June 1998

Integrin-Mediated Binding of Osteoblasts to Titanium Implant Materials

Antje Krause

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INTEGRIN-MEDIATED BINDING OF OSTEOBLASTS TO TITANIUM IMPLANT MATERIALS

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Dr.med.dent., Ludwig-Maximilians Universität München, 1993

A Thesis
Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Dental Science at the University of Connecticut 1998
APPROVAL PAGE

Master of Dental Sciences Thesis

INTEGRIN-MEDIATED BINDING OF OSTEOBLASTS TO TITANIUM IMPLANT MATERIALS

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I want to thank my advisor, Dr. Gloria Gronowicz, and my advisory comitee, Drs. Elizabeth Cowles and Ravindra Nanda. Gloria’s support and advice guided me through my research and Liz was always there to help me and teached me the techniques I needed to know to conduct my data. Dr. Nanda I want to thank for being my advocate throughout my residency. Working with all of them was a priviledge and a pleasure. Thank you to Dr. Mansur Ahmad and Mary-Beth McCarthy for their support and laboratory assistance. Thank you to Dr. Anna Napra, Dr. Anita Gohel and Marcia Nahounou for their help and friendship. I am thankful to Dr. Young Tze Kuah for pointing me in this direction and for his friendship and support. I also want to thank Dr. Zarah Ammari for her friendship and endless support throughout my residency. I am also grateful to Drs. Scott McElroy, Gary Opin, Robert Marzban, Sunil Wadhwa and Greg McKenna for their help and friendship. Finally a special thanks to my husband and my parents for their unconditional support and unending patience.
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Abstract

INTEGRIN-MEDIATED BINDING OF OSTEOBLASTS TO TITANIUM IMPLANT MATERIALS.
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Previous studies have shown that osteoblasts attach to orthopaedic implant materials in an integrin-dependent manner. The purpose of this study was to investigate how the integrin-mediated adhesion of osteoblasts to the orthopaedic implant material Ti6Al4V (TIV) differs from integrin-mediated adhesion to extracellular matrix proteins. Intracellular signaling and gene expression leading to an increase in osteoblast proliferation and differentiation were investigated. Primary osteoblasts from fetal rat calvaria were plated onto TIV, fibronectin (FN, positive control) and poly-L-lysine (PLL, negative control) for various time points (20 minutes to 24 hours). The protein levels of the intracellular signaling molecules focal adhesion kinase (FAK) and mitogen activated protein kinase (MAPK) and the protein and mRNA levels of AP-1 transcription factors, c-fos and c-jun, were compared on the three substrates at various time points by Western and Northern blot analysis, respectively. Cells on all substrates showed continuous FAK phosphorylation for 24 h with a small decrease in activated FAK at 24 h in cells on PLL and TIV but not FN. Therefore FAK was activated by all substrates. However the subsequent signal transduction pathway differed on PLL compared to TIV and FN. MAPK was activated in a similar manner by osteoblast attachment to FN and TIV, although the response to TIV was delayed. Cells on PLL showed no MAPK activation. On FN and TIV c-fos and c-jun message levels were maximal at 30 min and declined at 120 min but remained elevated on PLL. C-fos protein was increased at 60 min on FN and at 30 min on TIV and remained elevated while c-fos protein in cells on PLL was negligible and undetectable at 120 min. C-jun protein levels
were increased at 30 min on all three substrates, and only decreased on TIV at 120 min. C-
jun protein expression on PLL was less than on the other substrates and slightly increased. The actin-based cytoskeleton of the cells was observed at various time points by phalloidin fluorescence microscopy. The cytoskeletal changes were delayed at 1 h and 4 h on TIV compared to FN but by 24 h a well spread cytoskeleton with focal adhesion sites was apparent on TIV and FN. Cells on PLL were rounded and cell spreading was minimal even at 24 h. Cell proliferation was studied by monitoring cell number on days 1, 2, 4 and 6. Cell number on FN and TIV increased significantly by 2 days and cells continued to proliferate through day 6 although initial attachment on TIV was about 50% less than on FN. Cells on PLL attached but did not proliferate. We conclude that osteoblast adhesion to TIV implants is similar to osteoblast adhesion to FN and leads to osteoblast proliferation. These data provide evidence for the biocompatibility of TIV at an intracellular level.
Review of the Literature

Introduction

The development and maintenance of the bone-implant interface is characterized by direct apposition of bone matrix and osteoblasts to the implant surface without any intervening soft or fibrous tissue. This process is called osseointegration and is mandatory for secure association between the bone and the implant surface. Osseointegration was initially defined by Branemark as “a direct structural and functional connection between ordered, living bone and the surface of a load carrying implant” at the light microscope level (Branemark, 1985). Other authors described osseointegration as direct contact of the bony tissue with the implant material and the maintenance of this contact during physiological function of the implant (Plenk and Zitter, 1993). To achieve osseointegration of new implants, it is necessary to understand osteoblast responses to the implant material. Many histological studies have examined cell morphology, adhesion, proliferation and differentiation at the bone-implant interface. However little is known about the intracellular mechanisms of cellular responses to implant materials. Since cell signaling determines osteoblast proliferation and/or differentiation, it is important to understand the cell signaling pathways induced by the contact of osteoblasts with implant surfaces.

Since integrins have been shown to be involved in the attachment of osteoblasts to titanium implant surfaces (Gronowicz and McCarthy, 1996), we wanted to investigate the intracellular signaling events that are known to follow integrin activation and ultimately lead to gene expression. The signaling pathways activated through integrins are similar to those stimulated by growth factors, and induce genes affecting cell proliferation and differentiation. The time course of the expression of different proteins in this signaling pathway was compared between osteoblasts plated on Tivanium (Ti-6Al-4V, TIV), a commonly used implant material, and fibronectin (FN, positive control) and poly-L-lysine
AP-1 (activator protein-1), a transcription factor complex that modulates the expression of genes involved in proliferation and differentiation, is known to be activated by this signaling cascade. The time course of mRNA and protein levels of c-fos and c-jun, components of the AP-1 complex, was also measured and compared between the substrates. To determine whether the activation of this pathway actually alters osteoblast proliferation, cell number and morphology were investigated following attachment of osteoblasts to the implant surface.

**Titanium implants**

Metallic implants are widely used in medicine and dentistry. To achieve long-term stability they are required to be chemically stable, nontoxic and noncarcinogenic, to promote cell adhesion and to facilitate mineralization (Meenaghan et al., 1991). They must also demonstrate sufficient mechanical properties to withstand forces generated in their use for fracture fixation, joint replacements and dental implants by patients. Titanium and its alloys possess these qualities and are, therefore, considered to be superior to other implant materials. Since titanium's introduction as an implant material by Branemark in the 1960s (Branemark et al., 1985), extensive research has improved the clinical outcome. Today the success rate of titanium implants is more than 90%. Improved implant design, fabrication and quality control, advanced clinical procedures, and cleaning and sterilization procedures have contributed to their wide clinical use (Meenaghan et al., 1991). The most commonly used titanium alloy is Titanium-6Aluminium-4Vanadium (Ti-6Al-4V, Tivanium, TIV). Aluminum increases the strength and decreases the density of the material, and vanadium increases the corrosion resistance (Meffert et al., 1992). While commercially pure titanium (cpTi) is used for dental implants, TIV has been extensively used in orthopedics for hip and knee joint replacements.
Titanium oxide/tissue interface:

To determine the biocompatibility of an implant material, it is important to understand the events at the interface between the biomaterial and the biological environment. At this interface the molecular components of the biological environment interact with those of the implant material within a very narrow interface zone of less than 1 nm (Hanawa, 1991). Thus, surface properties on the atomic scale play important roles in interfacial phenomena (Weinländer, 1993).

An adherent, self-repairing surface oxide layer is associated with titanium and its alloys. Titanium implants have a very thin, amorphous, homogeneous and non-porous oxide layer mainly composed of TiO$_2$. The major differences between cpTi and TIV are that the oxide film on TIV is enriched with the alloying elements Al and V, and the TIV surface texture and microstructure are more complex and heterogeneous than that of cpTi (Kasemo and Lausmaa, 1991).

The oxide layer on a cut titanium surface forms within a millisecond of exposure to air, initially being 1-10 nm thick (Donley and Gillette, 1991). Under normal atmospheric conditions not only oxygen binds to the titanium surface but also nitrogen and hydrogen as well as other elements or ions in the air (Kasemo and Lausmaa, 1988). Preparative procedures may therefore result in contaminant films on the implant surface. It has been suggested that the most common source of both organic and inorganic deposits on metallic implants comes from the various sterilization procedures (Meenaghan et al., 1991). Cellular response to the implant material can be affected by absorbed surface molecules and ions because they affect the surface composition and charge of the implant material (Smith, 1991). For that reason the biocompatibility of the oxide layer depends upon the environment in which it was created. Sterilization and cleaning procedures that do not leave
residues on the implant surface include radiofrequency glow discharge treatment and proprietary UV light treatment (Meenaghan et al., 1991).

The thickness of this oxide layer increases with time. McQueen et al. (1982) reported a thickness of 200 nm, 8 years after implantation. Due to the chemical interactions of the titanium surface with water, hydroxyl, calcium and phosphate ions during and after implantation in bone tissue, the depth of the oxide layer increases faster than after exposure to air (Toth et al., 1985; Kasemo and Lausmaa, 1983). This oxide layer of a titanium implant is very stable and does not break down under physiological conditions (Parr et al., 1985). Therefore it is this stable, chemically inert, and corrosion-resistant oxide layer that interacts with the tissue rather than the metal itself.

In several in vitro and in vivo studies, calcium phosphate has been found to form in the titanium oxide film. Calcium phosphate aggregates similar to apatite were shown to form on titanium in neutral electrolyte solutions (Hanawa, 1991). In vivo this layer grows within an absorbed layer of proteins (Sundgren et al., 1986; McQueen et al., 1982; Meenaghan et al., 1991). Immediately after insertion of the implant into the bone, different organic macromolecules, such as glycoproteins and glycosaminoglycans, adhere to the implant surface (Kasemo, 1983; Meyer et al., 1991). Albrektsson and Hansson (1986) examined the titanium/bone interface in rabbit tibial bone after 3 months and found a 200-400 Å wide proteoglycan layer which separated the implant surface from collagen filaments and cells. Bone cells are able to attach to that layer of extracellular matrix (ECM) proteins through integrins, specific cell surface receptors to extracellular matrix proteins. The interface between epithelial cells and the titanium implant surface has been shown to be composed of glycoprotein-matrix similar to that seen between epithelium and natural tooth surfaces (Donley and Gillette, 1991). Previous work in this laboratory has shown that osteoblasts are also able to attach directly to the titanium surface through integrins (Gronowicz and
McCarthy, 1996). The mechanisms by which integrins interact with the nonbiological implant material are not known.

**Composition of bone matrix proteins**

Bone cells produce, mineralize and maintain the ECM, 90% of which is composed of type I collagen and the remaining 10% noncollagenous proteins. Bone matrix is capable of mineralizing calcium. The inorganic matter of bone consists of submicroscopic deposits of a form of calcium phosphate, very similar, but not identical, the mineral hydroxyapatite (\(\text{Ca}_{10} [\text{PO}_4]_6 [\text{OH}]_2\)) (Bloom and Fawcett, 1975). The calcium phosphate interacts with proteins to form a unique ECM.

Type I collagen is composed of three polypeptide chains, two \(\alpha 1\) chains and one \(\alpha 2\) chain, which form a triple helix structure. The collagen molecules assemble into microfibrils, which then assemble into collagen fibrils. Aggregates of collagen fibrils form a collagen fiber. Type I collagen affects the developmental expression of the osteoblast phenotype and is fundamental to the formation of the mineralized matrix (Owen et al., 1990). The accumulation of type I collagen is necessary for osteoblast differentiation and collagen synthesis is functionally coupled with the down-regulation of proliferation (Owen et al., 1990; Lynch et al., 1995). Associated with the osteoblast plasma membrane is alkaline phosphatase, which plays a vital but yet undefined role in bone mineralization and is an important differentiation marker of osteoblasts. Alkaline phosphatase is a 160 kDa glycoprotein composed of two identical subunits and is covalently bound to the plasma membrane.

The noncollagenous proteins produced by bone cells can be divided into four groups: proteoglycans, growth-related proteins, cell attachment proteins and \(\gamma\)-carboxylated (gla)
proteins (Termine and Robey, 1996). Proteoglycan, predominantly chondroitin sulfate and heparan sulfate, contain acidic polysaccharide side chains attached to a central core protein. Heparan sulfate is membrane associated and probably facilitates interaction of the osteoblast with ECM molecules and heparin binding growth factors (Termine and Robey, 1996). Osteonectin, the most abundant bone matrix glycoprotein, accounts for approximately 2% of the total protein of developing bone (Termine and Robey, 1996). Osteonectin is a phosphorylated glycoprotein which is produced by endothelial cells in developing capillaries (Sage, 1986; Bianco et al., 1989) and osteoblasts (Gehron Robey et al., 1992). Osteonectin contains two high affinity calcium ion binding sites. It also has a high affinity of binding physiologic hydroxyapatite and binds collagen and thrombospondin (Termine et al., 1981). Osteonectin has been associated with osteoblast growth and proliferation, as well as with matrix mineralization (Termine and Robey, 1996). Bone cells synthesize at least six proteins that affect cell attachment via integrin cell surface receptors: Type I collagen, fibronectin, thrombospondin, vitronectin, osteopontin and bone sialoprotein (Termine and Robey, 1996). Bone sialoprotein and osteopontin are acidic glycoproteins of similar size and are phosphorylated and contain an RGD sequence. Bone sialoprotein and osteopontin as well as thrombospondin are strong chelators of ionic calcium and are found in the mineralized bone matrix (Termine and Robey, 1996). The γ-carboxylated proteins are osteocalcin and matrix gla protein. Osteocalcin is produced by mature osteoblasts during mineralization and is found in the mature mineralized matrix which may facilitate the differentiation of osteoclasts (Glowacki et al., 1991; Ligget et al., 1994).

**Fibronectin**

Fibronectin (FN), a high molecular weight glycoprotein, is an abundant ECM component of many tissues and is important in cell attachment, spreading, migration,
development and wound healing. Addition of FN to transformed cells temporarily restored stress fibers while disruption of the actin cytoskeleton resulted in a loss of fibronectin from the cell surface (Burridge et al., 1988). In the mouse limb bud, FN is found in the mesenchyme prior to chondrogenesis during the active cell proliferation. The maturing chondrogenic regions gradually lose FN concurrent with collagen appearance (Silver et al., 1981). FN has been shown to be present in early bone formation (Weiss and Reddi, 1980), in adult rat bone (Nordahl et al., 1995) and in the periosteum as well as in the mature and immature human bone matrix in vitro (Grzesik and Robey, 1994). Primary osteoblast cultures expressed FN and c-fos messages during the phase of proliferation prior to differentiation markers such as type I collagen and alkaline phosphatase and osteocalcin (Owen et al., 1990; Lynch et al., 1995). Previous work in this laboratory has shown that FN is expressed during rapid cell proliferation and early intramembranous bone formation, prior to rapid bone growth and calcification. Thus FN appears to be involved in osteoblast precursor proliferation (Cowles et al., 1998).

The structure of FN is a dimer composed of two 250 kDa subunits which are disulfide bonded. FN has several cell and matrix binding domains (summarized in Moursi et al., 1996). The central cell binding domain of FN contains the RGD-sequence (arginine-glycine-aspartate) which is recognized by integrin receptors. Several studies have suggested that integrins participate in the assembly of soluble dimeric FN into insoluble fibrils. This is probably important in formation of the ECM in vivo (Schwartz et al., 1995). Heparin/fibrin and gelatin-binding regions can be found at the N-terminal end of the FN molecule. The FN C-terminus has heparin and fibrin binding sites (Hynes, 1990).
**Cell adhesion**

In tissue culture cells develop two types of adhesions: close contacts and focal adhesions or focal contacts. They are distinguished by the distance separating the cell surface from the underlying substratum. In close contacts the separation between plasma membrane and substratum ranges from 30 to 100 nm. Close contacts can extend over broad regions of the underside of cells and their protein composition has not been defined. In focal contacts (focal adhesion sites) the adhesions have a separation of 10-15 nm and they are composed of a well characterized set of proteins that form discrete regions of the ventral surface of the cell (Schneider and Burridge, 1994). Focal adhesion sites are regions of contact between the ECM and the inside of the cell. In focal adhesion sites ECM components are connected via integrins to the cytoskeleton which activate intracellular signaling molecules that influence cell proliferation and differentiation. ECM components that are involved in focal adhesion sites include: fibronectin, vitronectin, and heparan sulfate proteoglycan (Burridge et al., 1988). Cytoskeletal elements in focal adhesion sites include paxillin, talin, tensin, vinculin, α-actinin, and actin, which connect the integrins to the cytoskeleton (Horwitz, 1997; Clark and Brugge, 1995; Burridge et al., 1988). Signaling molecules found in focal adhesions are focal adhesion kinase (FAK or pp125FAK) and associated proteins.

**Integrins**

**Integrin structure**

Integrins are a large family of heterodimeric transmembrane glycoproteins, which are the principle receptors for ECM proteins, including collagen, fibronectin, and laminin.
Integrins are composed of two noncovalently associated trans-membrane glycoprotein subunits called α and β, both of which contribute to the binding to the ECM (Alberts et al., 1994). There are 16 known α-subunits and 8 β-subunits (Hynes, 1987; Clark and Brugge, 1995). The α-subunit determines the specificity of binding to a particular ligand. Various combinations of the α and β subunits produce receptors with different ligand specificities (see Table 1). Some of these integrin receptors are very specific and bind to only one ECM molecule like the fibronectin receptor α5β1 and the laminin receptor α6β1, others are able to bind different ECM molecules, like the α3β1, α4β1 and αvβ3 receptors derived from fetal rat calvaria which are able to bind fibronectin as well as other ECM components (Moursi et al., 1997; Gronthos et al., 1997). The same integrin molecule in different cell types can have different ligand binding activities as well, since additional cell type specific factors can interact with integrins to modulate their binding activity (Alberts et al., 1994). Integrin recognition sites in many proteins ligands consist of short linear sequences that contain an acidic residue. Additional discontinuous regions of the protein may provide secondary binding sites (Schwartz et al., 1995). One integrin subfamily has been shown to utilize an RGD sequence (Arg-Gly-Asp) to recognize and bind ligands (Alberts et al., 1994). The bone matrix contains many glycoproteins that have this integrin-binding sequence, such as type I collagen, fibronectin, vitronectin, thrombospondin and bone sialoprotein (Grzesik and Robey, 1994).
Integrins in osteoblasts

The integrin receptors $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_5$, and $\alpha_7\beta_3$ have been found in human bone cells (Saito et al., 1994; Gronthos et al., 1997; Clover et al., 1992; Grzesik and Robey, 1994; Hughes et al., 1993). Integrin expression was found to be uniform throughout the whole bone cell population (Gronthos et al., 1997) but the pattern of integrin expression varied according to the stage of bone development (Hughes et al., 1993). The $\beta_1$ integrins are the predominant adhesion receptor subfamily utilized by osteoblast-like cells and are found in the bone cell receptors for collagen, fibronectin, laminin and vitronectin (Clover et al., 1992; Hughes et al. 1993; Grzesik and Robey, 1994; Saito et al., 1994; Gronthos et al., 1997). The integrin fibronectin receptor, $\alpha_5\beta_1$, as well as three other integrins $\alpha_6\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_3$ have been identified in fetal rat calvarial tissue and cultured osteoblasts at all stages of differentiation (Moursi et al., 1997). Blocking of the $\alpha_5\beta_1$ receptor by addition of integrin specific RGD-peptide inhibited mineralization, disrupted osteoblast organization and the mineralized matrix, decreased the osteoclast number in a mineralizing organ culture system and decreased calcification (Gronowicz and DeRome, 1994). Dedhar (1989) found that inhibition of the $\alpha_5\beta_1$ function with monoclonal antibodies inhibited osteoblastic differentiation. Another study showed that osteoblasts interact with the central cell binding domain of endogenously produced FN containing the RGD sequence during early stages of differentiation (Moursi et al., 1996; Moursi et al., 1997). These interactions regulated bone nodule morphogenesis and the expression of mRNA for genes associated with osteoblast differentiation like alkaline phosphatase and osteocalcin. These studies suggest that FN may play an important role in bone cell
proliferation and early stages of differentiation, somehow mediating the maturational pathway of bone cells.

**Integrin function**

Integrins play an important role in cell anchorage, migration, proliferation, differentiation and apoptosis (programmed cell death). Integrins can mediate a large number of different cellular responses to the ECM depending on the cell type, the integrin receptors expressed by the cell, and the composition of the surrounding ECM. They are also mechanotransducers of force across the plasma membrane. The $\alpha$ and $\beta$ subunit cytoplasmic domains do not have any intrinsic enzymatic activity, but couple with cytoplasmic proteins that nucleate the formation of large protein complexes that contain both cytoskeletal and catalytic signaling proteins (Clark and Brugge, 1995). The assembly of integrins, the ECM proteins, and the intracellular protein complexes forms the focal adhesion site.

**Interactions with the cytoskeleton**

Integrins function as transmembrane linkers mediating the interactions between the cytoskeleton and the extracellular matrix. By influencing the cytoskeleton, integrins stabilize cell adhesion and regulate the shape, morphology, orientation and the movement of cells. The $\alpha$ cytoplasmic domain contains highly divergent amino acid sequences while the $\beta$ cytoplasmic domain shows partial sequence conservation. The $\beta$ cytoplasmic domain plays a role in affinity modulation of the integrins and is sufficient to target integrins to
focal adhesion sites. The α cytoplasmic domain regulates the specificity of the ligand-dependent interactions (Clark and Brugge, 1995). The β subunit connects to the cytoskeleton of actin filaments through proteins such as talin and α-actinin, and other intracellular attachment proteins like vinculin, paxillin and tensin, which form a complex or focal adhesion site. Talin and α-actinin bind to the integrin cytoplasmic domain in vitro (Alberts et al., 1994; Clark and Brugge, 1995, see table 2). These assemblies of structural proteins may serve as a framework for the association of signaling proteins that regulate signal transduction pathways leading to integrin-induced changes in cell function and cell behavior.

**Activation of intracellular signaling cascades**

Integrins are able to activate intracellular signaling cascades leading to changes in gene activity. Many of the signal transduction pathways identified previously for growth factors and cytokines are also activated by integrins. Signaling through both growth factor receptors and integrins seems to be necessary for optimal cellular responses like cell proliferation, adhesion and migration (Clark and Brugge, 1995). Current models postulate that the cytoplasmic tail of the β subunit of the integrin binds to signaling molecules, such as one or more kinases, which then initiate signaling cascades after integrin ligation and aggregation (Yamada and Myamoto, 1995). Protein phosphorylation within focal adhesion sites is one of the earliest events found in response to integrin stimulation. Tyrosine phosphorylation has been shown to be a common response to integrin activation in many cell types (Clark and Brugge, 1995). Intracellular signals can rapidly change integrin
function by altering the binding affinity for ligands and by influencing post-occupancy effects such as the lateral diffusion of integrin receptors (Schwartz et al., 1995).

**Focal adhesion kinase (FAK)**

Focal adhesion kinase (FAK or pp125FAK), located in focal adhesion sites, is one of the major substrates for integrin induced tyrosine phosphorylation (Schaller et al., 1992), and induces intracellular signaling. Although FAK activation may be not required for the maintenance of focal adhesions it has been suggested that FAK phosphorylation is important in regulating the formation of new focal adhesions (Richardson and Parson, 1996). Cellular adhesion to fibronectin and other ECM proteins and integrin clustering as well as a number of growth factors and hormones result in FAK tyrosine phosphorylation and FAK activation. In fibroblasts, phosphorylation and activation of FAK has been shown to require cell attachment to the ECM and integrin clustering. FAK activation is reduced in suspended cells (Burridge et al., 1992). When suspended cells are replated on ECM substrates, FAK is rapidly phosphorylated and shows high levels of kinase activity. This enhanced phosphorylation and activation does not occur when fibroblasts are plated on poly-L-lysine (PLL) (Illic et al., 1997). It is known that cells do not adhere to PLL via integrins and therefore do not form focal adhesions and do not spread (Brighton and Albelda, 1992; Hatai et al., 1994; Ouwens et al., 1996; Schlaepfer et al., 1997).

FAK is a tyrosine kinase which can be divided into three structural domains. The amino- and the carboxy-terminal domain, each approximately 400 amino acids in length, flank the central catalytic domain. The amino-terminal domain contains the integrin binding site. *In vitro*, FAK is able to bind directly to the β1-integrin cytoplasmic tail (Schaller and Parsons, 1994). Integrin engagement, either with ligand or with antibodies, induces the
autophosphorylation of FAK at tyrosine residues (Guan and Shalloway, 1992; Lipfert et al., 1992; Vuori and Ruoslahti, 1993). FAK has five tyrosine phosphorylation sites, including Tyr 379, an autophosphorylation site that generates a high affinity binding site for the SH2 domain of the Src family non-receptor protein tyrosine kinases. The interaction of Src with the FAK autophosphorylation site leads to the phosphorylation of other sites in the FAK protein and therefore increases FAK activation \textit{in vitro} (Ilic et al., 1997). However tyrosine phosphorylation itself does not necessarily result in FAK kinase activity (Ilic et al., 1997, Schaller and Parsons, 1994). Activation of FAK also requires the activation of protein kinase C and the integrity of the actin cytoskeleton (Schwartz et al., 1995).

Via its central catalytic domain activated FAK induces intracellular signaling by formation of complexes between FAK and other signaling molecules (Schaller and Parsons, 1994; Schwartz et al., 1995). Interaction with Src creates a Grb2-binding site which links FAK to the RAS/MAPK (mitogen activated protein kinase) pathway (Dedhar, 1995; Ilic et al., 1997) (see Figure 1).

The carboxy-terminal domain of FAK contains the focal adhesion targeting (FAT) sequence. This binding site overlaps with the paxillin and talin binding site. The tyrosine phosphorylation of paxillin may lead to recruitment of additional signaling molecules such as csk and crk, and mediate effects of FAK on growth stimulatory pathways. These PTKs phosphorylate cytoskeletal proteins that may regulate their assembly and disassembly in focal adhesions or transmit cytoplasmic signals. (Schaller and Parsons, 1994; Parsons, 1996; Schwartz et al., 1995; Ilic et al., 1997).
Mitogen activated protein kinases (MAPK)

The MAPK signal transduction pathway is activated by growth factors (Egan and Weinberg, 1993; Seger and Krebs, 1995), and integrins (Chen et al., 1994; Seger and Krebs, 1995; Morino et al., 1995; Zhu and Assoian, 1995; Dedhar, 1995; Clark and Brugge, 1995). The same pattern of MAPK signaling is used by different signaling pathways that involve distinct isoforms at each level of the cascade with little or no cross-reactivity between them (Blumer and Johnson, 1994; Seger and Krebs, 1995). Extracellular regulated kinase 1 and 2 (ERK1/2) are two 44/42 kDa isoforms of MAPK. The ERK superfamily is quite large and is comprised of the ERK 1/2, JNK, and p38 subfamilies. The ERK 1/2 subfamily is activated by hormones, growth factors and integrins, while ERK 3 and 5 are activated by cytokines and stress (Cobb and Goldsmith, 1995). ERK 1/2 have been shown to be activated in response to the adherence of human skin fibroblasts to fibronectin or by cross-linking of β1 integrins with antibody (Morino et al., 1995) as well as in NIH-3T3 cells adhering to fibronectin (Schaepjer et al., 1994; Zhu and Assoian, 1995). Chen et al. (1994) showed that adhesion of Swiss 3T3 cells to fibronectin, laminin and to a synthetic peptide containing the RGD sequence, but not to poly-D-lysine, caused activation of MAPK (ERK1/2). These experiments suggest that integrins are able to activate MAPK (ERK1/2). Inhibition of actin filament assembly blocked adhesion-induced MAPK activation indicating a critical role for the cytoskeleton (Chen et al., 1994; Morino et al., 1995). Therefore integrin-dependent cytoskeletal complexes seem to be involved in the activation of the MAPK pathway (Clark and Brugge, 1995).

One pathway of integrin-dependent MAPK activation may be initiated by association of FAK with Grb2 and SOS1 (see Figure 1). Then RAS, a small GTP-binding protein, is
activated by binding to Grb2 (Schaepjer et al., 1994; Clark and Brugge, 1995; Seger and Krebs, 1995). This is followed by the sequential stimulation of several cytoplasmic protein kinases. The serine/threonine protein kinases Raf and MEK kinase (MEKK) phosphorylate and activate MEK (MAPK/ERK kinase or MAPKK (MAPK kinase)). MEK, a specific threonine/tyrosine-directed kinase, activates MAPK by phosphorylation of threonine and tyrosine residues (Blumer and Johnson, 1994). Activated MAP kinase translocates to the nucleus and is able to phosphorylate and activate transcription factors (Clark and Brugge, 1995; Dedhar, 1995) including c-fos and c-jun, members of the activator protein-1 (AP-1) transcription factor complex, which control gene expression (Bernstein et al., 1994; Seger and Krebs, 1995).

**AP-1**

The AP-1 transcription complex modulates the expression of genes involved in proliferation, differentiation and neoplastic transformation (Bernstein et al., 1994). It was characterized by its ability to alter gene expression in response to growth factors, cytokines, tumor promoters, carcinogenesis and increased expression of various oncogenes such as *src* and *ras* (Angel and Karin, 1991).

The AP-1 complex is a multiprotein complex composed of the products of the fos and jun gene families. Fos and jun are phosphoproteins which combine to form hetero- or homodimer complexes. While jun proteins can form homodimers and heterodimers with the Fos proteins, Fos proteins can not form homodimers (Angel and Karin, 1991). Dimer formation is necessary to bind to the AP-1 recognition site on the DNA which stimulates the transcription of genes that contain AP-1 consensus sequences in their promoter regions. Regulation of AP-1 activity in response to extracellular signals is determined by the levels of transcription of the fos and jun genes as well as by posttranslational modification (Angel
and Karin, 1991). The AP-1 complex initiates early transcriptional events which lead to an increase in DNA synthesis and cell proliferation or can effect other cellular processes such as differentiation. AP-1 sites are known to be present in the promoters of many bone specific genes, such as type I collagen (Monson et al., 1982; Lichtler et al., 1989), alkaline phosphatase (Matsuura et al., 1990), osteocalcin (Owen et al., 1990; Schule et al., 1990) and osteopontin (Craig and Denhardt, 1991). Fos has been found to have a critical role in regulating the development and activities of bone and cartilage cells (reviewed in Grigoriadis et al., 1995). It can act both as a transforming oncogene and as cell type specific regulator of differentiation. Overexpression of fos in transgenic mice causes transformation of cells in the chondrogenic and osteogenic lineages leading to the development of chondrosarcomas and osteosarcomas. Fos knockout mice lack osteoclasts and their precursors and develop osteopetrosis (Grigoriadis et al., 1995). In vivo expression of c-fos has been shown to be associated with regions of fetal bone having the highest growth potential (Grigoriadis et al., 1995, McCabe et al., 1995). McCabe et al. (1995) found high levels of c-fos, c-jun and junB mRNA during the proliferative period of osteoblast development. These findings suggest a fundamental role of AP-1 in cell proliferation.

*Previous studies of cell/implant interactions*

Although a large number of studies have investigated bone’s reaction to titanium and its alloys, little is known about the intracellular events that take place in osteoblasts at the titanium surface. Investigators have looked at the synthesis of collagen, noncollagenous protein and alkaline phosphatase, DNA and at integrin expression, cell adhesion and cell morphology. No studies could be found investigating the intracellular signaling events that follow osteoblast attachment to titanium.
Several investigators looked at the expression of integrins on different implant surfaces. Gronowicz and McCarthy (1996) showed that attachment of osteoblasts to TIV is in part mediated by integrin binding to the implant surface. Antibodies to the fibronectin integrin receptor $\alpha_5\beta_1$ significantly inhibited the binding of cells to TIV while antibodies to other integrins did not, indicating that this integrin receptor may play a major role in cell attachment to TIV. In addition to that they demonstrated that the pattern of integrin expression changed within 24 h depending on the substrate to which the osteoblasts bind. Sinha and Tuan (1996), examined osteoblasts cultured on TIV and cobalt-chrome-molybdenum (Co-Cr-Mo) and found also that the nature of the metal alloy influenced the pattern of integrin expression. Schneider and Burridge (1994) investigated the effect of serum and fibronectin coating of titanium on the integrin expression in osteoblasts. They found increased levels of $\beta_1$ integrins in osteoblasts adhering to fibronectin-coated surfaces and accumulation of $\beta_3$ integrins in osteoblasts on the serum-coated titanium surfaces.

Conflicting results have been found on the effect of implant materials on collagen synthesis. Puleo et al. (1991) found that collagen synthesis was unaffected by the type of implant material used for osteoblast culture in case of 316L stainless steel, TIV, Co-Cr-Mo, poly(methylmetacrylate) (PMMA), hydroxyapatite, borosilicate glass, tissue culture polystyrene (PS). Gronowicz and McCarthy (1996) demonstrated that collagen synthesis, as determined by $^3$H proline incorporation into collagen (CDP) and noncollagen proteins (NCP) differed on TIV, Co-Cr-Mo and glass. CDP and NCP were increased on TIV, while mostly CDP synthesis was stimulated by Co-Cr-Mo. Glass and plastic had less CDP and NCP production than the two metal substrates. Alkaline phophatase activity was shown to be unaffected in cell/implant cultures by the type of substrate used, either titanium grade 1 and 4, and glass (Ahmad et al., submitted) or hydroxylapitite, TIV and Co-Cr-Mo (Vrouwenvelder et al., 1993).
Osteoblast attachment and growth differed depending on the substrate to which they attach. Puleo et al. (1991) found that cell adhesion and growth were similar on the nonapatitic materials tested after 2 h of cell culture, but attachment and growth of osteoblasts were significantly lower and slower on hydroxyapatite. Sinha et al. (1994) found greater numbers of osteoblasts adhering to TIV in comparison with Co-Cr-Mo and PS after 12 hours.

In contrast, conflicting data was found for osteoblast morphology on different substrates. Vrouvenvelder et al. (1993) demonstrated that osteoblast morphology was similar on hydroxyapatite, TIV and stainless steel at 1, 2 and 12 days in culture. Sinha et al. (1994) found that osteoblasts cultured on TIV were significantly larger and better spread than those on PS and Co-Cr-Mo. The rate of cytoskeletal reorganization and focal contact formation was enhanced on TIV. Schneider and Burridge (1994) found osteoblast attached to titanium but no cell spreading or focal adhesions were found without serum or fibronectin. Following incubation with serum it was shown that the titanium surfaces became coated with vitronectin.

It is difficult to compare these studies due to the diverse results and the various implant materials. Serum in the cell culture medium may make interpretation of some of the studies difficult. Furthermore a great variety in culture times, alloys and the cell lines exist so that it is not possible to draw definite conclusions from the findings of these previous studies.
General Objectives

In this work a primary culture of osteoblastic cells was chosen to study cell/implant interactions in a nonneoplastic system, while minimizing the influence of other cells present in the intact tissue. *In vivo* studies using animal models or retrieved implants from humans have been essential in providing information on biological responses to implants in bone, but the results have been difficult to interpret at the cellular level because of the complexity of the cell types and events that occur at the implantation site (Puleo et al., 1991). Our goals were to:

1. investigate the intracellular signaling mechanisms involved in osteoblast attachment to titanium implant surfaces and compare them to osteoblast attachment to fibronectin.
2. examine osteoblast proliferation and differentiation following cell attachment to the implant surface after several days in culture.

Hypothesis

Osteoblast attachment to TIV-implant surfaces utilizes the same signaling pathways and results in comparable osteoblast morphology and proliferation as osteoblast attachment to ECM proteins such as FN.
Specific Objectives

All experiments were performed using primary fetal rat calvarial osteoblasts.

1. Intracellular signaling was investigated at different time points after cell plating on Tivanium (TIV), fibronectin (FN, positive control) and poly-L-lysine (PLL, negative control). The phosphorylation of FAK and MAPK was determined. The induction of c-fos and c-jun mRNA were measured by Northern blot as well as c-fos and c-jun protein levels by Western blot analysis.

2. Cell morphology including cell spreading, cytoskeleton, focal adhesion site formation at different time points was examined by phalloidin fluorescence microscopy.

3. Cell proliferation was monitored up to 6 days after initial attachment.
Materials and Methods

F3 cell isolation and culture conditions

Rat primary osteoblasts were obtained from the third sequential digestion of 20 d fetal rat calvaria [fraction 3 (F3) cells] with 0.2% collagenase-0.1% hyaluronidase (Luben et al., 1976; Gronowicz, McCarthy, 1995). It has been shown that the first enzyme digestion (F1) is enriched in fibroblastic cells, while the third fraction is enriched in cells expressing the osteoblast phenotype (osteoblast progenitors and young osteoblasts): Cultures of F3 cells showed a high level of type I collagen synthesis and alkaline phosphatase activity in contrast to F1 cell cultures (unpublished data of this laboratory, McCarthy et al., 1988). F1 cells also have a spindle-shaped morphology and therefore resemble fibroblasts, while confluent F3 cell cultures exhibit a cobblestone-like morphology similar to osteoblast cultures. The cells were cultured in F-12 media containing 5% horse serum, 2% fetal bovine serum, and 1% kanamycin sulfate. The culture medium was changed every 7 days and only the first or second passage was used for experiments. Cells were plated at various densities in serum-free F-12 medium containing 1% BSA.

Protein coating or immobilization of protein on glass disks

Proteins were covalently bound to glass disks according to the method of Werb et al. (1989) as described below. Glass disks (Fisher Scientific; 22 mm) were soaked in 20% H$_2$SO$_4$ overnight, then rinsed with H$_2$O, washed with 0.1 N NaOH and rinsed with double distilled H$_2$O (ddH$_2$O). After drying on filter paper they were soaked for 4 min in 2% γ-
aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO) in acetone. The disks were rinsed in acetone, ddH₂O and phosphate-buffered saline (PBS) and incubated in 0.25% glutaraldehyde in PBS for 30 min at 22 °C. The disks were washed three times with sterile PBS and placed in 6 well Costar dishes (Costar, Cambridge, MA). They were coated with 10 μg/ml rat plasma fibronectin (FN) (Biomedical Technologies, Stoughton, MA) or poly-L-lysine (PLL) (Sigma) in a 10 mM sodium carbonate-30 mM sodium bicarbonate buffer (pH 9.6) and incubated for 1 h at 25 °C. The coverslips were washed twice with F-12 medium prior to use.

**Preparation of Tivanium disks**

Disks (22 mm diameter) composed of Tivanium (Ti6Al4V, TIV, American Society for Testing and Materials [ASTM] F-136) were provided by Zimmer (Warsaw, IN). According to the manufacturer, the disks were processed in the same way as clinical implants: a 32 μm machine finish was achieved, and the disks were sandblasted with 600 grit aluminum oxide and then 100 mesh glass beads. The disks were allowed to self passivate. In previous experiments, disks were reused with no differences in the adhesion properties of the cells (Gronowicz, McCarthy, 1996). Used disks were rinsed copiously in tap water, swabbed with 95% ethanol on a cotton applicator until no visible debris was present, soaked in detergent for 2-12 h, sonicated for 1 h in the same solution, rinsed extensively in ddH₂O, rinsed with 95% ethanol, and stored dry in a single layer. Prior to use the disks were sterilized by ultraviolet radiation for at least 20 min. Implant sterilization by ultraviolet radiation was shown to enhance the osseointegration of the implants and does not leave any organic residues that may affect results (Meenaghan et al., 1991).
Adhesion assay

F3 cells were trypsinized and washed with soybean trypsin inhibitor (final concentration of 1.5 μg/ml; Sigma) in serum-free F-12 medium. The cell suspension was centrifuged at 300 x g for 5 min, resuspended and the cells were counted in a Coulter Counter (Coulter Electronics, Hialeah, FL). The cells were plated onto substrates at concentrations of 2.5 x 10^4 cells/ml and 1 x 10^5 cells/ml (4 ml/well) and cultured for 1, 2, 4 and 6 days at 37 °C in F-12 medium with 1% BSA. To remove nonadherent cells prior to counting, the disks were washed once with F-12 medium and transferred to a clean well. Attached cells were trypsinized and counted. Since every well has a surface area of 9.6 cm^2, the cells were plated at densities of approximately 1 x 10^4 cells/cm^2 and 4 x 10^4 cells/cm^2. Each well held a disk 60% smaller in area than the well.

Rhodamine phalloidin fluorescence microscopy

After plating at a concentration of 4 x 10^4 cells/cm^2 for 1, 4 and 24 h cells were fixed with 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 30 min. The disks were washed with PBS, pH 7.4, after each subsequent step at room temperature. The cells were permeabilized with 0.1% Nonidet P-40 (Sigma) in PBS at 4 °C and then blocked with 0.2% BSA in PBS. The cells were labeled with a 1:200 dilution of rhodamine phalloidin (Molecular Probes Inc., Eugene, OR) in PBS for 1 h. The cells were visualized with 2.5% n-propyl gallate in a 1:1 mixture of PBS and glycerol in a Nikon Optiphot fluorescence microscope.
Northern blots

To determine the time course for induction of c-fos and c-jun mRNA, F3 cells were plated onto TIV disks, FN and PLL-coated glass disks. A concentration of 4 x 10^4 cells/cm^2 and 8 disks for each substrate were used for each time point. Cells in suspension were used as 0 time controls. RNA was isolated at 30, 60 and 120 min using the acid-guanidinium thiocyanate chloroform (GTC) extraction method of Chomczynski and Sacchi, (1987). GTC was added to the cells and the mixture was polytroned (Kinematica, Switzerland ) for 20 seconds. Phenol and chloroform:isoamyl alcohol in a 24:1 dilution were added to the samples which were then centrifuged at 13,000 x g at 4 °C for 10 min.

The nucleic acids which remain in the aqueous phase were removed gently and precipitated with isopropanol and sodium acetate at -20 °C for at least 1 h. After centrifugation at 13,000 x g at 4 °C for 10 min the supernatant was discarded. Ethanol (80%) was added to the pellet, the sample was centrifuged, and the supernatant was discarded twice.

RNA was quantitated by measuring the absorbance at 260 and 280 nm (UV/Vis spectrometer, Perkin-Elmer, Norwalk, CT). Samples containing 20 µg RNA each were lyophilized in a SpeedVac (Savant Instr. Inc., Farmingdale, NY). The RNA was denatured by adding 11.25 µl RNA denaturant and ethidium bromide (1µg in 1µl) to each sample. The samples were heated at 65 °C for 10 min, placed on ice and mixed with 1.25 µl of 26% glycerol, 0.125% bromphenol blue, 0.125% xylene cyanole, 2.5% SDS and 12.5 mM EDTA.
Twenty µg of RNA was added to each lane of a 1% agarose/2.2 M formaldehyde gel and separated for 3 h at 80V. The gel was viewed on a UV box and photographed. The RNA was transferred to a Genescreen nylon membrane (NEN life science products, Boston, MA) via PosiBlot (Stratagene, La Jolla, CA) at 80 psi for 1 h. RNA was immobilized by UV-crosslinking using a Stratalinker (Stratagene), prehybridized at 42 °C for 4 h and then hybridized at 42 °C overnight. The hybridization solution contained radioactively labeled (³²P) specific fos- or jun-DNA-probes. Positive bands were detected by autoradiography (Thomas, 1980). The 1.4 kb c-jun cDNA clone, ph-cJ-1, was kindly supplied by Dr. Peter Angel. The FBJ murine osteosarcoma viral oncogene analog c-fos probe was purchased from ATCC (Rockville, MD). Relative hybridization levels were determined by densitometry. To standardize for RNA loading, the densitometric scan of the RNA for each protein was compared to the ethidium bromide-stained 28S ribosomal RNA (rRNA) bands.

**Western blots**

To determine the time course of c-fos and c-jun protein expression, F3 cells were plated at concentrations of 4 x 10⁴ cells/ cm² onto TIV disks and onto FN and PLL-coated glass disks, and incubated for 30, 60 and 120 min. To determine the time course of FAK and MAPK activation by phosphorylation, F3 cells were plated onto the above substrates for 0, 20, 30, 45, 60, 120 min and 24 h.

**Protein isolation / Immunoprecipitation**

Protein was isolated from cells by solubilizing the samples in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, pH 7.4) containing protease inhibitors (10 µg/ml aprotinin, 2 µg/ml pepstatin, 2 µg/ ml leupeptin) (Boehringer...
Mannheim), and 0.5 mM phenylmethylsulfonyl fluoride (Sigma) (Pandey and Wang, 1995). In order to prevent dephosphorylation in experiments examining protein phosphorylation, the extraction buffer contained 1 mM Na orthovanadate. Protein concentration was determined by using a BCA protein assay kit obtained from Pierce (Rockford, IL, U.S.A.) and using BSA as a standard. Fifty μg of protein was immunoprecipitated with 1 μg of rabbit polyclonal IgG (Upstate Biotechnology, Lake Placid, NY) by gentle vortexing and incubation for 1 h on ice. Protein A immobilized on crosslinked 4% agarose (Sigma) was added at a concentration of 100 μg. After 1 h incubation at 4 °C with rocking, the mixture was centrifuged at 10,000 x g for 15 sec at 4 °C. The pellet was washed 3 times with lysis buffer and resuspended in sample buffer (2% SDS, 10% glycerol, 100mM Dtt in 60 mM Tris, pH 6.9 and 0.01% Bromphenol Blue).

**Western Blotting**

For Western blots other than FAK, 70 μg of protein was boiled in reducing sample buffer and loaded in each lane (Laemmli, 1970). The proteins were separated in 10% SDS polyacrylamide gel. The gels were run at 100 V for approximately 1.5 h. The protein was transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) in 25 mM TRIS base, 192 mM glycine and 15% methanol (vol/vol). The membranes were blocked in T-TBS (0.1% Tween-20 in 20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) with 5% (wt/vol) skim milk powder, or for incubation with FAK, anti-phosphotyrosine or MAP kinase antibodies, in T-TBS containing 2% BSA. After washing in T-TBS, the blots were incubated with the primary antibody. Anti-focal adhesion kinase (rabbit polyclonal IgG) was purchased from Upstate Biotechnology (Lake Placid, NY), used at a concentration of 1:250, and incubated with the immunoprecipitated blots for 2 h in T-TBS containing 2%
BSA. Anti-phosphotyrosine antibodies (mouse monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) were used at a concentration of 1:250 and incubated with the immunoprecipitated blots in T-TBS containing 2% BSA for 1 h. The antibody specific for MAP kinase (Erk1 and Erk2, rabbit polyclonal IgG, Santa Cruz Biotechnology) was used at a concentration of 1:500 in T-TBS with 5% skim milk powder and incubated for 1 h. The anti-phosphorylated (anti-active) MAPK (mouse monoclonal IgG, Promega, Madison, WI) was used at a concentration of 1:10,000 for a 3 h incubation in T-TBS containing 2% BSA. C-fos and c-jun antibodies (rabbit polyclonal IgG, Santa Cruz Biotechnology) were used at a concentration of 1:100 for 1 h in T-TBS with 5% skim milk powder. All incubations were performed at room temperature. After washing, the blots were incubated with horseradish peroxide-conjugated secondary antibody. The appropriate horseradish peroxidase conjugated secondary antibodies (Pierce, Rockford, IL) were used at a concentration of 1:20,000. For active-MAPK experiments, the concentration of the secondary antibody was increased to 1:10,000. Positive bands were detected using the ECL chemiluminescence kit (Amersham) and Fuji film. Blots were stripped by incubating in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.8, for 30 min at 50 °C.

All experiments were repeated at least twice or more with similar results.

**Statistical analysis**

Adhesion data were analyzed using a nonparametric one way analysis of variance (ANOVA) to evaluate differences between groups. Post hoc comparison of means was accomplished with the Student-Newman-Keuls test to determine significance between groups. P-values <0.05 were considered statistically significant.
Results

Cell adhesion

To study the effect of different substrates on cell adhesion, F3 cells were plated on FN and PLL-coated glass disks and on TIV disks for 1, 2, 4 and 6 days (Figure 2). In order to achieve an initial cell attachment rate which was optimal for cell proliferation, two different initial plating densities were used, since it was unknown how many cells would initially attach to the different surfaces.

Figure 2 A shows the cell proliferation at an initial plating concentration of $1 \times 10^4$ cells/cm$^2$. Initially (day 1) the plating efficiency on FN was 58%, on PLL 52% and on TIV 25%, being smaller on TIV, although not significantly, in comparison to the other substrates. Cells on FN showed an 84% increase in cell number by day 2, which was statistically significant ($p<0.05$), and maintained cell number through day 6. Cells on TIV showed a tendency to increase progressively over the 6 days but the numbers were not statistically significant. The cell numbers on PLL remained approximately at the same level from days 1 to 6, showing no significant increase or decrease. Figure 6 B shows the cell proliferation at an initial plating concentration of $4 \times 10^4$ cells/cm$^2$. At day 1 the plating efficiency on FN was 61%, on PLL 57% and on TIV 30%, which was significantly less in comparison to FN and PLL ($p<0.05$). Maximum cell attachment was found on FN, therefore no statistically significant increase in proliferation was found on days 1 to 6. Cell proliferation on TIV was more pronounced at this initial plating density. Cells on TIV increased significantly by 47% on day 2 and showed a tendency to further proliferate by day 4 although this increase was not statistically significant. Cell numbers remained unchanged by day 6. Cells on PLL showed a tendency to decrease progressively from day 2 to day 6, but the numbers were not statistically significant.
Cytoskeleton

The cytoskeleton and morphology of F3 cells was visualized by phalloidin fluorescence microscopy at 1, 4 and 24 h (Figures 2, 3 and 4). Phalloidin was used to stain the actin-containing microfilaments of the cytoskeleton. Thereby changes in cell morphology and organization of the cytoskeleton, as well as the formation of focal adhesion sites at different time points after cell attachment to the different substrates, were able to be studied.

At 1 h cells started to spread on FN and TIV, while cells on PLL were mostly rounded (Figure 3). At 4 h, cells on FN were well spread covering almost the entire surface of the disk with distinct staining of stress fibers (Figure 4). A few rounded cells were visible. Cells on PLL started to spread but most of the cells were rounded with heavy staining circling the nucleus and in the periphery of the cell but with no distinct stress fibers. On TIV a large number of cells were spread and flattened with finger-like processes at the periphery of cells, while some rounded cells were also present. Spread cells showed a stress fiber network, which was less pronounced than in cells on FN. Some areas in the cell membrane of spread cells showed intensive punctate staining, which appeared to be focal adhesion sites (arrowheads in Figure 4). By 24 h cells were contiguous and flattened on FN, with no rounded cells visible and a clear network of stress fibers and focal adhesion sites (Figure 5). Cells on PLL were spread and had some stress fibers. Rounded cells were still visible. On TIV cells were spread, no rounded cells were visible, and distinct regions in the cell membranes were stained extensively in a punctate pattern, indicating the presence of focal adhesion sites. The osteoblasts had a cobblestone-like appearance on TIV typical of an osteoblast phenotype. At 24 h, cells on FN were covering almost the entire surface of the disk, cells on TIV covered somewhat less surface area, and cells on PLL covered the least amount of disk surface.
FAK

FAK is one of the major substrates for integrin-induced tyrosine phosphorylation. Located in focal adhesion sites the activated form of FAK provides a link between the integrin receptor and the cytoskeleton by initiating intracellular signaling cascades like the RAS/MAPK pathway. The level of expression of FAK and activated (phosphorylated) FAK was determined at 20, 30, 45, 60 min, 2 and 24 h by Western blot analysis. The top lanes in Figure 6 A show the phosphorylated form of FAK for each substrate while the bottom lane demonstrates FAK protein levels. The densitometric scan of the top lane is shown in Figure 6 B and the bottom lane in Figure 6 C. As shown in Figure 6 C FAK protein levels are stimulated on all substrates showing a decrease on TIV and PLL at 24 h. In Figure 6 B cells on all substrates showed a maximal increase in FAK phosphorylation at 20 min compared to unplated cells. Cells on FN showed continuous FAK phosphorylation over the 24 h time period while cells on PLL and TIV demonstrated a decrease in FAK phosphorylation by 24 h.

MAPK

Integrin-mediated signal transduction pathways include MAPK activation which leads to activation of the AP-1 transcription factor complex. The level of expression of MAPK and activated (phosphorylated) MAPK was determined at 20, 30, 45, 60 min, 2 and 24 h by Western blot. The top lanes of Figure 7 A show the phosphorylated (active) form of MAPK for each substrate and the bottom lanes show the protein of MAPK (ERK1 and 2 isoforms). The densitometric scans of the top lanes are demonstrated in Figure 7 B and the bottom lanes in 7 C. As shown in Figure 7 C, MAPK protein levels were stimulated on FN and TIV and decreased on PLL. In Figure 7 B, cells plated on FN demonstrated an increase of MAPK activation at 20 min which was followed by gradual increase with maximal
expression at 120 min. MAPK was downregulated at 24 h. Cells on PLL did not show any MAPK activation over 24 h. Cells on TIV showed an increase in MAPK phosphorylation at 20 min followed by a gradual increase with a maximal expression at 24 h. Thus MAPK phosphorylation was slightly delayed on TIV compared to FN.

**AP-1 message levels**

The time course of c-fos and c-jun mRNA expression is shown by Northern blot. Previous work demonstrated that fos and jun is upregulated at 60 min in F3 cells plated onto FN in an integrin-dependent manner, and that cells on PLL do not upregulate AP-1 (Cowles et al., submitted). Therefore, F3 cells were plated onto FN, PLL and TIV for 30, 60 and 120 min. C-fos mRNA levels are shown in Figure 8 A and the densitometric scan of the blot normalized to the 28S ribosomal RNA is shown in Figure 8 B. C-jun mRNA levels are demonstrated in Figure 8 A with its respective densitometric scan in Figure 8 C. Before cells were plated, c-fos message levels (Figure 8 A and B) were hardly detectable at time 0 and were transiently and maximally expressed at 30 and 60 minutes on FN and TIV. By 120 min c-fos mRNA levels on both substrates had returned to 0 time levels. On PLL c-fos mRNA levels increased continuously over the entire time period. There was no downregulation of c-fos at 120 min. C-jun mRNA (Figure 8 A and C) was maximally expressed at 30 min on FN and TIV and remained elevated at 120 min. Cells on PLL showed a maximal increase in c-jun mRNA at 120 min in comparison to unplated cells.

**AP-1 protein induction**

To determine whether c-fos and c-jun mRNA is being translated into protein a Western blot was performed on cells after they were plated for 30, 60 and 120 min. Figure 9 A
demonstrates c-fos protein levels and Figure 9 B is the densitometric scan of that blot. The time course for c-jun expression is found in Figure 9 A with its respective scan in Figure 9 C. Cells on FN show maximal c-fos expression at 60 and 120 min, while cells on PLL did not express c-fos protein (Figure 9 A and B). Cells on TIV demonstrated maximal c-fos protein expression at 30 min and remained constant. C-jun protein was elevated at 30 and 60 min on FN and maximally expressed at 120 min (Figure 9 A and C). Cells on PLL showed a steady increase in c-jun protein levels over the time period. On TIV c-jun protein levels were increased at 30 min, maximally expressed at 60 min and downregulated at 120 min.
Discussion

Osteoblasts are able to attach to the ECM proteins at the bone/implant interface as well as to the implant material itself via integrins (Albrektsson and Hansson, 1986; Gronowicz and McCarthy, 1996). Integrins play an important role in cell proliferation and differentiation by activation of intracellular signaling cascades that lead to changes in gene expression. Integrins also influence cell adhesion and regulate the shape, morphology and the migration of cells through changes in the cytoskeleton. In this dissertation, the cytoskeletal changes as well as cell proliferation and integrin-dependent intracellular signaling pathways were examined following cell attachment to TIV, FN and PLL. These substrates were chosen for comparison because FN is an early and important ECM protein involved in osteoblast proliferation (Owen et al., 1990; Lynch et al., 1995; Cowles et al., 1998). Since the ultimate goal in biocompatibility is to activate similar responses as the native ECM a comparative study of osteoblast responses to FN and TIV was performed. Cells do not attach to PLL via integrins but rather via ionic interactions (Brighton and Albelda, 1992; Hatai et al., 1994; Ouwens et al., 1996; Schlaepfer et al., 1997; Ilic et al., 1997). Therefore PLL was chosen as a negative control to differentiate between integrin-independent cell adhesion and signaling, and cellular responses triggered by integrin-mediated adhesion and activation on the implant material. Serum and growth factors affect cell attachment, behavior, and signaling pathways. Therefore the experiments were performed in serum-free F12 medium. BSA was added to the medium at a concentration of 1%. BSA binds many lipids, cationic cofactors, and hormones that are either toxic at high doses or elicit their effect only when found at low concentrations or bound to BSA (Barnes and Saito, 1980).

The number of primary rat osteoblasts adhering to the different surfaces after one day in culture differed between TIV and the two other substrates. PLL had an initial plating
efficiency of 52% at an initial plating concentration 1 x 10^4 cells/cm^2 and 57% plating efficiency at an initial plating concentration of 4 x 10^4 cells/cm^2. Although many osteoblasts adhered on PLL, they did not proliferate over the 6 days of culture. At the higher initial plating concentration cell numbers on PLL showed a tendency to decrease at days 2, 4 and 6 suggesting that cell attachment in an integrin-independent manner probably via ionic charge, did not facilitate proliferation. The fact that even at day 6, cells survived on PLL may be explained by their production of ECM proteins, which would have triggered integrin activation, but perhaps not to the same levels as initial attachment to the ECM.

In contrast, cells on FN and TIV proliferated during 6 days of culture although the initial plating efficiency on the two materials was significantly different. Cells plated on FN had higher initial plating densities (58% and 64%) and continued to proliferate, more than doubling in cell number from day 1 to 6. At the higher initial plating concentration, cell numbers on FN remained unchanged indicating that maximal cell numbers had been reached on the disk which was confirmed by phalloidin fluorescence microscopy. Since the osteoblasts are from primary cell lines and exhibit contact inhibition, they did not proliferate. Primary osteoblasts adhered least to TIV, with a plating efficiency of only 25% at 1 x 10^4 cells/cm^2 and 30% at 4 x 10^4 cells/cm^2. At the higher initial plating concentration, cells on TIV had proliferated significantly by day 2, and showed a tendency to proliferate further by day 4, almost doubling the cell numbers from day 1 to 4. By day 6 the cell numbers remained unchanged from day 4. The cell numbers had reached a similar cell density as in cells on FN indicating that maximal cell attachment had been reached. At 1 x 10^4 cells/cm^2 cells were not able to proliferate significantly although a tendency to proliferate could be seen over the 6 days. This finding indicates that the initial plating density at this concentration might have been too low to allow optimal cell proliferation. Cells will proliferate on a substrate if they are able to condition the medium with enough growth factors and cytokines for cell survival. This was confirmed when more cells were
plated at a density of $2 \times 10^4$ cells/cm² on TIV. Cells continued to proliferate over the 6 day period similar to the proliferation seen at $4 \times 10^4$ cells/cm² (data not shown).

The organization of the cytoskeleton has been shown to be related to cell attachment and spreading (Sinha, 1994). Cell morphology and the organization of the cytoskeleton was visualized on the different substrates by rhodamine phalloidin fluorescence microscopy. Phalloidin stains the actin-containing microfilaments of the cytoskeleton which are involved in the determination of cell shape and in the generation of force for cell spreading and movement (Ingber et al., 1994). Bundling of actin microfilaments produces stress fibers that are visible with the light microscope. Integrins connect to the actin microfilaments through proteins such as talin and α-actinin in focal adhesion sites and thereby stabilize adhesion, regulate cell shape, morphology, orientation and migration of cells proteins (Alberts et al., 1994; Clark and Brugge, 1995). They also serve as the framework for the association of signaling molecules. Therefore, the structure of the cytoskeleton was examined on the three substrates and compared to the cell signaling capacity of the cells. Primary rat osteoblasts on FN rapidly adhered and spread with the development of numerous stress fibers by 1 h. Cells on TIV did not adhere in such large numbers and cell spreading advanced slower. This was reflected in the slight delay in FAK and MAPK phosphorylation of cells on TIV compared to cells on FN.

Initial adhesion to a substrate does not appear to predict the ability of that substrate to sustain cell proliferation or differentiation. Cells initially adhered to PLL in similar numbers as to FN, but remained mostly rounded at 1 h and 4 h with no visible organized cytoskeleton. The binding to PLL is mainly through ionic charges which may explain the poor spreading ability of cells on PLL. Our data demonstrated that cells did not proliferate on PLL but were able to divide on FN and TIV. At 24 h, osteoblasts on PLL seemed to have recovered with some spreading and the development of a few stress fibers. This is probably due to ECM protein production, which would restore integrin function and the
integrity of the cytoskeleton. This is in agreement with Gronowicz and McCarthy (1996) who found changes in integrin expression at 24 h on several substrates which was explained by the fact that cells had started to produce ECM proteins. Phalloidin staining demonstrated that cells on the FN-coated surfaces were fully spread and confluent at 24 h. Cells on TIV were also spread by 24 h and almost confluent. Morphologically cells on TIV appeared to have a cobblestone-like appearance by 24 h, which is typical of the osteoblast phenotype, suggesting that they are producing numerous ECM proteins. This was previously confirmed in our laboratory by results demonstrating increased collagen and noncollagen protein synthesis on TIV at 24 h (Gronowicz and McCarthy, 1996).

Extensive staining of distinct regions in the cell membrane of spread cells on TIV indicated the presence of focal adhesion sites. Osteoblasts on FN also displayed focal adhesion sites at 4 and 24 h. These are areas of integrin-mediated adhesion to TIV and FN. They are composed of a well characterized set of proteins which are involved in integrin-activated intracellular signaling cascades.

An important component of the focal adhesion site is FAK which becomes tyrosine phosphorylated upon integrin-mediated attachment to ECM proteins (Ilic et al., 1997). FAK phosphorylation is reduced in suspended cells. FAK is involved in the activation of various signaling cascades, and effects cytoskeletal organization and the formation of focal adhesions. Ilic et al., 1997, proposed a model for the phosphorylation cycle of FAK: Integrin-ECM interactions cause conformational changes in FAK and allow access of Src-family members to the FAK autophosphorylation site. Bound Src phosphorylates other tyrosine residues on FAK, which then is able to bind other signaling molecules such as Grb2. Bound Src also forms a complex with paxillin and Csk, which downregulates Src activity by phosphorylating its C-terminal tyrosine. The binding of Csk to paxillin in close proximity with FAK-associated Src may promote inactivation of Src. This, plus unknown signals that activate focal adhesion-associated phosphatases, could cause a
dephosphorylation of the FAK-associated signaling complexes. Some of the activated phosphotyrosine kinases (PTKs), which are activated via FAK, phosphorylate cytoskeletal proteins and thereby regulate their assembly and disassembly in focal adhesions. How all these factors contribute to changes in cell phenotypes and activity have yet to be explored.

In contrast to other groups (Burridge et al., 1992; Ouwens et al., 1996), but in accordance with Schlaepfer et al. (1997), we found that FAK was phosphorylated on all three substrates. Burridge et al. (1992) and Ouwens et al. (1996) found that FAK was not phosphorylated in fibroblasts plated onto PLL. Schlaepfer et al. (1997) showed FAK phosphorylation in fibroblasts plated onto PLL from 40 min to 6 h, although this FAK phosphorylation was less pronounced than in cells plated onto FN. In accordance with the literature FAK was not phosphorylated in un plated cells in our system (Burridge et al., 1992; Schlaepfer et al., 1997). FAK activation in primary osteoblasts cultured on PLL has not been previously studied. One explanation for our findings would be that primary osteoblasts are able to produce enough growth factors to lead to FAK activation. FAK is activated not only by integrins but also by a number of growth factors, hormones and several bioactive peptides, and serum factors such as lysophosphatidic acid (LPA) (Schaller and Parsons, 1994; Ilic et al., 1997). However, FAK phosphorylation on PLL did not lead to the activation of the MAPK-pathway of intracellular signaling with the stimulation of AP-1 transcription factors. Furthermore, the development of a well developed cytoskeleton, which is also mediated by FAK, is delayed in cells plated on PLL until 24 h. It has been shown that FAK tyrosine phosphorylation per se is not necessarily correlated with activity and signal transduction events (Ilic et al., 1997). FAK was shown to be phosphorylated in adherent, serum-starved fibroblasts but exhibited a low level of in vitro kinase activity. In vitro tyrosine phosphorylation of FAK had little effect on FAK kinase activity in chicken embryo cells (Schaller and Parsons, unpublished data in: Schaller and Parsons, 1994). It might be that in these cases the formation of the complex of FAK with Src family PTKs is not taking place. Since Src activation is necessary for the activation of
intracellular signaling pathways as well as for the FAK-induced organization of the cytoskeleton, this would cause the low level of FAK kinase activity. This will be explored in the future.

The adhesion of osteoblasts to FN and TIV resulted in MAPK activation, while adhesion to PLL did not allow MAPK activation. Even the levels of unphosphorylated MAPK decreased with time on PLL, although cells on all substrates displayed approximately equivalent amounts of MAPK at 20 min. These results demonstrate that although FAK was phosphorylated in cells plated on PLL, FAK activation did not result in activation of the MAPK signaling pathway. The MAPK signal transduction pathway is activated by integrins and growth factors. MAP kinases provide a link between cytoplasmic signaling molecules and transcription factors, such as c-fos and c-jun. It has been shown that MAP kinase activation by phosphorylation leads to upregulation of c-fos and c-jun (Bernstein et al., 1994; Seger and Krebs, 1995). The MAPK isoforms ERK1 and 2 have also been shown to be activated in response to cell adhesion to FN (Chen et al., 1994; Schaepjjer et al., 1994; Morino et al., 1995; Zhu and Assoian, 1995).

Our results demonstrate that FN and TIV activate similar signaling pathways. Since MAPK is activated by integrins, and cells on FN and TIV demonstrated MAPK activation in contrast to PLL, the results suggest that osteoblasts adhering to TIV, use the same signaling pathway involving MAPK as osteoblasts on FN. Adhesion to TIV was shown to depend partially on the fibronectin integrin receptor (Gronowicz and McCarthy, 1996) which would involve upregulation of MAPK as a result of integrin binding. The initial low levels of phosphorylated MAPK in cells on TIV in comparison to cells on FN may be due to a delay in attachment and spreading with the formation of a well-organized cytoskeleton as was confirmed by phalloidin staining. Integrin-dependent organization of the actin cytoskeleton has been shown to play a critical role in MAPK activation (Chen et al., 1994; Morino et al., 1995; Clark and Brugge, 1995). This might also explain why FAK
phosphorylation on PLL did not result in activation of MAPK, since cells on PLL did not exhibit a well developed cytoskeleton until 24 h. MAPK is an endpoint for a variety of signaling cascades in vertebrates, such as growth factors, hormones, stress induced-activation of Ras, PMA induced-activation of protein kinase C (PKC), progesterone induced-activation of Mos in Xenophus, heat shock, arsenite, interleukin 1 and integrin adhesion (Blumer and Johnson, 1994; Seger and Krebs, 1995).

The increase in AP-1 transcription factors appear to be an early indication of osteoblast phenotypic expression. Our results demonstrated that c-fos mRNA was transiently and maximally expressed at 30 and 60 min on FN and TIV. In primary osteoblast cultures, c-fos messages were expressed during the proliferative phase, prior to the expression of differentiation markers such as Type I collagen and alkaline phosphatase (Owen et al., 1990; Lynch et al., 1995). In our study c-fos message was downregulated on both substrates by 120 min, while c-jun mRNA remained elevated. On PLL, c-fos and c-jun mRNA levels increased continuously over 120 min. These results were in accordance with Cowles et al. (submitted) who found a similar pattern of c-fos and c-jun message induction after plating rat primary osteoblasts onto FN-coated glass disks.

Protein levels of c-fos and c-jun differed from the message levels. Attachment of osteoblasts to TIV and FN increased c-fos and c-jun protein. C-fos message was not translated into protein on PLL. However c-jun protein increased during 120 min of cell adhesion to PLL. Thus, the expression of c-fos and c-jun message and protein was similar on TIV and FN. These results show that integrin-mediated adhesion on TIV, upregulates the AP-1 transcription factor c-fos and c-jun, thereby leading to osteoblast proliferation, similar to findings on FN. Adhesion without integrins does upregulate c-fos and c-jun message levels but they are either not translated into protein (c-fos) or at a lower level (c-jun), as seen by the osteoblast response to PLL.
The finding that adhesion to TIV has a similar pattern of c-fos and c-jun message and protein expression as adhesion to FN may be due to the fact that adhesion to TIV is integrin-mediated. Adhesion to FN mainly involves the FN integrin receptor $\alpha_5\beta_1$. This integrin receptor appears to play a significant role in osteoblast binding to TIV, since antibodies to the FN receptor $\alpha_5\beta_1$ inhibited binding of cells to TIV by 63% (Gronowicz and McCarthy, 1996). The slight differences in the time course of c-fos and c-jun protein expression might be due to some differences in adhesion to the two substrates which need to be further studied. Cowles et al. (submitted) found that c-fos and c-jun pathways are both controlled by tyrosine phosphorylation but are induced by different, partly shared pathways. C-fos message expression in cells plated onto FN was controlled by PKC while c-jun mRNA expression was influenced by protein kinase A (PKA).

In conclusion, we examined cell proliferation, cytoskeletal organization, signaling pathways and gene expression on the implant material TIV. The results of this study demonstrate that the initial adhesion of primary osteoblasts to TIV involves the activation of similar intracellular signaling pathways and gene expression which allow cell proliferation. Comparable structural changes in the cytoskeleton were also seen in osteoblast adhesion to FN and TIV. This research provides evidence for the integrin-mediated adhesion of osteoblasts to the surface of TIV implants. The FN integrin receptor seems to be involved, what leads to the activation of intracellular signaling cascades in a similar way as cell adhesion to the ECM protein FN. Components of the AP-1 transcription factor complex, which play a major role in osteoblast proliferation and are also involved in osteoblast differentiation, were activated in a similar manner on TIV and on FN, which demonstrates the capability of TIV to enhance osteoblast proliferation.

In order to improve the material for future implants it would be desirable to enhance initial cell adhesion and spreading, which was found to be lower and less pronounced on
TIV in comparison to FN. Since the FN integrin receptor is not the only receptor responsible for cell attachment to TIV, future research will need to clarify the remaining components which contribute to cell adhesion to the implant material. In the future, it should be possible to change the molecular components of implant surfaces in a way that promotes optimal cell adhesion, proliferation, and differentiation.
<table>
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<th>Integrin receptor</th>
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<tr>
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**Table 1:** Ligand specificity of integrin receptors found in human bone cells (Saito et al., 1994)
**Cytoskeletal protein**

- **Actin**: Structural component of microfilaments
- **α-Actinin**: Homodimer that binds to and crosslinks actin filaments, binds to the integrin cytoplasmatic face, vinculin and phosphoinositide 3-kinase (PI-3K)
- **Talin**: Binds to the integrin cytoplasmatic face, binds to vinculin and phospholipids
- **Vinculin**: Binds to α-actinin, talin, paxillin, tensin, actin filaments, and phospholipids
- **Paxillin**: Tyrosine phosphorylated, binds to vinculin
- **Tensin**: Tyrosine phosphorylated, contains an SH2 domain, binds to vinculin and actin filaments

**Table 2**: Molecular components of focal adhesion sites (Clark and Brugge, 1995)
Figures
Figure 1

Integrin-mediated intracellular signaling pathway.
Figure 1
Figure 2

Proliferation of F3 cells on FN, PLL and TIV, cultured for 1, 2, 4 and 6 days.

A: Initial plating cell density $1 \times 10^4$ cells/cm$^2$.

Cell numbers on FN increased significantly by day 2 compared to day 1 ($*=p<0.05$).

B: Initial plating cell density $4 \times 10^4$ cells/cm$^2$.

Cell numbers on TIV increased significantly by day 2 compared to day 1 ($*=p<0.05$).

Initial attachment (day 1) was significantly lower on TIV in comparison to FN and PLL ($**=p<0.05$).
Figure 2
Figure 3

Phalloidin labeling of F3 cells grown on FN and PLL-coated glass disks and on Tiv disks for 1 h. Phalloidin stained the actin-based cytoskeleton and demonstrated cell morphology. The magnification was 25 X (left) and 100 X (right).
Figure 3
Figure 4

Phalloidin labeling of F3 cells grown on FN and PLL-coated glass disks and on TIV disks for 4 h. Phalloidin stained the actin-based cytoskeleton and demonstrated cell morphology. Arrowheads demonstrate focal adhesion sites. The magnification was 25 X (left) and 100 X (right).
Figure 4

FN

PLL

TIV

4 hrs
Figure 5

Phalloidin labeling of F3 cells grown on FN and PLL-coated glass disks and on TIV disks for 24 h. Phalloidin stained the actin-based cytoskeleton and demonstrated cell morphology. The magnification was 25 X (left) and 100 X (right).
Figure 5
Figure 6

Western blot analysis of FAK in F3 cells cultured on FN, PLL and TIV at 20, 30, 45, 60 min, 2 h and 24 h. The level of expression of FAK and phosphorylated (activated) FAK protein was determined using the appropriate antibodies as described in Methods.

A: Western blot of phosphorylated FAK and FAK protein are top and bottom panels, respectively.

B: Densitometric scans of the Western blot in (A), showing the time course of phosphorylated FAK expression.

C: Densitometric scans of the Western blot in (A), showing the time course of FAK expression.
Figure 6
Figure 6
Figure 7

Western blot analysis of MAPK in F3 cells cultured on FN, PLL and TIV at 20, 30, 45, 60 min, 2 h and 24 h. The level of expression of MAPK and phosphorylated MAPK protein was determined using the appropriate antibodies as described in Methods.

A: Western blot of phosphorylated MAPK and MAPK protein are top and bottom panels, respectively.

B: Densitometric scans of the Western blot in (A), demonstrating the time course of phosphorylated MAPK expression.

C: Densitometric scans of the Western blot in (A), demonstrating the time course of MAPK expression.
Figure 7
Figure 7
Figure 8

Northern blot analysis of c-fos and c-jun mRNA from F3 cells grown on FN, PLL and TIV for 30, 60, and 120 min.

A: Northern blots of c-fos and c-jun mRNA. The lower panel shows the corresponding 28S and 18S ribosomal RNA bands stained with ethidium bromide.

B: Densitometric scans of the time course of c-fos mRNA expression, corrected for RNA loading by comparison with 28S rRNA.

C: Densitometric scans of the time course of c-jun mRNA expression, corrected for RNA loading by comparison with 28S rRNA.
Figure 8
Figure 8
Figure 9

Western blot analysis of c-fos and c-jun protein from F3 cells cultured on FN, PLL and TIV at 30, 60 and 120 min.

A: Western blot of c-fos and c-jun.

B: Densitometric scans of the Western blot in (A), showing the time course of c-fos protein expression.

C: Densitometric scans of the Western blot in (A), showing the time course of c-jun protein expression.
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*Figure 9*
Figure 9
Literature


