydroxyapatite-binding proteoglycans
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HVEM	high voltage electron microscopy
KS	keratan sulfate
LRR	leucine-rich repeat
OPN	osteopontin
PBS	phosphate buffered saline
PG	proteoglycan
PMMA	polymethylmethacrylate

PMSF	phenylmethylsulfonyl fluoride
RR	ruthenium red
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TGFβ	transforming growth factor β
Ti-6-4	Ti-6%Al-4%V
TX	Triton X-100
XPS	X-ray photoelectron spectroscopy

INTRODUCTION

Forcibly inserting 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 (nonloaded) phase for healing of the bone (Albrektsson *et al.*, 1983). Ongoing 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. However, significant challenges remain. Premature loading causing excessive micromotion 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 this country alone, a delayed time for integration represents a sizable economic and social 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 surface. Adhesive biomolecules in the extracellular matrix are the likely source of much of the strength and stability of the bone-implant interface (Stanford and Keller, 1991). 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; that is, 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 process of mineralization and ossification (Gehron Robey, 1989). The goal of the studies described here was to better understand the molecular interactions between proteoglycans (PGs) and metallic implants in bone.

Structure of Proteoglycans

A number of excellent reviews (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 section will briefly summarize those proteoglycans considered 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 dependent 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 6-

carbon. Dermatan sulfate (DS) differs from CS in that most of the glucuronic acid residues have been epimerized 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, although 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, CSPG of bovine nasal cartilage, which bears a polypeptide portion of approximately 220,000 daltons. As many as 100 chains of CS, 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 KS are attached to the core, in most cases closer to the protein's amino terminus than the CS chains. A number of 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 glyco-proteins, 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 CSPGs may either inhibit (Dziewiatkowski and Majznerski, 1985; Chen and Boskey, 1986) or stimulate (Linde *et al.*, 1989a; Hunter and Szigety, 1992) mineralization, depending to some extent on whether the studies were conducted with soluble or immobilized PGs. Another large CSPG, dubbed versican, is found in developing and healing bone and is gradually replaced by smaller PGs as calcification proceeds. Versican (twelve 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 a number of 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 dentine (Branford-White, 1978); in these locales, HS 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) each have core polypeptides of approximately 37,000 daltons (Gehron Robey, 1989). Decorin has a single GAG chain 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 is addressed in the Discussion.

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 (hence the name decorin) consist of small dermatan sulfate PGs 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, although 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 (TGFβ) (Hildebrand *et al.*, 1994). TGFβ'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 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).

Evidence for Proteoglycans at the Interface: Retrievals

The presence of proteoglycan-like material at the implant interface was implied nearly 25 years ago when James and coworkers (1973) conducted histochemical studies on Vitallium implants retrieved from dog maxillae. When the specimens were stained with Periodic Acid Schiff-alcian blue and examined by light microscopy, they each revealed an acid mucopolysaccharide (GAG) 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 thin sectioning a bulk metal sample; this processing can easily disrupt the fine structure of the tissue in the first 10-100 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 in adult rabbit tibiae for 12 weeks. Examination of the retrieved and sectioned rods by transmission electron microscopy (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 pretreated with chondroitinase or hyaluronidase. The authors (Linder *et al*, 1983) concluded that the "amorphous" interface zone contains a mixture of CSPGs and HA 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 (Albrektsson *et al.*, 1985, 1986; Johansson *et al.*, 1989) subsequently repeated the cpTi studies and compared the tibial responses to polycarbonate plugs coated with zirconium, 316 low carbon stainless steel (316L SS), or titanium-6 aluminum-4 vanadium alloy. 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 nanometers thick. In the case of the titanium alloy, the PG layer ranged in width from 50-100 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 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 at a later point.

Some years later, Linder and coworkers (1989) analyzed the tissue reactions to implants of *bulk* metal retrieved from rabbit tibiae after 11 mos. 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; however, 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 (Albrektsson *et al.*, 1985, 1986; Johansson *et al.*, 1989), Linder *et al.* (1989) 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 no 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.*, 1992, p.102).

Sennerby *et al.* (1992) bolstered the case for the amorphous zone with his analysis of solid cpTi screws retrieved from rabbit tibiae. 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 to separate 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 (Sennerby *et al.*, 1992) 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 coworkers (1992, 1993) placed commercial root- and plate-form titanium implants into the mandibles of adult dogs and allowed a 5-mo unloaded healing period. One group of the animals were then sacrificed while a second group were prosthodontically loaded for an additional 6 mos. 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 (Steflik *et al.*, 1992, 1993) 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 6-mo loading period, the zone further constricted to 20-50 nm at sites where mature, densely mineralized bone approached the implant surface. The authors (Steflik *et al.*, 1992, 1993) 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 electron-dense, ruthenium-positive deposits similar to those seen on titanium. Osteocytes near the interface extended cellular processes which directly contacted 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 intervening PG layer. The authors (Listgarten *et al.*, 1992) 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. However, 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 DS, chondroitin 4-, and 6-sulfate PGs. 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 antidecorin 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 PGs (Nanci *et al.*, 1994). The presence of one should not exclude the presence of the other, as both are major noncollagenous 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 8 yrs, the oxide thickness increased from about 3.5 nm to as much as 100 nm. Sundgren *et al.* (1986) 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 (Sundgren *et al.*, 1986) attributed the sulfur peak to CSPGs which may each 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).

Evidence for Proteoglycans at the Interface: 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 coworkers (1990) seeded suspensions of rat bone marrow cells onto discs of commercially pure titanium that had been polished to 600 grit and then sterilized by steam autoclaving. After 3 weeks, the cultures were fixed and stained with ruthenium red, mounted in epon

resin, and briefly immersed in liquid nitrogen. This latter step freezefractured the interface and separated the metal disc from the fixed tissue. The authors (Davies *et al.*, 1990) 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, coatings of three average crystallinities (15%, 43%, and 69%) had been obtained. 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 (De Bruijn *et al.*, 1993) 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 to examine the interface of rat calvarial osteoblasts cultured on dense hydroxylapatite. Garvey and Bizios (1994) used 3 M HCl and 10 M hydrofluoric acid to carefully dissolve the hydroxylapatite coating and underlying glass coverslip away from the fixed and embedded 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 varying degrees of roughness. On the basis of radioactivity per microgram protein, the cells on each of the metal substrates incorporated less [^{35}S]-sulfate into nondialyzable material than did the controls grown on tissue culture plastic.

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 (1986) conclusions, El-Ghannam *et al.*, (1995) attribute this peak to the biosynthesis of PGs.

Evidence for Proteoglycans at the Interface: Adsorption Studies

The negative electrical charge of the titanium oxide coating at the implant surface (Ellingsen, 1991) 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 GAGs to titanium powder. Commercial preparations of HS, HA, 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 pretreated with calcium acetate. This finding is of particular interest since chondroitin-4-sulfate is the major GAG detected in bone PGs. The authors (Collis and Embery, 1992) pictured an electrostatic bridge (TiO_2^- --Ca^{++} -- C4S-) between the metal surface and PGs in the adjacent tissue. This conclusion was supported by Ellingsen's (1991) finding that calcium pretreatment of Ti surfaces enhances the adsorption of non-proteoglycan serum proteins, presumably via interactions of the polypeptides with the metal oxide. The same laboratory (Ellingsen and Pinholt, 1995) later reported that pretreatment of titanium with lanthanum ions increased albumin binding fivefold.

Adsorption to Ti surfaces should also be influenced by pretreatment of the metal with hydrogen peroxide. Inflammatory cells *in vivo* produce hydrogen peroxide as part of their response to foreign materials; H_2O_2 is also produced by cultured gingival fibroblasts (Rahemtulla and Rahemtulla, 1994). Tengvall and coworkers (1989a,b) have examined the aqueous peroxy gel that forms on titanium surfaces when the bulk metal is incubated with nonphysiological (~10M) concentrations of H_2O_2; much more dilute H_2O_2 can produce peroxy gels on powdered Ti. Spectrophotometric analysis using 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 peroxygels on titanium implant surfaces. However,

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 effect 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. 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). This property of Ti may provide a partial explanation for its remarkable biological interactions, although other factors, including surface energy, topography, and roughness (Kieswetter *et al.*, 1996), must also be considered.

HYPOTHESES

The overall goal of these studies was to better understand the interactions of proteoglycans with implant metal surfaces. Towards this end, four hypotheses were proposed and tested.

Hypothesis 1

Osteoblast-like cells cultured on titanium or titanium alloy synthesize significantly different types of proteoglycans than the same cells on stainless steel or CoCrMo.

Hypothesis 2

Pure titanium and Ti alloy differ from stainless steel and CoCrMo in the amounts and/or types of proteoglycans which adsorb to their surfaces.

Hypothesis 3

Chemical modifications of the titanium oxide surface layer significantly alter the adsorption of proteoglycans.

Hypothesis 4

Precoating titanium surfaces with proteoglycans enhances the attachment of osteoblast-like cells.

MATERIALS AND METHODS

Sample Preparation

Grade 2 (commercially pure) titanium rod stock (0.875" diameter; 0.016% C, 0.02% Fe, 0.002% H) was purchased from Metal Samples Co., Inc. (Munford, AL). Ti-6-4 ELI rod stock (7/8" diameter; ELI, ASTM-F-136; 5.94% Al, 3.82% V) was purchased from President Titanium (Hanson, MA). 316L stainless steel rod stock (0.875" diameter; 16.6% Cr, 11.63% Ni, 1.6% Mn) was purchased from Metal Samples Co., Inc. (Munford, AL). CoCrMo alloy discs (0.825" diameter, 0.125" thickness; ASTM F799) were obtained from Zimmer Inc., (Warsaw, IN).

Bars of the cpTi, Ti-6-4, and 316L SS rod stock were cut into discs 2-3 mm thick by the UAB Machine Shop. The discs were then deburred manually on 60 grit SiC paper and their edges ground such that the discs fit snugly into wells of 12-well tissue culture plates (Costar). Discs of all four alloys were then mounted in polymethylmethacrylate (PMMA) stubs and ground on a Buehler Ecomet 5 Polisher through a series of SiC grit papers (120, 240, 320, 400, 600, and 1200 grit). The discs were then polished sequentially with 1.0 micron and 0.3 micron alumina. After soaking for 24-48 hrs in acetone, the PMMA stubs were softened sufficiently to easily remove the polished discs. The discs were cleaned by sonication in a series of solvents (benzene; acetone; ethanol) and washed with distilled water. All were passivated in 40% nitric acid for 30 min at room temperature (ASTM F86), washed thoroughly with distilled water, and air dried. The surfaces were sterilized by exposure to UV light for 12-24 hrs. The discs were fastened to the bottom surfaces of the 12-well plates with 0.1 ml of molten, sterile 2% agarose. UV sterilization was continued for another 12-18 hrs.

Surface Characterization

XPS (X-ray Photoelectron Spectroscopy). Elemental compositions of the bulk metal samples were determined by XPS on a Perkin Elmer PHI 5400 ESCA System at the University of Alabama in Huntsville (UAH). This work was done under the direction of Dr. J. Weimer, Department of Material Sciences, UAH. Survey scans of each sample were conducted at an angle of 45 degrees, using a Mg filament at 325 W.

Profilometry. Average surface roughness of the polished metal discs was measured with a Federal Surfanalyzer System 4000 (Providence, RI) in the Department of Biomaterials, School of Dentistry, UAB. The discs were mounted with wax on a glass platform and scanned at 0.25 mm/sec. These analyses were done under the direction of Dr. William Lacefield, UAB.

Light Microscopy. The topography of the bulk surfaces was examined with a Micro-Vu Optical Measurement System (Windsor, CA) in the Orthopaedics Research Laboratory, UAB. Images of each disc were obtained at both 40% and 100% magnification, using a coaxial light source set at 15-20%.

Cell Culture

MC3T3-E1cells are a clonal osteogenic cell line established from newborn mouse calvaria by Kodama and coworkers (Sudo *et al.*, 1983). As these cells differentiate in culture, they display several osteoblastic characteristics such as high alkaline phosphatase activity and the capacity to deposit hydroxyapatite *in vitro.* The proteoglycans produced by these cells grown on tissue culture plastic have been described in detail (Takeuchi *et al.*, 1990), as have alterations in PG synthesis in response to a variety of factors including TGF-β (Takeuchi *et al.*, 1993). The excellent osteoblastic characteristics of MC3T3-E1 cells, together with their ease of handling and homogeneity of phenotype, make them the system of choice for the experiments described here. In addition, these cells grow well on

titanium and ceramic surfaces, as judged by their levels of total protein and DNA (Itakura *et al.*, 1988). These cells were routinely cultured on tissue culture plastic in alpha minimum essential medium containing 10% fetal calf serum, glutamine, and antibiotics.

Biosynthesis of PGs by MC3T3 Cells on Metal Substrates

MC3T3-E1 cells were seeded at a density of 50,000 cells/well onto polished, UV-sterilized metal discs (22 mm diameter) in 12-well tissue culture plates. For each metal, 3 discs were used for metabolic labeling studies; a second set of discs were assayed for hexosaminidase activity to determine cell number. When the cells in the control (plastic) wells reached confluence (4-5 days after seeding), the culture medium was replaced with fresh medium containing ^{35}S-sulfate (Amersham) at a concentration of 100 μCi/mL. After labeling for 48 hrs, the medium was removed and the cell layers were washed three times with 1 mL ice-cold phosphate buffered saline (PBS).

In some cases, the cell layer and extracellular matrix fractions were extracted together by incubation at 4°C overnight in extraction buffer (4 M guanidine HCl (GuHCl)/2% Triton X-100/10 mM EDTA/50 mM NaAc, pH 6.0/0.1 M 6-amino-hexanoate/5 mM benzamidine HCl/1 mM phenylmethylsulfonylfluoride (PMSF; Beresford *et al.*, 1987).

Analysis of the *total* cellular PGs might obscure subtle differences in the distribution of these molecules. Therefore, certain of these studies used extraction methods which divided the PGs into three cellular compartments: the culture medium, the cytoplasmic/cell membrane fraction, and the extracellular matrix. After removal of the culture medium and PBS washes of the attached cells, the cell layers were incubated for 15-30 min in cold PBS containing 2% Triton X-100 to solubilize PGs in the cytoplasmic/ cell membrane fraction. Next, the PGs remaining in the extracellular matrices on the various

substrates were solubilized overnight at 4°C in 4 M GuHCl/50 mM NaAc, pH 6, containing ethylenediamine tetraacetic acic (EDTA) and the protease inhibitor PMSF.

Ion-exchange and Gel Filtration Chromatography of Proteoglycans

The ^{35}S-macromolecules in each extract were separated from the free $^{35}SO_4$ precursor on PD10 columns (Pharmacia) in 7 M Urea/0.15 M NaCl/0.5% TX-100/50 mM NaAc, pH 6.0. The peak macromolecular fractions were pooled and counted in a Beckman LS1801 scintillation spectrometer with BioSafe II scintillation cocktail (RPI Co.). For analysis by ion exchange chromatography, samples (in 7 M urea/0.15 M NaCl/50 mM NaAc, pH 6/0.5% Triton X-100) were applied to 1 mL columns of DEAE-Sephacel and eluted with a linear gradient of 0.15-1.5 M NaCl in the urea buffer.

Gel filtration provides a general indication of the size distribution of a mixture of ^{35}S-proteoglycans. This method requires relatively low levels of radioactivity (5-10,000 cpm/mL) and the samples can be applied directly in a dissociative buffer such as 4 M guanidine. However, gel filtration cannot resolve these molecules into individual PG species. In the preliminary studies of PG biosynthesis and adsorption, PG distributions were analyzed on a column (1 x 45 cm) of Sepharose CL-4B in 4 M GuHCl/0.5% Triton X-100/50 mM Tris HCl, pH 7.4, at a flow rate of 10 mL/hr. Fractions (0.6 mL) were collected and counted in a scintillation spectrometer.

SDS-PAGE

Improved resolution of the various PGs was obtained using polyacrylamide gel electrophoresis. Proteoglycan extracts were concentrated by incubation (room temperature, 2 hrs) with 0.1 mL DEAE-Sephacel (Sigma) in Urea/NaCl buffer. This step effectively binds the ^{35}S-macromolecules to a small volume of resin. This material was then desorbed from the resin in a 50-100 µl 4 M GuHCl/0.1% TX-100/50 mM TrisHCl, pH 7.4. After

centrifugation, the supernatants were dialyzed against 0.1% Triton X-100 in 50 mM TrisHCl, pH 7.2 ("Tris/TX" buffer). Portions of each retentate were mixed with an equal volume of double-strength SDS-PAGE sample buffer and heated at 100°C for 4-5 min. These samples were then analyzed by SDS-PAGE on linear 4-15% acrylamide gradient gels (1.5 mm thickness; BioRad Protean II minigels) prepared essentially according to Hames (1981) and Beresford *et al.* (1987). Sample volumes of up to 30 µl were added to each of eight wells per gel; gels were usually electrophoresed at 200 V for 40-45 min. The gels were then fixed and stained in 0.1% Coomassie Brilliant Blue in 40% methanol and 10% acetic acid and destained for several hours in 3-4 changes of methanol/acetic acid.

Fluorography

Fluorography allows far more sensitive detection of radiolabeled proteins than simple autoradiography. The method used was essentially that of Bonner and Laskey (1974). The destained gels were soaked in water for 1hr and then in two changes of DMSO, each for 30 min. The gels were then incubated in 22% PPO (2,5-diphenyloxazole) in DMSO for 2-3 hrs and then washed with water for 1 hr and dried under vacuum for 2 hrs at 60°C. The dried gels were exposed to X-ray film at -80°C for periods ranging from 1 day to 2 weeks, depending on the level of radioactivity in the samples.

Enzymatic Digestions

Digestion with specific glycosidases was used to determine the glycosaminoglycan composition of the various mixtures of [^{35}S]-labeled proteoglycans. The assay buffer for both chondroitinase ABC and heparitinase contained 5 mM calcium chloride, 5 mM sodium acetate, 250 µg/mL bovine serum albumin, and 0.1% Triton X-100 in 100 mM Tris HCl, pH 7.2 (Beresford *et al.*, 1987). The assay buffer for chondroitinase ACII contained 100 mM sodium acetate, 250 µg/mL bovine serum albumin, and 0.1% Triton X-100 in 100

mM Tris HCl, pH 7.3 (Beresford *et al.*, 1987). In some cases, samples were exchanged into the appropriate assay buffer by chromatography on small columns of Sephadex G-50. Alternatively, samples were dialyzed against 50 mM Tris HCl, pH 7.3, containing 0.1% Triton X-100; the dialyzed samples were then supplemented with 5X concentrated assay buffer.

Stock solutions of the glycosidases (all from Seikagaku) were stored in aliquots of 20-50 µl at -20°C. Chondroitinase ABC, chondroitinase AC II, or heparitinase were added to the samples to final concentrations of approximately 1 milliunit per 50 µl. The mixtures were incubated for 3 hrs at 37°C. For SDS-PAGE, the incubation mixtures were then mixed with an equal volume of 2X SDS sample buffer and heated at 100°C for 4-5 min.

Digestion of the [^{35}S]-proteoglycans with Pronase E (final concentration 0.001% in heparitinase assay buffer) was used to distinguish relatively intact proteoglycans from free glycosaminoglycan chains.

Preparation of ^{35}S-labeled PGs for adsorption studies

MC3T3 cells were cultured to confluence in T75 tissue culture flasks. The cells were then fed with fresh culture medium supplemented with ^{35}S-sulfate (100 µCi/mL). After 48 hrs, the medium was removed and centrifuged to remove any floating cells. The medium was combined with solid GuHCl to a final concentration of approximately 4 M and with 200 mM PMSF (in methanol) to a final concentration of 1 mM.

After removal of the medium from the culture flasks, the cell layers were washed three times with ice-cold PBS. The cell layer PGs were then solubilized by incubation for 30 min on ice in a buffer containing 2% Triton X-100, 0.1 M 6-aminobenzoate, and PBS, pH 7.3. This extract was removed and the remaining PGs in the extracellular matrix (ECM) fraction were solubilized overnight at 4°C in the 4 M GuHCl extraction buffer described above. The ^{35}S-macromolecules in each fraction (culture medium, cell layer

extract, ECM extract) were separated from the free sulfate precursor by gel filtration on Sephadex G-50 (2.5 x 26 cm) in the 7 M urea buffer described above.

The G-50 excluded fractions were pooled, concentrated by adsorption to DEAE-Sephacel in urea buffer, and then eluted from the resin with the 4-6 M GuHCl/TX/Tris buffer described previously. This fraction, containing concentrated ^{35}S-PGs, was stored until needed at 4°C. Just prior to the adsorption studies, the PGs were dialyzed exhaustively against Tris/TX buffer in the cold.

Adsorption Protocol: Bulk Metals

Polished discs of the various metals (cp titanium, titanium 6Al-4V alloy, 316L SS, or CoCrMo; 22 mm diameter) were cleaned, passivated, and placed in 12-well culture dishes exactly as described above for the PG biosynthesis studies. Prior to the adsorption studies, each disc was washed twice with Tris/TX buffer. Portions of the ^{35}S-labeled PGs (~200,000 cpm) in a total volume of 0.5 mL Tris/TX buffer were incubated with the discs (3 discs for each metal; also 3 wells of tissue culture plastic) at room temperature for 18 hrs. Each supernatant was removed (the nonadsorbed fraction) and the discs were washed three times with Tris/TX buffer. The adsorbed PGs were recovered by incubation for 3 hrs in 4 M GuHCl/0.5 M ethylene diamine tetraacetic acid (EDTA)/0.1% TX-100/50 mM TrisHCl, pH 7.2. Aliquots of each nonadsorbed and GuHCl-desorbed fraction were counted. The desorbed PGs were exchanged into Urea/NaCl/TX/NaAc buffer on PD10 columns and concentrated on DEAE-Sephacel for SDS-PAGE as described above.

Surface Characterization

Elemental compositions of the Ti powders and of the bulk metal samples were determined by XPS at UAH. The variously treated powders were washed with water, dried, and then mounted on carbon conductive tabs (Ted Pella, Inc.). Survey scans (XPS) of each sample were conducted on a Perkin Elmer PHI 5400 ESCA System at an angle of

45 degrees, using an Mg filament at 325 W. This work was done under the direction of Dr. J. Weimer, Department of Material Sciences, University of Alabama in Huntsville.

Scanning electron microscopy of the carbon-disc mounted powders was performed on a Philips 515 SEM by Ms. Cherie Moss, Electron Optics Laboratory, Department of Materials and Mechanical Engineering, UAB.

Adsorption Protocol: Ti Powder

Ti powder (-100 mesh; 99.4% pure; Alfa Æsar) in 50 or 100 mg portions was placed in 1.5 ml Eppendorf tubes and incubated overnight at room temperature with either water (Control), 0.3 M $CaCl_2$, or 0.3 M $LaCl_3$. Additional batches were incubated overnight in the cold in 30% H_2O_2. After centrifugation, the powders were washed three times with several volumes of Tris/Triton Buffer. Samples of the ^{35}S-PGs (~40,000 cpm) were added to the variously treated powders, which were then agitated in an end-over-end mixer overnight at room temperature. After centrifugation, the supernatants were pooled with a Trix/TX wash of the pellet as the "nonadsorbed" PG fraction. The powders were then incubated overnight in 4 M GuHCl/0.5 M Na_4EDTA/0.1% TX-100/50 mM TrisHCl, pH 7.2. After centrifugation, portions of the supernatants were counted and pooled for further analysis. This was designated the "Gu/EDTA-desorbed" PG fraction.

In some cases, a *sequential* treatment, first with GuHCl and then with EDTA, was used to desorb proteoglycans from the metal surfaces. These agents differ in their specifity for disrupting protein-material interactions. High concentrations (4-6 M) of GuHCl would be expected to disrupt hydrophobic adsorption such as may occur between relatively nonpolar regions of the protein core and the material surface. In contrast, EDTA would be expected to chelate cations such as Ca and La, thereby disrupting primarily electrostatic interactions such as may occur between the anionic GAG chains and the negatively charged Ti oxide surface. If these agents differ significantly in their ability to release proteoglycans

from Ti surfaces, it would indicate whether these interactions are more hydrophobic or electrostatic in nature.

After removal of the nonadsorbed PG fraction, the powders were incubated overnight in 4 M GuHCl/0.1% TX-100/50 mM TrisHCl, pH 7.2. After centrifugation, the supernatants were pooled with a Gu/TX/Tris wash of the pellet as the "Gu-desorbed" PG fraction. Finally, the powders were incubated overnight in 0.2 M Na_4EDTA/0.1% TX-100/50 mM TrisHCl, pH 7.2 and centrifuged; the supernatants were pooled with an EDTA/TX/Tris wash of the pellet. This was designated as the "EDTA-desorbed" fraction. Portions of each fraction were counted.

The remainders of each fraction (nonadsorbed PGs; GuHCl/EDTA-desorbed PGs; GuHCl-desorbed PGs; EDTA-desorbed PGs) were exchanged into Urea/NaCl/TX-100/NaAc buffer by either dialysis or chromatography on PD10 columns. The samples were then concentrated on DEAE-Sephacel as described above and prepared for SDS-PAGE.

Zymography

Preliminary results of the adsorption studies indicated that incubation of the PGs with Ti powder for prolonged periods at room temperature caused limited degradation of the macromolecules. It was therefore necessary to consider the possibility that the PG preparations were contaminated with low levels of endogenous proteolytic enzymes. Zymography allows the detection of extremely low levels of protease activity and was therefore used to examine this issue. Briefly, 10% polyacrylamide gels (1.5 mm thickness) are cast with either casein or gelatin (appox. 7.5 mg/minigel) in the separating gel polymerizing mixture. After electrophoresis of the sample, the gels are washed sequentially for 20 min/wash in 1) Solution 1A--50 mM TrisHCl, pH 7.5, containing 3 mM azide and 2.5% Triton X-100; 2) Solution 2A--solution 1A + 5 mM $CaCl_2$ and 1 μM

$ZnCl_2$; 3) Solution 3A--Solution 2A without the Triton X-100. The gels are then incubated in Solution 3A overnight at 37°C and stained the following day with Coomassie Brilliant Blue. Areas of proteolytic activity appear as white bands on a blue background. The zymography protocol was kindly provided by Dr. Jack Windsor, UAB.

Titanium Oxide Powders

Titanium rapidly and spontaneously forms a surface oxide layer consisting primarily of TiO_2 (Ellingsen, 1991) which may exist in a number of crystalline forms, including rutile and anatase. Preliminary adsorption experiments were conducted using commercial stocks of the two titanium oxides. Titanium (IV) oxide powder, predominantly rutile (< 5 micron; 99.9% pure), and titanium (IV) oxide powder, anatase (-325 mesh; 99+% pure), were purchased from Aldrich. The adsorption of labeled PGs to these powders was examined using a protocol essentially identical to that used for the Ti powder. However, the average particle size of both oxides was significantly smaller than that of the Ti powder; therefore, attempts to relate PG adsorption to oxide surface chemistry would be confounded by the major differences in surface area.

Metal Sample Preparation

Titanium and titanium alloy rod stock (0.25 inch diameter) were machined to approximately 0.246-0.247 inch diameter and then cut with a diamond saw into discs approximately 2 mm thick. The discs were set into polymethylmethacrylate molds, ground and polished to 0.3 μ, and cleaned and passivated as described above. After sterilization by UV, the discs were placed in 96-well culture dishes.

Protein Coatings

The following PGs and matrix proteins were used for the attachment studies: bovine serum albumin (BSA; Sigma; Fraction V); fibronectin (Sigma F-0895; from human plasma; supplied as a 0.1% solution in Tris buffered saline); Collagen Type I (Sigma C8919; from calf skin; supplied as a 0.1% sterile solution in 0.1 N acetic acid; osteopontin (recombinant ratOPN with polyHis tail; generously provided by Dr. Pi-Ling Chang, Dept. Nutrition Sciences, UAB); decorin PG (Sigma D-8428; from bovine articular cartilage); biglycan PG (Sigma B-8041; from bovine articular cartilage); and aggrecan (Sigma A-1960; from bovine articular cartilage). Stock solutions were prepared for each protein (except for fibronectin and collagen) at a concentration of 1 mg/mL in Dulbecco's PBS; these were dispensed in aliquots and stored at -20°C until needed.

Immediately prior to the attachment assays, aliquots of the stock solution were thawed and diluted with D-PBS to a concentration of 0.01 mg/mL. The metal discs or control plastic wells were incubated with 100 µl (1 µg) of each protein or with buffer alone in the cold overnight. The solutions were then removed by aspiration and the wells gently washed three times with PBS. The wells were then incubated for 30 min at 37°C with a solution of 1% heat-inactivated BSA (inactivated at 80°C for 10 min). Prior to plating the cells, the plates were washed three times with PBS.

Cell Attachment Protocol

Subconfluent MC3T3 cells grown in plastic T75 flasks were washed with Hank's Buffered Saline (Ca and Mg free) and detached from the surface with Pronase E/EDTA. The cells were washed in sterile αMEM containing 0.2% BSA and cycloheximide (1 µg/mL) and resuspended in the same buffer to a final concentration of approximately 30,000 cells per 0.1 mL. Each of the sample wells (in triplicate) was seeded with 0.1 mL of the cell suspension, and the plates were incubated for 1-1.5 hrs at 37°C. After this interval, the wells were gently washed five times with PBS to remove the nonadherent

cells. When examined by light microscopy, the plastic wells coated with FN, collagen, or OPN were seen to have many attached and spread cells; few cells were visible in any of the control wells or wells treated with the other proteins.

Hexosaminidase Assay

In studies of cell attachment to plastic substrates, proteolytic enzymes are often used to detach the adherent cells; these can then be numbered with a hemacytometer or Coulter Counter. However, this approach is not suitable for cells cultured on metal substrates. Incubation with Pronase E/ EDTA, even for prolonged periods, very poorly releases MC3T3-E1 cells from titanium surfaces. As an alternative, a mild detergent was used to solubilize the cells attached to the various substrates. The cell lysates were then assayed for hexosaminidase activity, which is proportional to total cell number (Landegren, 1984).

The enzymatic substrate for this assay is p-Nitrophenyl N-Acetyl-β-d-glucosaminide (Sigma N9376). This substance is suspended at a concentration of 3.4 mg/mL in 0.1 M citrate, pH 5.0, and then solubilized by adding an equal volume of 0.5% Triton X-100. Cultures in 96-well plates are washed five times with PBS to remove the loosely adherent cells. Sixty microliters of the substrate are added to each well and the plate is incubated in a moist atmosphere for 1 hr at 37°C. Addition of 100 μl Stop Solution (50 mM glycine, 5 mM EDTA, pH 10.5) ends the reaction and maximizes the intensity of the yellow product p-nitrophenol. Each mixture is then carefully transferred to a fresh 96-well plate, which is scanned in a microplate reader at OD_{405} nm.

This procedure was altered slightly for cells cultured on larger areas. A 2X substrate solution was prepared by dissolving the p-Nitrophenyl N-Acetyl-β-d-glucosaminide in one half the volume of citrate/Triton described above. After 4-5 PBS washes, cells grown in 12-well plates were lysed in 0.6 ml 50 mM citrate, pH 5/0.25% Triton X-100. After 15 min at 37°C, 30 μl of each lysate were transferred to duplicate

wells of a 96-well plate. Thirty microliters of the 2X substrate were added to each well and the plate was incubated for 1 hr at 37°C. The reaction was stopped as above and the plate read at OD_{405}.

RESULTS

Hypothesis 1

Osteoblast-like cells cultured on titanium or titanium alloy synthesize significantly different types of proteoglycans than the same cells on stainless steel or CoCrMo.

The retrieval studies of Linder and coworkers (1983, 1989) suggest the possibility that implant materials induce metal-specific differences in the amounts and/or types of proteoglycans at the bony interface. The studies described here examined PG synthesis by osteoblastic MC3T3 cells on implant metals. In an initial set of experiments, ion-exchange and column chromatography were used to characterize and compare the PGs produced by these cells on cpTi and on tissue culture plastic. Analysis of the cell extract PGs on DEAE-Sephacel with a linear 0.15-1.5 M NaCl gradient (Figs. 1 and 2) showed that the cells grown on the two substrates produce nearly indistinguishable elution profiles. In both cases, a single symmetric peak eluted at at a sodium chloride concentration of approximately 0.4-0.5 M. Roughly 10% of the total recovered radioactivity does not bind to the resin and is likely to be non-PG sulfated glycoprotein in nature.

Gel filtration of the ^{35}S-cell extracts on Sepharose CL-4B yielded only slight differences in elution profiles for the plastic and cpTi substrates (Figs. 3 and 4). In both cases, a relatively small peak in the column void volume is followed by a broad included peak with a Kav of about 0.5. Treatment with chondroitinase ABC deletes the void volume peak and shifts the included peak to a slightly larger average molecular size. Further treatment with heparitinase converts the remaining ^{35}S-macromolecules to small fragments in the Vt. These results indicate that these cells, cultured on both plastic and cpTi, produce a diverse mixture of proteoglycans: 1) a very high molecular weight species,

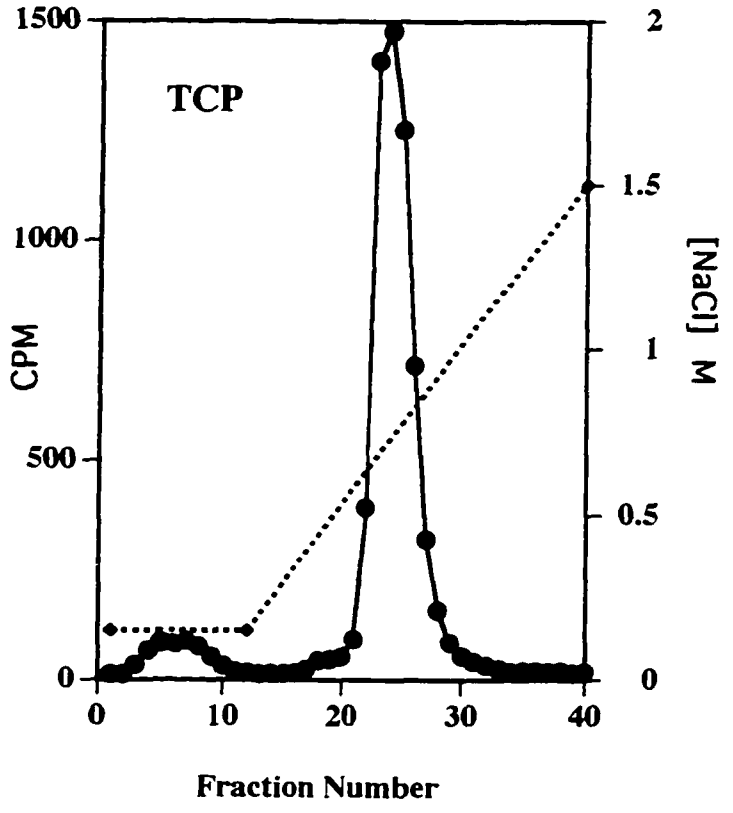

Figure 1. Ion exchange chromatography on DEAE-Sephacel: sulfated macromolecules from MC3T3 cells cultured on plastic.

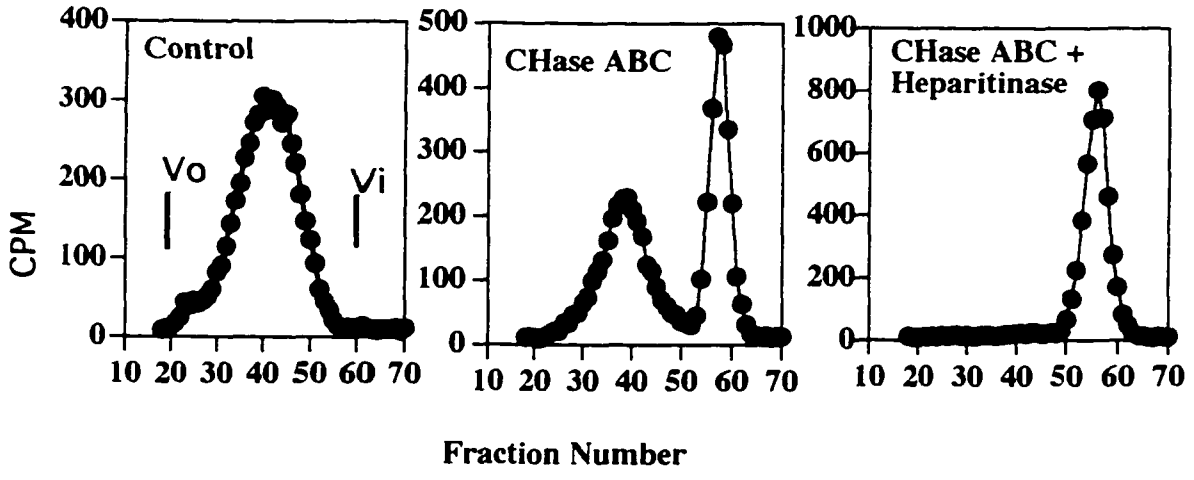

Figure 2. Gel filtration on Sepharose CL4B: sulfated macromolecules from MC3T3 cells cultured on plastic. Samples were treated with buffer, chondroitinase ABC, or chondroitinase ABC + heparitinase.

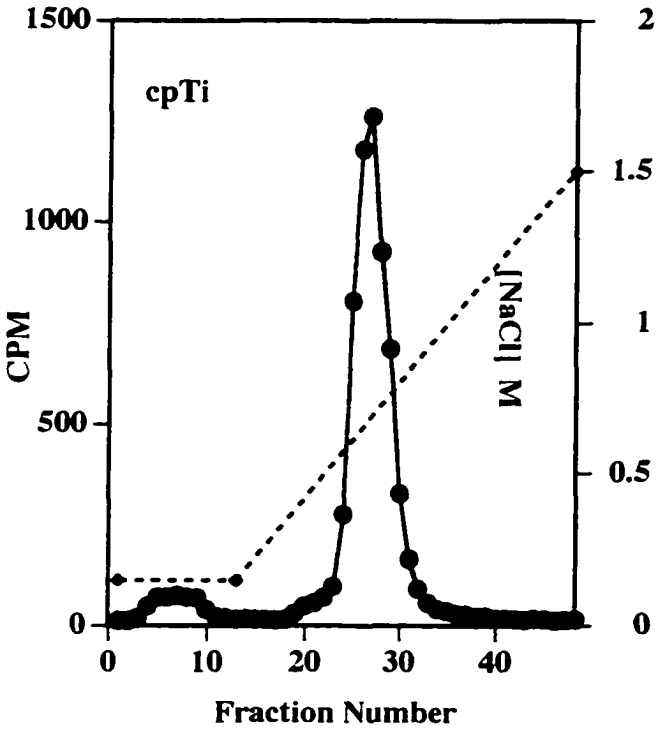

Figure 3. Ion-exchange chromatography on DEAE-Sephacel:sulfated macromolecules from MC3T3 cells cultured on cpTi.

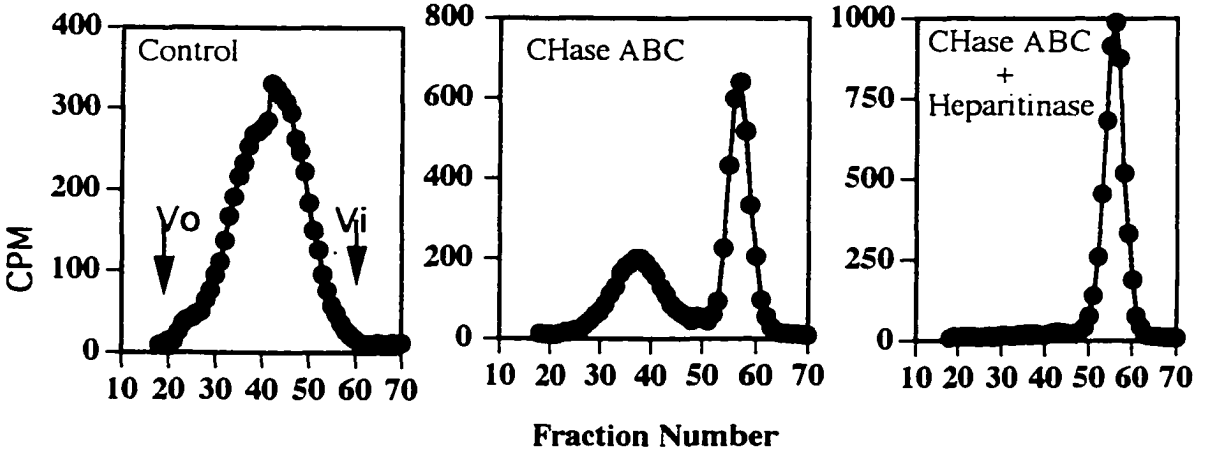

Figure 4. Gel filtration on Sepharose CL4B: sulfated macromolecules from MC3T3 cells cultured on cpTi.

akin to the large chondroitin sulfate PG of bone or cartilage; 2) a large heparan sulfate PG, and 3) a population of smaller CS/DS PGs, most likely consisting of decorin or biglycan. The PG profiles from the MC3T3 cells on plastic are comparable to those obtained by Takeuchi *et al.* (1990). In the present study, no dramatic differences were evident between the profiles from the two substrates; it was notable that the relative proportions of CS/DS and HS were approximately 45:55 on plastic and 55:45 on cpTi (Klinger *et al.*, 1995).

These results were obtained using methods that would lump the extracellular matrix PGs together with those in the cell layer. Since the matrix is the cellular compartment most likely to be critical for osseointegration, it seemed worthwhile to analyze the two populations separately from cells cultured on implant metals. MC3T3 cells were grown to confluence on plastic and on discs of polished cpTi, Ti-6-4, and 316L SS. After labeling with ^{35}S-sulfate, the culture medium was removed and the cell layer extracted by incubation with 2% Triton X-100. This treatment leaves the ECM still attached to the substrate. The matrix molecules can then be solubilized with a buffer containing GuHCl and EDTA. On a per cell basis, the results (Table 1; Klinger 1996a) demonstrate that the metals were not markedly different from each other but that all differed significantly from the PGs produced on plastic.

Table 1. PGs in cell layer and in ECM of MC3T3 cells cultured on implant metals

Substrate	Triton Extract (TX)	Guanidine Extract (GX)
TCP (plastic)	8600[a]	1580[a]
cpTi	13600	5760
Ti-6Al-4V	11560	5290
316L SS	12610	5650

[a] Average of duplicate samples; incorporated CPM/10^5 cells.

The Triton extracts, which consist of the cytoplasmic and membrane-associated

PGs, contained on average 46% more radioactivity per cell on the metal surfaces than fromthe same cells cultured on plastic. The Guanidine extracts, which consist primarily of the PGs associated with the extracellular matrix, contained an average of 3.5-fold more radioactivity per cell on the metal surfaces than from the same cells cultured on plastic.

The size distribution of the PGs in the Triton and Guanidine extracts from each of the substrates was analyzed by gel filtration on Sepharose CL-4B in 4 M GuHCl/0.5% Triton X-100, as shown in Fig. 5. Slight differences are evident in the profiles of the ECM PGs from the various substrates, while the PGs in the detergent extracts are not readily distinguishable.

A third series of experiments made use of SDS-PAGE/fluorography, which allows vastly greater resolution of the PGs found in cell extracts. Analysis of the PG mixtures on linear 4-15% gradient gels is presented in Fig. 6. No major differences are apparent in the PGs secreted into the culture medium (Fig. 6a) by MC3T3 cells cultured on plastic, cpTi, Ti alloy, stainless steel, or CoCr. Further analysis of this fraction from cells on cpTi (Fig. 7a) shows that PGs in the culture medium consist primarily of intermediate-size chondroitin and dermatan sulfate PGs, likely decorin and biglycan. The cells on plastic (Fig. 6a) appear to produce far less of a sulfated, non-PG molecule with an apparent molecular weight below 45 kD. It is tempting to suggest that this may be osteopontin; this remains to to be confirmed by immunochemical methods.

As before, the PGs in the cell layer + matrix from the four metals (Fig. 6b) gave quite consistent profiles with no gross distinctions. It is notable that plastic again differs from the metals as a group, this time in the relative migration of a PG in the MW range of 200-250 kD.

Spectroscopic examination of the bulk surfaces confirms the expected primary metal composition of the alloys. XPS analysis detected Ti, O, and C as the major components of the cpTi and Ti-6-4 discs (Fig. 8). In addition to oxygen and carbon, the 316L SS and CoCrMo samples showed a doublet for Cr at ~585 eV (Fig. 9). Neither Si nor Al was

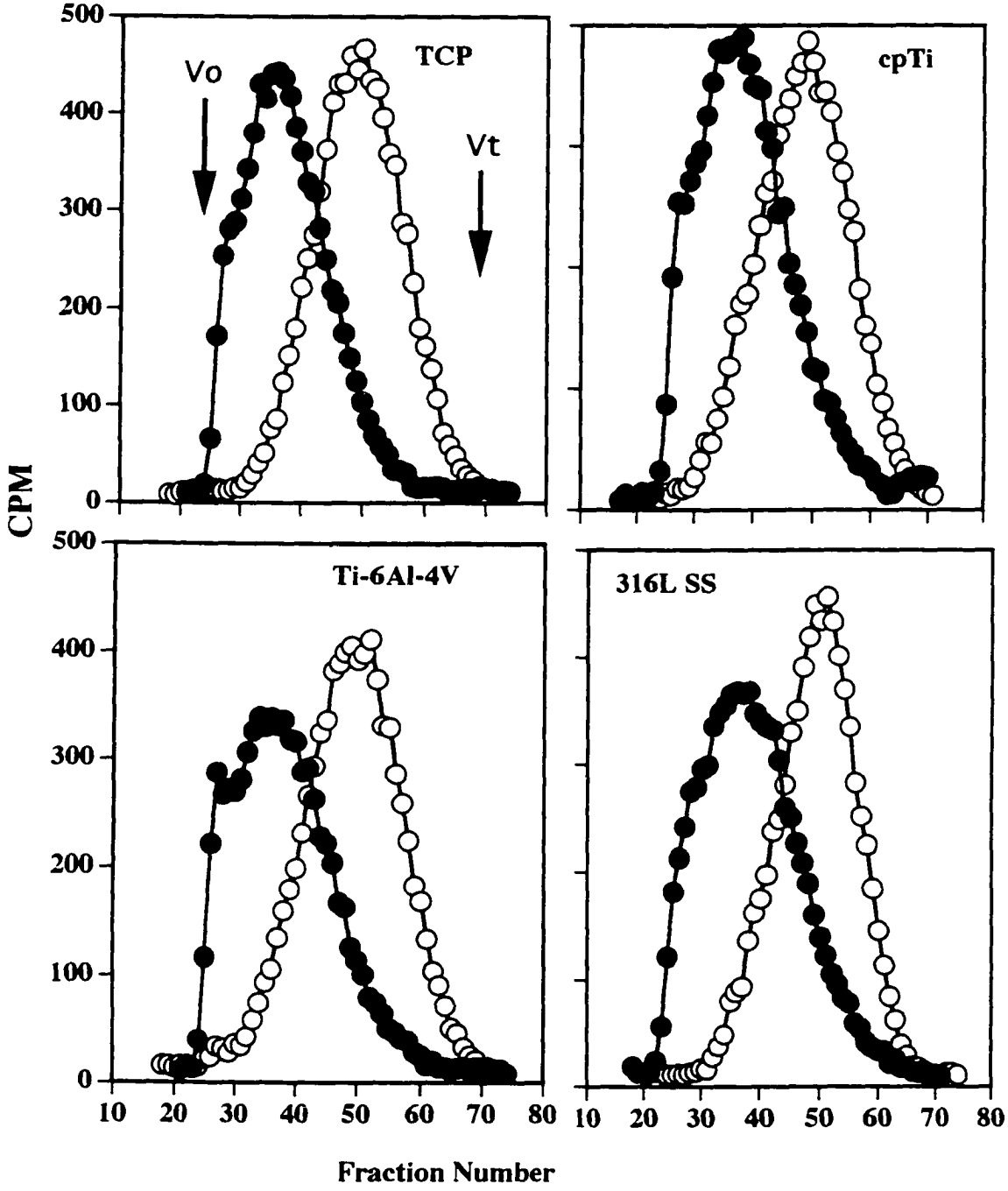

Figure 5. Gel filtration on Sepharose CL-4B of proteoglycans from MC3T3 cells cultured on tissue culture plastic, cpTi, Ti-6-4, and 316L SS. Open circles: cell layer fraction--extracted with 2% Triton X-100. Filled circles: extracellular matrix fraction--extracted with 4 M GuHCl.

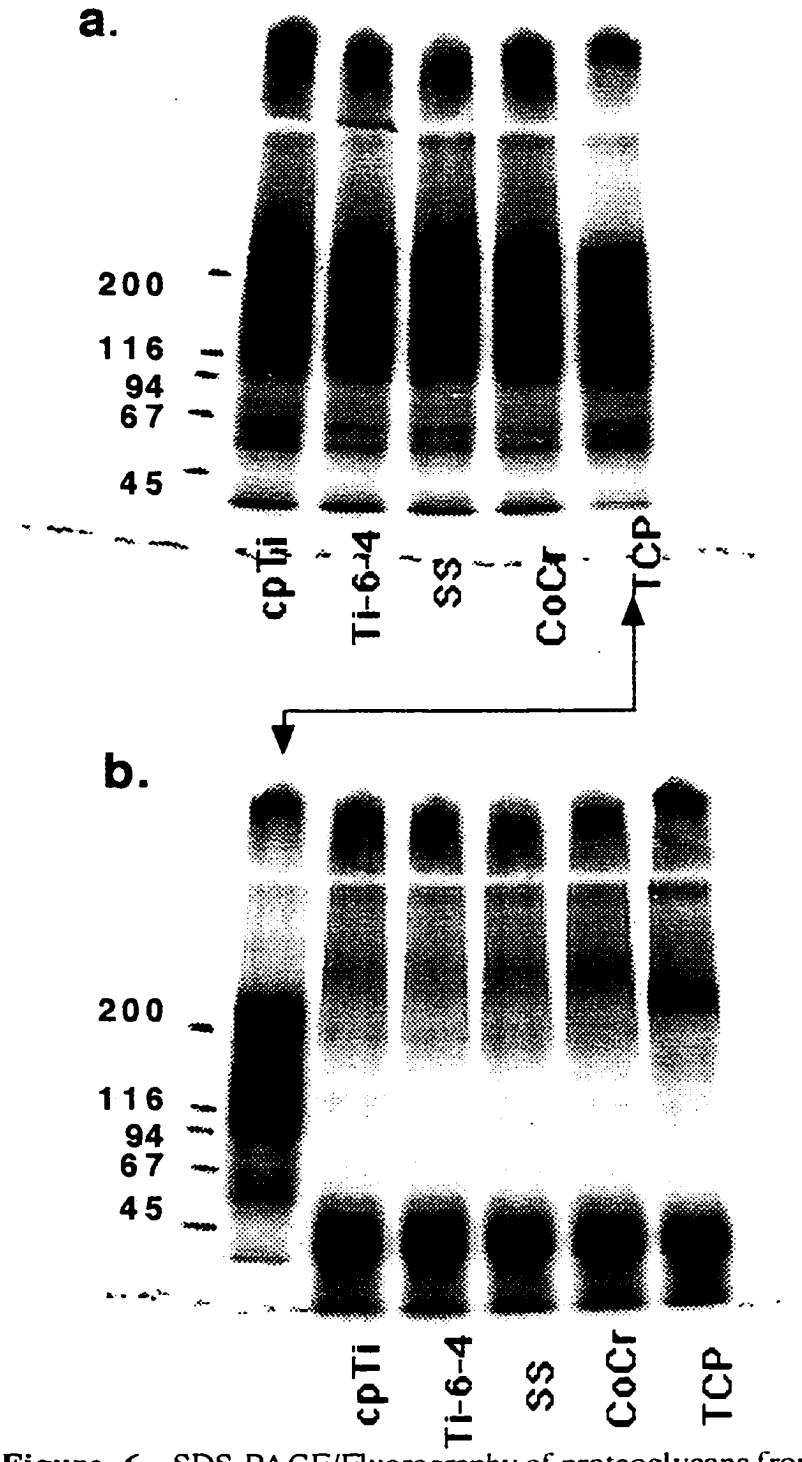

Figure 6. SDS-PAGE/Fluorography of proteoglycans from cells cultured on plastic and on metallic implant materials. 4-15% linear gradient of acrylamide. a. Proteoglycans secreted into culture medium. b. Proteoglycans extracted from cell layer and extracellular matrix fraction. Arrow indicates the same sample loaded in each of the two lanes.

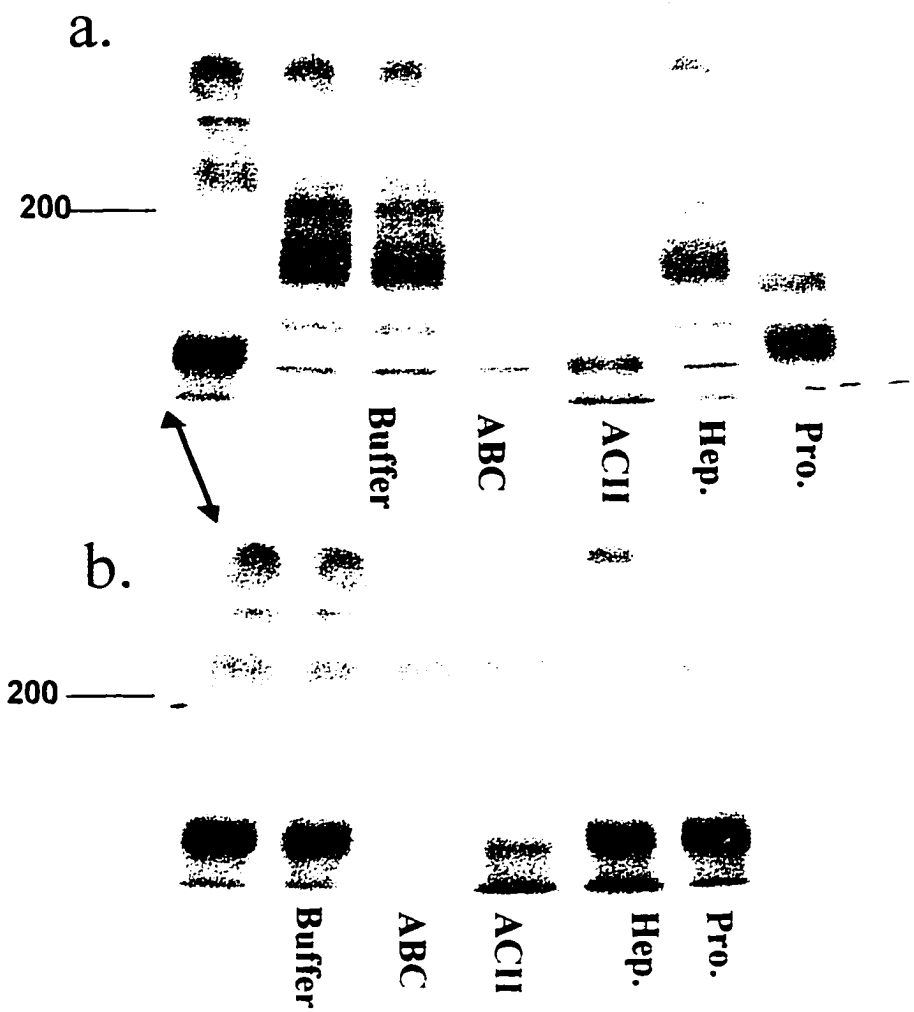

Figure 7. Proteoglycans synthesized by MC3T3 cells on cpTi: enzymatic digestion. Samples were treated for 3 hrs at 37°C with buffer alone, chondroitinase ABC, chondroitinase ACII, heparitinase, or Pronase E. Fluorogram of 4-15% linear gradient SDS-PAGE. a. PGs secreted into culture medium. b. PGs extracted from cell layers + ECM. First lane in each gel shows same sample for comparison.

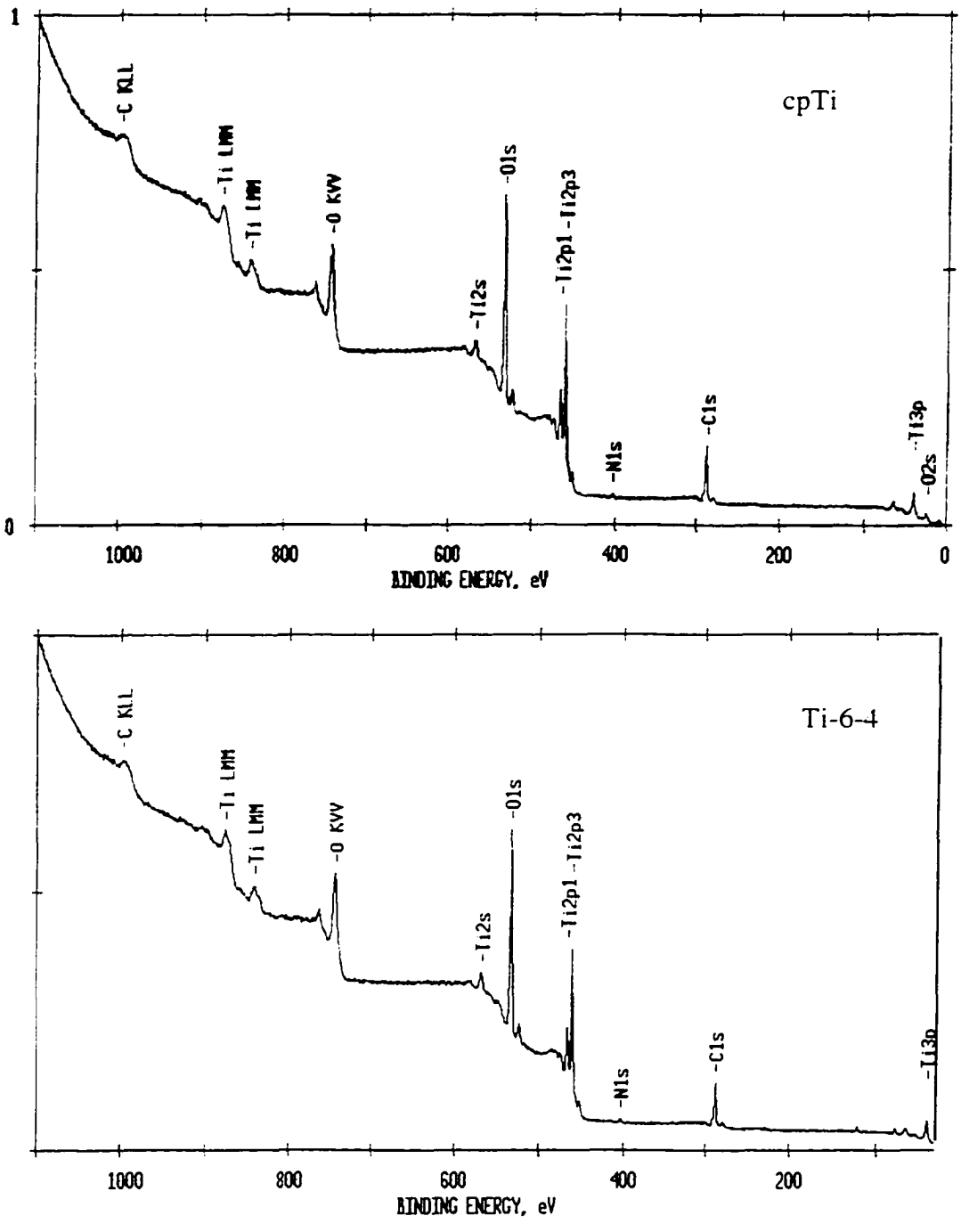

Figure 8. XPS survey scans of bulk metal surfaces: cpTi and Ti-6-4.

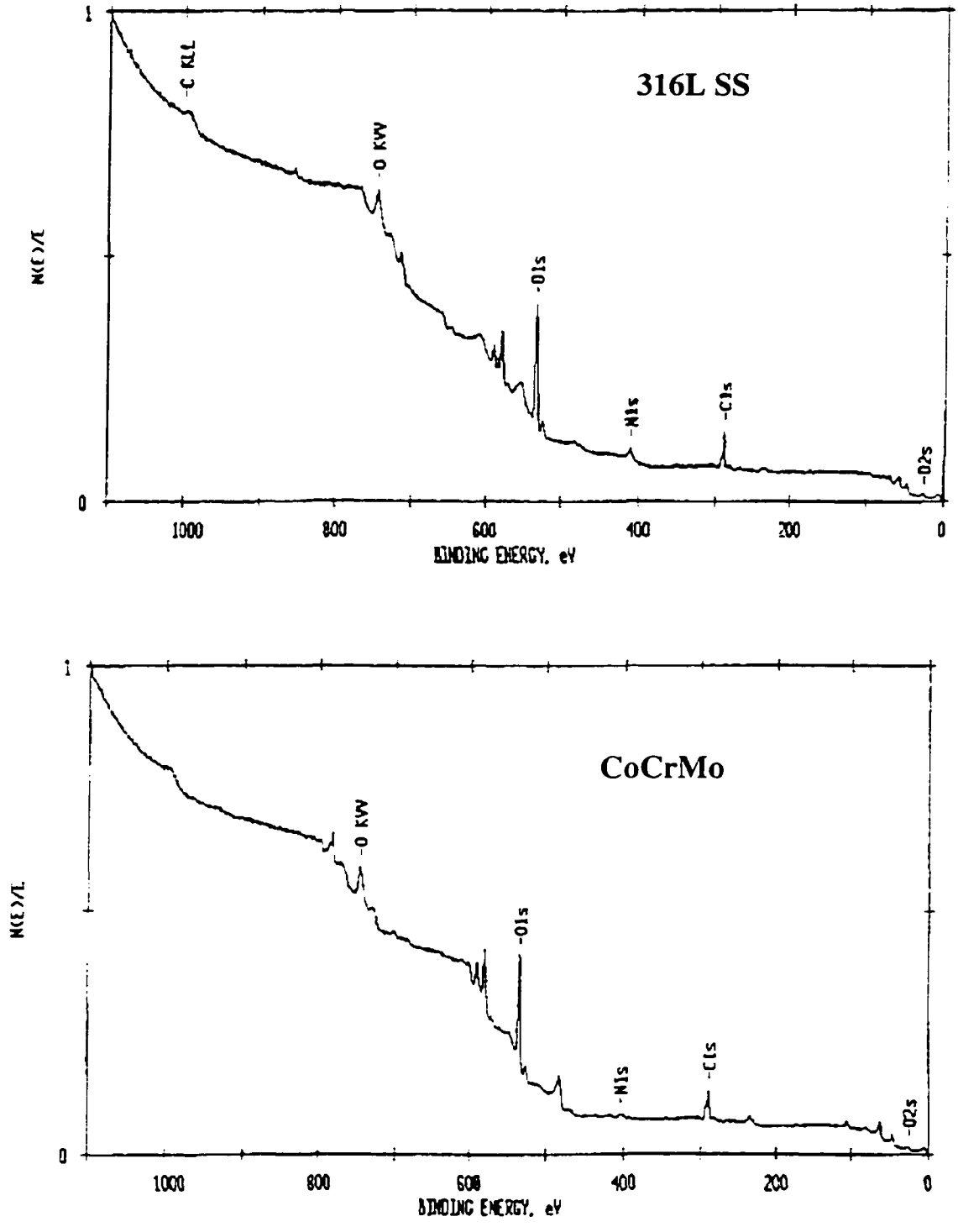

Figure 9. XPS survey scans of bulk metal surfaces: 316L SS and CoCrMo.

observed in any of the bulk metal surfaces, indicating that the cleaning process had effectively removed most of the residues deposited during the grinding and polishing steps.

Light microscopy of the surfaces (Figs.10 and 11) indicates considerable differences in surface topography on the alloys, despite the fact that all were polished to 0.3 μ and exhibited "mirror-like" finishes. Profilometry of these surfaces yielded an average roughness of 0.1-0.2 μ in all cases (Table 2). It is somewhat surprising that these measurements did not reflect the differences in morphology revealed by light microscopy.

Table 2. Average Roughness of Bulk Metal Surfaces

Alloy	Max.	R_A Avg.	Min.
cpTi	0.2 μm	0.1	0.1
	0.3	0.1	0.1
Ti-6-4	0.4	0.1	0.0
	0.5	0.1	0.0
316L SS	0.1	0.1	0.0
	0.2	0.2	0.2
CoCrMo	0.5	0.2	0.0
	0.1	0.1	0.0

If we assume that the different surface chemistries of the alloys should induce *dramatic* differences in bone cell proteoglycan metabolism, then the data thus far appear to reject the first hypothesis. Proteoglycan biosynthesis by MC3T3 cells on Ti and Ti alloy could not be distinguished from the profiles on stainless steel or CoCrMo. However, the four implant metals as a group did induce somewhat altered PG synthesis as compared to the tissue culture plastic surface. This difference may be critical in evaluating the effects of mechanical loading on PG metabolism; it is quite reasonable to suspect that PG metabolism by cells stressed on plastic substrates would differ from their responses to similar forces applied to metal surfaces.

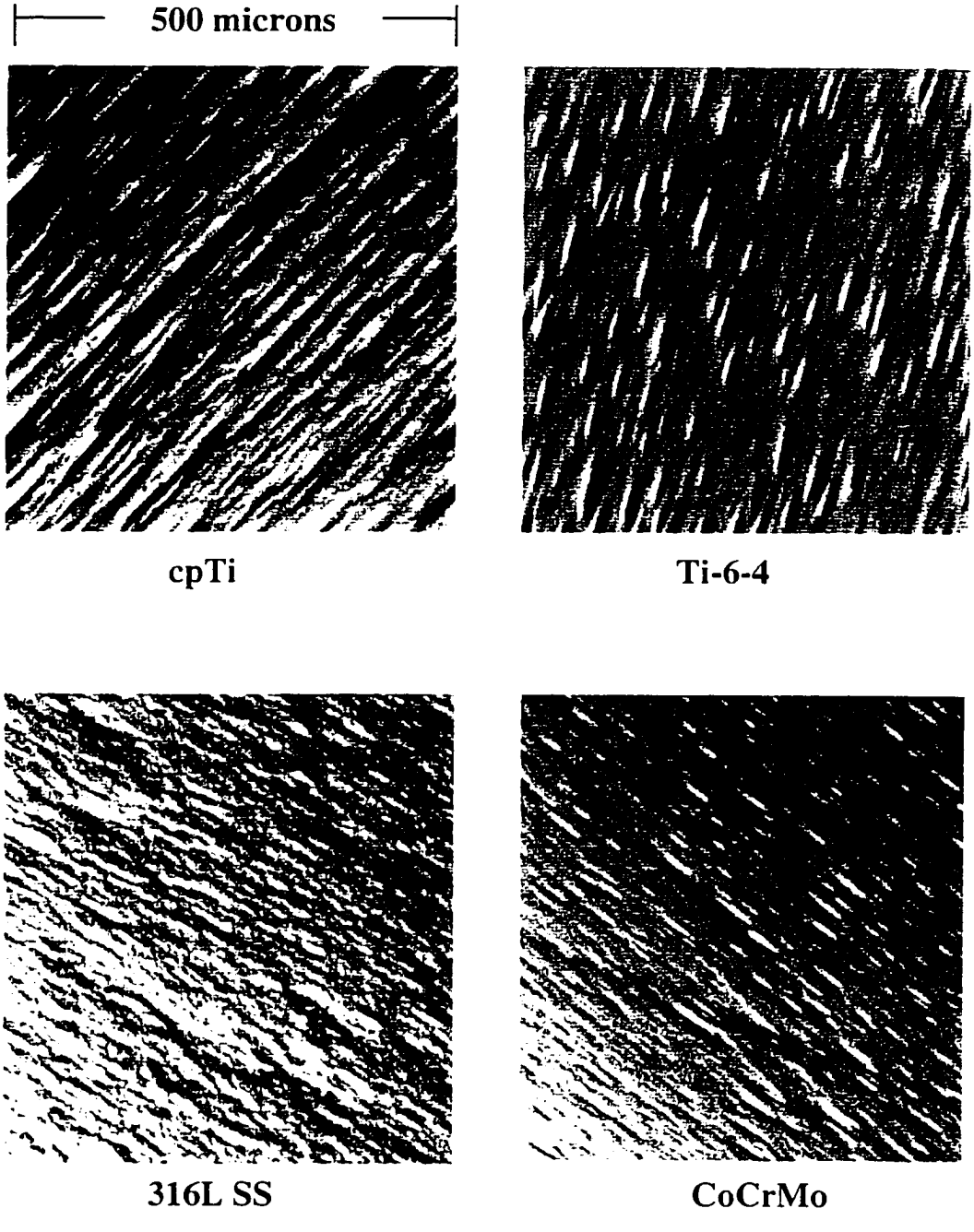

Figure 10. Bulk metal surfaces used for PG biosynthesis and PG adsorption studies. Micro-Vu 40% magnification.

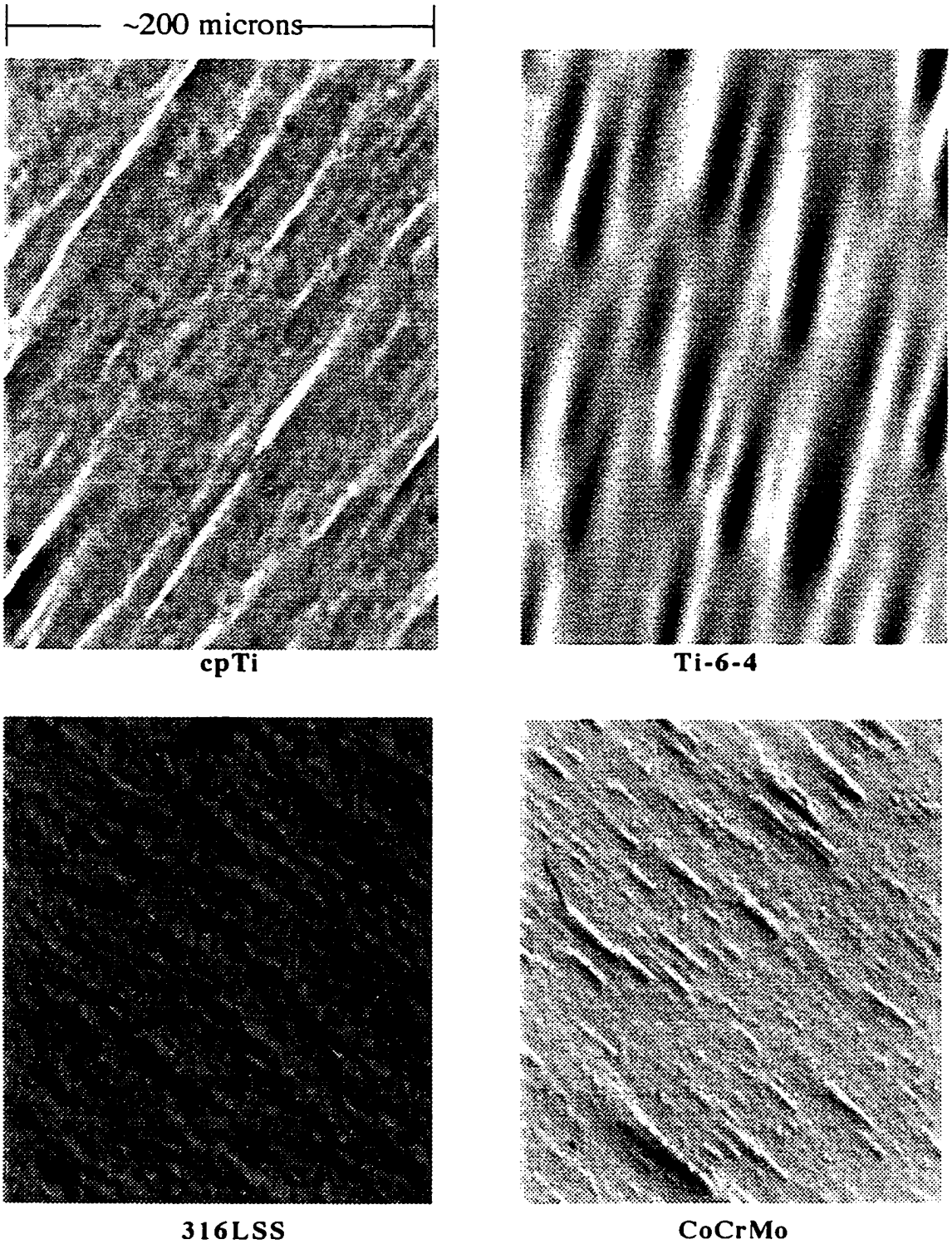

Figure 11. Micro-Vu images of bulk metal surfaces used for cell culture and proteo-glycan adsorption studies. 100% magnification.

Hypothesis 2

Pure titanium and Ti alloy differ from stainless steel and CoCrMo in the amounts and/or types of proteoglycans which adsorb to their surfaces.

Previous studies of protein adsorption to metals have generally relied on commercially available proteins such as albumin or fibronectin or on complex mixtures of serum or salivary proteins (Willliams and Williams, 1988; Ellingsen, 1991; Steinberg *et al.*, 1995). Proteoglycans have particularly complex structures due to their highly charged polysaccharide chains and, in the case of decorin and biglycan, to the unusual leucine-rich repeat sequences in their protein cores. Differences in the adsorption behavior of proteoglycans on the various metal surfaces could suggest reasons for the different abilities of these materials to support osseointegration.

Radiolabeled PGs from MC3T3 culture medium were incubated for 18 hrs at room temperature with 22 mm diameter discs of polished cpTi, Ti-6-4, 316L SS, or CoCrMo. Tissue culture plastic wells of the same diameter were used in parallel. In this study, only two fractions were measured: the nonadsorbed PGs and the PGs desorbed from the surfaces with a buffer containing both GuHCl and EDTA.

In each case, the great majority (> 95%) of the applied radioactivity was recovered in the nonadsorbed fraction. The PGs which bound to the metals and could be desorbed with GuHCl/EDTA accounted for only 3-4% of the starting material. Figure 12 presents the mean ± standard deviation of triplicate samples of each substrate. With the radioactivity desorbed from the tissue culture plastic normalized to 100%, the values for cpTi, Ti-6-4, 316L SS, and CoCrMo were 87.4%, 96.3%, 71.2%, and 65.6%, respectively. Single factor ANOVA (Excel 5.0) comparing each of the metals to plastic did not reveal significant differences for cpTi ($p < 0.07$) or for Ti-6-4 ($p < 0.45$) yet did show significant differences for 316L SS ($p < 0.02$) and for CoCrMo ($p < 0.002$).

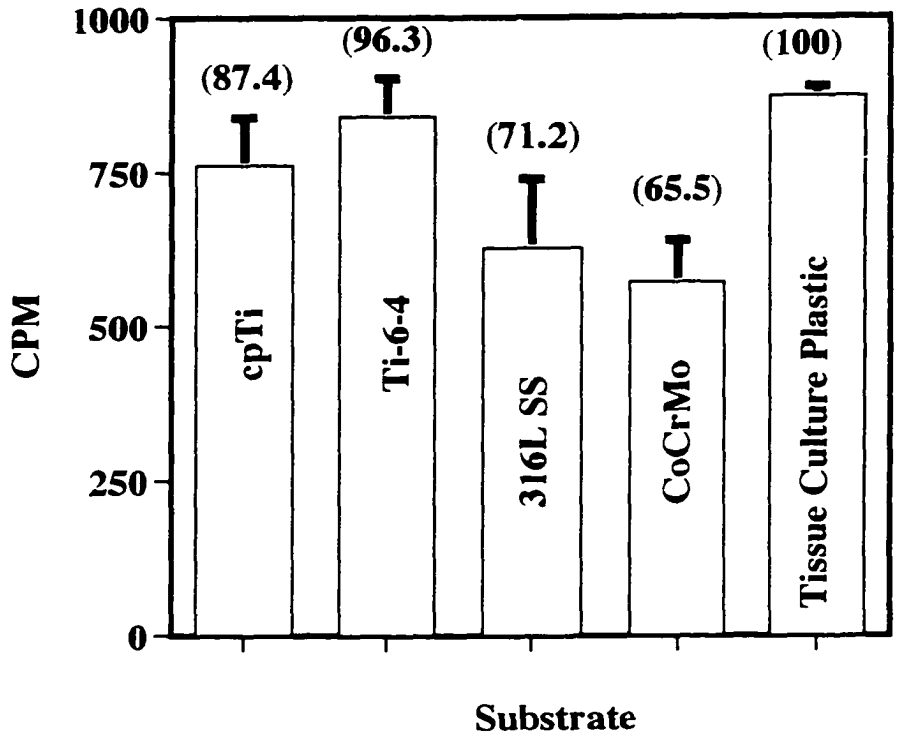

Figure 12. Desorption of proteoglycans from bulk metal surfaces. Culture medium ^{35}S-PGs were incubated with polished metals for 18 hrs at room temperature. Proteoglycans were desorbed with 4 M GuHCl/0.5 M EDTA/0.1% Triton X-100. Bars show mean ± standard deviation of triplicate values for each metal. Values normalized to those of tissue culture plastic (as 100%) are in parenthesis.

Proteoglycan binding to the Ti and Cr alloy surfaces appears to differ qualitatively as well as quantitatively. Results of SDS-PAGE of the various fractions are presented in Fig. 13. A subtle but definite distinction can be made between the gel profilesof the PGs desorbed from cpTi/Ti alloy vs. 316L SS/CoCrMo. A narrow band (MW ~120,000) is visible in the cpTi and Ti-6-4 lanes (as well as those from tissue culture plastic) but is not seen in the 316L SS or CoCrMo lanes. This band is quite discernable on the original X-ray film but cannot be readily seen in scanned or photocopied images.

These differences, although subtle, appear to confirm Hypothesis 2. The adsorption of PGs to Ti and Ti alloy is distinguishable from their adsorption to stainless steel and CoCrMo. The bulk surfaces used for these adsorption studies were identical in composition and surface preparation as those used for the PG biosynthesis experiments. Therefore, one cannot conclude that the differences were due strictly to surface chemistry and not to variations among the metals in surface roughness.

Hypothesis 3

Chemical modifications of the titanium oxide surface layer significantly alter the adsorption of proteoglycans.

These studies sought to determine whether pretreatment of titanium powders with certain cationic salts or with hydrogen peroxide alters the adsorption of PGs. Positive results would suggest relatively simple and inexpensive surface treatments in which the implants are pre-immersed in solutions of common salts or rare earths. Future studies of these various surface treatments may correlate PG adsorption with effects on cell attachment and/or mineralization.

The initial results (Fig. 14) using PGs from total cell extracts demonstrated that pretreatment of Ti powder with NaCl had no effect relative to the water-treated control. However, calcium and especially lanthanum caused marked increases in the amounts of total PGs adsorbed to the powders (Klinger *et al.*, 1997). Much but not all of the bound

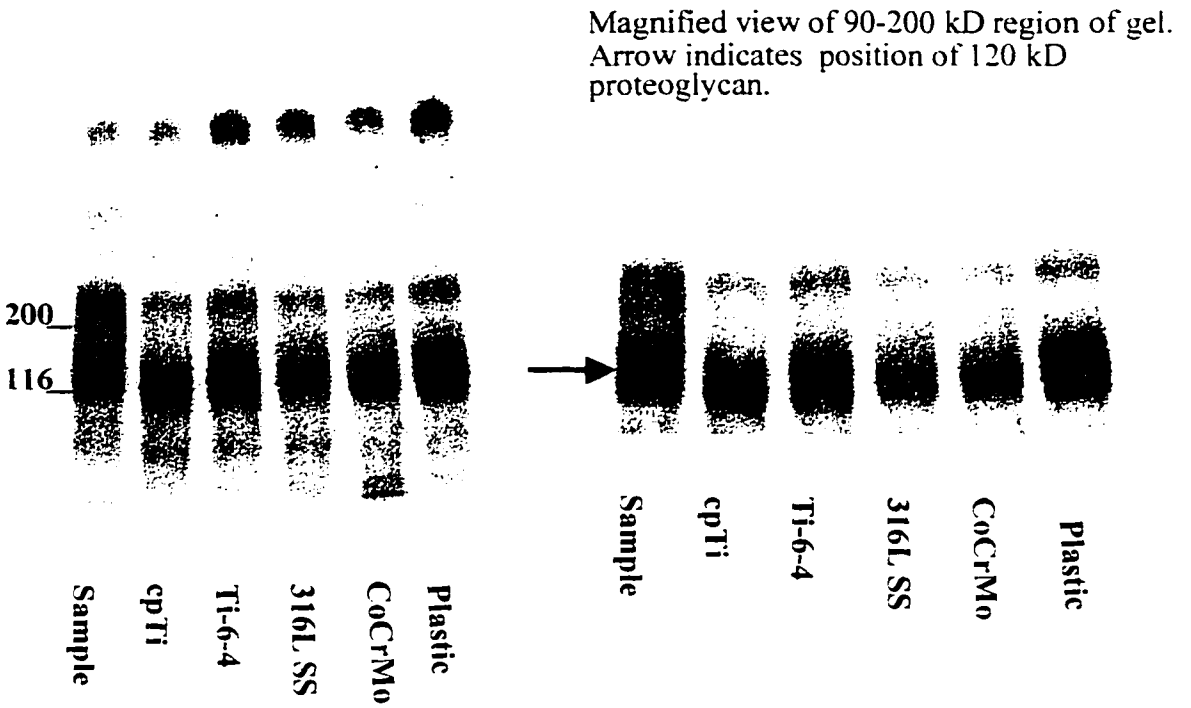

Figure 13. Proteoglycans desorbed from bulk metal surfaces. Culture. medium ^{35}S-PGs were incubated with polished metals for 18 hrs at room temperature. Proteoglycans were desorbed with 4 M GuHCl/0.5 M EDTA/0.1% Triton X-100. Fluorogram of 4-15% linear gradient SDS-PAGE.

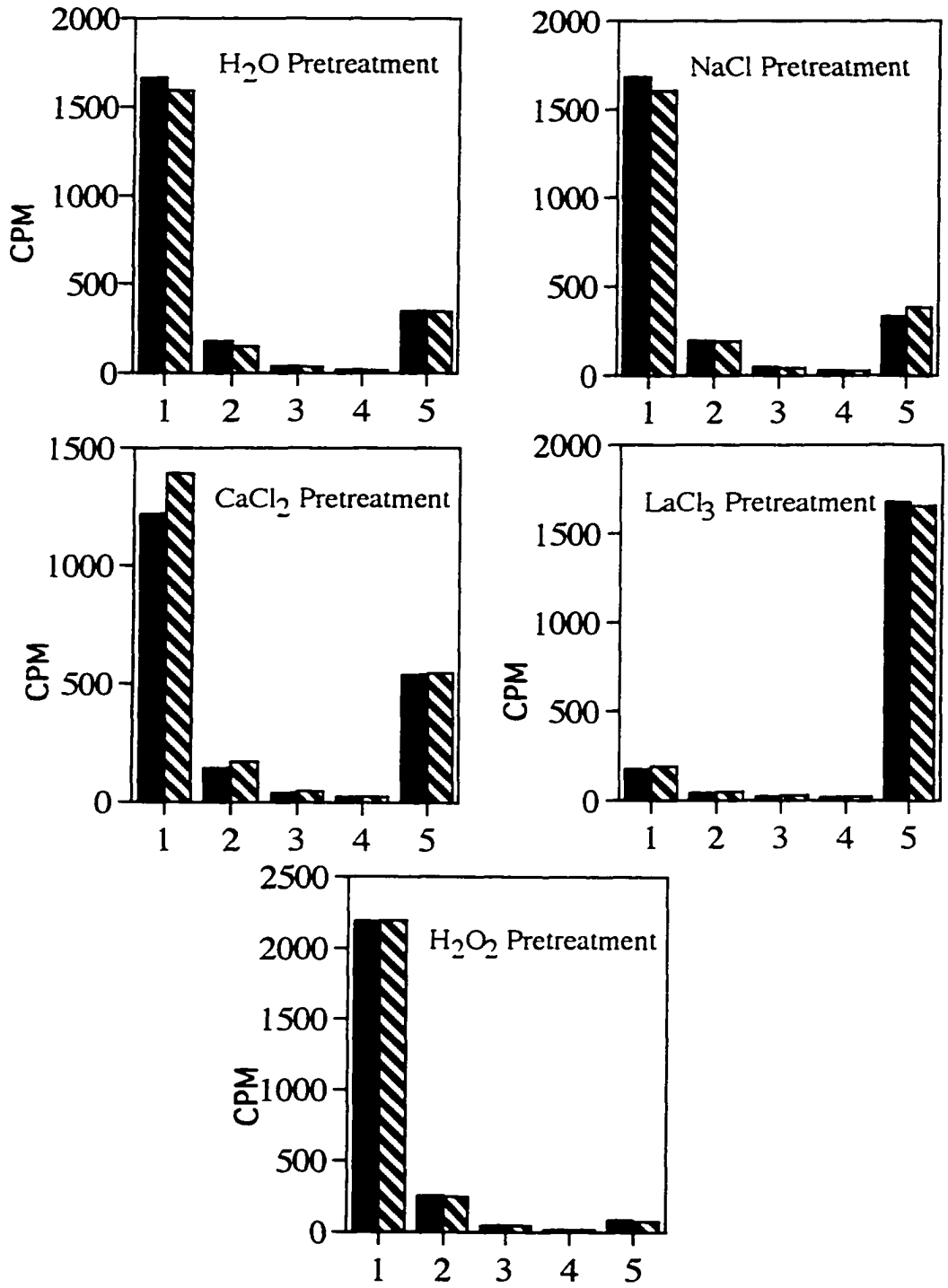

Figure 14. Adsorption of cell extract proteoglycans to Ti powder. Effect of pretreatments with 0.3 M salt solutions or 30% H_2O_2. Columns 1-4 in each panel indicate nonadsorbed fraction (PGs removed by buffer washes). Column 5 indicates fraction desorbed by GuHCl + EDTA. Filled and hatched bars show values for duplicate samples.

radioactivity could be released by soaking the washed powders in a buffer containing 4 M GuHCl and 0.5 M EDTA. Pretreatment with hydrogen peroxide had the reverse effect and decreased PG binding relative to the control.

This effect might have been due to a simple charge interaction between the oxide, cations, and sulfates on the PGs. This possibility was ruled out when the studies compared the adsorption of free sulfate with that of the labeled PGs (Figs. 15 and 16). Free sulfate did not adsorb to any appreciable degree to the Ti powders regardless of the various pretreatments. In contrast, the dramatic effects on PG adsorption were again observed.

The PGs used for these studies were not homogeneous populations of proteins but were instead complex mixtures of macromolecules. It was therefore important to determine if the salt pretreatments had qualitative, as well as quantitative, effects on PG binding. The PGs desorbed from the calcium- and lanthanum-pretreated Ti powders were separately chromatographed on a column of Sepharose CL-4B (Klinger *et al.*, 1996b). As can be seen in Fig. 17, the PGs from the Ca-Ti eluted earlier (and hence possessed a larger average molecular size) than the PGs desorbed from the La-Ti. This implied some specificity in cation effects of PG adsorption to Ti and suggested a possible means to control these interactions *in vivo*.

As these studies progressed, it became obvious that pretreatment of Ti powder with $CaCl_2$ often produced quite variable results, ranging from no effect at all to significant enhancements in PG binding. This discrepancy was eventually traced to differences in the lots of commercially available $CaCl_2 \cdot 2H_2O$ used for these studies (Fisher vs. Aldrich; new Fisher vs. old Fisher). The pH of 5% solutions of reagent grade $CaCl_2 \cdot 2H_2O$ varies hugely, apparently due to differences in the amounts of residual $Ca(OH)_2$ used in its manufacture. The mechanism for the resulting differences in PG binding was never fully resolved. In one study, however, the addition of small amounts of $Ca(OH)_2$ to a solution

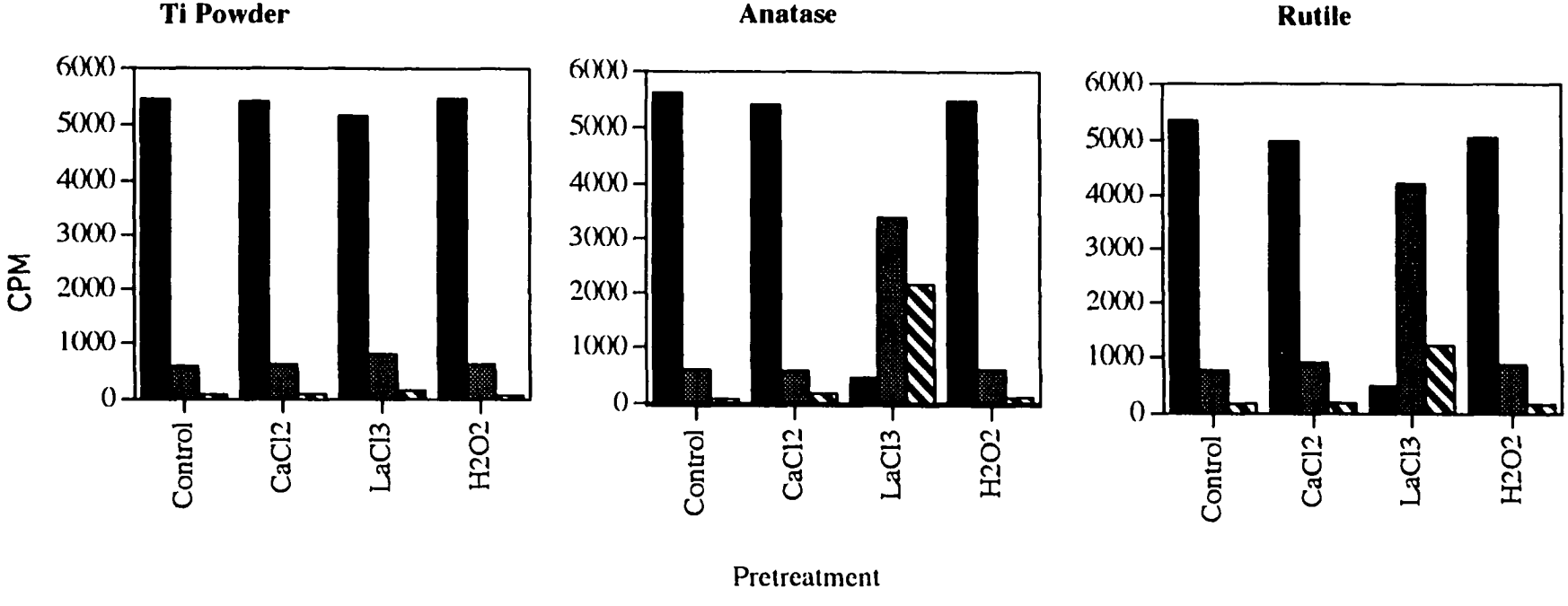

Figure 15. Adsorption of free $^{35}SO_4$ to Ti and Ti oxide powders. Portions (50 mg) of Ti, anatase, or rutile powder were incubated overnight at 4°C with water (Control), 0.3 M $CaCl_2$, 0.3 M $LaCl_3$, or 30% H_2O_2. After centrifugation, the powders were washed with Tris/Triton Buffer. Samples (~6000 CPM) of free $^{35}SO_4$ were added to the powders in parallel tubes and agitated in an end-over-end mixer overnight at 4°C. The mixtures were then centrifuged and the supernatants counted. This was the nonadsorbed fraction (filled bars). The powders were washed with Tris/Triton buffer to remove loosely adsorbed material (grey bars). Finally, the powders were incubated in a buffer containing 4 M GuHCl, 0.1 M EDTA, and 0.1% Triton X-100 (hatched bars).

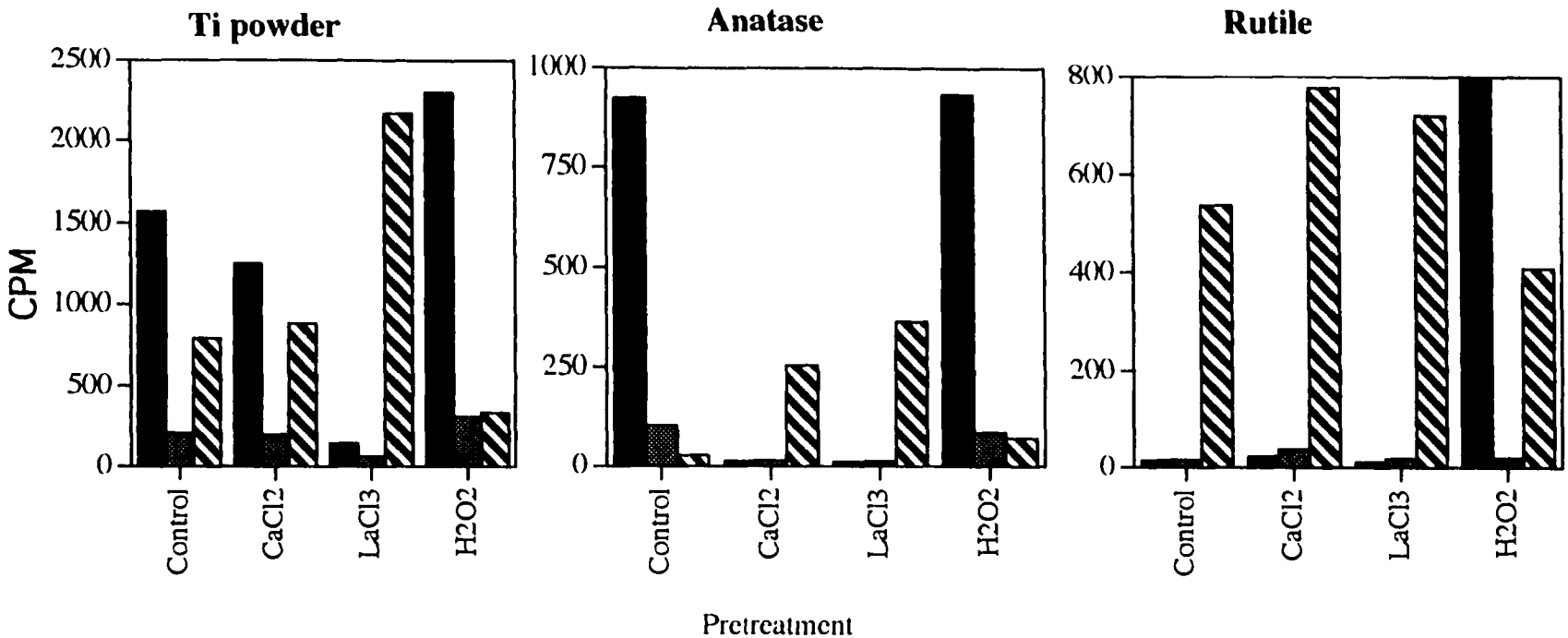

Figure 16. Adsorption of ^{35}S-proteoglycans to Ti and Ti oxide powders. Portions (50 mg) of Ti, anatase, or rutile powder were incubated overnight at 4°C with water (Control), 0.3 M $CaCl_2$, 0.3 M $LaCl_3$, or 30% H_2O_2. After centrifugation, the powders were washed with Tris/Triton Buffer. Samples (~5000 CPM) of the ^{35}S-PGs were added to the powders in parallel tubes and agitated in an end-over-end mixer overnight at 4°C . The mixtures were then centrifuged and the supernatants counted. This was the nonadsorbed fraction (filled bars). The powders were washed with Tris/Triton buffer to remove loosely adsorbed material (grey bars). Finally, the powders were incubated in a buffer containing 4 M GuHCl, 0.1 M EDTA, and 0.1% Triton X-100 (hatched bars).

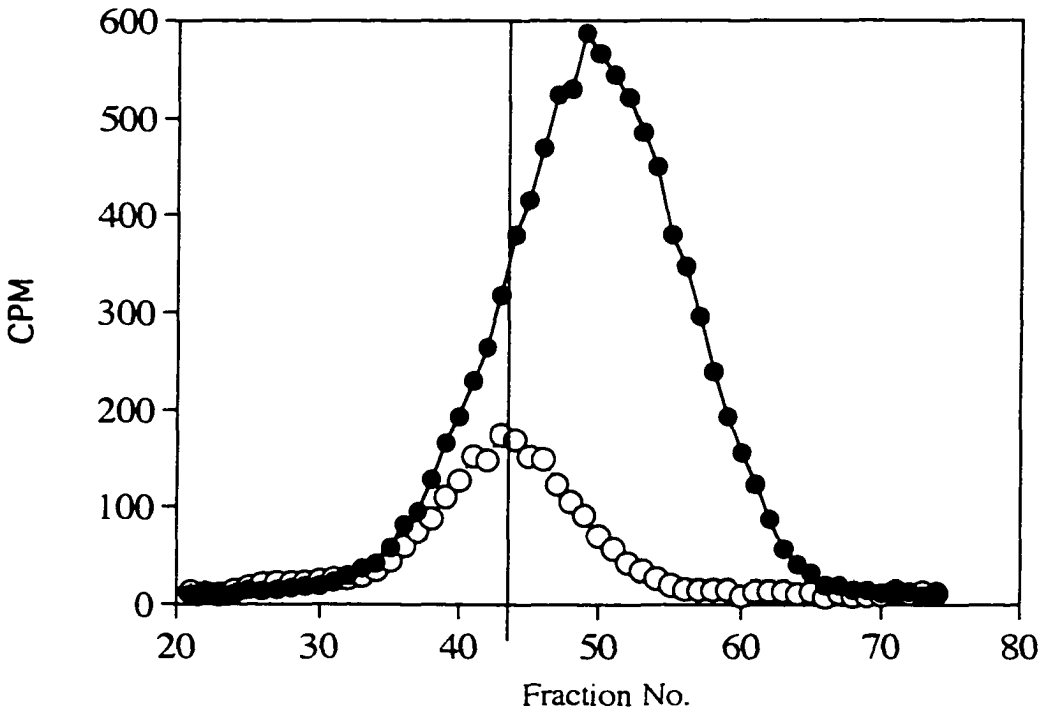

Figure 17. Gel filtration on Sepharose CL-4B of PGs desorbed from calcium- and lanthanum-treated Ti powder. Cell extract PGs desorbed with GuHCl/EDTA from Ti powders were analyzed on a column (1x47 cm) of Sepharose CL-4B in 4 M guanidine HCl/0.5% Triton X-100/50 mM Tris HCl, pH 7.2. Vertical bar indicates peak elution fraction of desorbed PGs from Ca-treated Ti. Filled circles: La-treated Ti powder; open circles: Ca-treated Ti powder.

of calcium chloride (Aldrich) altered its effects on subsequent PG adsorption to the Ti powder (Fig. 18).

Radiolabeled PGs are far more plentiful in cell culture medium than in the extracellular matrix fraction; thus, this material was used to further characterize PG binding to Ti powders. Following pretreatment with $CaCl_2$, $LaCl_3$, or 30% H_2O_2, Ti powders were incubated with ^{35}S-PGs at room temperature. Portions of each non-adsorbed fraction were removed at 10 min, 2 hrs, and 17 hrs for analysis by SDS-PAGE/fluorography (Fig. 19). Little effect is seen with the control and calcium treated powders for up to 2 hrs. However, after incubation overnight, a marked decrease is apparent in one of the major PGs in each sample; this is accompanied by the appearance of lower molecular weight fragments. Much the same effect is seen with the peroxide treated powder, except that a different PG disappears from the gel and is replaced by fragments.

The ^{35}S-PGs were rapidly and nearly quantitatively adsorbed to the La-treated powders; thus, the nonadsorbed PGs were not sufficient to analyze. Much of the adsorbed PGs were released from the La-Ti powders by sequential treatments with GuHCl and with 0.2 M EDTA. The PGs desorbed in this manner are shown in Fig. 20. The high molecular weight CSPG could not be detected in either fraction. The GuHCl-desorbed PGs had similar fragments to those seen with the other powders, but the EDTA-desorbed PGs had not been substantially degraded.

The most obvious explanation for the PG fragmentation is the presence of contaminating proteases in the PG mixture. If true, this would seriously compromise much of the results obtained from the adsorption studies. To test this possibility, portions of the PG mixture were incubated overnight at room temperature in the absence of Ti powder. Some of the sample mixtures were supplemented with $CaCl_2$, $LaCl_3$, or EDTA on the chance that the putative activity might be influenced by cations. A fifth tube contained Pronase E to determine the size of PG fragments resulting from proteolytic degradation.

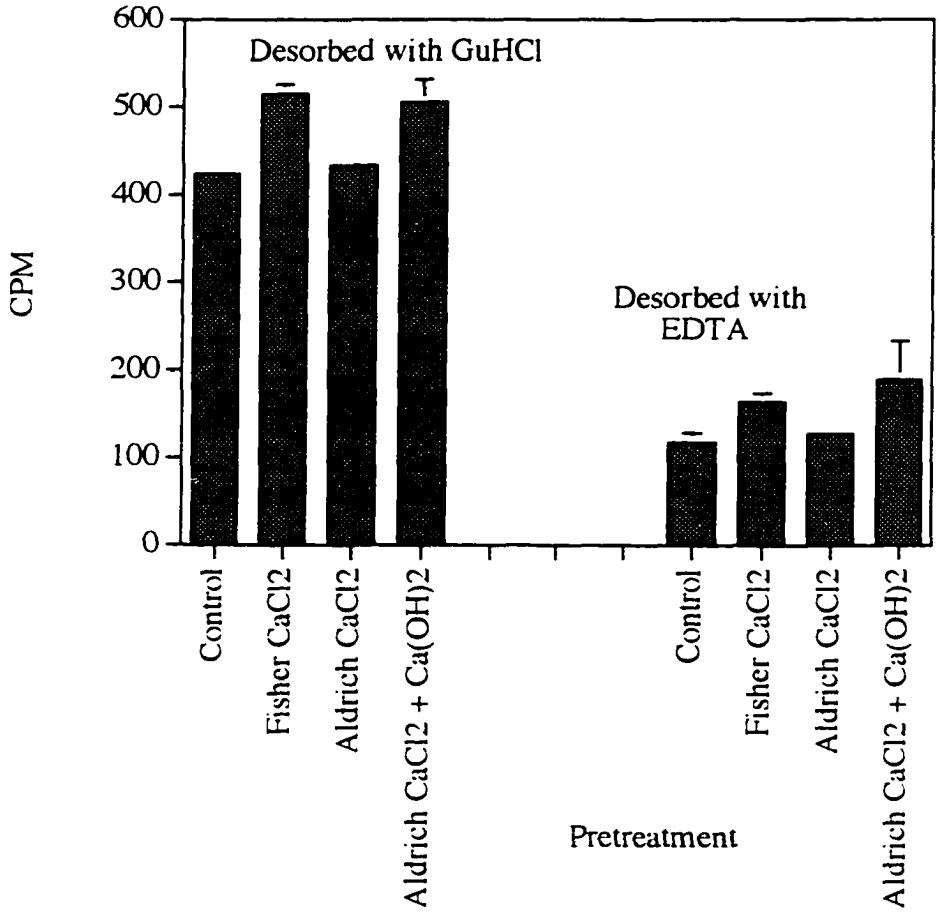

Figure 18. Proteoglycan adsorption to Ti powder: Effects of pretreatment with $CaCl_2$ and $Ca(OH)_2$. Triplicate portions (100 mg) of Ti powder were pretreated with water (control) or 0.3 M solutions of $CaCl_2.2H_2O$ from either Fisher Scientific or Aldrich Fine Chemicals. The latter solution was also supplemented with a saturating amount of $Ca(OH)_2$. After washing with buffer, all were powders were mixed with approx. 80,000 cpm ^{35}S-PGs (from culture medium). The bound PGs were desorbed sequentially with 4 M GuHCl in Tris/Triton buffer and then 0.2 M EDTA in Tris/Triton buffer.

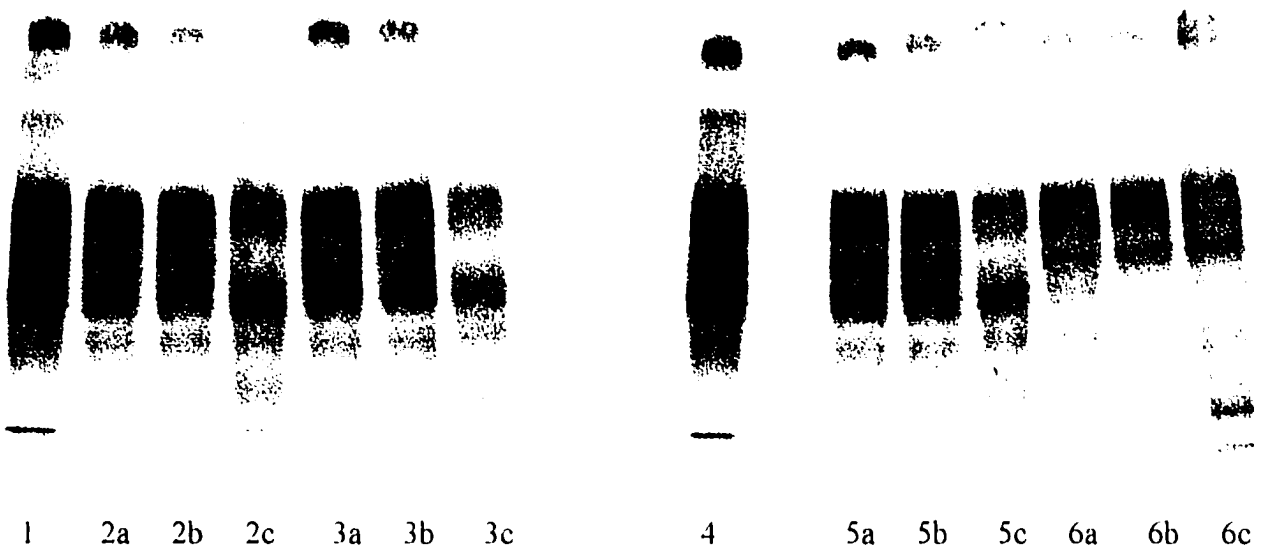

Figure 19. Time course of proteoglycan interactions with Ti powder: nonadsorbed fraction. Culture medium ^{35}S-PGs were incubated with treated Ti powders. Portions of the nonadsorbed fractions were removed at 10 min, 2 hrs, and 17 hrs and analyzed by linear gradient 4-15% SDS-PAGE. Lanes 1 and 4--^{35}S-PG sample. Pretreatments: 2--control; 3--0.3 M $CaCl_2$ (Fisher); 5--0.3 M $CaCl_2$ (Aldrich); 6--30% H_2O_2. Time point: a--10 min; b--2 hrs; c--17 hrs.

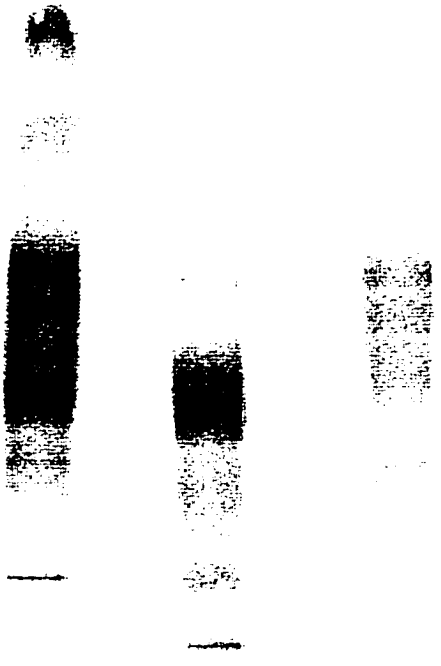

Figure 20. Proteoglycans desorbed from La-treated Ti powder. Culture medium ^{35}S-PGs which remained adsorbed after 17 hrs were treated sequentially with 4 M GuHCl and 0.2 M EDTA. These fractions were analyzed by 4-15% linear gradient SDS-PAGE (see legend to Fig. 19). Lane 1--^{35}S-PG sample; lane 2--PGs desorbed with GuHCl; lane3--PGs desorbed with EDTA.

The results (Fig. 21) show that incubation overnight with calcium or EDTA did not markedly affect the size distribution of PGs in the mixture. Pronase E completely degradedthe ^{35}S-labeled macromolecules to lower molecular weight products. Surprisingly, incubation with La seemed to cause much of the radiolabel to disappear from the mixture. This may have been due to an aggregation effect, with much of the precipitated PGs lost during centrifugation of the sample tubes; this possibility is supported by the appearance of a high-MW band in the stacking gel.

A second approach to test for protease activity in the PG mixtures was the use of casein and gelatin zymograms. Acrylamide gels are cast containing either of the two proteins in the polymerizing mix. Following electrophoresis of the PG sample mixes, the gels are incubated in buffer and stained with Coomassie Blue. This produces a negative image of the proteolytic enzymes in the gel, which appear as white bands in a field of blue (Fig. 22). As positive controls, commercially available protease enzymes (bromelain, papain, Pronase E) were run in parallel. Each of these proteases yielded large white areas in the stained gels. The gelatin zymogram contained a very faint band (~90 kD) in the lane with added PG sample; it is not clear if this activity is sufficient to explain the PG degradion observed with Ti powders.

The final series of experiments with the Ti powders compared the adsorption of PGs from the culture medium with those from the cell layers. Since the two mixtures contain quite distinct populations of PGs, this would provide additional information on the nature of Ti-PG interactions. Triplicate batches of Ti powder preincubated with Ca, La, or peroxide were incubated overnight at room temperature with samples of the ^{35}S-labeled PGs. The powders were then centrifuged and washed with buffer to remove the non- or loosely-adsorbed material. The powders were sequentially treated with 4 M GuHCl and then 0.2 M EDTA; at each step portions of the desorbed PGs were saved for analysis. By accounting for the total recovered radioactivity in each case, it was possible to calculate the

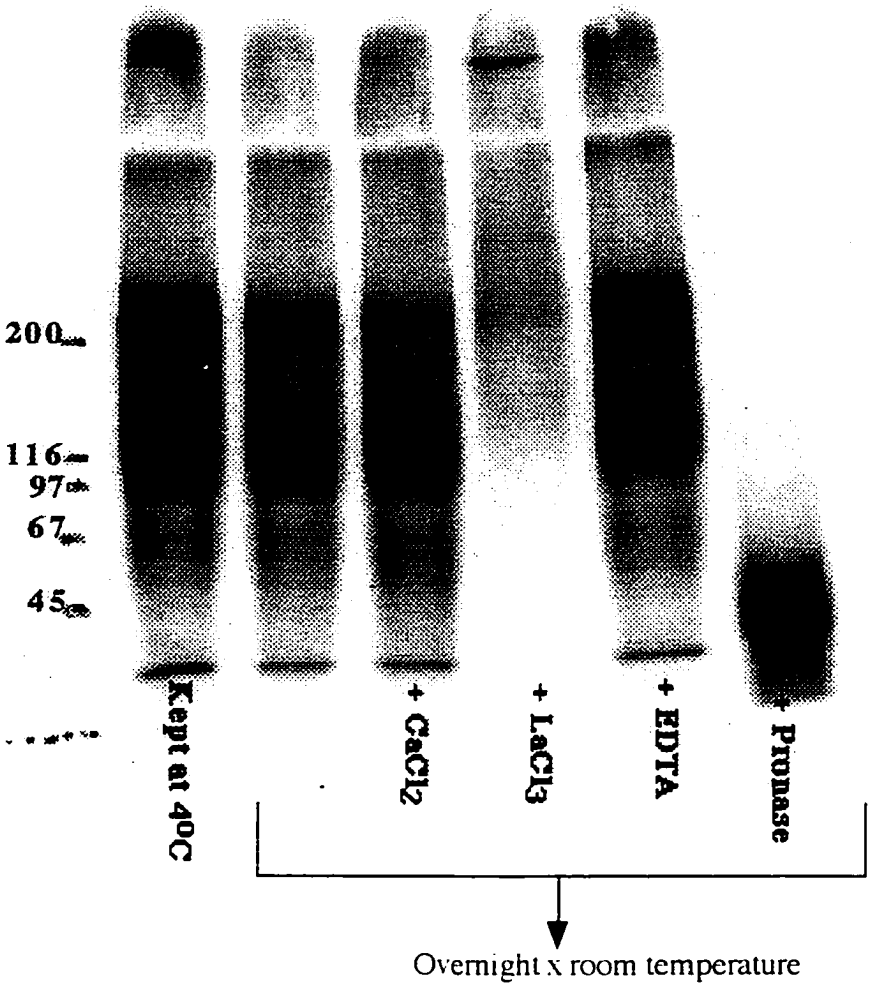

Figure 21. Stability of PG mixture: effect of overnight incubation at room temperature. ^{35}S-PGs were mixed (in the absence of Ti powder) with $CaCl_2$, $LaCl_3$, EDTA, or Pronase E. After overnight incubation at room temperature, the mixtures were analyzed on 4-15% linear gradient SDS-PAGE followed by fluorography.

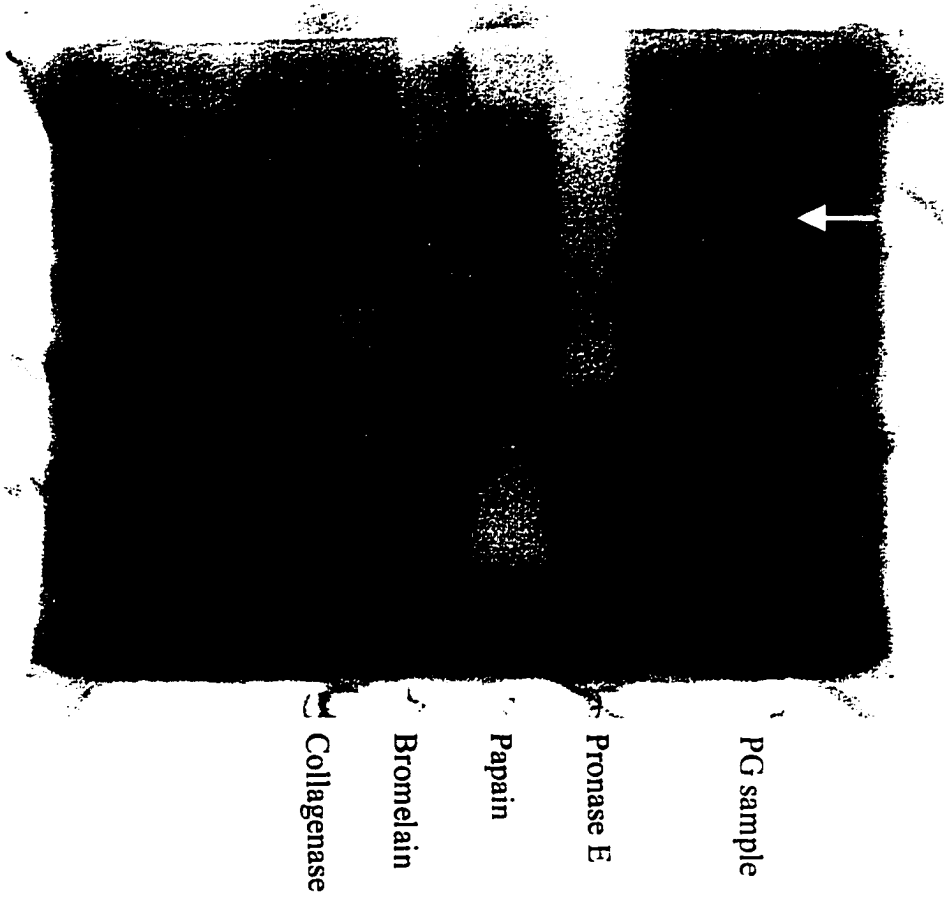

Figure 22. Gelatin zymogram of authentic proteases and of ^{35}S-PG sample. 10% SDS-PAGE. Arrow indicates faintly visible band in PG sample lane.

amount of material that remained on the powders and was thus "irreversibly" bound. The results (Figs. 23 and 24) demonstrate that La pretreatment of the powders markedlyaffected the adsorption of both PG mixtures; in contrast, peroxide pretreatment of the powders altered the binding of the PGs from the culture medium but not from the cell layers.

The various fractions from this study were examined qualitatively by SDS-PAGE (Figs. 25 and 26). As seen earlier, overnight incubation of the PGs with the powders produces some apparent degradation; again, the pattern of breakdown seen with the peroxide-treated Ti is somewhat different from that of the control or Ca-treated powders. PGs desorbed with EDTA from the La-treated powder showed relatively little degradation.

The effects of La and peroxide pretreatments on PG adsorption to Ti powder agree well with the results of the XPS analyses. The adsorption studies were based on the assumption that differences in PG binding were primarily due to modifications of the chemistry of the surface oxide layer. Survey scans of the control (water-treated) Ti powder (Fig. 27, top) confirmed that Ti, O, and C were the predominant elements at the particle surface. The control spectra showed no detectable signals for either Ca (~350 eV) or La (835-850 eV). Survey scans of the Ca-treated powders (Fig. 27, bottom) essentially matched those of the controls. The major elemental components were Ti, O, and C; at this level of resolution, Ca could not be detected.

As with the first two samples, Ti, O, and C were the major elements in the survey scans of the lanthanum- and peroxide-treated Ti powders (Fig. 28). Averaging the signals from repeated Multiplex scans show the presence of La in the lanthanum-treated but not the control powder (Fig. 29). Multiplex scans of the control powders yielded spectra with a doublet peak in the O1s region (530-536 eV), with maxima at approximately 532 and 534 eV (Fig. 30, top). By contrast, the O1s signal for the peroxide-treated powder (Fig. 30, middle) contained only a single major peak at 532 eV and lacked the second peak at 534 eV. These scans clearly indicate that overnight treatment with 30% H_2O_2 alters the surface

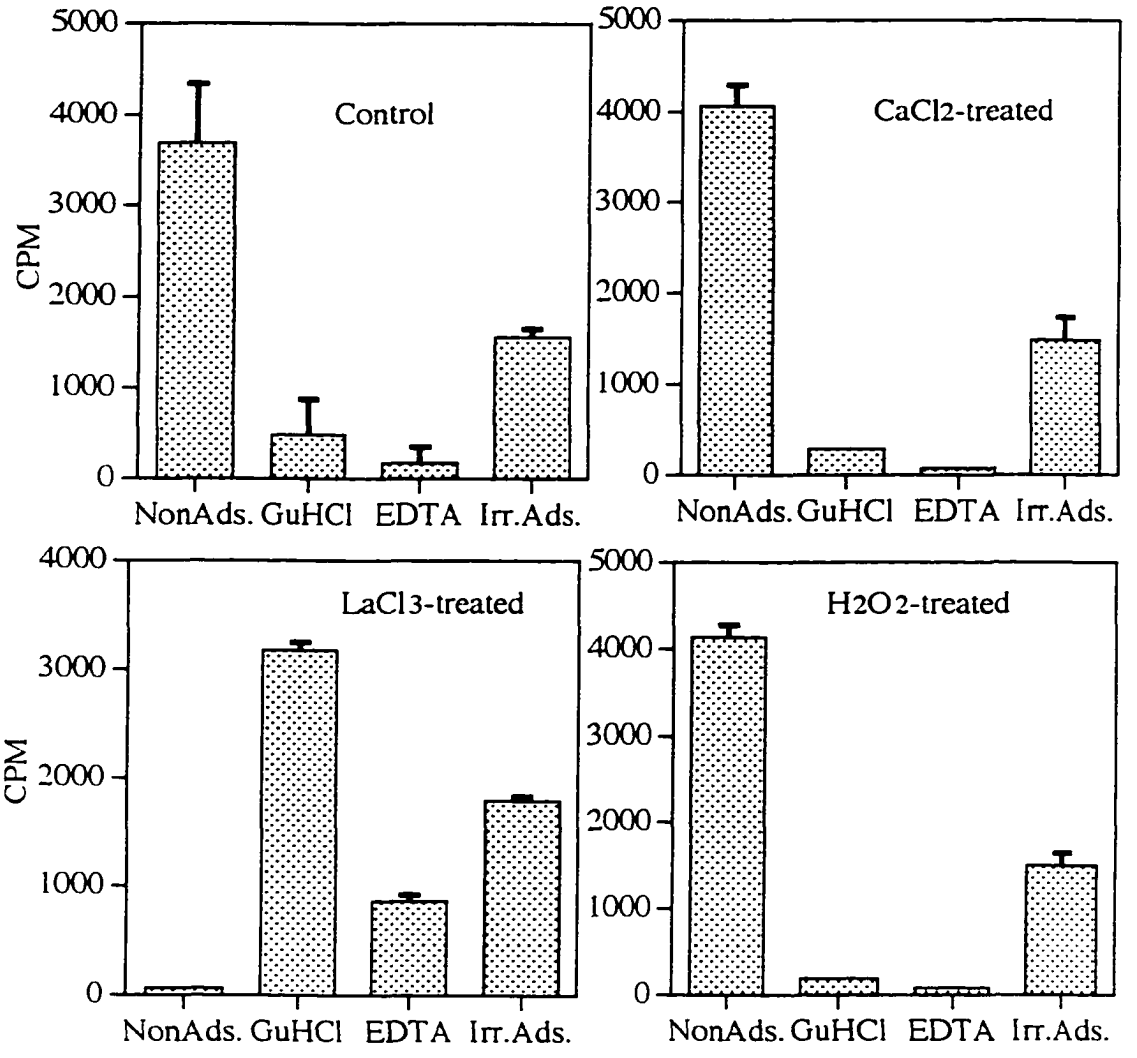

Figure 23. Adsorption of culture medium proteoglycans to Ti powder: sequential elution with GuHCl and EDTA. Triplicate portions (100 mg) of Ti powder were pretreated with water, 0.3 M solutions of $CaCl_2$ or $LaCl_3$, or 30% H_2O_2. After washing, the powders were mixed with ~38,000 cpm of culture medium 35S-PGs for 24 hrs at room temperature. The non-adsorbed PGs ("NonAds)" were removed with buffer washes of the powder. The adsorbed PGs were removed sequentially with 4 M GuHCl and then 0.2 M EDTA. Ten percent of the radioactivity in each fraction was used for countintg. The radioactivity which could not be desorbed by the GuHCl or EDTA treatments was termed irreversibly adsorbed ("Irr. Ads"). Indicated values are means +/- standard deviations.

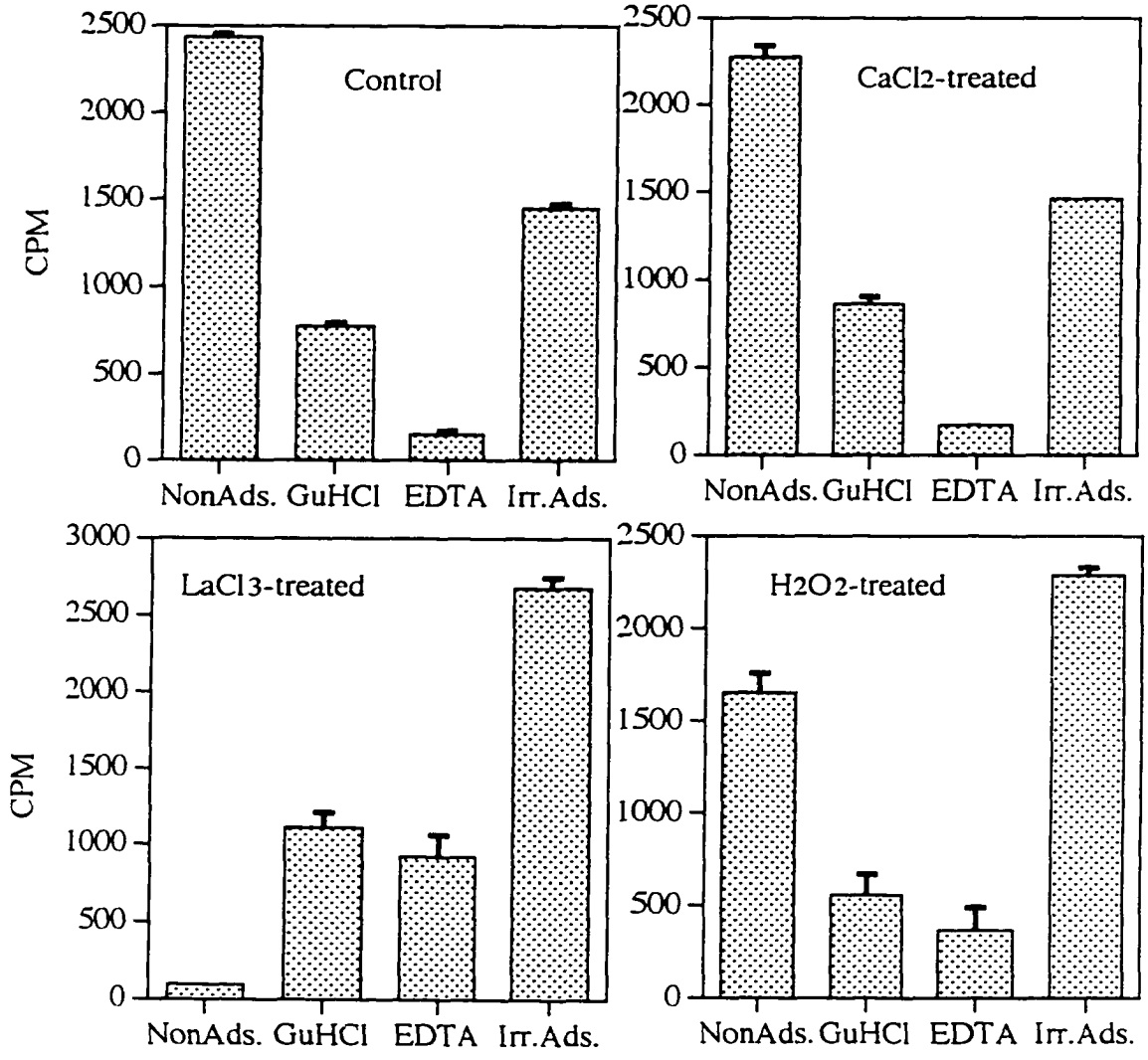

Figure 24. Adsorption of cell layer proteoglycans to Ti powder: sequential elution with GuHCl and EDTA. Triplicate portions (100 mg) of Ti powder were pretreated with water, 0.3 M solutions of $CaCl_2$ or $LaCl_3$, or 30% H_2O_2. After washing, the powders were mixed with ~38,000 cpm of culture medium 35S-PGs for 24 hrs at room temperature. The non-adsorbed PGs ("NonAds)" were removed with buffer washes of the powder. The adsorbed PGs were removed sequentially with 4 M GuHCl and then 0.2 M EDTA. Ten percent of the radioactivity in each fraction was used for countintg. The radioactivity which could not be desorbed by the GuHCl or EDTA treatments was termed irreversibly adsorbed ("Irr. Ads"). Indicated values are means +/- standard deviations.

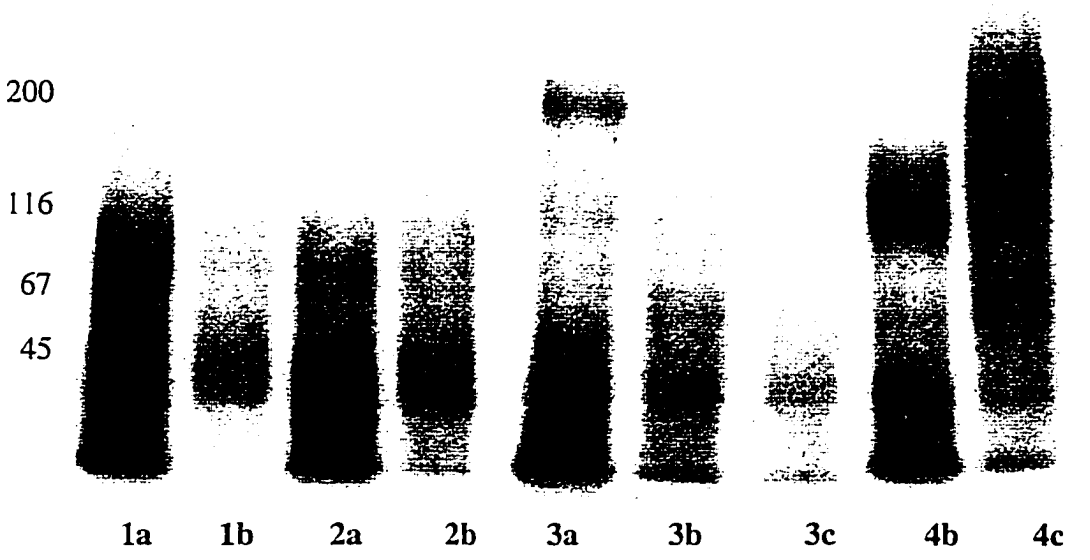

Figure 25. Adsorption of culture medium proteoglycans to pretreated Ti powders. Fluorography of 4-15% linear gradient SDS-PAGE. Pretreatments: 1--water; 2--0.3 M $CaCl_2$; 3--30% H_2O_2; 4--0.3 M $LaCl_3$. Fractions: a--nonadsorbed PGs; b--PGs desorbed with GuHCl; c--PGs desorbed with EDTA.

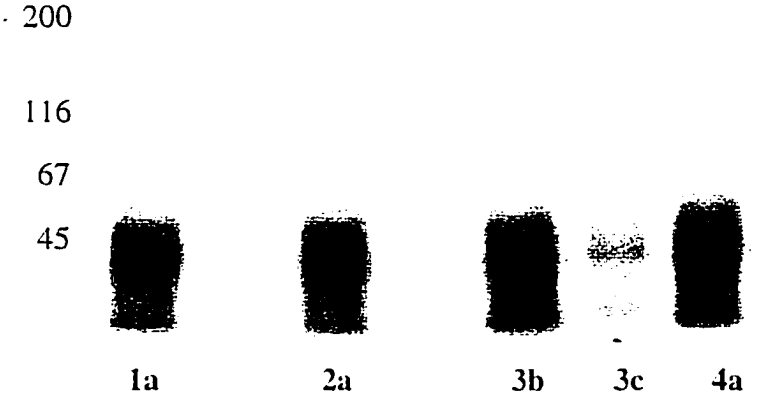

Figure 26. Adsorption of cell layer proteoglycans to pre-treated Ti powders. Fluorography of 4-15% linear gradient SDS-PAGE. Pretreatments: 1--water; 2--0.3 M $CaCl_2$; 3--0.3 M $LaCl_3$ 4--30% H_2O_2;. Fractions: a--nonadsorbed PGs; b--PGs desorbed with GuHCl; c--PGs desorbed with EDTA.

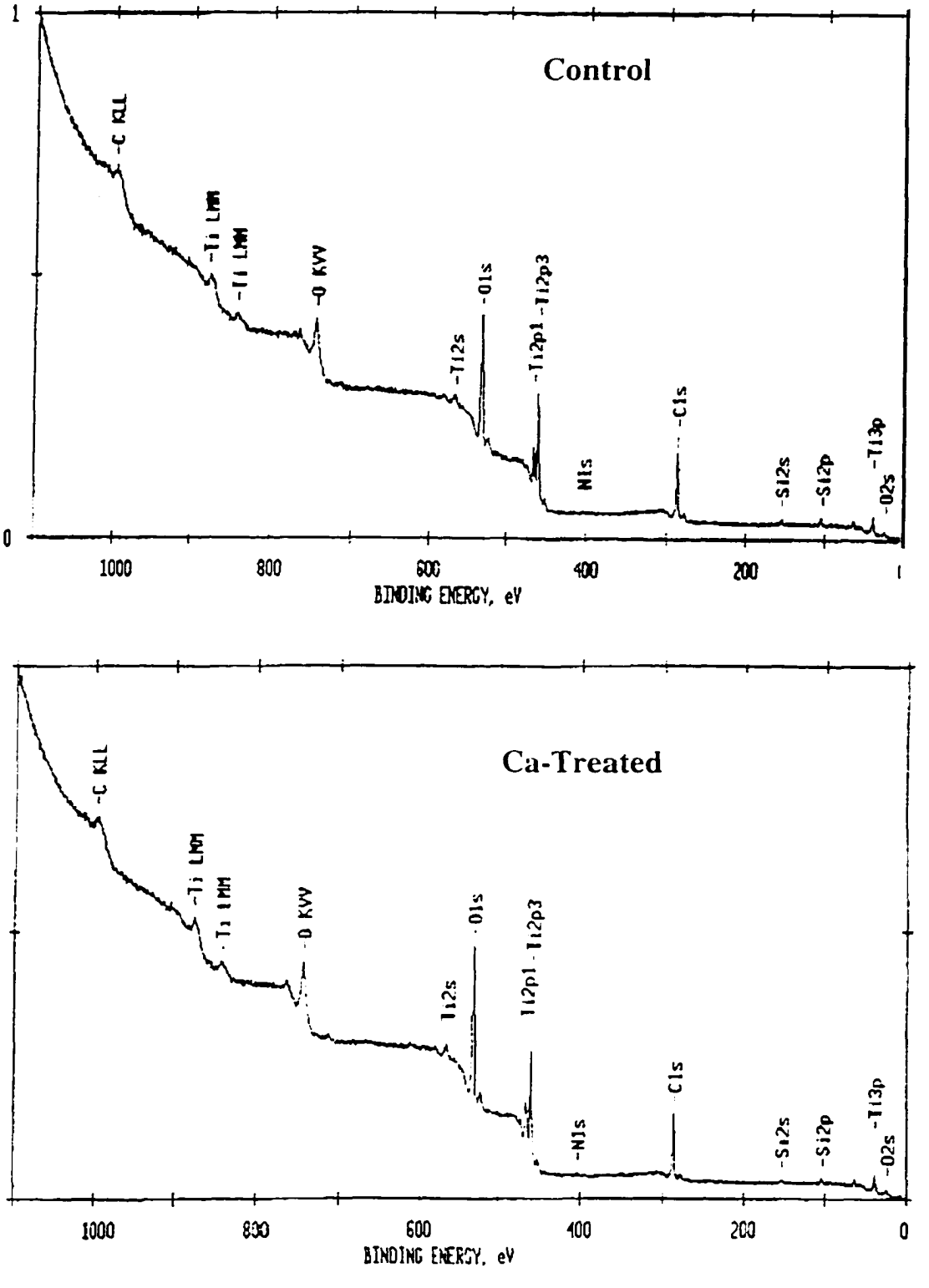

Figure 27. XPS survey scans of treated Ti powders: control and $CaCl_2$ treated.

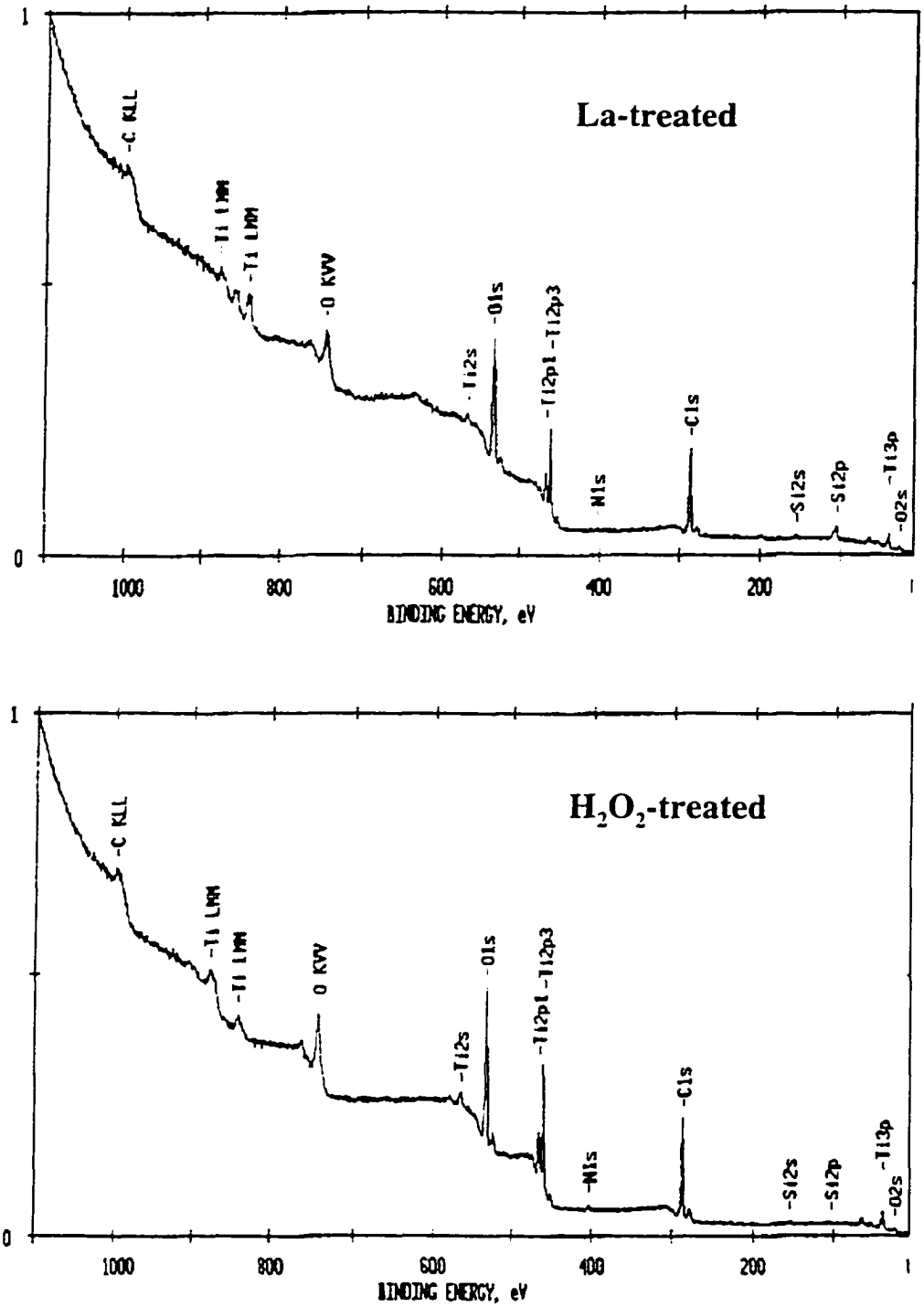

Figure 28. XPS survey scans of treated Ti powders: $LaCl_3$ and H_2O_2 treated.

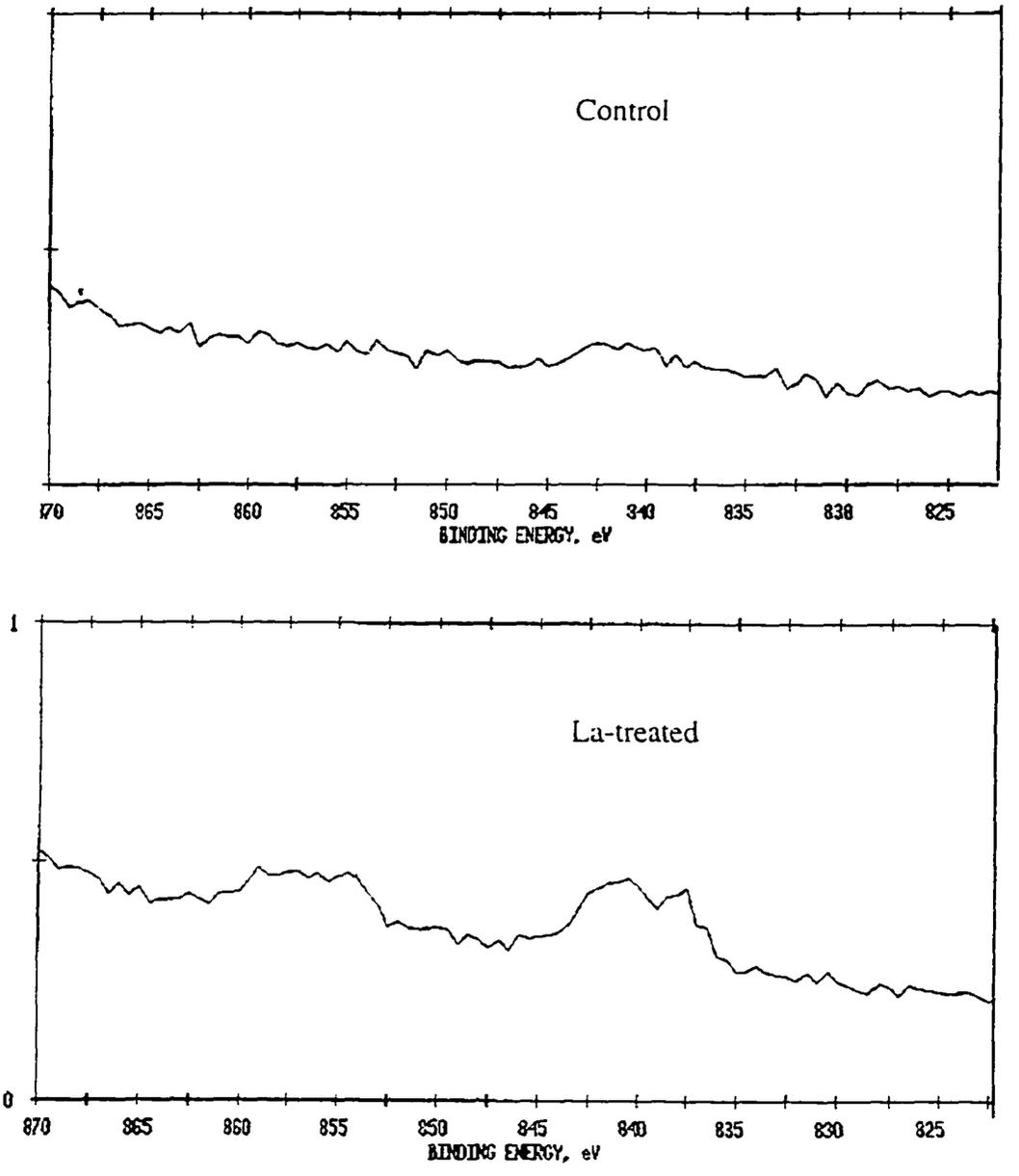

Figure 29. XPS multiplex scans of Ti powders: La region (820-870 ev)

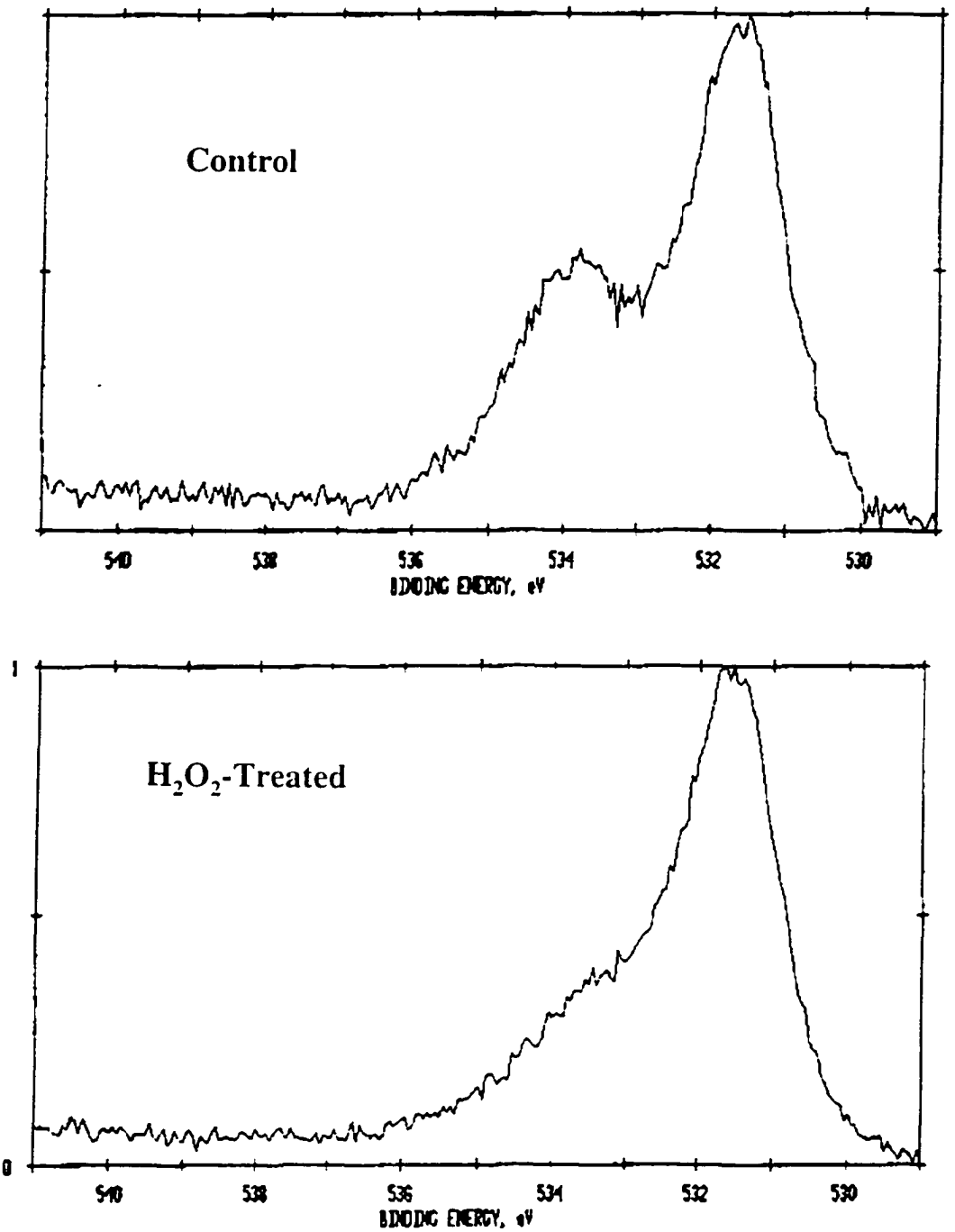

Figure 30. XPS multiplex scans of Ti powders: O1s region.

oxide composition of the Ti particles, but they do not provide information on the precise nature of the changes.

In both cases (La and peroxide) where significant effects were seen on PG adsorption, alterations were also seen in surface chemistry. Where no major changes were found in PG adsorption (*e.g.*, calcium-pretreatment), XPS revealed no detectable change in surface chemistry. Scanning electron microscopy of the variously treated powders shows no major effects on the morphology of the Ca- and La-treated powders but seems to indicate a decrease in particle size following pretreatment with H_2O_2 (Figs. 31 and 32).

Thus, the data appear to confirm Hypothesis 3. Detectable changes in surface chemistry of Ti powder are manifest as changes in PG adsorption.

Hypothesis 4

Precoating titanium surfaces with proteoglycans enhances the attachment of osteoblast-like cells.

When coated onto plastic or Ti surfaces, matrix proteins such as fibronectin and osteopontin significantly enhance the rate of cell attachment. It seemed reasonable that matrix PGs might similarly enhance the rate of osteoblast attachment to metals, an effect which could have important clinical applications. The studies described here made use of three proteoglycans which have recently become commercially available (Sigma). Decorin, biglycan, and aggrecan, all from bovine cartilage, were first analyzed for purity by SDS-PAGE (Fig. 33). Alcian Blue staining demonstrated that the biglycan and aggrecan were relatively homogeneous, but that the decorin preparation contained nearly equal parts of a second PG, apparently biglycan.

These PGs were used to coat 6.3 mm diameter discs of cpTi or Ti-6-4 fit snugly into 96-well plates. Parallel discs were precoated with either BSA, collagen Type I, fibronectin, or osteopontin. The results (Fig. 34) are fairly clear cut for the coatings on

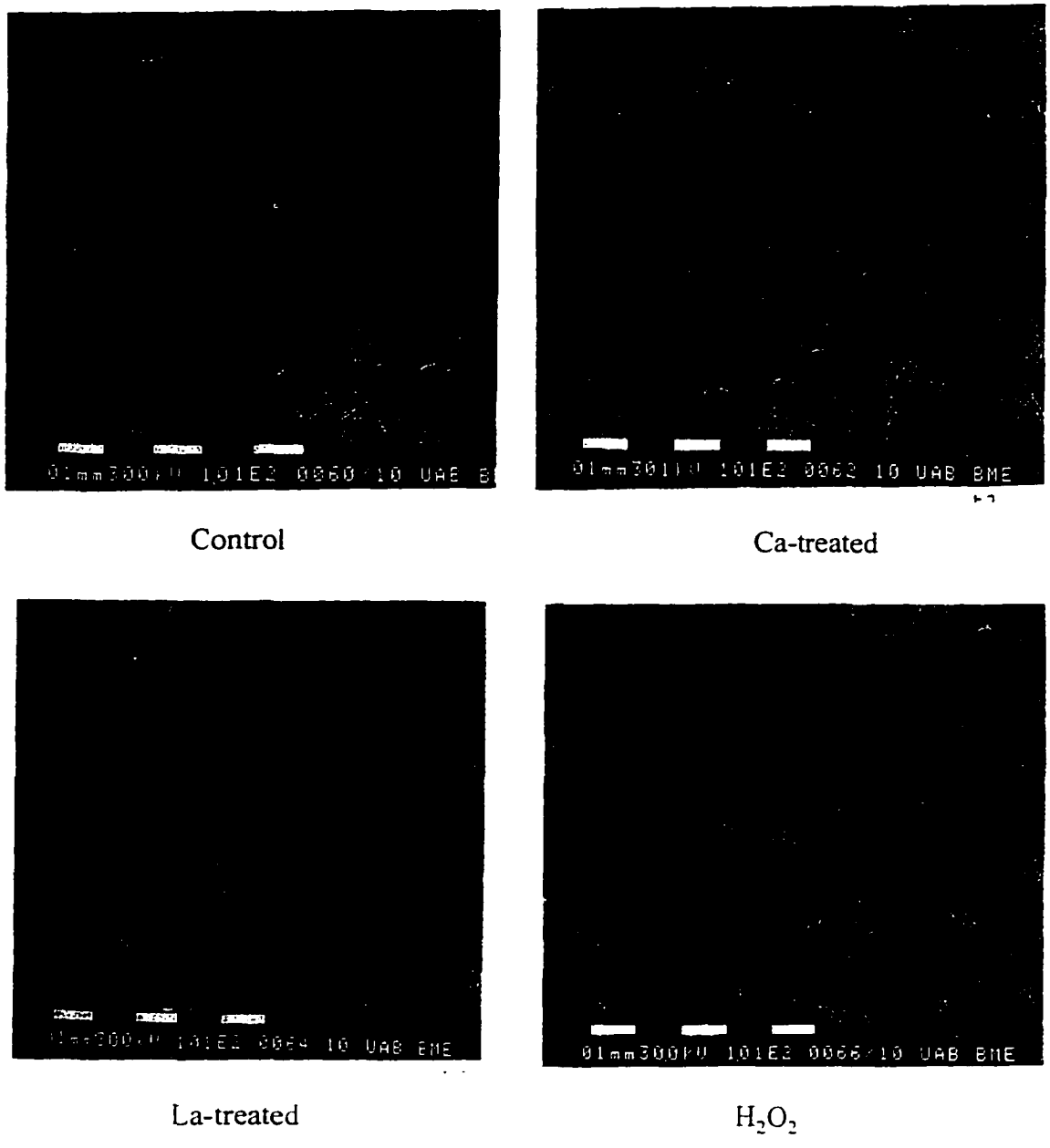

Figure 31. SEM of Ti powders for proteoglycan adsorption studies. 100 X Mag.

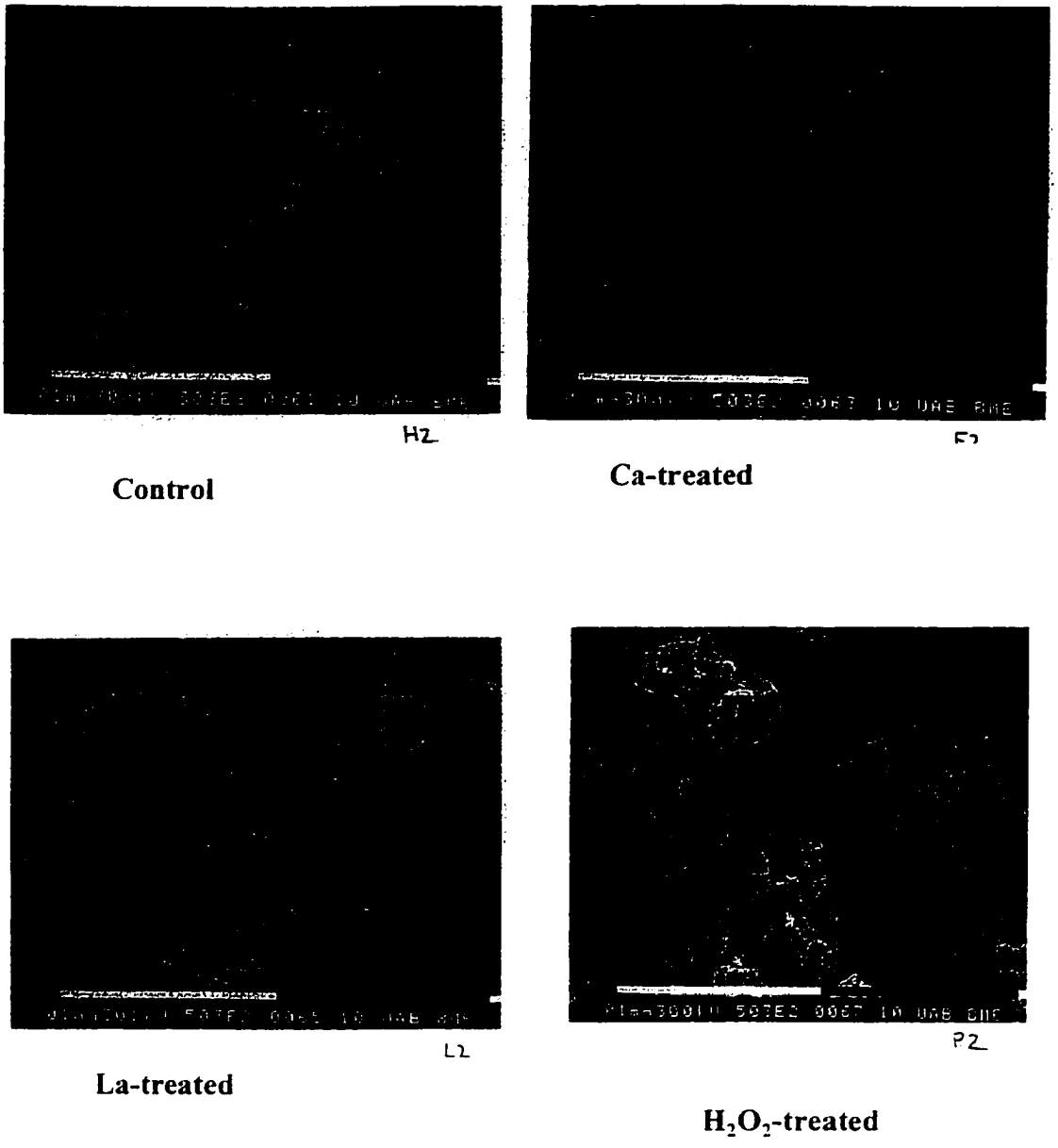

Figure 32. SEM of Ti powders for proteoglycan adsorption studies. 500 X Mag.

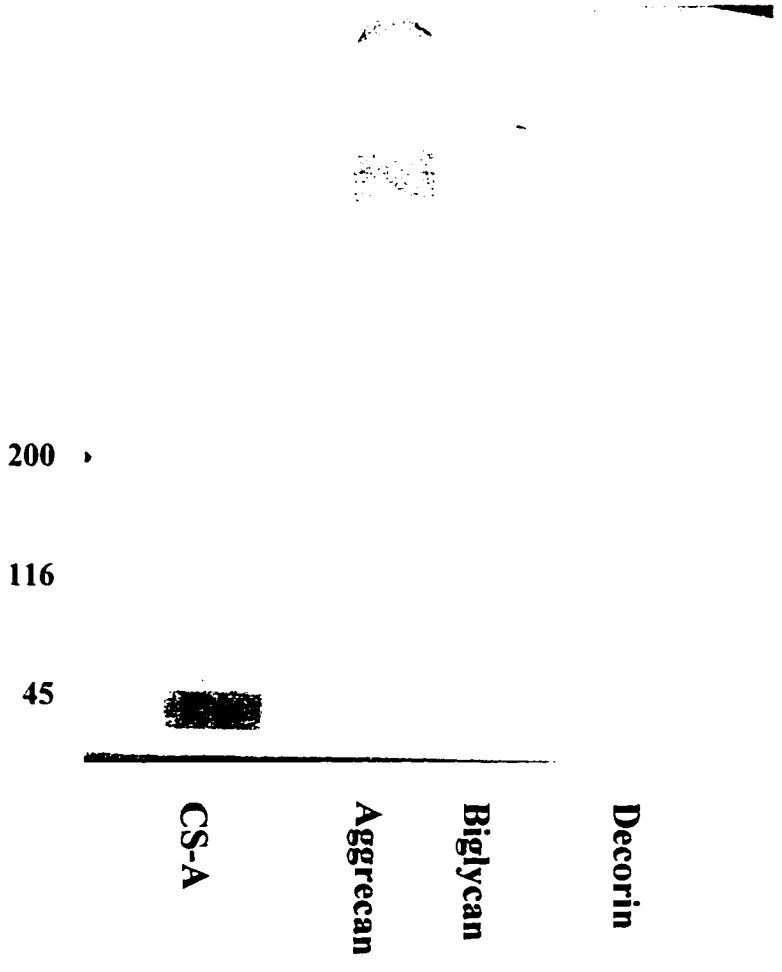

Figure 33. SDS-PAGE of proteoglycans used to precoat metal surfaces for cell attachment study. Stained with Alcian Blue. Samples were applied to 4-15% gradient SDS-PAGE and the gel was then stained with Alcian Blue. CS-A: chondroitin sulfate A.

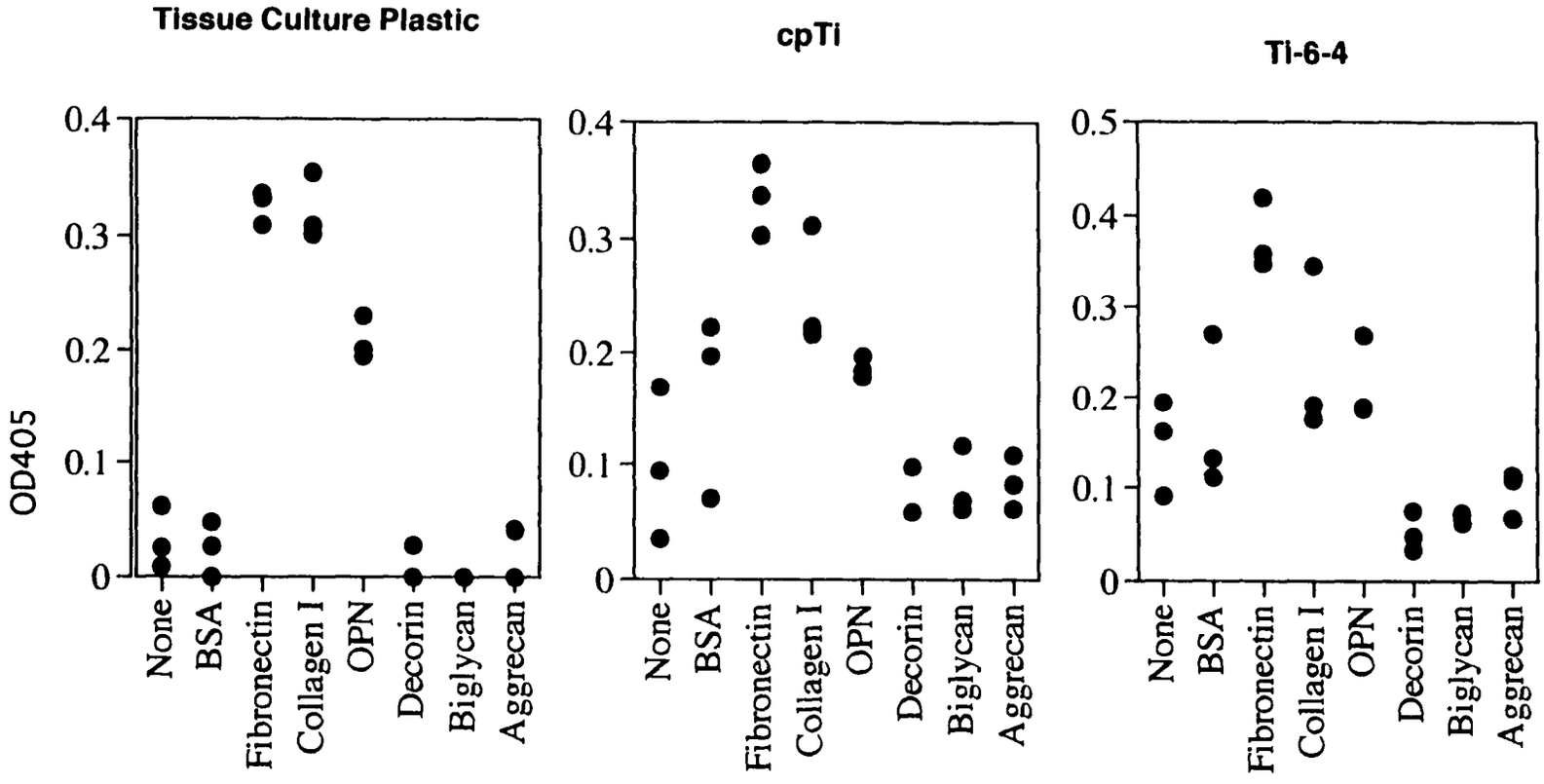

Figure 34. Attachment of MC3T3 cells to protein-coated cpTi and Ti-6-4. Polished discs of either alloy in 96-well plates were precoated with either of several matrix proteins. MC3T3 cells (30,000 cells/well) were plated on the discs and allowed to attach for 1-1.5 hrs. After removing the nonadherent cells by repeated buffer washes, the attached cells were assayed for hexosaminidase activity (OD405). Filled circles show values for triplicate samples.

plastic, in that fibronectin provided the greatest stimulation of attachment, followed closely by collagen I, and then by osteopontin. None of the three PGs appeared to affect attachment.

Variation within the groups of triplicates was far greater on the two metals than on plastic, perhaps due to slight differences between the individual discs in surface roughness. Nevertheless, some stimulation of cell attachment can clearly be attributed to fibronectin, collagen, and OPN. The three proteoglycans had either no effect or slightly decreased cell attachment to the two metals. Therefore, the data appear to reject Hypothesis #4.

DISCUSSION

Insights into the functions of proteoglycans in the formation and maintenance of the implant interface will ultimately yield clinically useful information. It is already commonplace 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. The results of the experiments described here provide information on several aspects concerning proteoglycan-metal interactions.

Proteoglycan Biosynthesis on Implant Metals

Osteoblastic MC3T3 cells produce *similar amounts and types* of proteoglycans whether they are cultured on cpTi, Ti alloy, stainless steel, or CoCrMo. While no major differences were seen among the four metals in effect on PG biosynthesis, these substrates as a group were *clearly distinct* from tissue culture plastic. Cells cultured on the metals not only produce more PGs per cell but also have differences in the sizes of certain PG molecules. This fits well with the observation by this worker and others (D. Rigsby, personal communication) that MC3T3 cells can be released rapidly from plastic surfaces with Pronase E/EDTA yet remain tenaciously bound to titanium surfaces treated identically. This supports the notion that PGs at the cell surface and in the extracellular matrix play a critical role in the attachment of these cells to material surfaces.

The methodologies described here are applicable to examinations of surface roughness as a factor in proteoglycan biosynthesis. Such differences in titanium surfaces

may alter bone cell metabolism (Martin *et al.*, 1995). This approach should also be extended to later stages of bone cell differentiation. Prolonged culture of MC3T3 cells in the presence of ascorbate allows these cells to differentiate into the mineralized state. These cells produce a different assortment of PGs in the mineralizing state as compared to the proliferative state (Takeuchi *et al.*, 1990). Chick embryonic calvarial osteoblasts exhibit enhanced matrix synthesis and mineralization when cultured on rough but not smooth titanium surfaces (Groessner-Schreiber and Tuan, 1992). Bone cells on the metals as compared to plastic also produce larger proportions of a sulfated, non-PG protein which may be related to osteopontin. This role of this molecule in cell attachment is well established (Prince *et al.*, 1987; Butler, 1989); it should be useful to determine how the underlying metal substrates influence its biosynthesis.

Mucopolysaccharide-rich zones have been observed at the interface of neonatal rat calvarial osteoblasts on hydroxylapatite discs (Garvey and Bizios, 1994). The current methods would be useful in characterizing PG synthesis on ceramic materials and their adsorption to calcium phosphate coatings.

Proteoglycan Adsorption to Bulk Metals

These results demonstrate that PG adsorption to cpTi and to Ti alloy is *distinguishable* from PG adsorption to stainless steel or to CoCrMo. Total PGs desorbed from cpTi or Ti alloy were 87% and 96%, respectively, of the levels desorbed from tissue culture plastic. In contrast, PGs desorbed from 316L SS or CoCrMo were 71% and 65%, respectively, of the amounts released from plastic. Moreover, SDS-PAGE analysis of these desorbed PGs shows that at least one identifiable sulfated macromolecule (MW ~120,000) binds more tightly to the Ti metals than to the Cr alloys.

These findings are important in that they point to a potential biochemical marker for evaluating new implant surface treatments *in vitro*. The 120 kD PG must be characterized in greater structural detail to reveal the nature of the GAG chains, as well as that of the core

protein. This PG may be related to the two novel PGs identified by Goldberg and coworkers (1988) in extracts of fetal porcine calvarial bone. These PGs both adsorbed to hydroxylapatite, had average Mr values of 110,000, and appeared to be unique to mineralized bone. Adsorption of the purified 120 kD molecule to bulk implant surfaces may provide clues to the molecular basis for the different clinical behaviors of the various alloys.

Proteoglycan Adsorption to Titanium Powders

Powders are especially attractive for studies of metal-biomolecular interactions because of their high surface area and relative ease of preparation. Others have used Ti powders to investigate the adsorption of serum proteins (Ellingsen, 1991), salivary proteins (Steinberg *et al.*, 1995), and free glycosaminoglycan chains (Collis and Embery, 1992). In all of these studies, certain species of the molecules bound better than others to the powders; a second common thread was that this interaction was significantly improved if the powders were pretreated with solutions of calcium salts. This suggests the possibility that calcium ions form electrostatic bridges between the negatively charged titanium oxide surface and the biomolecules (Parsegian, 1983). This phenomenon should be even more important for proteoglycan adsorption since glycosaminoglycan chains contain very high densities of anionic sulfate and carboxylate groups.

The results reported here suggest a quite different mechanism for PG binding to titanium. Pretreatment of titanium powder with calcium chloride yields at best a modest increase in PG binding, and this effect varies with the particular batch of $CaCl_2$. This is supported by the XPS analysis, which detects no substantial calcium bound to the Ti surface. By contrast, lanthanum ions dramatically increase PG binding to the powders, thus confirming the earlier report (Ellingsen and Pinholt,1995) of their effect on the adsorp-

tion of serum proteins. In this case, XPS analysis clearly shows La on the lanthanum-treated powders.

The differential desorption of the PGs with guanidine and EDTA solutions implies that the mechanism of attachment of these molecules to the oxide surface is not a simple charge interaction. Proteoglycans are desorbed from control or calcium-treated powders far more efficiently with guanidine than with EDTA; this could mean that the interaction is more hydrophobic than electrostatic in nature; the protein portion of the PGs, rather than the GAG chains, would thus be bound to the metallic oxide.

This fits logically with the observation that fragmentation of certain PGs gradually occurs when the molecules are incubated with Ti powders. This effect does not seem to be due to contamination of the sample with proteases, although this cannot yet be totally ruled out. An alternative explanation is that certain PG proteins, in close proximity to a Ti oxide surface, are subject to slow, metal-dependent, oxidative cleavage. This notion is supported by the data showing that fragmentation of PGs by H_2O_2-treated Ti is faster and qualitatively different from the degradation on control or calcium-treated Ti. The XPS analysis of the peroxide-treated Ti clearly shows alterations in the composition of oxides at the surface.

The results of the Ti powder studies may be combined with previous knowledge of osseointegration and PG structure to suggest a model for molecular interactions at implant surfaces. As discussed earlier, osseointegrated sites on titanium implants are characterized by 20-50 nm thick zones which stain intensely with ruthenium red. It must be recognized that there is little definitive information on the composition and orientation of PGs in the amorphous zone observed on titanium surfaces. Nevertheless, we can speculate on these features based on known properties of PGs and collagen proteins. The data suggest a model in which two parallel layers of decorin PGs oppose each other across a gap of 20-50 nm (Fig. 35). One layer is bound through its core proteins to a network of

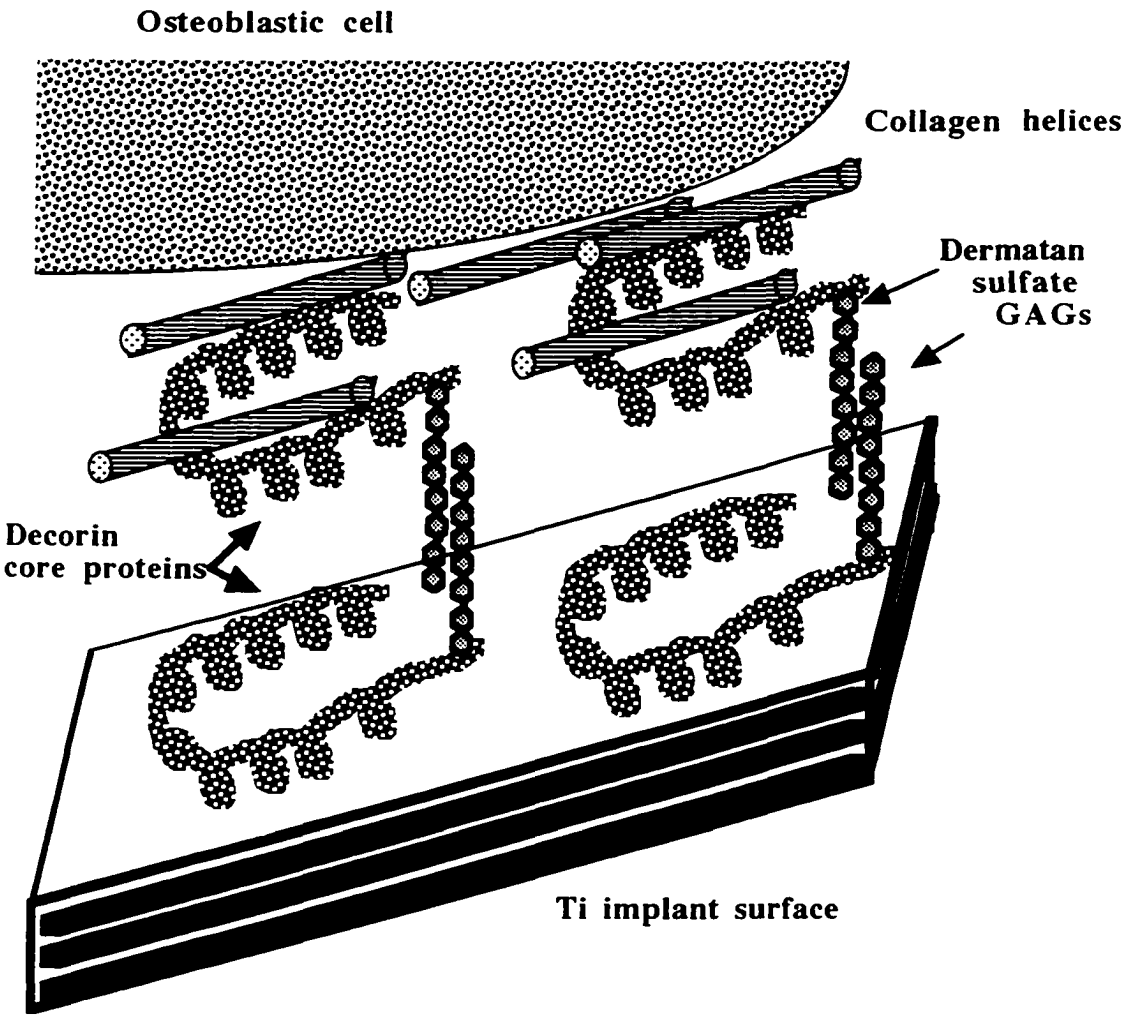

Figure 35. Model for proteoglycan interactions at the implant interface.

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 (Klinger *et al.*, in press).

This model is based on Scott's (1992a,b) vision of PG:collagen interactions in corneal stroma. By staining tissue preparations with Cupromeronic blue and then uranyl acetate, Scott (1992b) obtained high resolution images of individual GAG 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 PGs bind to collagen molecules on the surfaces of adjacent fibrils. Dermatan 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 nonbiological material surface. Decorin belongs to a select family of structurally related molecules known collectively as small leucine rich proteoglycans, or SLRPs (pronounced "slurps") (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 10 and 8 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 surface 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 to RI's nonglobular, 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 reexamined 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 10 tandem LRRs forming multiple attachment sites to the surface oxide. The GAG chain at the amino terminal would outstretch 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 reaggregate into cohesive, spherical masses, a process which is enhanced by a large, species-specific complex of cell adhesion PGs. 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.

Osteoblastic Cell Attachment to Titanium Precoated with Proteoglycans

Proteoglycan coatings *did not enhance* cell attachment to titanium surfaces. These conclusions are strengthened by positive controls showing that cell attachment to Ti is markedly stimulated when the metals are precoated with fibronectin, collagen Type I, or osteopontin. Proteoglycans may adsorb more poorly than the other matrix proteins to the metal surface, and/or less of these molecules may be available for cell binding. Isolation of these PGs from tissue using denaturing salt solutions may alter their biological properties; decorin PG loses much of its fibroblast adhesion properties when it is transiently exposed to high concentrations of guanidine (Winnemöller *et al.*, 1991). Moreover, these molecules may require the presence of other matrix proteins to exert an effect on cell attachment. Decorin binds avidly to collagen I (Pogány *et al.*, 1994); thus, titanium coated with combinations of these proteins may have a synergistic effect on osteoblast adhesion.

SUMMARY AND CONCLUSIONS

A sizable body of histochemical, spectroscopic, and biochemical data points to PGs as a vital factor at the bone-implant interface. Taken as a whole, the evidence for a "proteoglycan-rich" zone at the interface is highly suggestive but must be confirmed by the use of monospecific antibodies and more selective histochemical probes. If these findings are verified, then it is extremely likely that PGs, as major components of the extracellular matrix, play a special role in events leading to osseointegration.

It is essential to understand why certain biomaterials such as pure Ti and Ti alloy perform better in general than others such as stainless steel and CoCrMo. It seems reasonable to expect that the metabolism of bone PGs would differ on these two types of alloys. Such differences could then be applied towards developing new implant surfaces yielding better clinical outcomes. The experiments described here were designed with this goal in mind.

The results of these studies may be distilled into a few brief statements.

Differences in the surface oxides of titanium- and chrome-based alloys do not cause significant differences in the biosynthesis of PGs by proliferating osteoblastic cells on these materials. However, these metals as a group are distinguishable from tissue culture plastic in this regard.

Pretreatment of cpTi and Ti-6-4 with certain PGs (decorin, biglycan, aggrecan, all derived from bovine cartilage) does not enhance osteoblast attachment to the metal surfaces. It is possible that similar PGs, purified without exposure to denaturing solvents, may be more effective. Alternatively, these molecules may enhance attachment if coated in combination with certain other matrix proteins such as collagen or osteopontin.

Pretreatment of titanium powders with lanthanum chloride or hydrogen peroxide significantly alters their capacity to adsorb PGs.

The mechanism of PG binding to titanium oxides is highly complex. It is not a simple electrostatic interaction between the surface and the GAG chains; rather, it also appears to involve hydrophobic interactions between the surface oxide and the polypeptide portion of the molecules.

Titanium- and chrome-based alloys differ qualitatively and quantitatively in the adsorption of PGs. The difference is subtle; however, at least one sulfated protein, Mr approximately 120,000, binds detectably to cpTi and to Ti-6-4 and not to 316L SS or to CoCrMo.

FUTURE DIRECTIONS

Using these results as a starting point, one can pursue a number of research avenues which should yield clinically-useful information.

The structure and adsorption properties of the 120 kD sulfated-proteoglycan must be investigated further. It will be important to determine if the *purified* PG exhibits different affinities for Ti/Ti-6-4 as compared to 316L SS/CoCrMo. If this turns out to be the case, then this protein may serve as an *in vitro* predictor of clinical implant performance.

A limitless number of Ti alloy surface treatments can be developed by using various combinations of anodization and pretreatment with specific cations (including rare earths) and/or hydrogen peroxide. These treatments are especially attractive because they are relatively inexpensive compared to many protein coatings. Several of these surface treatments should be analyzed to determine their relative affinities for osteoblastic proteoglycans and to determine their *in vivo* performance as implant materials in an animal model (*e.g.*, rabbit tibiae). Correlations between these two parameters should suggest more rational approaches to preparing new surface modifications.

These studies must be extended to include PG binding to ceramic coatings such as hydroxyapatite and fluoroapatite. The oxide composition on these surfaces is quite different from that on the metallic alloys and could be expected to yield very different patterns of PG adsorption.

The matter of PG biosynthesis on metals will not be truly resolved until we examine proteoglycan biosynthesis by *mineralizing* MC3T3 cells on various implant materials.

Surface analyses of bulk metal surfaces consistently indicate the presence of carbon, often at significant levels. This element's effect on PG interactions at the surface may be a critical factor in understanding the factors leading to osseointegration. The polypeptide portions of many PGs have considerable hydrophobic properties. Thus, metal surfaces pretreatedwith simple common hydrocarbons, both aliphatic and aromatic, may exhibit altered affinities for proteins in general and particularly for proteoglycans. If true, this could lead to additional, inexpensive surface treatments designed to enhance the strength of the bone-metal interface.

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I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this students are such that _he may be recommended for the degree of Doctor of Philosophy.

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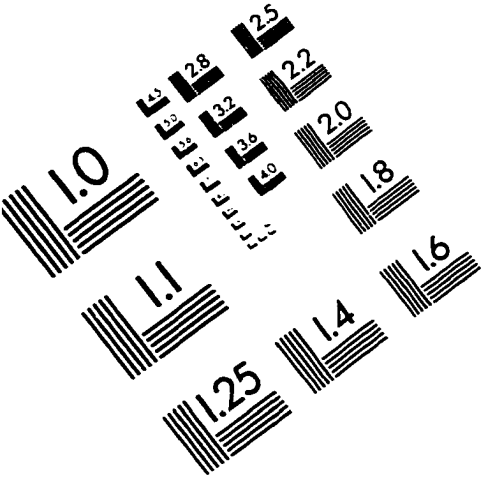

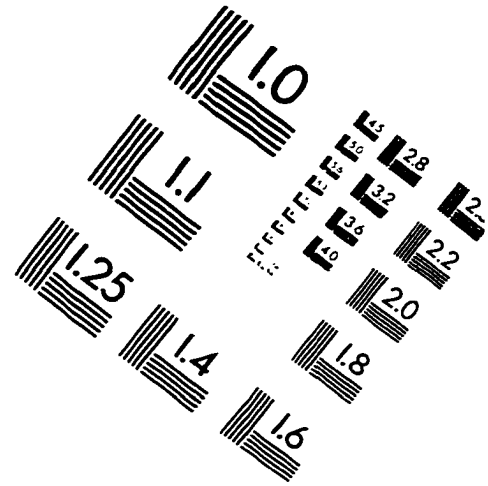

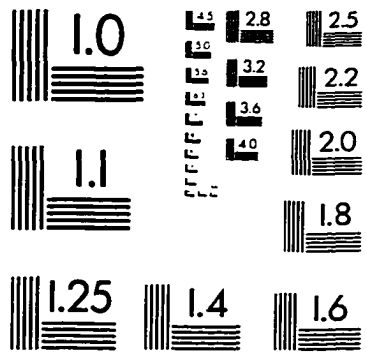

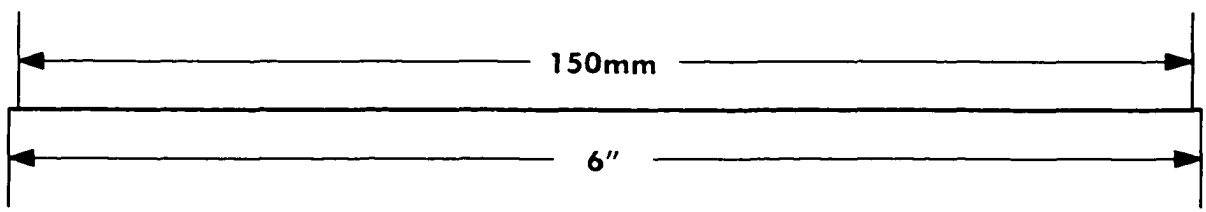

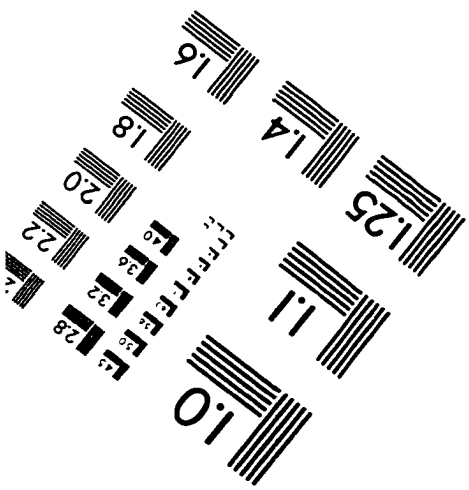

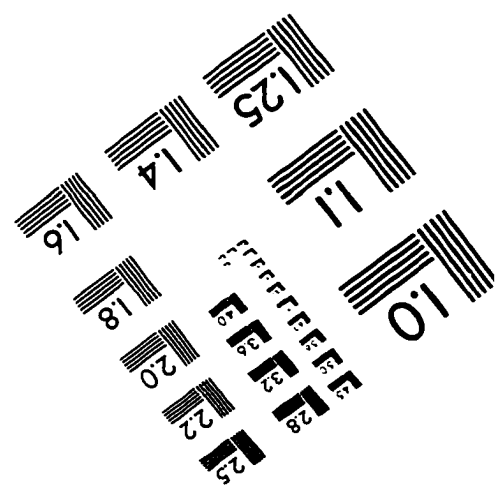

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