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ATTACHMENT KINETICS AND DIFFERENTIATION OF OSTEOBLASTS ON DIFFERENT BIOMATERIALS

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Abstract

Primary bovine osteoblast cell cultures were used to study the response of osteoblasts ton three different biomaterials, ionomeric cement (IC), tri-calcium phosphate poly-L-lactic acid composite foil (TCP) and poly-L-lactate-polycitric acid composite foil (PLA). The cells which grew on the materials produced all typical bone matrix proteins and were osteoblast-like as shown by immuno-staining. Analysis of the cell attachment kinetics revealed significant differences within the first 7 hours between the various materials. The best rate of cell attachment was found on the IC surface, followed by the TCP and then the PLA surface. The kinetics of attachment appeared to be due to different surface wettabilities and could be expressed by a linear equation. Further investigations showed an ion mediated attachment of osteoblasts on the surfaces. Quantitative analysis of non-collagenous protein matrix production and DNA content per cell was carried out, showing the best results for PLA, followed by IC and TCP, indicating that the material with the best primary attachment characteristics is not necessarily that on which the cells differentiate the best. Scanning electron microscopy observations of primary outgrowth cultures showed close contact of osteoblasts on all surfaces. It was concluded that such techniques may be developed as a means of batch testing bioactive biomaterials and investigating bone cell/biomaterial interactions.

Key Words: Biocompatibility, osteoblasts, plating efficiency, kinetics.

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Introduction

The number of implants using new biomaterials steadily increase every year world-wide. Most biomaterial testing has been performed it *in vivo* in a variety of species. Usually the results of the animal tests have been evaluated histomorphologically after the material was implanted in either a bony or soft tissue implantation bed. These methods are difficult to analyze quantitatively, and due to cost considerations usually only a minimal number of experiments are performed. Therefore, the potential for pertinent and reliable evaluation is limited, and a comparison between different materials is difficult.

Jones *et al.* [21] developed a quantitative osteoblast cell culture system to evaluate the bioactivity of potential orthopaedic prosthetic materials, demonstrating that cell culture testing of potential biomaterials can be made under defined conditions. The cell culture parameters, plating efficiency, matrix production and DNA-content per cell could reliably predict the biocompatibility of biomaterials when compared with parallel animal tests. Gross and Strunz [12, 13] showed that implants are capable of releasing substances which may influence differentiation of cells in the peri-implant compartment. These early effects on the processes of bone healing are considered to be more important than long term bone-remodeling effects [14]. As a single cell type is responsible for the elaboration of bone tissue, an understanding of osteoblast reaction to synthetic surfaces is of central importance in explaining the bioreactive pathways affected by bone-substitute biomaterials [9]. From studies of cell culture on different surfaces it is known that wettability of the surface plays an important role for cell adhesion [23].

Morphological *in vitro* studies have shown that osteoblasts have the potential for variable phenotypic expression, which may be critically influenced by the presence of an artificial material [26], but little is known about the causal relationship between morphological appearance and cellular activity and differentiation. Thus the shape of the cell might not reflect its activity and stage of differentiation. Jones and Matthiass [22] have described several types of osteoblasts which differ in

morphology and response to various factors, but produce the same matrix proteins.

The purpose of this study was to evaluate the initial and later response of osteoblasts on three material surfaces with different wettabilities to develop a quantitative biocompatibility test. Experiments were performed under different conditions to gain insight into the mechanisms of cell attachment. Cell attachment kinetics, the type of attachment and quantification of differentiation of the cells, investigated by biochemical parameters, were compared with phenotypic expression of osteoblasts, migrating over the bulk biomaterials.

Materials and Methods

Materials

Three different biomaterials were evaluated. One was a commercially available ionomeric cement (Ketac-0, Ionos, Germany). The cement is formed by an acid-base reaction between the ion-leachable glass and a polyalkenoic acid. The glass composition by mass is SiO_2 - 35%; Al_2O_3 - 30%; CaO - 15%; fluorine - 18%; Na_2O - 3%; and P_2O_5 - 7% (calculated as the oxides). The material properties have been described by Jonck and Gobbelaar [20]. The material was delivered with a smooth glass-like surface (surface roughness less than 0.5 μm). The tri-calcium phosphate-poly-L-lactic acid composite contents by mass 70% tri-calcium-phosphate and 30% poly-L-lactic acid. The poly-L-lactate-polycitric acid composite foil consists of 95% poly-l-lactate and 5% polycitric acid, with a smooth, glass-like surface and a rough surface. The smooth surface was used in our studies. The two latter materials were prepared by H. Heide by dissolving bulk polylactic acid and polycitric acid (Boehringer) in chloroform, to which tri-calcium phosphate (TCP) granules were mixed. All materials were delivered and tested as bulk materials. Materials were immersed in 70% alcohol for 30 minutes, air dried aseptically and, just before placement into the culture system, washed in Ham's medium containing 10% fetal calf serum.

Cell culture

Cells were prepared using an outgrowth method previously described by Jones *et al.* [22] from metacarpels of 18 month old steers obtained at the local slaughterhouse. Periosteum pieces were cultured in High Growth Enhancement Medium (HiGem, Flow Laboratories) medium containing 10% fetal calf serum for 4 to 5 weeks. Culture medium was changed once a week. After confluence the cells were harvested by collagenase treatment (0.4 g collagenase and 98.8 mg Ham's F10 in 10 ml HEPES buffer) for 20 minutes followed by Tyrode's solution treatment {300 mg ethylenediaminetetraacetic acid (EDTA)-Na salt in 1000 ml solution, containing 200 mg KCl, 8 g NaCl, 1 g NaHCO, 56.5 mg NaH_2PO_4 and 1 g glucose}. Cells were counted in a Coulter Counter and used for the experiments.

Plating efficiency

The plating efficiency was defined as the percentage of attached cells on the surface after 24 hours. Preliminary experiments established the plating efficiency for each surface, so that the same numbers of attached cells were used to compare the growth and production of matrix proteins on the different surfaces. For the kinetic attachment experiments the same number of *viable* cells were used (as determined by the tyrpan blue exclusion test) for each material.

'Flexiperm' silcone wells (Heraeus, Hanau, Germany) were attached to the material to form 8 separate culture chambers and 500 μl Ham's medium including 160,000 viable cells was added to each chamber to give 70% confluency. The chamber area was 0.64 cm^2. Cells were incubated at 37°C and 5% CO_2. After 1, 4, 7, 11 and 24 hours the supernatant was removed and cells were measured in a Coulter Counter. The cells remaining in suspension are more easily and more accurately counted than measuring cells attached to the surfaces, although control experiments determined that this number is the inverse of the number attached. Two aliquots each from three chambers were counted four times and the results expressed as the mean of the cell count ($\pm$ standard-deviation, n = 3, 24 measurements).

Rate of cell attachment

The experiment was performed after 4 hours incubation under the same conditions except, that 200,000 viable cells were used per chamber. Aliquots from the supernatant were counted. After 4 hours the cells attached to the surface were treated alternatively by incubation with a Ca^{++} and Mg^{++} free EDTA solution (0.03%) for 20 minutes (to determine the attachment due to calcium and magnesium mediated attachment), or by incubation with a Ca^{++} and Mg^{++} free EDTA solution containing 0.5 mg pronase in 1 ml EDTA solution (to determine the number of cells attached to the surfaces by adhesion proteins). Detached cells were then counted in a Coulter Counter and the surfaces were viewed under a microscope equipped with Nomarski differential contrast to control for cells remained attached to the surface.

Immunostaining

Periosteum pieces were attached to the material surfaces and incubated with HiGem for 21 days. The periosteum pieces were then removed and the cells were stained with polyclonal antibodies against specific bone matrix proteins reacted with alkaline phosphatase-anti alkaline phosphatase (APAAP) complex [6]. Polyclonal anti-osteocalcin, anti-osteopontin (BSPI), anti-bone sialoprotein II (BSPII), anti-N terminal type I procollagen and anti-N terminal type III procollagen antibodies (gifts of Dr. L. Fisher, Bone Research Laboratory NIH, Bethesda, Maryland, USA) were used. An anti-actin antibody was used as positive control, the negative control was incubated with non-specific serum. Stained cells were photographed on the material surface using a photomicroscope (Zeiss model 67937, Oberkochem) to control for the morphology of the stain either inside the

cell or associated with the extracellular matrix. The preparations were then mounted and photocopied to show the entire surface of the material. This preparation is called a cell blot.

Protein and DNA measurement

Materials were placed into petri dishes. About 60,000 cells per cm^2 were plated out on the material surfaces. Three replicate cultures for each material were used in two identical experiments. Cells were incubated in Ham's F10 medium for 10 days. After 10 days the cells were detached (as described in cell culture methods above), centrifuged at 400 rpm for 10 minutes, counted in the Coulter Counter and prepared for the DNA- and protein measurement. The cells were lysed with 0.3% EDTA pH 12 at 37°C for 30 minutes. A small aliquot (5% of the volume) was removed for protein determination. Protein measurement was performed on this aliquot by the method of Bradford [4] with Coomassie blue. The sample was then neutralised with a small volume of 4 N HCl and then with 20 mM HEPES in 1 mM $CaCl_2$ and incubated with 0.5% pronase for 20 minutes at 30°C. DNA measurement was performed by a modified method of West *et al.* [36], using the Hoechst bisbenzimide dye (No. 33258) by measuring fluorescence in a Titretek Fluoscan II. The protein and DNA concentrations were calculated and adjusted taking into account the volumes used.

Outgrowth experiments

Periosteum pieces were placed on the different materials and cultured for 3 weeks as described in cell culture methods above. The cultures were then prepared for scanning electron microscopy. After removal of the periosteum the specimen were first fixed by replacing the culture medium with 2.5% glutaraldehyde for 1 hour at 4°C. Probes were washed three times in Sørensen buffer for 30 minutes followed by incubation in 0.1 M cacodylate buffer (pH 7,4) for 1 hour at 20°C. Thereafter, cells were dehydrated in an ethanol-water series of 30, 50, 70, 80 and 95% ethanol, each step taking 30 minutes, followed by 1 hour in 100% ethanol, critical point dried (in a Balzer CDP 010 critical point drier) and coated with a thin layer (approximately 20 nm) of gold. They were examined in a Philips scanning electron microscope operated at 20 kV. Cracks developed in the ionomeric cement during this process to the change in volume due to the water content of the material. It was not possible to prepare this material in a way to prevent crack formation.

Results

Immunostaining

The differentiation of periosteal cells into osteoblast-like cells on the material surfaces was demonstrated by immunostaining with antibodies against bone specific matrix proteins. The cells produced in addition to a type I collagen matrix, an extracellular non-collagenous protein matrix consisting of osteocalcin, osteopontin (bone sialoprotein I or BSPI), and bone sialoprotein II (BSPII) which are also shown by immunostaining. Over 99% of the cells stained positively to all of the bone matrix proteins investigated, while skin fibroblasts were positive only to BM-40, decorin, biglycan and types I and III N terminal pro-collagens (data not shown). Figure 1 shows the presence of osteocalcin on the different surfaces after 10 days of culture. The presence of osteocalcin demonstrates a late stage of differentiation of periosteal cells. These results indicate that the cells growing out of the periosteum pieces differentiate into osteoblast-like cells.

Plating efficiency

In Figure 2, the results of plating efficiency of bovine osteoblasts on IC-, TCP- and PLA- surfaces are shown. During the first 7 hours, a steep decrease in cell number in the supernatant on all material surfaces was measured, which was exactly paralleled by the number of cells attached to the surface (data not shown). The rate of cell adhesion was fastest on the IC-surface, followed by TCP- and PLA-surfaces. Between 7 and 24 hours the rate of attachment was less on the IC- and TCP-surfaces and higher on PLA-surface. After 24 hours the number of non-adherent cells was below 12.5% of the initial cell number on all surfaces. Differences between the material dependant rate of attachment was observable during the first 11 hours of the experiment, but followed a logarithmic function in the first 7 hours of the experiment. Figure 3 shows the cell attachment kinetics of the different materials over a period of 7 hours. The rate of cells leaving suspension (attachment) follows a logarithmic curve (ln-log^e) and can be described by a linear equation:

$$y = (m * t) + x \qquad (1)$$

where: x = initial cell number, t = time, and m is a material constant.

Values of m for the three materials are:

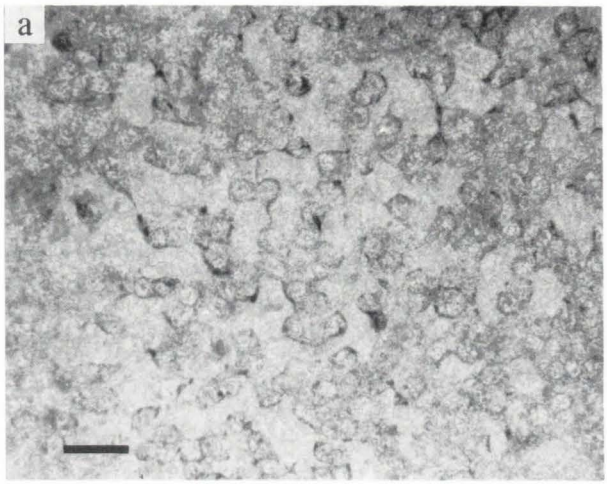
m (IC) = -0.33
m (TCP) = -0.22
m (PLA) = -0.13

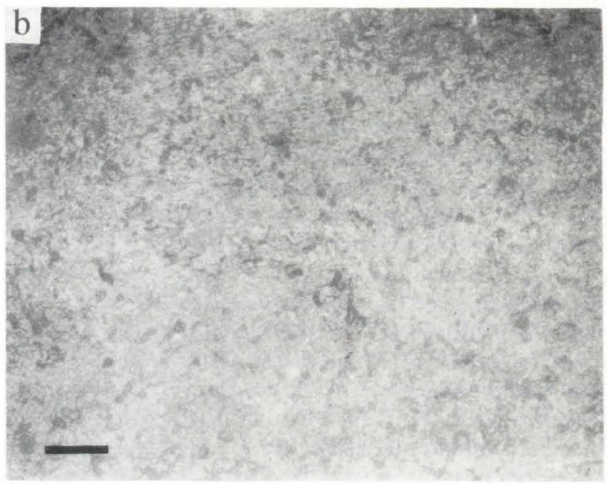
The correlation coefficients lie in the range of -0.96 (PLA) to -0.99 (IC, TCP). The inverse of the rate of disappearance of cells from the suspension is the number of cells attached, or cells settled on the surface (COS), which can be described by the formula:

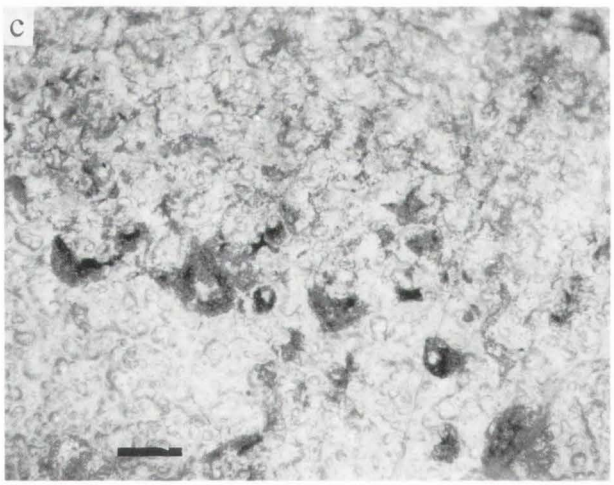
$$COS = x - e^{(-\{m * t\} + \ln x)} \qquad (2)$$

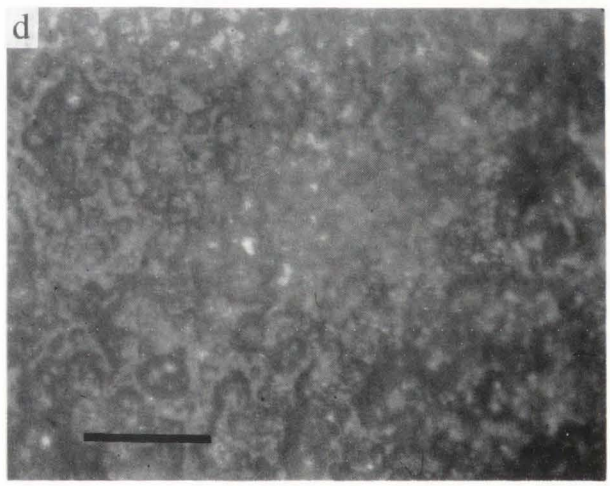
Cell detachment

Comparison between detachment of osteoblasts on the material surfaces by EDTA treatment and by a combined pronase/EDTA treatment after 4 hours incubation showed no statistically significant differences. Nearly all cells were detached by EDTA treatment and additive enzymatic treatment had no further effect, indicating that little attachment was mediated by adhesion proteins at that time. Figure 4 shows that most cells were adherent on the IC-surface and that a poorer attachment on the TCP- and PLA-surfaces occurred.









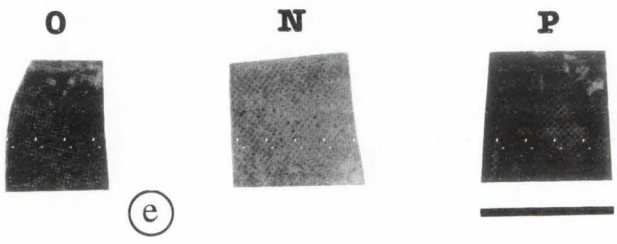


Figure 1. Presence of osteocalcin in osteoblasts on the various materials after 14 days in culture. The cultures were stained using the APAAP method described in **Materials and Methods**. For the cell blot, the cultures were first investigated microscopically for the distribution of the stain intracellularly and then scanned into a computer to show the intensity of stain over the entire culture surface. **a**) Osteoblasts on IC material stained by the APAAP method for osteocalcin. Bar = 20 μm. **b**) Negative control for a. **c**) Osteoblasts on TCP material stained by the APAAP method for osteocalcin. Bar = 20 μm. **d**) Negative control for c. **e**) Cell blot for osteocalcin on PLA also showing positive control against actin (P), negative control with non-specific serum (N) and using the specific polyclonal antibody against osteocalcin (O). Bar = 1 cm.

Protein and DNA measurement

Figure 5 shows the amount of matrix production per cell and the DNA content per cell after 10 days in culture. Cells on PLA produced the highest amount of matrix proteins (510 pg/cell). Growth on the TCP surface led to a significant lower protein production (326 pg/cell) whereas IC had a protein/cell ratio in between the other materials (381 pg/cell; Figure 5a). The pattern of DNA content per cell on the different materials was similar to the protein measurement (Figure 5b). Cells on the PLA-surface had the highest DNA content (25 pg/cell), followed by cells on IC- (19 pg/cell) and TCP- (14.2 pg/cell) surfaces. The protein/DNA ratios of the

Figure 4 (on facing page, bottom). Cell detachment of adherent osteoblasts on different materials after 4 hours of incubation. Columns indicate the number of detached cells by different treatments. Columns A and C indicate the cell number in the supernatant, column B indicate the cell number detached by Tyrode's solution treatment and column D indicate the cell number detached by a combined pronase/Tyrode's solution treatment.

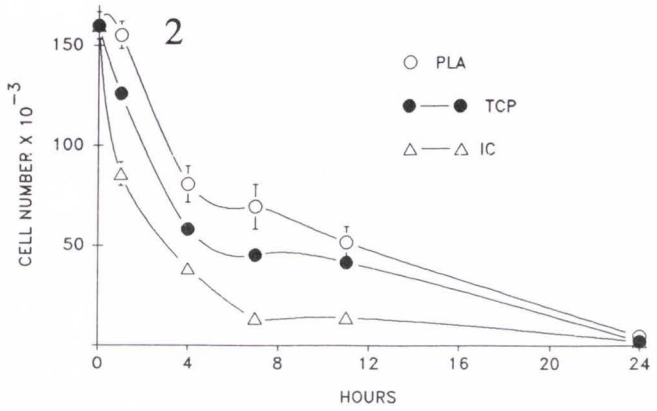


Figure 2. Plating efficiency kinetics of bovine osteoblasts on the different materials. Cell number indicates absolute number of non-adherent cells in the supernatant, measured after 1, 4, 7, 11 and 24 hours of incubation. Data are mean values of three determinations (± standard deviation).

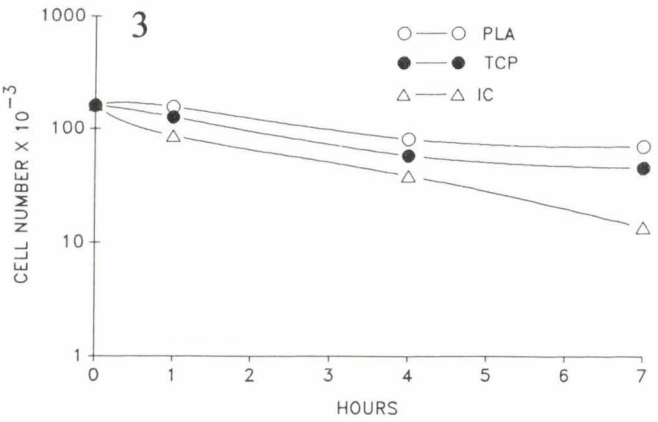
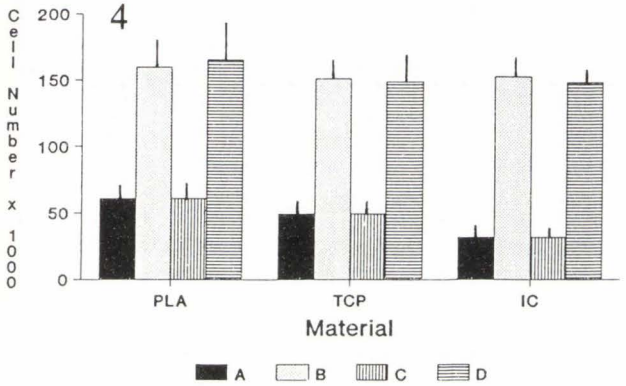
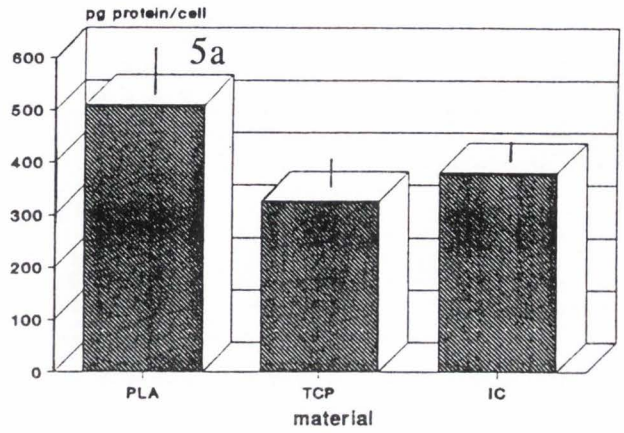
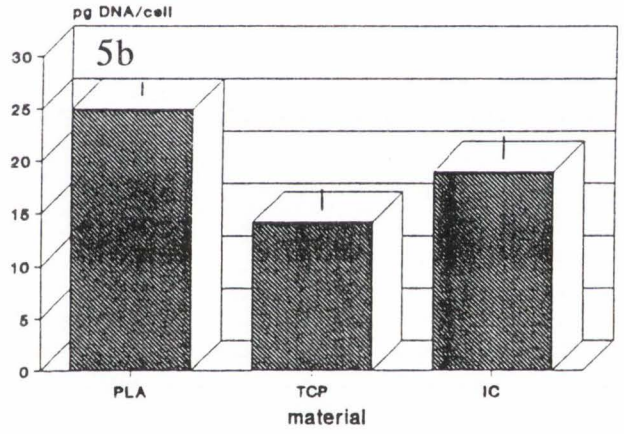


Figure 3. Cell attachment kinetics of cells during the first 7 hours of cell/surface contact. The values of Figure 2 are shown over time as a logarithmic function (base 10).







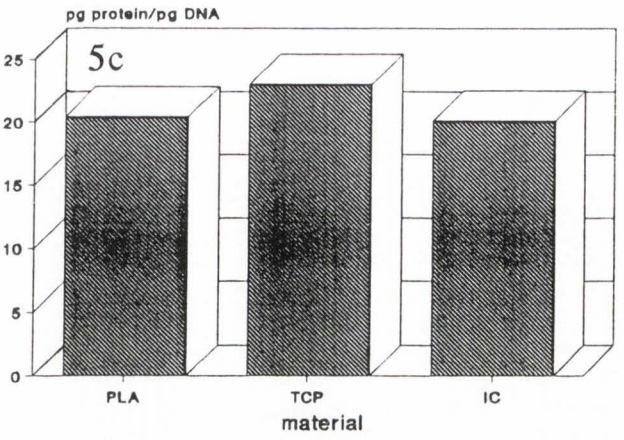
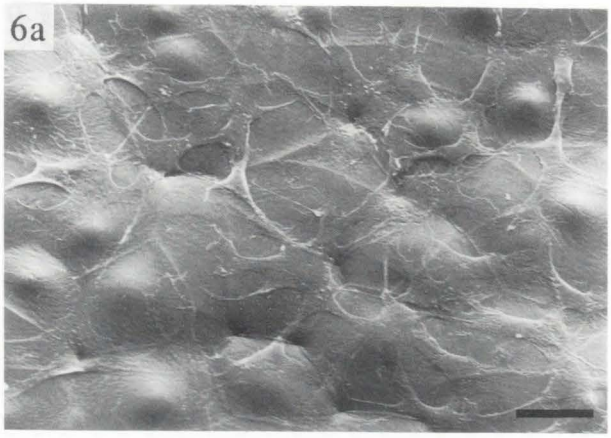


Figure 5a. Protein measurement of matrix production of osteoblasts after 10 days culture on PLA, TCP and IC. Cells were counted with a Coulter Counter and protein production was measured as described in **Methods**.
Figure 5b. DNA content per cell after 10 days of culture. The same cells as shown in Figure 5a (protein) were analysed.
Figure 5c. Protein/DNA ratio of cells. Values indicate the relation of protein production in relation to the DNA content of the cells.



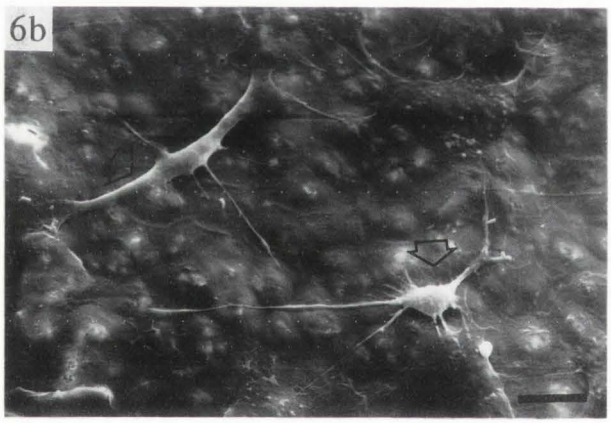


Figure 6. Scanning electron micrographs of 3 week old samples. Cells have formed a monolayer on the different materials. (**a**) Osteoblasts express a flattened morphology on the PLA surface. (**b**) On the TCP surface some cells can be seen in a 'stand-off' position (arrows). (**c**) On the IC surface cells are similar to TCP surface in morphology. Bar = 10 μm.

cultures were similar on all materials (Figure 5c). In contrast to the amount of matrix production and DNA content per cell, cells on TCP showed a slightly higher ratio (factor 46) than on IC and PLA (factor 40), where no statistically significant difference could be found.

Scanning electron microscopy

Osteoblast-like cells migrated over all three material surfaces. Cells spread out from the periosteum pieces to form a monolayer on the surface of the materials. Figure 6 shows the morphology of cells in the growing front after 3 weeks of periost culture. The flattened shapes of the osteoblasts indicate an intimate contact with the material. The cells also contacted each other via cellular extensions. Figure 6a shows a large number of extremely flattened cells spreading over the PLA-surface. The star-like shape of the cells was due to cellular extensions and filopodia, which are in contact with other cells. A further characteristic of cells on these substrates was an almost complete lack of dorsal cell-surface membrane activity in form of blebs and ruffles. Figure 6b shows osteoblasts on TCP. The shape of the cells is not the same as cells spread over the PLA-surface. Cells in intimate contact with the material can be distinguished from cells in a 'stand-off' position. The center of the cells in a 'stand-off' position is round shaped with dorsal cell-surface membrane activity. These cells have very long filopodia in comparison to flattened cells, probably due to having contracted. Osteoblasts migrating over IC express flattened and 'stand-off' positions. Some cells exhibit filopodial extensions, having intercellular contact or attaching to the material surface (Figure 6c). Intracellular protrusions can be observed in the peripheral area of some osteoblasts exhibiting dorsal cell-surface membrane activity. The cracks in the electron micrographs are material-specific for IC, caused by the critical point drying process due to the binding of water by ions in the material.

Discussion

In this study the attachment kinetics of osteoblast-like cells on different implant material surfaces was studied in relation to their subsequent differentiation and growth. We have used primary cells as these cells do not have the anchorage-independent growth phenotype typical of many cell lines and therefore most closely represent the physiological situation. Bovine osteoblasts show very few differences to human osteoblasts in their response to environmental factors and are more convenient to obtain in large, homogenous, quantities and vary little between cultures, which is not the case with human cell cultures.

The kinetics of cell attachment, the amount of non-collagenous matrix production and the DNA content of cells was compared with phenotypic expression as investigated at the light and electron microscopical level. Three different materials (IC, TCP, PLA), currently used as orthopaedic implant materials [11, 17, 19], were investigated. The osteoblast-like cells were derived

from outgrowth from periosteum pieces. Periosteum pieces were also used for migration experiments over the different materials and differentiation into osteoblast like cells was investigated. Production of osteocalcin and other bone matrix proteins indicated differentiation into bone forming cells, as previously shown [21].

Differentiation into bone forming cells is an important parameter to test for an improved contact between the material and the bony implantation bed. Another good parameter of biocompatibility is the ability of cell attachment to the surface, perhaps important in the first period of implantation. It is unknown which surface characteristics determine cell adhesion to different material surfaces and which mechanisms lead to cell attachment. Several characteristics have been proposed, including chemical groups [24, 29], interfacial free energy [1, 34] and surface charge [15, 33]. Results of studies concerning the mechanism of cell attachment are sometimes contradictory or suggest that unknown factors may play a role.

During the first 7 hours, the rates of attachment followed first order kinetics on all tested materials. Although the kinetics describe only a time dependant behaviour of a reaction, this leads to the suggestion of a simple mechanism of cell attachment in the first period of cell/surface interaction.

The rate of attachment over this time appeared to be related to an unknown property of the material, which preliminary studies indicate might be the wettability of the material, as measured by the water surface contact angle (preliminary data, not shown).

The significance of a high rate of cell attachment could be that it is necessary for recruitment of cells onto the material surface during the first period of implantation and, therefore, it is possibly important in determining the cellular pattern of growth on the implant. As the wettability of the materials was different (best for IC, followed by TCP and PLA), our results are similar to those of Weiss and Blumenson [35] and Dekker *et al.* [10], who showed that an improved wettability leads to a higher cell attachment. Cell attachment on the surfaces showed no differences after 24 hours incubation, indicating that other factors than wettability may begin to play an important role after this time.

To gain a better insight into the mechanism during this period we detached cells after 4 hours incubation by an EDTA-treatment and compared it with a combined EDTA/pronase-treatment. Data showed that EDTA-treatment alone leads to a detachment of all adherent cells and that no additional effect was obtained by enzymatic treatment. The fact that the most of the cells were detached by EDTA-treatment on all surfaces strongly suggests that the adhesion of cells was mediated by calcium and magnesium ions. The fact that plating efficiency was higher on surfaces with higher wettabilities (88% on IC, 78% on TCP, 73% on PLA) might support this hypothesis since it is to be expected that affinity of calcium and magnesium ions will be higher on more wettable surfaces. With regard to cell-substrate kinetics, Ca^{++}-containing biomaterials are specially interesting since reports in the literature point out that divalent cations such as Mn^{++} [25, 28], Mg^{++} and in particular, Ca^{++} [7, 14], enhance cell adhesion and spreading. Adhesion molecules such as fibronectin also have calcium binding sites, occupation of which are required for the function of the protein [2].

After 24 hours, the plating efficiency of all materials showed values in the range of 87% to 95% for all materials, comparable with results of Jones [21], who reported a relative plating efficiency for osteoblasts in the range of 80% on potassium glass to 98% on hydroxylapatite and cell culture polystyrol surfaces. Reports in the literature have pointed variously to attachment proteins, such as fibronectin or vitronectin, as being important for anchorage of cells to synthetic implant materials during the first phase of attachment [32]. The spreading of transformed cells and the amount of fibronectin adsorption was correlated with surfaces properties by Horbett and Schway [16]. We have not investigated the role of these proteins in these studies and the ion dependant attachment we have observed might be mediating an anchorage protein-surface interaction. Studies using protein free media are planned for the future to investigate this point further.

Matrix production and DNA-content per cell are important parameters of physiological activity of cells, indicating cellular stimulation of cells after some days of cell/surface contact. DNA to protein ratio in cells grown on synthetic surfaces is a valuable indicator of surface biocompatibility as judged by comparison to the results of independently conducted animal trials [20]. Osteoblast like cells produced most protein on PLA, followed by cells grown on IC and TCP. Jones [21] reported values of 180 to 850 pg protein per cell for bovine osteoblasts on synthetic surfaces like hydroxyapatite/calcium phosphate composites and hydroxyapatite ceramics. Values between 326 and 510 pg protein per cell in our experiments indicate a moderate physiological activity. DNA measurement per cell showed the same pattern as protein measurement, with highest DNA content in cells growing on the PLA-surface, followed by cells on the IC- and TCP-surface. The DNA content of the cells used in this experiment was between 15 and 25 pg DNA per cell. This is compatible with values quoted by Olander *et al.* [27], who quoted a value of 15 pg/cell for bovine endothelial cells, and Jones [21], who reported values of 19 to 40 pg/cell for bovine osteoblasts on different synthetic material surfaces, indicating different stages of the cell cycle. However other authors have found values for bovine fibroblasts and monocytes of 7-10 pg/cell [18, 31], which are probably too low. The protein/DNA ratios have similar values for all surfaces, due to the different DNA contents per cell. Since production of matrix is a feature of differentiation in osteoblasts, which is correlated with a slow down in the rate of cell division, an increased amount of matrix produced per cell can be taken as a positive sign of increased differentiation in the cells, as previously described [22].

We note that plating efficiency and protein/DNA-measurement can lead to different evaluation of the materials, indicating that adhesion and cell differentiation are distinct properties of a material. Obviously not only one of these parameters determines the biocompatibility of biomaterials. Accordingly to test biomaterials for improved biocompatibility, we suggest that a combination of experiments lead to a better prediction of biocompatibility. Osteoblast like cells were seen to colonize all three tested substrata. The phenotypic response of cells varied on the different surfaces, showing flattened cells on PLA and a combination of flattened cells and cells in a 'stand-off' position on TCP and IC. It is evident from this result, that osteoblasts in cell culture migrate over materials of different wettabilities. No signs or evidence of toxicity were apparent. However, comparison of osteoblast morphology on these substrata, maintained in identical cell culture conditions, would indicate that surface parameters play a critical role in determining migratory morphology. The effect of surface topography on our results is thought to be minimal since we used surfaces of similar roughness (smooth surfaces of rugosity of less than 0.5 μm). Therefore, the difference in wettability may lead to different morphologic expression. Similar results are reported by Davies *et al.* [8]. They demonstrated that surface charge determines the morphology of osteoblasts. In their experiments, positively charged surfaces led to extreme flattening of the cells and resulted in less dorsal cell-surface membrane activity. They assumed that though protein adsorption was probable, the charge of the underlying substrate is the major factor in influencing the behaviour of the migrating cells. Bagambisa and Joos [3], and Cheung and Haak [5] cultured transformed osteoblast cell lines directly on hydroxyapatite and calcium phosphate ceramics, demonstrating the growth, proliferation and secretion of extracellular matrix without any impairment of their cell physiology. Intercellular contact via cellular extensions, seen on all materials, could contain gap junctions. Gap junctions have been found it *in vitro* in different animal species, including rat calvarial cells and bovine periosteal cells [30]. It is suggested that they may be a prerequisite for a metabolic coupling between osteoblasts in an extended network.

Conclusions

The present study shows that the various implant materials can support the growth and differentiation of periosteal cells into osteoblast-like cells. Quantification of the rate of cell attachment reveals a dependance, within the first 7 hours of culture, on a material constant, which might reflect the wettability. This method might allow a quantitative evaluation of biomaterials as a means of batch testing bioactive biomaterials. Cell attachment by Ca^{++} ion mediated-attachment might be responsible for this result.

Measurement of the plating efficiency after 24 hours revealed less significant differences between the materials tested.

A quantitative measurement of the degree of differentiation indicates that the material property responsible for the different rates of attachment is not the same as that influencing differentiation on the material.

Measurement of physiological activity of cells combined with investigations of phenotypic expression may elucidate the mechanism by which material surfaces can effect bone cell behaviour. In this way, we hope to establish a quantitative it *in vitro* test, which is important for the selection or design of synthetic biomaterials prior to it *in vivo* testing.

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Discussion with Reviewers

U. Gross: Figure 6b demonstrates two cells in 'stand off' position. Do these cells eventually belong to another cell species, e.g., osteoblast-like cells? What is the evidence for classification of these cells as osteoblast-like cells?

Authors: These cells are osteoblast-like on the basis that cells with the same morphology stain for osteoblast matrix proteins (procollagen I, osteocalcin etc). Time lapse photography of osteoblast cultures on biomaterials show that during cell division, osteoblasts will raise themselves up out of the cell sheet, divide, wander around to find a place to attach again, and either not reattach or attach on top of other cells, especially when a lot of collagen is produced.

U. Gross: Figure 6c provides areas with cells in two layers or in multilayers. Is there evidence for production of collagen and for mineralisation between multilayered cells?

Authors: Yes, a little collagen and a little mineral. We

are looking into it more closely. However, we think that cells are attached through hyaluronic acid containing matrix proteins and their hyaluronic acid receptors.

U. Gross: Did you observe dead or dying cells, or remnants of cells being attached to the artificial surfaces?
Authors: The number of dead cells in the cultures is dependant on the way they have been handled. In our system we can get a very high level of viability (sometimes up to 98%), and, depending on the substrate, a corresponding high level of plating efficiency (up to 97%). Sequential digestion cultures of osteoblasts, a common way of preparing cultures, usually have very high mortalities. During culture, and depending on the medium, and even the medium-surface interaction, osteoblasts will leave the surface to divide and find a less crowded place, if they cannot re-establish themselves on a surface they can live for a while as a rounded-up cell and then eventually die. In the time course of our experiments, this does not usually happen.

P.B. van Wachem: It seems to me that a control material (preferably tissue culture polystyrene, TCPS) is missing. How do these cells perform on TCPS? I expect adhesion and spreading to be similar (or better?), but what about matrix protein production?
Authors: An important point; we have previously described the behaviour of cells on cell culture plastic referred to in the text (ref 22). Since we can reliably produce quantitative results from the surfaces, we now choose the TCPS surface as our 'control' surface, as this produces better matrix attachment than the cell culture plastic. Further, the handling of the culture plastic is not as simple for these tests as the materials we use.

P.B. van Wachem: Can your method represent a quantitative measurements of osteocalcin?
Authors: We did not quantify the amount of osteocalcin produced by the cells, only the total amount of non-collagenous protein. The purpose of the immune staining is to show the presence of this positive marker and to show that a very high percentage of the cells are positive for it. Osteocalcin measurement by a radioimmunoassay (RIA) was beyond our budget and would require larger numbers of cells, for which our system is not adapted.

P.B. van Wachem: In general, it seems to be a problem to present results of an 'explant culture' (as done here with immunostaining) and SEM. There seems to be no possibility to clearly compare the (matrix protein production and morphology) reaction to these three materials since the cell numbers are not known. Or in case of confluent layers, it is not known how long these have been confluent? Differences in confluency might be reason for differences in morphology.
Authors: We have carried out three studies. In the first we have measured the kinetics of attachment. In the second we have determined the amount of protein per cell and the DNA content per cell, and in the third we have studied the development of the cell on the materials. The first two are quantitative, and as pointed out by the reviewer, the third is not. However, as explained in the text, it does demonstrate the important point that osteoblast precursor cells (from the periosteum) can differentiate on the material surfaces.

P.B. Van Wachem: Why not show, as a control, the naked materials? And why was 24 hours not chosen as the moment of SEM?
Authors: In view of the results showing that at 24 hours there was little difference between the materials and that after 10 days differences could be seen, it seemed more interesting to show the morphology at a later point in the development of the culture. Also it takes at least 8 to 10 days before the first cells grow out of the periosteum pieces. Photography of the naked material will give no further information since smooth surfaces were used in our experiments.

P.B. van Wachem: I am surprised that such different time periods were chosen for different tests. Was it not possible to do immunostaining, DNA-measurements, and SEM at the same time point (e.g., always after 14 days)?
Authors: The time points chosen reflect the different phases of the culture. The cell cultures are differentiated after 5 days, and the explant cultures differentiated after 3 weeks! Thus we have investigated the cells at the same stage of differentiation, due to the different culture systems.

PB van Wachem: Why covering about 70% of chamber area?
Authors: We used a cell number to give 70% coverage because the larger number of cells gives more accurate results, and a higher number of cells than this would interfere with the settling out due to competition. A cell number that gives 70% coverage is the best compromise to measure plating efficiency.

P.B. van Wachem: What additional information is provided given by equations 1 and 2 since they are not further discussed in the paper?
Authors: We were interested in the rate of attachment of cells to the different surfaces since they had different wettabilities. We have shown, by analysis of the attachment kinetics, that the rate of settling was related to the wettability but, as discussed in the text, this does not effect the degree of differentiation as determined by the protein/cell ratio. The equations are of a simple first order form and, as shown in the text, the kinetics of settling correlate strongly with the formula. Hence we show that the approach of cells to the surface is related to only one parameter, and that this parameter appears to be the wettability of the surface. The question is then what is the significance of hydrophobicity and what does this mean in terms of the measurement made, since the techniques available for measuring surface wettability are at a scale far greater (mm^2) than the scale on which the

cells 'see' the surface (tens of nanometers). The equations we derive from our results, therefore, represent a phase of cell settling related to a surface parameter and are an essential part of understanding the phenomena.

F.B. Bagambisa: Can the authors substantiate the statement "The fact that the most of the cells were detached by EDTA-treatment on all surfaces, strongly suggests that the adhesion of cells was mediated by calcium and magnesium ions"?
Authors: EDTA is a divalent cation chelator. Hence if EDTA removes cells from the surface within a few minutes, this must be due to chelation of these ions. As implied in the **Culture methods** section, the medium we used contained mainly calcium (1 mM) and some Mg and Mn (50 μM).

F.B. Bagambisa: The authors interpret their results to mean that high cell attachment rates were related to the wettabilities of the materials they tested. However, it is known from the literature that in serum-containing media (and the present authors subjected their specimens to serum treatment), the disparity between the adhesion capacity of wettable compared to non-wettable substrates is compromised to the extent that it disappears at 100% serum content [35]. This is presumably due to the adsorption of serum proteins, which can take place within the first seconds to minutes of contact with serum-containing media [16; and Reddi AH: Implant-stimulated interface reactions during collagenous bone matrix-induced bone formation. J Biomed Mater Res **19**, 223-39, 1985]. How do the authors explain that the wettability of the materials they tested is not affected by the adsorption of such proteins?
Authors: As you say, wettability will be affected by serum proteins, since hydrophobic surfaces will bind hydrophobic proteins (most proteins have hydrophobic regions). It is indeed interesting that in our experiments using 10% serum, the correlation with surface wettability was made. However, it has been suggested that cells only interact with proteins. In view of the short time that we have measured the kinetics of attachment, the question becomes either: what proteins are absorbed onto the surface that intermediates the observed attachment (Vroman effect?) or, as our results appear to imply, that during the first few hours, that there is a direct interaction with the surface. We made no statements about the wettabilities of the materials due to reservations about the method for making these measurements (what does bulk measurement over a large area have to do with the microenvironment of the cell?). We are trying to get better methodology for these measurements. Attachment kinetics under serum free conditions are being investigated at present. We have recently measured the surface potential of these materials, which are in fact quite different (work in progress), so the wettability remains the major correlation.

A. Dekker: In order to determine the plating efficiency, the authors counted the numbers of cells in the supernatant. An alternative method could have been the one used for the determination of cell attachment, namely, counting the number of adherent cells. Why did the authors choose the first method?
Authors: Counting cells in the suspension is less prone to methodological error than to count the cells settled on the surface. This is because a certain percentage will be damaged by the stripping procedure, and a certain percentage remains on the surface. A certain percentage of cells which are 'in between' settling will not be counted by measuring those bound to the surface as they will be easily washed out. We wanted to count those cells interacting with the surface. Also during the early part of the experiment there are far more cells in suspension than on the surface, so sampling error is less. The experiments were carried out in batch mode, not continuously. We have previously carried out experiments to satisfy ourselves that the number in suspension is the reciprocal of those settled out, hence the sampling of the suspension.

A. Dekker: In order to determining the importance of proteins for the adhesion of osteoblast-like cells, the authors used pronase. Why did they use pronase instead of collagenase which was also used for harvesting the cells?
Authors: Cells will attach to collagen after it is excreted. This occurs after about 7 days in culture. Before this happens, the cells are attached to the surface by fibronectin. Pronase is used to remove the attachment due to adhesion proteins, and collagenase the adhesion due to collagen attachment (which pronase will not effect). We used the appropriate protease for the appropriate type of attachment. This is a well known phenomenon in osteoblast cultures.

A. Dekker: In this study bovine osteoblast like cells were used. From literature it is known that, in general, homologous cell types from various species differ from each other. Do the authors think that bovine osteoblast-like cells are representative for human osteoblasts?
Authors: There is very little difference between bovine and human osteoblasts. Rat osteoblasts, for instance, are more different in some details from human osteoblasts, but only in some minor aspects of molecular biology. There is greater difference between a cell line derived from a human osteosarcoma and a human osteoblast than between a normal bovine or rat osteoblast and a normal human osteoblast.

J.E. Davies: Why do the authors use pronase to prepare the cells, and not collagenase?
Authors: During the rapid growth phase of osteoblast-like cells in culture, very little matrix is produced. As the cells start to differentiate they increase alkaline phosphatase activity, and then start to produce collagen. As the collagen secreted from the cell forms fibers and attaches to the substrate, the cells start to attach to the

collagen in preference to the fibronectin that first mediates cell attachment to surfaces (see e.g., Chinn *et al.* J. Colloid. Interface Sci. 1989, **127**: 67-73; and Dekker *et al.* Biomaterials 1991, **12**: 130-138). We find that removal of the cells from the primary outgrowth culture for re-plating before the collagen production phase gives a better yield of viable cells. It is normal in cell culture techniques to use EDTA-trypsin or EDTA-pronase solutions to loosen cells from the culture surface. We find that in our cultures, after 2-3 weeks, EDTA-pronase E (0.02% and 0.03% in calcium magnesium free Tyrodes solution, pH 7.4) loosens the cell sheet, but the cells are still attached to one another and to the collagen sheet. Collagenase helps disperse the cells at this stage. We did not culture the cells for so long in this study, hence there is no need for collagenase treatment. In addition, we have recently investigated the type of cell adhesion molecules responsible for mediating attachment on some of the surfaces used in the present study and found that the integrins $\alpha 5$ and $\beta 1$ were present (fibronectin receptor) and not the $\alpha 7\beta 1$ (collagen/laminin receptor) as is found at a later stage in the culture, when collagen is present (paper in preparation).

J.E. Davies: Why do the authors not show the collagen immunostaining results?

Authors: We used antibodies directed against the intracellular N and C terminals of pro-collagens I and III. This was mainly to investigate whether collagen production had started at the time of our investigation. Since osteocalcin is a very specific marker for osteoblast-like phenotype than types I and III collagens, which are found in most fibroblast lineage cells, we did not think that there would be much gained in showing the cell blots, which were as positive as those for the osteocalcin.