Effects of Osteopromotive and Anti-infective Membranes on Bone Regeneration: An Experimental Study in Rat Mandibular Defects

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Purpose: *Reconstructive surgical treatment with osteopromotive membranes has become an integral part of implant dentistry. Nevertheless, there are still instances in which this technique alone is of limited or no benefit. The aim of the present study was to determine whether a combination of titanium membranes coated with transforming growth factor β1 (TGF-β1) and insulin-like growth factor I (IGF-I) is of value in the regeneration of so-called critical-size defects in the rat model. An analysis was made of whether or not locally administered antibiotics are deleterious to bone regeneration.* ***Materials and Methods:*** *A total of 24 rats were included in the study and were divided into 4 groups, each with 6 animals. Critical-size defects were created bilaterally and covered by titanium membranes coated with (1) polylactide, (2) polylactide and clindamycin, (3) polylactide and growth factors, or (4) polylactide, clindamycin, and growth factors. All 24 contralateral defects were covered by titanium membranes without any substrate (controls). Four weeks after treatment the animals were sacrificed.* ***Results:*** *In groups 3 and 4, most defects showed thin but almost complete bridging of the defects with new bone formation. In particular, clindamycin had no inhibitory effect on the regeneration of bone. Nevertheless, after 28 days, there was no significant difference between the individual groups (including controls) with respect to the total amount of newly formed bone.* ***Discussion and Conclusion:*** *These results support the hypothesis that coating titanium membranes with TGF-β1/IGF-I leads to almost complete bony bridging of critical-size defects without voluminous carrier materials. Moreover, simultaneous administration of clindamycin seems possible.* (Int J Oral Maxillofac Implants 2003;18: 369–376)

Key words: *antibiotics, artificial membranes, bone, growth factors, guided tissue regeneration, implant dentistry*

In recent years, the submerged-membrane technique has been recommended frequently to enhance bone regeneration in oral surgery.[1–5] Previous studies have demonstrated that membranes can promote osseous regeneration around experimentally created so-called critical-size defects, ie, bone defects that will not heal with bone during the lifetime of the animal.[2,5]

Another way of achieving new bone formation is the local administration of growth factors, especially those belonging to the bone morphogenetic protein (BMP) family.[1,6–8] Considerable interest has been focused on the role of recombinant human bone morphogenetic protein 2 (rhBMP-2)[3] and transforming growth factor β1 (TGF-β1).[4,5,9] It has been shown in vitro that TGF-β1 is synthesized by bone cells[10] and acts primarily on cells that are already committed to the osteoblastic lineage.[4,11,12] However, in the absence of periosteum, recombinant human TGF-β1 clearly has an inhibitory action on low-differentiated mesenchymal cells.[4]

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Table 1 Treatment of Defects in Each Group

Group no.	Carrier (PDLLA "R 203")	IGF-I	TGF-β1	Clindamycin
1 (n = 6)	1.6675 mg	—	—	—
2 (n = 6)	1.6675 mg	—	—	0.0667 mg
3 (n = 6)	1.6675 mg	0.0667 mg	0.0133 mg	—
4 (n = 6)	1.6675 mg	0.0667 mg	0.0133 mg	0.0667 mg

Another growth factor of considerable interest is human insulin-like growth factor I (IGF-I), which is produced by the liver.[4] It has also been shown in vitro that IGF-I has a minor positive effect on the proliferation of osteoblasts[13] and enhances the function of osteoblasts by increasing the expression of Type I collagen as well as the rate of bone matrix apposition, both of which contribute to increased bone formation.[13–15]

Consequently, the 2 factors could counteract each another. Nevertheless, the effects of growth factors in vivo have not been investigated thoroughly, particularly not in combination with the submerged-membrane technique. However, it has been shown recently that the combination of TGF-β1 with membranes is able to enhance significantly the amount of newly formed bone.[5] The latter growth factors were delivered with different carriers, rather than from the membranes themselves, because of the lack of a resorbable membrane coating that could act as a slow-release device.

During the last few years, a new biodegradable polylactide coating has been developed by the authors. It can absorb most drugs, especially growth factors and antibiotics, which could be helpful in preventing local infection. It has also been shown by this group that a combination of TGF-β1 and IGF-I results in a statistically significant acceleration of bone healing.[11] Nevertheless, it is not yet known whether titanium membranes coated with TGF-β1/IGF-I and clindamycin are of value in alveolar ridge augmentation. It is known from the literature that growth factors, especially TGF-β1, can have both osteopromotive and inhibitory effects on bone regeneration, depending on the species, anatomic region, and age and sex of the animals studied.[4,5] Accordingly, the purpose of this study was to analyze the effects of growth factor–coated titanium membranes in combination with a locally delivered antibiotic on bone healing in the rat mandibular model.

MATERIALS AND METHODS

Titanium Membranes and Drug Delivery

A total of 48 custom-made round titanium membranes (20 µm thick, 10 mm in diameter; Friadent, Mannheim, Germany) were used. For the solvent casting procedure, a poly (D,L-) lactide carrier (PDLLA) (R 203; Boehringer, Ingelheim, Germany), 30,000 Dalton molecular weight, was dissolved in chloroform. The following substances were incorporated according to the groups that were examined: recombinant human IGF-I (R&D Systems, Wiesbaden, Germany) (5% w/w); recombinant human TGF-β1 (R&D Systems) (1% by weight); and clindamycin (Pharmacia & Upjohn, Erlangen, Germany). The membranes were immersed into the respective solutions and dried under sterile conditions.

The following groups were examined:

- Group 1: Six titanium membranes coated with the polylactide carrier (PDLLA) alone
- Group 2: Six titanium membranes coated with PDLLA and clindamycin
- Group 3: Six titanium membranes coated with PDLLA and a mixture of TGF-β1 and IGF-I
- Group 4: Six titanium membranes coated with PDLLA and a mixture of TGF-β1 and IGF-I and additionally supplemented with clindamycin

Another 24 uncoated titanium membranes were used as controls.

The incorporated dose of growth factors in the coating and in the groups examined is shown in Table 1.

Animals and Anesthesia

The experimental study was approved by the appropriate institutional ethical committee (Regierung von Oberbayern) on November 3, 1999. A total of 24 female Sprague-Dawley rats were used (body

weight 450 to 500 g; Charles River, Cologne, Germany). The animals were maintained in standardized plastic cages with 12-hour day/night cycles. They were allowed free access to water ad libitum and standard laboratory pellets.

Prior to surgery, the rats were sedated with an intraperitoneal injection (3.3 mL/kg body weight) consisting of 25% fluanison (Hypnorm, 10 mg/mL; Janssen Pharmaceuticals, Beerse, Belgium); 25% midazolam (Dormicum, 5 mg/mL; Hoffman-La Roche, Basel, Switzerland); and 50% water ad injectabilia. Sedation was supplemented during surgery when needed.

In all cases, a local anesthetic was injected at the operation site (0.5 mL articain/adrenalin solution: Ultracain DS 1:200000; Aventis, Frankfurt, Germany). During the first week after surgery, the animals were given subcutaneous injections of buprenorphine twice a day for pain relief (Temgesic, 0.3 mg/mL; Reckitt & Colman, Hull, United Kingdom; 0.03 mg/kg body weight).

Surgical Procedure

Before the surgical intervention, both submandibular regions were shaved and disinfected with an iodine solution (Braunol; Braun, Melsungen, Germany). Extraorally, bilateral submandibular skin incisions were made. The mandibular rami were exposed through preparation of the soft tissues, muscles, and periosteum at both the buccal and the lingual aspects of the mandible. With a standardized round trephine bur (Friadent), transosseous defects 5 mm in diameter were created on both sides of the jaw during extensive cooling with saline (Fig 1). To prevent violation of the medial soft tissues, a raspatorium was positioned at the lingual aspect of the exposed mandible. The term "critical-size defect" implies that the lesion will not heal spontaneously during the lifetime of the animal.[2]

The animals were randomly divided into 4 groups of 6 rats each and allowed to heal for 28 days. In each group, each animal received 1 of the aforementioned coated membranes, and an uncoated membrane was placed contralaterally. The membranes were positioned randomly on the left- or right-side defect. Because the membranes were 10 mm in diameter, they extended about 2 mm outside the margins of the defect. They were adapted tightly to the surrounding bone and fixed by means of titanium nails (Frios-System; Friadent). Periosteum, soft tissues, and skin were carefully redraped and closed in layers.

Tissue Processing and Evaluation

After a healing period of 28 days, the animals were sacrificed by an overdose of Narcoren (Rhone Merieux, D-Laupheim, Germany) (80 mg/kg pentobarbital sodium). The mandibles, including the membranes, were resected without the surrounding soft tissues. After macroscopic photo documentation, the membranes were elevated and removed. Again, the regions of the defects were photographed.

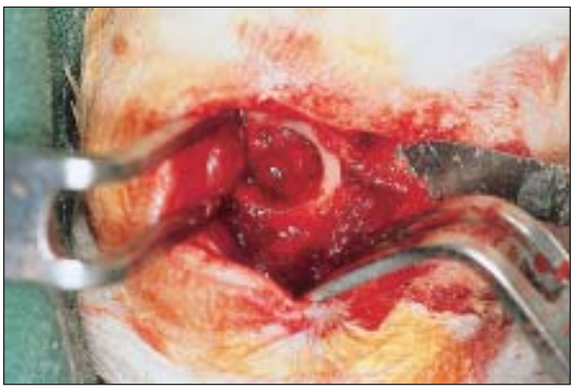

Fig 1 Transosseous critical-size defect (diameter 5.0 mm).

The specimens were immersed in 10% neutral buffered formalin, followed by decalcification in EDTA, dehydration in ethanol, and embedment in paraffin. Then the specimens were cut in horizontal sections (5 µm) and stained with hematoxylin-eosin.[4,5]

From each defect, the 3 most central sections were analyzed histologically. Bone ingrowth was qualified by means of light microscopy (Zeiss, Oberkochen, Germany). Important findings were compared first within each group, then among all 4 groups.

Histometry was performed by computerized image analysis (Quantimed 500 MC; Leica, Cambridge, United Kingdom). The amount of bony regeneration was expressed as a percentage of the total area.

Statistical analysis was performed using a commercial computer program (Excel, version 97; Microsoft, Munich, Germany). Data are presented as means ± standard deviation, maxima, and minima. Two-tailed Student *t* tests permitted comparison of the newly formed bone in the 4 treatment groups. A *P* value less than .05 in the 2-tailed test was considered to indicate statistical significance.

RESULTS

Clinical Observations

All 24 animals recovered well after surgery. Macroscopically, there were no signs of infection. After resection of the soft tissues, all titanium membranes could be seen in their proper positions. Accordingly, all 48 operation sites could be evaluated.

Fig 2a Specimen of the control group (macroscopic view). Minimal new bone formation is apparent on the rims of the defect.

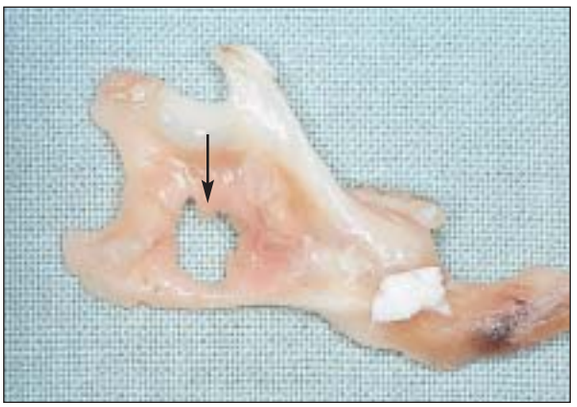

Fig 2b Specimen of group 1 (macroscopic view). There is evidence of new bone formation along the rims of the defect.

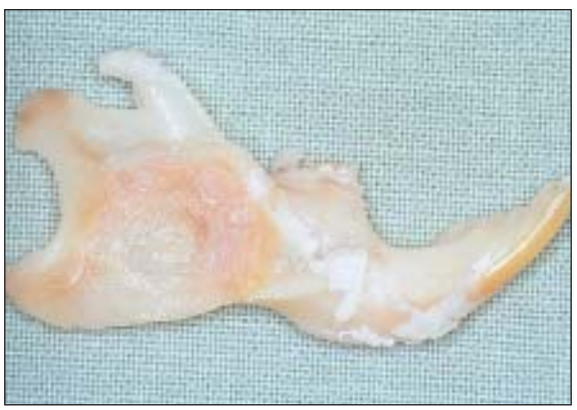

Fig 2c Specimen of group 2 (macroscopic view). Thin but complete bony regeneration of the defect is evident.

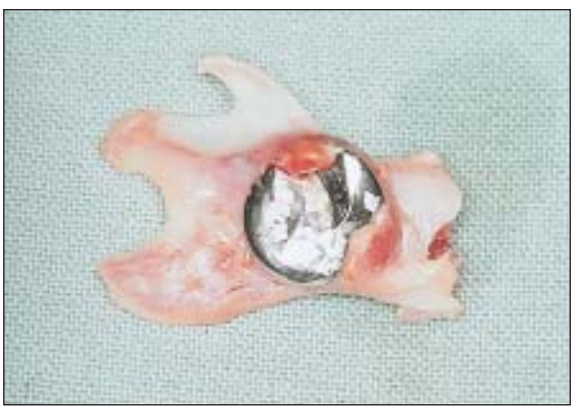

Fig 2d Specimen of group 3 (macroscopic view). Complete bony regeneration of the defect can be seen, with extraosseous bone formation on the buccal aspect of the membrane.

Macroscopically, most specimens of the control group and group 1 showed no bone regeneration at all (Figs 2a and 2b). In group 2, bone bridging was more advanced (Fig 2c). In group 3 (titanium membranes coated with a bioresorbable polylactide and a mixture of TGF-β1 and IGF-I), the membranes were partially covered with newly formed bone (Fig 2d). No other group, including group 4, showed a similar clinical result.

Histologic Observations

Microscopically, in most of the animals in the control group (contralateral defects covered with uncoated titanium membranes), no bony bridging at all was detected (Table 2). A complete bony bridging was found in only 1 specimen (n = 24). Nevertheless, on the rims of the defects, considerable amounts of new bone formation could be seen. Centripetally, most defects were filled with soft tissue that was relatively free of inflammatory cells.

In group 1 (titanium membranes coated with a bioresorbable polylactide), moderate amounts of new bone were also found at the rims of the defects. Newly formed woven bone was seen with active osteoblasts and, sporadically, osteoclasts. Centripetally, the defects were still filled with a fibrous tissue with vessels and hemosiderophageous cells. One section displayed inflammatory cells (lymphocytes, plasma cells, macrophages, neutrophile granulocytes, giant cells), which was consequently regarded as infection on the microscopic level.

In group 2 (titanium membranes coated with polylactide and clindamycin), bone bridging was more advanced, although only minor to moderate amounts of bone formation were detected at the rims of the defects. Some areas showed evidence of new lamellar bone adjacent to woven bone.

In group 3 (titanium membranes coated with a bioresorbable polylactide and a mixture of TGF-β1 and IGF-I), thin but almost complete bridging of

Table 2 No. of Defects Per Group Showing Complete, Incomplete, and No Bridging

Result	Controls (n = 24)	Group 1 (n = 6)	Group 2 (n = 6)	Group 3 (n = 6)	Group 4 (n = 6)
Complete bridging	1	1	2	4	3
Incomplete bridging	2	2	2	1	3
No bridging	21	3	2	1	0

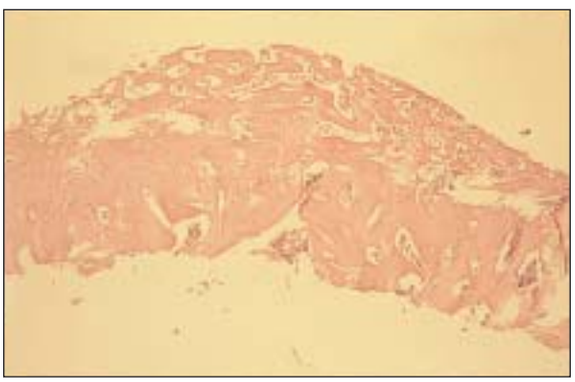

Fig 3 Specimen of group 3. Vivid formation of new woven bone is evident, with active osteoblasts and almost complete bony bridging of the defect (hematoxylin-eosin; magnification ×100).

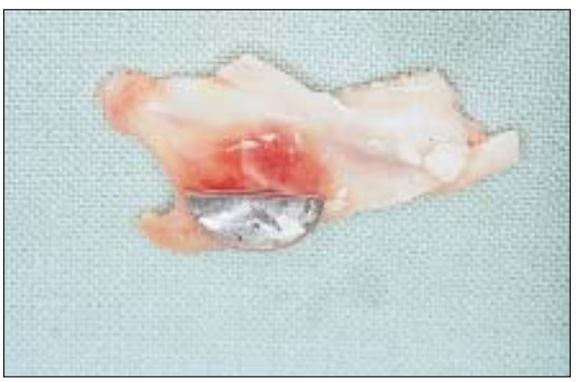

Fig 4 Specimen of group 4 (macroscopic view). Complete bony bridging of the defect is apparent.

the defects with new woven bone was seen in 4 of 6 specimens, partially transforming into lamellar bone and exostotic bone formation (Figs 2d and 3). The fifth specimen demonstrated complete bridging with soft tissue, whereas in the sixth specimen, minimal bone formation was seen only at the rims of the defects.

In group 4 (titanium membranes coated with a bioresorbable polylactide and a mixture of TGF-β1 and IGF-I and additionally supplemented with clindamycin), a vivid formation of new woven bone with active osteoblasts was seen, along with thin but almost complete bony bridging of the defects, partially transforming into lamellar bone (Figs 4 and 5). Only 1 specimen showed minimal formation of new woven bone. Nevetheless, exostotic reactions similar to those in group 3 were not seen.

A comparison of groups 1 to 4 and the control group with regard to numbers of bony bridgings showed similar results in groups 3 and 4 (thin bony bridgings), whereas most cases from groups 1 and 2 and the control group showed incomplete bridgings of the defects but considerable new bone formation on the rims of the defects (Table 2). With respect to the bone quality yielded in these groups 28 days after surgery, there were no significant differences at the light microscopic level. In all groups as well as in the controls, some areas showed new woven bone, with signs of remodeling toward a more compact structure, surrounding a developing marrow.

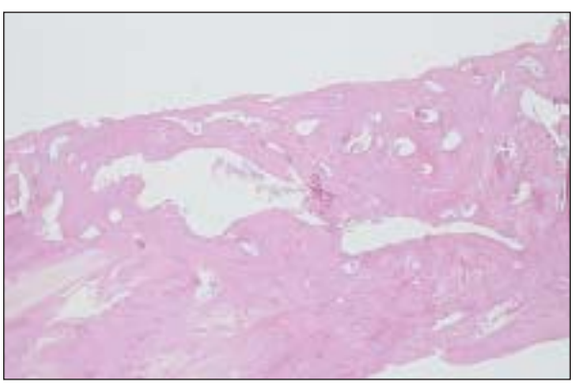

Fig 5 Specimen of group 4. Complete bridging of the defect can be seen, along with active osteoblasts and vivid formations of new woven and lamellar bone (hematoxylin-eosin; magnification ×200).

Histometry and Statistical Analysis

The amounts of new bone growth in the defects were evaluated as percentages of the former defect area, as shown in Table 3 and as bar graphs in Fig 6. In the group 1 specimens, the defects displayed an average percentage of bone fill of 22%; in group 2,

Table 3 Bone Ingrowth (%) of Critical-size Defects, Expressed as Percentage of the Total Area

Group no.	Mean	Standard deviation	Minima	Maxima
1 (n = 6)	22	11.7	20	80
2 (n = 6)	38	11.7	40	100
3 (n = 6)	32	16.4	20	100
4 (n = 6)	33	13.6	20	100
Control (n = 24)	25	17.2	0	100

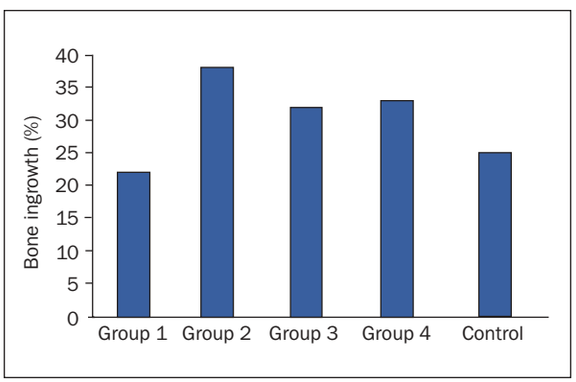

Fig 6 Graphical overview of the newly formed bone in groups 1 to 4 and controls. Concerning the amounts of newly formed bone after 24 days, no statistically significant difference could be found.

Table 4 Statistical Analysis of Bone Ingrowth

Tested correlation	Mean ± SD	z value	Difference significant?
Group 1/control	22 ± 11.7/25 ± 17.2	–0.59	No
Group 2/control	38 ± 11.7/25 ± 17.2	2.48	No
Group 3/control	32 ± 16.4/25 ± 17.2	0.95	No
Group 4/control	33 ± 13.6/25 ± 17.2	1.31	No

According to the Student *t* test, there were no statistically significant differences in osseoregeneration between all groups at the .05 level.

average bone fill was 38%; in group 3, 32% bone fill was achieved; and in group 4, 33% bone fill was seen. The control specimens demonstrated an average bone fill of 25%.

No significant differences in bone fill were detected among all 4 experimental groups and the controls after 28 days ($P \geq .05$) (Table 4).

DISCUSSION

In this study, titanium membranes coated with growth factors and clindamycin were tested in critical-size defects in rat mandibles. The aims of the study were to evaluate *(1)* the osteopromotive potential of a combination of the growth factors TGF-β1 and IGF-I, *(2)* whether clindamycin itself has an influence on bone healing, and *(3)* whether the quality of the newly formed bone is affected by these substances.

To date, there have been only a few studies dealing with TGF-β1 in vivo, whereas most interest has been focused on rhBMP-2. It was shown by Kübler and coworkers in the rat mandibular model[3] that carrier materials have a significant influence on the volume of bone that is newly formed by osseoinductive substances. A total of 10 µg rhBMP-2 added to granulates (such as resorbable β-tricalcium phosphate)

resulted in significantly greater bone volumes than materials consisting of smooth fleeces (such as L-lactide/polyglycolide/polydioxanon). Nevertheless, all carrier materials soaked with BMP-2 resulted in a total bony bridging of the critical-size defects. On the other hand, none of these carriers was able to induce a bony regeneration of the defects without BMP-2.

Accordingly, in the present study, histology demonstrated only 1 specimen each that was totally bridged with new bone in both the control group (uncoated membranes) and in group 1 (titanium plus PDLLA). However, it should not be concluded that titanium membranes, either alone or in combination with a polylactide coating, are of more value in bone regeneration in the rat mandibular model than carriers filled into the defects.

On the light microscopic level, there was a difference between the histometrically evaluated bone ingrowth of the defects (related to the total defect area) and the number of complete bony bridgings. This can be explained by the observation that in some cases histologic analysis showed very thin but complete bony bridgings, and in others there was extensive formation of new bone but only at the borders of the defects. This discrepancy accounts for the fact that no statistically significant differences in percentage of new bone growth were found between groups in corresponding histologic sections, whereas the number of complete bony bridgings clearly differed between groups (Tables 2 to 4).

Total bony bridging could be seen in most of the defects treated with membranes that were coated with polylactide and a combination of TGF-β1 and IGF-I (group 3). It was reported by Zellin and Linde[5] that TGF-β1 has a clearly inhibitory effect on bone regeneration in combination with e-PTFE membranes. In that study, periosteal cells were totally excluded from bone healing, as the defects were covered by the membranes from the medial aspect as well as from the lateral aspect. In the present study, an inhibitory effect was not found, because cells from the medial periosteum could reach the defects. Consequently, it can be concluded that titanium membranes coated with polylactide and a combination of TGF-β1 and IGF-I may also be of value in the regeneration of critical-size defects in the presence of periosteal cells. Nevertheless, in a future study, the combination of TGF-β1 and IGF-I should also be assessed in the absence of periosteal cells.

In another recent study, rhTGF-β1 and resorbable carriers were placed in surgically prepared canine alveolar ridge defects.[16] A healing time of 2 months was allowed. In contrast to Zellin and Linde's results, no statistically significant difference was noted between low-dose and high-dose treatments. Inhibitory effects were not seen. The authors concluded that the use of rhTGF-β1 in conjunction with a barrier membrane greatly enhances bone regeneration in osseous oral defects. It is not yet clear whether these differences were a result of the different species that were used or the different healing times.

Concerning the osseoregenerative potentials, it was shown by Zellin and coworkers[4] that rhBMP-2 is significantly more effective than rhTGF-β1. The authors found that rhTGF-β1, irrespective of carrier materials and total dose applied, was able to facilitate a maximum of 30% bony regeneration in rat mandibular critical-size defects after 24 days.[4] These results are very similar to those yielded by histometry after 28 days in the present study. Therefore, according to the literature, it may be assumed that the combination of TGF-β1 and IGF-I is not more effective in bone healing than TGF-β1 alone after 28 days.

Schmidmaier and coworkers[9] reported that fractures of tibiae that were stabilized by intramedullary steel pins and coated with PDLLA and the same combination of TGF-β1 and IGF-I that were used in this study showed complete bony regeneration in the rat model after 42 days. That study used juvenile rats (5 months of age), whereas the present authors used adult animals. The differences in the healing process, therefore, could be caused by both a longer follow-up period on the one hand and a different healing biology related to the younger age of the animals on the other hand.

Titanium membranes that were additionally coated with clindamycin (groups 2 and 4) also yielded bony regeneration of critical-size defects. Thus, it can be additionally concluded that moderate concentrations of clindamycin are not detrimental to bone healing.

In summary, it can be concluded that titanium membranes coated with a combination of TGF-β1 and IGF-I in the concentrations employed in this study, with or without additional coating with clindamycin, may be of value in the regeneration of so-called critical-size defects. Moreover, it should be pointed out that, in contrast to the study of Kübler and associates,[3] the osteopromotive factors were not filled into the defect. The bone cells could only be reached by diffusion. Consequently, this new approach to the application of growth factors can be considered effective in the rat model.

With regard to the histologic results, the combination of growth factors tested had a favorable influence on the time required for bony bridging of

the defects. However, none of the pharmacologically active combinations used in this study had an influence on the quality of bone yielded. Furthermore, there are no reports in the literature indicating that growth factors render optimization of newly formed bone with regard to the ratio of woven bone to lamellar bone after 24 days.[4] Consequently, it must be concluded that the combination of TGF-β1 and IGF-I does not enhance the bone quality within the first 28 days of bone healing. Nevertheless, bioactively coated membranes can accelerate the healing process of bony defects in the rat mandibular model.

CONCLUSION

The results of this study show that titanium membranes coated with a combination of TGF-β1 and IGF-I are of value in the bone regeneration of critical-size defects in the rat mandibular model. Furthermore, it could be demonstrated that an additional coating of the membranes with clindamycin was not deleterious to osseoregeneration. Nevertheless, it was not possible to enhance the quality of bone within the time period examined. These in vivo results will require clinical verification.

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