Tailor-made implant surfaces: the effects of surface roughness
and calcium phosphate coating

Paranimfen: Anne ter Brugge

Natasja Jannink

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Petra Josepha ter Brugge

geboren 10 januari 1972 te Almelo.

Promotores: Prof. dr. J.A. Jansen

               Prof. dr. C.G. Figdor

Manuscriptcommissie: Prof. dr. P. Spauwen (voorzitter)

                         Prof. dr. K. de Groot (Universiteit Leiden)

                         Dr. H. Vondenhoff

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Chapter 1

General introduction

1.1: Introduction:

Biomaterials can be defined as materials that are used to replace or reconstruct damaged tissues. With respect to bone replacing biomaterials, the term osseointegration has been used to describe an optimal biological interface between the material and surrounding bone. Bone formation on an implanted material can be considered as the result of a series of separate stages, involving conditioning of the surface by serum adsorption, followed by cell attachment, proliferation, osteogenic differentiation and eventually bone remodeling.[1] Among the factors influencing this process, biomaterial surface characteristics play an important role. Therefore, knowledge of the precise effect of material characteristics is essential when designing bone implant materials.

In this chapter, the bone forming cells as well as the effect of different material surface characteristics on bone formation will be described. Further, the integrin family of receptors, transmembrane proteins that generate intracellular signals in response to environmental cues will be discussed.

1.2: The osteogenic lineage:

Bone is a tissue that serves a dual function, i.e. providing support for the body and acting as a calcium reservoir. Bone is a very dynamic tissue, which is constantly broken down and rebuild by specialized cells associated with the mineralized bone matrix, called osteoclasts and osteoblasts respectively. Although from different lineage, both cell types originate in the bone marrow.

Within the bone marrow, two major cell systems are found: the hematopoietic lineage, which gives rise to blood cells and also to osteoclasts, and the marrow stroma.

Bone marrow stroma consists of a heterogeneous population of cells, containing marrow adipocytes, stromal fibroblasts and, near bone surfaces, osteoblasts and bone-lining cells. All these cell types are thought to be the progeny of a common stromal stem cell that resides within the stromal compartment. This stem cell would by definition be characterized as a cell that shows unlimited potential for self-renewal, and gives rise to committed progenitors of the different cell lines.[2,3]

Little is know about the mesenchymal stem cell (MSC), largely because of the lack of known markers or distinctive morphological characteristics. The term MSC is often used to describe a population of adherent marrow cells with a potential to differentiate along different lineages, called colony forming units fibroblasts (CFU-F).[4] Use of the term MSC to describe CFU-F is based on the fact that colonies derived from single marrow cells will, under the appropriate stimuli, differentiate into several cell types, an indication for the multipotential nature of the cells. However, the capacity for self-renewal has not yet been shown in culture, and therefore it is unclear whether the CFU-F contain stem cells, or

consist of early uncommitted progenitors.

Attempts to characterize CFU-F in other ways than using functional assays have been made in the last few years. These studies have resulted in the development of antibodies reacting with markers found on undifferentiated stromal cells. Expression of these markers is lost when the cells differentiate into the different stromal lineages.[5-7] Unfortunately, these markers are not specific for stromal cells, but are also found on other tissues.

The precise characteristics of stromal fibroblastic cells in vivo are also unclear. Stromal fibroblasts (also called reticular cells, adventitial reticular cells or Weston-Bainton cells) are cells expressing high levels of alkaline phosphatase that are found in the prenatal animal in the primitive marrow, where they provide a reservoir of precursors for prenatal osteogenesis. In the postnatal marrow, the cells generate the hematopoietic microenvironment and support hematopoiesis. In postnatal marrow, the cells also show characteristics of pre-adipocytes. Thus, the cells are myelosupportive elements with a multipotential nature.[2,8]

With progression along the mesenchymal lineage, the precursors are thought to progressively loose their multipotential nature, which eventually leads to the formation of committed progenitor cells. In vitro studies of bone marrow cells show the existence of a precursor that can differentiate into the adipogenic, chondrogenic or osteogenic lineage.[9,10] The cells progressively loose the adipogenic and chondrogenic potential with increased cell doubling.[9]

Differentiation along the osteoblastic lineage has been described to proceed along a number of specific stages (Figure 1). These stages were described based on morphological features, requirement for inducers and expression of markers in vitro and in vivo.

Committed osteoprogenitors are identified by functional assays of their capacity to form bone nodules in vitro. The cells show limited self-renewal and an extensive capacity for proliferation.[11,12] Osteoprogenitor cells are relatively rare, forming less than 0.1% of the total marrow cell population.[13] In vitro studies indicate that there are two types of osteoprogenitor cells. The immature type will only undergo osteoblastic differentiation in the presence of specific inducers, whereas the mature type will show spontaneous differentiation in the absence of this inducer.[14,15]

The osteoprogenitor stage is followed by the stage of the pre-osteoblasts, cells that stain for alkaline phosphatase, but have not yet acquired many of the other characteristics of osteoblasts. Pre-osteoblasts still possess limited capacity for proliferation. The mature osteoblast is described as a post-proliferative, strongly alkaline phosphatase positive cell found at sites of active matrix production.[16,17] The osteoblast phenotype is further characterized by the synthesis of bone matrix proteins such as collagen I, osteocalcin, bone salioprotein and osteopontin and by the ability to mineralize this matrix.

Decreasing Proliferation and increasing Differentiation
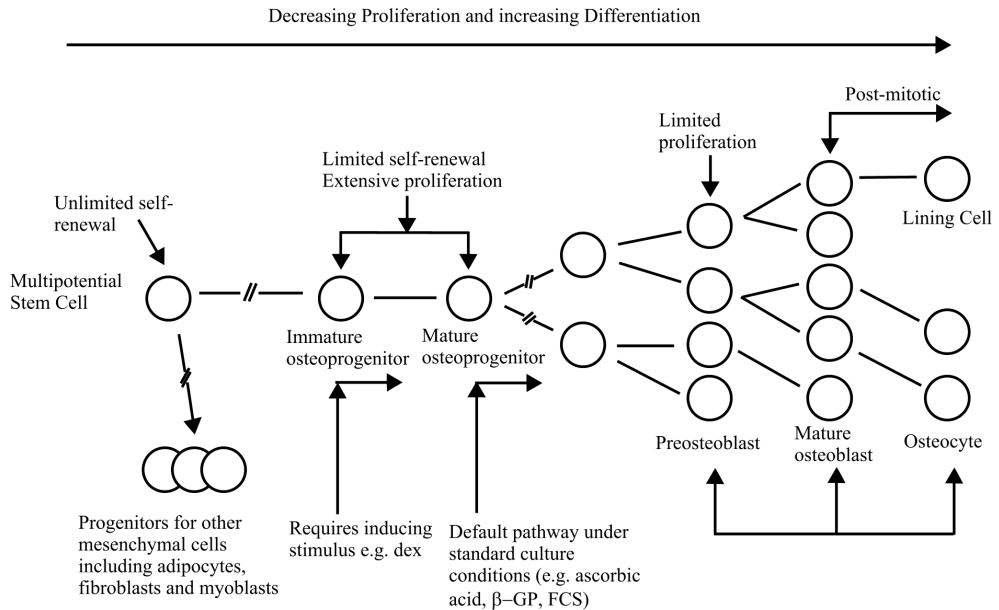
Figure 1: Postulated steps in the osteogenic lineage.[17]

1.3: Substrate surface characteristics:

The success or failure of a bone implant is determined by many variables, including patient variables such as bone quality, surgical technique, mechanical loading and material surface characteristics.[18]

An event that takes place almost immediately upon implantation of a material is the adsorption of proteins, lipids, minerals and sugars from the blood and tissue fluids. Cells from the osteogenic lineage attach, proliferate and differentiate, eventually resulting in bone formation on the implant. In the last step, osteoclasts are recruited and bone is remodeled.[1]

Many different materials have been designed as biomaterials in bone. These materials differ in characteristics such as surface composition, surface energy and surface roughness. Undifferentiated mesenchymal cells are the first cells supposed to encounter the implant material. When an implant is placed into the bone bed, committed osteoprogenitors may also be found around the implant.[1,19,20] The ability of the cells to attach, migrate, proliferate, differentiate and eventually form bone depends on the combination of various surface characteristics of the implant material.

1.3.1: Surface composition: titanium vs. calcium phosphate.

Titanium is the most widely used material for bone implants, due to its excellent biocompatibility. Titanium and its alloys are sometimes classified as bioinert, indicating that they do not induce a severe inflammatory response. Bone formation around titanium implants is called contact osteogenesis: bone is formed in close contact with, but not attached to the implant. Titanium implants are often provided with a coating layer made out of calcium phosphate (CaP). These materials are considered bioactive, meaning that they interact with the surrounding tissues to generate a chemical bond between the bone and the implant, so-called bonding osteogenesis.[19,21] Many studies have shown good bone formation around CaP coated implants, often better than around uncoated titanium implants.[22-29]

The precise reason for the effect of CaP on bone formation is not clear. One hypothesis is that dissolution of ions from bone-bioactive ceramics, followed by a precipitation reaction lead to the formation of a carbonated apatite layer. This new apatite layer, resembling bone mineral would then stimulate bone formation.[30,31] Further, high calcium and phosphate concentrations could directly affect cellular activity.[32-35] However, it has been shown that in the presence of serum, dissolution/precipitation reactions around some ceramics are significantly hampered by the presence of the proteins adsorbed to the material.[36,37] In these materials, formation of carbonated apatite is only found in the presence of cells, suggesting cell-mediated mechanisms.[37]

A second explanation for the bioactivity of CaP is its high affinity for many proteins and growth factors that play a role in bone formation.[38-40] Also, initial cell attachment may be differentially regulated by CaP or titanium surfaces, due to differential adsorption of proteins. For example, substrate surface composition may directly influence the type or amount of proteins adsorbed on the material immediately after implantation.[41-47] Surface composition may also affect protein conformation, which results in changes in the biological activity of the protein.[48-52]

Direct comparison of CaP and titanium surfaces showed that CaP adsorbs a larger amount of protein from serum than titanium and also adsorbs proteins not found on titanium.[53-55] Since many of these proteins are involved in cell adhesion to materials, differences in the adsorbed protein layer could explain the different biological effects of CaP and titanium substrates.

One important fact to mention is that the term CaP is used to describe a number of different materials that vary in composition, crystallinity and method of manufacturing. Many aspects of the material may be determined by the production method. For instance, magnetron sputtering generates a homogeneous CaP layer,[56] whereas a layer produced by plasma spraying consists of mixed amorphous and crystalline phases, depending on the parameters used.[57] This will result in differences in degradation between layers made using

different methods. We know already that the dissolution of magnetron sputtered coatings decreases with increased crystallinity.[56,58] In contrast, more crystalline plasma-sprayed coatings may degrade faster than amorphous layers, due to release of crystalline particles with dissolution of the amorphous phase.[57,59,60] Variations in CaP composition can lead to different dissolution/ precipitation behavior and may also affect in bone response.[36,61-63] Finally, we have to notice that in many studies the used materials differed not only in surface composition, but in surface roughness as well. This may also affect bone response.

1.3.2: Surface roughness.

A second surface characteristic that can affect bone response is implant surface roughness. Several in vivo studies show improved bone formation with increased surface roughness.[29,64-66] A large number of in vitro studies show that increased surface roughness enhances attachment, proliferation and expression of different markers in osteogenic cells.[67-72] On the other hand, there are also reports that show no effect of increased surface roughness or describe that it decreases cell function and bone formation.[73-77] Obviously, the precise reaction of tissues and cells to surface roughness is influenced by additional factors. These include area of implant insertion,[26] method of material cleaning,[72,78,79] cell maturation state[80] and surface geometry (regular vs. irregular).[81-83]

Several studies report that a surface roughness of 0.81, 4.0 or 1.1-1.4 μm (Ra value) respectively elicits an optimal response from bone cells and tissue.[71,84,85] However, it is impossible to compare the measured values with each other and with other studies, since differences in the method used to measure roughness lead to different results. In view of this, several different measurement techniques have been developed each with their own advantages and disadvantages.[86] The choice of the measuring method and the size of the measured area will greatly influence the values that are found.[87-89] Furthermore, depending on the type of implant, different locations on the same implant may show different roughness.[86] Therefore, in order to be able to compare roughness measurements in different studies, a consensus will have to be reached on the precise method of measurements.

There are several explanations for the effects of surface roughness on bone response. Increasing surface roughness leads to increased release of metal ions.[69] Above certain concentrations these ions inhibit proliferation and differentiation of osteogenic cells, which may explain the negative effect of high surface roughness on bone formation.[90,91]

On the other hand, roughening of the surface results in a better mechanical interlocking of the implant with the surrounding bone, increasing the fixation of the implant.[29,85] Surface roughness may also directly influence cell response. It has been implied that surface roughness changes the type and amount of protein adsorbed to the material.[67] Further, surface roughness may also influence cell spreading. Some authors have suggested that on

rough materials, the cells form attachment sites on different peaks of the material.[71] In contrast, cells on a smooth material would form all of their attachment sites in one plane, on the ventral side of the cells, resulting in larger lateral spreading. Attachment sites are linked to the cytoskeleton, which is involved in the generation of mechanical tension within the cell. The degree of spreading may influence the amount of force generated within the cells, which may directly influence biochemical signals in the cell.[92,93] In this way, surface roughness may directly influence the phenotype of cells on the surface.

1.4: Integrins:

Cells interact with an implant material through the layer of proteins adsorbed to the material immediate upon implantation. A large family of cellular adhesion receptors is the group of the integrins. Integrins are large transmembrane glycoproteins, consisting of a heterodimer of two subunits, α and β. The combination of the subunits determines the ligand specificity.[94,95]

Integrins are found in specialized adhesion sites called focal adhesions or focal contacts. In these structures, integrins span the membrane and interact with proteins on both site of the membrane. The extracellular domain of the integrin heterodimer forms a ligand-binding site, which recognizes specific sequences in extracellular matrix (ECM) proteins.[96] Many integrins recognize several proteins, while many ECM proteins act as ligands for more than one integrin. This pattern of overlapping specificity is probably due to the fact that most integrins recognize the sequence arginine-glycine-aspartic acid (RGD) found in many proteins. Several other more restricted recognition sequences are also found.[94,97]

The intracellular domain of the β subunit interacts with cytoskeletal proteins and signal transduction proteins. Ligand binding results in the generation of intracellular signals, which regulate cellular functions such as adhesion, spreading, proliferation, migration, differentiation and apoptosis.[98-100]

Integrins have been found on all cell types, with the exception of erythrocytes. Many studies into the expression of integrins on cells of the osteogenic lineage have been performed. These studies show that osteogenic cells express a wide variety of integrin subunits.[101-109] Many of these studies show different integrin expression patterns, probably due to the use of cells from different species, different anatomical locations, cultured cells or cells in vivo. Furthermore, integrin expression has been shown to change with lineage progression.[101,104,110,111]

Interactions of integrins with ECM molecules regulate many cellular functions in osteogenic cells. Interfering with the activity of integrins inhibits attachment of marrow stromal, human or rat osteoblasts to a variety of ECM proteins.[108,109,112] Increases in proliferation and expression of osteopontin in osteoblasts are both associated with cell

attachment to ECM proteins via integrins.[113,114] Further, the expression of osteogenic differentiation markers, such as alkaline phosphatase expression, nodule formation and mineralization are all inhibited by blocking the activity of specific integrins.[111,112,115-117]

Since integrins mediate adhesion of cells and transduce signals from the substrate into the cells, it is possible that integrins mediate the effect of surface characteristics on the cell. It has been shown that initial interactions of osteogenic cells with different materials may be regulated by different integrins.[55,118] Integrin expression may be differentially regulated upon adhesion to surfaces with different material characteristics.[119,120] Finally, formation of focal contacts can be altered depending on the surface characteristics of the materials the cells attach to.[121,122]

1.5: Objective of the study:

Substrate surface characteristics are among the variables that determine the response of cells and tissues to an implanted material. Substrates may affect the cells around an implant in different ways. For instance, surfaces adsorb different amounts or types of proteins and growth factors, which can stimulate osteogenic differentiation, possibly by acting as receptors for integrins. In that case, the integrins expressed by the cells may strongly influence the possible cellular response. Alternatively, the dissolution of calcium from calcium phosphate containing implants can directly stimulate the differentiation of cells around the implant. In that case, the dissolution rate of the material can be an important factor when designing an implant material.

In this study, the effects of various substrate surface characteristics on the responses of osteoblast-like cells in vitro were studied. In order to gain more insight into the mechanisms in which materials with different characteristics affect cellular response, the following questions were addressed:

* What is the effect of dexamethasone and serial subculturing on the expression of osteogenic markers by rat bone marrow cells.

* What is the effect of the crystallinity of RF magnetron sputtered coatings and substrate surface roughness on the proliferation and expression of osteogenic markers by rat bone marrow cells.

* What is the effect of surface roughness and presence of a CaP coating on the initial attachment, spreading and integrin expression of human osteosarcoma cells and rat bone marrow cells during their initial interaction with the material.

* What is the effect of surface roughness and the presence of a CaP coating on the expression pattern of integrins during prolonged culturing of human osteosarcoma cells and rat bone marrow cells.

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Chapter 2

In vitro osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone.

P.J. ter Brugge, J.A. Jansen

Introduction:

Bone tissue engineering depends on the combination of osteogenic cells with appropriate carrier materials. Both in vitro and in vivo research are important parts in the assessment of the safety and applicability of these constructs.[1] For the in vitro evaluation of bone substituting materials, several cell types are commonly used.[2]

However, many studies in this field require vast amounts of cells, which may present a problem when using primary cells, since the total number of cells generated in primary culture is often limited. A way to obtain a sufficient number of cells would be to pool cells isolated from different donors. However, osteogenic cells isolated from different donors show large variation in proliferation and osteogenic potential.[3-5] Another method would be to expand the cell population by serial passaging of cultures. This also presents a problem, since serially passaged osteogenic cells have been shown to loose their capacity to differentiate.[6-8]

A solution to the above mentioned problems would be culture expansion of undifferentiated cells and addition of differentiation-inducing media as soon as the desired cell numbers are reached. Differentiation into the osteoblastic lineage after extensive subculturing has already been shown for human marrow derived cells.[5,9,10] Similar results are found for rat bone marrow (RBM) cells, which show differentiation into several cell types after culture expansion.[11-13]

On the other hand, there are signs that the osteogenic potential of undifferentiated bone derived cells decreases during subculturing. In vitro culture expansion of human bone marrow cells, as well as rat calvarial osteoprogenitor cells results in loss of osteogenic capacity.[14,15] There are indications that the same applies to the culture of RBM cells.[16,17]

In our lab, one of the main research areas is the formation and regeneration of bone. We routinely use the RBM cell culture system in our investigations. As far as we know, continuous serial subculture and assessment of osteogenic potential of these cells in vitro has not been investigated before. Therefore we examined the in vitro osteogenic potential of RBM cells that were culture-expanded before differentiation. We also subcultured RBM cells in the continuous presence of dexamethasone which induces osteogenic differentiation.[18-20] We then examined and compared the osteogenic potential of cultures with or without dexamethasone at different subcultures.

Materials and methods:

Cell culture:

Cells were isolated using the method described by Maniatopoulos.[18] Briefly, bone marrow cells were obtained from femora of male Wistar rats. Femora were washed 3 times in α-MEM with 0.5 mg/ml gentamycin and 3 μg/ml fungizone. Epiphyses were cut off and

diaphyses flushed out with 15 ml α-MEM, supplemented with 10% FCS, 0.05 mM ascorbic acid-2-phosphate, 10 mM Na β-glycerophosphate and 50 μg/ml gentamycin (-*Dex* medium). The cell suspension was counted using a Coulter Counter and cell density was adjusted to $1x10^6$ cells/ ml.

Figure 1 shows the complete protocol for subculture and assaying of the cells. Two experimental tracks were followed. In track 1, cells were seeded in 12-well plates in a density of $5x10^5$ to study the potential for osteogenic differentiation and in 1 culture flask (75 cm^2). The medium added did not contain dexamethasone (-*Dex* medium). These primary cells are referred to as P0. After 24 hours the medium was changed to remove the non-adherent cells. After this, medium was changed every other day. Cells were subcultured every 7 days. Cells were removed from the culture flask by trypsinization, counted and seeded in new culture flasks in a density of $2x10^5$ cells. This was repeated for as long as cells showed proliferation. These cultures are referred to as P1, P2 etc. Cell counts obtained during passaging were used to calculate the cumulative population doubling (CPD). Population doublings were calculated as logN/ log2, where N is the number of cells generated from the culture flask, divided by the number of cells seeded.[8] During subculturing, cells were also seeded in 12-well plates at a density of $2,5x10^4$ cells per well. The cells were cultured in the wells for up to 24 days to study the osteogenic differentiation. Culture occurred in -*Dex* medium (-/- cultures) or in +*Dex* medium (-/+ cultures). +*Dex* medium is -*Dex* medium supplemented with dexamethasone (10^{-8}M).

In track 2, a similar procedure was followed. However, the cells were provided with dexamethasone in all subcultures (+/+ cultures).

To assess osteogenic differentiation of the cells seeded in the 12-well plates during all passages, cell morphology, alkaline phosphatase expression and calcium content were studied.

Cell morphology:
The cell morphology was studied every two days, using phase contrast microscopy (Leica). Cultures were photographed at a magnification of 10x.

Alkaline phosphatase:
Alkaline phosphatase (AP) was measured at day 4, 8, 12, 16 and 24. Medium was removed and cell layers were washed in PBS. Cell layers were scraped in 0.5 ml deionized water (MilliQ) and sonicated for 10 minutes, then centrifuged at 2000 rpm, room temperature. Aliquots of the supernatant were removed for protein determination. The supernatant was stored at –20°C until the assay was performed.

For the assay, 80 μl of sample and 20 μl of alkaline buffer (Sigma) were added to 100 μl of substrate solution (5mM paranitrophenylphosphate: Sigma). For the standard curve, serial
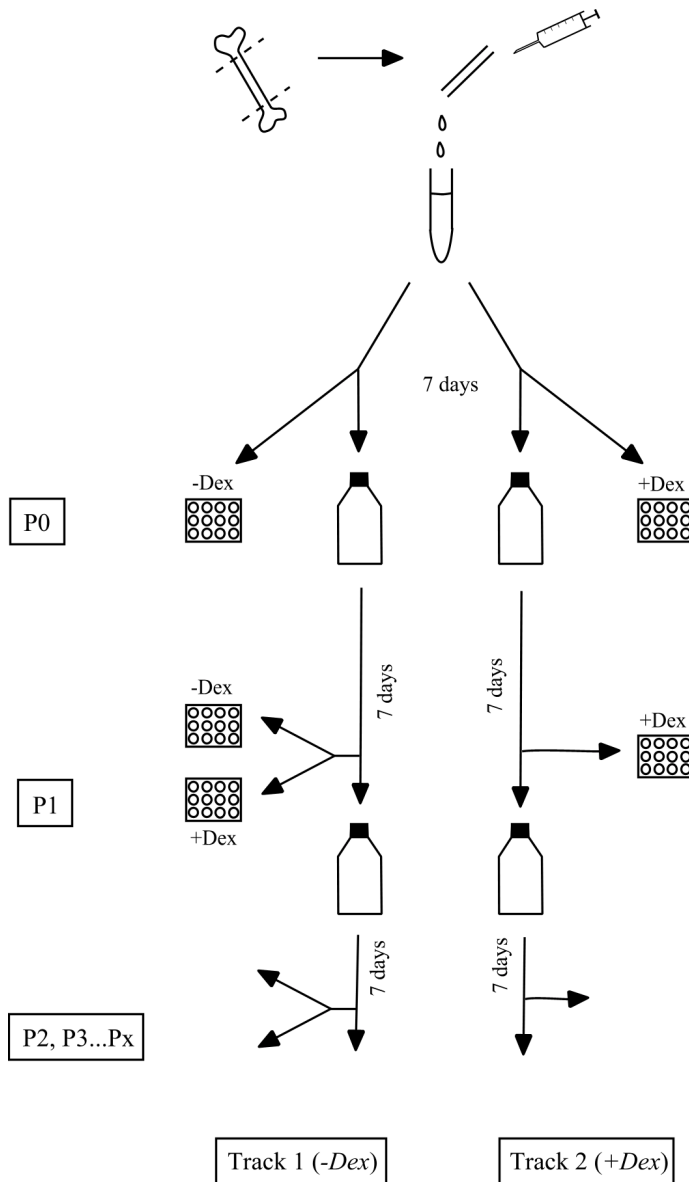
Figure 1: Schematic representation of cell culture protocol. Rat bone marrow cells were isolated and cultured in the absence (Track 1) and the presence (Track 2) of dexamethasone. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C and subcultured every 7 days.

dilutions of 4-nitrophenol (0-25 nmol) were made. The plate was incubated at 37°C for 60 minutes. The reaction was stopped by addition of 0.3M NaOH. The plate was read in an ELISA reader (Bio-Tek, USA). Samples and standards were assayed in duplicate. Measurements were then normalized for protein concentrations.

Cellular protein:

Cellular protein concentrations were measured with a micro BCA protein assay (Pierce) after 4, 8, 12, 16 and 24 days of culture. Cell layers were scraped in 0.5 ml MilliQ, sonicated for 10 minutes, and then centrifuged at 2000 rpm. BCA working solution was prepared according to manufacturers instructions. 100 μl of sample was incubated with 100 μl BCA working solution for 2 hours at 37°C. Serial dilutions of BSA (0-200 μg/ml) were used for a standard curve. The plate was read at 570 nm in an ELISA plate reader.

Calcium content:

Calcification was determined by measuring the amounts of calcium in cell layers after 8, 16 or 24 days of culture. Calcium content was measured using the OCPC (ortho-cresolphthalein complexone) method. Cell layers were washed in PBS and incubated in 1 ml 1N acetic acid for 24 hours.

OCPC solution was prepared by adding 80 mg OCPC to 75 ml MilliQ with 0.5 ml 1N KOH and 0.25 ml 1N acetic acid. Working solution was prepared by adding 5 ml OCPC solution to 5 ml 14.8M ethanolamine/boric acid buffer (pH 11), 2 ml 8-hydroxyquinoline (5 g in 100 ml 95% ethanol) and 88 ml milliQ. 300 μl working solution was added to 10 μl culture sample. To generate a standard curve, serial dilutions of $CaCl_2$ (0-100 μg/ml) were made. Samples were incubated for 10 minutes at room temperature, then read at 570 nm in an ELISA plate reader. Samples and standards were assayed in duplicate.

Statistical analysis:

The complete experiment was performed two times. In both experimental runs, all measurements were performed on quadruple samples in P0. In P1, P2 and P3, all measurements were performed on triplicate samples. Statistical analysis was performed using the Mann Whitney U test. Calculations were performed in Statmost32 (DataMost Corporation, USA).

Results:

Continuous subculture and cell numbers:

Figure 2 shows cell numbers and cumulative population doubling of RBM cells during continuous subculturing. Results shown are from one run as absolute cell number differed

between the 2 runs. However, similar trends were found in both performed runs. No population doubling could be calculated for primary cells, since it is not known how many adherent cells were seeded. In both runs, cells cultured continuously in the presence of dexamethasone stopped dividing after 4 passages. On the other hand, cells cultured without dexamethasone could be passaged at least 6 times, without apparent decrease in population doublings.
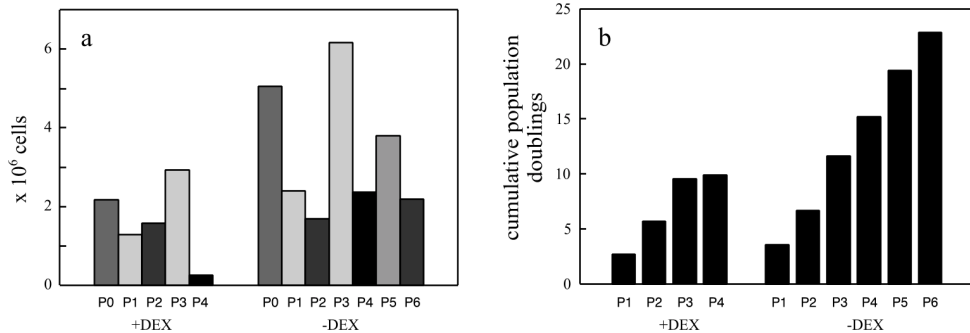

Figure 2: Cell number and Cumulative Population Doubling (CPD) in cultures without dexamethasone or continuously supplemented with dexamethasone. (a) Cell numbers, as function of passage number. Cells were counted at the time of passaging. (b) CPD, as function of passage number. Cell numbers from (a) were used to calculate population doublings.

Cell morphology:

After removal of non-adherent cells, clusters of adherent cells were seen in primary culture (P0). Between 10-15 clusters were seen in each well. Cells proliferated, with most of the cells exhibiting an elongated, fibroblast-like morphology for the first 6 days. Thereafter, areas with cuboidal, osteoblast-like cells appeared in the *+Dex* cultures, which later mineralized (Figure 3a). In the *-Dex* cultures, cells remained fibroblast-like and did not show mineralization. Cultures reached confluence in approximately 12 days. Around 17 days of culture, cells in *-Dex* cultures started to detach in layers from the well surface. These layers curled up at the sides of the wells. From these layered areas, cells started to proliferate again (Figure 3b).

Cells in P1 proliferated and formed areas with cuboidal cells that mineralized in all cultures supplemented with Dex. Nevertheless, mineralization occurred later and less extensive in -/+ than in +/+ cultures. Cells in -/- cultures remained fibroblast-like during the entire culture period.

In P2, cells in all cultures showed a fibroblast-like morphology (Figure 3c), although +/+

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cultures showed some cuboidal cells and mineralization. In all cultures, cells became flat and spread out after 20 days.

In P3 -/- and -/+ cultures, cells initially showed fast proliferation. After 5 days of culture, parts of the cell layers started to detach and subsequently were lost during medium changes. The remaining cells showed a flat, broad morphology and proliferated slowly. In +/+ cultures, cells proliferated for the first half of the culture, after which parts of the cell layer detached from the well surface and also were lost. The cells remaining in the wells were flat and spread out on the surface. In none of the cultures mineralization was observed.

Morphology of cells in later passages was similar to P3, with no signs of osteogenic differentiation.
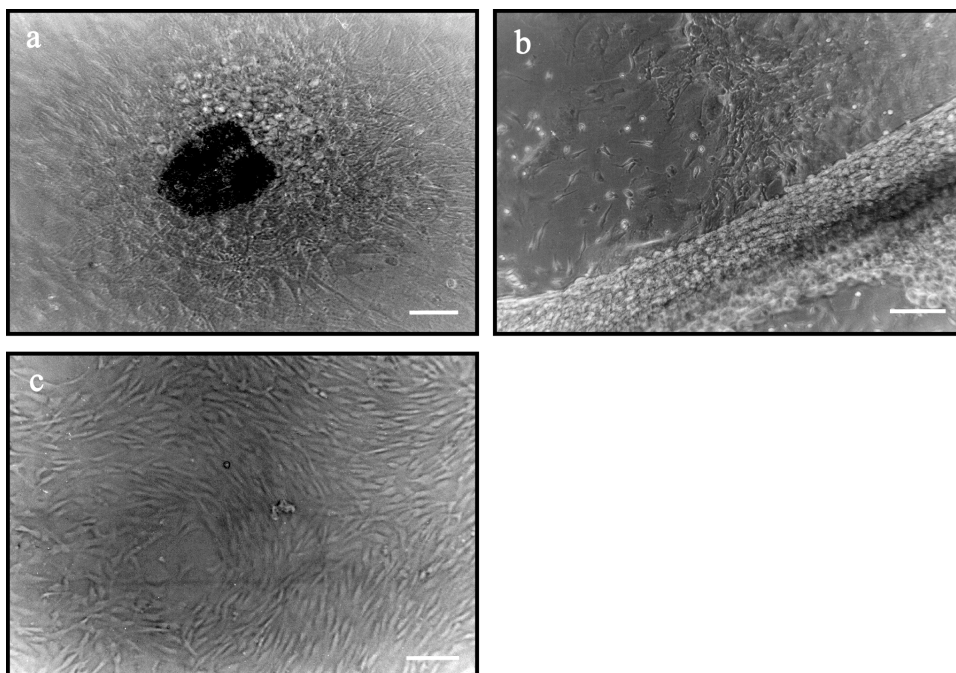

Figure 3: Morphology of RBM cells. (a) Primary RBM cells after 14 days of supplementation with dexamethasone, areas with cuboidal cells appear. The dark region is an area of calcification. Outside the area with cuboidal cells, fibroblast-like cells are seen. (b) Primary RBM cells cultured for 20 days without dexamethasone. Part of the cell layer has detached and has formed layered sheets of cells (bottom right-hand corner). Cells can be seen proliferating out of these layers. (c) RBM cells after 2 passages, continuously supplemented with dexamethasone, after 14 days of culture. Only fibroblast-like cells are seen in this region of the culture. Scale bar in all figures: 100 μm.

Alkaline phosphatase:

Results for the AP specific activity are shown in Figure 4. Results from one run are shown. Large differences were found in the absolute values for AP expression between the two runs. However, the general trend in expression was similar for both runs.

For P0, AP activity increased from day 8, to a maximum at day 12 for both -/- and +/+ cultures and decreased after that (Figure 4a). After 8, 12 and 16 days, AP in +/+ cultures was significantly higher than in -/- cultures ($p<0.05$).

In P1 cultures, AP activity increased to a maximum on day 8 in all cultures (Figure 4b; $p<0.05$). AP activity in +/+ cultures was significantly higher than in -/+ and -/- cultures on day 8, 12 and 16. For -/+ cultures, AP activity was higher than -/- cultures ($p<0.05$).

Similar to P1, AP activity in P2 increased from day 4 to day 8 in all cultures (Figure 4c). At

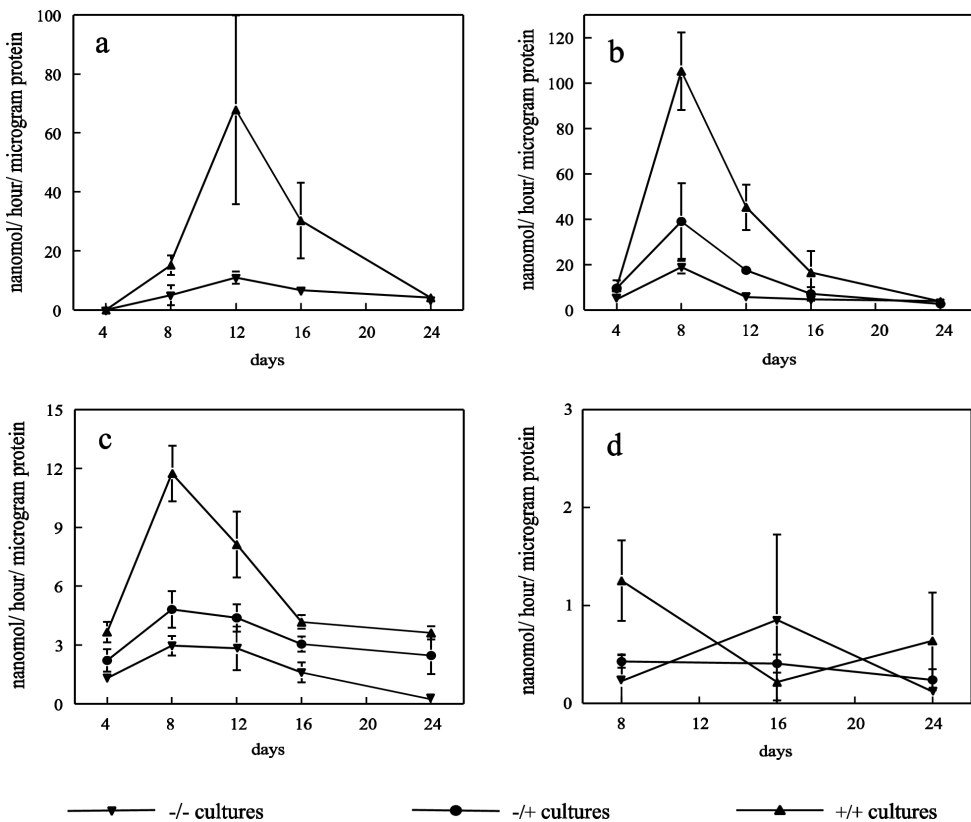
Figure 4: AP expression by RBM cells after 4, 8, 12, 16 and 24 days of culture, in -/-, -/+ or +/+ cultures. AP expression was measured in P0 (a), P1 (b), P2 (c) and P3 (d). Expression was normalized for protein content. Values are mean ± SD.

all timepoints, AP activity was different between culture conditions, with +/+ > -/+ > -/-
(p<0.05). Further, AP activity in all cultures was lower in P2 than in P1.

In P3 and later passages, AP activity was extremely low (Figure 4d). In some cultures, AP
activity was not even detectable.

Calcium content:

Calcium content of the cultures is shown in Figure 5. Results from one run are shown.
Differences were found in the absolute values for calcium content between the two runs.
However, the general trend in expression was similar for both runs.

In P0, no mineralization was found in -/- cultures. In +/+ cultures, mineralization apparently
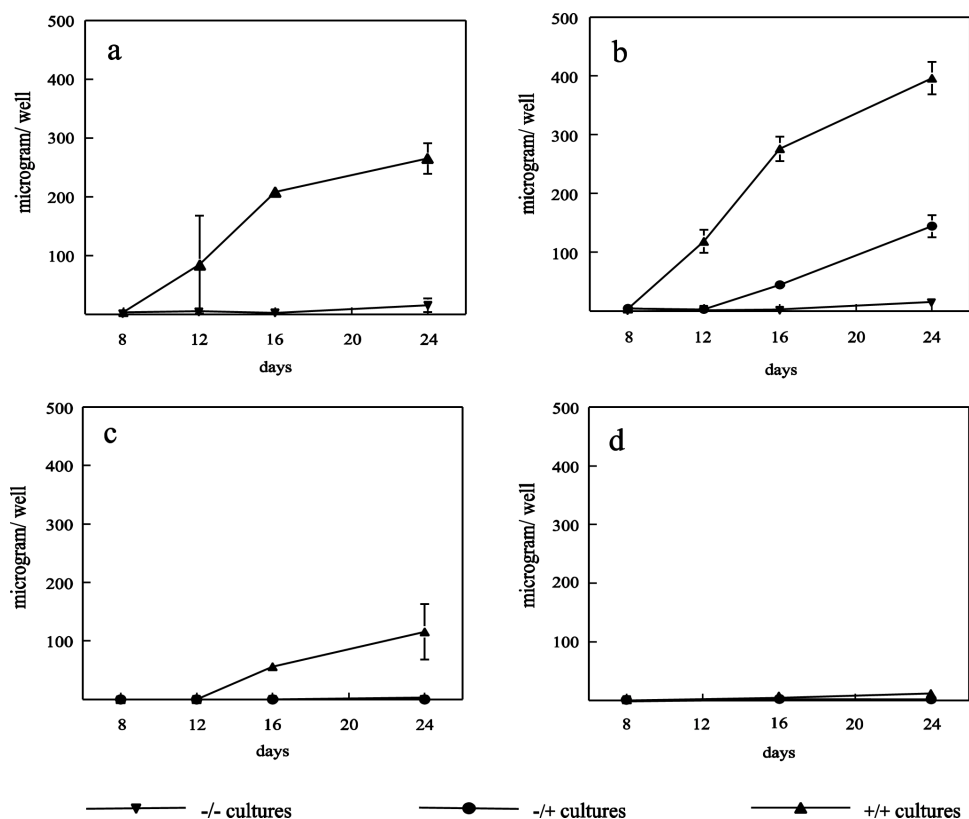started after 12 days (Figure 5a).

Figure 5: Calcium content in cells layers after 8, 12,16 and 24 days of culture, in -/-, -/+ or +/+ cultures. Calcium was measured in P0 (a), P1 (b), P2 (c) and P3 (d). Values are mean ± SD.

In P1, again no calcification was found in the absence of dexamethasone. Cells continuously cultured in the presence of dexamethasone (+/+) showed extensive mineralization. In -/+ cultures, calcification was found after 12 days of incubation. Mineralization was significantly lower than in +/+ cultures (Figure 5b).

Cells in +/+ cultures did show mineralization in P2, but calcium content was decreased compared to P1 (Figure 5c).

In P3 and later passages, no clear mineralization was found in any of the culture conditions (Figure 5d).

Discussion:

Bone marrow is thought to contain a population of undifferentiated cells that are referred to as mesenchymal stem cells. By definition, stem cells have the capacity for unlimited self-renewal. Furthermore, mesenchymal stem cells generate the progenitors for osteogenic, adipocytic, chondrogenic and myogenic cells.[1,21,22]

In vitro, bone marrow cells have the potential for adipogenic, chondrogenic or osteogenic differentiation, depending on the culture conditions.[23] Differentiation into the osteogenic lineage is induced by the addition of dexamethasone. RBM cultures supplemented with dexamethasone show clusters of cuboidal cells, high expression of alkaline phosphatase and mineralization of the cell clusters.[18,20] We used these markers to assess osteogenic differentiation in cultures of RBM cells. Our RBM cells cultured with dexamethasone showed expression of differentiation markers, especially in primary cells and after first passage. Mineralization was closely associated with areas of cuboidal cells and was never found in cultures without dexamethasone, indicating that calcifications were not the result of spontaneous precipitation of calcium phosphate crystals.

Expression of osteogenic markers increased between P0 and P1, with earlier maximal alkaline phosphatase expression and onset of mineralization in P1. This is probably due to the low number of cells seeded in primary culture. In P0, 5×10^5 freshly isolated cells were seeded. Many of these cells were non-adherent cells and were washed away during the first medium changes. Non-adherent cells were not removed before seeding, since this was also not done with cultures from which passaged cells were generated. In the adherent fraction, only a small number of cells are osteogenic progenitors. This can explain the lower and later expression of osteogenic markers in P0, since the onset of RBM cell differentiation is cell seeding-density dependent.[24] After the first passage, we observed a decrease in osteogenic expression in cultures continuously subcultured with dexamethasone. Similar results have been reported earlier for RBM cells[7] as well as other osteoblast-like cells.[8,14,25] We know that after passaging, the seeded cells will be a combination of differentiated and undifferentiated cells. However, the differentiated cells only have a limited capacity for

proliferation.[7,26] Consequently the cells proliferating out of the seeded cells will mostly be undifferentiated cells. This results in a decrease of the percentage of osteogenic cells in the culture. The finding that the osteogenic potential decreases with passaging indicates that the cells do not have the capacity to continually generate osteogenic precursors.

In our study, first passage RBM cells cultured in -*Dex* medium and subcultured with dexamethasone (-/+ cultures) show expression of differentiation markers. This indicates (partial) retention of osteogenic potential. Further subculturing of undifferentiated RBM cells leads to a quick loss of osteogenic potential of the cultures. Nevertheless, many studies using subcultured bone marrow cells show bone formation when these cells are seeded in porous ceramic cubes and implanted.[27-31] However, in most studies cells were only subcultured for a limited number of passages. Furthermore, we know that bone formation in porous carriers is not always related to the presence of osteogenic cells, since the occurrence of spontaneous bone formation has been described too.[32]

The retention of osteogenic potential of culture-expanded bone marrow cells has been examined in several other studies. In vivo and in vitro experiments with rat or canine cells showed a decrease in osteogenic potential with increasing number of passages.[27,30] Our observations agree with these studies. On the other hand, our results do not corroborate those found with human bone marrow cells. In vitro research showed that culture-expanded human bone marrow cells retain their ability to differentiate into osteoblasts, until they approach cellular senescence.[9,10] Nevertheless, the cells loose the ability to differentiate into chondrocytes and adipocytes, indicating loss of multipotentiality.[10,33] Furthermore, the in vivo bone forming efficiency of subcultured cells is remarkably reduced compared to that of fresh bone marrow cells.[15]

In our study, continuous addition of dexamethasone to the culture medium resulted in higher AP expression and mineralization compared to other culture conditions. Dexamethasone is thought to increase the number of osteoprogenitor cells in culture, compared to cultures without dexamethasone.[17,34] Evidently, continuous addition of dexamethasone results in a more differentiated secondary culture. Our findings are in agreement with previous reports of in vitro RBM cell differentiation.[17,35] Besides, this is confirmed by in vivo implantation experiments with cell-seeded constructs. Culturing of the bone marrow cells with dexamethasone before implantation increased the amount of bone formed.[31,36,37]

We observed a fast reduction in osteogenic potential. We have to emphasize that this could be due to the relative low number of cells as used for serial subculture. Since only a small percentage of the cells are osteoprogenitor cells, subculture in low density could quickly deplete these cells from the culture. Consequently, it is possible that subculture in higher densities could result in expression of differentiation markers for a larger number of passages.

Finally, we have to notice that apparently irrelevant modifications in the cell culture protocol can still have a significant effect on the final observations. For example, we know that the expression of osteogenic markers is influenced by conditions, such as culture medium, the addition of growth factors,[25,38] scaffold material[1] or serum used.[5,39,40] Culture of human osteoblasts in medium containing a defined serum substitute prevents dedifferentiation of the cells with subculture.[41] In addition, cells derived from different strains of the same species may show large variations in growth and differentiation.[42] Further, a close relationship exists between hematopoietic and osteogenic cells in vivo. In vitro, hematopoietic bone marrow cells have been shown to alter the expression of cytokines by human osteoblasts in culture.[43] In rat cultures, the number of bone nodules is increased when adherent bone marrow cells are co-cultured with the non-adherent fraction.[17] The non-adherent fraction of human bone marrow contains cells that stimulate the in vitro development of osteogenic precursors.[44] Co-culture of the adherent fraction with non-adherent fraction of bone marrow may therefore directly affect the differentiation potential of the osteogenic cells.

Based on our results we conclude that RBM cells in vitro retain part of their potential for osteogenic differentiation when subcultured. Nevertheless, this potential quickly decreases with increasing subculture, both when cultured with or without dexamethasone. This indicates that the used cells do not possess complete self-renewal, and cannot be considered true stem cells. This also implies that RBM cells used for biocompatibility testing or tissue engineering have to be used at the lowest passage possible. Furthermore, RBM cells subcultured without dexamethasone express lower level of osteogenic markers in every passage than RBM cells continuously supplemented with dexamethasone. Consequently, addition of dexamethasone has to be preferred over subculture of undifferentiated cells.
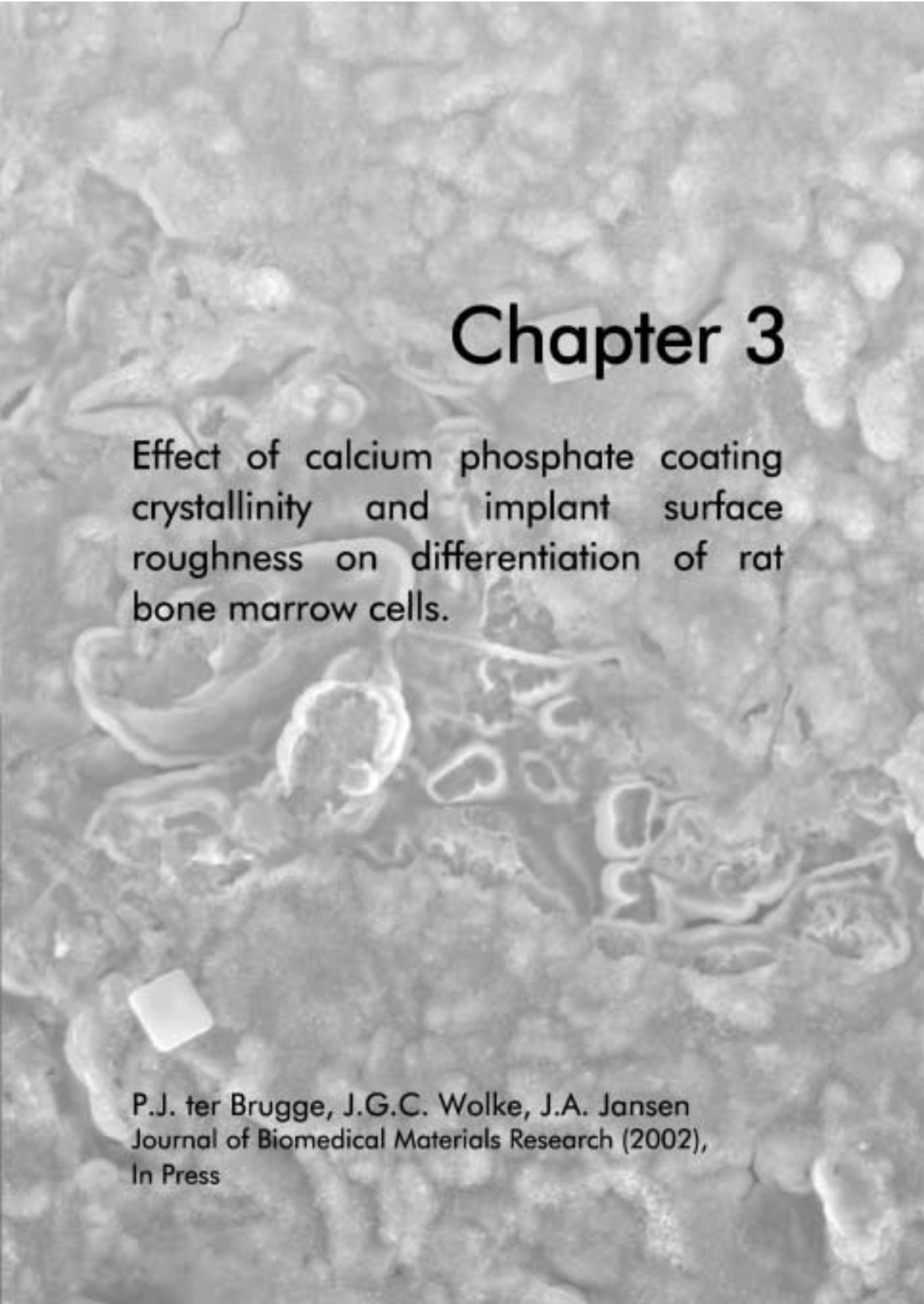
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Chapter 3

Effect of calcium phosphate coating crystallinity and implant surface roughness on differentiation of rat bone marrow cells.

P.J. ter Brugge, J.G.C. Wolke, J.A. Jansen

Introduction:

The reaction of tissues to a biomaterial is influenced by characteristics such as surface composition, energy and topography.[1] In view of this, calcium phosphate (CaP) coated materials have been demonstrated to favor bone formation around implants when compared to non-coated titanium implants.[2-4] This effect of CaP ceramics was also demonstrated in in vitro studies with osteogenic cells. Differences were found in the regulation of attachment and spreading of human and rat osteoblast-like cells cultured on calcium phosphates and titanium.[5,6] Also, differentiation of the cells was stimulated when cultured on calcium phosphate surfaces.[7,8] Furthermore, it was shown that calcium phosphates affect the expression of connective tissue proteins[9] and accumulation of glycosaminoglycans in the extracellular matrix (ECM).[10-12]

A hypothesis to explain this effect of calcium phosphates is dissolution of calcium and phosphorus, leading to interfacial supersaturation of Ca^{2+}. This then leads to reprecipitation of calcium phosphates, resulting in a transformed surface that would stimulate differentiation of osteogenic cells.[13] We know that the dissolution characteristics of CaP ceramics are determined by the crystallinity of the material.[14-16]

A problem in many of the above-mentioned studies is that the tested materials not only differed in composition, but also in surface roughness.[10,17] Occasionally, surface roughness was not determined at all.[8,12,18] This makes interpretation of the results difficult, since we know that tissue reactions are not only determined by the substrates' surface composition, but also by surface roughness.[1,19,20]

In this study, we evaluated the effect of CaP coating crystallinity on the growth and differentiation of rat bone marrow (RBM) cells. We compared uncoated rough titanium substrates to substrates that were provided with a thin (2 μm) CaP coating using a RF magnetron sputtering technique.[21] Such a thin coating does not affect the surface roughness properties of the substrate. Crystallinity of the coatings can be controlled by heat-treatment of the deposited amorphous coatings.[22]

Materials and methods:

Cell culture:

Cells were isolated and cultured using the method described by Maniatopoulos.[23] Briefly, bone marrow cells were obtained from femora of male Wistar rats. Femora were washed 4 times in α-MEM with 0.5 mg/ml gentamycin and 3 μg/ml fungizone. Epiphyses were cut off and diaphyses flushed out with 15 ml α-MEM, supplemented with 10% fetal calf serum (FCS), 50 μg/ml ascorbic acid, 50 μg/ml gentamycin, 10 mM Na β-glycerophosphate and 10^{-8} M dexamethasone. Medium was changed 3 times a week. Cells were incubated in a

humidified atmosphere of 95% air, 5% CO_2 at 37°C. Cells were used in experiments after 7 days of primary culture.

Substrates:

Commercially pure titanium (cpTi) disks (diameter 12 mm) were used. Disks were used as machined (Ti-s) or subjected to a TiO_2 gritblasting procedure, ultrasonically cleaned in acetone for 15 minutes and placed in 100% boiling ethyl alcohol. After drying, gritblasted substrates were left uncoated (Ti-r) or provided with a RF-magnetron sputtered CaP coating.

The coating procedure was performed using a commercially available RF magnetron sputter unit (Edwards ESM 100). The target material used in the deposition process was a copper disk provided with a plasma-sprayed hydroxyapatite coating (CAMCERAM7). The process pressure was $5x10^{-3}$ mbar and the sputter power was 400W. The deposition rate of the films was 100-150 nm/min. The thickness of the coating was 2 μm. After deposition, coatings were left as sputtered (CaP-as) or subjected to an additional heat treatment at temperatures of 500°C (CaP-500) or 700°C (CaP-700) in an infrared furnace (E4-10-P, Research Inc.).

Deposited coatings were characterized by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), X-ray diffraction (XRD) and Fourier transform infrared spectrometry (FTIR). CaP-as had an amorphous structure, CaP-500 an amorphous-crystalline structure and CaP-700 showed a crystalline structure. Ca/P ratios of the coatings were measured with EDS. Ca/P ratios of all coatings were between 1.77 and 1.83.

Surface roughness of the substrates was measured using a noncontact laser profilometer (Rodenstock Metrology RM600®). The measuring length was 4 mm and scan speed was 40 mm/ min. 5 Scans were performed and measurements were averaged. The results of surface roughness measurements are shown in Table 1.

	Ra (μm)	Sm (μm)
Ti-s	0.24	7.8
Ti-r	1.31	21.2
CaP	1.40	21.3

Table 1: Results of substrate surface roughness measurements.
Ra value indicates mean hight variation, Sm value indicates mean spacing of surface peaks.

Disks were autoclaved for 15 minutes at 120°C before use in cell culture and were placed on the bottom of 24 well-plates. Cells were released by trypsin treatment and 1ml cell suspension ($1x10^4$ cells/ml) was added to the substrates. Cells were cultured according to

the same method as primary cells. Two experimental runs were performed. In each run, all materials were present in triplicate.

Protein:

To determine cellular proliferation, cellular protein concentrations were measured with a micro BCA (bicinchoninic acid) protein assay (Pierce) after 4, 8 and 16 days of culture. Cell layers were scraped in 0.5 ml milliQ and sonicated for 10 minutes and then centrifuged at 2000 rpm. 100 μl of sample was incubated with 100 μl working solution for 2 hours at 37°C. Serial dilutions of BSA (3.12-200 μg/ml) were used for a standard curve. The plate was read at 570 nm in an ELISA plate reader (Bio-Tek).

Alkaline phosphatase:

Alkaline phosphatase (AP) was measured at day 4, 8 and 16. Medium was removed and cell layers were washed in PBS. Cell layers were scraped in 0.5 ml milliQ and sonicated for 10 minutes, then centrifuged at 2000 rpm, room temperature. Aliquots of the supernatant were removed for protein determination. The supernatant was stored at –20°C until the assay was performed.

For the assay, 80 μl of sample and 20 μl of alkaline buffer (Sigma) were added to 100 μl of substrate solution (5mM $MgCl_2$, 0.5 M 2-amino-2methyl-1-propanol). For the standard curve, serial dilutions of 4-nitrophenol were made. The plate was incubated at 37°C for 60 minutes. The reaction was stopped by addition of 0.3M NaOH.

The plate was read in an ELISA reader. Samples and standards were assayed in duplicate.

Osteocalcin:

Osteocalcin (OC) was measured in the cell layers by enzyme immunoassay (EIA; Biomedical Technologies, Inc).

After 8 and 16 days of culture, cell layers were collected by scraping in EIA sample buffer and sonicated for 10 minutes. 100 μl of sample was added to the wells and the plate was incubated at 4°C for 18 hours. The plate was washed 3 times, 100 μl antiserum was added and the plate was incubated for 1 hour at 37°C. After washing 3 times, 100 μl of donkey anti-goat IgG peroxidase was added and incubated for 1 hour at room temperature. The plate was washed, 100 μl of substrate solution (1 volume TMB (3,3[1],5,5[1]-tetramethylbenzidine)/ 1 volume H_2O_2) was added and the plate incubated for 30 minutes. 100 μl stop solution was added and the plate was read at 450 nm in an ELISA plate reader. Samples and standards were assayed in duplicate.

Prostaglandin E$_2$ EIA:

PGE$_2$ was measured in the cell culture medium by enzyme immunoassay kit (Cayman Chemical). 1 ml of cell culture medium was harvested on day 4, 8 and 16 and frozen at –80°C until EIA was performed. EIA was performed according to manufacturer's instructions. Briefly, 50 μl of PGE$_2$ acetylcholinesterase, 50 μl of antiserum and 50 μl of the sample or standard (375-5000 pg/ml) was added. The plate was incubated for 18 hours at room temperature, then washed and 200 μl of Ellman's reagent was added. After 60 minutes of incubation, the plate was read at 405 nm, using an ELISA plate reader (Bio-Tek). Samples and standards were assayed in duplicate.

Calcification:

Calcification was determined by measuring the amounts of calcium in cell layers after 8, 16 or 24 days of culture. Substrates without cells were incubated in culture medium for 8, 16 or 24 days and calcium content was also measured.

Calcium content was measured using the OCPC (ortho-cresolphthalein complexone) method. Substrates were washed in PBS and incubated in 1N acetic acid for 24 hours. Samples were incubated several times, until no calcium was dissolved from the substrate anymore (calcium assay was negative).

OCPC solution was prepared by adding 80 mg OCPC to 75 ml milliQ with 0.5 ml 1N KOH and 0.25 ml 1N acetic acid. Sample solution was prepared by adding 5 ml OCPC solution to 5 ml 14.8M ethanolamine/boric acid buffer (pH 11), 2 ml 8-hydroxyquinoline (5 g in 100 ml 95% ethanol) and 88 ml milliQ. 300 μl sample solution was added to 10 μl sample. To generate a standard curve, serial dilutions of CaCl$_2$ (1.56-100 μg/ml) were made. Samples were incubated for 10 minutes at room temperature, then read at 570 nm in an ELISA plate reader. Samples and standards were assayed in duplicate.

Scanning electron microscopy (SEM):

Samples for SEM were taken after 8 and 16 days of culture. Samples were fixed in 2% glutaraldehyde, washed twice in 0.1M sodium-cacodylate buffer (pH 7.4) and dehydrated using a graded series of ethanol. After drying with tetramethylsilane, samples were sputter-coated with carbon and photographed using a Jeol 6310 SEM with an acceleration voltage of 15 kV.

Statistical analysis:

For all assays, measurements from the two different experimental runs were pooled. Differences between experimental groups were calculated using the Mann Whitney U test with Bonferroni correction. Calculations were performed in Statmost32.

Results:

Protein assay:

The results of the protein measurements from two runs were pooled. Results are depicted in Figure 1. RBM cells showed no proliferation on CaP-as or CaP-500. On Ti-r, Ti-s and CaP-700, protein content increased from day 4 to day 16 of culture. On day 8 and 16, protein content on these substrates was significantly higher ($p < 0.05$) than on CaP-as and CaP-500. Statistical testing revealed that after 8 days, protein content on Ti-s was significantly higher than on CaP-700 ($p < 0.05$). No significant differences were found between protein levels on Ti-s, Ti-r and CaP-700 after 16 days of incubation.
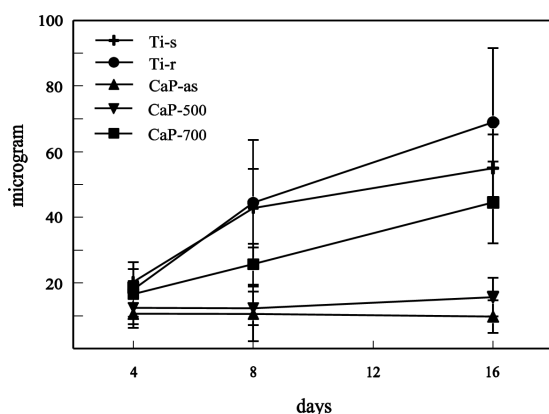

Figure 1: Cellular protein content of RBM cells after 4, 8 or 16 days of culture on various substrates. Values are mean ± SD.

Alkaline phosphatase:

Large differences were found in the absolute values for AP expression between the two runs. However, since the general trend in expression was similar for both runs, relative AP specific expression was calculated for each run (Ti-s at day 16 was considered to be 1) and data was pooled (Figure 2).

Cells on CaP-as and CaP-500 expressed very low levels of AP at all culture times. AP expression on Ti-s, Ti-r and CaP-700 substrates increased between days 4 and 16. On day 8, expression on Ti-r was significantly higher than on Ti-s and CaP-700 disks ($p < 0.05$). No difference between these 3 materials was found on day 16.

Osteocalcin:

Differences were found in the absolute values for OC expression between the two runs. Therefore, relative osteocalcin expression for each run was calculated (Ti-s at day 16 was considered to be 1) and the results were pooled. Relative expression is shown in Figure 3. On CaP-as and CaP-500 no osteocalcin expression was found at all. Osteocalcin expression
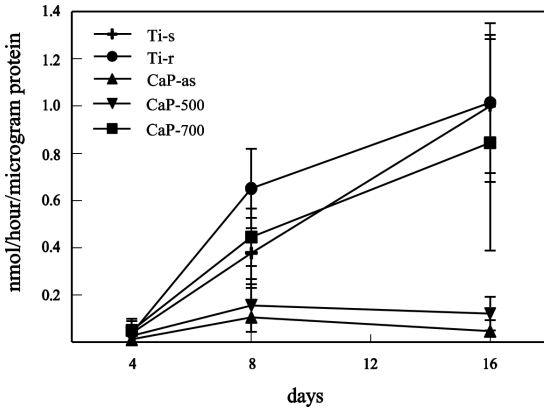
Figure 2: AP expression by RBM cells after 4, 8 and 16 days of culture. Expression was normalized for protein content. Values are mean ± SD.

remained low on Ti-r, while expression on Ti-s and CaP-700 increased from day 8 to day 16. Significant differences ($p < 0.05$) existed between these materials: CaP-700>Ti-s>Ti-r.

Prostaglandin E_2:

PGE_2 measurements from two runs were pooled, results are shown in Figure 4.

Low expression of PGE_2 was found on Ti-s, Ti-r and CaP-700 substrates at all culture periods. At day 8, expression of PGE_2 was significantly higher on all coated substrates than on non-coated substrates and was elevated on CaP-as compared to CaP-700 ($p < 0.05$). A large variation was found in the PGE_2 expression on CaP-as, with 2 out of 3 samples in a run showing increased expression, while the third sample showed no increase. This pattern was seen in both runs and the same was found for PGE_2 expression on CaP-500 after 16 days. At day 16, the PGE_2 level was significantly higher only for CaP-500 specimens ($p < 0.05$). No differences were found in PGE_2 expression between the other substrates.
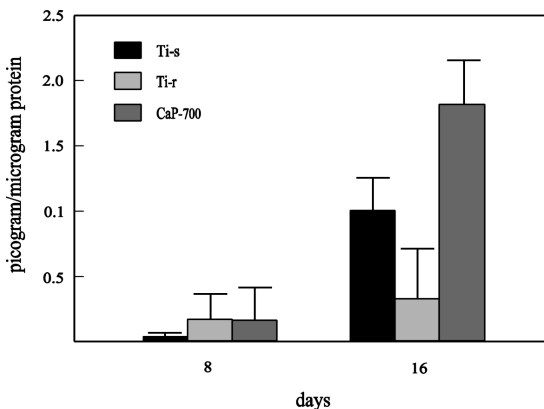

Figure 3: Osteocalcin expression by RBM cells after 8 and 16 days of culture. Expression was normalized for protein content. Values are mean ± SD.
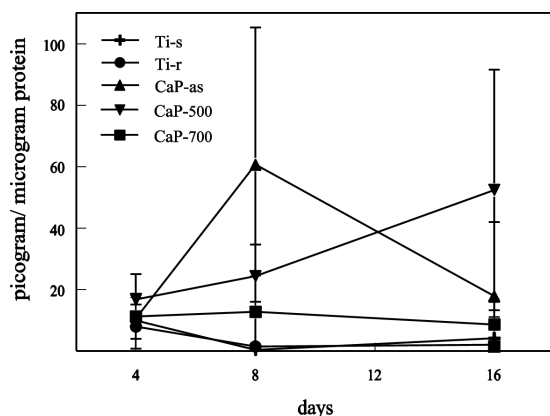
Figure 4: PGE$_2$ expression of RBM cells after 4, 8 and 16 days of culture, normalized for protein content. Values are mean $\pm$ SD.

Calcification:

Substrates with or without cells were incubated in culture medium and calcium content was measured. On the titanium substrates without cells, calcium content was increased slightly by day 8 and 16, but this precipitate disappeared by day 24. When amorphous coatings without cells were incubated in culture medium, calcium content decreased, with significantly lower ($p<0.01$) calcium content by day 24, compared to day 0. On the crystalline coating without cells, calcium content was significantly increased ($p<0.05$) during the entire incubation period (Figure 5).

When cells were cultured on the substrates (Figure 6), calcium content on titanium substrates was increased by day 8, with a maximum increase on day 16. Calcium content on the amorphous coatings was decreased significantly by day 24 ($p<0.01$, compared to day 0), while on the crystalline coating with cells a significant increase was found by day 8, with a maximum increase by day 24 ($p<0.05$). The maximum increase in calcium on CaP-700 was significantly higher than the maximum increase on the titanium substrates ($p<0.05$).

Calcium content was significantly higher on titanium substrates with cells on day 16 and 24, and on CaP-700 on day 24, compared to substrates without cells ($p<0.05$). Amorphous coatings showed no difference in calcium content between substrates with or without cells.

SEM:

On CaP-as and CaP-500 coatings, only a limited number of cells were found. Both coatings showed severe signs of degradation by day 8 (Figure 7a). Some areas of precipitation were found, consisting of calcium phosphates, as determined by EDS. EDS measurements also revealed that the Ca/P ratios of the coatings had dropped to 1.532 ± 0.024 for CaP-as and 1.64 ± 0.049 for CaP-500 coatings.

By day 16, the CaP-as coating had a smooth appearance, without any surface pits or areas of precipitation. The CaP-500 coating showed many surface pits and areas of precipitation.
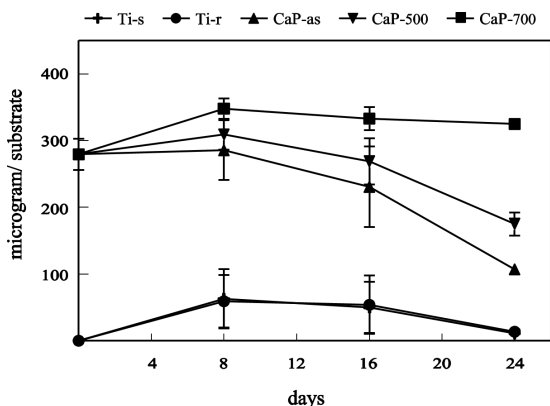
Figure 5: Calcium content of substrates without cells after incubation in culture medium for 8, 16 and 24 days. Values are mean ± SD.
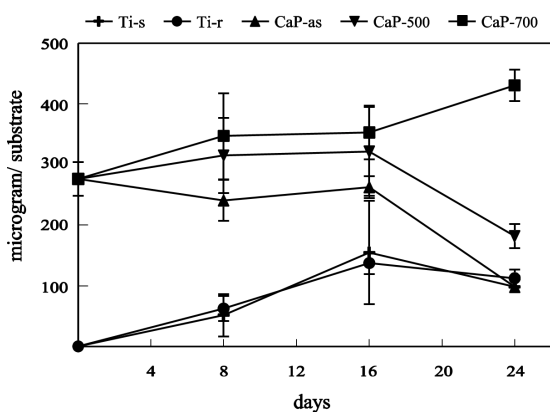

Figure 6: Calcium content of substrates with cells after incubation in culture medium for 8, 16 and 24 days. Values are mean ± SD.

Occasionally, areas resembling the original coating were still found. The Ca/P ratios for both coatings remained at the same level as day 8. On the other substrates, cells formed a multilayer at day 8 (Figure 7b). After 16 days, the cells had formed a layer of calcified globular accretions associated with collagen bundles (Figure 7c). CaP-700 showed only limited dissolution with some small areas of precipitation during the entire incubation period (Figure 7d). No changes in the Ca/P ratio were found.

Discussion:

In this study, the effect of CaP coating crystallinity and substrate surface roughness on growth and differentiation of RBM cells was evaluated. The results demonstrate that RBM cells cultured on a heat-treated CaP coating showed higher expression of osteogenic markers

than cells cultured on uncoated titanium. On the other hand, on thin amorphous CaP coatings, cells showed no proliferation and differentiation at all.

The calcium measurements revealed that a small amount of calcium was precipitated from the medium on titanium substrates incubated in culture medium without the addition of cells. This precipitate disappeared after longer incubation periods, indicating that the precipitate is not stable. As demonstrated before, precipitation of calcium phosphates is in the form of carbonated apatite[24] with a high solubility. This explains the shift in the equilibrium towards dissolution found after longer incubation periods in our experiments.
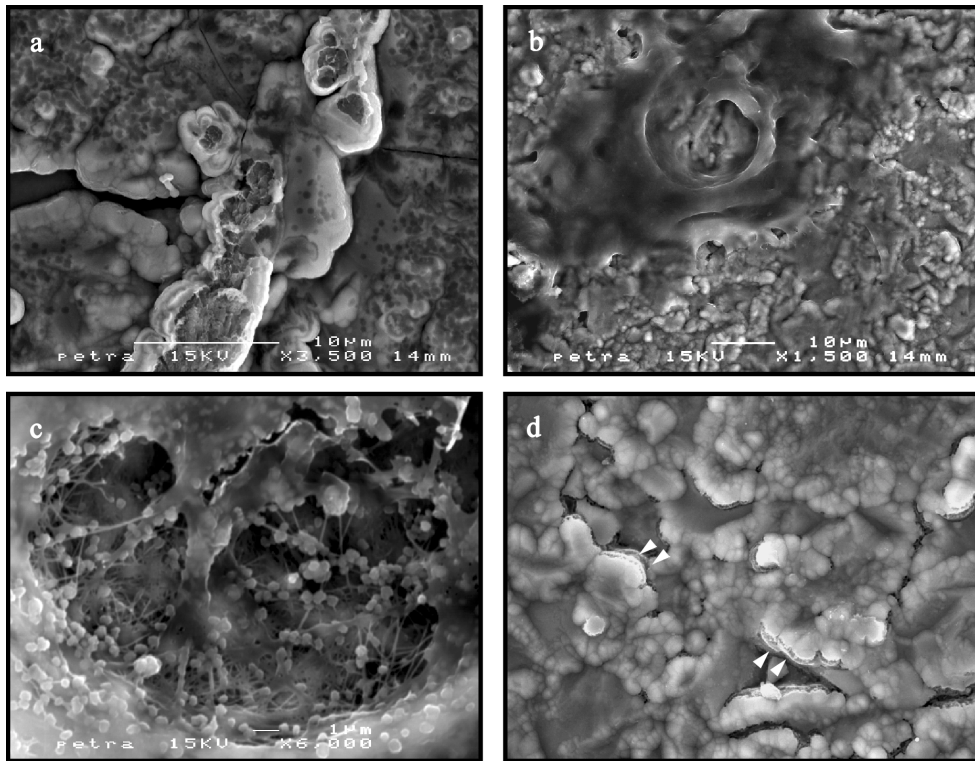
Figure 7: (a) SEM picture of CaP-500, incubated in culture medium for 8 days, showing dissolution of the coating, with surface pits. (b) SEM picture of RBM cells cultured on CaP-700 for 8 days. Cells have started to form a multilayer. No signs of coating dissolution can be found in this picture. (c) Formation of multilayer with collagen fibers and globular accretions after 16 days of culture on Ti-s. (d) SEM picture of CaP-700 coating after 8 days of incubation in culture medium. The coating shows signs of limited dissolution (arrowheads).

On Ti-s, Ti-r, CaP-as and CaP-500 substrates, this shift occurs both in the absence and presence of osteogenic cells. In contrast, on CaP-700, the precipitate deposited during early culture is stable and is observed during the entire culture period. In the presence of cells, the initially deposited precipitate is probably not cell-mediated. After prolonged culture times the crystalline coating and precipitate appear to form a continuous stimulus for mineralization of the osteogenic cells. Increased mineralization is accompanied by increased alkaline phosphatase and osteocalcin expression. Furthermore, the calcified globules are closely associated with collagen fibers. This indicates that the mineralization identified in these specimens is not the result of CaP precipitation due to the presence of β-glycerophosphate in the culture medium, but is cell-mediated.

The amorphous coatings showed extensive dissolution and reprecipitation during the first 2 weeks of culture, as can be seen in SEM analysis and analysis of calcium content. The calcium content did not change significantly, suggesting equilibrium between dissolution and precipitation. During later culture periods, calcium content did decrease. Similar dissolution curves were described by Anselme[25], who showed an increase in dissolution after several days of immersion in culture medium.

It has been suggested that amorphous coatings show more rapid bone formation, since the surface is modified faster.[15] Clearly, this is not true for our coatings. In view of this, it appears appropriate to mention the difference between our coatings and the plasma-sprayed coatings as used in other studies.[15,25] Depositing calcium phosphate coatings by RF magnetron-sputtering results in a layer with a uniform structural and morphological appearance. During plasma spraying, (partially) melted calcium phosphate particles are deposited on a substrate. Completely melted particles may result in an amorphous coating, while partial melting of the particles during deposition may lead to a more crystalline coating.[26] However, these coatings consist of an amorphous phase with crystalline regions. Degradation of the coating is the result of dissolution of the amorphous phase and release of the attached crystalline regions, with the removal of larger crystalline areas after a longer period.[15] Dissolution of these coatings is therefore not only dependent on overall crystallinity, but also on the size of the crystalline particles in the amorphous areas. This makes it difficult to compare results from dissolution assays performed with crystalline plasma-sprayed and crystalline magnetron sputtered coatings.

Considering the absence of cell proliferation on the CaP-as and CaP-500 substrates, it is possible that the dissolution of calcium from the coatings leads to the release of calcium phosphate particles. This could negatively affect the cells growing on the surface. For example, we know that the presence of hydroxyapatite particles in culture inhibits proliferation of rat osteogenic cells, but stimulates expression of PGE_2.[27,28] This would also explain the increase in PGE_2 expression we observed on the coated substrates. This

hypothesis about release of particles from the amorphous coatings is supported by our SEM findings. The increase in PGE_2 expression was highest on CaP-as by day 8, at which time the SEM pictures of the coating showed a heavily pitted surface. By day 16, the surface looked smooth, without any pits, and PGE_2 expression had decreased. In contrast, the surface of CaP-500 still showed many pits and holes and PGE_2 expression was still elevated. PGE_2 is an important local factor in the regulation of bone metabolism that can inhibit or stimulate bone formation and bone resorption.[29-31]

Besides implant surface chemistry, implant surface roughness has also been reported to influence PGE_2 expression.[32,33] Our study showed no effect of surface roughness on the expression of PGE_2 by RBM cells. On the other hand, we did find differences in osteocalcin expression by osteogenic cells cultured on either smooth or roughened titanium substrates. Surface roughness was earlier shown to affect expression of osteocalcin by MG-63 cells[32-34], rat calvarial osteoblasts[20,35] and mouse osteogenic cells. In contrast with our results, these studies show an increase in osteocalcin expression on rougher surfaces. On the other hand, reports can be found of a higher osteocalcin expression by rat calvarial osteoblasts on smooth surfaces than on rough surfaces.[36] Furthermore, surface roughness has no effect on the osteocalcin expression by human fetal osteogenic cells.[37]

Several explanations can be given for the differences in PGE_2 and osteocalcin expression between the various studies. MG-63 is a human osteogenic cell line that shows osteogenic characteristics like expression of alkaline phosphatase and osteocalcin. Expression of osteocalcin by MG-63 is similar to expression by bone derived cells.[38] Nevertheless, proliferation of MG-63 cells, alkaline phosphatase expression and expression of several matrix proteins was different from non-transformed cells.[38,39] Therefore, MG-63 osteosarcoma cells are not the best model to study PGE_2 expression. Studies with rat calvarial cells showed that cells are capable of sensing an optimum surface roughness, at which the cells show a maximal expression of osteocalcin and alkaline phosphatase. Surfaces with either a lower or a higher surface roughness resulted in lower expression of these markers.[20] The geometry of the substrate is also supposed to be an important factor influencing cell reactions. MG-63 cells cultured on rough surfaces with a regular geometry showed an increase in cell number comparable to cells cultured on smoother surfaces, whereas matrix production was similar to that of cells grown on rough surfaces with irregular geometry.[40] Finally, the treatment of the substrate before culture can affect the cell reactions. When titanium substrates are plasma cleaned, an inverse relationship is found between surface roughness and osteocalcin expression by rat calvarial cells.[36] With other sterilization treatments, there is no difference in expression on materials with different surface roughness. The precise effect of surface roughness on cell phenotype expression is therefore not always clear. In our experiments, we saw a decrease in osteocalcin expression

on rough titanium compared to smooth titanium surfaces. However, cells cultured on CaP coatings with a surface roughness comparable to the rough titanium showed an increase in osteocalcin expression. It seems that the CaP coating had a positive effect on osteocalcin expression that could overcome the negative effect of increased surface roughness.

Based on the results mentioned above, we can conclude that surface characteristics of the tested substrates can alter the osteogenic expression of RBM cells cultured on the surface. The cells expressed osteogenic markers on both smooth and rough titanium and on heat-treated CaP coatings. The crystalline coatings had a positive effect on expression of osteogenic markers. The amorphous coatings showed no positive effect on the differentiation of the cells, probably due to the high rate of dissolution of the coatings.

Despite our positive results with the crystalline coating, a remark has to be made on the extrapolation of in vitro results to the in vivo situation. In the in vivo situation, the implant is placed in an area of surgical trauma that cannot be mimicked in vitro. Furthermore, the implant will encounter a wide variety of cell types, whereas in vitro studies are usually performed with a limited number of cell types, often of animal origin. The cell type and the species of animal used to isolate cells may affect the specific results of the study.[38,41] However, these problems may also be encountered in vivo, since in vivo experiments are mainly animal studies and since implant location could influence the final bone response.[42] The advantage of in vitro systems is that they provide a standardized, simplified system, which can be used to elucidate the mechanisms involved in the tissue response to a biomaterial.

A final comment has to be made on the setup of the performed experiments. In this study, cells cultured on CaP-700 show higher expression of osteogenic markers within the tested period. However, for many of the tested markers, it is not clear if the maximal expression is reached already. Therefore, more tests, with extended culture periods are required. Alternatively, experiments may be performed with higher seeding densities. Osteogenic cells require a well defined, three-dimensional structure for differentiation to occur. With more cells present, this structure will be formed at an earlier time point.

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Chapter 4

Initial interaction of U2OS cells with non-coated and calcium phosphate coated titanium substrates.

P.J. ter Brugge, S.C. Dieudonné, J.A. Jansen

Introduction:

The initial interactions of cells with a biomaterial involve their attachment and spreading on the biomaterial surface. We know that cells do not attach directly to a material, but interact with the material through an intervening protein layer. These proteins may be synthesized by the cells, or adsorb to the material from culture medium in vitro. Matrix proteins act as ligands for a family of transmembrane cell signaling receptors, called integrins. Integrins consist of two subunits, i.e. α and β. The extracellular domain of both subunits forms a ligand-binding site. Interaction of the integrin with a ligand results in the formation of focal contacts, structures containing signaling and cytoskeletal proteins that interact with the intracellular domain of the β subunit.[1-3] In this way, integrins transduce signals from the environment into the cell. Signaling through integrins can regulate cellular functions, such as cell growth, differentiation, migration, extracellular matrix (ECM) synthesis and apoptosis.[2,4-6] Therefore, variations in the composition or organization of the protein layer can induce changes in the final cellular response to the material. The type and amount of proteins adsorbed and their conformational state are all affected by substrate surface characteristics.[7-11] Referring to the earlier described signaling pathways this will have consequences for various cellular processes. For instance, both cellular attachment and spreading are influenced by material characteristics such as composition and roughness. [9,12-17]

In order to obtain more knowledge about the relationship between implant surface properties and cellular behavior, it is relevant to identify expression of integrins on the cell surface. Only by elucidating the interactions of cells with materials, we are able to design safe and purposeful implants.

In previous studies, we already investigated the longterm (1-2 weeks) effect of calcium phosphate (CaP) coated titanium substrates on the proliferation, differentiation and integrin expression of osteoblast-like and osteosarcoma cells.[18,19] In the current study, we focussed on the initial interaction of osteosarcoma cells with CaP-coated and non-coated titanium substrates. Therefore, we examined the effect of the substrates on the attachment, spreading and integrin expression of osteosarcoma cells.

Materials and Methods:

Substrates:

Commercially pure titanium (cpTi) disks (diameter 25 mm) were used. Disks were used as machined (Ti-s) or subjected to an Al_2O_3 gritblasting procedure. Gritblasted substrates were passivated in 20% HNO_3 and cleaned in 100% boiling ethyl alcohol. Substrates were left uncoated (Ti-r) or provided with a 2 μm thick RF-magnetron sputtered calcium phosphate

coating.[20] After deposition, coatings were subjected to an additional infrared heat treatment at 700°C in an infrared furnace (E4-10-P, Research Inc.) (CaP-ht). The apatite-like crystalline structure of the coatings was confirmed by X-ray diffraction. Ca/P ratios of the coating as measured with energy dispersive spectroscopy were between 1.77 and 1.83. Surface roughness of the substrates was measured using Atomic Force Microscopy (Digital Instruments). Per substrate, 3 areas of 50x50 μm were scanned. Three sections were made in each direction (horizontal, vertical and diagonal) and analyzed using the Nanoprobe program. The results of the roughness measurements are shown in Table 1.

Disks were autoclaved for 15 minutes at 120 °C before use in cell culture.

	Ra-value (μm)	Sm-value (μm)
Ti-s	0.60 ± 0.26	5.78 ± 1.94
Ti-r	1.64 ± 0.59	4.30 ± 1.11
CaP-ht	1.62 ± 0.67	4.17 ± 1.64

Table 1: surface roughness of Ti-s, Ti-r and CaP-ht. Ra value indicates mean height variation; Sm value indicates mean spacing of surface peaks.

Cell culture:

In all experiments, an osteosarcoma cell-line (U2OS) was used. Cells were cultured in α-MEM, supplemented with 10% FCS and 50 μg/ml gentamycin. Medium was changed every second day. After one week of culture, cells were trypsinized and added to the substrates for attachment study, scanning electron miroscopy (SEM), FACS analysis or fluorescent microscopy.

Attachment assay:

Cells were seeded onto the substrates ($2x10^5$ cells/ substrate). After 0.5, 1, 2, 3, 4, 7 or 24 hours, cells were released by trypsinization and counted. The percentage of attached cells was calculated as the number of attached cells divided by the number of seeded cells.

Two runs of the assay were performed, in each run all substrates were present in triplicate.

Scanning electron microscopy:

Cells were seeded on the substrates ($2x10^5$ cells/substrate) and cultured for 3, 7 or 24 hours. Samples were rinsed in PBS and fixed in 2% glutaraldehyde. Samples were washed twice in 0.1M sodium-cacodylate buffer (pH 7.4) and dehydrated using a graded series of ethanol. After drying with tetramethylsilane, samples were sputter-coated with carbon and photographed using a Jeol 6310 SEM with an acceleration voltage of 15 kV.

FACS analysis:

Antibodies for FACS analysis were purchased from Chemicon ($\alpha1$, $\alpha6$), Serotec ($\alpha2$, $\alpha4$, $\alpha5$, $\alpha v \beta3$, $\beta1$) and Immunotech ($\alpha3$). Antibodies against αv, $\beta3$, $\beta4$ and $\beta7$ were obtained from the Dept. of Tumor Immunology, University Medical Center, Nijmegen. FITC-labeled goat-antimouse was purchased from Sigma.

Cells were added to the substrates in a density of $2x10^5$ cells per substrate and were cultured for 3, 7 or 24 hours. An aliquot of the seeding suspension was used to study integrin expression of the cells at the time of seeding. These measurement are referred to as T=0.

Cells were released by trypsinization, suspended in 0.5% PBA (PBS, 0.5% BSA) and counted. Released cells were plated in a 96-wells plate in a concentration of $0.5x10^5$-$1x10^5$ cells per well. The plates were centrifuged for 1 min. at 1400 rpm, washed with 100 μl PBA and incubated with 25 μl/well of Mab in PBA for 30 min. at 4°C. After washing with 100 μl PBA cells were incubated with a FITC labeled secondary Ab for 30 min. at 4°C. After washing cells were resuspended in 100 μl PBA and transferred to FACS tubes. Analysis was performed on a FACscan flow cytometer (Becton Dickenson). The experiment was repeated 4 times. In each run, samples were present in triplicate. Cells from triplicate samples were pooled before staining.

Fluorescent microscopy:

Cells were seeded onto the substrates ($1x10^5$ cells/ substrate) and cultured for 0.5, 1, 2, 3, 4, 7, 16 and 24 hours. At the end of the culture time, medium was removed from the substrates and samples were fixed in 2% paraformaldehyde for 15 minutes. Samples were washed in PBS, permeabilized with 1% Triton X for 5 minutes and washed. Samples were incubated with TRITC labeled phalloidin for 30 minutes. Samples were washed in PBS and dried. A Biorad 1024 confocal microscope was used to make optical vertical sections through cell layers and substrate.

For measurements of cell shape and size, samples were studied under a microscope equipped with epifluorescent light (Leica) using a magnification of 20x. Images of 5 randomly selected fields per substrate were taken with a digital camera (Leica DC200). In this way, at least 75 cells were measured per substrate. Size of the cells in the image was calculated Leica Qwin Pro. The area of the cells was expressed as square pixels. The cell shape was calculated according to: (area/ perimeter^2) x 4π.

Two runs of the experiment were performed.

Statistical analysis:

For all measurements, the data from the separate runs was pooled for statistical analysis. Statistical analysis on FACS was performed using the Mann Whitney U test. Statistical

analysis on results from attachment assay, cell shape and cell size measurements was performed using analysis of variance (ANOVA), followed by a Tukey test.

Results:

Attachment assay:

The results of the attachment assay are shown in Figure 1. The graph shows that the number of attached cells quickly increases within the first 90 minutes. After 90 minutes, cell attachment still increased, but at a slower rate. The percentage of attached cells was significantly higher on Ti-r than on Ti-s at all times, except at 30 minutes (p<0.05). At 90 minutes, the number of attached cells was significantly different between all materials (Ti-r>CaP-ht>Ti-s). No differences in cell numbers were found at 24 hours.
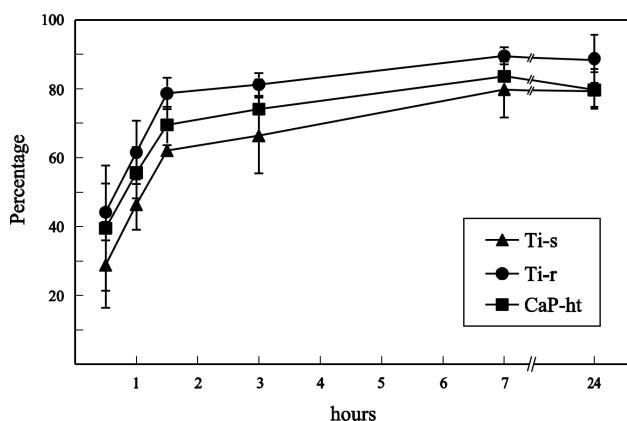

Figure 1: Attachment of U2OS cells on different substrates, at 0.5, 1, 1.5, 3 7 and 24 hours. Attachment is calculated as percentage of cells originally seeded. Values represent the mean ± SD of 2 experiments.

SEM:

For the first 3 hours, cells on Ti-r and CaP-ht showed a round morphology, with a smooth appearance of the cell membrane. Cells attached to the surface through the formation of lamellipodia (Figure 2a). Cells on Ti-s were also rounded, but these cells showed a ruffled cell membrane, with many fingerlike protrusions (Figure 2b). At 7 hours, most cells were in the process of spreading. Some cells still had a round shape, while others were already fully spread (Figure 2c). Again, cells on Ti-r and CaP-ht showed a smooth cell membrane, whereas many cells on Ti-s still showed a ruffled membrane. At 24 hours of culture, cells were fully spread on all substrates (Figure 2d). No differences in morphology were found.
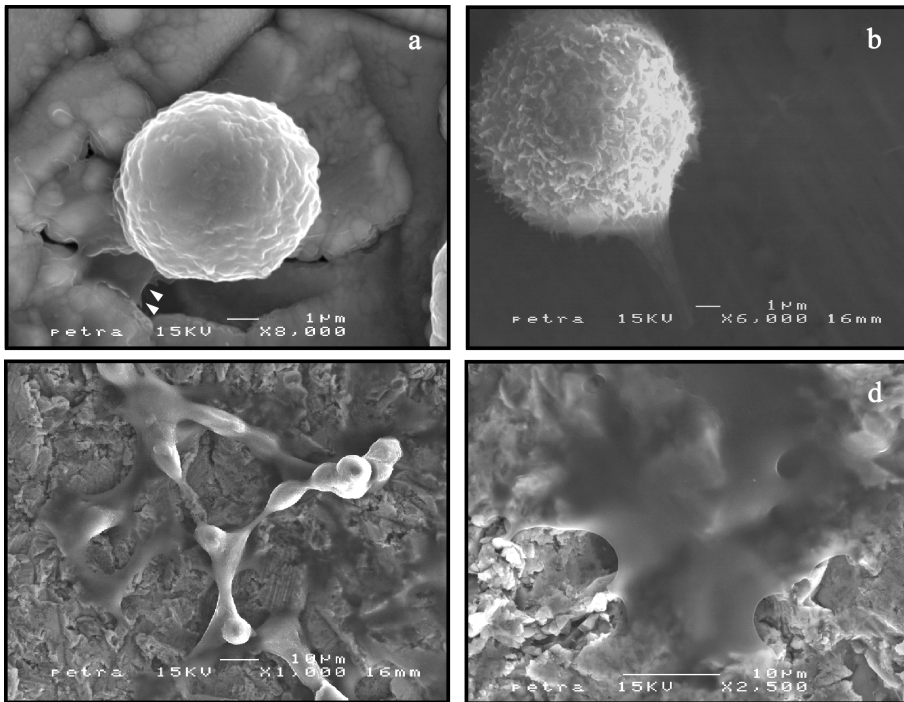
Figure 2: Attachment and spreading of U2OS on Ti-s, Ti-r and CaP-ht. (a) SEM image of U2OS cells, after 3 hours on CaP-ht. Arrowheads indicate lammellipodia. (b) U2OS cells, after 3 hours on Ti-s. (c) SEM image of a number of U2OS cells on Ti-r, after 7 hours. (d) U2OS cells cultured for 24 hours on Ti-r.

FACS analysis:

Integrin expression by U2OS is shown in figure 3, 4 and 5. At the time of seeding, the cells express $\alpha 2$, $\alpha 5$, $\alpha 6$, and αv subunits. Mean fluorescence for $\alpha 1$, $\alpha 4$, $\alpha v \beta 3$, $\beta 3$, $\beta 4$ and $\beta 7$ was low to not detectable (Figure 3). The cells show high mean fluorescent intensity for $\alpha 3$ and $\beta 1$ (Figure 4). When we compared the integrin expression on the various materials, we found that $\beta 1$ expression at 3 hours was significantly higher on CaP-ht than on Ti-s and Ti-r ($p < 0.05$: Figure 4a). This difference was not seen at 7 and 24 hours. Figure 4b shows the $\alpha 3$ expression on the various materials at different times. The expression of the $\alpha 3$ subunit was increased at 3 hours compared to T=0 on all substrates ($p < 0.05$). Mean fluorescent intensity decreased significantly from 3 to 7 hours on Ti-s and Ti-r ($p < 0.05$), but not on CaP-ht. No difference was found in $\alpha 3$ expression between 7 and 24 hours.

The expression of $\alpha 5$, $\alpha 6$ and αv was increased at 24 hours, compared to T=0 on CaP-ht

(p<0.05: Figure 5). The expression of α2 was increased at 24 hours compared to T=0, 3 hours and 7 hours. On Ti-s and Ti-r, the mean fluorescent intensity for α2, α5, α6 and αv did not change during culture, but remained at the level of T=0.

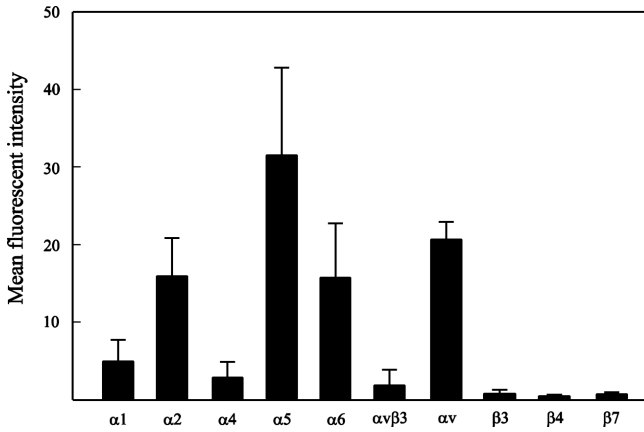**Figure 3:** Integrin expression by U2OS cells at T=0. Figure shows the mean fluorescent intensity measured for α1, α2, α4, α5, α6, αvβ3, αv, β3, β4 and β7 at T=0. Values represent the mean ± SD of 5 experiments.
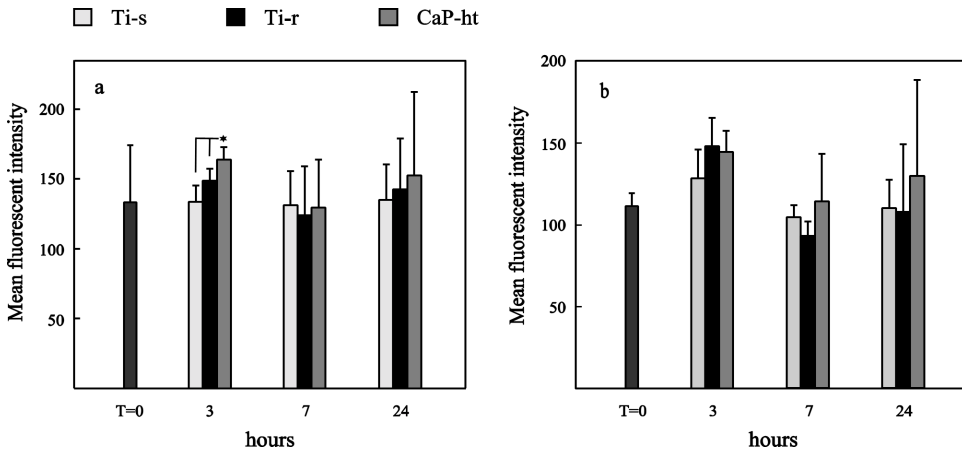

Figure 4: Expression of α3 and β1 subunits. (a) Mean fluorescent intensity for β1 at T=0, and at 3, 7 and 24 hours on Ti-s, Ti-r and CaP-ht. (b) Mean fluorescent intensity for α3 at T=0, and at 3, 7 and 24 hours on Ti-s, Ti-r and CaP-ht. Values represent the mean ± SD of 5 experiments.
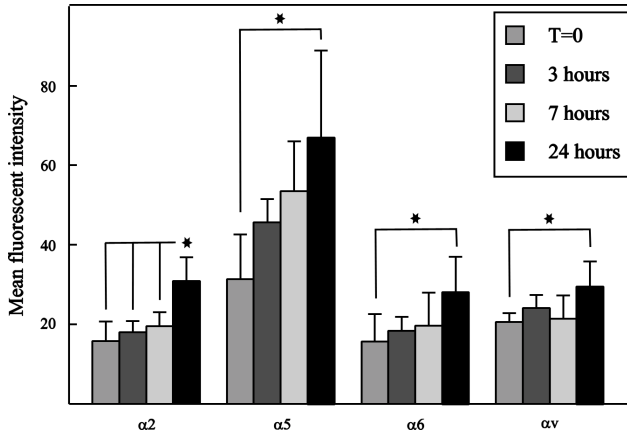
* indicates p < 0.05.

Figure 5: Mean fluorescent intensity of $\alpha 2$, $\alpha 5$, $\alpha 6$ and αv at T=0 and after culture on CaP-ht for 3, 7 and 24 hours. Values represent the mean $\pm$ SD of 5 experiments.

* indicates $p < 0.05$.

Fluorescent microscopy:

The results of cell shape and size measurements are shown in Figure 6. As indicated by the bar graphs, the cells showed a round appearance for the first three hours, i.e. a shape factor close to 1.0 (Figure 6a). No differences in cell shape existed for the first three hours between the various materials. At 4 hours, shape factor decreased significantly on Ti-s and Ti-r (P<0.01), but not on CaP-ht. At 7 hours, cells on CaP-ht also became more spindle-shaped. Still, the shape factor was significantly higher than on Ti-s and Ti-r (p<0.01). At 16 and 24 hours, the cells had the same spindle-shaped appearance on all materials. Additional fluorescent micrographs showed that at 16 and 24 hours, cells on Ti-s showed a preferential orientation along the grooves that resulted from the machining of the substrates (Figure 7a). Such a specific orientation was not found on the rough surfaces. The vertical section of cells and substrates show that the cells conformed to the roughness of the substrates and followed the surface contours (Figure 7b).

Similar to cell shape, cell size did not change during the first 3 hours of culture on the various materials (Figure 6b). Thereafter, cell size increased on all materials. On Ti-s and CaP-ht, cell size remained constant after 16 hours. This in contrast to Ti-r, where cell size was increased at 24 hours, compared to 16 hours. At 4, 7, 16 and 24 hours, cell size differed significantly between the three materials, with Ti-s> Ti-r >CaP-ht (p<0.01).
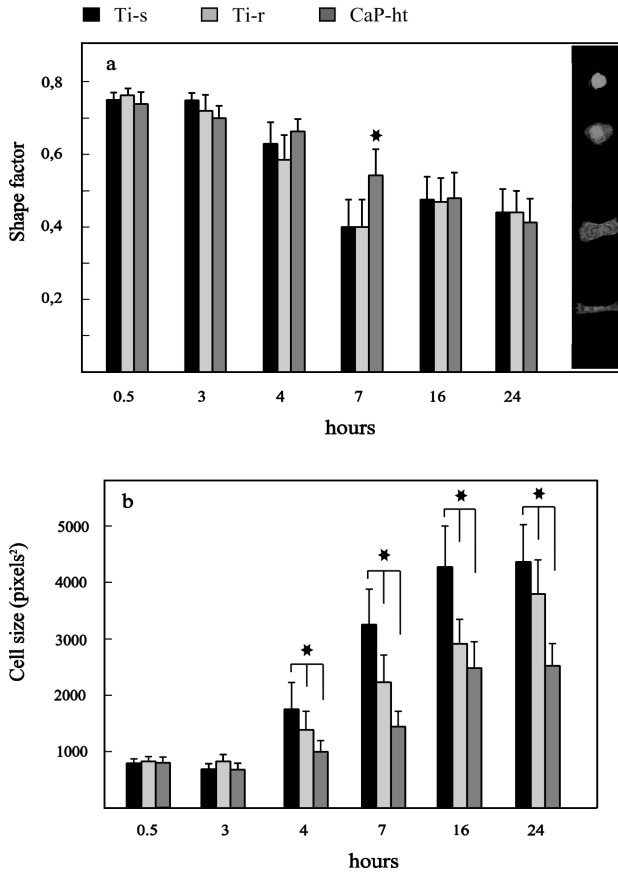
Figure 6: Effect of different substrates on the shape and size of cultured U2OS cells. (a) Shape of U2OS cells cultured on Ti-s, Ti-r and C-ht. Significant differences between the materials are indicated by * (p < 0.01). A completely round object is described by a shape factor of 1.0, a straight line approximates a shape factor of 0. This means that more elongate cells have a lower shape factor, as can be seen from the inset. (b) Size of U2OS cells cultured on Ti-s, Ti-r and C-ht. * indicates p < 0.01. Cell size is described as square pixels.

Discussion:

In this study, we investigated the effect of substrate surface characteristics on the initial interaction of U2OS cells. We showed differences in attachment, spreading and integrin expression between surfaces, which differed in roughness and/ or surface composition.

First, a remark has to be made about the use of the U2OS cell-line. Untransformed osteoblast-like cells isolated from different donors show a large variability in differentiation, attachment and integrin expression,[17,21-23] which severely complicates the evaluation of obtained results. This problem is not encountered when using U2OS cells, even though these cells have lost many of their osteogenic features. U2OS cells express a matrix that has some characteristics of an osteoblast-like matrix.[24] On the other hand, the
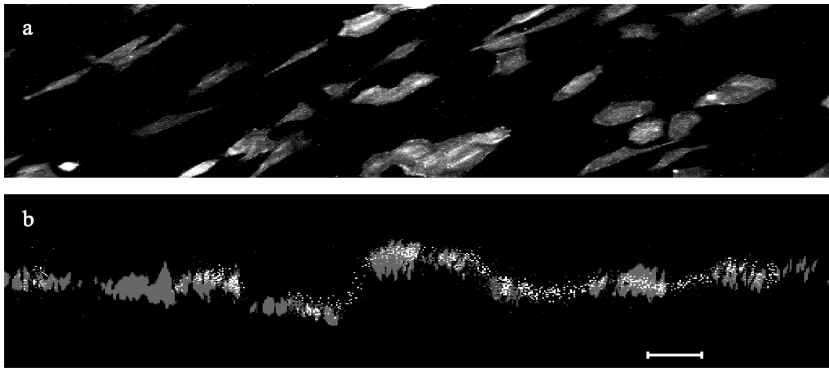
Figure 7: (a) U2OS cells cultured for 16 hours on Ti-s. The cells show alignment with the machining grooves in the substrate. Original magnification: 20x. (b) U2OS cells cultured for 24 hours on CaP-ht. CLSM was used to make a vertical section through cells and substrate. Phalloidin-labeled actin (white dots) and substrate surface reflectance (gray areas) were acquired in the same section. Actin staining is shown to overlap with the areas of surface reflectance. Bar =20 μm.

cells show low basal alkaline phosphatase activity, and no mineralization of the matrix.[19] However, the cell population does show limited variability, in contrast to untransformed osteoblast-like cells. Furthermore, osteosarcoma cell lines can be used as appropriate model for studying some aspects of cell function, such as integrin expression.[25] As a consequence, as part of our studies, we used U2OS as a basic model to analyze the effect of modulation of integrin subunits by substrate surfaces.

We observed that attachment of U2OS was decreased on smooth titanium compared to the rough surfaces. This effect of surface roughness has been shown before, with rat[26] and human[15,17] osteoblast-like cells, and also with human osteosarcoma cells.[27] The cells on the smooth substrate showed a cell membrane with many protrusions in all directions during attachment and spreading, whereas the cells on rough materials showed a smooth cell membrane. Spreading involves the formation of extensions containing actin filaments, like microspikes. The association with focal precursor contacts stabilizes these actin filaments. We suppose that on the smooth substrate, the cells encounter only a limited number of adhesion sites, and membrane extensions are not stabilized. On the other hand, on the rough substrates, the cells encounter adequate numbers of attachment sites, resulting in stabilization of actin filaments and association of the cytoskeletal elements with focal contacts.[28]

Cell spreading and shape are important modulators of cellular function. Restriction of spreading in human osteoblasts is associated with lack of integrin receptor clustering and

focal adhesion formation.[29] In many cell types, changes in cell function are accompanied by changes in cell shape. In these cells, inhibition of cell spreading results in induction of apoptosis and proliferation only takes place in cells that are spread out. When spreading is restricted, differentiation-specific function is enhanced.[30,30-32]

The properties of proteins adsorbed onto a material may directly affect cell spreading behavior.[14] Therefore, we examined the shape and size of U2OS cells on Ti-s, Ti-r and CaP-ht substrates. Spreading started at 4 hours on all materials and was associated with the acquisition of a more elongated cell shape. This effect was somewhat delayed on CaP-ht. Evaluation of cell size on the various substrates by fluorescent microscopy revealed that cell size was clearly affected by surface characteristics. Cells on the smooth surface spread out to a larger cell area than cells on the rough substrates. This can be due to the three dimensional structure of the rough substrates. By conforming to the surface roughness, cells achieve a large surface contact, without the need for extensive lateral spreading.[12] However, this cannot explain the difference between cell size on CaP-ht and Ti-r, substrates with similar surface roughness. Apparently, the substrate surface composition also plays a role in this process.

Besides cell size, integrin expression is also affected by the surface characteristics of our experimental substrates. It has been shown before that integrin properties may change, depending on substrate surface characteristics. Conformational changes in adsorbed proteins may result in the protein being recognized by different integrins or may change the binding properties of the integrins.[33] Furthermore, cells can modulate their integrin expression profile when attaching to different substrates.[34-36] Evaluation of our cultures demonstrated that integrin subunits expressed by the U2OS cells include α2, α3, α5, α6, αv and β1. Expression of α1, α4, αvβ3, β3, β4 and β7 was extremely low or absent. The subunits α2, α3, α5, α6 and αv all combine with the β1 subunit, forming receptors for a variety of ECM proteins, including collagen, fibronectin, vitronectin and laminin.[1] The α subunit has been shown to determine integrin ligand specificity, whereas the cytoplasmic domain of the β subunit interacts with signaling and cytoskeletal proteins.[1] Cell spreading appears to depend on activity of the β1 subunit.[37-39]

In our experiments, we only found differences in β1 expression between titanium substrates and CaP-ht even though cells showed differences in adhesion and spreading on all three materials. As we obtained information about the amount of β1 expression and not about its spatial distribution or activation state, it is difficult to relate the expression of the β1 subunit to changes in attachment and spreading in our study. At 24 hours, α2, α5, α6 and αv subunit expression increased on CaP-ht, but not on titanium. It is possible that at 24 hours, U2OS cells on CaP-ht have started synthesizing ECM proteins such as collagens, laminins, fibronectin and/ or vitronectin, which results in upregulation of the receptors for these

proteins. Upregulation of these integrin subunits may also explain why CaP coated have been shown to increase bone formation compared to uncoated substrates.[40] Both α2β1 and α5β1 integrins have been shown to play a critical role in osteoblast differentiation and blocking of the interaction of these integrins with their ligands suppresses the development of osteoblastic phenotype.[41-44]

However, at this point it appears appropriate to emphasize that FACS analysis is only used to quantify integrin expression, and does not exclude the possibility of differences in activation of the integrins present. Integrins are often expressed in an inactive form that does not interact with its ligand. For ligand-integrin interaction to occur, the integrin has to switch to an activated state.[45] Ligand binding activity of integrins may be regulated by intracellular signals or the presence of divalent cations.[46-48] Moreover, integrins that play a minor role in adhesion can have a major effect on intracellular signaling events.[49] Additional studies will have to be performed to see if U2OS cells also show similar mechanisms of integrin regulation, and whether U2OS cells use these mechanisms on the materials we studied.

In conclusion, we found that U2OS cells show differences in attachment and spreading on materials with different surface characteristics. We also showed that integrin expression, can be influenced by substrate surface composition, i.e. the presence of a CaP coating on the material.
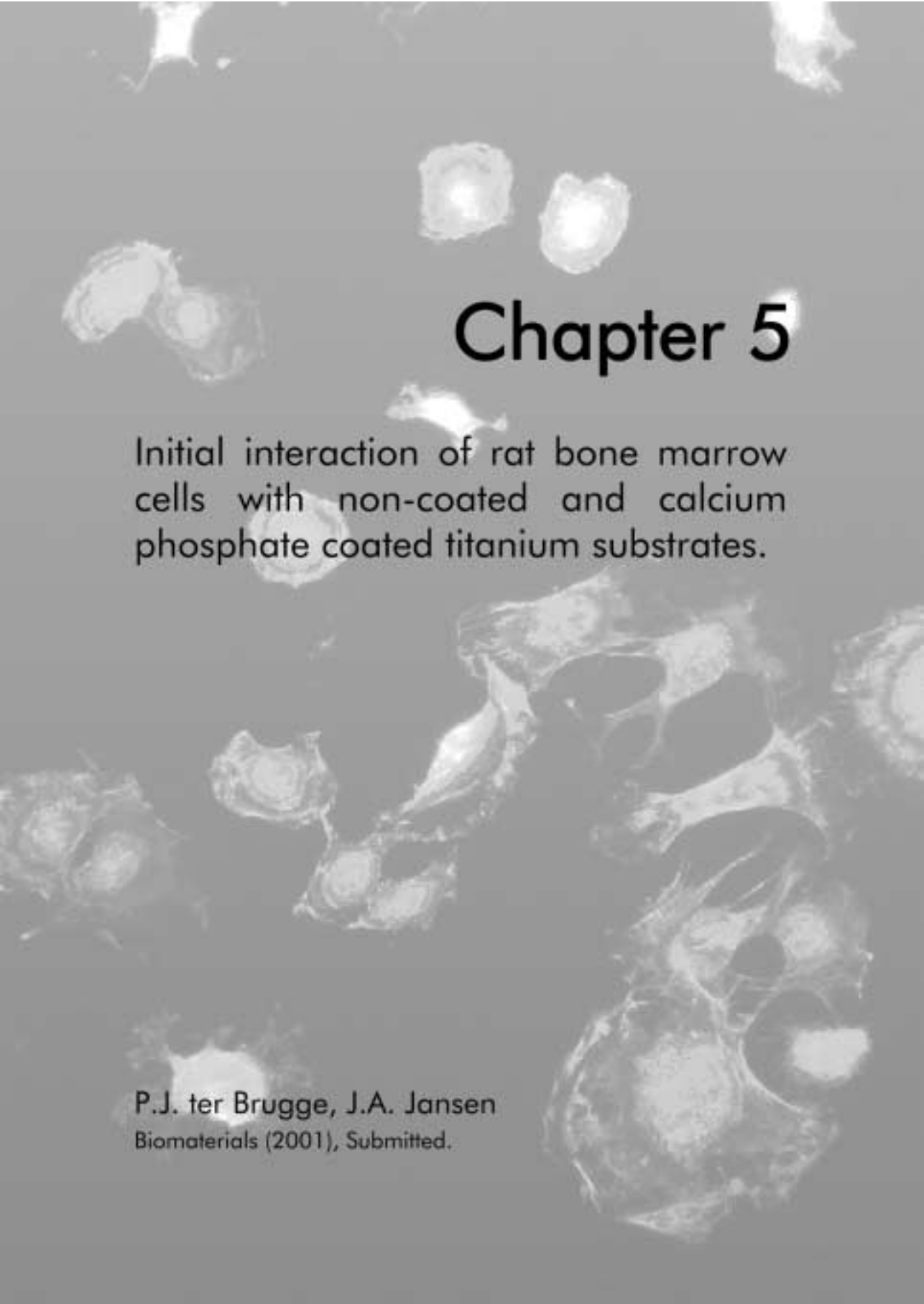
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Chapter 5

Initial interaction of rat bone marrow cells with non-coated and calcium phosphate coated titanium substrates.

P.J. ter Brugge, J.A. Jansen

Introduction:

Substrate surface characteristics have been shown to affect many aspects of cellular behavior, such as attachment, spreading, proliferation and differentiation. For example, in studies performed in our laboratory, we observed higher expression of osteogenic markers by rat bone marrow (RBM) cells cultured on calcium phosphate (CaP) ceramic compared to smooth and rough commercially pure titanium substrates.[1,2] So far, we still do not know what specific surface property causes this difference in cellular reaction.

Cells interact with a biomaterial through a layer of (extracellular matrix) ECM proteins. This layer may be adsorbed from fluids surrounding the material, or may be synthesized by the cells.[3-6] These proteins act as ligand for a family of cell surface proteins called integrins. These receptors consist of two transmembrane subunits, i.e. α and β, and ligand specificity is determined by the combination of α and β subunit. The intracellular domain of the β subunit associates with cytoskeletal and signaling proteins. Interaction of an integrin with its ligand results in generation of intracellular signals. These signals regulate many cellular functions, such as proliferation, migration, ECM synthesis, differentiation and apoptosis.[7]

Differences in the substrate adsorbed protein layer may result in changes in integrin signaling, which can modify subsequent cellular behavior, such as attachment, spreading, proliferation and differentiation.[3,4,8,9] The precise nature of the protein layer (type, amount of protein and conformational state) is determined by the characteristics of the substrate surface.[10] For instance, differences are found in the type and amount of adsorbed proteins between CaP and titanium.[4] This may explain why calcium phosphates differently affect osteogenic cell differentiation compared to titanium. To prove this hypothesis, we investigated the effect of non-coated and CaP coated titanium substrates on the initial reaction of RBM cells. We compared the attachment and spreading behavior of the cells on the various materials. Further integrin expression of attached and non-attached RBM cells was studied.

Material and Methods:

Substrates:

Commercially pure titanium (cpTi) disks (diameter 25 mm) were used. Disks were used as machined (Ti-s) or subjected to an Al_2O_3 gritblasting procedure. Gritblasted substrates were left uncoated (Ti-r) or were provided with a 2 µm thick radio frequent-magnetron sputtered calcium phosphate coating.[11] After deposition, coatings were subjected to an additional infrared heat treatment at 700°C in an infrared furnace (E4-10-P, Research Inc.) (CaP-ht). The apatite-like crystalline structure of the coatings was confirmed by X-ray diffraction. The Ca/P ratio of the coating, as measured with energy dispersive spectroscopy, was

between 1.77 and 1.83. Surface roughness of the substrates was measured using Atomic Force Microscopy (Digital Instruments). Per substrate, 3 areas of 50x50 µm were scanned. Scans were analyzed using the Nanoprobe program. The results of the roughness measurements are shown in Table 1. All disks were autoclaved for 15 minutes at 120°C before use in cell culture.

	Ra value (µm)	Sm value (µm)
Ti-s	0.60 ± 0.26	5.78 ± 1.94
Ti-r	1.64 ± 0.59	4.30 ± 1.11
CaP-ht	1.62 ± 0.67	4.17 ± 1.64

Table 1: surface roughness of Ti-s, Ti-r and CaP-ht. Ra value indicates mean height variation; Sm value indicates mean spacing of surface peaks.

Cell culture:

Rat bone marrow (RBM) cells were isolated and cultured using the method described by Maniatopoulos.[12] After 7 days of primary culture in α-MEM supplemented with 10% fetal calf serum, 50 µg/ml ascorbic acid, 50 µg/ml gentamycin, 10 mM Na β-glycerophosphate and 10^{-8} M dexamethasone, cells were removed from the culture flasks by trypsinization. Cells were resuspended in supplemented medium. Substrates were placed at the bottom of 6 well-plates and cells were seeded on the substrates for attachment assay, fluorescent microscopy and FACS analysis. To determine the integrin expression pattern of primary RBM cells, the remainder of the cell suspension was used for FACS analysis. These measurements are referred to as T=0.

Attachment assay:

Cells were seeded onto the substrates ($2x10^5$ cells/ substrate). After 15, 30, 60, 120, 240 or 360 minutes cells were released by trypsinization and counted. The percentage of attached cells was calculated as the number of attached cells divided by the number of seeded cells. Two runs of the assay were performed, in each run all substrates were present in triplicate.

FACS analysis:

Antibodies for FACS analysis were purchased from Pharmingen (α1, α2, α4, α5, β1, β3: goat anti hamster IgM; goat anti-mouse IgG), Serotec (α6), Bender Medsystems (α3), Sigma (goat anti-rabbit IgG) and Jackson (goat anti-hamster IgG).

Cells were seeded on the substrates in a density of $2x10^5$ cells/ substrate and incubated at 37°C. After 30, 60, 120 or 240 minutes, non-attached cells were removed, washed in PBS containing 1% BSA (PBA) and counted. Attached cells were removed by trypsinization (0.25% trypsin, 0.01% EDTA; 5 minutes), suspended in PBA and counted. Attached and

non-attached cells were plated in a 96-wells plate in a concentration of 1×10^5 cells per well. The plate was centrifuged for 1 min. at 1400 rpm at 4°C. Cells were washed with 100 µl PBA and incubated with 25 µl/well of the anti-integrin antibody in PBA for 30 min. at 4°C. After washing with 100 µl PBA cells were incubated with an appropriate FITC labeled secondary Ab for 30 min. at 4°C. After washing cells were resuspended in 100 µl PBA and transferred to FACS tubes. Analysis was performed on a FACscan flow cytometer (Becton Dickinson).

For α3 staining, the protocol described above was modified, since the antibody recognizes the cytoplasmic domain of the subunit. Cells were permeabilized before addition of the primary antibody. 50% Ethanol (1ml FCS, 1ml α-MEM, 6ml 70% ethanol) was added to the cells and incubated on ice for 30 min. Cells were washed with PBA and stained for FACS according to the protocol mentioned above. The complete experiment was performed nine times.

Fluorescent staining:

Cells were seeded onto the substrates (1×10^5 cells/ substrate) and cultured for 30, 60, 120 or 240 minutes. At the end of culture, medium was removed from the substrates and samples were fixed in 2% paraformaldehyde for 15 minutes. Samples were washed in PBS, permeabilized with 1% Triton X for 5 minutes and washed. Samples were stained for actin by incubation with TRITC labeled phalloidin for 30 minutes. Samples were washed in PBS and dried. Morphology of the cells was studied using a Biorad 1024 confocal microscope.

For measurements of cell shape and size, samples were studied under a microscope equipped with epifluorescent light (Leica) using a magnification of 20x. Images of 6 randomly selected fields per substrate were taken with a digital camera (Leica DC200). In this way, at least 50 cells per substrate were measured. An image analysis program (Leica Qwin Pro) was used to trace the outline of the cells in the image. Cell size was defined as the area within the outline and was expressed as square pixels. The cell shape factor was calculated according to the formula: (cell area/ cell perimeter^2) x 4π.

Four individual runs of the experiment were performed.

Statistical analysis:

Statmost 32 (Datamost corporation) was used to perform statistical analysis. Differences between groups were tested using an ANOVA. Post-hoc testing was performed with a Student-Newman-Keuls test.

Results:

Attachment assay:

In both runs, the RBM cell attachment started within 15 minutes. In one run, the number of attached cells increased quickly for the first 60 minutes and reached a maximum at 240 minutes (Figure 1a). In the second run, cell attachment proceeded at a slower rate, with maximal attachment at 360 minutes (Figure 1b). At 360 minutes, percentage of attached cells is similar in both runs. No significant difference in attachment was found between the experimental materials at any studied time point in either run ($p > 0.05$).
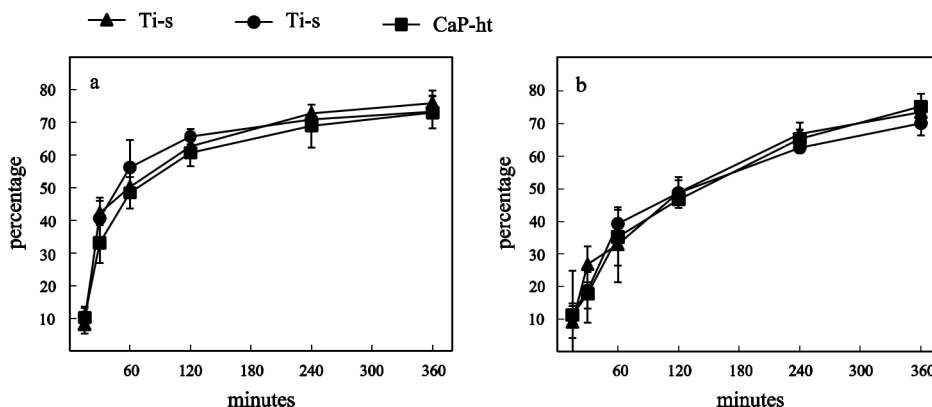

Figure 1: Attachment of RBM cells to Ti-s, Ti-r and CaP-ht. Two separate runs (a and b) of the experiment are shown.

FACS analysis:

Integrin expression in primary RBM cells (T=0) varied greatly between individual experimental runs, as is illustrated in Figure 2. The cells always expressed $\alpha1$, $\alpha5$, $\alpha6$ and $\beta1$ subunits. Expression of $\alpha3$ was found in all but one run and was very low in a second run. Expression of $\alpha2$, $\alpha4$ and $\beta3$ subunits was absent or was very low.

Similar variability in integrin expression was found after seeding of the cells onto the substrates. Expression of $\alpha1$, $\alpha3$, $\alpha5$, $\alpha6$ and $\beta1$ subunits was always found after seeding. Similar to primary cells, expression of $\alpha2$, $\alpha4$ and $\beta3$ subunits was absent or was very low.

In five of the performed experimental runs, integrin expression fluctuated during the experiment, with large differences in fluorescence between materials (Figure 3a). In these runs, integrin expression increased during early culture times, then decreased again after longer culture periods. In the four other runs, integrin expression remained at the same level at all incubation times, and no difference was found between the various substrates for any of the studied subunits (Figure 3b).
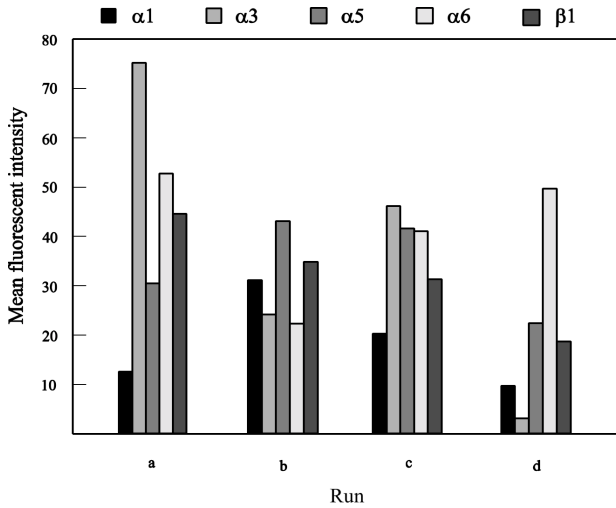
Figure 2: Integrin expression by primary RBM cells (T=0) cultured for 7 days on tissue culture plastic. Fluorescent intensity measured in four (a-d) of the nine performed runs is plotted.

For $\alpha 1$, $\alpha 5$, $\alpha 6$ and $\beta 1$ we found that in four runs expression was different between attached and non-attached cells (Figure 3a). In the other five runs, this effect was not observed (Figure 3b). Different results were obtained for $\alpha 3$. At early incubation times, no reproducible differences were found in expression between attached and non-attached cells (Figure 4a). After longer periods, $\alpha 3$ expression decreased for attached cells, whereas expression for non-attached cells remained high (Figure 4b). This pattern was seen in all performed runs.
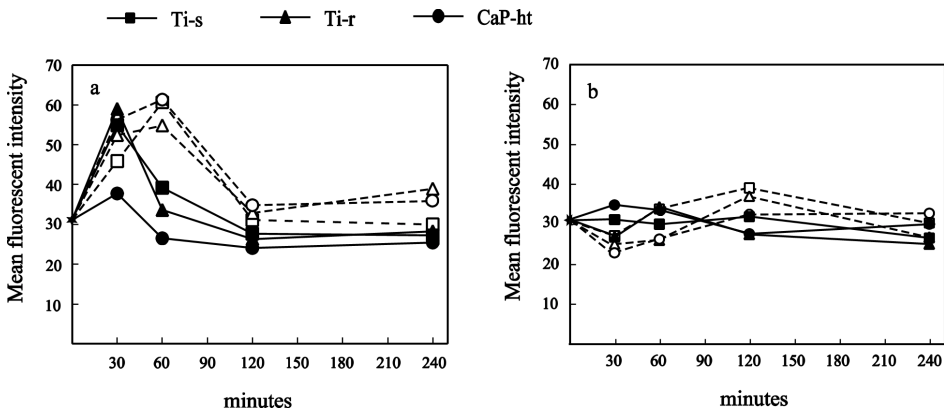

Figure 3: Expression of $\beta 1$ on attached and unattached cells on Ti-s, Ti-r and CaP-ht. Two separate runs (a and b) of the experiment are shown. Closed symbols show $\beta 1$ expression on attached cells, open symbols show integrin expression on unattached cells.
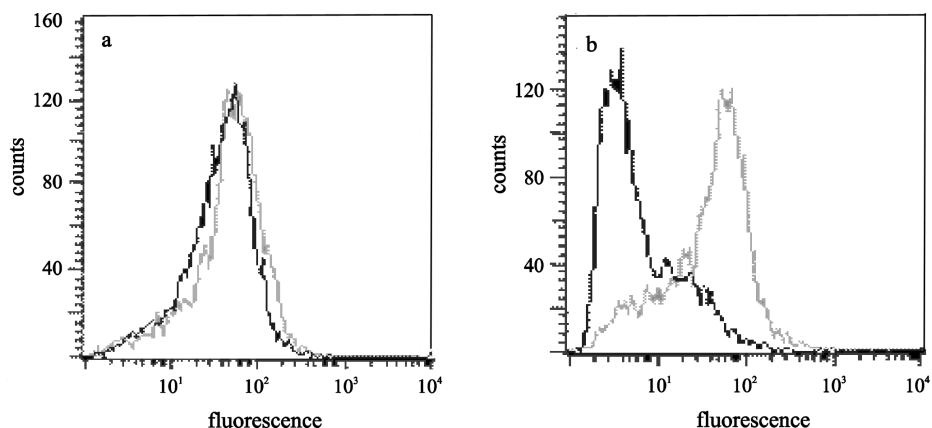
Figure 4: Expression of α3 on Ti-r after 30 minutes (a) and 240 minutes (b). Results are from one run. Frequency histograms for unattached cells (gray line) and attached cells (black line) were superimposed.

Fluorescent staining:

Data from the four experimental runs was pooled and the results are shown in Figure 5.

RBM cell spreading started as early as 30 minutes on all materials. The size of the cells on Ti-r and CaP-ht increased between 30 and 120 minutes, then remained at the same level (Figure 5a). Although cells on Ti-r were spread less at early incubation times, no difference was found in cell size between Ti-r and CaP-ht at 120 and 240 minutes. The size of the cells on Ti-s increased between 30 minutes and 240 minutes. The cells on Ti-s spread out to a significantly larger size than the cells on Ti-r and CaP-ht ($p<0.05$).

At 30 minutes, cells on Ti-r had a significantly higher shape factor than cells on Ti-s and CaP (Figure 5b). Shape factor decreased on all materials between 30 and 240 minutes. At 240 minutes, cells on Ti-r and CaP-ht showed a significantly lower shape factor than cells on Ti-s ($p<0.05$).

Further evaluation of the specimens revealed that spreading at early culture times was usually associated with the formation of short cellular extensions (Figure 6a). After longer culture periods, many cells on Ti-r and CaP-ht elongated and formed long processes extending away from the cell body (Figure 6b). Nevertheless, cells with a completely different morphology were also found (Figure 6c). Cells on Ti-s mostly showed a compact cell body, with little formation of cellular extensions. However, variability in cell size and shape was found (Figure 6d).

In addition, spreading was associated with the formation of stress fibers (Figure 6d). These stress fibers were only seen in part of the cell population.
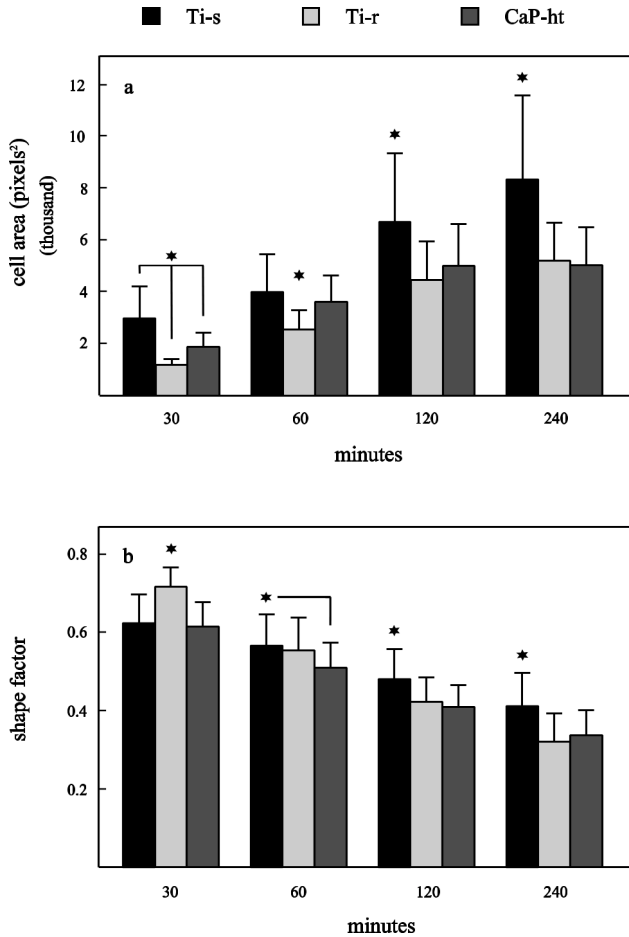
Figure 5: Size and shape of RBM cells on Ti-s, Ti-r and CaP-ht. (a) Size of cells on Ti-s, Ti-r and CaP-ht. Results from 4 separate runs were pooled. (b) Shape factor of cells on Ti-s, Ti-r and CaP-ht. A completely round object is described by a shape factor of 1.0, a straight line approximates a shape factor of 0. This means that elongated cells or cells with many extensions have a lower shape factor. Results from 4 separate runs were pooled.

* indicates a significant difference ($p < 0.05$).

Discussion:

 In this study we investigated the effect of substrate surface characteristics on initial attachment, spreading and integrin expression of RBM cells. We found no differences in cell attachment or integrin expression between the various materials. The materials did affect the cell spreading behavior.

The cell attachment rate differed between the two experimental runs. Nevertheless, we found no difference in final attachment of RBM marrow cells between the coated and non-coated materials. These studies do not correlate with other studies that show higher cell attachment on hydroxyapatite than on titanium substrates.[13-15] On the other hand, higher cell attachment on titanium has also been found.[16] Cell attachment is mediated through proteins adsorbed onto the substrate surface. Some studies show differences in protein adsorption
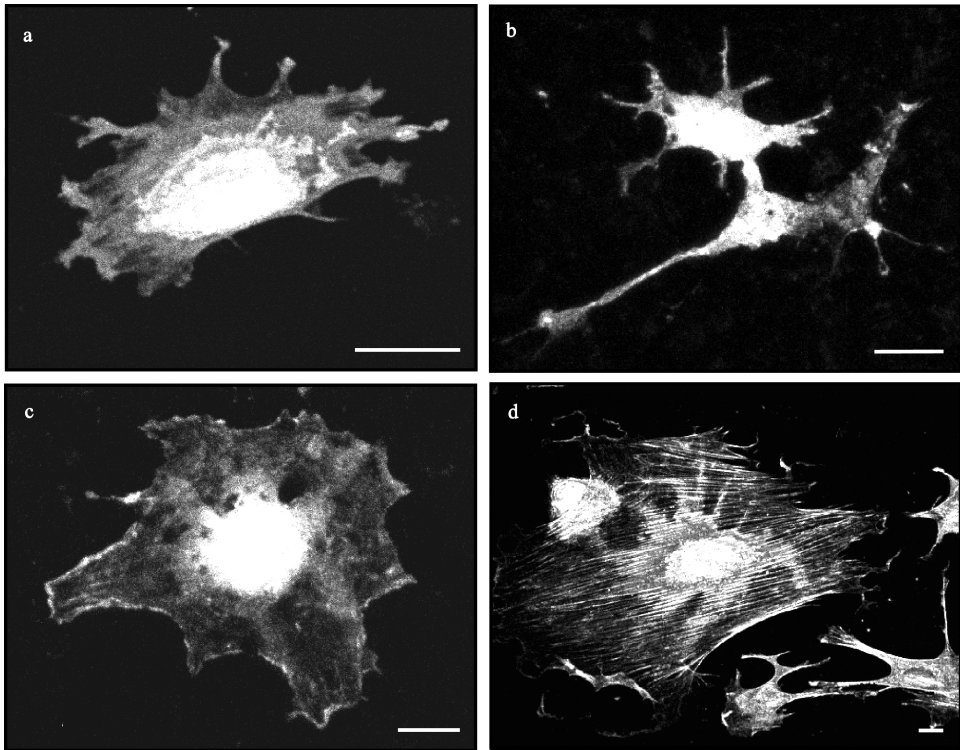
Figures 6: Morphology of RBM cells on Ti-s, Ti-r and CaP-ht. (a) RBM cell after 30 minutes on Ti-s. Cell shows a compact cell body with short cellular processes. No stress fiber formation is seen (b) Two cells after 240 minutes on CaP-ht. Cells show long, thin cellular extensions and no stress fibers. (c) RBM cell after 240 minutes on CaP-ht. Cell shows a compact cell body, with short cellular extensions. (d) Extensive stress fiber formation in RBM cells on Ti-s after 240 minutes. A large variation is seen in the shape and size of the different cells in the image.
Bar in all images: 15 μm.

between CaP and titanium, whereas other studies do not show these differences.[4,17] Our surfaces do not appear to elicit differential protein adsorption. However, we can not exclude the possibility that RBM cells are not sensitive to possible differences in the protein layer, for instance because the cells lack a receptor to a differentially adsorbed protein.

We also found no effect of surface roughness on cell attachment. Still, many studies describe this effect. For instance, a positive effect of surface roughness has been shown for human osteoblast[18], human marrow-derived cell[19] and rat calvarial osteoblast attachment.[20,21] On the other hand, no effect of surface roughness was seen using the Saos-2 cell line.[22,23] This could indicate that the effect of surface roughness is cell type dependent.

Further, we have to emphasize that a comparison between various studies dealing with surface roughness and cell response is not as straightforward as occasionally suggested. For example, the various studies not only differ in cell type used, but also in applied surface roughness. In this context, we know that there are various methods to measure and define surface roughness. The observed values are dependent on measuring equipment and technique.[24] Also, often different roughness parameters are used. This may explain why studies on the effect of surface roughness on cellular attachment differ so widely in outcome.[18,19,25-27,27] Therefore, it is essential that arrangements are made that surface roughness is defined and characterized by all investigators in the same way.

Since cell adhesion is mediated through integrins, we used FACS to study the expression of these molecules during attachment and spreading of RBM cells. Changes in integrin expression related to differences in substrate characteristics have been shown before.[28,29] In our study, we found a large variation in integrin expression between individual runs, both in primary cells and in cells cultured on the materials. Although differences in expression on various materials were found in individual runs, these differences were not consistently found in all runs. It is unlikely that the lack of differential integrin expression between the various materials is due to the release of the cells from the materials by the use of trypsin. Although trypsin releases cells by proteolytic degradation of matrix proteins, integrins are not affected trypsin in concentrations similar to the one we used.[30,31] Therefore, it appears that the expression of integrins by RBM cells during attachment and spreading is not modulated by the surface characteristics of the materials we used.

In all experimental runs, expression of α1, α5, α6 and β1 remained roughly at the same level on both attached and nonattached cells. Expression of the α3 subunit decreased on attached cells at later times of incubation. This is a specific effect due to attachment of the cells, since expression of nonattached cells remained at similar levels as found in early culture.

The α3 subunit combines with β1 to form a receptor for collagen, fibronectin and laminin. Expression of α3 has been found in both human and rat osteoblast-like cells.[32-34] Interactions of α3β1 with matrix proteins appears to be involved in regulation of osteogenesis.[35] Unfortunately, at the moment no other information is available about the function of α3 in osteoblasts, especially during attachment and spreading. In our study, the decrease of α3 expression is found at the same times that the cells are in the process of spreading. Therefore, we speculate that in RBM cell spreading is associated with downregulation of α3 expression.

Cell spreading is involved in the regulation of many cellular functions. Inhibition of spreading results in apoptosis and changes in cell spreading are associated with changes in proliferation, migration and differentiation of several cell types.[36-40] Spreading may be accompanied by development of mechanical tension by the cytoskeleton, which results in

generation of intracellular signals.[37,41]

In our study, cells reached the largest size on Ti-s, with no difference in size between cells seeded on Ti-r and CaP-ht. In a previous study, we found that osteoblast-like cells on Ti-r and CaP-ht followed the roughness of the surface, thereby maximizing the area of surface contact, and minimizing the need for lateral spreading. This resulted in a smaller cell size than on Ti-s.[42] Cells on Ti-s usually had a compact cell body, with limited cellular extensions. In contrast, cells on Ti-r and CaP-ht often showed elongated shapes, with many long extensions. Shah et. al. hypothesized that cells on some materials may extent many cell processes in order to form sufficient anchors for attachment, when they are unable to conform to a material surface.[43] However, cells with long cellular extensions also showed a larger cell size and a lower attachment than cells with a compact cell shape. This is in contrast with our study, where cells with many long processes had a smaller size, and no differences were found in attachment. Therefore, in the cells we used, the formation of extensive cellular processes is probably not related to the ability of cells to conform to the surfaces.

In our study, we found large variations in cell behavior between the different performed run. Variability in the attachment, proliferation and differentiation of primary marrow derived cells has been shown before.[18,44,45] We know that several cell types are present in RBM cell cultures and also wide variations in the ratio of cell type in replicate cultures do occur.[46] This would account for many of the differences we found in our study between RBM cells in the different experimental runs.

Despite the variations in RBM cells from different runs, some general conclusions can be drawn. Attachment of RBM cells is not affected by the surface characteristics of the studied materials. Further, integrin expression on RBM during attachment and spreading is apparently not modulated by the materials. Evidently, cell spreading and morphology are influenced by surface characteristics. Nevertheless, the final effect of this phenomenon on osteogenesis needs further investigation.

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Chapter 6

Analysis of integrin expression in U2OS cells cultured on various calcium phosphate (CaP) ceramics.

J.E. de Ruijter, P.J. ter Brugge, S.C. Dieudonné, S.J. van Vliet, R. Torensma, J.A. Jansen.

Introduction:

Currently many different materials are being used for repair and replacement of bone tissue. Nevertheless, our understanding how these materials interact with cells in general and bone cells in specific is still very limited.

We recently found that titanium coated with a thin calcium phosphate (CaP) coating modulated the proliferation and differentiation of osteoblast-like cells. For instance, CaP coated substrates enhanced calcified matrix formation by rat bone marrow stromal cells compared to cells grown on uncoated specimen.[1] The underlying mechanism that explains the influence of CaP ceramics on cellular behaviour is still unknown. We know that extracellular matrix macromolecules, as deposited on a substrate surface, have striking effects on the behaviour of cells in culture.[2] They influence their shape, polarity, movement, metabolism, development, and differentiation status.[3,4,5] The composition and conformational state of the absorbed extracellular matrix molecules depends on the substrate surface properties.

The information residing within the substratum that controls events such as differentiation, proliferation and biosynthesis must be transmitted into the cell via surface molecules.[6] Each cell has a specific combination of cell-surface adhesion molecules (cell-surface receptors) that enables the cell to bind to other cells and to the extracellular matrix. The major cell surface receptors on animal and human cells for binding to matrix proteins are a family of transmembrane proteins, the integrins.[7-11] Integrins are heterodimeric transmembrane glycoproteins that consist of noncovalently associated α and β subunits. These subunits can combine to form at least 21 distinct integrins receptors. Integrins represent a major family of transmembrane molecules that report the status of the ECM to intracellular moieties. Cells are capable of expressing several integrins, in culture and in situ.[11-20] Cell proliferation and differentiation have been shown to depend on the nature of implant material.[12,21,22] The nature of the substrate has been shown to affect the expression of integrins by cells attaching to the materials.[23-26]

Consequently, the aim of our experiments was to investigate whether well-defined CaP substrates can influence cellular proliferation and integrin expression. For this first study, we used an osteosarcoma cell line, U2OS.

Materials and Methods:

Substrates:

Commercially pure titanium (cpTi) disks with a diameter of 12 mm were used. The disks were polished to 320 grit with abrasive papers, ultrasonically cleaned for 15 min. in acetone and dried in 100% boiling ethyl alcohol. After drying, the disks were left untreated or were

provided with two different 2.5 µm thick CaP coatings. The CaP coatings were produced using an RF magnetron sputter technique as described earlier.[27] The sputter process was performed at a power level of 400 W and a process pressure of 1.5×10^{-2} mbar using argon gas. The titanium disks were mounted on a water-cooled substrate holder. The coatings were produced with a rotating substrate holder.

Finally, five groups of substrates were created:

  - cpTi disks with an amorphous CaP sputtercoating (0% O_2, Ca/P ratio 1.8: TiHA-0%)
  - cpTi disks with an amorphous CaP sputtercoating (5% O_2, Ca/P ratio 2.2: TiHA-5%)
  - Non coated cpTi disks: Ti
  - Dense sintered hydroxyapatite disks: HA
  - Thermanox® (Nunc products by Gibco) was used as reference material: Th.

All deposited coatings and the dense HA-ceramics were characterised by Scanning Electron Microscopy (SEM), X-ray Diffraction (XRD), and Fourier Infrared Absorption Spectrometry (FTIR). The structural and compositional features of these materials were found to be consistent with previously described substrates.[1,28]

Before use in cell culture experiments, all disks were autoclaved for 20 min. at 120°C.

Cell culture:

The osteosarcoma cell line U2OS was cultured in Iscove's Dulbecco medium (Gibco) containing 10% FCS and 0.5 mg/ml gentamycin (Gibco). At confluence, the cells were detached by trypsinization with 0.25% (w/v) crude trypsin and 0.02% (w/v) EDTA, pH 7.2.

Cell proliferation assay:

U2OS cell suspension, containing 1×10^4 cells/ml, was seeded on the different substrates, which covered the bottom of 24-wells plates (Greiner). The cultures were incubated for 1, 3, 5 and 8 days at 37°C in 5% CO_2 air atmosphere. The medium was changed every other day. At the end of the various incubation times, the cultures were washed using Isoton II azide free balanced electrolyte solution (Coulter) to remove non-attached cells. The substrates were taken out of the well and placed into a counting tube. The cells were detached by trypsinization with trypsin/EDTA and counted using a cell counter (Coulter). Complete removal of cells was determined by staining the substrates with methylene blue.

Two runs of experiments were carried out. In each run all materials were present in quadruplicate.

Alkaline phosphatase assay:

The substrates were placed at the bottom of a 24-well plate (Greiner) and U2OS cells

($1x10^4$ cells/ml) were added to the wells.

After 5 and 8 days the cell cultures were rinsed twice in PBS then 1 ml milliQ was added to each well, and the specimens were put on ice. The cells were harvested with a cell scraper and the cell suspension was transferred in a 10 ml tube. The cells were sonicated for 10 min. and centrifuged at 2000 rpm for 10 min. Subsequently, the supernatants were transferred to eppendorf tubes and stored at -20^oC until further analysis.

Samples of the supernatants were incubated with assay mixture (0.5 M 2-amino-2-methyl-1-propanol, pH 10.3, 5mM $MgCl_2.6H_2O$, 5mM paranitrophynylphosphate) for 60 min. at 37^oC. The reaction was stopped with 0.3M NaOH. Finally, enzyme activity was measured with a spectrophotometer at 405 nm. Two runs of experiments were carried out. In each run all materials were present and cultures were done in duplicate.

To determine relative amounts of alkaline phosphatase (ALP) activity, protein content from the same lysate was measured using the Pierce BCA protein assay (Pierce, Rockford, IL, USA).

Fluorescence activated cell sorting (FACS) analysis:

To determine integrin expression in the cells under the various conditions, FACS analysis was performed. First, we determined the expression pattern of individual integrins on Thermanox®, the culture reference material. The integrins that were expressed were tested for the various biomaterials. Cell suspension ($1x10^4$ cells/ml) was added to the various substrates as previously described in the cell proliferation assay. After 5 and 8 days of incubation, the cells were harvested by short trypsinization of subconfluent monolayers and suspended in PBA (PBS containing 0.5% BSA and 0.02% azide). Subsequently, the cells were counted with the Coulter counter and cells were put in a V-bottom 96-wells plate (Greiner) in a concentration of $5x10^4 – 2x10^5$ cells per well. The plate was centrifuged for 1 min., 1400 rpm. After washing with PBA, they were incubated with 25 μl/well monoclonal antibodies in PBA for 30 min. at 4^oC. After washing with PBA, the cells were incubated with 25 μl of the appropriate fluorescein-isothiocyanate-conjugated secondary antibody (FITC) (DAKO, Glostrup, Denmark) for 30 min. at 4^oC. After washing with PBA, cells were suspended in 100 μl PBA and transferred in FACS tubes. Analyses were performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Five separate runs of experiments were carried out. In each experimental run, cells from the various substrates were tested in sixfold.

Monoclonal antibodies:

The following antibodies were used for the FACS experiments: TS2/16 (anti-β1 chain, CD29), Dr. T. Springer, Boston, MA[29]; J134 (anti-α3 chain, CD49c), Dr. E. Klein, Ulm, FRG[30]; HP2/1 (anti-α4 chain, CD 49d), Dr. F. Sanchez-Madrid, Madrid, Spain[31]; Sam-1

(anti-α5 chain, CD49e) and GoH3 (anti-α6 chain, CD49f) Serotec, United Kingdom; AMF-7 (anti-αv chain), Dr. C.G. Figdor, Nijmegen, The Netherlands.

Scanning electron microscopy:

For scanning electron microscopy (SEM), cell suspension (1×10^4 cells/ml) was added to the different substrates as above in the cell proliferation assay. The cultured cells were incubated for 5 and 8 days at 5% CO_2, 95% air at 37^oC. After incubation, non-attached cells were removed by rinsing with PBS. Attached cells were fixed in situ with 2% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer for 15 min. at room temperature, rinsed twice in cacodylate buffer for 10 min., followed by dehydration through a graded series of ethanol. Subsequently, the specimens were dried with tetramethylsilane. Finally, after sputtercoating with gold, substrates were examined using a JEOL scanning microscope.

Statistical analysis:

For the proliferation data, a two-way analysis of variance (ANOVA), and a multiple comparison test (Student-Newman-Keuls test) were used. For FACS analysis, the nonparametric Mann-Whitney test was used.

Results:

Proliferation:

Figure 1 shows the proliferation curve of the U2OS cells cultured on the various substrates. Adherent cells were removed by treatment with trypsin and counted at day 1, 3, 5, and 8. Complete removal of the cells by trypsinization was deduced from methylene blue staining.
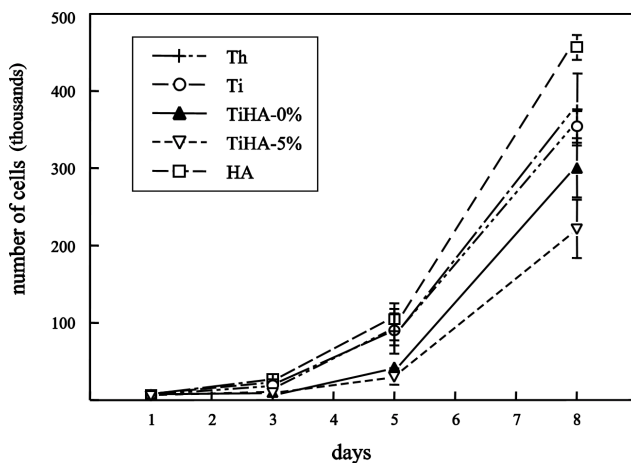

Figure 1: Proliferation curves of osteosarcoma cells (U2OS) cultured on various substrates. Average cell numbers and SD are shown, n=4.

At day 5, cell numbers were significantly lower for both types of CaP coated titanium substrates (TiHA-0% and TiHA-5%) compared with the other substrates (P<0.05). There was no significant difference between HA, Ti and Th substrates.

At day 8, cell numbers for uncoated Ti were similar to standard culture conditions (Thermanox®). In contrast, cell numbers were significantly different (P<0.05) among the various biomaterial substrates, HA > Th= Ti > TiHA-0% > TiHA-5%.

Integrin expression:

First, integrin expression was determined on Thermanox®. FACS analysis showed a high percentage of cells expressing β1, α3, α5, α6 and αv (Figure 2a). High mean fluorescence for β1, moderate levels for α3 and αv, and low levels for α4, α5 and α6 integrin subunits were observed (Figure 2b). No expression was found for β2, β3 and β4 subunits (not shown). The levels of these integrins significantly (P<0.05) decreased with culture time except for β1, α3 and αv.

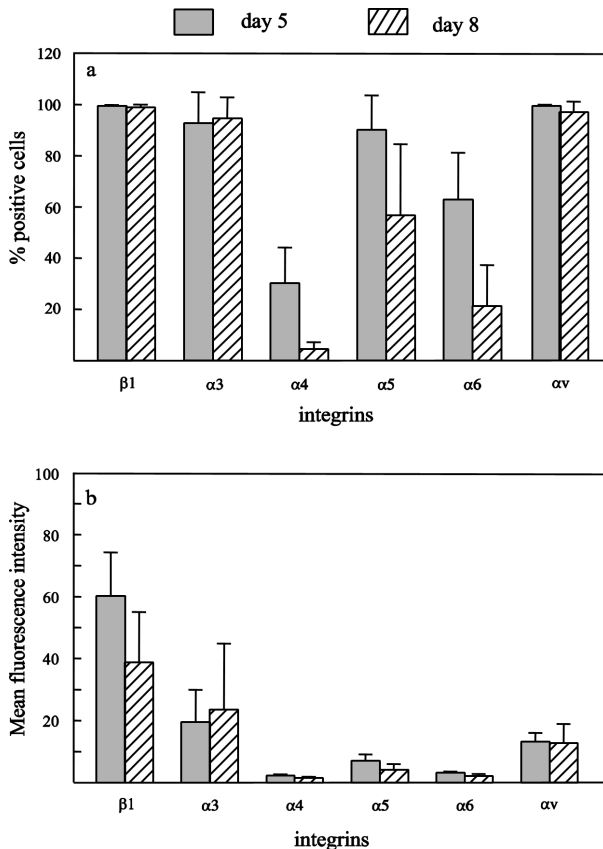**Figure 2:** Quantification of flow cytometric analysis after 5 and 8 days on thermanox. (a) The percentage of cells stained with intensity greater than a threshold set with the control is plotted for each anti-integrin monoclonal antibody. (b) The mean fluorescence intensity of the cells is plotted. Negative controls have been subtracted. Data indicate the mean (+/- SD) of six replicate runs.

In Figures 3 and 4, integrin expression by U2OS cells cultured on the biomaterial substrates are shown. At day 5 (Figure 3a) nearly all the cells were positive for β1, α3, α5, and αv integrin subunits, while 80% of the cells were positive for α6 and 50% for α4 integrin subunits.

The percentage of cells positive for the integrins under study decreased from day 5 to day 8, except for β1, α3, and αv which remained high at around 100% (Figure 4a). Integrin subunit expression of α4 was decreased with culture time on all substrates ($P<0.05$), while the percentage of positive cells for α5 was decreased on Th only ($P<0.05$). The percentage of cells expression α6 was significantly decreased on Th, Ti and HA ($P<0.05$).

At day 5 (Figure 3b), the mean fluorescence for α4 and α6 was extremely low for all substrates. The mean fluorescence from the cells positive for β1 integrin subunit showed significant differences between the various substrates. Statistical analysis showed a higher

Figure 3: Quantification of flow cytometric analysis after 5 days on various substrates. (a) The percentage of cells stained with intensity greater than a threshold set with the control is plotted for each anti-integrin monoclonal antibody. (b) The mean fluorescence intensity of the cells is plotted. Negative controls have been subtracted. Data indicate the mean (+/- SD) of six replicate runs.

expression level of β1 by cells cultured on uncoated Ti and Th compared to TiHA-0% (P<0.05) and Ti compared to TiHA-5% (P<0.05).

Between the coated titanium substrates TiHA-5% induced the highest expression level for β1, α3, and αv, while TiHA-0% induced higher expression levels for α5, although these differences were not significant. Also, for the other integrin subunits no significant differences between the various substrates were found.

The mean fluorescence of cells positive for β1, α4, α5, and α6 decreased from day 5 to day 8. Statistical significant differences were found in a few cases. A significant decrease (P<0.05) was found for the Th and Ti substrates (Figure 4b) with the exception for Th on which the β1 level at day 8 remained at the 5 day level. Furthermore, the expression of the α4 integrin subunit decreased significantly from day 5 to 8, for cells cultured on the coated titanium substrates TiHA-0% and TiHA-5% (P<0.05).
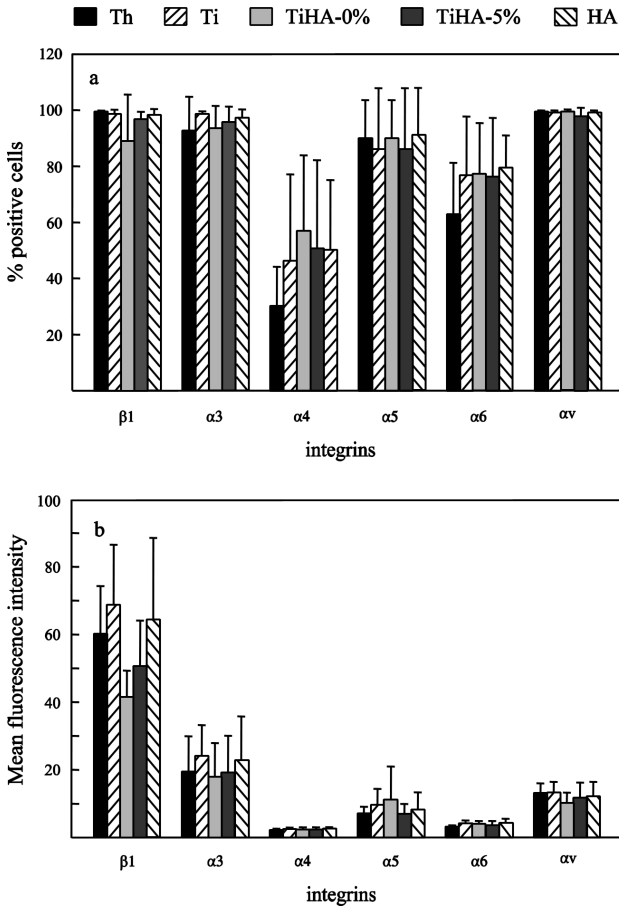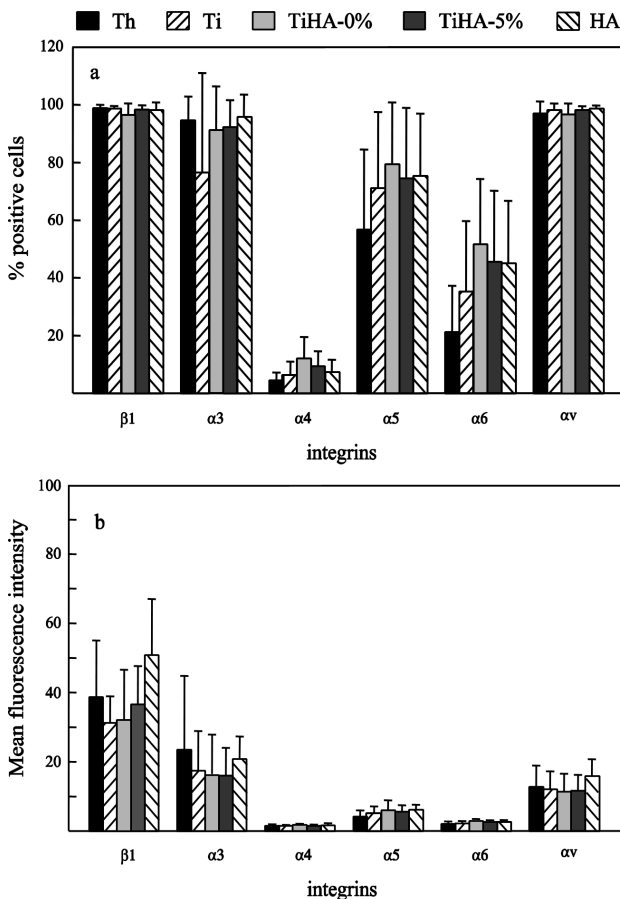

Figure 4: Quantification of flow cytometric analysis after 8 days on various substrates. (a) The percentage of cells stained with intensity greater than a threshold set with the control is plotted for each anti-integrin monoclonal antibody. (b) The mean fluorescence intensity of the cells is plotted. Negative controls have been subtracted. Data indicate the mean (+/-

When comparing the mean fluorescence intensity at day 8 (Figure 4b), only the β1 integrin subunit showed significant differences. HA had a higher β1 integrin expression than Ti (P<0.05), while the coated substrates and Ti exhibited similar expression levels.

Alkaline phosphatase:
There was no alkaline phosphatase activity found on the U2OS cells (data not shown).

SEM:
Scanning electron microscopy showed that all substrates were covered with osteosarcoma cells, U2OS, after 8 days of incubation (Figure 5). Cells growing on the Thermanox® substrates appear to have more pseudopodia compared to the other substrates. A cell sheet was observed on the CaP coated substrates. This cell layer appeared denser on the coated substrates compared to Th, Ti, and HA. Nevertheless, this was not a uniform observation. In general the cells on the coated substrates were lying in clusters. Also spots without any cells were seen. The coating could be seen in those cell-free areas. Cells did not show signs of mineralization**.**

Discussion:

The aim of our study was to investigate whether there were significant differences in the cellular proliferation and expression of integrin receptors as produced by osteosarcoma cells on CaP substrates.

One difficulty in the *in vitro* study of the bone response to biomaterials is the availability of cells. Many investigators use primary osteoblast cultures, either isolated from bone marrow or complete bone. Despite the clear advantage that these cells are extensively characterized for osteoblastic criteria,[32] the disadvantage of such cells is that they show a wide variance in behaviour. Therefore, we decided to use a more standardized osteosarcoma cell line (U2OS) for our initial experiments. Nevertheless, we know that there are well-known differences between cancerous and primary cells regarding kinetics, expression of adhesion molecules and ECM synthesis.[32] For instance, the osteosarcoma cell line U2OS did not show any expression of alkaline phosphatase activity in our experiments. Furthermore, although U2OS cells have previously been shown to produce an osteoblast-like matrix,[33] our SEM observations did not show any mineralization of the matrix. We suggest that this observation is due to the loss of some osteogenic properties by these cells. Also, integrin expression pattern of osteosarcoma cells is clearly different from the pattern of primary bone cells.[15,20] However, Clover et al.[15] demonstrated that osteosarcoma cell lines can still be used to study specific aspects of cell function. Consequently, although part of the osteogenic character of these cells has dissapeared, they still provide a basic model to
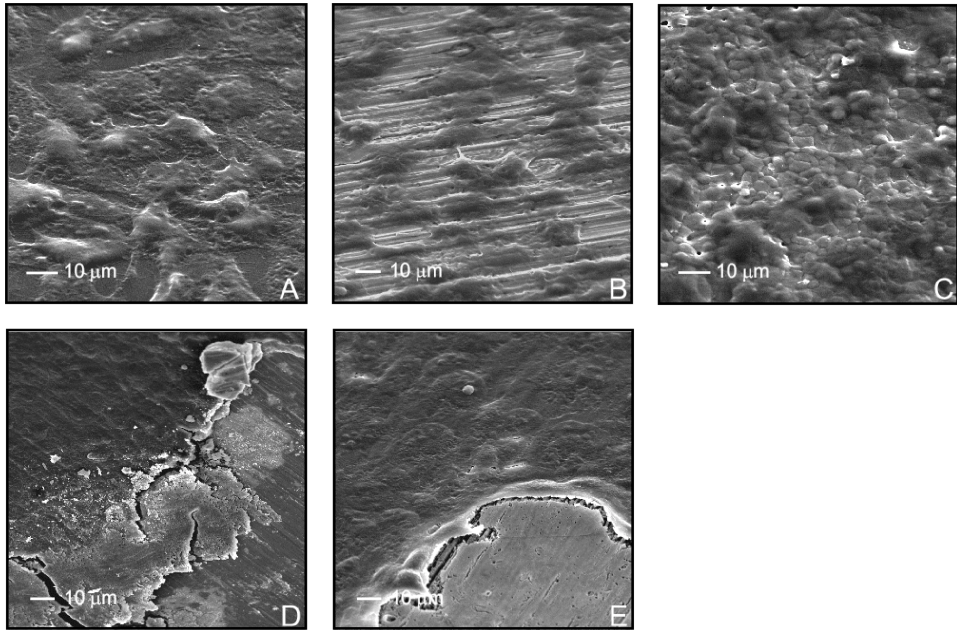
Figure 5: SEM pictures of U2OS cells on different substrates after 8 days of incubation: (a) Thermanox, (b) Titanium, (c) Hydroxyapatite, (d) TiHA-0% coating, (e) TiHA-5%

analyse modulation of integrin expression induced by a substrate environment.

Our results show significant differences in proliferation between the various substrates at day 5 and 8. An explanation for this phenomenon is that cellular proliferation on the amorphous CaP coatings is affected by dissolution of the coating. This suggestion is supported by ongoing SEM studies in our laboratory, in which we observed that the coating had partially disappeared at some sites.

To determine whether substrate-induced differences in proliferation and morphology can be related to differential integrin expression, we performed flow cytometric analysis. This analysis showed that the U2OS cells were characterized by high expression levels of $\beta 1$, moderate expression levels of $\alpha 3$, αv, and low levels for $\alpha 4$, $\alpha 5$, and $\alpha 6$. Significant substrate-induced differences for mean fluorescence were found for the expression of the $\beta 1$ integrin both at day 5 and day 8. Our data confirm that $\beta 1$ plays an important role in tissue organization and remodelling in bone.[5] In view of this we have to emphasize that the dense HA substrates revealed significantly higher $\beta 1$ expression at 8 days than all other substrates. This finding confirms again the beneficial biological properties of calcium phosphate ceramics. On the other hand, both sputter coatings did not show a similar response. We suggest that this again is due to an early dissolution of these amorphous coatings.

Our results on integrin expression agree with those of several other investigators.[11,12] We have to emphasise that they used non-quantitative methods and other cell types (Saos-2 and human osteoblasts) which still possess the capacity to express the osteoblast phenotype and to mineralize in vitro. These properties have been lost in the U2OS cell line. Alterations in phenotype are associated with changes in integrin expression. This can explain our limited results, but also why we found low mean fluorescence in general. Limited differences in expression were found underlying the importance of the functional properties of integrin molecules. The differences found for the different substrates could therefore reside in the functional properties of the expressed integrins. For example, integrin function is critically dependent on bivalent cation concentration.[8,9,10,34,35] Therefore, calcium ions, as released from a CaP surface, may directly alter the ligand binding affinity of the receptor, thereby regulating cellular responses including proliferation, differentiation, cytokine production and matrix modelling.

Finally, since the initial events in cell adhesion are very relevant for the proliferation and differentiation from cells,[36,37] we recommend to determine integrin expression at early time-points.

On basis of our results, we conclude that there are indeed indications that CaP ceramics can affect the integrin expression of osteosarcoma cells. Still, more research has to be performed with other bone cell types and at shorter incubation periods. Consequently, the current study can be considered as a first step in a series of studies in which we intend to elucidate the bone forming mechanism of CaP ceramics.

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Chapter 7

Modulation of integrin expression on rat bone marrow cells by substrates with different surface characteristics.

P.J. ter Brugge, R. Torensma, J.E. de Ruijter,
C.G. Figdor, J.A. Jansen

Introduction:

Previous research demonstrated that attachment of undifferentiated cells to an implant surface is mediated via an adsorbed intervening protein layer.[1] Immersion of a material in a solution containing proteins leads to adsorption of proteins to the material.[2,3] The presence of specific proteins and confirmation of these proteins play an important role in final cell spreading, proliferation and differentiation. In this process, transmembrane cell signaling receptors, like integrins, are involved. Integrins consist of two subunits, i.e. α and β. The extracellular domain of both subunits forms a ligand-binding site, whereas the intracellular domain of the β subunit has regions that form signalling complexes with proteins.[4] Currently, more than 20 different integrin heterodimers are known to exist.[1] Interaction of integrins with adsorbed substrate proteins can generate intracellular signals.[5-8] These signals are essential in the regulation of growth and differentiation of cells.[6,9]

Integrin expression has already been studied for various tissues and cell types, including bone and bone cells.[10-19] All these studies showed that osteogenic cells can express a large number of integrins.

Since integrins can regulate cellular behavior by transmitting signals across the cell membrane, changes in integrin expression modify the signals sent into the cell, resulting in changes in cell phenotype. This opens another way to evaluate the relationship between implant surface properties and cellular response. For example, we observed that rat bone marrow cells (RBM) show increased mineralization and osteocalcin expression when cultured on calcium phosphate ceramic (CaP) compared to smooth and rough commercially pure titanium.[20,21] Until now, we still do not know what specific surface property causes this difference in cellular reaction. Therefore, the purpose of this study was to determine whether differences in rat bone marrow cell response on smooth, rough, and calcium phosphate coated titanium substrates could be related to differences in integrin expression.

Materials and Methods:

Substrates:

Commercially pure titanium (cpTi) disks (diameter 25 mm) were used. Disks were used as machined (Ti-s) or subjected to an Al_2O_3 gritblasting procedure. Gritblasted substrates were left uncoated (Ti-r) or were provided with a 2 µm thick RF-magnetron sputtered calcium phosphate coating.[22] After deposition, coatings were subjected to an additional infrared heat treatment at 700°C in an infrared furnace (E4-10-P, Research Inc.) (CaP-ht). The apatite-like crystalline structure of the coatings was confirmed by X-ray diffraction. Ca/P ratios of the coating as measured with energy dispersive spectroscopy were between 1.77 and 1.83.

Surface roughness of the substrates was measured using Atomic Force Microscopy (Digital Instruments, USA). Per substrate, 3 areas of 50x50 µm were scanned. Scans were analyzed

using the Nanoprobe program. In each measured area, 4 sections were taken. The sections were used to measure the height variation (Ra) and spacing of surface peaks (Sm). The results of the roughness measurements are shown in Table 1.

Disks were autoclaved for 15 minutes at 120°C before use in cell culture.

	Ra-value (µm)	Sm-value (µm)
Ti-s	0.60 ± 0.26	5.78 ± 1.94
Ti-r	1.64 ± 0.59	4.30 ± 1.11
CaP-ht	1.62 ± 0.67	4.17 ± 1.64

Table 1: Surface roughness of Ti-s, Ti-r and CaP-ht. Ra value indicates mean height variation; Sm value indicates mean spacing of surface peaks.

Cell culture:

Rat bone marrow (RBM) cells were isolated and cultured using the method described by Maniatopoulos.[23] After 7 days of primary culture in α-MEM supplemented with 10% FCS, 50 µg/ml ascorbic acid, 50 µg/ml gentamycin, 10 mM Na ß-glycerophosphate and 10^{-8} M dexamethasone, cells were removed from the culture flasks by trypsinization. Substrates were placed at the bottom of 6 well-plates and 1 ml cell suspension ($1x10^5$ cells/ml) was added to the substrates. To determine the integrin expression pattern by primary RBM cells, the remainder of the cell suspension was used for FACS analysis. These samples are referred to as day 0.

Cells were cultured on the substrates for 8 or 16 days under similar conditions as primary cells, removed from the substrates by trypsinization (0.25% trypsin, 0.01% EDTA, 10 minutes) and integrin expression was studied by FACS analysis.

To examine whether all the cells were removed, substrates were studied by SEM after trypsinization.

FACS analysis:

Antibodies for FACS analysis were purchased from Pharmingen (α1, α2, α4, α5, β1, β3: goat anti hamster IgM; goat anti-mouse IgG), Serotec (α6), Bender Medsystems, (α3), Sigma (goat anti-rabbit) and Jackson (goat anti-hamster IgG).

After removal from the substrates, cells were suspended in PBS containing 1% BSA (PBA) and counted. Cells were plated in a 96-wells plate in a concentration of $1x10^5$ cells per well. The plate was centrifuged for 1 min. at 1400 rpm at 4°C. Cells were washed with 100 µl PBA and incubated with 25 µl/well of the anti-integrin antibody in PBA for 30 min. at 4°C. After washing with 100 µl PBA cells were incubated with an appropriate FITC labeled

secondary Ab for 30 min. at 4°C. After washing cells were resuspended in 100 μl PBA and transferred to FACS tubes. Analysis was performed on a FACscan flow cytometer (Becton Dickinson).

For α3 staining, this protocol was modified, since the antibody recognizes the cytoplasmic domain of the subunit. Cells were permeabilized before addition of the primary antibody. 50% Ethanol (1ml FCS, 1ml α-MEM, 6ml 70% ethanol) was added to the cells and cells were incubated on ice for 30 min. Cells were washed with PBA and stained for FACS according to the protocol mentioned above.

A total of eight replicate runs were performed to study integrin expression in primary RBM cells. In three of these runs, α3 expression was studied. Expression of α1, α2, α4, α5, α6, β1 and β3 was studied in all runs.

To study integrin expression on the titanium and CaP-coated substrates, ten replicate runs were performed. A scheme of the runs, with the studied subunits and times is provided in Table 2.

Table 2: Integrin subunits studied on RBM cells cultured on the test substrates in the different runs.

no. of runs	day 8								day 16							
	α1	α2	α3	α4	α5	α6	β1	β3	α1	α2	α3	α4	α5	α6	β1	β3
1	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-
3	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-
3	-	-	+	-	+	+	+	-	-	-	+	-	+	+	+	-

+ indicates this subunit was studied, - indicates the subunit was not studied in this run.

Scanning electron microscopy:

After culture for 8 or 16 days on coated and non-coated substrates, cell layers were studied with SEM and EDS. Substrates were also prepared for SEM after trypsin treatment, to study the removal of the cells.

Samples were washed in PBS and fixed in 2% glutaraldehyde, washed twice in 0.1M sodium-cacodylate buffer (pH 7.4) and dehydrated using a graded series of ethanol. After drying with tetramethylsilane, samples were sputter-coated with carbon and photographed

using a Jeol 6310 SEM with and acceleration voltage of 15 kV.

Statistical analysis:

Statistical analysis was performed using Statmost32 (DataMost Corporation). The Kruskall-Wallis test was used to calculate significant differences in integrin expression between the studied materials.

Results:

Integrin expression in RBM cultured on tissue culture polystyrene:

In the series of experiments to study integrin expression by primary RBM cells (day 0), a large variation in expression pattern was found between the different runs. Examples of four independent runs (a, b, c and d) are plotted in Figure 1. In two runs, fluorescence was low for all studied subunits. No expression of α4 was found, or fluorescence was extremely low. Fluorescent staining for α2 and β3 was found in only one run and staining was absent in the other runs. Fluorescence for α1 was low in four of the runs, intermediate in the remaining runs. Fluorescence for α5 was low in three runs, intermediate to high in five runs. The cells expressed α6 and β1 in all runs. Expression of α3 was found in three out of three runs studied.
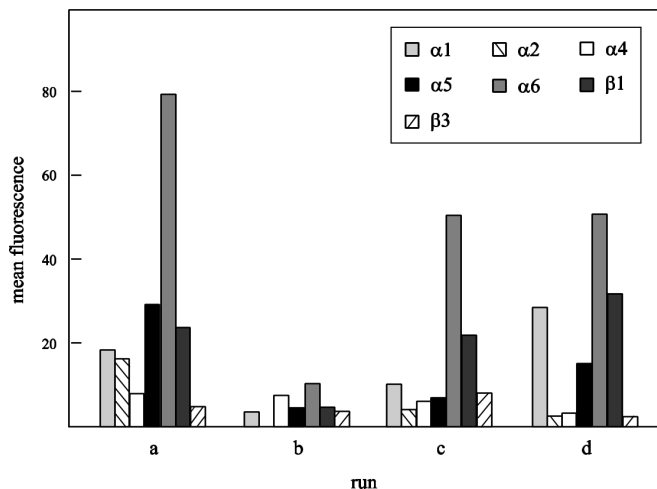

Figure 1: Integrin expression by primary RBM cells cultured for 7 days on tissue culture plastic. Fluorescent intensity measured in 4 (a-d) of the 8 performed runs is plotted.

Integrin expression in RBM cells cultured on titanium and CaP-ht:

In the series of experiments to study integrin expression of RBM cells cultured on Ti-s, Ti-r and CaP-ht substrates, no expression of $\alpha2$ and $\alpha4$ was found both after 8 and 16 days of culture. Expression of $\alpha1$ and $\beta3$ was found in half of the runs performed (data not shown). However, the measured fluorescence was low and no major difference in expression on the tested materials was found.

Expression of $\alpha5$, $\alpha6$, $\alpha3$ and $\beta1$ was found in all the runs performed. Fluorescence measured for $\alpha3$ and $\alpha5$ was low in some of the runs. Fluorescence measured for $\alpha6$ was high in most runs. In general, fluorescence measured was lower on day 16 than on day 8.

We used the mean of the fluorescent measured for statistical analysis of differences between expression on the different materials. For convenience of reference, we included the data of all runs in the form of a schematic representation of the integrin expression patterns on the different materials in the separate runs, after 8 and 16 days (Table 3). Substrates were assigned a rank, depending on the fluorescent intensity that was measured using FACS analysis, with +++ indicating highest fluorescent intensity.

After 8 days of incubation, no significant difference was found in mean fluorescence for $\alpha3$ between the 3 materials, even though highest fluorescence was found on CaP-ht in 4 out of 6 performed runs. On the other hand, in the other two runs, fluorescence was highest on Ti-s and lowest on CaP-ht. Similar results were found after 16 days of culture.

Significant differences were found in $\alpha5$ expression between the three tested materials (CaP-ht > Ti-r > Ti-s; $P<0.05$). In seven of the performed runs, expression of $\alpha5$ after 8 days was highest on CaP-ht. In most runs fluorescence for $\alpha5$ was higher on day 8 than on day 16. An example of such a run is shown in Figure 2a. In one run, fluorescence was low on day 8 and increased by day 16, with highest expression on CaP-ht again (Figure 2b).
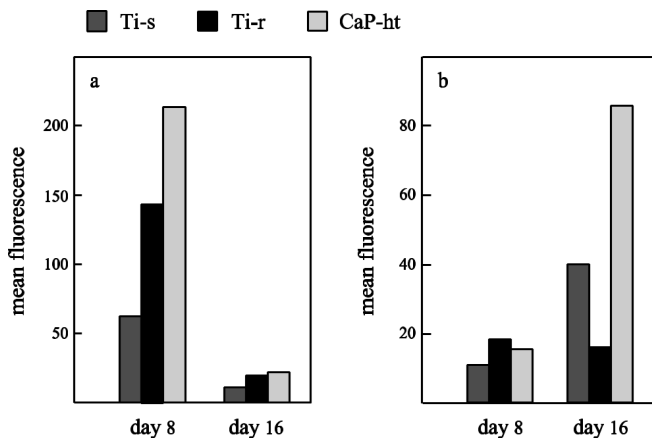

Figure 2: Expression of the $\alpha5$ subunit on Ti-r, Ti-s and CaP-ht after 8 and 16 days of culture. Fluorescent intensity measured in two different runs (a and b) is plotted.

	run	day 8			day 16		
		Ti-s	Ti-r	CaP-ht	Ti-s	Ti-r	CaP-ht
α3	1	++	+	+++	nd	nd	nd
	2	+	++	+++	nd	nd	nd
	3	+	++	+++	+++	+	++
	4	+++	++	+	++	+	+++
	5	+++	++	+	++	+	+++
	6	+	++	+++	+	+++	++
α5	1	++	+	+++	nd	nd	nd
	2	+	+++	++	nd	nd	nd
	3	+	++	+++	nd	nd	nd
	4	+	++	+++	+	++	+++
	5	+	+++	++	++	+	+++
	6	+	++	+++	+	++	+++
	7	+	++	+++	++	+	+++
	8	+	++	+++	+	++	+++
	9	+	++	+++	+	++	+++
	10	+	+++	++	+	+++	++
α6	1	+	++	+++	nd	nd	nd
	2	+++	++	+	nd	nd	nd
	3	+	++	+++	nd	nd	nd
	4	+	++	+++	+	+++	++
	5	++	+++	+	+++	++	+
	6	++	+++	+	+	++	+++
	7	++	+++	+	+	++	+++
	8	+	++	+++	+	++	+++
	9	+	++	+++	+	++	+++
	10	++	+++	+	+	+++	++
β1	1	+	++	+++	nd	nd	nd
	2	+++	+	++	nd	nd	nd
	3	++	+	+++	nd	nd	nd
	4	+	++	+++	+	++	+++
	5	++	+++	+	++	+	+++
	6	++	+++	+	++	+	+++
	7	++	+	+++	++	+	+++
	8	+	++	+++	+	++	+++
	9	++	+	+++	+	++	+++
	10	++	+++	+	+	++	+++

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Table 3: Schematic representation of α3, α5, α6 and β1 expression after culture for 8 or 16 days on Ti-s, Ti-r and CaP-ht. + indicates lowest expression, +++ indicates highest expression of a subunit on a material in a run. nd = not done.

After 16 days, mean α5 expression was significantly higher on CaP-ht than on Ti-s and Ti-r, with no differences between the titanium substrates (P<0.05).

No significant differences in α6 expression between Ti-r and CaP-ht were found after 8 and 16 days of culture. On the other hand, Ti-s showed significantly lower α6 expression than Ti-r after 8 days (P<0.05). Fluorescent intensity decreased between day 8 and 16, although in some runs only slightly. In four of the measurements performed on day 16, highest α6 expression was found on CaP-ht. Expression of α6 on Ti-s was significantly lower than on CaP-ht after 16 days of culture (P<0.05).

In six out of ten measurements on day 8, highest fluorescence for β1 was found on CaP-ht, in three runs highest fluorescence was found on Ti-r, in one run on Ti-s. Nevertheless, mean β1 expression on the three materials showed no significant differences. Similar to α5, the measured fluorescence decreased in most runs between day 8 and 16. Only occasionally, an increase was found. For day 16, highest mean expression of β1 was found on CaP-ht (P<0.05), with no difference between Ti-s and Ti-r.

Overall, fluorescence for α3, α5, α6 and β1 was highest on CaP-ht in many runs.

In some runs, all the subunits were upregulated on a specific material. An example of such a run can be found in Figure 3a. However, in other runs, we found no relation in upregulation of the subunits on one specific material, as can be seen in Figure 3b. Also, no relation could be found between the expression patterns of the primary cells and of the cells passaged onto the coated and non-coated substrates.
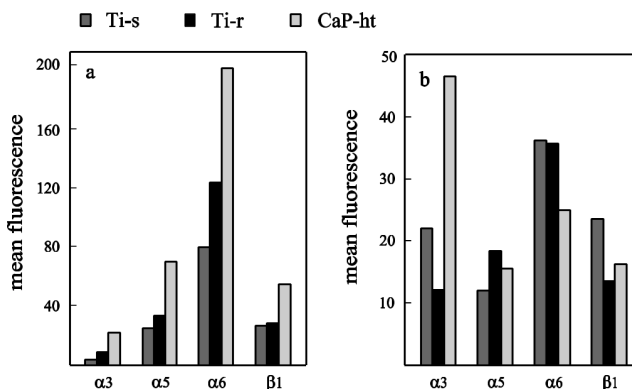

Figure 3: Expression of α3, α5, α6 and β1 after 8 days of culture on Ti-s, Ti-r and CaP-ht. Fluorescent intensity measured in two different runs (a and b) is plotted.

SEM:

SEM evaluation revealed that after 8 days of culture, all substrates were covered with multilayers of cells. By day 16, these layers contained collagen fibers and associated globules. EDS analysis indicated that these globules consisted of calcium and phosphate.

After trypsinization, most of the cell layer of the 8-day cultures was removed. Only occasionally, small areas with cells were detected (not shown). In contrast, on day-16 samples large areas of the substrate remained covered with a layer of collagen fibers. In some instances, partially detached, rounded cells could be seen lying on this layer (Figure 4).
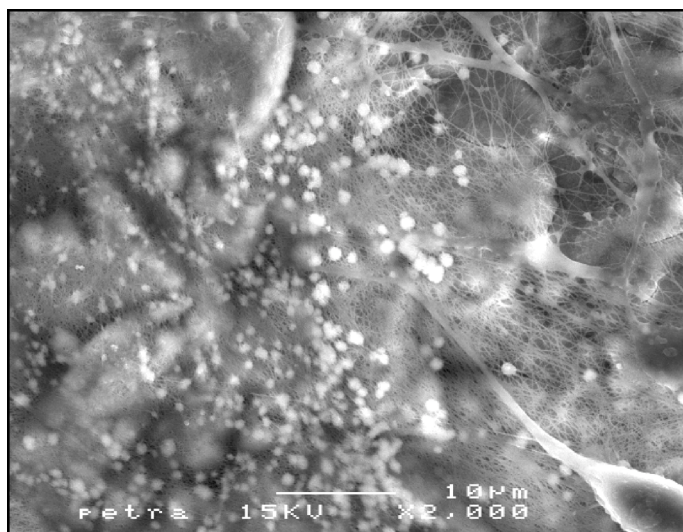
Figure 4: RBM cell culture, after 16 days on CaP-ht, after treatment with trypsin. The matrix shows many collagen fibers and associated mineralized globules. In the bottom right hand corner, a rounded cell can be seen.

Discussion:

Our analysis of the expression pattern of primary RBM cells on tissue culture polystyrene revealed that the cells always expressed the $\alpha3$, $\alpha6$ and $\beta1$ subunits. On the other hand, $\alpha1$, $\alpha2$, $\alpha5$, and $\beta3$ were expressed only in some experimental runs. The subunits $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$ and $\alpha6$ all combine with $\beta1$, forming different integrins that recognize collagens, laminins and fibronectin. The $\beta3$ subunit, associated with αv is a receptor for several ECM proteins, including osteopontin and vitronectin.[4,9] These results demonstrate that primary RBM cells are capable of adhering to a wide variety of ECM proteins. Nevertheless, we have to notice that the combination of expressed subunits as well as the overall

fluorescence varied greatly between the performed runs. Donor variation in integrin expression on bone marrow derived cells is a phenomenon that has been described before.[24] We suppose that in our study this variability is due to the differentiation phase of the cells from the different isolates that were used for the different runs. Cells from one individual isolate may reach a certain stage faster than the next individual isolate. Marrow derived cells from different donors have been shown to differ in growth and differentiation potential in several species.[25,26]

Our suggestion is further supported by the findings in various other studies of integrin expression in osteoblast cell cultures. For example, in rat osteogenic cultures, $\alpha5\beta1$, $\alpha3\beta1$ and $\alpha8\beta1$ were found to be expressed in both early and late culture. However, the expression of $\alpha5\beta1$ remained constant all through the culture period, while expression of $\alpha3\beta1$ and $\alpha8\beta1$ was downregulated in more differentiated cultures.[27] Further, in bovine osteoblast culture, $\beta3$ was only expressed during very early culture periods.[28] A similar finding was done for mRNA expression for $\beta1$ in rat osteoblasts cultured on collagen.[29] In human osteoblasts, integrin expression may be modulated during differentiation.[30]

We also observed a large variation in the expression patterns of integrin subunits on the CaP-coated and non-coated titanium substrates. Therefore, we included the results of all the separate experiments, in Table 3. Since we only measured integrin expression at 8 and 16 days of incubation, we cannot exclude that transient up- or downregulation of expression takes place at earlier or intervening culture times. This can only be proven by additional studies.

We see a downregulation of the integrin expression with longer culture periods. This could be due to the addition of dexamethasone to the cultures, since glucocorticoids downregulate integrin expression on osteogenic cells after prolonged culture.[31,32]. It is also possible that this decreased fluorescence after 16 days is associated with incomplete removal of the cells from the substrates, resulting in selection of a population of cells with low overall fluorescence. It is unlikely that differences in integrin expression are due to the method used to remove the cells, by using trypsin. Trypsin used in concentrations similar to the one we used has been shown not to affect expression of integrins.[33,34] Also, all samples were subjected to the same trypsin treatment, which further indicates that differences in expression are not due to release of cells by the use of trypsin.

At this point, a remark has to be made about the suitability of FACS analysis for these studies. We decided to use this method as it allows quantification of integrin expression. This is in contrast to other methods, which only provide information about presence or absence of integrin subunits. Nevertheless, the FACS method is associated with two serious problems for our kind of research. Firstly, FACS analysis requires removal of the cells by a technique that leaves the cells intact, such as the use of trypsin. However, after 16 days of culture the ECM, as deposited by the RBM cells, showed extensive calcification. As a

consequence, trypsin could not remove the entire cell layer. Therefore, it is possible that the released cells may not represent the entire cell population as present, but form a specific subpopulation. In view of this, care has to be taken in the interpretation of the integrin measurements after 16 days of culture. We recommend focusing mainly on the results obtained after 8 days of culture.

Secondly, FACS analysis requires a relatively large number of cells, especially when several antigens are studied. In our experiments, we cultured cells on several substrates and pooled the cells before staining to obtain a sufficient amount of cells for analysis. Measuring at additional incubation times will result in problems in preparing sufficient substrates for the experiments. This also means that changes in integrin expression cannot be correlated to changes in cell growth or differentiation in the same cell population, since additional measurements would require even more substrates.

On basis of our results, we conclude that substrate surface composition and microgeometry modulate integrin expression of RBM cells. Expression of α5, α6 and β1 is often higher on CaP-ht than on titanium. Comparison of the results of the smooth and rough titanium substrates shows that expression of α5 and α6 is upregulated on rough titanium compared to smooth titanium after 8 days.

The α5β1 and α6β1 integrins are receptors for fibronectin and laminin respectively. The α3β1 receptor recognizes laminin, collagen and fibronectin.[4,9] The observation that these receptors were expressed in most runs could indicate that the cells always express laminin and fibronectin. Both matrix proteins are essential in regulation of osteogenesis. Laminins are involved in the attachment of calvaria-derived osteogenic cells[35] and in the differentiation and survival of the cells. Disruption of endogenous laminin results in decreased mineralization of the matrix.[36,37] Interactions between integrins and fibronectin are required for the differentiation and survival of osteogenic cells. Interfering with these interactions suppresses osteoblast gene expression.[27,38,39]

Although our results appear to indicate that integrin expression is affected by the substrate surface properties, we have to emphasize that the cells can regulate adhesion and signaling through integrins in a way other than up- or downregulation of receptors. Affinity of the receptor and intracellular signaling events may be modulated by intracellular proteins[40,41] or cations.[42-44] The CaP coated substrates may then directly influence signaling by the release of Ca-ions and differences in expression could be of secondary importance.

We have to stress that differences in integrin expression at earlier times may be even more important than the differences at late incubation periods as used in the current study. Several biomaterials have already been shown to be capable of regulating focal contact formation and integrin expression in attaching osteogenic cells.[45-49] The signals generated during initial attachment may then determine subsequent phenotype of the cells. Consequently, the next step in our studies will be the study of initial attachment and

integrin expression of RBM cells on the same materials as used in the present study.

In conclusion, RBM cells modulate the level of integrin expression depending on the substrate characteristics. Expression of integrins that act as receptors for laminin, collagen and fibronectin is often higher on CaP-ht than on titanium substrates. Since these matrix proteins are involved in regulation of osteoblast differentiation, this could explain the positive effect on matrix mineralization frequently associated with CaP-coated implants.

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Chapter 8

Summary, address to the aims, conclusions.

Summary, address to the aims and closing remarks.

In order to be able to design oral and medical implants that elicit a specific tissue response, extensive knowledge of the interaction of an implant material with its surrounding tissue is essential. The surface characteristics of the material, as well as the cellular characteristics of the tissue govern the precise interaction of a material with the tissue. Although many implant materials show a favorable bone response, the mechanisms involved in this bone response are still poorly understood. The studies described in this thesis are aimed at elucidating the mechanisms by which RF magnetron sputtered calcium phosphate (CaP) coatings and implant surface roughness affect the bone response. In the first chapter, an introduction into the formation and differentiation of bone cells and the effects of different material surface characteristics on bone cell reactions are presented. In the subsequent chapters, the questions asked in the scope of the study are addressed.

What is the effect of dexamethasone and serial subculture on the expression of osteogenic markers by rat bone marrow (RBM) cells.

In chapter 2, the possibility of serial subculture, and the effect of the differentiation inducing agent dexamethasone on RBM cells were investigated. RBM cells were isolated and cultured in the absence or presence of dexamethasone. Cells were subcultured every 7 days and tested for the expression of alkaline phosphatase and the formation of mineralized matrix, markers of osteogenic differentiation. RBM cells continuously cultured in the absence of dexamethasone showed no expression of osteogenic markers at all. Cells cultured in the continuous presence of dexamethasone showed high levels of alkaline phosphatase expression and mineralized matrix formation after the first passages. However, the expression of markers for osteogenic differentiation decreased with increasing number of passages. Cells that were cultured in the absence of dexamethasone in primary culture, than subcultured with dexamethasone also showed alkaline phosphatase expression and mineralization. Osteogenic expression also decreased in these cells after a larger number of passages. Further, the alkaline phosphatase expression and mineralization remained lower than that of cells cultured with dexamethasone in each passage.

From this we conclude that RBM cells retain part of their osteogenic potential when subcultured in the absence of dexamethasone. However, the osteogenic potential is decreased compared to cells subcultured in the presence of dexamethasone. Also, RBM cells loose their osteogenic potential with increased subculturing, indicating that in studies on the osteogenic differentiation of RBM cells, extensive subculturing should be avoided.

What is the effect of the crystallinity of RF magnetron sputtered coatings and substrate surface roughness on the proliferation and expression of osteogenic markers by RBM cells.

Chapter 3 described the proliferation and differentiation of RBM cells on materials with different surface characteristics. Rough titanium substrates were compared with smooth titanium substrates and with rough substrates provided with a CaP coating. The CaP coated substrates were left as sputtered or were subjected to heat treatments to generate coatings with different crystallinities, from amorphous to crystalline. RBM cells were cultured on the substrates for up to 16 days and examined for the expression of proliferation and differentiation makers. Coated and uncoated substrates without cells were also incubated in culture medium, in order to study the effect of material characteristics on dissolution/precipitation phenomena. Amorphous CaP coated substrates showed extensive dissolution and precipitation, whereas only limited dissolution was found for the crystalline coating. Some CaP precipitation was found on the crystalline coatings and also on the titanium substrates. However, this precipitate was not stable and disappeared after longer culture periods. Cells on the crystalline coating showed the highest expression of differentiation markers, followed by the titanium substrates. In contrast, no proliferation and differentiation of RBM cells was seen on the amorphous coatings. This was probably due to the high level of dissolution and precipitation. These studies show that rough titanium substrates provided with a crystalline CaP coating induce more osteogenic differentiation of RBM cells than substrates without coating. Further, the high level of dissolution of the amorphous coatings inhibits RBM cell proliferation and differentiation.

What is the effect of surface roughness and presence of a CaP coating on the attachment, spreading and integrin expression of human osteosarcoma (U2OS) and RBM cells during their initial interaction with the material.

In a study described in chapter 4, U2OS cells were used to study initial cellular interaction with different materials. Smooth and rough titanium substrates were used, as well as rough titanium substrates provided with a crystalline CaP coating. U2OS cells were used since they show limited variability, in contrast to RBM cells. The cells showed lower initial attachment on the smooth titanium substrates than on the rough titanium substrates. U2OS cells expressed the $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, αv and $\beta 1$ integrin subunits. After 3 hours, the expression of $\beta 1$ was higher on the CaP coated substrate than on the other substrates. Further, at 24 hours, the expression of $\alpha 2$, $\alpha 5$, $\alpha 6$ and αv subunits was upregulated compared to the expression of cells that were originally seeded on the coated substrates.

Cells spread to a different size on the various materials, with smooth titanium > rough titanium > CaP coated titanium. The cells on the smooth material showed alignment with the machining grooves. The cells on the rough material conformed to the roughness of the material. Based on our results we concluded that surface roughness is capable of regulation U2OS attachment and cell spreading. The presence of a CaP coating can modulate the integrin expression and also affect cell spreading. The latter phenomenon cannot completely be explained by the presence of surface roughness, since the rough titanium and the CaP coated substrates show similar roughness, but differ in cell size.

In a similar study the initial interactions of RBM cells with different substrate surfaces were studied (chapter 5). Again we used smooth and rough titanium substrates and rough CaP coated substrates. RBM cells were seeded onto the substrates. Subsequently attachment, integrin expression and spreading were studied. Most cells attached to the substrates within two hours. No differences were seen in the attachment rate of the RBM cells to the different materials. A large variability was seen in the expression of integrins in different experimental runs. However, no differences in integrin expression were seen between the various materials. Cells on the smooth titanium spread out to a larger size than cells on the rough materials. On the rough materials, cells showed many long cellular extensions, whereas cells on the smooth titanium only had some short cellular processes. In conclusion, RBM cell attachment and integrin expression during attachment appeared to be insensitive to the different substrate characteristics of the materials as used. In contrast, the material characteristics induce differences in spreading behavior between the smooth and rough materials.

What is the effect of surface roughness and the presence of a CaP coating on the expression pattern of integrins during prolonged culturing of RBM cells and U2OS cells.

In chapter 6, the effect of surface characteristics on the integrin expression of U2OS cells during culture was investigated. Therefore, titanium substrates were left uncoated or were provided with CaP coatings sputtered in the presence or absence of oxygen. Dense hydroxyapatite disks were also used. Integrin expression was studied by FACS analysis. Besides integrin expression, U2OS proliferation was also studied. After 5 days of incubation, no difference was seen in proliferation. At day 8, significant differences were seen in proliferation, with the highest cell number found on hydroxyapatite.
The cells expressed $\alpha3$, $\alpha5$, αv and $\beta1$ subunits, whereas the expression of $\alpha4$ and $\alpha6$ was low or not detectable on all substrates. At day 5, expression of $\beta1$ was significantly higher on the uncoated titanium substrates than on the coated substrates. No other differences in

expression were found at day 5. Integrin expression decreased from day 5 to day 8. At day 8, the cells on hydroxyapatite expressed higher levels of $\beta1$ than the cells on uncoated titanium.

In conclusion, substrates with different surface characteristics affect the response of U2OS cells. Both proliferation and integrin expression are affected, although the effect on integrin expression is limited.

The effect of substrate surface characteristics on the integrin expression during proliferation and differentiation of RBM cells was studied (chapter 7). Similar substrates were used as in the studies described in chapter 4 and 5. Cells were cultured for one week of primary culture, then subcultured on the substrates for 8 or 16 days. Integrin expression was studied by FACS analysis. RBM cells always expressed the $\alpha3$, $\alpha5$, $\alpha6$ and $\beta1$ subunit. Expression of $\alpha1$, $\alpha2$ and $\beta3$ was also found occasionally. A large variability was found in the expression patterns between individual runs, both in primary cultures and in culture on the substrates. After 8 days of culture on the substrates, expression of $\alpha5$, $\alpha6$ and $\beta1$ was significantly higher on the CaP coated substrate than on the titanium substrates. No difference was seen in $\alpha3$ expression. A problem was encountered in the measurements after 16 days of culture, since the cells were difficult to remove from the substrates. It is possible that the cells released from the substrates at day 16 consist of a cell population with different integrin expression. We therefore recommend focusing on the results from day 8. These results demonstrate that the substrates are capable of influencing integrin expression by RBM cells during prolonged culture.

Concluding remarks, future perspectives:

In a series of investigations the effect of substrate surface characteristics on in vitro osteoblast response was studied.

The results of the studies showed a clear effect of the presence of crystalline RF magnetron sputtered CaP coatings on the response of osteoblastic cells. The coating increased the expression of osteogenic markers compared to that of cells cultured on either smooth or rough titanium. In contrast, the amorphous coatings showed a negative effect on osteogenic cells. This was most likely due to their high dissolution rate. On the other hand many other studies show a positive effect of amorphous coatings on osteogenic cells, while crystalline coatings do not increase osteogenic differentiation. However, these studies generally use plasma-sprayed coatings, which have a completely different dissolution behavior compared with the coatings we used. Therefore we have to emphasize that complete characterization in terms of chemical composition, crystallinity, but also method of manufacturing is very important when developing and reporting about CaP coated implants.

Further, we observed that surface roughness only had a limited effect on the response of osteogenic cells. This is in contrast to many other studies. Although a definite explanation for this discrepancy is difficult to give, we know that surface roughness measurements are very complex. The different methods used in the various studies can result in different data. This hampers a correct comparison of the obtained results. To overcome this problem, a standardized method for measuring and describing surface roughness will have to be developed.

Beside the effect of surface characteristics on general cellular response, we also found that they can influence the expression of integrins, even though the observed differences were limited. These differences in integrin expression can be related to differences in osteogenic expression. Still, a final conclusion cannot be made. Although U2OS cells make a good model for integrin expression studies, these cells do not differentiate. Therefore a relation with expression of osteogenic markers cannot be established. On the other hand, the RBM cells used do show differentiation. However, they display a wide variation in integrin expression pattern. Consequently, we cannot confirm that modulation of integrin expression is the major mode by which various materials affect bone response. For example, besides integrin expression, integrin function may also be modulated. Consequently, we recommend that subsequent studies focus on this aspect of the interaction of cells with their environment. Studies on the effect of Ca^{2+} on integrin function on osteogenic cells or on intracellular signaling as a result of interaction with various materials will help to understand cellular responses to specific materials. Additional methods to further improve our understanding in the relations of cells with materials could be the coating of materials with ECM proteins, or the blocking of interactions of integrins with ECM proteins and the effect on the cell response.

Eventually, all these studies have to lead to more insight into the bone-biomaterial response and will enable the manufacturing of tailor-made implants that generate a predicted tissue response.

Samenvatting, evaluatie van de doelstellingen en afsluitende opmerkingen.

Om in staat te zijn orale en medische implantaten te ontwerpen die een specifieke weefselreactie oproepen, is uitgebreide kennis van de interactie van geïmplanteerde materialen met omliggende weefsels van groot belang. Zowel de oppervlakte karakteristieken van het materiaal, als de karakteristieken van de weefselcellen beïnvloeden deze interactie. Hoewel veel implantatie materialen een goede botreactie laten zien is nog weinig bekend over de mechanismes achter deze reactie. De studies beschreven in dit proefschrift zijn gericht op het ophelderen van de vraag op welke manier RF magnetron gesputterde calciumfosfaat coatings en implantaat ruwheid de reactie van bot beïnvloeden. In het eerste hoofdstuk wordt een inleiding gegeven over differentiatie van botcellen en over de effecten van verschillende materiaal eigenschappen op de reacties van botcellen. In de volgende hoofdstukken worden de doelstellingen zoals beschreven in hoofdstuk 1 nader besproken.

Wat is het effect van dexamethasone and van serieel doorkweken op de expressie van osteogene markers door rattebeenmerg (RBM) cellen.

In hoofdstuk 2 wordt het effect van serieel doorkweken en van de botdifferentiatie inducerende stof dexamethasone op RBM cellen onderzocht. RBM cellen werden geïsoleerd en gekweekt met of zonder dexamethasone. Cellen werden eens in de 7 dagen overgezet en getest op expressie van alkalische fosfatase en op de vorming van een gemineraliseerde matrix, beide markers van osteogene differentiatie. RBM cellen die gekweekt werden zonder dexamethasone vertoonden geen expressie van osteogene markers. Cellen die gekweekt werden met dexamethasone vertoonde hoge expressie van alkalische fosfatase en sterke vorming van een gemineraliseerde matrix. De expressie van osteogene differentiatiemarkers werd echter steeds minder bij vaker overzetten van de kweken. Cellen die tijdens de primaire kweek zonder dexamethasone werden gekweekt en daarna overgezet werden in een kweek met dexamethasone vertoonden ook expressie van alkalische fosfatase en mineralisatie van de matrix. Ook in deze kweken verminderde de osteogene expressie bij vaker doorkweken. Voorts bleek dat de expressie van alkalische fosfatase en mineralisatie door deze cellen lager was dan bij cellen die constant waren gekweekt met dexamethasone.

Deze resultaten leiden tot de conclusie dat RBM cellen een deel van hun osteogeen potentieel behouden wanneer ze doorgekweekt worden zonder dexamethasone. Dit osteogene potentieel is echter verminderd vergeleken met cellen die doorgekweekt zijn met dexamethasone. Verder verliezen RBM cellen hun osteogene potentieel wanneer ze vaker worden overgezet, wat betekent dat langdurig doorkweken vermeden moet worden in

studies naar osteogene differentiatie van RBM cellen.

Wat is het effect van de kristalliniteit van RF magnetron gesputterde coatings en substraat oppervlakte ruwheid op de proliferatie en expressie van osteogene markers door RBM cellen.

Hoofdstuk 3 beschrijft de proliferatie en differentiatie van RBM cellen op materialen met verschillende oppervlakte karakteristieken. Ruwe titanium substraten werden vergeleken met gladde substraten en met ruwe substraten met een calciumfosfaat coating. De calciumfosfaat coating werd onbehandeld gelaten of werden hittebehandeld zodat coatings ontstonden van verschillende kristalliniteit, van amorf tot kristallijn. RBM cellen werden maximaal 16 dagen op de substraten gekweekt en de expressie van proliferatie- en differentiatiemarkers werd onderzocht. Ook werden gecoate en ongecoate substraten zonder cellen in kweek medium geïncubeerd, om het effect van materiaal karakteristieken op oplos- en neerslag gedrag te onderzoeken. Amorfe calciumfosfaat gecoate substraten vertoonden duidelijke sporen van oplossen en neerslaan, terwijl kristallijne coatings maar in beperkte mate oplosten. Een kleine hoeveelheid calciumfosfaat neerslag werd gevonden op de kristallijne coatings en ook op de titanium substraten. Deze neerslag was echter niet stabiel en verdween bij langer doorkweken. Op de kristalline coatings vertoonden de cellen de hoogste expressie van differentiatiemarkers, gevolgd door de titanium substraten. Op de amorfe coatings werd daarentegen geen proliferatie en differentiatie van RBM cellen gevonden. Dit was waarschijnlijk te wijten aan de sterke mate van oplossen en neerslaan van deze coatings. Deze studies laten zien dat ruwe titanium substraten met een kristallijne calciumfosfaat coating de osteogene differentiatie van RBM cellen meer stimuleren dan substraten zonder coating. De hoge mate van oplossen die de amorfe coatings vertonen remt de proliferatie en differentiatie van RBM cellen.

Wat is het effect van oppervlakte ruwheid en de aanwezigheid van een calciumfosfaat coating op de hechting, spreiding en integrine expressie van humane osteosarcoma (U2OS) en RBM tijdens de initiële interactie met het materiaal.

In de studie beschreven in hoofdstuk 4 werden U2OS cellen gebruikt om de initiële cellulaire interactie met verschillende materialen te bestuderen. Gladde en ruwe titanium substraten werden gebruikt, en ruwe titanium substraten gecoat met een kristallijne calciumfosfaat coating. U2OS cellen werden gebruikt omdat ze een beperkte variabiliteit vertonen, in tegenstelling tot RBM cellen. De cellen vertoonden een lagere hechting op de

gladde dan op de ruwe titanium substraten. U2OS cellen brachten α2, α3, α5, α6, αv en β1 integrine subunits tot expressie. Na 3 uur was de β1 expressie hoger op de calciumfosfaat gecoate substraat dan op de andere substraten. Verder bleek dat de expressie van α2, α5, α6 en αv subunits op de gecoate substraten na 24 uur verhoogd was ten opzichte van cellen die oorspronkelijk gezaaid waren. De mate van celspreiding was verschillend op de verschillende materialen, met glad titanium > ruw titanium > calciumfosfaat gecoat titanium. Op de gladde substraten oriënteren de cellen zich in de richting van de groeven die ontstaan zijn door polijsten. De cellen op de ruwe materialen volgden de oppervlakte ruwheid van de materialen. Op basis van deze resultaten concluderen we dat oppervlakte ruwheid de hechting en spreiding van U2OS cellen kan reguleren. De aanwezigheid van een calciumfosfaat coating beïnvloedt de integrine expressie, en ook de cel spreiding. Dit laatste fenomeen kan niet volledig verklaard worden door de ruwheid van het oppervlak, want hoewel calciumfosfaat en ruw titanium vergelijkbare ruwheid hebben, verschilt de cel spreiding op deze twee materialen.

In een vergelijkbare studie werden de initiële interacties van RBM cellen met verschillende substraat oppervlakten bestudeerd. Opnieuw werden gladde en ruwe titanium substraten en ruwe calciumfosfaat gecoate titanium substraten gebruikt. RBM cellen werden op de substraten gezaaid. Daarna werden hechting, integrine expressie en cel spreiding bestudeerd. De meeste cellen hechtten binnen twee uur aan de substraten. Er werden geen verschillen gevonden tussen de verschillende materialen in de snelheid waarmee cellen hechten. Een grote variatie werd gevonden in integrine expressie tussen de verschillende experimentele runs. Er werd echter geen verschil gezien in integrine expressie tussen de verschillende materialen. Op de gladde materialen vertoonden de cellen een grotere spreiding dan op de ruwe materialen. Op de ruwe materialen vertoonden de cellen lange cellulaire uitsteeksels, terwijl de cellen op glad titanium alleen korte uitsteeksels hadden. In conclusie, RBM hechting en integrine expressie lijken ongevoelig te zijn voor de verschillen in substraat karakteristieken van de materialen die gebruikt zijn. Materiaal karakteristieken veroorzaken daarentegen wel verschillen in spreiding van cellen op gladde of ruwe substraten.

Wat is het effect van oppervlakte ruwheid en aanwezigheid van een calcium fosfaat coating op het integrine expressie patroon van RBM cellen of U2OS cellen tijdens langere kweekperioden.

In hoofdstuk 6 werd het effect van substraat eigenschappen op de integrine expressie van U2OS cellen tijdens langere kweektijden onderzocht. Titanium substraten werden ongecoat

gebruikt, of werden voorzien van een calciumfosfaat coating gesputterd in de aan- of afwezigheid van zuurstof. Ook werden schijfjes hydroxyapatiet gebruikt. Naast integrine expressie werd ook U2OS proliferatie bestudeerd. Na 5 incubatiedagen werd geen verschil gevonden in proliferatie. Na 8 dagen werden significante verschillen gevonden in proliferatie, met het grootste aantal cellen op hydroxyapatiet. De cellen brachten α3, α5, αv en β1 subunits tot expressie, terwijl α4 en α6 expressie laag tot niet detecteerbaar was. Na 5 dagen was de expressie van β1 op de ongecoate titanium substraten significant hoger dan op de gecoate substraten. Geen andere verschillen werden gevonden na 5 dagen. Integrine expressie daalde tussen 5 en 8 dagen. Na 8 dagen was de β1 expressie op hydroxyapatiet hoger dan op de ongecoate titanium substraten.

Concluderend, substraten met verschillende oppervlakte eigenschappen beïnvloeden de respons van U2OS cellen. Zowel de proliferatie als de integrine expressie worden beïnvloed, hoewel het effect op integrine expressie beperkt is.

Het effect van substraat oppervlakte eigenschappen op de integrine expressie door RBM tijdens proliferatie en differentiatie werd bestudeerd (hoofdstuk 7). Substraten zoals in hoofdstuk 4 en 5 beschreven werden ook in deze studie gebruikt. Primaire cellen werden een week gekweekt, en daarna 8 of 16 dagen doorgekweekt op de substraten. Integrine expressie werd bestudeerd door middel van FACS. RBM cellen brachten altijd de α3, α5, α6 en β1 subunit tot expressie. Ook werd soms expressie van α1, α2 en β3 gevonden. Veel variatie werd gevonden in expressie patronen tussen de verschillende experimentele runs, zowel in primaire kweek als tijdens kweek op de substraten. Na 8 dagen van kweek op de substraten was de expressie van α5, α6 en β1 significant hoger op de gecoate substraten dan op de titanium substraten. Er was geen verschil in α3 expressie. Een probleem ontstond bij het meten na 16 dagen, omdat de cellen moeilijk van de substraten te verwijderen waren. Het is mogelijk dat de cellen die na 16 dagen van de cellen gehaald werden een cel populatie is met een ander integrine expressie patroon dan de cellen die niet van de substraten verwijderd konden worden. We raden daarom ook aan om voornamelijk naar de resultaten van dag 8 te kijken. De resultaten tonen aan dat substraten in staat zijn integrine expressie door RBM cellen te beïnvloeden.

Conclusies, toekomstperspectief:

In een serie van studies werd het effect van substraat oppervlakte eigenschappen op in vitro osteoblast respons bestudeerd.

De resultaten tonen een duidelijk effect van een kristallijne calciumfosfaat coating op de respons van osteoblasten aan. De coating stimuleerde de expressie van osteogene markers in vergelijking met cellen gekweekt op glad of ruw titanium. Amorfe calciumfosfaat

coatings daarentegen lieten een negatief effect zien op osteogene cellen. Dit was waarschijnlijk het effect van de grote oplosbaarheid van deze coatings. Dit is in tegenspraak met een groot aantal andere studies, die een positief effect van amorfe coatings laten zien, terwijl kristallijne coatings osteogene differentiatie niet stimuleren. Deze studies gebruiken echter vaak plasma gespoten coatings, met een kompleet ander oplosgedrag dan de coatings die wij gebruikten. Daarom moeten we benadrukken dat een complete karakterisering op het gebied van chemische compositie, kristalliniteit, maar ook fabricage methode bijzonder belangrijk is wanneer het gaat om het ontwikkelen en onderzoeken van calciumfosfaat gecoate implantaten.

Verder vonden we dat oppervlakteruwheid een beperkt effect heeft op de respons van osteogene cellen. Dit is in contrast met veel andere studies. Hoewel het moeilijk is een verklaring te geven voor deze tegenstrijdige vondsten, is het bekend dat het meten van oppervlakte ruwheid zeer complex is. Verschillen in de meetmethoden gebruikt in de studies kunnen resulteren in verschillen in data. Dit bemoeilijkt het goed vergelijken van de verschillende resultaten. Om dit probleem te voorkomen, is het ontwikkelen van een gestandaardiseerde methode voor meten en beschrijven van oppervlakte ruwheid van groot belang.

Naast het effect van oppervlakte eigenschappen op de algemene celreactie vonden we ook dat deze eigenschappen de expressie van integrines kunnen beïnvloeden, zelfs al zijn de verschillen beperkt. Verschillen in integrine expressie kunnen gerelateerd zijn aan verschillen in osteogene expressie. Het is echter niet mogelijk een definitieve conclusie te trekken. Hoewel U2OS cellen een goed model zijn voor integrine expressie, vertonen deze cellen geen differentiatie. Daarom kan een relatie met de expressie van osteogene markers niet worden gemaakt. RBM cellen daarentegen differentiëren wel, maar vertonen een grote variatie in integrine expressie patroon. Dit betekent dat we niet met zekerheid kunnen zeggen dat modulatie van integrine expressie de belangrijkste manier is waarmee verschillende materialen botrespons beïnvloeden. Naast integrine expressie kan bijvoorbeeld ook integrine functie gemoduleerd worden. Daarom stellen we voor dat toekomstig onderzoek zich moet richten op dit aspect van de interactie van cellen met hun omgeving. Studies naar het effect van Ca^{2+} op integrine functie bij osteogene cellen of op intracellulaire signalering na interactie van de cellen met verschillende materialen zullen helpen cellulaire reacties op materialen beter te begrijpen. Andere manieren om de relatie van cellen met materialen beter te leren kennen zouden het coaten van materialen met extracellulaire matrix (ECM) eiwitten, of het blokkeren van de interactie van integrines met ECM eiwitten om het effect op de cel reactie te meten. Uiteindelijk moeten al deze studies leiden tot meer inzicht in de reactie van bot met biomaterialen en moet het mogelijk worden implantaten te ontwerpen waarbij de weefselreactie voorspeld kan worden.

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Curriculum Vitae

Petra ter Brugge werd geboren in Almelo op 10 januari 1972. In 1990 haalde zij het VWO diploma aan de Pius X te Almelo. In dat zelfde jaar werd begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen.

Haar hoofdvakstage liep zij bij afdeling Dierfysiologie, en bijvakstages werden uitgevoerd op de afdelingen Pathologie en Medische Microbiologie, waarvoor zij 6 maanden in Tanzania verbleef. In 1997 studeerde zij af in de Fysiologische/ Biochemische richting. In dat jaar werd zij aangesteld als Assistent in Opleiding aan de afdeling Biomaterialen van de Medische Faculteit van de KUN. Dit proefschrif beschrijft de resultaten van het onderzoek uitgevoerd aan deze afdeling.