Substrate Modulation of Osteoblast Adhesion Strength, Focal Adhesion Kinase Activation, and Responsiveness to Mechanical Stimuli

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Abstract: Osteoblast interactions with extracellular matrix (ECM) proteins are known to influence many cell functions, which may ultimately affect osseointegration of implants with the host bone tissue. Some adhesion-mediated events include activation of focal adhesion kinase, and subsequent changes in the cytoskeleton and cell morphology, which may lead to changes in adhesion strength and cell responsiveness to mechanical stimuli. In this study we examined focal adhesion kinase activation (FAK), F-actin cytoskeleton reorganization, adhesion strength, and osteoblast responsiveness to fluid shear when adhered to type I collagen (ColI), glass, poly-L-lysine (PLL), fibronectin (FN), vitronectin (VN), and serum (FBS). In general, surfaces that bind cells through integrins (FN, VN, FBS) elicited the highest adhesion strength, FAK activation, and F-actin stress fiber formation after both 15 and 60 minutes of adhesion. In contrast, cells attached through non-integrin mediated means (PLL, glass) showed the lowest FAK activation, adhesion strength, and little F-actin stress fiber formation. When subjected to steady fluid shear using a parallel plate flow chamber, osteoblasts plated on FN released significantly more prostaglandin E2 (PGE2) compared to those on glass. In contrast, PGE2 release of osteoblasts attached to FN or glass was not different in the absence of fluid shear, suggesting that differences in binding alone are insufficient to alter PGE2 secretion. The increased adhesion strength as well as PGE2 secretion of osteoblasts adhered via integrins may be due to increased F-actin fiber formation, which leads to increased cell stiffness.

keyword: Osteoblasts, Extracellular matrix, Focal adhesion kinase, Adhesion, Mechanotransduction.

1 Introduction

Interaction of osteoblasts with various substrates through adhesion is known to modulate osteoblast functions such as proliferation, differentiation, signaling, adhesion strength, protein production, mineralization, and cell morphology (Carvalho, Kostenuik, Salih, Bumann and Gerstenfeld 2003; Cowles, Brailey and Gronowicz 2000; Geissler, Hempel, Wolf, Scharnoweber, Worch and Wenzel 2000; Globus, Doty, Lull, Holmuhamedov, Humphries and Damsky 1998; Grzesik and Robey 1994; Kaiser and Chandrasekhar 2003; Kim, Jang, Chung and Ku 2003; Krause, Cowles and Gronowicz 2000; Rezania and Healy 1999; Sommerfeldt, McLeod, Rubin and Hadjiargyrou 2001; Stephansson, Byers and Garcia 2002). Therefore, it is not surprising that many studies have been performed to examine the enhancement of bone cell function on implant materials coated with various extracellular matrix (ECM) proteins (Becker, Geissler, Hempel, Bierbaum, Scharnoweber, Worch and Wenzel 2002; Degasne, Basle, Demais, Hure, Lesourd, Grolleau, Mercier and Chappard 1999; Geissler, Hempel, Wolf, Scharnoweber, Worch and Wenzel 2000; van den Dolder, Bancroft, Sikavitsas, Spauwen, Mikos and Jansen 2003). However, few studies address the effect of ECM modulation of mechanotransduction (Ponik and Pavalko 2004). The attachment of osteoblasts to ECM proteins initiates signaling responses, such as focal adhesion kinase (FAK) activation, which in turn, induces filamentous actin (F-actin) remodeling (Burridge, Turner and Romer 1992). These changes in the actin cytoskeleton contribute to alterations in cell morphology, adhesion, and stiffness. Such changes in the mechanical and structural properties of the cell should have significant influence on mechanosensation of the cell to external mechanical stimuli such as fluid induced shear. In this study, we explore the role of ECM proteins in modulating osteoblast adhesion (quantified by adhesion strength), cytoskeleton organization, and cellular responsiveness to mechanical stimulation.
Bone tissue contains several ECM proteins such as type I collagen (Coll), fibronectin (FN), and vitronectin (VN) that adhere osteoblasts via integrins (Burridge, Turner and Romer 1992; Grzesik and Robey 1994; Guan and Shalloway 1992; Nykvist, Tu, Ivaska, Kapyla, Pihlajaniemi and Heino 2000; Tuckwell, Calderwood, Green and Humphries 1995). Integrins are heterodimeric transmembrane receptors that co-localize with signaling proteins and the actin cytoskeleton to form focal adhesions, and are thought to play an important role in implant osseointegration (Geissler, Hempel, Wolf, Scharnweber, Worch and Wenzel 2000; Shah, Lazatin, Sinha, Lennox, Hickok and Tuan 1999). Integrins have also been shown to directly transmit mechanical stimulation (Bierbaum and Notbohm 1998; Maniotis, Chen and Ingber 1997; Peake, Cooling, Magnay, Thomas and El Haj 2000; Salter, Robb and Wright 1997; Wang, Butler and Ingber 1993) through the ECM proteins to the inside of the cell. In addition, integrin mediated binding enhances cell adhesion strength. Specifically, surfaces modified with proteins or peptides containing the RGD integrin ligand motif have been shown to increase adhesion strength in a concentration dependent manner (Garcia, Ducheyne and Boettiger 1998; Rezania, Thomas, Branger, Waters and Healy 1997). Therefore, it is likely that an intimate relationship exists between the adhesion of cells to various ECM proteins, changes in cell shape, reorganization of the cytoskeleton, and cell response to mechanical stimulation.

One of the signaling molecules associated with integrins and is activated upon cell adhesion to ECM proteins is focal adhesion kinase (FAK) (Guan and Shalloway 1992; Krause, Cowles and Gronowicz 2000). FAK activation has also been implicated in cell adhesion (Burridge, Turner and Romer 1992), which is often used as an indicator of implant biocompatibility (Gronowicz and McCarthy 1996). For instance, inhibition of FAK phosphorylation has been shown to retard cell spreading (Abbi, Ueda, Zheng, Cooper, Zhao, Christopher and Guan 2002; Richardson and Parsons 1996), and reduce cell adhesion (Hassid, Yao and Huang 1999; Lee, Hee Kim, Boong Park, Xu, Cance, Block and Scully 2003; Xu, Yang, Craven and Cance 1998) in various cell types. FAK activation also plays a role in mechanotransduction, since mechanical stimulation has been shown to increase FAK phosphorylation and association with the cytoskeleton (Li, Kim, Hu, Jalali, Schlaepfer, Hunter, Chien and Shyy 1997; Seko, Takahashi, Tobe, Kadowaki and Yazaki 1999; Toma, Ashkar, Gray, Schaffer and Gerstenfeld 1997). FAK activation is also involved in changes in the F-actin cytoskeletal architecture. While inhibition of FAK phosphorylation severely limits F-actin fiber formation (Burridge, Turner and Romer 1992), disruption of F-actin fiber formation has also been shown to prevent FAK activation (Haimovich, Lipfert, Brugge and Shattil 1993). FAK mediated cytoskeletal reorganization may change the mechanical properties of the cell, since F-actin contributes to cell stiffness (Rotsch and Radmacher 2000), as well as cell morphology, and these factors in turn may influence adhesion strength. Despite the important relationship between cell adhesion and FAK activation, few studies have examined the relation between FAK activation, subsequent cytoskeletal reorganization, and cell adhesion strength (Asthagiri, Nelson, Horwitz and Lauffenburger 1999).

In addition to alterations in adhesion strength, changes in cell shape and stiffness of osteoblasts plated on various substrates may lead to changes in the local mechanical response (ie. deformation, stress) of the cell to a bulk stimulus, such as fluid shear. It has also been demonstrated that the transmission of mechanical signals in bone cells is dependent on an intact actin cytoskeleton (Ajubi, Klein-Nulend, Nijweide, Vrijheid-Lammers, Alblas and Burger 1996; Pavalko, Chen, Turner, Burr, Atkinson, Hsieh, Qiu and Duncan 1998; Toma, Ashkar, Gray, Schaffer and Gerstenfeld 1997). For example, prostaglandin E2 (PGE2) release in response to fluid shear, which is a well documented signaling pathway in osteoblasts (Bakker, Soejima, Klein-Nulend and Burger 2001; Klein-Nulend, Burger, Semeins, Raisz and Pilebeam 1998; Smalt, Mitchell, Howard and Chambers 1997), is abolished by disruption of the actin cytoskeleton (Ajubi, Klein-Nulend, Nijweide, Vrijheid-Lammers, Alblas and Burger 1997; Reich, McAllister, Gudi and Frangos 1997; Saunders, You, Trosko, Yamashita, Li, Donahue and Jacobs 2001; Small, Mitchell, Howard and Chambers 1997), is abolished by disruption of the actin cytoskeleton (Ajubi, Klein-Nulend, Nijweide, Vrijheid-Lammers, Alblas and Burger 1996). Thus bone cell responses to mechanical stimuli are likely to be modulated by differences in mechanical perturbation of the cytoskeleton and focal adhesion proteins, depending on the ECM substrates.

In this study we have examined the adhesion strength, FAK activation, and cytoskeletal organization, as well as differences in prostaglandin E2 (PGE2) release in response to fluid shear of osteoblasts adhered to substrates...
that bind cells via integrin and non-integrin mediated means.

2 Materials and Methods

2.1 Adhesion Strength Assay

Osteoblast adhesion to seven substrates were examined: glass slides (Fisher Scientific, Pittsburgh, PA) coated with 10\,\mu g/mL (Cowles, Brailey and Gronowicz 2000) calf skin type I collagen (Coll, Cohesion Technologies, Palo Alto, CA); bovine plasma fibronectin (FN, Gibco Life Technologies Inc., Rockville, MD); bovine plasma vitronectin (VN, Gibco Life Technologies Inc.); poly-L-lysine (PLL, Sigma, St. Louis, MO); 55% fetal bovine serum (FBS, Sigma); or phosphate buffered saline (PBS, bare glass), and tissue culture treated plastic slides (TC) coated with PBS. Flexiperm silicone wells were attached to the glass slides and the slides were covered with 500\,\mu L of the various coating solutions and incubated at 37\,\degree C for 90 minutes. To block non-specific binding of the cells, coated glass slides were blocked with 2% bovine serum albumin (BSA) in PBS for 1 hour at 37\,\degree C and rinsed with PBS thoroughly immediately before use.

Several different quantitative methods have previously been used to determine adhesion strength such as uniform laminar flow (parallel plate flow chamber) (van Kooten, Schakenraad, van der Mei, Dekker, Kirkpatrick and Busscher 1994), radial flow (Rezania, Thomas, Branger, Waters and Healy 1997; Rezania, Thomas and Healy 1997), spinning disc (Garcia, Ducheyne and Boettiger 1997; Garcia, Ducheyne and Boettiger 1998), dynamic stretching (Lacouture, Schaffer and Klickstein 2002), and centrifugation (Asthagiri, Nelson, Horwitz and Lauffenburger 1999). In this study, a parallel plate flow chamber, similar to that used by Hung et al., was used to assess cell adhesion and responsiveness on different substrates, since this method allows the application of a uniform wall shear stress over a large area (Hung, Pollack, Reilly and Brighton 1995). A parallel plate flow chamber with a transparent viewing window was attached to a magnetic gear pump (Micropump, Vancouver, WA) and placed under a light microscope equipped with a video camera and image acquisition software Metaview 4.1\,TM (Universal Imaging Corp., West Chester PA). Confluent osteoblast-like MC3T3-E1 cells were detached using trypsin/EDTA and suspended in g-Minimal Essential Medium (\alpha-MEM; Gibco Life Technologies) supplemented with 2% charcoal-stripped fetal bovine serum that was heat inactivated as per manufacturer’s instructions (CS-HI FBS Hyclone Laboratories Inc., Logan, UT), and allowed to recover in suspension for 45 minutes at 37\,\degree C. Osteoblast-like MC3T3-E1 cells were then plated onto the prepared slides at a density of 50,000 cells/cm\(^2\) and allowed to attach for either 15 (n=6) or 60 minutes (n=5). The slides were placed in the flow chamber and subjected to a wall shear stress of 395 dynes/cm\(^2\) for 10 minutes, using \alpha-MEM + 2% CS-HI FBS as the perfusate. Images of the cells in a field 822x614 mm\(^2\) were captured prior to and after flow, and the number of cells in each image was manually counted. The percentage of cells remaining adherent after flow was calculated as the number of cells in a field after flow divided by the number of cells in the same field before flow.

2.2 Focal Adhesion Kinase (FAK) Activation Assay

To investigate FAK activation in MC3T3-E1 cells adhered to different substrates, glass coverslips (48x65 mm) were coated with the various solutions as described above. The coverslips were then placed into 100mm dishes coated with 1% agarose to ensure correct plating density (to prevent cells from migrating underneath coverslips). MC3T3-E1 cells were plated at a density of 50,000 cells/cm\(^2\) on the coated, blocked coverslips, or on TC plastic (not blocked with BSA) after a 45 minute recovery period in suspension at 37\,\degree C. After allowing cells to bind for either 15 (n=3) or 60 minutes (n=4), the cells were harvested and lysed on ice using a buffer solution containing protease inhibitors (5\,\mu g/mL leupeptin and 1M phenylmethylsulfonyl fluoride, Sigma) and a tyrosine phosphatase inhibitor (1mM sodium orthovanadate). The protein concentration in each sample was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). The lysates were subjected to SDS-polyacrylamide gel electrophoresis (8% polyacrylamide gel) with 20\mu g of protein loaded per lane, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford MA). Western blots were performed using an anti-phospho-Y397 FAK (pFAK, BioSource Int., Camarillo, CA) primary antibody and alkaline phosphatase conjugated secondary antibody (Biosource Int.), and detection was performed using CDPstar chemiluminescent substrate (New England Biolabs, Beverly, MA). Cells prior to plating but after the recovery period served as
pre-binding (PB) negative controls, since cells in suspension have decreased tyrosine phosphorylation (Burridge, Turner and Romer 1992). The fluorescent intensities of the bands were detected as arbitrary light units using a Lumi-imager chemiluminescent imaging system (Roche Diagnostics, Indianapolis, IN).

2.3 Staining of the F-actin Cytoskeleton

To examine cytoskeletal organization, osteoblastic cells were plated on various coated slides in a manner similar to the adhesion assay, and fixed with 3.7% formaldehyde after 15 or 60 minutes of plating. Cells were then stained for F-actin using FITC-phalloidin (Sigma). Confocal images of the cells were taken using a Fluoview confocal microscope system (Olympus, Melville, NY) with a 60x objective lens, approximately 2µm from the attachment surface.

2.4 Prostaglandin E2 (PGE\textsubscript{2}) Release on Different Substrates in Response to Flow

MC3T3-E1 cells were plated on glass slides coated with either 10µg/mL FN or PBS (bare glass) and as described above, at a density of 5x10\textsuperscript{4} cell/cm\textsuperscript{2} for 1 hour. Half of the slides were then placed in a Streamer Gold\textsuperscript{TM} (Flexcell Int., Hillsborough, NC) parallel plate flow chamber. The osteoblastic cells in the flow chamber were then subjected to 16 dyne/cm\textsuperscript{2} fluid shear using α-MEM supplemented with 2% CS-HI FBS as the perfusate for 15 minutes (n=6 for FN and glass). Control specimens were placed next to the flow chamber in a 37°C incubator (n=6 for FN and glass). Osteoblastic cells were post-incubated with 200µL of media for 1 hour, and PGE\textsubscript{2} was measured in this media using a PGE\textsubscript{2} enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). The cells on each slide were harvested in PBS, lysed, and the DNA concentration was determined using the Hoechst dye (Bisbenzimide) (Labarca and Paigen 1980). The total PGE\textsubscript{2} released was then normalized to the total amount of DNA per slide, to account for any differences in the number of cells per slide, particularly in those slides subjected to fluid shear.

2.5 Statistics

The difference in the number of adherent cells and pFAK levels between substrate groups was analyzed by one-way analysis of variance (ANOVA) with a Fisher’s post-hoc test for both flow and control (no-flow) conditions. A linear correlation was performed to examine the relationship between FAK activation and adhesion strength. For all statistical analyses, a p value of less than 0.05 was considered statistically significant.

3 Results

3.1 Osteoblast Adhesion Strength to Various Substrates

The adhesion strength of MC3T3-E1 cells on ColI, FN, VN, FBS, PLL, glass, and TC was quantified after 15 and 60 minutes of plating. After both 15 and 60 minutes of plating, FN promoted the strongest adhesion, while cell adhesion to glass and TC was the weakest (Figure 1). FBS and VN, which also adhere osteoblasts through integrin-mediated binding, facilitated high adhesion strength at 15 minutes with higher adhesion compared to glass, TC, and ColI (p<0.05). Interestingly, osteoblast adhesion to ColI was not different from PLL, glass, or TC at 15 minutes, but the percentage of adherent cells increased nearly 3 fold by 60 minutes of plating, and became significantly higher compared to glass (p=0.01). Overall, there was increased adhesion on all substrates between 15 to 60 minute plating.

3.2 Focal Adhesion Kinase Activation

FAK activation on various substrates showed that after both 15 and 60 minutes of plating, FN had the highest pFAK levels. VN and FBS, which also bind osteoblasts via integrins, showed trends of high pFAK after 15 minutes of plating with significantly higher pFAK levels compared to glass, TC, and ColI (p<0.05). Interestingly, osteoblast adhesion to ColI was not different from PLL, glass, or TC at 15 minutes, but the percentage of adherent cells increased nearly 3 fold by 60 minutes of plating, and became significantly higher compared to glass (p=0.01). Overall, there was increased adhesion on all substrates between 15 to 60 minute plating.

FAK activation was positively correlated with adhesion strength (Figure 3), and this correlation was significant (p=0.02, R\textsuperscript{2}=0.7) after 15 minutes of plating. However, this significant correlation was lost at longer adhesion
Figure 1: Percentage of cells remaining after flow on various substrates. ColI=type I collagen; PLL=poly-L-lysine; FN=fibronectin; VN=vitronectin; FBS=fetal bovine serum; TC=tissue culture plastic. A) 15 minute plating. \( * p < 0.001 \) compared to all others, \( ** p < 0.03 \) compared to PLL, FN, VN, and FBS; B) 60 minute plating. \( * p < 0.02 \) compared to all others except FBS, \( ** p < 0.04 \) compared to all except PLL and TC; \( *** p < 0.01 \) compared to FN and FBS.

3.3 Cytoskeletal Architecture

Osteoblasts adhered to FN coated surfaces were well spread, and F-actin staining showed extensive fiber formation extending (Figure 4). In contrast, osteoblasts plated on bare glass showed a more rounded morphology and exhibited minimal F-actin fiber formation. Similar to osteoblasts adhered to FN, osteoblasts plated on VN and FBS were well spread, with many prominent F-actin stress fibers. Interestingly, ColI, which is also an ECM protein, promoted only a few fine F-actin fibers and a stellate morphology. Osteoblasts adhered to PLL were, in general, more well spread than osteoblasts on glass, but showed minimal F-actin fiber formation only in the periphery of the cell. These trends were seen after both 15 and 60 minutes of plating.

3.4 PGE\(_2\) Release

PGE\(_2\) release per microgram DNA in osteoblasts plated on FN was 63% higher compared to that of osteoblasts plated on glass, when subjected to fluid shear (Figure 5; \( * p < 0.001 \)). However, there was no difference in PGE\(_2\) levels of osteoblasts plated on the two substrates in the
absence of flow (control group). For osteoblasts plated on either substrate, the application of fluid shear significantly increased PGE$_2$ release.

4 Discussion

In this study we have demonstrated that osteoblast adhesion strength, focal adhesion kinase (FAK) activation, F-actin fiber formation, and response to fluid shear are all modulated by the substrates to which they adhere. Generally, ECM proteins that bind cells via integrins, FN, VN, and FBS (which contains ECM proteins such as FN, and VN) increased the adhesion strength, pFAK levels,
and F-actin fiber formation in osteoblasts, compared to glass and PLL, which bind cells through non-integrin mediated binding (Cowles, Brailey and Gronowicz 2000; Gronowicz and McCarthy 1996; Guan and Shalloway 1992; Krause, Cowles and Gronowicz 2000; Schneider and Burridge 1994). These findings are in agreement with previous reports of elevated pFAK levels in cells adhered to FN and VN, and low FAK activation levels in cells adhered to PLL (Burridge, Turner and Romer 1992; Guan and Shalloway 1992; Krause, Cowles and Gronowicz 2000). Also, in agreement with this study, the adhesion strengths of osteoblasts plated on FN or VN coated surfaces were found to be higher than that of osteoblasts plated on a surface that adhered cells via non-integrin mediated means, when subjected to cyclic stretch (Lacouture, Schaffer and Klickstein 2002). Increased F-actin fiber formation observed on ECM protein coated substrates, and minimal fiber formation on glass and PLL surfaces is also consistent with previous studies of osteoblasts on FN or PLL coated titanium surfaces (Krause, Cowles and Gronowicz 2000).

FN, which elicited the highest pFAK levels also promoted the strongest adhesion strength. In general, substrates that promoted higher adhesion strenghts and FAK activation also showed more prominent F-actin fiber formation. This higher adhesion strength observed in osteoblasts adhered via integrins is most likely due to alterations in the cytoskeleton, such as increased F-actin fiber formation following FAK phosphorylation, since FAK activation has been implicated in the remodeling of the actin cytoskeleton. Specifically, inhibition of FAK phosphorylation has been shown to disrupt F-actin stress fiber formation (Burridge, Turner and Romer 1992), and FAK is known to phosphorylate and co-localize with the cytoskeletal proteins paxillin (Richardson and Parsons 1996; Schaller and Parsons 1995) and tensin (Richardson and Parsons 1996). The actin cytoskeleton plays an important role in cell adhesion strength in that disruption of actin polymerization significantly reduces the adhesion strength of fibroblasts (Lotz, Burdsal, Erickson and McClay 1989). FAK activation was positively correlated with adhesion strength after 15 minutes of plating, but this correlation was diminished by 60 minutes of adhesion. The loss of correlation between FAK activation and adhesion strength over time may be due to the time course of F-actin remodeling, since enhanced adhesion strength due to F-actin fiber formation has been reported to occur within 15 minutes after initial adhesion in fibroblasts (Lotz, Burdsal, Erickson and McClay 1989).

Since the actin cytoskeleton has been found to contribute to the stiffness of cells (Rotsch and Radmacher 2000), differences in actin stress fiber formation due to adhesion to different substrates should alter the stiffness of cells. Indeed, osteoblasts adhered to FN were found to be stiffer compared to those adhered to glass or PLL (Takai, Costa, Shaheen, Hung and Guo 2005). This increase in cell stiffness may contribute to the increased adhesion strength of osteoblasts plated on FN. The insertion of stress fibers into the focal adhesion is also thought to increase the local stiffness of the adhesion site and to distribute the load more evenly across the adhesion site (Garcia and Gallant 2003). Furthermore, changes in the cytoskeleton occurred concurrently with changes in cell morphology, where osteoblasts adhered to ECM proteins generally exhibited a more well spread (flattened) morphology compared to glass or PLL. These alterations in cell shape are also likely to influence adhesion strength through modulation of local applied forces. Stronger cell binding through integrin mediated binding itself may also contribute to the observed increase in adhesion strength with integrin-mediated binding. In line with this idea, the work needed to separate an atomic force microscope probe tip coated with VN from the surface of osteoblasts was found to be significantly higher than the work needed to separate an uncoated probe tip, suggesting that the adhesion force of integrins are higher than non-integrin surface interaction forces (Kim, Arakawa, Osada and Ikai 2003).

In contrast to other ECM proteins examined, type I collagen, which also binds cells via integrins (Tuckwell, Calderwood, Green and Humphries), overall showed lower adhesion strength, FAK activation, and less fiber formation, at shorter plating times. Collagen interaction with cells has been shown to also involve non-integrin receptors such as discoidin domain receptors (DDR) (Abdulhussein, McFadden, Fuentes-Prior and Vogel 2004; Agarwal, Kovac, Radziejewski and Samuelsson 2002), where overexpression of DDR1α or β in leukocytes resulted in increased adhesion (Kamohara, Yamashiro, Galligan and Yoshimura 2001). Also, maintenance of the native conformation of collagen was found to be necessary for integrin-mediated binding of type I collagen through α1β1 and α2β3 integrins (Gullberg, Gehlsen, Turner, Ahlen, Zijenah, Barnes and Rubin 1992), which

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is RGD independent (Davis 1992; Geissler, Hempel, Wolf, Scharnweber, Worch and Wenzel 2000). Since the collagen used in this study was denatured, it is possible that osteoblast adhesion to the collagen-coated surfaces was partly through non-integrin mediated interactions, and thereby elicited different adhesion mediated interactions from the other ECM proteins. Alternatively, the adhesion to the collagen may also be through αβ3 integrins, which bind to the RGD sequence of denatured type I collagen (Davis 1992). Short-term binding has been reported to be through both α2β3 and αvβ3 integrins, while longer-term binding is mostly through αvβ3 integrins (Rezania and Healy 1999). This finding is consistent with our observations that with longer plating time the adhesion strength of osteoblasts plated on collagen increased to be significantly higher than that of osteoblasts adhered to glass, and that the increase in pFAK level over time was the greatest in osteoblasts plated on collagen.

Osteoblast response to mechanical stimuli in terms of PGE2 secretion was also modulated by cell-surface interactions. We have examined PGE2 secretion in response to fluid shear of osteoblasts adhered to glass or FN because these substrates promoted the lowest and highest adhesion strengths, FAK activation, and F-actin fiber formation, respectively, of the substrates examined. Osteoblasts adhered via integrins (FN), with prominent actin fibers, released significantly more PGE2 compared to osteoblasts adhered via non-integrin mediated means (glass), with few fibers, when subjected to fluid shear. This is consistent with previous findings, which showed that cyclic stretch induced membrane depolarization (Salter, Robb and Wright 1997; Salter, Wallace, Robb, Caldwell and Wright 2000) and osteopontin expression (Carvalho, Schaffer and Gerstenfeld 1998) were inhibited or diminished by blocking integrin-mediated binding. Also, inhibition of focal adhesion formation has been shown to diminish flow induced PGE2 release (Ponik and Pavalko 2004). Taken together, our results, as well as these previous studies, support the idea that integrin-mediated binding, focal adhesion formation, and subsequent cytoskeletal reorganization play an important role in mechanotransduction. Our observation that increased F-actin fibers accompany increased PGE2 secretion is in line with a previous finding that increased PGE2 secretion and cyclooxygenase-2 expression were blocked by disruption of the actin cytoskeleton (Ajubi, Klein-Nulend, Alibas, Burger and Nijweide 1999; Ajubi, Klein-Nulend, Nijweide, Vrijheid-Lammers, Alibas and Burger 1996; Pavalko, Chen, Turner, Burr, Atkinson, Hsieh, Qiu and Duncan 1998), suggesting that mechanically induced activation of the PGE2 pathway is modulated by the degree of F-actin fiber perturbation. Osteoblast cell stiffness is dependent on the substrate to which they bind (Domke, Dannöhl, Parak, Müller, Aicher and Radmacher 1997), and adhesion mediated increases in stiffness are due to F-actin stress fibers (Takai, Costa, Shaheen, Hung and Guo 2005). This increase in cell stiffness has been found to increase the shear stress within the cell under the same applied (macroscopic) wall shear stress (Guo, Takai, Liu and Wang 2001). Therefore, the increase in PGE2 secretion of osteoblasts adhered to FN compared to those on glass, may be due to increased local shear in cells plated on FN under the same applied shear stress, since osteoblasts plated on FN have increased stress fibers and increased cell stiffness.

The modulation of PGE2 secretion on different substrates was only seen in the presence of mechanical stimulation, which suggests that integrin occupancy alone is insufficient to alter PGE2 secretion. Integrin occupancy with fibronectin alone, in contrast, has been shown to upregulate OPN gene expression in osteoblasts, while adhesion to vitronectin did not (Carvalho, Schaffer and Gerstenfeld 1998). This illustrates that both the nature of integrin adhesion, as well as concurrent mechanical stimulation play a role in modulating downstream signaling events. The necessity of mechanical stimulation, in addition to integrin mediated binding to elicit changes in some bone cell responses may in part explain the lack of increased osteoblast anabolic activities or differentiation observed in some studies of implant surfaces coated with ECM proteins (Becker, Geissler, Hempel, Bierbaum, Scharnweber, Worch and Wenzel 2002; van den Dolder, Bancroft, Sikavitas, Spauwen, Mikos and Jansen 2003). Also, for future studies, it would also be of interest to examine modulation of osteoblast response to mechanical stimuli due to long-term osteoblast-substrate interactions, since this study has focused on short-term osteoblast-substrate interactions.

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