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Influence of RGD-Loaded Titanium Implants on Bone Formation *in Vivo*

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ABSTRACT

Little is known about the ability of peptide-coated surfaces to influence cell responses *in vivo*. Many studies have demonstrated that peptide-modified surfaces influence cell responses *in vitro*. Integrins, which bind specifically short peptide sequences, are responsible for these cell responses. In this way, information can be transmitted to the nucleus through several cytoplasmic signaling pathways. The peptide sequence Arg-Gly-Asp (RGD peptide) plays an important role in osteoblast adhesion. The present study was designed to investigate new bone formation in a porous titanium (Ti) fiber mesh implant, which was coated with cyclic RGD peptide. The RGD-Ti implants were inserted into the cranium of a rabbit and were compared with porous titanium fiber mesh disks without RGD sequence (Ti) and with an open control defect. Histologic and histomorphometric examinations were performed 2, 4, and 8 weeks postoperatively. A significant increase in bone formation, or bone ingrowth, was seen in the RGD-Ti group compared with the Ti group after 4 and 8 weeks. All control defects stayed open in all three periods. It was concluded that the use of cyclic RGD peptide in combination with titanium fiber mesh has a positive effect on bone formation *in vivo* in a rabbit animal model.

INTRODUCTION

IN THE FIELD OF BONE REGENERATION, naturally derived and synthetic polymers, ceramics, metals, and composites are being used. On occasion, these so-called scaffold materials are also provided with growth factors as well as stromal cells in order to enhance their bone-forming capacity. Demands on the scaffold properties largely depend on the site of application and the function that must be restored. For example, the ideal scaffold material is mechanically strong, biocompatible, and biodegradable; can be shaped easily; possesses interconnective porosity; is osteoconductive or osteoinductive; and promotes angiogenesis.¹

Unfortunately, none of the currently used materials meets all of the properties postulated. Some of the ma-

terials (e.g., polymers and ceramics that degrade quickly) show an undesirable inflammatory response or foreign body reaction. These reactions are associated with a reduced osteoinductive response. Other materials show a lack of structural support and good mechanical characteristics.

In view of the above, in our laboratory a series of studies is ongoing in which we explore the feasibility and efficacy of titanium fiber mesh as a scaffold material for bone reconstructive purposes. Titanium is well known for its excellent biocompatibility. This is expressed by two major observations: (1) a favorable response of tissues to titanium surfaces, and (2) the absence of allergic reactions to titanium.^{2,3} For example, bone cells and mineralized bone matrix are laid down on titanium surfaces without interposition of other tissues.²

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The porous titanium fiber mesh as used in our studies has several advantages relative to bulk titanium, such as its flexibility and interconnective porosity. Flexibility helps presumably to eliminate focal stresses by distributing the stresses between implant and tissue over a larger area.³ The porosity of the fiber mesh, which can be varied during fabrication, can influence the amount of bone ingrowth into the material and allows a more normal restoration of the bone, unlike nonporous implant materials.³

Proper bone growth also requires initial stability. The frictional characteristics of porous titanium fiber mesh when contacting bone exceed those of solid metal materials presently available. In the early postoperative period these frictional and structural properties allow a high initial stability of the construct.

Although porous titanium fiber mesh is nondegradable, it still offers several advantages over other materials by its uniformity and structural continuity, as well as by its strength, low stiffness, high porosity, corrosion resistance, and high coefficient of friction.⁴ Nevertheless, previous studies have shown that the osteoconductive properties of the material are still insufficient to allow complete closure of cranial defects in rats.⁵ Stromal bone marrow cells have been seeded into the mesh porosity to overcome this problem.⁵ Although the data confirmed that this technique supports bone formation inside the mesh, this cell-based approach is laborious and the outcome is not completely predictable. Therefore, titanium mesh has also been treated with bone growth-stimulating factors, such as bone morphogenetic protein and transforming growth factor- β_1 .⁶⁻⁸ Despite favorable results in rats, the disadvantage of the use of such morphogens is the cost and reproducibility of the results in larger animals and humans.

An alternative to the above-mentioned approaches may be the covalent coupling of ligands, such as peptides, to the titanium mesh surface.⁹

The organic component of bone is composed of numerous extracellular matrix proteins that serve multiple roles in bone formation and homeostasis, ranging from simple cell attachment to binding of hydroxyapatite. These extracellular matrix proteins interact with a heterodimeric cell membrane receptor family, known as integrins, that use multiple intracellular signaling pathways. Arginine-glycine-aspartic acid, or RGD, is a small peptide ligand that has high affinity for these integrins and is the most extensively studied integrin-stimulating peptide. It is known that RGD peptides increase the overall adhesiveness of the surface for osteoblasts.^{10,11} In this way, they can essentially mimic cell attachment activity of bone cells.^{12,13} It has also been suggested that RGD peptide coating enhances titanium rod osseointegration in the rat and goat femur.^{14,15}

In the current study, the influence on bone formation of a cyclic RGD peptide sequence coated on porous titanium fiber mesh was investigated. We evaluated the osteoconductive properties of porous titanium fiber mesh, coated or not coated with covalently coupled RGD sequence, in a rabbit non-critical-size cranial defect model. We hypothesized that adding this cycled RGD peptide to the titanium fiber mesh would enhance the osteoconductive properties of this carrier material.

MATERIALS AND METHODS

Implant preparation

Sintered titanium fiber mesh implants (Bekaert, Zwegem, Belgium) with a volumetric porosity of 86%, a density of 600 g/m³, and a fiber diameter of 40 μ m, were used.

The prepared implants were disk-shaped with an outer diameter of 8.0 mm, a thickness of 0.8 mm, and a weight of approximately 33.5 mg. All implants were ultrasonically cleaned with isopropanol and 70% ethanol for 15 min, and then sterilized by gamma sterilization. Sixty implants were prepared.

Subsequently, 30 of the titanium disks were coated with 100 μ M cyclic RGD peptide (provided by Biomet Deutschland [Darmstadt, Germany] and first synthesized by the group of H. Kessler [Technical University of Munich, Munich, Germany]) containing a phosphonate anchor, in phosphate-buffered saline (PBS, pH 7.4). The RGD was connected with a covalent bond, which creates a connection. The titanium meshes were soaked in the coating solution and the peptide was allowed to immobilize overnight. After three washes with PBS the meshes were dried and sterilized by gamma sterilization (\approx 25 kGy) and ready for implantation. Gamma sterilization was confirmed by the manufacturer (Biomet Deutschland) and was shown not to affect the activity of the peptide coating.

Experimental study design

Thirty adult female New Zealand White rabbits (2.5–3.5 kg) were used. National guidelines for the care and use of laboratory animals were observed. In each rabbit three cranial defects with an outer diameter of 8.0 mm were made (Fig. 1). The distance between the defects was at least 5 mm. Treatments were titanium fiber mesh (Ti), titanium fiber mesh loaded with RGD peptide sequence (RGD-Ti) and an open control defect to follow the regular healing process of the non-critical-sized cranial defects.

The rabbits were killed at 2, 4, and 8 weeks (respectively, groups I, II, and III; Table 1), and in each case the skull with the implants was retrieved so that histologic evaluation could be performed.

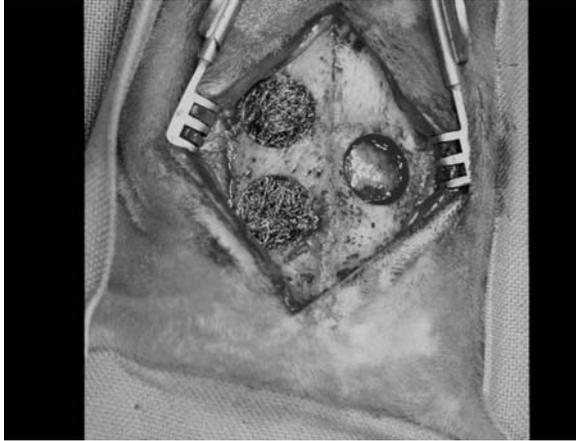


FIG. 1. Three cranial full-thickness bone defects with an outer diameter of 8 mm (RGD-Ti, Ti, and open).

Surgical procedure

Surgery was performed under general inhalation anesthesia. Anesthesia was induced by an intravenous injection of Hypnorm (fentanyl citrate [0.315 mg/mL] and fluanisone [10 mg/mL]) and atropine, and maintained by a mixture of nitrous oxide, isoflurane, and oxygen through a constant volume ventilator. To reduce the perioperative infection risk, antibiotic prophylaxis (2.5% Baytril) was given to the rabbits.

After anesthetization, the animals were immobilized on their abdomen. Hair from the cranium was shaved and the skin was disinfected with povidone-iodine.

From the nasal bone to the occipital protuberance, a longitudinal incision was made. Thereafter, a midline incision was created in the periosteum. Subsequently, the periosteum was removed gently and lifted from the parietal skull with a blunt instrument. Three cranial full-thickness bone defects with an outer diameter of 8 mm were prepared with a dental trephine bur at low rotation speed (1000–1500 rpm) in both sides of the parietal bone. The drill depth was limited to 1.5 mm and the cranial bone disks were carefully removed. During the drilling procedure, saline was supplied as a coolant. After this procedure, the RGD-Ti implant was inserted according to a randomized implantation schedule in one of the three defects in the skull (Table 2). The titanium

fiber mesh without RGD was placed in the second defect (Ti). The third defect was left open as a control in order to monitor the regular healing capacity of this non-critical-sized cranial defect in rabbits (Fig. 1). The periosteum and skin were closed (tensionless) with Pro-lene 4.0 sutures.

Histologic and histomorphometric evaluation

Skull bones containing the implants were retrieved for histologic examination at the various times cited above. Directly after retrieval, skulls were fixed in 4% buffered formalin solution and dehydrated in a graded series of ethanol. Before embedding in methylmethacrylate (MMA), the skulls were divided with a sawing machine into three separate specimens containing one implant each. Finally, thin sections (10 μm) were prepared by a sawing microtome technique after polymerization.¹⁶ All sections were made in the frontal-caudal direction. The slides were stained with methylene blue–basic fuchsin and observed with a light microscope for histologic examination (Leica Microsystems, Rijswijk, The Netherlands). At least three sections from the central part of each implant were evaluated.

Histologic evaluation consisted of a concise description of the observed specimens and a histomorphometric analysis of the tissue response.

Histomorphometric evaluation was carried out by computer-based image analysis (Leica QWin Pro image analysis system; Leica Microsystems). From each implant, data were pooled and the mean was used for statistical analysis.

The following histomorphometric parameters were assessed for the Ti and RGD-Ti cranial implants (Fig. 2):

- The surface area where the titanium implant was inserted (the so-called region of interest [ROI])
- The bone surface area in the ROI (expressed as a percentage of the ROI)
- The distance of bone ingrowth into the titanium fiber mesh. This was calculated as the sum of ingrowths at the right and left sides of the implants, divided by the total length of the implant

For the open control defects only the maximum gap as left at the end of the respective implantation times was measured.

TABLE 1. NUMBER OF ANIMALS, DEFECT SIZE, AND IMPLANTATION TIME

Group	Defect size (mm)	Number of defects (RGD-Ti, Ti, open)	Implantation time (weeks)
I ($n = 10$)	8.0	3	2
II ($n = 10$)	8.0	3	4
III ($n = 10$)	8.0	3	8

TABLE 2. RANDOMIZED IMPLANTATION SCHEDULE

Rabbit no. and location skull	RGD-Ti location	Ti location	Control defect location	No defect
1, 5, 9, 13, 17, 21, 25, 29	1	2	3	4
2, 6, 10, 14, 18, 22, 26, 30	2	3	4	1
3, 7, 11, 15, 19, 23, 27	3	4	1	2
4, 8, 12, 16, 20, 24, 28	4	1	2	3

Statistical analysis

All measurements were statistically evaluated with GraphPad Instat 3.05 software (GraphPad Software, San Diego, CA), using an unpaired Student *t* test with Welch correction to compare untreated Ti with RGD-treated Ti specimens for each implantation time. All data were tested for normality. Differences were considered significant at *p* values less than 0.05.

RESULTS

Macroscopic evaluation

All 30 animals survived the implantation period and remained in good health. At retrieval no macroscopic signs of inflammation or adverse tissue reaction were seen around any of the 90 defects. All implant sites and empty control defects could be easily located. Each implant remained seated at the original site and they were all covered by periosteum. Bone formation could be seen macroscopically in most of the specimens. The newly formed bone appeared normal. No gross macroscopic differences could be observed between the RGD-Ti and Ti groups.

Histologic and histomorphometric evaluation

Two weeks. All of the control defects (10) were open after 2 weeks. New bone formation started mainly at the edges of the defect (Fig. 3). The morphological appearance and thickness of the newly formed bone differed from the original bone. Histomorphometry revealed that

the bone had covered $52 \pm 8\%$ of the length of the original control defect size.

Also, none of the titanium defects were closed (covered with bone over the whole defect). In all Ti and RGD-Ti implants, blood vessel ingrowth and fibrous tissue formation were present. Blood vessel ingrowth in the RGD-Ti group appeared to be more prominent than in the Ti group. No inflammatory cells or fibrous tissue were present in or around the surface of both types of implant.

After 2 weeks, $12 \pm 5\%$ of the RGD-Ti fiber mesh

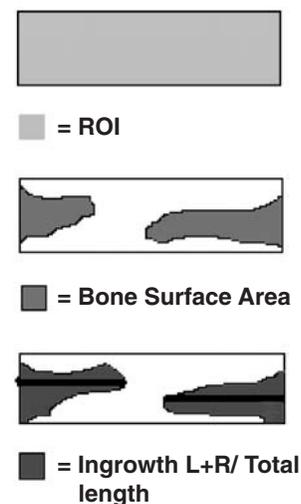


FIG. 2. Histomorphometric parameters of Ti and RGD-Ti cranial implants (region of interest [ROI], bone surface area, and bone ingrowth in length).

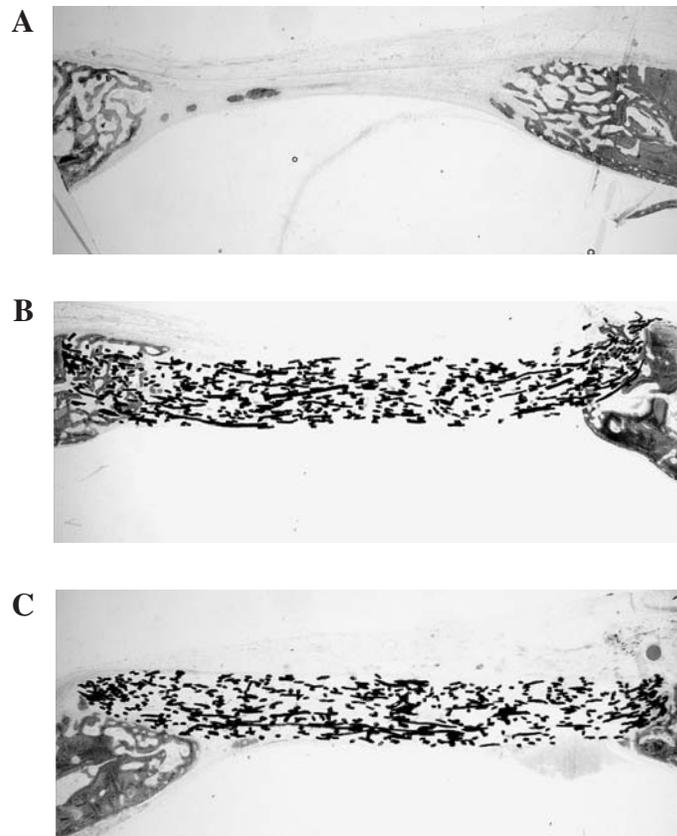


FIG. 3. Light microscopy overview of (A) control defect, (B) RGD-Ti scaffold, and (C) Ti scaffold, 2 weeks postimplantation (original magnification, $\times 1.6$).

was covered with bone with a trabecular structure, starting at the edges, and $8 \pm 6\%$ of the Ti fiber mesh without RGD was covered (no significant difference; Table 3). There was also no significant difference between the total ingrowth (percent ingrowth of the total length of the implant) of the RGD-Ti group ($58 \pm 24\%$) and the Ti group ($44 \pm 26\%$) (Table 4).

Representative examples of light microscopic sections of control, RGD-Ti, and Ti implants 2 weeks postimplantation are depicted in Fig. 3.

Four weeks. All of the empty control defects were still open after 4 weeks ($n = 10$). The newly formed bone covered $52 \pm 12\%$ of the total length of the original control defect size (Fig. 4). The contour, that is, thickness, of the ingrown bone differed from the original bone. Inside the empty defect space, fibrous tissue was present and blood vessel invasion had occurred. Always, a fibrous tissue capsule could be observed at the periphery of the bone defect.

The Ti and RGD-Ti mesh implants were also surrounded with a fibrous tissue capsule. No inflammatory cells were seen in or around the surfaces of the implants. There was no observable increase in thickness of the capsule from week 2 to week 4.

After 4 weeks there was significantly more bone surface area in the RGD-Ti group ($12 \pm 4\%$) compared with the Ti group ($6 \pm 5\%$; Table 3). Further, total bone ingrowth in the RGD-Ti group was $71 \pm 17\%$ and in the Ti group it was $50 \pm 20\%$, which is significantly different as well (Table 4).

Figure 4 shows typical histologic slides of control, RGD-Ti, and Ti implants 4 weeks postimplantation.

Eight weeks. All of the empty defects were still open after 8 weeks. New bone formation covered $64 \pm 12\%$ of the total length of the control defect (Fig. 5). The newly

TABLE 3. BONE SURFACE AREA IN RGD-TI AND TI SCAFFOLDS 2, 4, AND 8 WEEKS POSTIMPLANTATION

Time postimplantation (weeks)	Area of bone (%) ^a	
	RGD-Ti	Ti
2	12 ± 5	8 ± 6
4	12 ± 4	6 ± 5
8	18 ± 10	10 ± 7

^aRelative to region of interest (ROI).

TABLE 4. BONE INGROWTH^a IN RGD-Ti AND Ti SCAFFOLDS 2, 4, AND 8 WEEKS POSTIMPLANTATION

Time postimplantation (weeks)	Bone ingrowth (right + left)/ total length of implant (%)	
	RGD-Ti	Ti
2	58 ± 24	44 ± 26
4	71 ± 17	50 ± 20
8	71 ± 21	52 ± 16

formed bone still differed in thickness compared with the original bone. A fibrous tissue capsule was maintained at the outside of the gap.

One of the RGD-Ti implants was completely covered with bone over its total length. Further, blood vessel ingrowth and fibrous tissue encapsulation were comparable between the 2- and 4-week specimens.

After 8 weeks, 18 ± 10% of the RGD-Ti fiber mesh surface area was occupied by bone, compared with 10 ± 7% of the titanium fiber mesh without RGD (Table 3).

This difference was not significant ($p = 0.057$). However, the total bone ingrowth percentage in the RGD-Ti group (71 ± 21%) was significantly greater than in the Ti group (52 ± 16%) (Table 4).

Figure 5 shows the light microscopic appearance of control, RGD-Ti, and Ti implants 8 weeks postimplantation.

DISCUSSION

In this study, we investigated the effect of RGD-coated titanium fiber mesh on bone formation *in vivo* at three different times postimplantation. Histomorphometric analysis determined that 18 ± 10% of the RGD-Ti fiber mesh was occupied with bone after 8 weeks compared with 10 ± 7% of the titanium fiber mesh without RGD (not significant; $p = 0.057$). Nevertheless, after 4 weeks there was significantly more bone surface area in the RGD-Ti group (12 ± 4%) compared with the Ti group (6 ± 5%; Table 3). A significant increase in ingrowth (length) after 4 and 8 weeks occurred as well, when compared with the reference material (uncoated titanium fiber

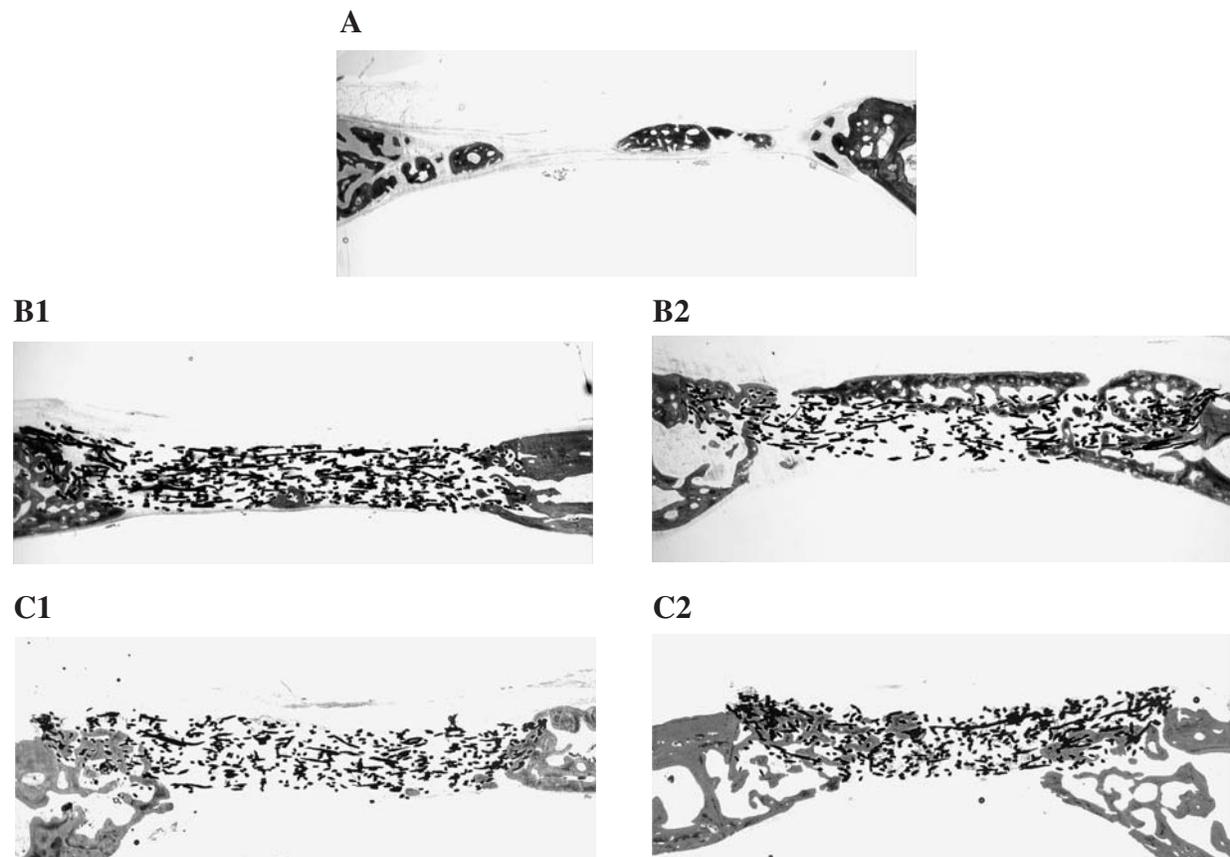


FIG. 4. Light microscopy overview of (A) control defect, (B) RGD-Ti scaffold (B1 shows limited bone ingrowth; B2 shows bone overgrowth of mesh structure), and (C) Ti-scaffold (C1 shows limited bone growth; C2 shows deep bone penetration in mesh porosity), 4 weeks postimplantation (original magnification, ×1.6).

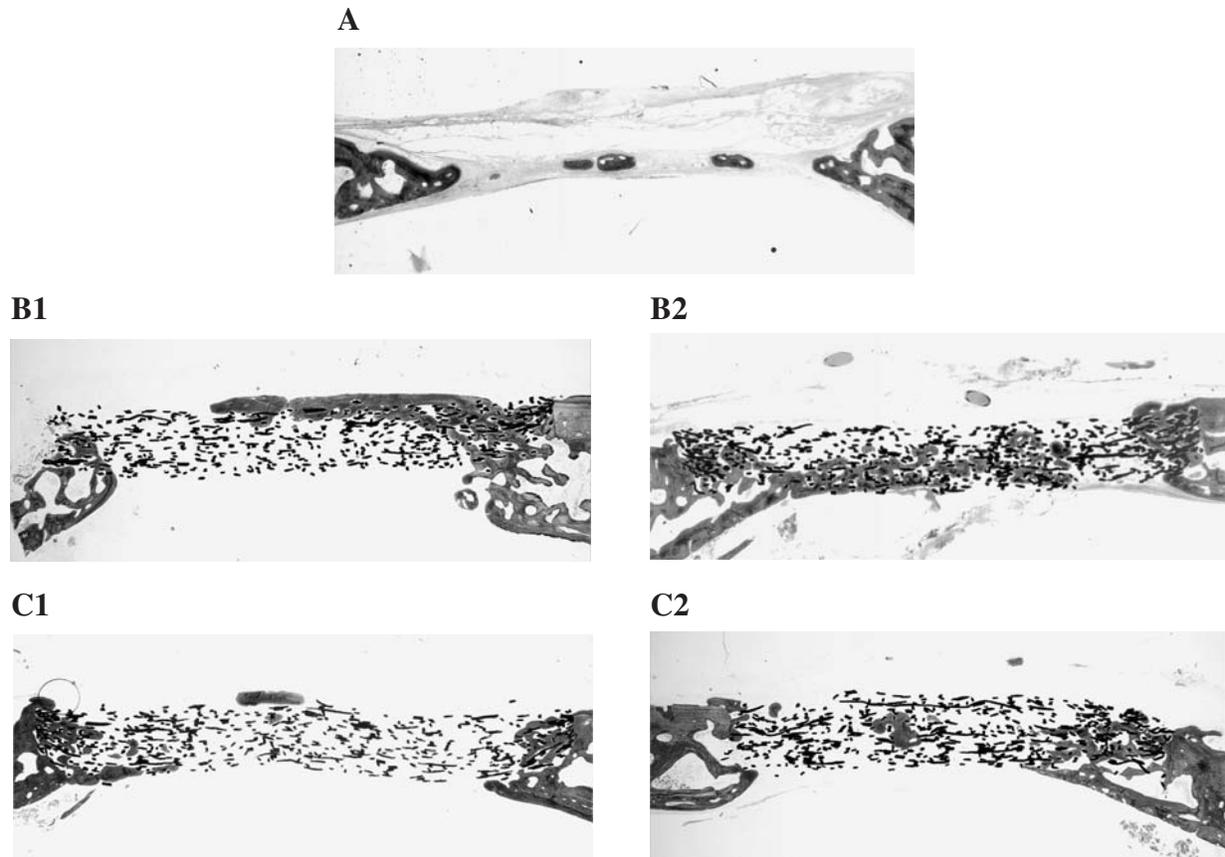


FIG. 5. Light microscopy overview of (A) control defect, (B) RGD-Ti scaffold (B1 shows bone overgrowth; B2 shows bone ingrowth in mesh structure), and (C) Ti-scaffold (C1 shows no bone ingrowth; C2 shows limited bone ingrowth in mesh porosity), 8 weeks postimplantation (original magnification, $\times 1.6$).

mesh). We must also note that all control defects stayed open until 8 weeks postimplantation. This emphasizes the relevance of our model. Evidently, the Ti scaffolds also did not hamper or interfere in a negative way with the bone-healing process.

Bone cell adhesion and migration are known to play an important role during the biological processes underlying bone healing and bone regeneration. Cell adhesion peptides, such as RGD, are supposed to play a role in the control of bone cell proliferation and differentiation. The peptides must be covalently attached to a suitable carrier material in order to be used in implant surgery. The results, as obtained in the current study, confirm the hypothesis that coating cyclic RGD peptide on titanium fiber mesh will enhance the osteoconductive properties of this carrier material. The results corroborate with other studies. For example, Ferris *et al.*¹⁴ demonstrated a significant increase in new bone thickness around RGDC (Arg-Gly-Asp-Cys)-modified surfaces of polished titanium rods in rat femur 2 and 4 weeks after implantation. They measured a significantly thicker shell of newly formed bone around the implant as soon as 2 weeks

postimplantation, but using another animal model and another titanium implant and implantation site.

Elmengaard *et al.*¹⁷ showed that a cyclic RGD coating on unloaded press-fit titanium implants has an osteoconductive effect only directly at the interface 4 weeks postimplantation (implanted in the tibia of a dog model). Also, Schliephake *et al.* found an increase in bone-implant contact from 1 to 3 months postoperatively in a group of RGD-coated implants, using a combination of collagen and RGD on dental implants in the mandibles of 10 beagle dogs.¹⁸ However, similar to our study, Schliephake *et al.* showed a large degree of variation in bone contact rates within the various experimental groups, resulting in a decreased level of significance. The experimental model can be an important variable for this phenomenon. Tripeptide RGD has been shown to enhance *in vitro* the adhesion and spreading of fibroblasts, endothelial cells, smooth muscle cells, and osteoblasts when this biomolecule was grafted on various surfaces.^{19–22} Concerning the effect of RGD peptides on osteoblast cell differentiation, integrin-mediated cell binding seems to be the essential parameter. In particular,

$\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin-selective RGD peptide ligands are responsible for mediating the initial adhesion and increased synthesis of mineralized matrix.²³ Bernhardt *et al.* also indicated that RGD peptides were capable of generating signals for the specific microenvironment around titanium implants in the femur of a goat and thus accelerated the bone formation process.¹⁵ Nevertheless, a decisive factor in the cell attachment-promoting activity of surface-bound RGD peptide is, of course, the availability of a sufficient amount of free bone-forming cells. This may have been the major problem in our *in vivo* study. Periosteal osteo- and chondrogenesis is important for bone defect healing. In our study design, the periosteum was first lifted from the skull bone and at the end of the procedure was sutured back over the mesh implant to its original position. The periosteum consists of two layers: a fibrous outer layer and, bordering the bone, a cambium layer, which contains specific osteogenic and chondrogenic precursor cells.²⁴ Therefore, maintenance of this cambium layer may be essential for bone healing to occur in the mesh implant after closure of the periosteum at the end of the implant installation. However, in rabbits this layer is difficult to retain during surgery, resulting in delayed healing.²⁵ Besides preservation of the cambium layer, skull bone is composed mainly of cortical bone with a minimal portion of marrow-rich cancellous bone. In addition, the amount of contact between the mesh scaffold and bone defect wall was limited to a few 40- μm -thick titanium fibers because of the specific morphological structure of the mesh material. Apparently, this also did not promote the migration of bone cells into the mesh porosity.

Besides the experimental model, another critical issue dealing with the modification of implant surfaces by peptides is the final selectivity of the peptides used. Besides bone cells, the adhesion of other cell types can be promoted. Also, multiple ligands may be required for a full cell adhesion response.²⁶ In view of this, two relevant parameters for the final biological effect of grafted RGD peptides are the competitive adsorption of a second protein from the surrounding body fluid and the RGD peptide surface concentration.²⁷ The second adsorbed protein can result in inefficient cell adhesion, and an increase in RGD peptide surface concentration has been shown to result in increased cell proliferation with, at the same time, reduced matrix production.²⁷ Unfortunately, we do not know whether optimum surface conditions existed for early differentiation and phenotypic expression on our RGD-coated titanium fiber meshes.²⁸ In view of the above, it must also be noted that other implant materials are available with perhaps better osteoconductive properties as compared with Ti mesh. Most of these materials belong to the class of calcium phosphate (CaP) ceramics. Unfortunately, these materials lack the advantageous mechanical properties

of titanium, the result being that titanium, with its improvement in osteoconductive behavior, is still attractive from the final clinical application perspective.²⁹

CONCLUSION

In conclusion, cyclic RGD peptide in combination with titanium fiber mesh has a positive effect, compared with titanium fiber mesh alone, on bone formation *in vivo* after 4 and 8 weeks when implanted in the skull of New Zealand White rabbits. Nevertheless, the amount of bone formation inside the mesh pores was still limited and further studies are required to optimize the bone biological effect of cyclic RGD peptide grafted on titanium fiber mesh scaffold.

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