Induced tissue integration of bone implants by coating with bone selective RGD-peptides *in vitro* and *in vivo* studies

P. SCHAFFNER, J. MEYER, M. DARD, R. WENZ, B. NIES
Merck Biomaterial GmbH R&D, D-64271 Darmstadt, Germany
E-mail: joerg.meyer@merck.de

S. VERRIER,
Unite INSERM U 443, 146 rue Leo Saignat, F-33076 Bordeaux, France

H. KESSLER, M. KANTLEHNER,
Institut für Org. Chem. und Biochem., Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany

The optimal function of medical implant materials used in tissue substitution is often limited due to its healing properties. This effect is linked to reduced interactions of the implants with the surrounding tissue. Implant surfaces biologically functionalized with arginine-glycine-aspartic acid (RGD) peptides, a class of cellular adhesion factors, are described in this paper. The RGD-peptides are either bound via bovine serum albumin linking on culture plastic dishes as a model surface or via acrylic acid coupling on PMMA surface as a potential implant material. Resulting functionalized surfaces aquire the capability to bind cultured osteoblasts in high levels and show high proliferation rates *in vitro.* These results are observed for osteoblast cultures as well as from different species with different preparation procedures. A critical minimum distance between the bioactive portion of the RGD-peptides and the implant surface of 3.0–3.5 nm is crucial for the induction of an optimum cell binding process. *In vivo* animal studies in the rabbit show that newly formed bone tissue generated a direct contact with the RGD-peptide coated implants. In contrast uncoated implants are separated from newly formed bone tissue by a fibrous tissue layer thereby preventing the formation of a direct implant–bone bonding.

1. Introduction

Although indicated as being biocompatible, many implant materials do not actively interact with the surrounding healthy and regenerating tissue thereby preventing the generation of a mechanical and functional stable biomaterial–tissue bonding. The aim of this study is to demonstrate that the presented strategy of surface coating with tailor-made bone-selective arginine-glycine-aspartic acid (RGD)-peptides may induce an enhanced and accelerated tissue integration of bone implants with respect to an improved long-term stability.

2. Materials and methods

Primary human osteoblasts, primary human osteoprogenitor cells, primary rat osteoblasts and mouse calvaria osteoblastic cells of the line MC3T3H1 were prepared or provided as described [1–4]. The α_v-selective RGD-peptides cyclo (-RGDfK) with thiol anchor was synthesized according to Jonczyk *et al.* [5]. Cyclo (-RGDfK) with acrylate anchor were generated similar to Gurrath *et al.* [6]. The covalent coating of the thiol peptides on bovine serum albumin (BSA)-coated cell culture 48-well plates was carried out according to Ruoslahti *et al.* [7].

The covalent grafting of the acrylate peptides onto prepolymerized polymethylmethacrylate (PMMA) implants was performed by incubation with a peptide solution in dimethylsulfoxide (DMSO)/isopropanol/ 0.2% (w/v) campherchinon and subsequent u.v.-radiation. The determination of cell adhesion was performed as described by Landegren [8]. The cell proliferation assay was observed by using the WST-1 colorimetric test.

For animal studies RGD-peptide-coated bone implants and uncoated control implants were implanted in a rabbit model in the patella groove. PMMA implants (8 mm length/4.6 mm in diameter) with interconnective porosity (Hard Tissue Repair material (HTR), Walter Lorenz, Jacksonville, USA) were press-fit implanted with the diamond bone-cutting system (DBCS®) from Merck Biomaterial GmbH, Germany. After 2 and 4 weeks the

implants were explanted and newly formed bone was examined after histological staining according to Goldner–Masson in cross-sections.

3. Results and conclusions

The integrin α_v-directed active thiol cyclo (-RGDfK) peptides after covalent grafting to BSA-precoated cell culture wells strongly and dose-dependently stimulate the cell adhesion of cultured α_v-positive primary human osteoblasts, primary human osteoprogenitor cells, primary rat osteoblasts, and mouse MC3T3H1-osteoblasts. In contrary the effect on α_v-deficient human melanoma cell line M21L is negligible (Fig. 1). We prove that the cell adhesion is mediated by cyclo (-RGDfK) by treating osteoblasts with soluble cyclo (-RGDfK). Depending on the concentration of the soluble peptide, cells pretreated with peptide could be prevented from binding on a peptide surface. Additionally, as negative control we coated BSA surfaces with the inactive peptide cyclo (-R(βA)DfK), a derivative that is not able to bind integrin receptors. In this case no cell adhesion was observed, proving the extremly high selectivity of the binding process.

Covalent grafting of acrylate cyclo (-RGDfK) peptides with different molecular length onto prepolymerized PMMA discs stimulated the osteoblast cell adhesion similar to the BSA coating (Fig. 2). The acrylate peptides differ in length: acrylate–peptide 1 = 2.7 nm, acrylate–peptide 2 = 3.5 nm, acrylate–peptide 3 = 3.7 nm and acrylate–peptide 4 = 4.3 nm. The shortest peptide showed a minimum biological response, while the other peptides had a comparable high bioactivity. We conclude a critical minimum distance between the cell recognition sequence of the RGD-peptide and the material surface of 3.0 to 3.5 nm to promote successful cell adhesion. In addition cell proliferation of MC3T3H1-osteoblasts over a period of 22 days is dose-dependently stimulated by the coating of PMMA-surfaces with acrylate peptide cyclo (-RGDfK) (Fig. 3).

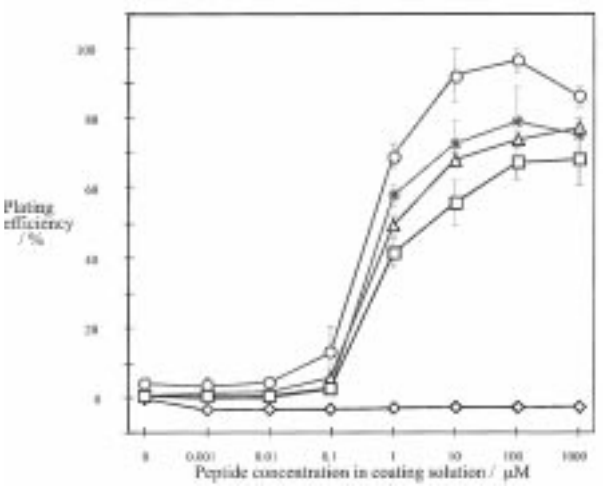


Figure 1 Graph of cell adhesion on BSA peptide coating shows plating efficiency at different peptide concentrations. (○) MC3T3 H1 mouse osteoblasts; (□) primary rat osteoblasts; (*) primary human osteoprogenitor cells; (△) primary human osteoblasts; (◇) human melanoma (M21L). $n = 3$.

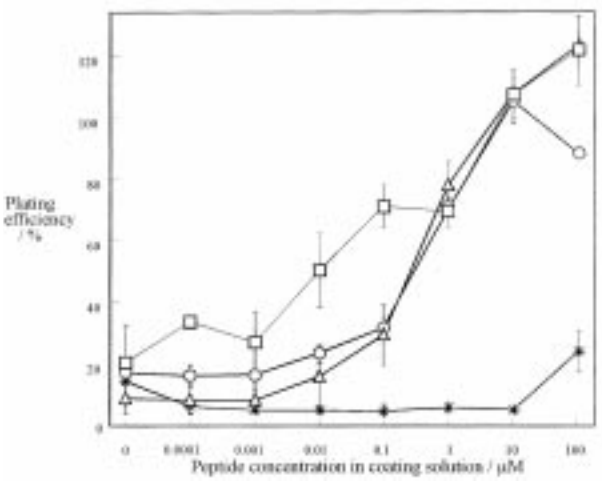


Figure 2 Graph of cell adhesion assay on PMMA discs coated with different acrylate-peptides. (*) acrylate–peptide 1; (○) acrylate–peptide 2; (□) acrylate–peptide 3; (△) acrylate–peptide 4. $n = 3$.

These data prove the potential of adhered osteoblasts to proliferate strongly, thereby increasing the cell number at least 10-fold within this period, resulting in a PMMA surface completely covered by osteoblasts.

In animal studies in the rabbit RGD-peptide-coated porous PMMA implants induce an enhanced and accelerated tissue bone ingrowth. Newly formed bone directly contacts the implant surface, even growing into the center of porous material (Fig. 4a). In contrast uncoated implants were separated from newly formed bone by a fibrous tissue layer (Fig. 4b). The implant coating with tailor-made RGD-peptides indicates an attractive strategy for generating a new generation of implants bearing the biological information for selective activation of target cell species performing tissue regeneration. This strategy might serve as a generally useful key technology for tissue engineering.

Acknowledgments

The authors thank B. Blessing and G. Fleißner for the excellent technical assistance. For constructive discussions and peptide synthesis we express many thanks to B. Diefenbach and A. Jonczyk.

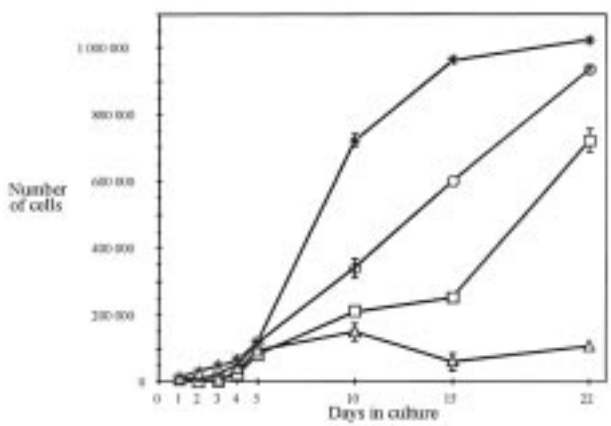
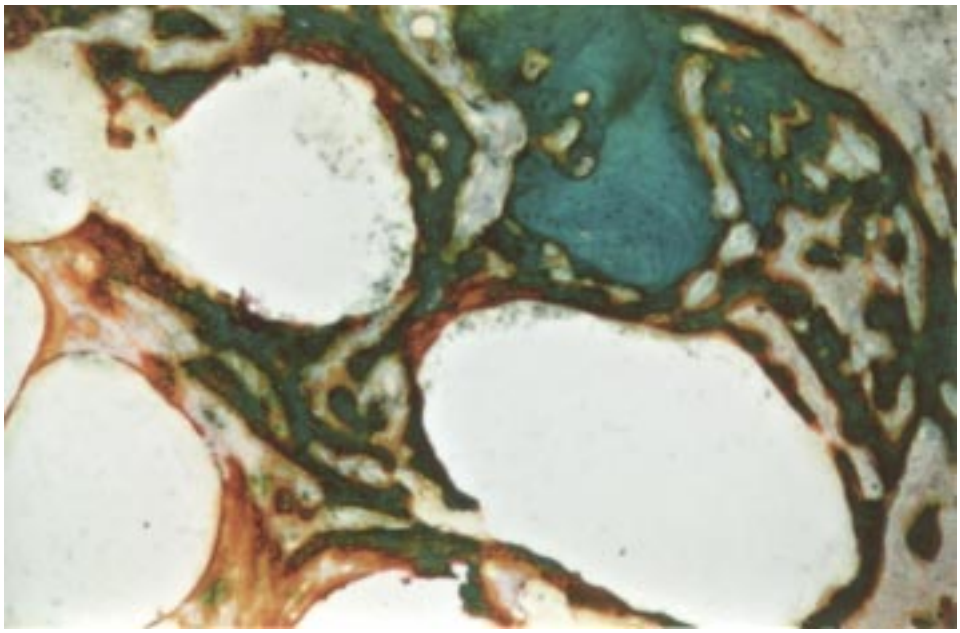


Figure 3 Graph of cell proliferation assay shows number of cells over the time period. (*) 100 µM acrylate–peptide 3; (○) 1 µM acrylate–peptide 3; (□) 0.01 µM acrylate–peptide 3; (△) control. $n = 3$.

(a)



(b)

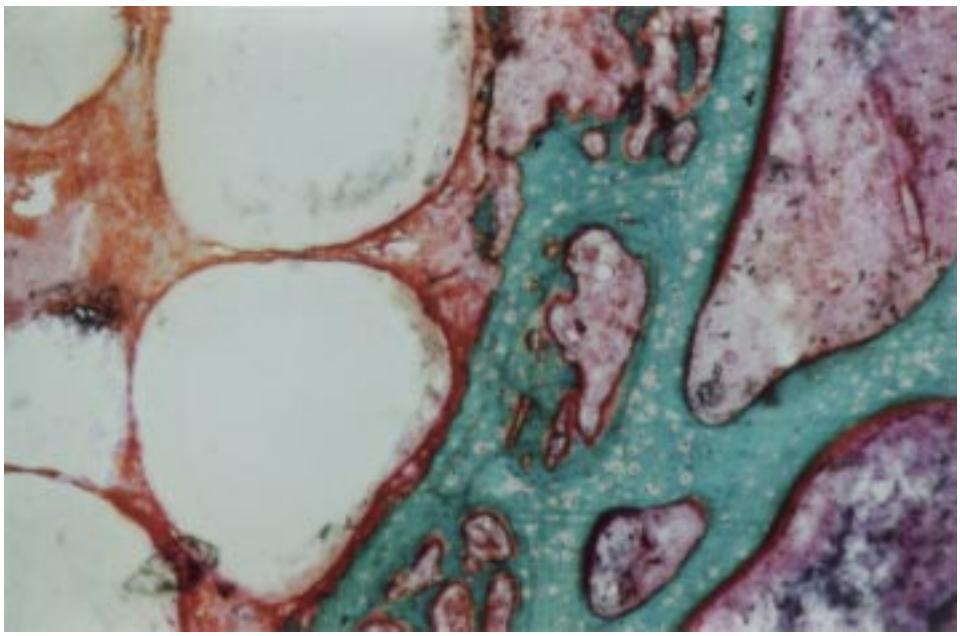


Figure 4 Cross-section of implanted PMMA implants (× 60). (a) RGD-peptide-coated PMMA implant. (b) Uncoated PMMA implant. Colour code: purple, fibrous tissue; green, existing bone; dark green/black, newly formed bone; red-brown, newly formed osteoid; white, PMMA beads.

References

1. H. SIGGELKOW, D. HILMES, K. REBENSTORFF, W. KURRE, I. ENGEL and M. HÜFNER, *Clin. Chim. Acta* **272** (1998) 111.
2. J. VILAMITJANA-AMEDEE, R. BAREILLE, F. ROUAIS, A. I. CAPLAN and M. F. HARMAND, *In Vitro Cell. Dev. Biol.* **29** (1993), 699.
3. J. A. YAGIELA and D. M. WOODBURY, *Anat. Rec.* **188** (1977) 287.
4. K. HEERMEIER, M. SPANNER, J. TRÄGER, R. GRADINGER and J. SCHMIDT, *Cells Mater.* **5** (1995) 309.
5. A. JONCZYK, S. GOODMAN, B. DIEFFENBACH, A. SUTTER and H. KESSLER, patent DE 19538741.
6. M. GURRATH, G. MÜLLER, H. KESSLER, M. AUMAILLEY and R. TIMPEL, *Eur. J. Biochem.* **210** (1992) 911.
7. E. RUOSLAHTI, E. G. HAYMAN, M. PIERSCHBACHER and E. ENGVALL, *Methods Enzymol.* **82** (1982) 803.
8. U. LANDEGREN, *J. Immunol. Methods* **67** (1984) 379.

Received 14 May
and accepted 2 June 1999