CLINICAL ORTHOPAEDICS AND RELATED RESEARCH
Number 427, pp. 220–225
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# Molecular Regulation of Osteoblasts for Tissue Engineered Bone Repair

*Saadiq F. El-Amin, MD, PhD*; Michelle D. Kofron, BA*; Mohamed A. Attawia, MD†; Helen H. Lu, PhD¶; Rocky S. Tuan, PhD‡; and Cato T. Laurencin, MD, PhD*§‖¶*

**The use of biodegradable polymers in medicine and biomedical research is increasing. A key growth area has been the use of these materials in tissue engineering, especially for guided regeneration of bone and cartilage. Our interest has been in determining the mechanisms by which cellular attachment and growth occurs on these materials. In the current study, we examined human osteoblast cell adhesion, growth, and morphologic changes on polymeric scaffolds composed of polylactic-co-glycolic acid and polylactic acid materials. We examined these characteristics in association with measurements of levels of key adhesion integrin receptors in the presence and absence of antibodies against $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$ subunits, and the adhesion ligand peptides RGD (Arg-Gly-Asp) and RGE (Arg-Gly-Ser). At 2 hours, results showed initial cell adhesion was considerably decreased on polylactic-co-glycolic acid and polylactic acid in the presence of the $\alpha_2$ and $\beta_1$ antibodies with a 70% adhesion rate difference observed among the groups evaluated. Higher levels of inhibition were observed on polylactic-co-glycolic acid relative to polylactic acid, which may be correlated to a higher number of cells being able to interact with the surface initially. The presence of known competitive peptide (RGD) at 2 hours, revealed its ability to block cellular adhesion to these matrices relative to the control and noncompetitive peptide RGE on polylactic-co-glycolic acid matrices. Overall adhesion rate was affected by the presence of the integrin antibodies to the $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$ subunits with highest differences among polylactic-co-glycolic acid relative to its control, therefore suggesting that initial osteoblastic cell adhesion to commonly used biomaterials is regulated through integrin binding.**

Received: October 4, 2003
Revised: April 7, 2004
Accepted: July 7, 2004
From the *Department of Orthopaedic Surgery, the§ Department of Chemical Engineering, and the ‖Department of Biomedical Engineering, University of Virginia, Charlottesville, VA; †Depuy, Johnson & Johnson, Raymond, MA; ‡the National Institute of Musculoskeletal Research, National Institutes of Health, Bethesda, MD; and the ¶Department of Chemical Engineering, Columbia University, New York, NY.
This research was supported by the National Institutes of Health, Grant number: NIH (AR08562–01, AR44051, DE12864, AR39740), NSF (BES-9896282, BES-9817872). Dr. Laurencin was a recipient of the Presidential Faculty Fellowship Award from the National Science Foundation.
Correspondence to: Cato T. Laurencin, MD, PhD, Department of Orthopaedic Surgery, University of Virginia, 400 Ray C. Hunt Drive, Suite 330, Charlottesville VA, 22903. Phone: 434-243-0250; Fax: 434-243-0252; Email: ctlf3f@virginia.edu.
DOI: 10.1097/01.blo.0000137556.51604.0c

In tissue engineering, polymeric materials have been suggested for use for regeneration of tissues such as bone and cartilage.[5,16] As a result, this has encouraged a concerted effort to further understand cellular interactions with polymeric matrices. Studies have shown that human cells can successfully grow on various matrices constructed from the degradable polyester polymers, polyglycolic acid (PGA), polylactic acid (PLA), and the copolymer polylactic-co-glycolic acid (PLAGA).[3,6,10,12,20,30] The polymers have gained increasing attention because of their approval by the Food and Drug Administration for use in applications ranging from sutures to bone fixation devices.[19] In addition, their biocompatibility, workability, and degradation characteristics make their use appealing for various tissue replacement procedures.[23]

For biomaterials to continue to be successful in tissue engineering and orthopaedics, their interaction with cellular tissue must be understood. One way to closely examine the interaction of a polymeric material with its surrounding tissue is to study the receptors involved in cell adhesion, the integrins. Integrins are transmembrane heterodimeric proteins that contain α and β subunits that localize to form a receptor.[14,22] Integrins have been shown to be involved in cell adhesion, signal transduction, and gene regulation.[13,29] Investigators have reported integrins to bind to the extracellular matrix (ECM) component sequence RGD (Arg-Gly-Asp).[8,24,25] The RGD sequence is found in various proteins such as collagen and fibronectin.[25]

Integrin localization and expression has been shown to be affected by the surface in which the cell is grown.[29] For example, Sinha and Tuan reported that various orthopaedically relevant metals influence expression of different integrin subunits on the surface of human osteoblastic cells.[29] In addition, El-Amin et al[9] examined integrins expressed on biodegradable polymers and suggested that

polymeric materials may influence the receptors expressed by human osteoblastic cells seeded on these materials. However, the precise determination of integrins specifically involved in initial cellular adhesion is unclear. Few studies have examined the expression of the complex pattern of integrin expression together with competitive proteins and antibodies.[7,11,21] Anselme[1] examined adhesion rate along with the ECM proteins and antibodies against the integrin subunits on plastic surfaces, and reported there was a variation in the adhesion rate. Gronowicz and McCarthy[13] also examined osteoblast adhesion to metal alloys along with the RGD peptide and the integrin antibody to the fibronectin receptor, $\alpha_5\beta_1$. Their results showed that by blocking the fibronectin receptor, adhesion to metals such as Zimaloy and Tivanium could be altered. To date, no studies have examined whether inhibition of integrin receptor subunits can affect human osteoblast cell adhesion to orthopaedically relevant biodegradable polymers.

Using antibodies to integrin subunits $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$, we examined integrins responsible for supporting adhesion at initial interactions between polymeric matrices and cellular tissue. We also investigated the integrin ligand RGD that is expressed in key extracellular matrix molecules to determine its role in osteoblast adhesion in vitro to polymeric matrices composed of PLAGA and PLA. We hypothesized that integrins and ECM proteins play a key role in human osteoblast cell adhesion to polyermic materials through the integrin-RGD interaction on the surface of the material.

## MATERIALS AND METHODS

Polylactic-co-glycolic acid (ratio of 50:50 Mw = 50,000; American Cyanamid, Sunnyvale, CA) and PLA (L-isomer form, Mw = 60,000; PURAC, Gorichem, The Netherlands) were obtained and stored in a nitrogen glove box to prevent hydrolysis of the material. To make polymeric matrices for adhesions studies, 1 g of each polymer was measured and placed in a 10 mL solution of methylene chloride (Aldrich, Milwaukee, WI) in a 20 mL scintillation vial. The polymer was mixed at a constant vortex speed for 3 hours. The dissolved polymer then was poured into a Teflon-coated petri dish and placed under a vacuum hood until the matrix solidified. The matrix then was allowed to solidify slowly overnight at −20° C. After evaporation of the solvent, thin film matrices were bored into circular discs 14 mm in diameter and 4 mm in height. The bored matrices then were lyophilized (Labconco 12, Kansas City, KS) for at least 24 hours to remove residual solvent and stored at −20° C under argon to prevent surface changes of the polymer. Before using the matrices they were placed under UV light for 15 minutes on each side to minimize contamination.[18]

Human osteoblastic cell culture was done through a protocol using trabecular bone isolated from the femoral head (IRB approval obtained).[29] Under aseptic conditions, trabecular bone was dissected and removed from the femoral head. The dissected bone was placed in vials containing calcium-free Dulbecco's Modified Essential Medium (DMEM)/F12K medium (Specialty Media, Phillipsburg, NJ). The mixture of bone was minced extensively using surgical scissors until the bone chips were between 3–5 mm diameter. The solution containing bone chips then was washed in medium several times. After several washes, the bone chips were digested in a collagenase P enzyme media (Sigma, St Louis, MO) and stirred constantly at 37° C in 5% $CO_2$ for several hours. The bone chips were washed repeatedly in sterile buffered saline (pH 7.4). To assist in removal of fibrous material, the bone chips were vortexed between washes at a low constant speed. The bone chips then were observed under an Olympus CK2 light microscope (Hitech Instruments, Edgemont, PA) to determine if sufficient removal of fibrous material occurred. The bone chips were plated in a 125-mm cell culture flask (COSTAR, Cambridge, MA) containing calcium-free DMEM/F12 medium, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 50 μg/mL ascorbate, and 50 μg/mL penicillin/streptomycin at 37° C in 5% $CO_2$. Confluency of the osteoblasts was reached at 3–4 weeks, at which time the media was supplemented with 110 mmol/L $CaC1_2$ and the cells were maintained.[29]

For normal adhesion studies, human osteoblastic cells were seeded on the polymeric matrices PLAGA and PLA, and the control tissue culture polystyrene for 2 hours. Confluent cells were removed from the cell culture flask using 10 mL of 0.5% trypsin (Sigma) at 37° C in 5% $CO_2$. The cell suspension was neutralized with the addition of culture media and centrifuged at 1000 rpm for 5 minutes. To remove residual trypsin, the media were replaced, and the cells were counted using a hemocytometer and plated at a density of $2.0 \times 10^4$ cells/disc on sterilized matrices and the control surface of the control tissue polystyrene, and incubated at 37° C in 5% $CO_2$. At the predetermined time of 2 hours after plating, a Bovine Serum Albumin protein dye (BSA) was used to determine the total protein concentration, which then was used to determine the total percent of adhered cells (Sigma). At each specific time, cells grown on the matrix were washed using a phosphate-buffered dye which was added at 37° C in 5% $CO_2$ for 30 minutes. The colorimetric dye was read using a Spectrofluor Plus instrument (Tecan US Inc, Research Triangle Park, NC) at a wavelength of 485 nm. The cell number was determined by comparison of the dye intensities from a standard curve of known cell numbers.[28] A parallel sample of osteoblasts adhered on PLAGA at 2 hours was evaluated using immunofluorescence to observe the morphologic features of the cells using a previously published method.[28]

Primary human osteoblastic cells were obtained as described previously, grown to confluency in DMEM/F12 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 μg/mL ascorbate, and 50 μg/mL penicillin/streptomycin at 37°C in 5% $CO_2$. Cells were removed from the cell culture flask using a 2% trypsin solution, designed not to interfere with the integrin receptors. Cells then were neutralized in media, centrifuged, and counted using a hemocytometer. The $4.0 \times 10^4$ cells then were placed in sterilized Eppendorf tubes in serum-free medium. One of the following monoclonal antibodies to $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$ (Chemicon International, Temecula, CA) at 1:1000 dilution, was added to the cell suspension and incubated at 37°C in 5% $CO_2$

for 30 minutes. The solution containing the cells with the bound antibodies was plated on various polymeric matrices of PLAGA, PLA, and the control tissue culture polystyrene. Parallel cultures also were plated on these surfaces that did not contain antibodies as a comparison. At 2 hours the number of adhered cells was determined following the colorimetric BSA protein assay previously described.

Cells were pretreated with either 40 μg/mL of the competitive peptide, RGD, or the noncompetitive ligand, RGE, and plated on matrices of PLAGA, PLA, and control tissue culture polystyrene, and then incubated at 37° C in 5% $CO_2$ for 2 hours. At 2 hours, the number of adhered cells was determined using the cell adhesion assay described previously.

Statistical analysis was done using JMP IN 3.2.1 software (Cary, NC). One-way ANOVA was used to determine any statistically significant relationship among polymer matrices of PLA, PLAGA, and the control tissue culture polystyrene with respect to expression of integrin subunits. Statistical significance was studied at the $p = 0.05$ level. Three to four scaffolds were analyzed at each quantitative assay and repeated four times.

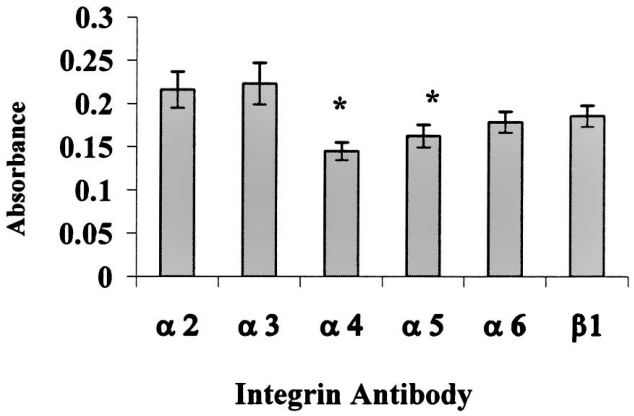


**Fig 1.** The human osteoblastic cell adhesion rate on tissue culture polystyrene at 2 hours, in the presence of the monoclonal inhibitory integrin antibodies ($\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$) was compared with adhesion without the inhibitory antibody. n = 6 and * denotes $p < 0.05$

## RESULTS

Human osteoblastic cells were isolated from trabecular bone chips and grown to confluency. The morphologic and cellular appearances were consistent with those previously reported.[28]

Our first goal was to examine cellular adhesion on tissue-engineered matrices of PLAGA and PLA at 2 hours relative to the control tissue culture polystyrene. After plating, cellular attachment was initiated by 2 hours on all surfaces. Micrographs showed that cellular growth by 2 hours started toward the center of the material and spread outward (data not shown). There was a notable increase in the number of cells adhered to PLAGA initially relative to PLA and the control tissue culture polystyrene.

To assess which integrin receptors were necessary for cellular adhesion on tissue-engineered matrices, human osteoblast cells were grown in the presence of the following antibodies: $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$. The cells then were plated onto PLAGA, PLA, and the control tissue culture polystyrene. The results showed that cells grown on all surfaces showed a change in the number of adhered cells in the presence of the antibodies. On the control tissue culture polystyrene, the material designed for cellular adhesion, the percent of cells adhered was relatively equal among the integrins (Fig 1). The integrin profile showed that $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$ affected cellular adhesion compared with $\alpha_2$ and $\alpha_3$. For cells grown on PLAGA in the presence of the antibodies, $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$, the initial adherence rate was affected more in the presence of the antibodies $\alpha_2$, $\alpha_4$, $\alpha_5$, and $\alpha_6$, relative to $\alpha_3$, and $\beta_1$ (Fig 2). There was a significant decline seen when comparing the number of adhered cells in the presence of the integrins $\alpha_2$, $\alpha_4$, $\alpha_5$ to $\alpha_6$, and $\beta_1$ ($p < 0.05$). When evaluating the effect of integrins on cellular adhesion to PLA, we again saw the same trend that was observed on the control tissue culture polystyrene with lower numbers on groups treated with $\alpha_4$ and $\alpha_5$ and slightly less for $\alpha_2$ relative to $\alpha_3$, $\alpha_6$, and $\beta_1$ (Fig 3).

When comparing the effect of integrins on cellular adhesion with different polymers, PLAGA, and PLA, and the plastic surface control tissue culture polystyrene, the results showed that a lower number of cells overall adhered to PLA and control tissue culture polystyrene relative to the PLAGA. This was evident by the decrease in the percent observed on the two surfaces. However, among the groups, cell adhesion was affected at 2 hours at a higher rate on PLAGA.

To study the effect of integrin activation on cellular attachment to the tissue-engineered matrices, investiga-

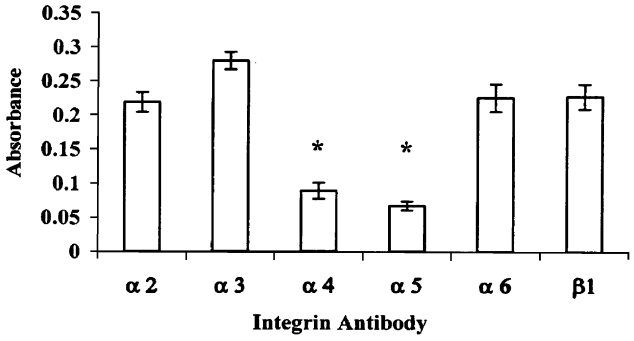


**Fig 2.** The human osteoblastic cell adhesion rate on PLAGA at 2 hours, in the presence of the monoclonal inhibitory integrin antibodies ($\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$) was compared with adhesion without the inhibitory antibody. n = 6 and * denotes $p < 0.05$

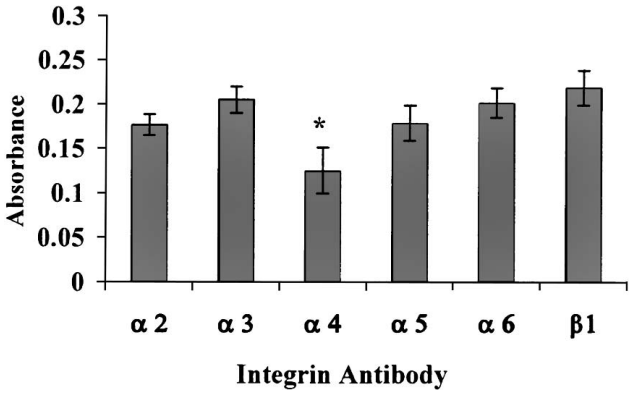


**Fig 3.** The human osteoblastic cell adhesion rate on PLA at 2 hours, in the presence of the monoclonal inhibitory integrin antibodies ($\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_6$, and $\beta_1$) was compared with adhesion without the inhibitory antibody. n = 6 and * denotes $p < 0.05$

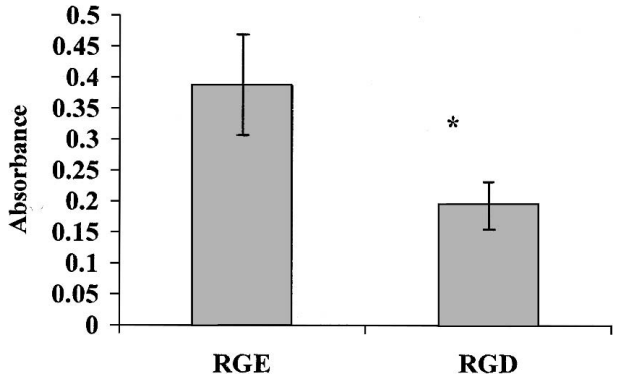


**Fig 5.** The human osteoblastic cell adhesion rate on PLAGA at 2 hours, in the presence of the cell binding peptides RGD(Arg-Gly-Arp) was compared with RGC(Arg-Gly-Ser). n = 6 and * denotes $p < 0.05$

tions were done with the adhesion peptide RGD (Arg-Gly-Asp), and the noncompetitive binding peptide, RGE (Arg-Gly-Ser). With the RGD molecule we found cellular adhesion at 2 hours to be less than with RGE. On tissue culture polystyrene, the adhesion rate had a difference of 20% when comparing RGD with RGE (Fig 4). For cells grown on PLAGA with RGD and RGE peptides, we found a significant decrease in the number of cells adhered to the matrix (Fig 5). There was a difference of approximately 50% when comparing the two groups. When observing the effect of the peptides on human osteoblastic cells seeded on PLA, the difference in cellular adhesion was slightly less for the RGD treated group relative to RGE (Fig 6).

When comparing the polymeric groups among each other, the lower adhesion rates were seen for the control tissue culture polystyrenes, with both treatment groups being equally represented. Next, PLA had a lower total number of cells adhered relative to PLAGA groups when examining overall adherence among matrices.

To observe the effect of integrin binding and peptides on osteoblastic cell adhesion, the morphologic features of the osteoblastic cells were studied. With the $\alpha_2$, $\alpha_4$, and $\alpha_5$ integrin antibodies, cellular morphologic features were consistent with those previously reported for osteoblastic cells in the Type I shape,[29] which usually is circular and has an unorganized cellular framework (data not shown). The normal morphologic reponse of osteoblasts to PLAGA matrix is shown in Figure 7. Cells have a normal star-shaped pattern with focal contact points, which are reported to be generated by integrin-ECM contact.[28]

## DISCUSSION

The objective of the current study was to gain additional knowledge of the parameters involved in osteoblast adhesion to materials for tissue engineering through examination of integrin receptor interactions. Inhibition studies were done by blocking the receptor-using antibodies and

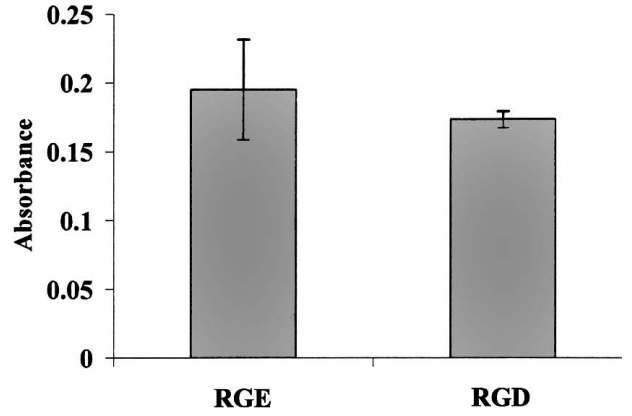


**Fig 4.** The human osteoblastic cell adhesion rate on tissue culture polystyrene at 2 hours, in the presence of the cell binding peptides RGD(Arg-Gly-Arp) and RGC (Arg-Gly-Ser) is shown. n = 6 and * denotes $p < 0.05$

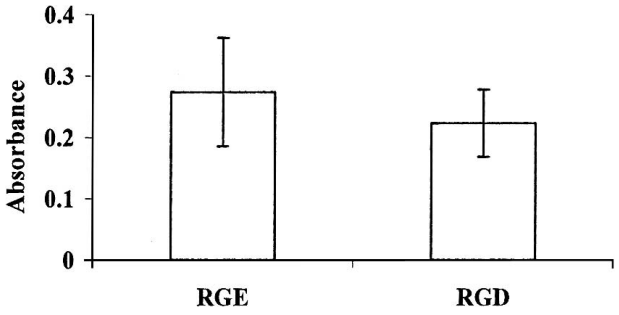


**Fig 6.** The human osteoblastic cell adhesion rate on PLA at 2 hours, in the presence of the cell binding peptides RGD(Arg-Gly-Arp) was compared with RGC (Arg-Gly-Ser). n = 6 and * denotes $p < 0.05$

with a competitive peptide recognition sequence RGD (Arg-Gly-Asp) and a noncompetitive peptide RGE (Arg-Gyl-Ser). In a previous study, investigators from our laboratory examined which receptors were expressed on biodegradable matrices, the extracellular matrix produced by the matrices, and the long-term growth and mineralization on these materials.[9] In the current study, understanding the molecular interactions involved when cells come into contact with various polymeric surfaces was the focus. This is one of the first studies to investigate integrin receptor-mediated adhesion by osteoblast cells on biodegradable polymers.

The initial adherence rate of human osteoblastic cells on the tissue-engineered matrices was investigated first. The results showed that human osteoblastic cells isolated from trabecular bone were able to adhere within the first 2 hours of contact with the polymeric surface. Despite differences among the groups, with PLAGA having a higher adhesion rate initially, cell adherence was observed on all surfaces. Other investigators studying human osteoblast cell adherence on biomaterials reported that the surface can affect cellular adhesion.[2,4,20,29] In addition, metals such as Ti or CoCr have different adhesion rates.[2,4,20,29] As for human osteoblast cell growth on the degradable polymers, few studies have been reported.[9,29] Studies using either an osteoblast cancer line (SaOS-2) or an osteoblast-like cell line that resembles osteoblast activity (MC3T3-E1), have shown differences in initial cellular adhesion among various polymeric groups.[3,10,20]

To assess the molecular interaction that occurs when a cell adheres to a biomaterial surface, we studied the receptors involved in cell adhesion, the integrins. El-Amin et al[9] investigated which integrins were present with time on the surface of cells grown on tissue-engineered matrices made from PLAGA and PLA. Osteoblastic integrin expression was detectable on both surfaces. In addition, human osteoblastic cell integrin expression was dependent on surface composition of the polymer. For instance, there was a higher expression on PLAGA relative to PLA, showing that the surface of PLAGA more favorably influenced the repertoire of receptor expression. Furthermore, among the integrins there was an increase in the receptors reported to bind to the extracellular matrix molecules, collagen, and fibronectin. We examined regulating cellular attachment through blocking the receptor sites either through antibodies specific to the integrin subunits or through the use of a competitive peptide (RGD), which has been reported to have integrin-binding activity.[8,13,14,28] Our results showed that initial cellular adhesion was affected when human osteoblastic cells were plated along with key integrin antibodies, especially on the PLAGA material as opposed to PLA and control tissue culture polystyrene. These results provide additional evidence that the integrins play a crucial role in cellular adherence to these polymeric materials and stress the importance of their actions early during cellular attachment. Among the integrins studied, the highest percent of change seemed to be involved with the integrin subunits of $\alpha_4$ and $\alpha_5$, and to a lesser extent $\beta_1$. The ability to reduce cellular adherence using antibodies against integrin subunits shows their importance in initial cellular adhesion. We believe that the integrin subunits, $\alpha_2$, $\alpha_4$, $\alpha_5$,and $\beta_1$ are specific to key extracellular matrix proteins, collagen, fibronectin, and vitronectin.[13,17,26] Given that there may be correlation between their importance during the early stages of adhesion and the production of the ECM. Studies have shown that the ECM is crucial to osteoblastic cellular adhesion to metals and to biodegradable polymers.[28,29] Shah et al[28] reported that when an osteoblast cell comes in contact with a surface, key ECM materials are secreted by the cells and are laid down on the surface. Once the network is formed, cells upregulate key integrin receptors to bind more efficiently to the surface.[1] Since it has been reported that the ECM proteins expressed initially may be collagen and fibronectin and to a lesser degree vitronectin, it is not surprising that the integrins found to specifically adhere to these materials are the ones that affect initial cellular attachment.[13,31] Therefore, we believe osteoblast cells initially rely on the integrins that contain the subunits $\alpha_2$, $\alpha_4$, $\alpha_5$, and $\beta_1$ to make contact with the surface of a material and help guide the adhesion process.

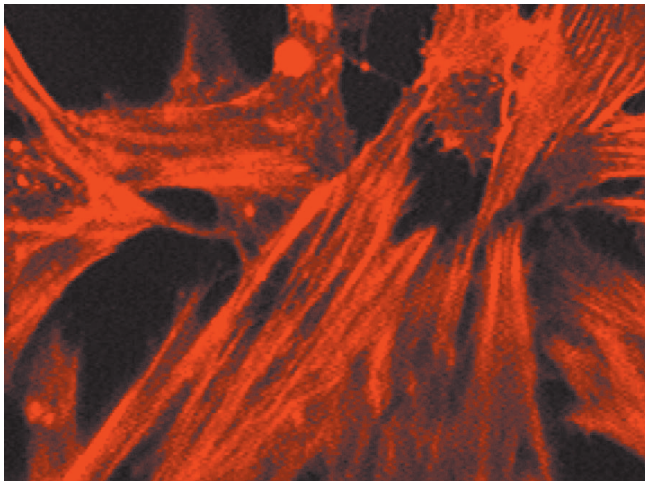

**Fig 7.** The human osteoblastic cell adhesion on PLAGA matrix as a representative micrograph shows osteoblast adhesion and morphologic features at 2 hours. Cells were stained with immunfluorescence antibody (rhodaimine-phalloidin) and observed under a confocal laser microscope (Magnification, ×20).

To further understanding of the critical role integrins play during the initial adhesion of osteoblastic cells to

biodegradable polymeric material, we examined the specific binding peptide RGD and the nonspecific ligand RGE to determine whether specific binding of the peptide to the integrin receptor affected initial adherence. The RGD sequence has been reported to be specific for the integrin subunits for $\alpha_2$, $\alpha_4$, $\alpha_5$, and $\beta_1$[8] The amino acid sequence Arg-Gly-Asp first was detected in the large fibronectin protein and later was discovered to be responsible for integrin binding.[13,25,27] Several studies have shown that with the RGD molecule, adhesion of osteoblast cell can be affected.[13,26,27] Gronowicz and McCarthy[13] reported that with peptide-coated surfaces, osteoblastic cell adhesion could be altered either positively or negatively depending on whether the peptide adhered to the surface to promote adhesion or if added to the cells to prevent adhesion. This showed that the specific binding of RGD to the integrin receptor is crucial and determines the fate of cellular adhesion initially. This also was supported with the use of a nonspecific peptide that revealed no effect on cellular adhesion. Few studies have examined this effect on osteoblast cells grown on biodegradable polymers.[13,26,29] Therefore, the introduction of such a system is novel and enhances the understanding of the mechanisms of adhesion to biomedical polymers. Our results showed that with the RGD peptide, initial cell adhesion was less than in the untreated and nonspecific groups (RGE). This was especially apparent on cells seeded on PLAGA, the polymer with an increase in integrin expression on the cellular surface of seeded cells. This trend also was apparent on PLA and control tissue culture polystyrene, but to a lesser extent.

Our goal was to gain better understanding of the process involved in cellular adhesion to biodegradable polymeric materials fabricated as tissue-engineered scaffolds for use in orthopaedic applications. Adhesion events occurring when osteoblast cells come in contact with these surfaces are highly dependent on integrin expression and binding potential. Additional studies will focus on understanding the surface interactions involved from the polymeric surface.

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