Selective RGD-Mediated Adhesion of Osteoblasts at Surfaces of Implants**

Martin Kantlehner, Dirk Finsinger, Jörg Meyer, Patricia Schaffner, Alfred Jonczyk, Beate Diefenbach, Berthold Nies, and Horst Kessler*

The nonphysiological character of synthetic materials often leads to poor integration after implantation into human or animal tissues. Graft rejection, low mechanic stability of the biomaterial–tissue interface, infections, and inflammations are undesired side effects of insufficiently active interactions between implant and surrounding tissue. They often make a revision of the graft necessary. The boundary between implant and tissue can be strengthened by coating of such implants with integrin-specific and cell-selective molecules to bind and activate the integrin-expressing cells, the osteoblasts. These processes of tissue remodeling require a tuned interaction of bone-forming osteoblasts with bone-resorbing osteoclasts whose activities are in a natural equilibrium.[1] Natural full-length adhesion proteins of the extracellular matrix (fibronectin, vitronectin, collagen)[2] as well as short peptide sequences which contain the adhesion-mediating sequence (e.g. the RGD sequence) can be used for this purpose.[3–9] Here we report a new optimized method for the coating of implants using integrin-specific peptide ligands and the direct covalent anchoring of these peptides to the common graft material poly(methyl methacrylate) (PMMA). We demonstrate that these surfaces bind osteoblasts, stimulate their proliferation, and therefore trigger biological tissue regeneration (Figure 1).

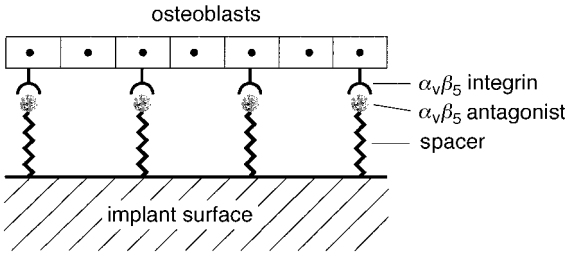

Figure 1. Adhesion of osteoblasts on PMMA surfaces mediated by $\alpha_v\beta_5$ integrin.

We used the cyclic pentapeptide c(-RGDfX-) (f = D-phenylalanine),[10–15] which is selective for the $\alpha_v\beta_3$ and the $\alpha_v\beta_5$ integrin receptors. The residue X is a lysine residue, which allows us to link the peptide over a length-optimized spacer through an acrylic acid functional group to the PMMA graft used as bone implant. For clinical application it is essential that the pentapeptide with a D-phenylalanine residue following the binding sequence RGD exhibits high activity as well as higher α_v selectivity compared to the platelet receptor $\alpha_{IIb}\beta_3$ to induce the prefered adhesion of osteoblasts rather than of platelets. We could show that RGD peptides which do not fulfill these criteria, such as linear peptides or cyclic peptides containing the D-amino acid in another position, do not possess α_v selectivity and often have lower activities as well.[11, 14]

In a pilot study we bound c(-RGDfK-) through a *N*-succinylcysteamide linker (→thiol peptide **A**)[16] or through a 3-sulfanylpropionic (3-mercaptopropionic) acid linker (→thiol peptide **B**)[16] and maleinimide to surfaces coated with bovine serum albumine (BSA) and studied the adhesion of different osteoblast cultures (primary human osteoblasts, primary human osteoprogenitor cells, primary rat osteoblasts, and mouse MC3T3H1 osteoblasts).[17] Using immuno fluorescence with integrin-specific antibodies, we could prove that all investigated osteoblast cultures express $\alpha_v\beta_5$ and even $\alpha_v\beta_3$ integrin to only a limited extent. It turned out that all osteoblast cultures bind to the c(-RGDfK-)-coated surfaces, while the control cell line M21L, which does not express $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, does not bind (Figure 2).

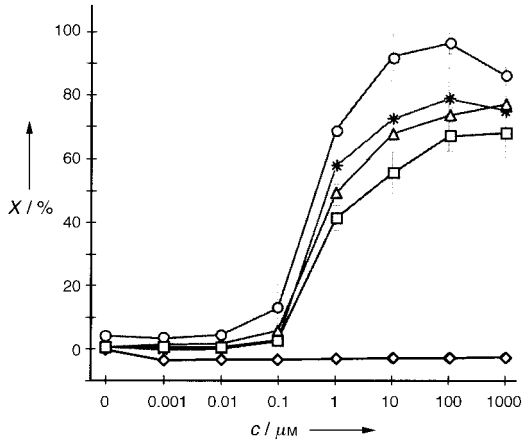

Figure 2. Dependence of the cell plating efficiency X of different osteoblast cultures on the concentration c of thiol peptide **A** used for the coating protocol. M21L cells which do not express $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin receptors do not bind to the surface. △: primary human osteoblasts, ∗: primary human osteoprogenitor cells, □: primary rat osteoblasts, ○: MC3T3H1 mouse osteoblasts, ◇: human melanoma cells (M21L).

Binding of cells occurs through the $\alpha_v\beta_5$ integrin and through $\alpha_v\beta_3$. Cyclopeptide c(-RGDfK-) binds to both receptors.[11, 12] Adherent cells can be removed from the surface by addition of dissolved c(-RGDfK-). The negative control peptide c(-RβADfK-) (control thiol peptide), for which the insertion of only a single methylene group at the glycine residue (β-alanine) prevents integrin binding completely, does not stimulate any adhesion of osteoblasts (Figure 3). This is additional proof for integrin-RGD-peptide interaction to be the cause for the observed cell adhesion. A

[*] Prof. Dr. H. Kessler, M. Kantlehner, Dr. D. Finsinger
Institut für Organische Chemie und Biochemie der Technischen Universität München
Lichtenbergstrasse 4, D-85747 Garching (Germany)
Fax: (+49) 89-2891-3210
E-mail: kessler@ch.tum.de
Dr. J. Meyer, P. Schaffner, Dr. B. Nies
Merck Biomaterial GmbH, Forschung, Darmstadt (Germany)
Dr. A. Jonczyk, Dr. B. Diefenbach
Merck KGaA, Präklinische Forschung, Darmstadt (Germany)

[**] The authors acknowledge technical assistance from B. Blessing, G. Fleissner, H.-G. Kreysch, and Mona Wolff. Financial support was provided by the Fonds der Chemischen Industrie.

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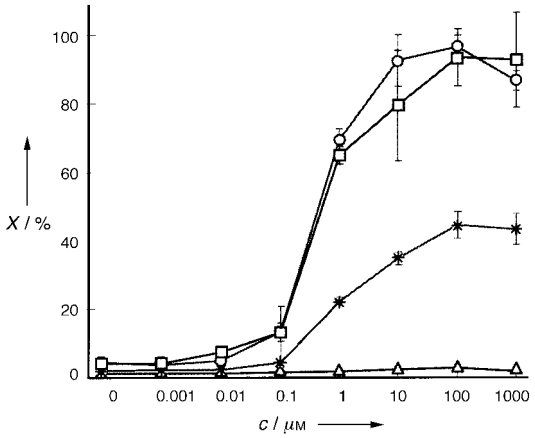


Figure 3. Dependence of the cell plating efficiency X of MC3T3H1 mouse osteoblasts on the concentration c of peptide in the coating solution. The effect of peptide sequence and linker length was studied with thiol peptides A (○), B (□), C (∗), and the control thiol peptide (△). The thiol peptides **A** and **B** contain c(-RGDfK-) as highly active and selective integrin antagonist, whereas thiol peptide **C** has c(-RGDEv-) as a ligand. The control thiol peptide contains β-alanine instead of glycine (c(RβADfK)) and is inactive.

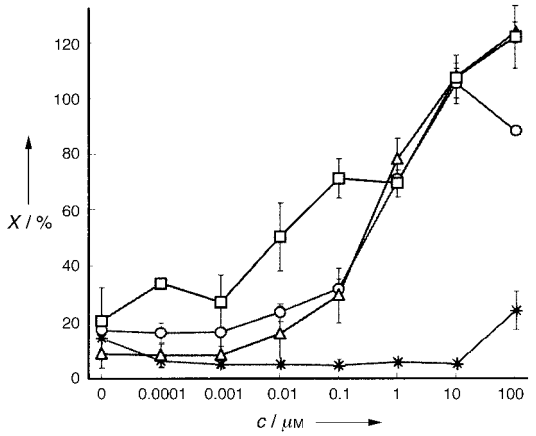
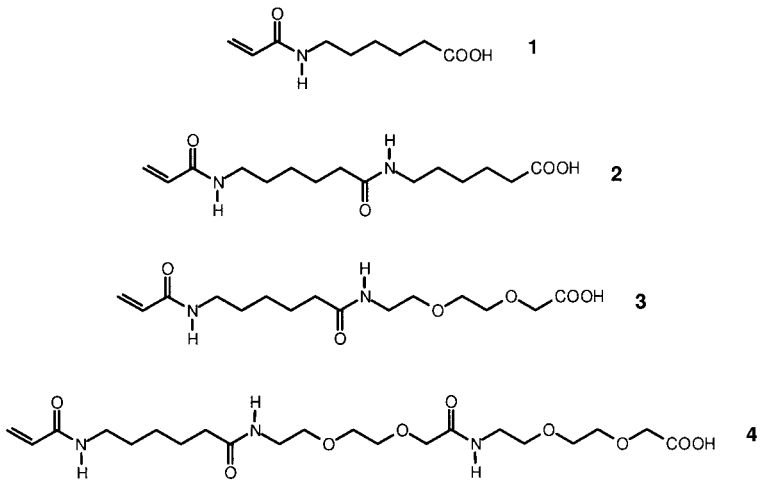


Figure 4. Dependence of the cell plating efficiency X of MC3T3H1 mouse osteoblasts on the concentration c of peptide in the coating solution. Different acrylate peptides were tested which differ only in the nature of the spacer **1**–**4**. ∗: acrylate peptide 1, ○: acrylate peptide 2, □: acrylate peptide 3, △: acrylate peptide 4. Spacer **1** is too short for effective adhesion of osteoblasts.

comparison the ligand c(-RGDfK-) containing linkers **A** or **B** with the peptide c(-RGDEv-) synthesized by Delforge et al.[7] (thiol peptide **C**) clearly indicate an improvement in cell adhesion with c(-RGDfK-) as ligand (Figure 3).

To coat the PMMA surface with c(-RGDfK-) the peptide was linked through a spacer to acrylic acid and then radically polymerized onto the PMMA graft.[18] Apparently, free double bonds of the polymer are sufficient for this cross-linking.

Initially linker **1**[19] was used for the RGD peptide. However, the resulting surfaces did not bind osteoblasts (Figure 4). Therefore, we synthesized RGD peptides with longer linkers



2, **3**, and **4**, which also differ in their hydrophilicity/hydrophobicity profile. All three acryl peptides[21] stimulate adhesion of osteoblasts, and there are no significant differences between them (Figure 4). We therefore conclude that for effective integrin-mediated cell adhesion on surfaces a minimum distance of about 3.5 nm between the ligand and the surface is required. As expected the cell adhesion rate (expressed as a percentage; ratio of the number of adherent cells to the number of seeded cells × 100) rises with increasing ligand density at the surface. Even with the relative high numbers of suspended cells (50 000 per cm^2 of surface) used in these experiments, all cells bind to the surface: Cell adhesion rates of up to 100 % are obtained (Figure 5). Adhesive cells are tightly bound and cannot be removed from the surface by washing or mechanical shaking.

In a study over 22 days the adherent osteoblasts bound through the RGD peptide to PMMA surfaces were stimulated to proliferate at different ligand densities at the surface. The number of proliferated cells increased over this time span by a factor of 10 in comparison to the untreated PMMA surface

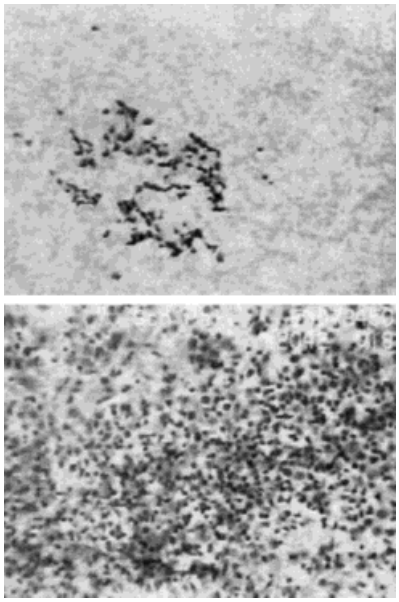


Figure 5. Optical microscopy image of attached MC3T3H1 mouse osteoblasts (dark) on uncoated PMMA surfaces (top) and on PMMA bone cement treated with acrylate peptide 3 (spacer **3**). The peptide concentration in the coating solution was 100 μM. While the top image is representative for the coated surface, the bottom image shows the only area of the untreated surface where cell adhesion was observed at all.

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(Figure 6).[22] This indicates the potential to cover the surface completely and to obtain a natural bonding from implant to the biological tissue.

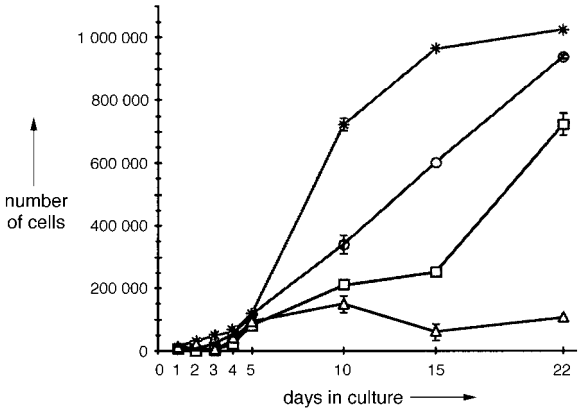

Figure 6. Stimulated proliferation of PMMA-attached MC3T3H1 mouse osteoblasts over a time span of 22 days as a function of ligand density (acrylat peptide 3): ∗: 100 μM, ○: 1 μM, □ 0.01 μM acrylate peptide 3. An untreated surface served as a control (△).

The results presented here demonstrate an attractive strategy for the development of cell-free and bioactive implants that carry the biological information for the selective activation of those target cells which are needed for selective tissue regeneration.

Received: August 3, 1998 [Z12237IE]
German version: *Angew. Chem.* **1999**, *111*, 587 – 590

Keywords: bioorganic chemistry · integrins · materials science · peptides · surface chemistry

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