Comparison of efficacies of different bone substitutes adhered to osteoblasts with and without extracellular matrix proteins

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Abstract
Background/purpose: Regeneration of bony tissue is a major goal in periodontal and implant surgery. The adhesion efficacy of osteogenic cells to bone substitutes is a determinant for osteogenesis. The adsorbed proteins have a significant impact on the behavior of cells and the biological activity of the grafting materials. The aim of the present study was to compare the adhesion ability of different grafting materials with and without extracellular matrix (ECM) protein coating.

Materials and methods: MG63 human osteosarcoma cells were cocultured with the same volume of different bone substitutes (Bio-Oss, Cerasorb, MBCP, and BoneCeramic). The nonadhesion cells in the suspension were counted. The medium pH values were measured. The viability of attached cells was measured by the dye WST-1 (water-soluble tetrazolium salts-1). The same procedures for cell viability were repeated for each bone substitute coated with ECM proteins (fibronectin, vitronectin, type I collagen) separately.

Results: Adsorption of ECM proteins increased cell adhesion to the bone substitutes. Vitronectin and fibronectin increased cell attachment to Cerasorb or BoneCeramic significantly more than type I collagen did. Type I collagen was better in increasing cell attachment to Bio-Oss, which had the lowest attached cell count both on dry material and with protein coating.

Conclusion: The results indicated that ECM proteins increased cell attachment to bone substitutes in vitro. The preferential affinity of different bone substitutes to certain ECM proteins...
Introduction

Osteoconductivity of bone substitutes are related to the adherence of osteoblasts to the surface of the substitutes as well as the cell’s migration and proliferation later along these scaffolds. Accordingly, the adhesion efficacy of osteogenic cells is critical. Cell adhesion is the first interaction between cells and materials and the quality of this phase influences further cell functions. In fact, immediately after the bone substitute is implanted into an organism or comes into contact with cell culture environments, protein adsorption to its surface occurs. These proteins include fibronectin, vitronectin, fibrinogen, immunoglobulins, albumin, and others. The adsorption takes place within seconds, long before the first cells reach the surface. Hence, cells almost never come into direct contact with the material surface; rather, they interact with the layer of adsorbed proteins. This layer mediates the cell adhesion and provides signals to the cell through the cell adhesion receptors, determining the cellular response to the bone substitute. Fibronectin, an abundant glycoprotein in the extracellular matrix (ECM), is present at its highest concentration during osteogenesis. It binds to osteoblasts preferentially and more strongly than other ECM proteins. It contributes to osteoblastic adhesion, differentiation, and matrix mineralization. Vitronectin also has high affinity for osteoblasts as compared to other cells. This would generate a clinical advantage in osteogenesis by preventing connective tissue formation after grafting material was implanted. Both fibronectin and vitronectin could bind to the integrins on the surface of the osteoblast, which improves cell attachment to the bone substitutes or spreads cells on the surface of these biomaterials. Expressed from early stages of differentiation onwards, type I collagen is another ECM protein that is essential for bone formation and matrix production. The spontaneous and competitive adsorption of certain proteins from surrounding body fluids after implantation has a significant impact on the behavior of cells. The type, amount, and geometrical conformation of adsorbed proteins influence further cell functions and strongly depend on the physicochemical properties of the material surface, such as its wettability, electrical charge, surface roughness, and topography, mechanical properties (rigidity or flexibility), crystallinity, porosity, solubility, pH, or presence of certain atoms or chemical functional groups. This investigation was to compare the adhesion efficacies of four bone substitutes with and without coating of the above-mentioned three ECM proteins. Among the four substitutes of clinical interest, one is Bio-Oss (cancellous bovine bone), a non-synthetic xenograft, and the rest are synthetic, which are Cerasorb [pure phase β-tricalcium phosphate (TCP)], BoneCeramic [40% β-TCP, 60% hydroxyapatite (HA)], and MBCP (40% β-TCP, 60% HA). In addition, the pH values of each medium were monitored because the adhesion substrates can release calcium and phosphate ions that induce strong alkalinization of the cell culture media leading to cell death in the conventional static cell culture system.

Materials and methods

Cell culture osteoblast-like cells (MG63) were cultured in sterile Falcon wells (Becton Dickinson, Franklin Lakes, NJ, USA) containing ATCC-formulated Eagle’s Minimum Essential Medium (Catalog No. 30-2003), which was supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO, USA) and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL; Sigma Chemical Co.). Medium renewal was performed two to three times per week. The cultures were maintained in a 37°C atmosphere of 95% air and 5% carbon dioxide. Observation of MG63 cell attachment cells at a density of 2 × 10⁴ were seeded on culture plates. Half of the plates were coated with polystyrene and the rest without. Cell morphology was observed directly under a microscope (Olympus (100 Lauman Lane, Suite A Hicksville, NY 11801), 10 × 20 magnification) after 24 hours. Cell attachment to bone substitutes 100 µL of MG63 cells at a density of 2 × 10⁴ were seeded into culture wells coated with and without polystyrene as the control group. The experimental group consisted of four wells without polystyrene coating. Each well contained different bone substitutes of 100 mm³. This volume was used to represent a clinical alveolar bony defect. Those bone substitutes were Bio-Oss (Geistlich, Wolhusen, Switzerland, 0.25–1 mm granules), Cerasorb (Riemsar Arzneimittel AG, Germany, 500–1000 microns), MBCP (Citagenix, Laval, Canada, 0.5–1 mm granules), and BoneCeramic (Straumann, Wolhusen, Switzerland, 500–1000 µm). After 24 hours, all of the suspension medium of each well was aspirated to count floating cells. The pH value of the medium was measured by a pH meter (Mettler-Toledo SevenEasy, Wolhusen, Switzerland). Cell viability was assayed by using a colorimetric assay. The principle was based on the cleavage of WST-1 (water-soluble tetrazolium salts-1) tetrazolium salt (Roche, Mannheim, Germany) by mitochondrial dehydrogenases in viable cells, which changed absorbance. Ten microliters of WST-1 and 90 µL of MG63 cells at a density of 2 × 10⁴ were added into each well after the suspension medium was aspirated. The wells were incubated for a further 2 hours in a humidified atmosphere at 37°C. The numbers of viable cells were determined by measuring the absorbance of samples (A450) using an enzyme-linked immunosorbent assay (ELISA) reader. Experiments were repeated six times in six replicates. MG63 cells attachment to bone substitutes that were wetted with ECMs. The bone substitutes were wetted with 100 µL of 99% ethanol for 1 hour at room
temperature. Then, ethanol was discarded. The wetted bone substitutes were washed with 100 μL of PBS at room temperature three times within 30 minutes. One hundred microliters of 5 μg/mL fibronectin (Sigma), vitronectin (Sigma), and type I collagen (Sigma) were each mixed with 100 μL of 10% FBS (Gibco BRL, Grand Island, NY, USA) and were added into the washed bone substitutes, respectively. They were left stand at 4°C for 12 hours. The fluid of FBS and the protein were then removed, and 100 μL of PBS was used to rinse the bone substitutes three times in 5 minutes under gentle agitation. Finally, the protein-wetted bone substitutes were put into new wells. One hundred microliters of 2 × 10^4 MG63 cells were seeded into each well. The control group had MG63 cells only. All the wells were not coated with polystyrene. After 24 hours, the culture medium that contained nonattached cells was discarded. The 90-μL new culture medium and 10 μL WST-1 were added. Cells were incubated for 2 hours in a humidified atmosphere at 37°C. The numbers of viable cells were determined by measuring the absorbance Amax 450 nm with an ELISA reader. Experiments were repeated six times in six replicates. Statistics data are reported as representative or mean ± standard error of the mean (SEM) of six experiments. Data were analyzed by two-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC, USA). A P value < 0.05 was considered significant.

**Results**

Cell attachment was noticeable after 24 hours in a culture plate coated with polystyrene. As shown in Fig. 1, the circle shape represented a nonattached viable cell, the spindle an attached cell, and the irregular a dead cell. By contrast, cell attachment was not evident in a culture plate without polystyrene coating, as indicated in Fig. 2 where the spindle shapes were hardly found. As shown in Fig. 3, the suspension cell count of Bio-Oss was the highest. The cell count of MBCP was higher than BoneCeramic and Cerasorb. Fig. 4 indicates that there was no difference in cell adhesion efficacies between Cerasorb and BoneCeramic; both of them were significantly better than MBCP, and MBCP was better than Bio-Oss. The pH values of the media aspirated from each well were between 7.93 and 8.08 as illustrated in Fig. 5. The pH values of Bio-Oss and BoneCeramic were higher than the others. Fig. 6 shows that type I collagen significantly increased the cell attachment extent of
Cerasorb and BoneCeramic. There were statistical differences between BoneCeramic and Cerasorb. Cell attachment extent in the order from high to low is BoneCeramic, Cerasorb, MBCP, and Bio-Oss. In Fig. 7, vitronectin showed a trend the same as that of type I collagen and had the same order. Fibronectin could also increase the cell attachment extent of Cerasorb and BoneCeramic (Fig. 8). By comparing the cell attachment extent of the protein-wetted bone substitutes with that of the notwetted in Table 1, the following findings were evident. Type I collagen increased the cell attachment extent of Bio-Oss. All three ECM proteins increased the cell attachment extent of Cerasorb. The order from high to low was fibronectin, vitronectin, and type I collagen. Neither of the three ECM proteins could increase the cell adhesion capacity for MBCP. The cell attachment extent of BoneCeramic was doubled by vitronectin and fibronectin, and was nearly doubled by type I collagen. The bone substitutes wetted with the selected ECM proteins that achieved the largest cellular attachment.

Figure 4  Cell viability on materials. Cells were cultured with 100 mm³ of bone substitute for 24 hours. Data are expressed as percentage of control. The nonpolystyrene-coated well served as control. Data are mean ± standard error of the mean (SEM) of six experiments. The wells containing all the bone substitutes were nonpolystyrene-coated. * P < 0.05 compared to control.

Figure 5  pH values of the medium after 24 hours. The “control” contained the culture medium only. The “cells” were added with MG63 cells, and the rest with the cells and the respective bone substitute. Data are mean ± standard error of the mean (SEM) of six experiments. *P < 0.05 compared to control.

Figure 6  Cell viability on type I collagen-adsorbed bone substitutes. Cells were cultured with 100 mm³ of type I collagen-wetted bone substitutes for 24 hours. Data are expressed as percentage of control. The “control” contained the medium only. Data are mean ± standard error of the mean (SEM) of six experiments. *P < 0.05 compared to control.

Figure 7  Cell viability on vitronectin-adsorbed bone substitutes. Cells were cultured with 100 mm³ of vitronectin-wetted bone substitutes for 24 hours. Data are expressed as percentage of control. The “control” contained the medium only. Data are mean ± standard error of the mean (SEM) of six experiments. *P < 0.05 compared to control.
Bone substitutes adhered to osteoblasts

Discussion

Human osteoblasts are anchorage-dependent cells. The focal contact between the intracellular cytoskeleton of these cells and ECM proteins can initiate signal transmission. These signals are further transmitted to the cytosol and nucleus and then they go through cell proliferation, matrix formation, maturation, and mineralization. The MG63 cell is a permanent human osteosarcoma cell line. It has been used as an osteoblast model to study cell differentiation. In particular, polystyrene is one of the most widely used polymeric materials for cell culturing and as scaffolds for tissue regeneration. The attachment of osteoblasts to bone substitutes would not be as good as to polystyrene culture plates. This was verified by the result of the cell viability on materials (Fig. 4). However, to exclude the possible interference of polystyrene, the ensuing experiments used the wells without coating instead. Data in Fig. 4 led to the impression that MG63 cells adhered to MBCP poorly and to Bio-Oss even worse. Although MBCP and BoneCeramic are both β-TCP/HA (40%/60%), the MG63 cells adhere to MBCP far less than BoneCeramic. This biological outcome might result from the subtle manufacturing differences of the following parameters: homogeneity of the TCP/HA, crystallinity of HA, sintering temperature, mean size of macro pores, and percentage of total porosity. For example, BoneCeramic is 90% porous, Cerasorb (500–1000 μm) has a total porosity of 80%, and MBCP has a 70% overall porosity. The larger surface area allows interaction of the bone substitutes with bone-forming cells, thus promoting cell adhesion. Bio-Oss is deproteinized, sterilized bovine bone. The deproteinization and inactivation of pathogen processes of Bio-Oss were shown to have adverse biological effects on cells due to oxidative stress. Vitronectin and fibronectin increased the cell attachment of BoneCeramic and Cerasorb but they did not provide the same effect for Bio-Oss. Fibronectin even had a negative impact on Bio-Oss. Type I collagen was the only one of the three proteins that could enhance the cell adhesion capacity of Bio-Oss. However, it reduced the cell adhesion capacity of Bio-Oss.

![Figure 8](image)

**Figure 8** Cell viability on fibronectin-adsorbed bone substitutes. Cells were cultured with 100 mm² of fibronectin-wetted bone substitutes for 24 hours. Data are expressed as percentage of control. The “control” contained the medium only. Data are mean ± standard error of the mean (SEM) of six experiments. * P < 0.05 compared to control.

<table>
<thead>
<tr>
<th>Bone substitutes</th>
<th>Nonwetted</th>
<th>+ Type I collagen</th>
<th>+ Fibronectin</th>
<th>+ Vitronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Oss</td>
<td>11.1 ± 0.4</td>
<td>14.9 ± 0.8</td>
<td>6.0 ± 0.5</td>
<td>9.8 ± 0.9</td>
</tr>
<tr>
<td>Cerasorb</td>
<td>118.6 ± 12.4</td>
<td>156.0 ± 12.8</td>
<td>232.0 ± 14.5</td>
<td>189 ± 13.6</td>
</tr>
<tr>
<td>MBCP</td>
<td>39.8 ± 4.3</td>
<td>23.6 ± 1.6</td>
<td>33.2 ± 6.5</td>
<td>36.2 ± 4.7</td>
</tr>
<tr>
<td>Bone Ceramic</td>
<td>108.1 ± 9.4</td>
<td>187 ± 2.4</td>
<td>216.0 ± 6.5</td>
<td>238.0 ± 11.3</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of the total attachment (100%) of the MG63 cells in noncoated wells.
attachment of MBCP. These variations might result from the different affinities of the proteins to the bone substitutes. The findings suggest potential applications in surface modification of different bone substitutes for osteo-conduction. Further studies are needed to investigate the affinities of the ECM proteins to the bone substitutes and to osteoblasts. Attention should also be given to possible interactions among the ECM proteins that might be synergistic in increasing adhesion efficacy. From the perspective of an ideal bone substitute, the adhesion efficacy of Bio-Oss was far lower than that of Cerasorb and BoneCeramic, although it could be improved by type I collagen in this study and other investigations. Bio-Oss did not yield the kind of clinical effectiveness that was displayed by BoneCeramic and Cerasorb, which was surprising for a bone substitute. This might be because the TCP-based materials (MBCP, Cerasorb, and BoneCeramic) resorbed faster causing micromovements and inflammatory reactions induced by chemical dissolution of TCP. Both factors might affect bone formation negatively in vivo, whereas Bio-Oss did not. Further studies are needed to investigate how new bone is generated after osteoblasts attach to bone substitutes, that is through proliferation, maturation, and mineralization, and the differences between synthetic (BoneCeramic and Cerasorb) and nonsynthetic (Bio-Oss) bone substitutes.

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References

7. Petrie TA, Capadona JR, Reyes CD, Garcia AJ. Integrin specificity and enhanced cellular activities associated with surfaces presenting a recombinant fibronectin fragment compared to RGD supports. Biomaterials 2006;27:5459–70.