

Integrin Expression and Osteopontin Regulation in Human Fetal Osteoblastic Cells Mediated by Substratum Surface Characteristics

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ABSTRACT

Integrin-mediated adhesion of anchorage-dependent cells to scaffolds is a critical component of tissue engineering. We investigated integrin expression by the human fetal osteoblastic cell line, hFOB 1.19 (hFOB), as a function of substratum surface wettability. The influence of surface wettability on bone cell phenotype was also examined. Plasma-treated quartz (PTQ) and glass (PTG) (hydrophilic, contact angles of 0°), octadecyltrichlorosilane-treated quartz (STQ) and glass (STG) (hydrophobic, contact angles above ca. 100°), and tissue culture polystyrene were used for cell culture. hFOB cells cultured on hydrophilic substrata displayed well-developed actin stress fibers relative to cells on hydrophobic substrata. Western blot analysis revealed that hFOB cells cultured on hydrophobic substrata (STQ or STG) express lower levels of α_v and β_3 integrin subunits than cells on hydrophilic substrata (PTQ or PTG). This effect was more pronounced in cells on STQ than on STG. These variations in integrin expression were lessened by extended culture time. Double-labeled integrin/actin immunofluorescence confirmed western blot results, i. e., cells cultured on PTQ displayed distinct, large plaques of α_v and β_3 subunits and integrin $\alpha_v\beta_3$, as well as their colocalization with actin stress fiber ends, whereas cells on STQ did not display integrin plaques after 24 h and only displayed minimal plaque formation after 3 days. Vinculin, a focal adhesion protein that mediates binding between the integrin and actin cytoskeleton, appeared in western blot to mimic the variations of α_v and β_3 expression with respect to surface wettability. Interestingly, real-time RT-PCR analysis showed that hFOB cultured on hydrophobic substrata, which have downregulated α_v and β_3 integrin subunits, displayed greater steady-state mRNA levels of osteopontin, an extracellular matrix (ECM) protein containing Arg-Gly-Asp (RGD) integrin recognition sequence, than cells cultured on hydrophilic substrata. Our results imply that substratum surface wettability regulates integrin-mediated bone cell adhesion and further influences the expression of bone cell-ECM complexes.

INTRODUCTION

Tissue engineering has received increasing attention during the last decade as a potential method for healing traumatized tissue. Many tissue engineering strategies have utilized three-dimensional scaffolding materials for in vitro cell seeding and growth followed by in vivo transplantation.¹ Recently, considerable progress has been made in the attempt to regenerate various tissues including bone, cartilage, blood vessel, liver, myocardium, etc.²⁻⁸ Despite this progress, scaffolding materials have yet to be optimized, especially as regards their surface characteristics (chemistry, topography, surface energy, etc.) and morphology (porosity, pore size, pore connectivity, etc.).^{9,10}

We have investigated bone cell-substratum interaction focusing on substratum surface energy (wettability) by comparing the growth of a human fetal osteoblastic cell line, hFOB 1.19 (hFOB), on glass and quartz substrata with and without octadecyltrichlorosilane (OTS)-treatment. These paired specimens represent the extremes of water wettability, representing hydrophilic and hydrophobic categories, respectively.¹¹ We previously demonstrated that hFOB adhesion and proliferation positively correlated with substratum wettability and that cells exhibited a strong preference for relatively hydrophilic substrata over hydrophobic counterparts.¹² These trends mirrored results reported for soft-tissue cells,¹³⁻¹⁵ except for a cell preference for hydrophilic quartz over hydrophilic glass. Apparently, glass is somewhat cytotoxic whereas crystalline quartz facilitates hFOB adhesion and proliferation. Perhaps more interesting was that substratum surface chemistry/energy influenced cell phenotype through as-yet-undefined mechanisms that profoundly affect cell physiology.

Long-term cell-substratum compatibility is mediated by various biological molecules such as extracellular matrix (ECM) proteins, membrane proteins, and cytoskeleton proteins. Of particular interest are the transmembrane integrins that act as receptors for the ECM and are linked to the intracellular actin cytoskeletons via focal adhesion proteins such as vinculin, talin, and paxillin.^{16,17} Integrins, molecules at the interface between intracellular and extracellular compartments, play a role in signal transduction and gene regulation influencing various cell functions.¹⁸⁻²⁸ Integrins are heterodimers composed of α and β

subunits, and their combinations determine the ability of a cell to attach to particular ECM. A variety of integrin heterodimers including $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$, have been identified in bone cells and cultured osteoblastic cells.²⁰⁻²⁴ These integrins bind to various bone ECM proteins including type I collagen (Col-I), osteopontin (OP), vitronectin, fibronectin, and bone sialoprotein. This binding is dependent on specific interactions between integrin receptors and Arg-Gly-Asp (RGD) integrin recognition sequences in ECM.²⁹⁻³¹ Studies of bone cells cultured on ECM-coated surfaces have demonstrated several such interactions including $\alpha_2\beta_1$ -collagens, $\alpha_5\beta_1$ -fibronectin, $\alpha_v\beta_3$ -vitonectin, and $\alpha_v\beta_3$ -osteopontin, and demonstrated their significance in bone cell adhesion, growth, differentiation, and mechanotransduction.³²⁻³⁸

In addition to studies investigating the effect of ECM proteins on integrin expression, some studies have investigated the effect of substratum per se, in the absence of ECM proteins, on integrin expression.³⁹⁻⁴¹ For example, primary human osteoblasts grown on titanium and cobalt-chrome exhibited a notable absence of α_3 , α_5 , and α_6 subunits compared to cells on ECM-coated tissue culture polystyrene (TCPS).⁴⁰ Integrin expression by primary human osteoblasts cultured on two biodegradable polymers was examined, and expression of α_2 , α_5 , and β_1 subunits was greater on poly(lactide-co-glycolide) (PLGA) than on polylactide (PLA).⁴¹ These results strongly suggest that osteoblast adhesion to various substrata is mediated by differential integrin expression profiles. Consequently, we hypothesized that substratum surface energy effects on hFOB adhesion and proliferation would positively correlate with integrin expression and potentially mediate osteoblastic differentiation. We examined the expression of integrin subunits by hFOB cells cultured on substrata having extremes of water wettability and investigated the potential effect of differential integrin expression on bone cell-ECM interactions.

MATERIALS AND METHODS

Cell culture

hFOB 1.19 cells, originally provided by Dr. Steven Harris (Bayer, West Haven, CT),⁴² were

cultured in DMEM-Ham's F-12 1:1 media (GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone), 1% (v/v) penicillin-streptomycin, 10^{-8} M menadione (Sigma), 100 $\mu\text{g/ml}$ ascorbic acid (Sigma), and 10^{-8} M 1,25-dihydroxy vitamin D₃ (Biomol) in TCPS dishes and incubated at 37°C in 5% CO₂ in air. Media was changed every three days. Cells were routinely removed from TCPS by rinsing in phosphate buffered saline (PBS) and incubating in trypsin-EDTA solution. Cells were seeded on each test substrata at 2×10^4 cells/cm² in the same media for all assays. Cells were allowed to adhere for 3 h, rinsed with PBS, and adherent cells allowed to proliferate until the time of assay with media changed every three days.

Hydrophilic and hydrophobic substrata

Monolithic quartz and glass substrata in the form of microscope slides were rendered fully-water-wettable by air-plasma-discharge treatment at 13.56 MHz and 100 mtorr in an inductively-coupled plasma cleaner after consecutive washing in distilled water, isopropyl alcohol, and chloroform. Plasma-treated quartz (PTQ) and glass (PTG) substrata were hydrophobized by reaction with a 5% (v/v) OTS solution in chloroform at reflux temperature, minimizing the contact with air by keeping surfaces in chloroform vapor. OTS-treated quartz (STQ) and glass (STG) were rinsed three times with fresh chloroform to remove non-reacted OTS and were dried in air. TCPS served as a control.

The water contact angles of test surfaces were quantified by using a commercially available automated tensiometer (First Ten Angstroms Inc., Portsmouth, VA). A liquid-handling robot deposited 10 μL of distilled-deionized water (18 M Ωcm obtained from a Millipore Simplicity System) onto a test surface that was supported on a rotary table inside a humidified (> 99% RH) chamber. Droplets were viewed with a magnifying camera and images were captured by a frame grabber at 1 image/s while the rotary table was tilted at a rate of 10°/min. Advancing and receding contact angles were measured at a final substrate tilt angle of 35° after wetting dynamics had come to steady-state. Means and standard deviations ($n = 10$) are reported.

Western blot analysis

hFOB cells were collected on day 3 and 6 and lysed in a 0.1% (v/v) Triton X-100 Tris-EDTA solution with protease inhibitors (Calbiochem). Approximately 40 μ g of protein was fractionated by SDS-PAGE on a 10% gel (Gradipore) and electro-transferred to a polyvinylidene fluoride film (Bio-Rad). The blots were blocked with 1% (w/v) bovine serum albumin (BSA) solution in TBS-T (10 mM Tris, 150 mM NaCl, and 0.05% (v/v) Tween 20) for 2 h at pH 7.5 to minimize non-specific protein-protein interactions. The blots were then incubated for 1 h in 1% (w/v) BSA in TBS-T solution containing 1:500 of either rabbit antibodies specific to integrin subunits of α_2 , α_3 , α_4 , and α_5 or mouse antibodies specific to α_v , β_1 , and β_3 (α_2 : AB1936, α_3 : AB1920, α_4 : AB1924, α_5 : AB1949, β_1 : MAB1965, Chemicon; α_v : sc-9969, β_3 : sc-13579, Santa Cruz Biotechnology). After washing, the blots were incubated with appropriate HRP-conjugated secondary antibodies. The blots were stringently rinsed and bands were then visualized by reacting blots with Amersham ECL detection reagents and exposing to Kodak X-Omat AR film. Blots were stripped by incubation at 55°C in a 0.1 M glycine solution at pH 2.8 and probed for the loading control GAPDH by using a mouse monoclonal antibody (Accurate Chemical and Scientific Corporation, YBG46999555) at 1:5000. The abundance of integrin and control GAPDH were quantified by densitometry. For each of the integrin subunits, three western blot experiments were performed by using protein samples from different experiments. For each blot, integrin expression was normalized to GAPDH by dividing optical density (OD) of integrin subunit by OD of GAPDH. Average and standard deviations of three GAPDH-normalized values were obtained and are presented as a percentage ratio compared to the TCPS result on day 3 (therefore, percentage of integrin expression for TCPS on day 3 is set to 100%). A representative blot from three blots completed is shown and the variation between blots can be seen as standard deviation. Western blot analysis for vinculin expression in hFOB cells was performed in the same way by using mouse antibody specific to vinculin (Sigma, V4505) at 1:1000.

Immunofluorescence assay

Actin protein formation by hFOB cells on different substrata was examined 3 and 24 h post-seeding. Cells were washed with PBS, fixed with 4% (w/v) formaldehyde in PBS for 10 min, rinsed with PBS, and permeabilized for 5 min with 0.1% (v/v) Triton X-100 (Sigma) in PBS. After washing, cells were preincubated for 20 min with 1% (w/v) BSA solution in PBS to minimize non-specific protein-protein interactions. Actin was stained for 20 min using a Rhodamine Phalloidin (Molecular Probes, R-415) solution in PBS (1:100) with 1% (w/v) BSA. After washing, cells were observed using a Nikon OPTIPHOT-2 fluorescent microscope with an ex 541 nm filter.

For integrin subunits displaying significant changes by western blot analysis, results were confirmed by immunofluorescence assay. After 1 and 3 days of culture, cells were fixed, permeabilized, and incubated with a BSA solution as described above. Cells were exposed for 1 h to PBS solution with 1% (w/v) BSA and mouse monoclonal antibodies (1:100) for either α_v and β_3 integrin subunits or $\alpha_v\beta_3$ integrin (α_v : sc-9969, β_3 : sc-13579, Santa Cruz Biotechnology; $\alpha_v\beta_3$: MAB1976Z, Chemicon). Cells were rinsed and incubated for 1 h with FITC-conjugated goat anti-mouse IgG secondary antibody (Chemicon, AP124F) solution in PBS (1:50) with 1% (w/v) BSA. To simultaneously detect actin, cells were double-labeled with TRITC-conjugated phalloidin (Chemicon, FAK100 kit-90228) at 1:250 in the same secondary antibody solution for integrin. Double-labeled immunofluorescence of α_v /actin, β_3 /actin, and $\alpha_v\beta_3$ /actin was observed using filters appropriate for TRITC and FITC.

Real-time RT-PCR analysis

RNA was isolated on day 3 and 6 using a RNeasy kit (Qiagen). Real-time RT-PCR was performed with a Perkin-Elmer ABI Prism 7700 sequence detector. Human OP and Col-I (α) cDNA primers and probes were designed from previously reported sequence data^{43,44} using real-time RT-PCR probe/primer design software, Primer Express 1.0 (Perkin-Elmer). The sequences designed for OP were 5'-CGC GGA CCA AGG AAA ACT CAC TAC CA-3' (probe), 5'-TTG CAG CCT TCT CAG CCA A-3'

(forward primer), and 5'-CAA AAG CAA ATC ACT GCA ATT CTC-3' (reverse primer). The sequences used for for Col-I were 5'-CAA GTC GAG GGC CAA GAC GAA GAC A-3' (probe), 5'-CGC ACG GCC AAG AGG A-3' (forward primer), and 5'-ACG CAG GTG ATT GGT TGG G-3' (reverse primer). We followed previously published protocols for reverse transcription reaction, PCR reaction, and calculation of the ratio of each mRNA to 18S mRNA by using 2.5 μ l of mRNA (20 ng/ml).⁴⁵

Statistics

Western blot and real-time RT-PCR experiments were performed three times, each sample obtained from three different cultures. Means and standard deviations are reported. Statistical significance between the hydrophilic and hydrophobic counterpart substrata assessed by a student's t-test (*: $p < 0.05$ and **: $p < 0.01$) is shown.

RESULTS

Water wettability of test substrata is reported in Table 1 as advancing and receding water contact angles (θ) and corresponding water adhesion tensions ($\tau = \gamma \cos \theta$, where γ is water surface tension = 72.8 dyne/cm, computed for both advancing and receding θ) as a measure of substratum surface energy.¹² PTQ and PTG were fully-water-wettable ($\theta = 0^\circ$, high surface energy herein termed hydrophilic), whereas STQ and STG were poorly-water-wettable ($\theta \sim 100^\circ$, low surface energy herein termed hydrophobic). These substrata are representatives of extremes of water wettability between which most tissue engineering scaffolding materials fall. For comparison, Table 1 shows the surface energetics of two biodegradable polymers of PLA and PLGA that are FDA-approved and currently the most widely used materials for tissue engineering scaffolds as well as for implant materials. The wettability of test substrata was maintained for the long-term culture period, because cell culture did not alter chemistry of the substrate.

After 3 h of adhesion, hFOB cells on PTQ and PTG displayed a more spread-out morphology relative to cells on STQ and STG. Immunofluorescence of samples 3 h post-seeding demonstrated that

hFOB cells on PTQ had actin stress fibers, while cells on STQ did not (Fig. 1). After 24 h of culture, cells on PTQ displayed a well spread and inter-connected morphology with well-developed actin stress fibers. However, a small number of cells adhered to STQ, proliferated uni-directionally, became spindle-shaped, and actin stress fibers began to form. Similar differences were observed for PTG and STG, and cells on TCPS displayed similar cell morphology and actin stress fibers to PTQ and PTG cultured cells (not shown).

Western blot analysis revealed that, regardless of substrata examined, hFOB cells expressed, in the presence of serum, all the integrin subunits examined in this study (α_2 , α_3 , α_4 , α_5 , α_v , β_1 , and β_3). However, the relative abundance of expression was affected by substratum surface energy (Fig. 2). The α_2 subunit composing collagen I-IV receptor,^{33,35} α_3 composing multiple receptors for collagen, laminin, and fibronectin,³⁷ and α_4 known to bind fibronectin,²² did not show statistically significant differences with substratum wettability. The α_5 subunit, a component of the fibronectin receptor $\alpha_5\beta_1$,^{29,30} displayed higher expression in hFOB cells on STQ relative to cells on PTQ, but only on day 3 ($p < 0.05$). The α_v subunit, which together with β_3 composes the vitronectin receptor,^{21,23,38} exhibited the most dramatic variation with respect to surface energy. hFOB cells cultured for 3 days on both STQ and STG showed significantly lower α_v expression than cells grown on PTQ and PTG, e. g., there was a three-fold decrease of α_v in hFOB on STQ relative to cells on PTQ on day 3 ($p < 0.01$). α_v expression increased in cells with time in culture regardless of the substrata (day 6 versus day 3). On day 6, α_v expression remained higher in hFOB cultured on PTQ relative to STQ ($p < 0.05$), however expression on PTG was similar to STG. The β_1 subunit, which forms heterodimers with numerous α subunits (α_2 , α_3 , α_4 , α_5 , and α_v) and binds to various ECM components,^{20,21,31} showed no significant differences in expression correlated with substrate wettability. On the other hand, β_3 , which binds specifically with α_v , varied with substratum wettability in a manner similar to the variations of α_v . This effect was particularly pronounced in hFOB cells cultured on quartz samples, i. e., significantly higher β_3 levels were detected in cells on PTQ relative to those on STQ on day 3 ($p < 0.05$). As with α_v , these differences became less pronounced with extended time in

culture (on day 6). hFOB cells cultured on TCPS displayed a less obvious trend in integrin expression. However, cells on TCPS displayed significantly higher α_v expression relative to cells cultured on STQ for 3 and 6 days, and on STG and PTG for 3 days.

The changes in integrin α_v and β_3 subunit levels observed in western blot analysis were confirmed by immunofluorescence assays (Fig. 3). hFOB cells cultured on PTQ displayed distinct, large plaques of integrin α_v and β_3 subunits for both 24 h (not shown) and 3 days. Whereas cells cultured on STQ did not display integrin plaques after 24 h (not shown) and only displayed minimal plaque formation after 3 days. This is consistent with the western blot results. Cells displayed the same immunoreactivity variation in the full unit of integrin $\alpha_v\beta_3$ on PTQ and STQ (Fig. 3). Furthermore, double-labeling with antibody against cytoskeletal actin protein revealed that α_v , β_3 , and $\alpha_v\beta_3$ plaques colocalize with the actin stress fiber ends, and this was also more pronounced in cells on PTQ than on STQ.

Expression of vinculin, one of the focal adhesion proteins that acts to link membrane proteins to actin fiber networks, was significantly lower in hFOB cells cultured on STQ relative to those cultured on PTQ on day 3 ($p < 0.05$) (Fig. 4). However, this difference was not significant on day 6. For cells cultured on PTG and STG, no significant difference in vinculin expression was found.

Real-time RT-PCR analysis (Fig. 5) revealed that hFOB cells grown for 3 days on either STQ or STG exhibited significantly higher steady-state OP mRNA levels than those grown on PTQ and PTG, respectively ($p < 0.01$ for quartz and $p < 0.05$ for glass samples, respectively). In cells grown on STQ the upregulation of OP mRNA still reached levels of significance on day 6 of culture (STQ versus PTQ, $p < 0.05$). Extended periods of culture (day 12 or 15) resulted in decreased OP mRNA levels on the whole and the differences with respect to surface wettability became insignificant (not shown). Col-I mRNA expression showed no significant differences with respect to substratum surface wettability.

DISCUSSION

We previously observed that hFOB adhesion and proliferation exhibit a strong correlation with

substratum surface energy.¹² These data showed hFOB adhesion to be faster and the rate of proliferation greater on hydrophilic surfaces ($30 < \tau < 73$ dyne/cm) compared to hydrophobic surfaces ($-40 < \tau < 30$ dyne/cm). Adherent cell number at 3 h post-seeding was over six-fold higher on PTQ (72%) compared to STQ (11%) and two-fold higher on PTG (39%) than on STG (19%) (in these experiments 55% of cells adhered to TCPS after 3 h). While it is generally accepted that hydrophilic substrata support cell adhesion and proliferation better than hydrophobic substrata,^{9,12-15} contrary observations have been reported for poly(ethylene glycol) (PEG) modified PLA.⁴⁶ The adhesion and proliferation of marrow stromal cells cultured on PEG-PLA diblock copolymer films, which have lower contact angles than PLA films, was shown to be lower than on PLA films. However, PEG-PLA films enhanced both the alkaline phosphatase activity and mineralization ability of marrow stromal cell cultures more strongly than did PLA films. These contradicting findings suggest that the effect of substratum water wettability on bone cell adhesion, proliferation, and differentiation must be re-examined in more detail in order to develop optimal scaffolds for bone tissue engineering. As a first step in this direction, we examined integrin expression by osteoblastic cells on materials of different surface energy.

Integrin-mediated osteoblast adhesion, because of its significant effects on bone cell growth and differentiation,²⁹⁻³¹ is one of the more important considerations in pursuing optimal bone cell-material interactions. From a tissue engineering perspective, potential effects of scaffold surface characteristics (topography, wettability, surface chemistry, etc.) on bone cell behavior are fundamental considerations in developing optimal biomaterial scaffolds for bone tissue regeneration. In this study, integrin expression by hFOB cells was examined as a function of substratum surface energy (hydrophilicity/ hydrophobicity) and its potential effect on osteoblastic differentiation was investigated.

A variety of integrin subunits (α_2 , α_3 , α_4 , α_5 , α_v , β_1 , and β_3) were expressed by hFOB cells, in the presence of serum, on all the substrata examined in this study. Several contradictory observations as to the repertoire of integrins expressed by osteoblasts have been reported.^{20-24,40} For instance, α_2 , α_3 , and β_3 subunits were not detected in either human osteoblasts or bone lining cells *in vivo*.²¹ However, these subunits have been shown to be expressed by primary human osteoblasts cultured *in vitro* using serum

supplemented media.⁴⁰ Thus, human osteoblastic cell integrin expression appears to adapt differently to serum used in vitro than to the in vivo milieu. This is not surprising since interstitial fluid in vivo has different components, growth factors, mitogens, etc. than serum used in vitro. These differences would be expected to alter integrin expression.

Previous studies suggest that substratum per se substantially influences integrin expression.³⁹⁻⁴¹ For example, α_3 , α_5 , and α_6 subunits were absent when primary human osteoblasts were cultured on titanium or cobalt-chrome,⁴⁰ and PLGA supported higher α_2 , α_5 , and β_1 expression than did PLA.⁴¹ In this study, we investigated whether a well-defined substratum surface characteristic, surface wettability, can regulate integrin expression. The integrin expression profile (Fig. 2) is summarized below. α_v and β_3 subunit expression was significantly lower in hFOB cells cultured on hydrophobic substrata than on hydrophilic counterparts. This was more pronounced for α_v than β_3 and between quartz samples than between glass samples. These effects were more pronounced on day 3 than on day 6. The other integrin subunits did not show any statistically significant differences correlated to surface wettability except for α_5 , which on day 3 showed higher expression on STQ than PTQ. Consult the surface energetics in Table 1 for ‘hydrophilic’ and ‘hydrophobic’ terms used herein.

We observed distinct differences in cell morphology and cytoskeletal organization depending on substratum wettability, i. e., a more spread-out cell morphology with well-developed actin stress fibers was seen in cells cultured on hydrophilic substrata relative to cells on hydrophobic substrata (Fig. 1). We speculate that differences in adhesion preference observed previously¹² and in cell morphology might be explained by different integrin expression profiles. For example, the lower α_v and β_3 expression in hFOB cells on hydrophobic substrata likely contributes to decreased attachment, a less spread cell morphology, and subsequently decreased proliferation. This interpretation is consistent with the observation by Pistone et al.,²⁹ in which primary human osteoblasts cultured in the presence of serum on non-ECM-coated glass (the same conditions as our study) only showed localization of α_v and β_3 subunits in focal contacts, while α_5 and β_1 were observed in focal contacts when cells were cultured in serum free media on fibronectin-

coated surfaces. These results imply that $\alpha_v\beta_3$ is the major integrin responsible for focal contacts in bone cell adhesion on non-ECM-coated surfaces in the presence of serum. Therefore, the reduction in α_v and β_3 expression may partly explain the decreased adhesion of hFOB cells on hydrophobic substrata.

Interestingly, variations in vinculin expression as a function of substratum wettability resembled those of α_v and β_3 integrin subunits (Fig. 4). Focal adhesion proteins, such as paxillin, talin, vinculin, etc., link the actin cytoskeleton to integrins at sites of focal contacts. Thus, higher expression of vinculin in hFOB cells on PTQ on day 3 correlates with higher α_v and β_3 at the same time point. Again, vinculin expression suggests that $\alpha_v\beta_3$ is a major integrin responsible for focal cell adhesion, since both integrin subunits α_v and β_3 and vinculin are regulated by substratum surface energy in a similar manner.

A role for integrin $\alpha_v\beta_3$ and vinculin in focal adhesion of hFOB cells is strongly supported by integrin/actin immunofluorescence data (Fig. 3). Colocalization of actin stress fiber ends with α_v , β_3 , and $\alpha_v\beta_3$ indicate that focal contacts are formed at integrin $\alpha_v\beta_3$ plaques, that is, cytoskeletal proteins are anchored at integrin $\alpha_v\beta_3$ plaques and tensioned to form actin stress fibers. Such anchoring processes are mediated by linker proteins such as vinculin. Therefore, our results indicate that the integrin-vinculin-actin signal transmitting structure is regulated by substratum surface energy and that integrin $\alpha_v\beta_3$ plays a more important role in hFOB cell adhesion relative to other integrins. Similarly, even though higher α_5 expression was observed in cells cultured on STQ relative to cells on PTQ on day 3, we suggest that α_5 is probably not dominant in mediating the formation of focal adhesion structures, relative to $\alpha_v\beta_3$. This is supported by the lack of correlation between α_5 variation and cell adhesion, spreading, and vinculin and actin variation.

We also demonstrated potential effects of substratum wettability on the expression of bone cell phenotypic markers. hFOB cells cultured on hydrophobic substrata displayed significantly higher steady-state OP mRNA levels relative to cells on hydrophilic substrata (Fig. 5). It has been shown that integrin $\alpha_v\beta_3$, the so-called vitronectin receptor, also mediates osteoblast adhesion to other non-collagenous ECM proteins including OP, fibronectin, and bone sialoprotein.^{21,23,34,38} The observation that hFOB cultured on

hydrophobic substrata downregulate α_v and β_3 expression while upregulating OP mRNA levels suggests that cell adhesive proteins, other than OP, present in serum may be initially incorporated for immediate bone cell adhesion. After an extended time in culture the role of serum ECM proteins in hydrophobic culture may become redundant due to the upregulation of OP secretion. This suggestion also explains the reduced effects on integrin and mRNA expression seen over time between hydrophobic and hydrophilic cultures. Similarly, it was suggested that focal adhesion of primary human osteoblasts via $\alpha_v\beta_3$ is most likely due to serum vitronectin, since other components of serum, such as fibrinogen, thrombospondin, and Von Willebrandt factor, did not support cell spreading.²⁹ In this regard, again, the use of serum proteins in cell adhesion, proliferation, and differentiation is another important consideration in developing bone tissue engineering protocols.

Since OP levels are increased in cells on hydrophobic substrata, to which cells adhere poorly, an intriguing possibility is that cells, by upregulating OP containing RGD integrin recognition sequences, are trying to condition the surface for optimal cell growth. This is partly supported by the observation that differences in α_v and β_3 integrin subunits, vinculin, and OP expression in hFOB cells as a function of surface wettability decreased with culture time, suggesting that substrata had indeed been conditioned to overcome differences in surface energy. This is consistent with our previous observation that hFOB cells cultured on substrata with different surface energies for 9-12 days, presumably long enough for cells to condition the substrate, had similar alkaline phosphatase activities.¹² On the other hand, steady-state Col-I mRNA, which binds to $\alpha_2\beta_1$ integrin,^{33,35} did not show significant variation with substratum wettability, as was also the case for α_2 and β_1 integrin subunits.

It is important to note that differences in α_v , β_3 , vinculin, and OP expression between cells on PTQ and STQ were greater than those seen between cells on PTG and STG, even though the surface wettability differences between those two pairs were similar. This is also consistent with our previously reported data on attached cell number. For example, the difference between cells adhering to PTQ versus STQ is far greater than that between cells on PTG versus STG.¹² This suggests that additional surface

characteristics other than surface energy, for example, surface chemistry or crystalline order, contribute to differences in integrin-mediated adhesion and osteopontin mRNA expression.

In conclusion, our results suggest that substrate surface energy (hydrophilicity/hydrophobicity), one of the more well-defined material surface parameters, affects integrin-mediated osteoblast adhesion by regulating specific integrin expression ($\alpha_v\beta_3$). In addition, combined with our previous results,¹² these data indicate that specific aspects of bone cell differentiation and interactions with ECM are mediated by substratum surface wettability possibly via regulation of specific integrins. Therefore, biomaterial surface wettability is an important consideration not only for initial cell adhesion but also for subsequent cell differentiation, both being important in developing optimized biomaterials for bone tissue engineering applications.

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TABLE 1. SURFACE CHARACTERISTICS OF TEST SUBSTRATA USED FOR CELL CULTURE AND TWO BIODEGRADABLE POLYMERS WIDELY USED FOR TISSUE ENGINEERING SCAFFOLDS

<i>Substrata</i>	<i>Contact angle^a</i> <i>(θ, °)</i>		<i>Water adhesion tension^a</i> <i>(τ, dyne/cm)^b</i>	
	<i>Advancing</i>	<i>Receding</i>	<i>Advancing</i>	<i>Receding</i>
Plasma-treated quartz (PTQ)	0±0.0	0±0.0	72.8±0.0	72.8±0.0
OTS ^c -treated quartz (STQ)	112.7±1.7	98.1±0.8	-28.1±2.0	-10.3±1.0
Plasma-treated glass (PTG)	0±0.0	0±0.0	72.8±0.0	72.8±0.0
OTS-treated glass (STG)	105.9±0.4	97.1±2.7	-19.9±0.6	-9.0±3.4
Tissue culture polystyrene (TCPS)	55.0±1.5	45.0±0.8	41.8±1.6	51.5±0.7
Poly(lactide) (PLA) ^d	78.0±0.8	66.3±0.6	15.1±1.0	29.3±0.7
Poly(lactide-co-glycolide) (PLGA) (7/3) ^d	73.5±2.2	64.4±1.8	20.7±2.7	31.5±2.1

^a The unit of variability is standard deviation ($n = 10$).

^b Water adhesion tension (τ) was calculated from water contact angle (θ) ($\tau = \gamma \cos \theta$, where γ is water surface tension of 72.8 dyne/cm) and was used herein as a measure of substratum surface energy.

^c OTS: octadecyltrichlorosilane.

^d Surface energetics are quoted from previous report.¹²

LIST OF FIGURE LEGENDS

Fig. 1. Actin immunofluorescence in hFOB cells cultured on PTQ and STQ for 3 h and 24 h (See Table 1 for substrata notation). At 3 h, distinct actin stress fibers are visible within cells on PTQ, while they are rare in cells on STQ. At 24 h, cells cultured on PTQ display a more spread-out cell morphology with well-developed actin stress fibers, whereas a more spindle-shaped cell morphology with less-developed actin stress fibers is displayed by cells on STQ. Bars for 3 h and 24 h are 20 μm and 100 μm , respectively.

Fig. 2. Integrin protein subunit expression, as assessed by western blot, in hFOB cells cultured on substrata with varying surface energy (See Table 1 for substrata notation): (a) α_2 , (b) α_3 , (c) α_4 , and (d) α_5 . GAPDH-normalized values ($n = 3$) are shown relative to TCPS result on day 3 (see MATERIALS AND METHODS). The α_5 subunit displays higher expression in hFOB cells cultured on STQ than on PTQ on day 3 (*: $p < 0.05$).

Fig. 2. (Continued) (e) α_v , (f) β_1 , and (g) β_3 . α_v subunit expression is significantly lower in hFOB cells cultured on STQ than on PTQ (**: $p < 0.01$) and on STG than on PTG (*: $p < 0.05$) on day 3. The β_3 subunit displays lower expression in cells on STQ than on PTQ on day 3 (*: $p < 0.05$). On day 6, only α_v shows lower expression in cells on STQ than on PTQ (*: $p < 0.05$).

Fig. 3. Integrin/actin double-labeled immunofluorescence in hFOB cells cultured for 3 days on PTQ and STQ: (a) integrin α_v subunit/actin, (b) integrin β_3 subunit/actin, and (c) integrin $\alpha_v\beta_3$ /actin (See Table 1 for substrata notation). Cells cultured on PTQ display distinct, large plaques of integrin α_v and β_3 subunits and the full $\alpha_v\beta_3$ unit, all of which are colocalized with actin stress fiber ends. Cells on STQ display much less pronounced development in those structures.

Fig. 4. Vinculin protein expression, as assessed by western blot, in hFOB cells cultured on substrata with varying surface energy (See Table 1 for substrata notation). GAPDH-normalized values ($n = 3$) are shown relative to TCPS result on day 3 (see MATERIALS AND METHODS). Vinculin expression was significantly lower in cells cultured on STQ relative to cells on PTQ on day 3 (*: $p < 0.05$).

Fig. 5. Steady-state OP (a) and Col-I (b) mRNA levels, assessed by real-time RT-PCR, in hFOB cells cultured on substrata with varying surface energy (See Table 1 for substrata notation). 18S-normalized data reveal that steady-state OP mRNA levels are higher in cells cultured on STQ than cells on PTQ (**: $p < 0.01$) and in cells on STG than cells on PTG (*: $p < 0.05$) on day 3. On day 6, OP mRNA levels are higher in cells on STQ than in cells on PTQ (*: $p < 0.05$) but not for glass samples. No significant differences are found for Col-I.

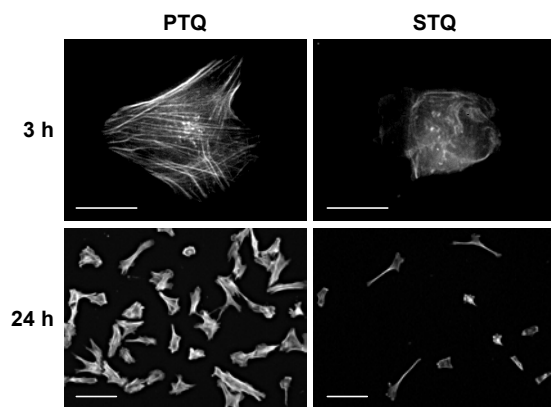


Fig. 1

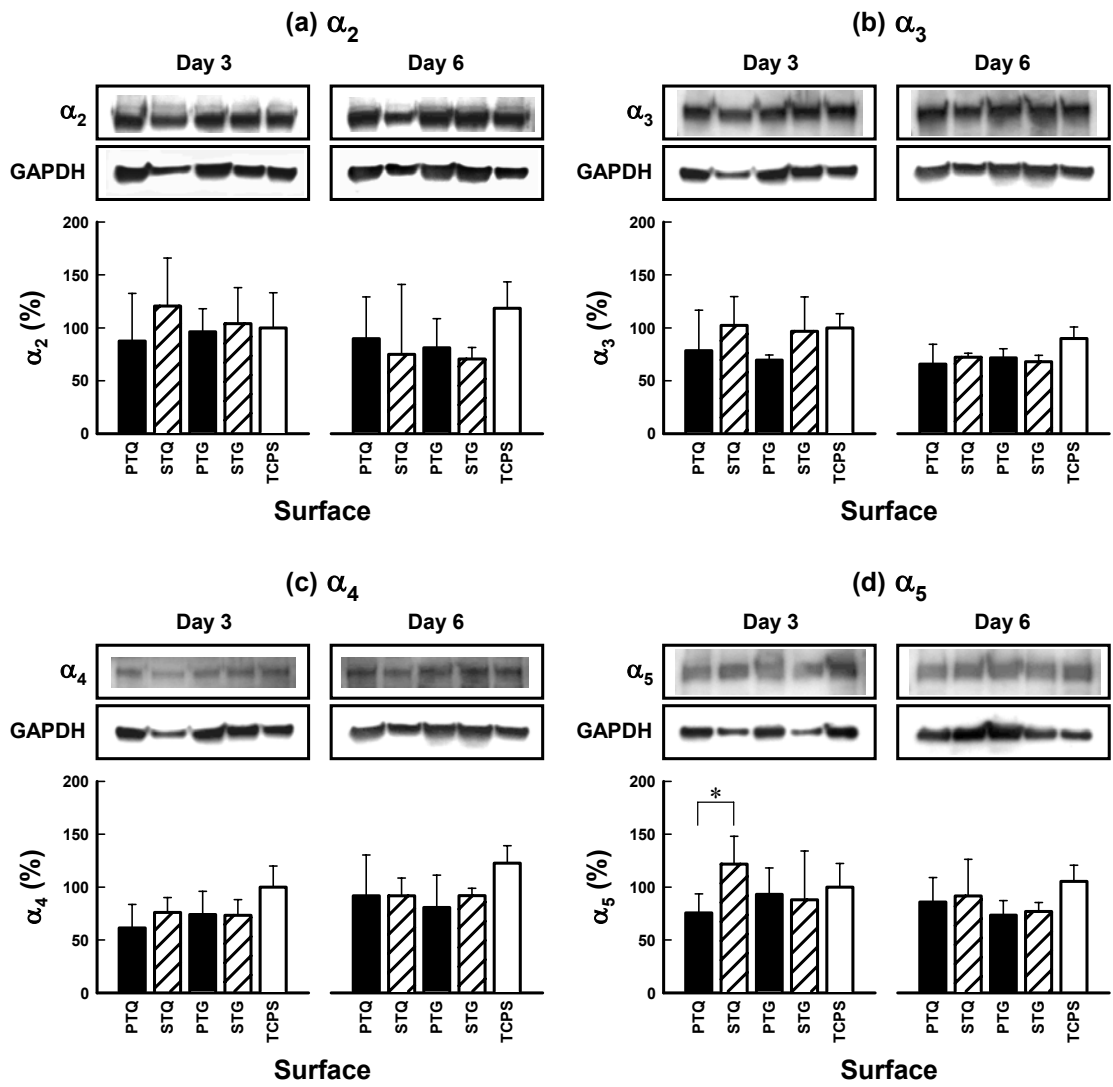


Fig. 2

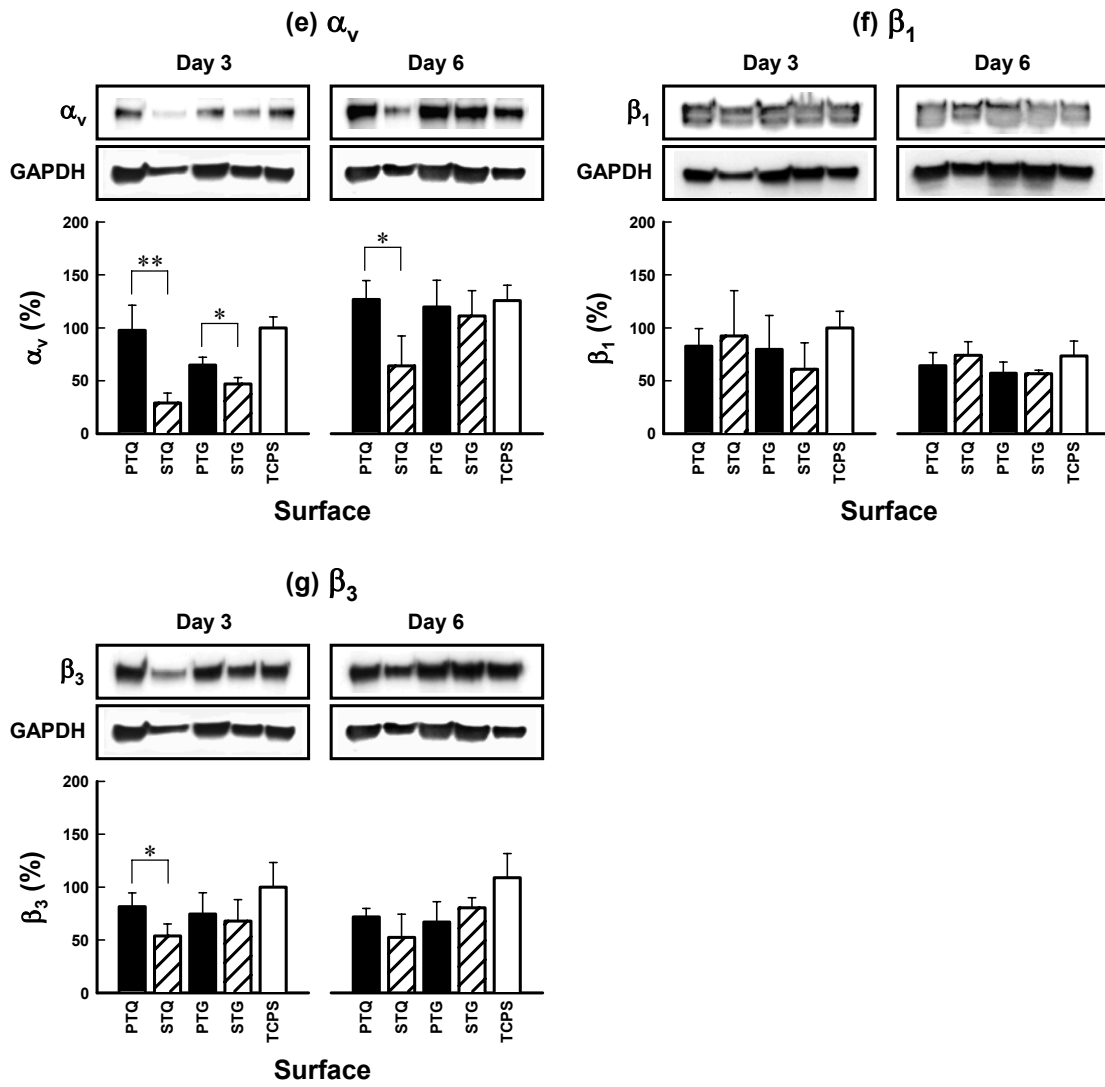


Fig. 2 (Continued)

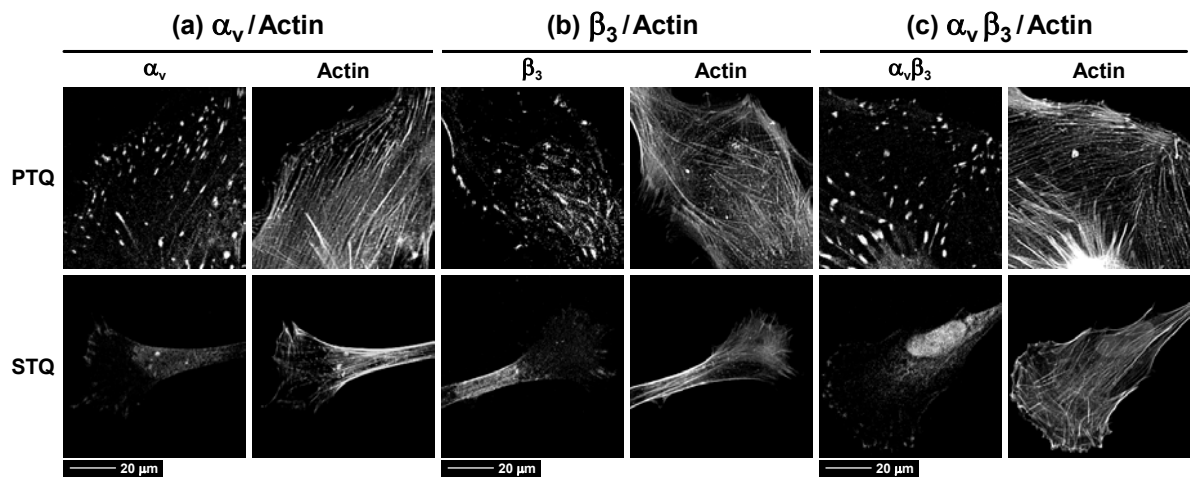


Fig. 3

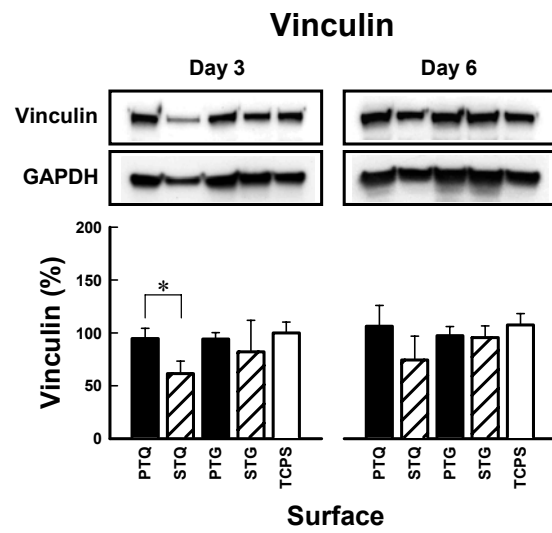


Fig. 4

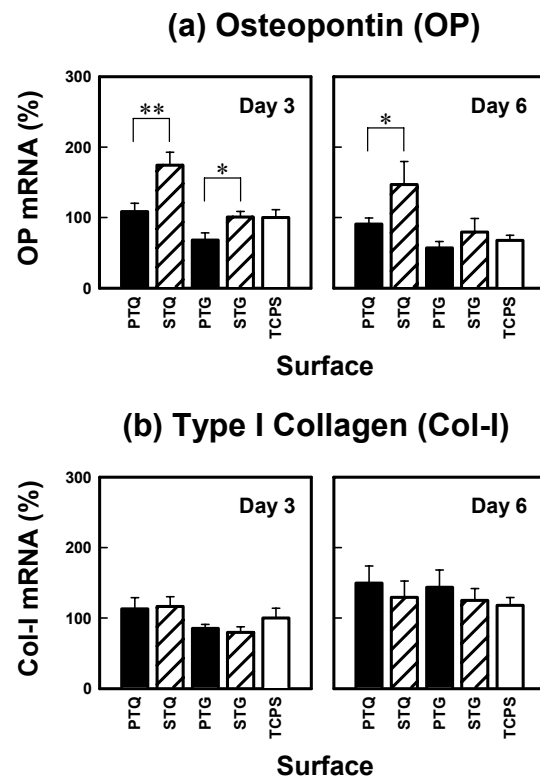


Fig. 5