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# Biocompatibility and integrin-mediated adhesion of human osteoblasts to poly(DL-lactide-co-glycolide) copolymers

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**Abstract**

The biocompatibility of polylactic acid (PLA) and polyglycolic acid (PGA) copolymers, employed in manufacturing bone-graft substitutes, is affected by their chemical composition, molecular weight and cell environment, and by the methods of polymerization and processing. Their in vitro bioactivity on human osteoblasts has been investigated very little. We first evaluated the behavior of primary human osteoblasts cultured in close contact with 75:25 and 50:50 PLA–PGA copolymers for 14 days adopting a cell culture system that allowed us to evaluate the influence of direct contact, and of factors released from polymers. The copolymers had no negative influence on cell morphology, cell viability and proliferation. Alkaline phosphatase (ALP) activity and osteocalcin production were also not affected. The initial adhesion of osteoblasts on implant surfaces requires the contribution of integrins, acting as a primary mechanism regulating cell-extracellular matrix (ECM) interactions. We observed that adhesion of osteoblasts to PLA–PGA copolymers, 2 h after plating, was reduced by ≈70% by antibodies capable to block integrin $\beta_1$ and $\alpha_5\beta_1$ complex and only by ≈30% by an anti-integrin $\alpha_v$ antibody. Therefore, $\beta_1$ integrins may represent a predominant adhesion receptor subfamily utilized by osteoblasts to adhere to PLA–PGA copolymers. These materials do not show any negative influence on cell proliferation and differentiation.


*Keywords:* Osteoblasts; Cell proliferation; Cell adhesion; Integrin; Poly(lactide-co-glycolide)

## 1. Introduction

Polyesters, including polylactic acid (PLA), polyglycolic acid (PGA) and their copolymers (polylactide-co-glycolide (PLGA)) are employed in manufacturing internal devices (Athanasiou et al., 1996, 1998; Leenslag et al., 1987; Zislis et al., 1989; Schakenraad and Dijkstra, 1991). The biocompatibility of a biodegradable polymer is affected by its chemical composition, molecular weight, and crystallinity and by the cell environment where it has been placed (Park and Cima, 1996; Ikarashi et al., 2000a). A loss of biocompatibility of these polymers has been related to their degradation rate (Ignatius and Claes, 1996). Schakenraad et al. (1989), for instance, observed a more intense tissue reaction for polymers containing PLA, with higher degradation rates. This process ultimately leads to the release not only of degradation products, but also of residual monomers, catalysts and additives (Taylor et al., 1994). Moreover, degradation of PLA and PLGA polymers may be influenced by other factors, including polymer characteristics, the methods of polymerization and processing, and sterilization and storage conditions (Vert et al., 1992, 1994).

Possible interactions between bone cells and biomaterial should be evaluated in cell culture systems in order to see how osteogenic cells respond to these materials (for a review see Morrison et al., 1995). In vitro biocompatibility of PLA and PLGA polymers has been mainly evaluated in immortalized cell lines such as mouse fibroblasts or osteogenic cells (Elgendy et al., 1993; Morrison et al., 1995; Schwartz et al., 1999). However, in these models several cellular processes such as proliferation, apoptosis and expression of

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differentiation markers may be modified (Puleo et al., 1991; Xynos et al., 2000; Ikarashi et al., 2000b).

In this study we investigated the behavior of primary human osteoblasts cultured in the presence of PLGA copolymers for 14 days. We adopted a cell culture system that has been employed to evaluate the influence of direct contact and of factors released from bone-graft substitutes (Winn and Hollinger, 2000). To evaluate biocompatibility we assessed cell viability, proliferation and morphology. We also measured the alkaline phosphatase (ALP; E.C. 3.1.3.1) and osteocalcin. ALP is involved in preparing the extracellular matrix (ECM) for the ordered deposition of mineral and serves as an early marker of differentiation for osteoblasts (Attawia et al., 1995). Osteocalcin is the most abundant non-collagenous protein of the mineralized extracellular bone matrix, and increases during late osteoblastic differentiation (Desbois and Karsenty, 1995).

Events leading to integration of polymeric materials into bone take place primarily at the polymer interface and require different factors (for a review see Ziats et al., 1988; Puleo and Nanci, 1999). These include the initial cell adhesion and spreading over implant surfaces through extracellular matrix proteins, such as fibronectin, vitronectin, fibrinogen and collagen (Buck and Horwitz, 1986; Grzesik and Robey, 1994; Sinha and Tuan, 1996; Chang et al., 1998). In fact, adhesive cells utilize ECM proteins to attach and migrate on substrates, exchange signals that block apoptosis and allow cell cycle progression and the appearance of tissue-specific phenotypes (García et al., 1999; Zhu et al., 1996; Cutler and García, 2003). Cell adhesion to ECM proteins is primarily mediated by integrins, transmembrane receptors composed of α and β subunits, with at least 12 different α and nine β subunits described to date (for a review see Ivaska and Heino, 2000; van der Flier and Sonnenberg, 2001). Upon ligand binding, integrins cluster together and organize into complexes that contain structural and signaling proteins (Jockusch et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Damsky, 1999; Stephansson et al., 2002). Human osteoblasts grown on tissue culture polystyrene express primarily the integrin subunits $\alpha_{1-6}$, $\alpha_v$ and $\beta_{1,3}$ (Hughes et al., 1993; Gronthos et al., 1997; Bennett et al., 2001). Integrin expression appears to be modified in osteoblasts grown on surfaces such as titanium and cobalt-chrome or on biodegradable polymers such as PLA and PLGA (Gronowicz and McCarthy, 1996; Geissler et al., 2000; El-Amin et al., 2002). The contribution of any specific integrin to osteoblast adhesion to metal materials has been recently evaluated by functional blocking antibodies (Krause et al., 2000; Matsuura et al., 2000; Schneider et al., 2001; Zreiqat et al., 2002; Cutler and García, 2003); however, to date, little is known about their involvement in cell adhesion to PLGA. Therefore, in the second part of this study we evaluated osteoblast adhesion to PLGA copolymers in the presence of antibodies capable to block integrins $\beta_1$, $\alpha_5\beta_1$ and $\alpha_v$.

## 2. Materials and methods

### 2.1. Preparation of polymer scaffolds

Two blends of PLGA copolymers, obtained from Sigma–Aldrich (Milan, Italy), were investigated: poly(DL-lactide-co-glycolide) 75:25 (PLGA (75:25); inherent viscosity 0.19 dl/g in chloroform; Mw 75,000–120,000) and poly(DL-lactide-co-glycolide) 50:50 (PLGA (50:50); inherent viscosity 1.00 dl/g in hexafluoroisopropanol; Mw 50,000–75,000). The polymers are amorphous and were dissolved in tetrahydrofuran (10% (w/v)) and stirred for 4 h at room temperature. After evaporation of the solvent, polymer scaffolds ($100 \pm 1$ mg) were compression-molded as round plates under a pressure of 41,340–55,120 kPa (diameter $= 50$ mm; thickness $\approx 200$ μm) and subsequently dried (50 °C; 2 h). Each polymer was bonded on the central area of the bottom of tissue culture polystyrene flasks (TCPS; 75 $cm^2$) or of 100 mm polystyrene dishes purchased from Bibby Sterlin (Milan, Italy), using 50 μl chloroform to prevent it from floating in the growth media. The flasks were left open at room temperature for 24 h to allow the residual solvent to evaporate, then sterilized using $^{60}Co$ γ-irradiation at a dose of 2.0 Mrad. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Gibco-Life Technologies (Grand Island, NY, USA), unless otherwise indicated.

### 2.2. Cell culture system

Human osteoblasts from hip bone were obtained from PromoCell™ GmbH (Handschuhsheimer Landstr. 12, D-69120 Heidelberg, Germany). The normal—untransformed—osteoblasts have cellular functions comparable with those in the natural environment. All osteoblast cultures underwent a wide spectrum of tests and validation studies at PromoCell™. Amongst others, the following were checked on a routine basis: proliferation rate, morphology, absence of HIV-1 and Hepatitis B DNA, absence of bacteria (including mycoplasma), fungi and yeast. A certificate of analysis was issued for each batch and sent with every delivery of cells.

The cells were grown in an osteoblast growth medium obtained from PromoCell™ and maintained at 37 °C in a humidified atmosphere at 5% $CO_2$ in air. They were subcultured at least once before use. Cells were removed and resuspended by mechanical desegregation (using a cell scraper) in the solution contained in the cell detachment kit supplied by PromoCell™. The cells used in the experiments were from passages 1–3. Osteoblasts were seeded at a density of 3000 cells/$cm^2$ (equivalent to approximately 10% confluency) in 75 $cm^2$ flasks, with the polymers placed in the flasks before addition of the cells (as previously described), or in empty TCPS. The cells were evenly distributed over the surface of each flask by gentle horizontal rotation, and cultured for 14 days. The medium from each flask was removed and

replaced with fresh medium on day 7, so as to assess the influence of any material slowly released from the polymer on the cell parameters). All experiments were performed in triplicate.

### 2.3. Cell morphology

Each culture was examined daily with a light microscope (Zeiss Axioplan). The cells were examined microscopically to assess any changes of morphology and to evaluate cell proliferation around the test material. Any change from normal morphology was recorded. Vacuolization, detachment, cell lysis and membrane damage were assessed. Any change was rated as: none; slight (less than 10% of the cell population was involved); moderate (between 10 and 50% of the cell population was involved); severe (more than 50% of the cell population was involved). Microphotographs were taken after 14 days of culture.

### 2.4. Cell viability

Dyes whose uptake depends on cellular membrane integrity are commonly used. For routine purposes Trypan blue is used and a visual count is made of unstained "live" cells. Cells were cultured for 14 days, then culture medium was aspirated and the cells were washed with 10 ml of phosphate buffered saline (PBS), detached with a cell scraper and centrifuged (3000 × *g*; 5 min). The pellet was suspended in 1 ml of PBS and 10 μl of the cell suspension were transferred to an Eppendorf tube (1.5 ml) and 90 μl of PBS was added. Five microliters of cell suspension were mixed with 5 μl of Trypan blue solution (Trypan blue vital stain Sigma–Aldrich T-8154; 0.4% (w/v)), and transferred to the hemocytometer. The number of living cells was ascertained by the Trypan blue exclusion. Data are expressed as % of living cells per flask.

### 2.5. Lactate dehydrogenase (LDH) assay

This assay measures the activity of lactate dehydrogenase (LDH) released from the cytoplasm of treated and control cells. A commercial kit was used (CytoTox 96®; non-radioactive cytotoxicity assay, Promega, Madison, WI, USA). This assay quantitatively measures lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis. LDH release is quantified with an assay in which a tetrazolium salt is converted into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Two factors in the tissue culture medium can contribute to background absorbance in this assay: phenol red and LDH from fetal calf serum. This background absorbance is corrected for by including a culture medium control. The absorbance from this control is used to normalize the values from other samples. Maximum LDH release is also required in calculations to determine 100% release of LDH; this was evaluated in cells grown in empty flasks. Cells were collected from a control flask adopting the procedure described. After a cell count, approximately 5000 cells were suspended in 100 μl of PBS and 10 μl of 10× lysis solution was added. The cell suspension was incubated in a humidified chamber at 37 °C, 5% $CO_2$, for 45 min, then centrifuged (250 × *g*; 4 min) and LDH was evaluated on 50 μl as described (Song et al., 1997). Cytotoxicity is expressed as arbitrary absorbance values.

### 2.6. MTT assay

In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay (purchased from Sigma–Aldrich), this compound is actively absorbed into cells and reduced in a mitochondrial-dependent reaction to yield a formazan product that accumulates within the cell since it cannot pass through the cell membrane. Upon addition of isopropanol, or another suitable solvent, the product dissolves and is released, so it can be readily quantified colorimetrically. The ability of the cells to reduce MTT provides an indication of mitochondrial integrity and activity, which is also interpreted as a measure of cell viability. During the final 4 h of incubation with the test material, the test medium was replaced by a 0.5% (w/v) MTT solution in growth medium at 37 °C in the incubator. Then the culture medium was discarded and 10 ml per flask of PBS were added; the cells were detached with a cell scraper and pelleted by centrifugation (3000 rpm; 5 min; room temperature). After the addition of isopropanol (containing 0.004 M HCl) for 30 min, the optical density (OD) of each sample was determined at 550 nm and a reference wavelength of 690 nm was adopted. The absorbance values are reported.

### 2.7. Measurement of cell proliferation

For radiochemical determination of DNA synthesis, the cells were pulsed with [$^3$H]thymidine (final concentration 50 μM) for 4 h at the end of the exposure time with the test materials (14 days), washed twice with PBS, scraped with a cell scraper into 1 ml of PBS, and centrifuged (3000 × *g*; 5 min). The supernatant was discarded and the pellet was suspended in ice-cold 10% (w/v) aqueous trichloroacetic acid (TCA; 500 μl per tube) and left in ice for 20 min. Then the cells were centrifuged and the pellet washed with ice-cold methanol, suspended and centrifuged again. After decantation of the methanol, the pellet was suspended in 300 μl 0.1 M NaOH (60 °C; 5 min); then 300 μl of 1 M HCl were added and the radioactivity was determined on an aliquot of the final suspension by liquid scintillation counting. The incorporated radioactivity (counts per minute (cpm)) is expressed as a percentage of the controls (cells grown in empty flasks).

### 2.8. Osteogenic phenotypes

#### 2.8.1. Alkaline phosphatase activity (ALP)

The cultures were incubated for 14 days. After removal of the culture medium, the cell layers were rinsed in PBS. Distilled water was added to each substrate and specimens were put on ice. Finally, the cells were harvested with a cell scraper and the cell suspension was transferred to a 10 ml tube. The cells were sonicated for 2 min and centrifuged at 2000 rpm for 10 min (4 °C). Subsequently, 100 μl of cell lysate were added to 100 μl of working solution in a 96-well culture plate and incubated for 1 h at 37 °C. The working solution consisted of 0.5 M 2*S*-Amino-2-methyl-1-propanol (Sigma), 5 mM *p*-nitrophenol phosphate (Sigma) and 5 mM magnesium chloride (1:1:1 (v/v/v)). The reaction was stopped using 100 μl of 0.3 M sodium hydroxide and the absorbance was read at 405 nm using a microplate reader (Wallac mod. 1420). Alkaline phosphatase activity (ALP) activity (expressed as μmol of converted *p*-nitrophenol/min) was normalized by the total intracellular protein content (determined using the Pierce BCA protein Assay; Pierce, Rockford, IL) and expressed as μmol of *p*-nitrophenol/(min mg protein).

#### 2.8.2. Osteocalcin production

Osteoblast cells were cultured for 13 days as described, then the medium was removed and replaced with serum free Ham's F-12 medium containing $2 \times 10^{-8}$ M Vitamin $D_3$ (Sigma). Several studies, in fact, have shown that osteocalcin synthesis can be stimulated in primary bone cell cultures by Vitamin $D_3$ (Desbois and Karsenty, 1995). After 24 h of incubation in serum free medium, 1 ml of supernatant was collected and mixed with 7 μl of protease inhibitor containing 1.72 mM phenylmethylsulphonylfluoride, 0.376 M EDTA and 0.663 M *n*-ethylmaleinimide. The samples were stored at −80 °C until measurement. The production of osteocalcin by osteoblasts was evaluated using a commercially available enzyme-linked immunosorbent assay (Alexis Biochemicals, Basel, Switzerland). The osteocalcin concentration was normalized to the protein content of each flask.

### 2.9. In vitro degradation studies

Hydrolytic degradation assays were performed in 20 ml of sterile water or in cell culture medium added to the flasks containing the copolymers. The flasks were left up to 20 days in an incubator at 37.0 ± 0.5 °C. For each data point three specimens were analyzed. Water uptake and weight loss were determined by weighing, respectively, the swollen specimen after aspirating the water or the cell culture medium and wiping the surface with paper (weight $m_s$), and after thorough vacuum drying (50 °C for 24 h; weight $m_t$). The following equations were used: water absorption (%) $= 100[m_s - m_t]/m_t$; weight loss (%) $= 100[m_0 - m_t]/m_0$ ($m_0$ being the initial weight of the polymer).

### 2.10. Cell adhesion studies

For adhesion studies, human osteoblasts were cultured as previously reported and plated on PLGA copolymers (placed in 100 mm tissue culture dishes) or in empty polystyrene dishes at a density of $5.0 \times 10^4$ cells per dish and then incubated at 37 °C in 5% $CO_2$ over a 8 h period. At 2, 6, and 8 h after plating, cell adhesion was measured using the dye 2′7′-bis(2-carboxyethyl)5-carboxyfluorescein acetyloxymethylester (BCECF-AM, Molecular Probes, Junction City, OR, USA) which was internalized within the membrane of living cells and releases a fluorescent component upon lysis of the membrane (El-Amin et al., 2002, 2003). Thirty minutes prior to harvesting, cells were washed in PBS (pH 7.4) and incubated with BCECF-AM in cell culture medium for 30 min at 37 °C in 5% $CO_2$. Cells were washed twice with PBS and lysed using 1% Triton X-100 buffer (Sigma–Aldrich). Dye release was determined spectrofluorimetrically (Jasco, Japan, mod. FP6200) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. In parallel, serial dilutions of BCECF-AM-labeled cells were prepared to make a standard curve from which the number of adherent cells was calculated.

To evaluate the role of specific integrins in mediating adhesion, osteoblasts were detached from tissue culture flasks, suspended in culture medium (1 ml/$5 \times 10^4$ cells) and incubated for 30 min with antibodies under gentle agitation (37 °C; 95% air/5% $CO_2$) prior to seeding for 2 h on copolymers. The following antibodies (final concentration 10 μg/ml) were used: anti-integrin $\beta_1$ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-integrin $\alpha_5\beta_1$ and anti-integrin $\alpha_v$ antibody (Calbiochem, San Diego, CA, USA). Control cells were treated with 10 μg/ml human immunoglobulin G1 (IgG; Calbiochem). In preliminary studies, we confirmed that anti-integrin $\beta_1$ and anti-integrin $\alpha_5\beta_1$ antibodies (10 μg/ml) blocked by ≈90% mouse fibroblast adhesion to fibronectin-coated tissue culture dishes; similarly, anti-integrin $\alpha_v$ antibody blocked by ≈80% M21 human melanoma cell adhesion to vitronectin-coated tissue culture dishes (data not shown).

### 2.11. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined by Newman–Keuls test after ANOVA using GraphPad Prism, Version 3.0 (GraphPad Software, Inc., San Diego, CA). P values $<0.05$ were considered significant.

## 3. Results

### 3.1. Morphology

Morphologic evaluations of cells were done daily using phase contrast microscopy. No obvious differences

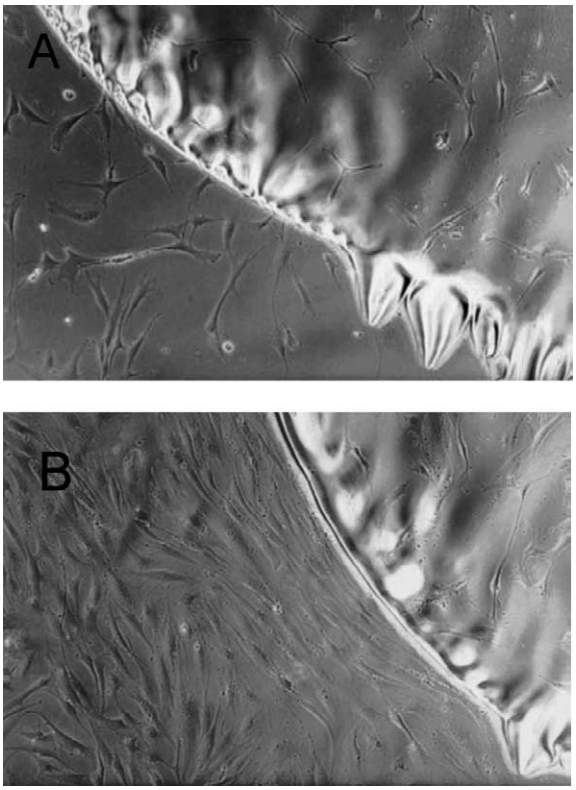


Fig. 1. Phase contrast microphotographs of primary human osteoblasts cultured in close contact with PLGA copolymers. (A) Cells cultured for 7 days in the presence of PLGA (50:50); (B) cells cultured for 14 days in the presence of PLGA (50:50). The polymer glued on the bottom of the flask is visible. Osteoblasts grown in proximity or on the surface of the polymer matrices. Original magnification 40×.

in morphology were observed between osteoblasts grown in the presence of the two polymers and controls. In general, the cells grown in proximity to the polymers and adhered on the surface of the polymer matrices. As shown in Fig. 1, osteoblasts present a typical polygonal morphology with extended filopodia which may reach the polymer. Microphotographs show cells cultured in empty flasks or in the presence of the copolymer PLGA (50:50) for 7 days or 14 days. Similar findings were observed for copolymer PLGA (75:25) (data not shown).

### 3.2. Cell viability and proliferation

Throughout the entire study the number of cells grown in the presence of the polymers remained similar to the controls. The assays to ascertain the viability and proliferative activity of osteoblasts grown in the presence of the polymers for 14 days indicate that these latter did not affect the number of living cells evaluated by Trypan blue exclusion, LDH leakage and the MTT assay (Fig. 2). The incorporation of [$^3$H]thymidine in osteoblasts grown the presence of the polymers and in control cells was similar (Fig. 3).

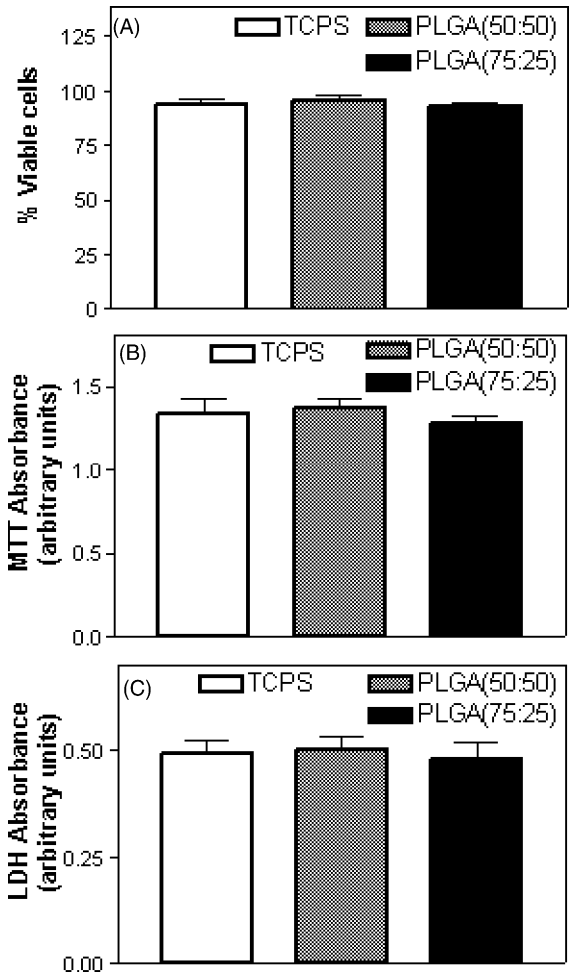


Fig. 2. Viability (A), mitochondrial integrity assay (B) and LDH leakage (C) of primary human osteoblasts cultured for 14 days in close contact with PLGA copolymers or in empty tissue culture polystyrene flasks (TCPS). The percentage of viable cells was assessed by Trypan blue exclusion. Mitochondrial integrity (MTT assay) and LDH leakage were evaluated as described in Section 2. Values are the mean ± S.E.M. of at least three independent samples.

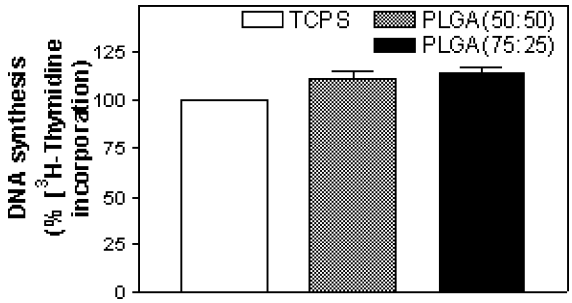


Fig. 3. Proliferation of primary human osteoblasts cultured for 14 days in close contact with PLGA copolymers or in empty tissue culture polystyrene flasks (TCPS). The percentage of [$^3$H]thymidine incorporated into DNA was taken as an index of cell proliferation, as described in Section 2. Values are the mean ± S.E.M. of at least three independent samples.

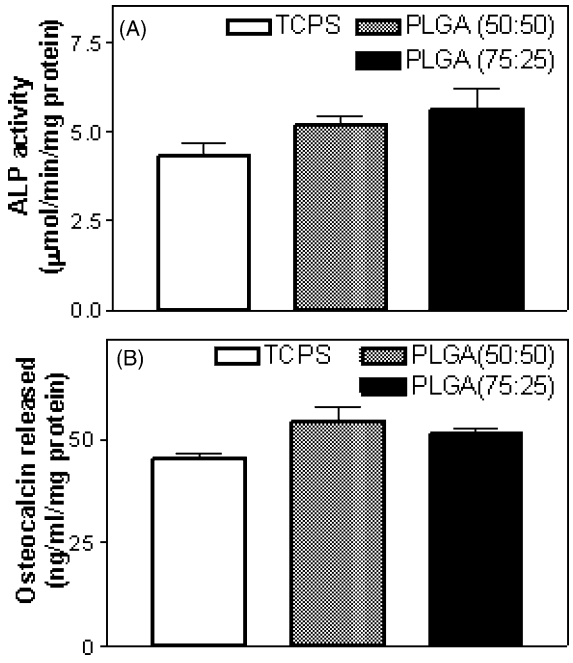


Fig. 4. Alkaline phosphatase (ALP) activity (A) and osteocalcin release (B) of primary human osteoblasts cultured for 14 days in close contact with PLGA copolymers or in empty tissue culture polystyrene flasks (TCPS). ALP activity and osteocalcin release were normalized to cell protein content and determined as described in Section 2. Values are the mean ± S.E.M. of at least three independent samples.

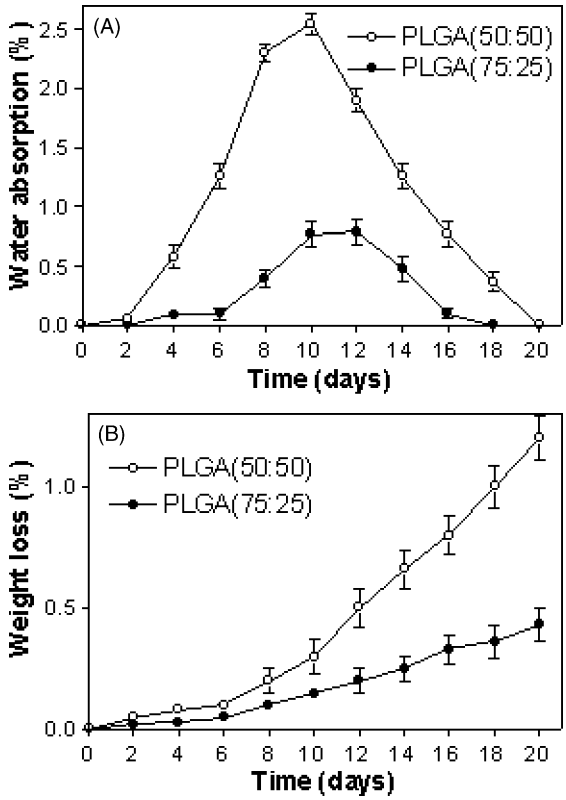


Fig. 5. Water absorption (A) and weight loss (B) of PLGA copolymers in relation to time. Values are the mean ± S.E.M. of three independent samples.

### 3.3. ALP activity and osteocalcin release

ALP enzyme activity of osteoblasts grown for 14 days in the presence of the polymers was quantified using the *p*-nitrophenol phosphate assay. No significant differences were found between the control cells and those grown with the polymers (Fig. 4(A)). Osteocalcin levels measured in the media collected from osteoblasts grown with the polymers did not show any significant difference from controls (Fig. 4(B)).

### 3.4. Water absorption and weight loss

Water absorption studies of the copolymers, after immersion in distilled water, showed that PLGA (50:50) had a moderate uptake of water which was maximal (2.6%) after 10 days then it gradually declined; on the contrary, water uptake of PLGA (75:25) was only 0.8% after 12 days and then it slowly declined (Fig. 5(A)). Weight loss of the two copolymers gradually increased over time and after 20 days it was ≈1.2% for PLGA (50:50) and ≈0.4% for PLGA (75:25) (Fig. 5(B)). The two copolymers showed a similar profile in cell culture medium (data not shown). Water absorption was the result of the balance between water uptake and the dissolution of oligomers generated by hydrolysis. Water-soluble degradation products accumulated within the film and the increasing hydrophilic end groups generated by the degradation had the potential to increase water absorption. Initially, water is absorbed and degradation products accumulate until the peak time after which the dissolution of the oligomers is increased and these diffuse from the surface of the sample into the water (Ara et al., 2002; Hurrell et al., 2003).

### 3.5. Cell adhesion on PLGA copolymers

As shown in Fig. 6, after 2 h, a higher number of cells adhered to PLA–PGA copolymers than to control cells seeded on TCPS. However, at 6 and 8 h, the adhesion of osteoblasts plated on PLGA copolymers was comparable to controls and cells became increasingly adherent to materials by increasing incubation time. Osteoblast adhesion to PLGA copolymers was significantly reduced by treatment with certain integrin-specific antibodies. In cells exposed to antibodies directed against the $\beta_1$ integrin subunit or the complex $\alpha_5\beta_1$, adhesion was reduced by ≈70% in comparison with the values for IgG-treated controls. Blocking of $\alpha_v$ integrins significantly decreased adhesion of osteoblasts but only by ≈30% (Fig. 6(B)).

## 4. Discussion

PLA and PLGA polymers are employed to prepare biodegradable internal bone fixation devices (Leenslag

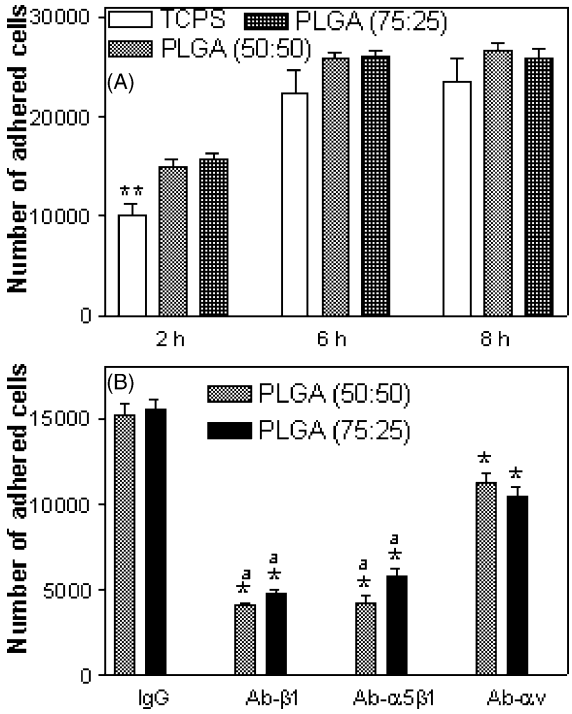


Fig. 6. Adhesion of human osteoblasts on PLGA copolymers or on tissue culture polystyrene flasks (TCPS). (A) Cell adhesion over 8 h. (B) Effect of exposure to specific antibodies (10 μg/ml) directed against the $\beta_1$ integrin subunit or raised against $\alpha_5\beta_1$ or $\alpha_v$ integrins. Control cells were exposed to immunoglobulin G1 (IgG; 10 μg/ml). Osteoblasts were treated as described in Section 2. Values are the mean ± S.E.M. of at least six different samples.** $P < 0.01$ vs. cells exposed to PLGA (50:50) or PLGA (75:25); * $P < 0.01$; vs. TCPS-plated cells; [a] $P < 0.01$ vs. cells exposed to antibodies against $\beta_1$ integrin subunit or $\alpha_5\beta_1$ integrin complex (Newman–Keuls test after ANOVA).

et al., 1987; Athanasiou et al., 1996, 1998). Their biocompatibility can be influenced by molecular weight, preparation processes and degradation rate (Park and Cima, 1996; Ikarashi et al., 2000a). Therefore, novel mixtures of lactide and glycolide polymers need to be investigated in vivo and in vitro. We employed human osteoblasts to evaluate the biocompatibility of two PLGA copolymers. The cell culture system enabled us to assay the influence of direct contact of the polymer with the cells, as well as any effect of soluble factors released from them (Schwartz et al., 1999). So far, only a few studies have employed these latter cells to assay the biocompatibility of PLA and PLGA polymers over time. The cells selected for biocompatibility studies are crucial and must be representative of the cell population in the tissue exposed to the biomaterial in clinical use. In vitro models are useful for examining cell interactions with polymers since in vitro conditions provide a controlled environment in which to investigate the direct effect of the polymer on cell adhesion, viability and proliferation, without the influence of other systemic events. For biocompatibility assays, it is essential to select a very sensitive index of cytotoxicity. The material being assayed often causes only slight toxicity over time so it is necessary to differentiate between these mild cellular responses to choose the most biocompatible materials (Macnair et al., 1997; Ikarashi et al., 2000b).

We did not find any negative influence of PLGA copolymers on cell morphology, viability and proliferation. We allowed a prolonged exposure of cells to soluble products released by the polymers by plating a low number of cells and only changing the culture medium once a week. For orthopedic and dental materials it is essential to evaluate the effects of a direct interaction of biomaterials with bone-forming cells. Osteoblasts, in fact, are more sensitive to these interactions than fibroblasts (Winn and Hollinger, 2000).

It is also important to examine the retention of proper phenotypes when examining the long term biocompatibility of osteoblasts cultured with synthetic materials, to ensure normal cell activity. In this study, we measured ALP activity and osteocalcin levels to rule out any effect of the polymers on the production of proteins characteristic of the osteoblastic phenotype. Several authors, in fact, have recommended that the assessment of osteoblast responses to biomaterials in vitro should include assays of the processes involved in bone mineralization, including ALP activity (Lian et al., 1999) and osteocalcin levels (Desbois and Karsenty, 1995).

In vitro degradation studies indicated that only the PLGA (50:50) copolymer swelled appreciably, probably because of its lower hydrophobicity of than PLGA (75:25) (Morrison et al., 1995). The PLGA copolymers do have a low degradation profile which increases significantly after several weeks of incubation at 37 °C (Ikarashi et al., 2000b). This process, as reported in previous studies (Hurrell et al., 2003), can be separated in several stages. In stage I, small quantities of water diffuse into the sample. In stage II, little further water is absorbed, but polymer hydrolysis causes the molecular weight to decrease. In later stages, oligomers begin to diffuse from the surface of the sample.

In order to determine any mechanism responsible for cell adhesion to PLGA copolymers, a study aimed to characterize integrins' role was carried out by measuring osteoblast adhesion on copolymers. We observed that the $\beta_1$ integrin subunit and integrin $\alpha_5\beta_1$ significantly contribute to cell adhesion to PLGA copolymers. These data are in agreement with studies showing that fibronectin, a major ECM component in bone, interacts with the $\alpha_5\beta_1$ integrin complex to regulate osteoblast adhesion, differentiation and survival (Globus et al., 1998; Moursi et al., 1997; Cowles et al., 2000). On the contrary, $\alpha_v$ integrins seem to play a minor role as well as suggested by Gronthos et al. (1997). In fact, these authors have reported that the attachment of normal human bone cells to collagen, laminin, fibronectin and vitronectin was unaffected in the presence of a function-blocking antibody to $\alpha_v\beta_3$ integrin whereas $\beta_1$ integrins appear to be the predominant adhesion receptor subfamily utilized by osteoblast-like cells to adhere to collagen and laminin and in part to fibronectin. El-Amin et al. (2002, 2003) have recently reported that osteoblasts adhere to PLA and PLGA copolymers in vitro inducing changes in the production of ECM proteins and of several integrins.

Our results, for the first time, demonstrate the crucial role played by $\alpha_5\beta_1$ integrin and by the $\beta_1$ integrin subunit during the early adhesion of osteoblasts to PLGA copolymers. Therefore, in addition to evaluate integrin expression in cultured osteoblasts it is relevant to evaluate any functional role played by these molecules on the complex interactions that take place at the cell-polymer interface. In agreement with this idea, integrins have been demonstrated to play a key role in osteoblast adhesion to various orthopaedically relevant substrates (Krause et al., 2000; Matsuura et al., 2000; Schneider et al., 2001; Zreiqat et al., 2002; Cutler and García, 2003). Future studies will further explore the role of specific integrins in events controlling osteoblast adherence and growth on polymers suitable for bone tissue engineering.

In conclusion, this study ascertained that human osteoblasts can adhere, grow and proliferate in vitro in the presence of PLGA copolymers.

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