ORIGINAL ARTICLE

Osteoblast proliferation and differentiation on a barrier membrane in combination with BMP2 and TGFβ1

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Abstract

Objectives Bioresorbable collagen membranes are routinely utilized in guided bone regeneration to selectively direct the growth and repopulation of bone cells in areas of insufficient volume. However, the exact nature by which alveolar osteoblasts react to barrier membranes as well as the effects following the addition of growth factors to the membranes are still poorly understood. The objective of the present study was therefore to investigate the effect of a bioresorbable collagen membrane soak-loaded in growth factors bone morphogenetic protein 2 (BMP2) or transforming growth factor β1 (TGFβ1) on osteoblast adhesion, proliferation, and differentiation.

Material and methods Prior to experimental seeding, membranes were soaked in either BMP2 or TGFβ1 at a concentration of 10 ng/ml for 5 min.

Results Human osteoblasts adhered to all soak-loaded membranes as assessed by scanning electron microscopy. Growth factors BMP2 and TGFβ1 increased osteoblast proliferation at 3 or 5 days post-seeding when compared to control collagen membranes. Analysis of real-time PCR revealed that administration of BMP2 increased osteoblast differentiation markers such as osterix, collagen I, and osteocalcin. BMP2 also increased mineralization of primary osteoblasts as demonstrated by alizarin red staining when compared to control and TGFβ1 soak-loaded membranes.

Conclusion The combination of a collagen barrier membrane with growth factors TGFβ1 and BMP2 significantly influenced adhesion, proliferation, and differentiation of primary human osteoblasts.

Clinical relevance The described in vitro effects following the combination of collagen barrier membranes with growth factors TGFβ1 and BMP2 provide further biologic support for the clinical application of this treatment strategy in guided bone regeneration procedures.

Keywords Barrier membranes · Growth factors · Guided bone regeneration · GBR · GTR

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Introduction

In guided tissue regeneration (GTR) and guided bone regeneration (GBR), a barrier membrane is utilized to selectively direct the growth and repopulation of periodontal ligament and bone cells in periodontal and bone defects [1, 2]. Regenerative surgery involving the use of GTR has proven to be an effective method for periodontal and bone regeneration and is widely used for the treatment of periodontal and bone defects [1, 3–7]. Since its first clinical use in the early 1980s [8, 9], new innovative materials have been designed to increase the effectiveness of the barrier membranes. Such membranes should be capable of facilitating cell attachment, increasing cell proliferation, and

promoting cell migration to the surface of the underlying defect [10–13].

A variety of synthetic and natural bioresorbable barriers have been fabricated and studied both in vitro and in vivo [3–6, 14–17]. Such materials must provide biocompatibility, tissue integration, cell occlusivity, space-making ability, and clinical ease of use [18]. The first generation of barrier membranes was fabricated from nonbioresorbable expanded polytetrafluorethylen but required a second surgery to remove the barrier thus bearing the possibility of damaging the newly formed tissue and increasing patient morbidity. Chance of reinjury of the newly formed periodontal and bone tissues combined with crestal resorption of the alveolar bone and bacterial colonization were commonly reported [19, 20]. More recently, synthetic bioresorbable membranes were fabricated primarily from polylactic, polyglycolic acids, and collagen filaments fabricated in multiple cross-linking patterns and techniques, such as ultraviolet light, glutaraldehyde, diphenylphosphoryl azide, and hexmethylene diisocyanate, in order to prolong degradation of collagen filaments by enzymatic activity of infiltrating macrophages and leukocytes [21–27]. Many of the commercially available GTR membranes fabricated from porcine collagens I and III showed similar clinical results without the need of a second surgery [3, 18, 28–30] being fully resorbable 6 months postsurgery [14, 31].

A novel approach to GBR is the use of growth factors which promote the regeneration of selective tissues. Of particular relevance are the use of bone morphogenetic protein (BMP) and transforming growth factor β (TGFβ); both BMPs and TGFβ have been shown to increase the proliferation and differentiation of mesenchymal cells and osteoblasts in vitro [32–39] and improve the speed and quality of new bone formation in vivo [40–46]. In an attempt to further improve clinical outcomes, dental clinicians recently introduced the combination of GTR barrier membrane procedures with growth factors such as BMP2 and TGFβ1 [47–50]. However, the exact nature by which alveolar osteoblasts react to barrier membranes as well as the effects following addition of growth factors to the membranes are still poorly understood. Therefore, the aim of the present study was to investigate the effect of a bioresorbable collagen membrane soak-loaded in growth factors BMP2 or TGFβ1 on osteoblast adhesion, proliferation, and differentiation.

Methods

Membrane coating with BMP2 and TGFβ1

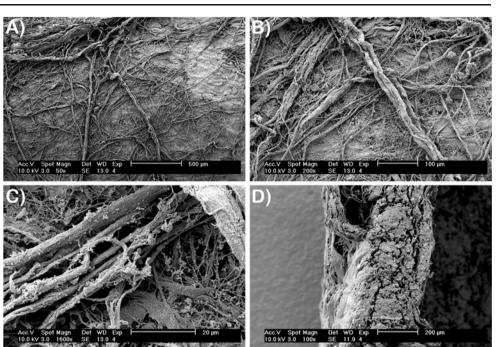The GTR membranes used in this study were a 30×40-mm bioresorbable bilayer collagen membrane (Geistlich Bio-

Gide®, Wolhusen, Switzerland). It is obtained from porcine collagen under standardized and certified procedures. For in vitro experiments, membranes were cut under sterile conditions to fit in the bottom of 24- and 6-well culture plastic dishes. Prior to experimental seeding, membranes were soaked in either BMP2 (Part # 892143, R&D Systems, Minneapolis, USA) or TGFβ1 (Part # 891127, R&D Systems, Minneapolis, USA) at 10 ng/ml [51, 52] for 5 min to simulate clinical application. All images viewed in this study are oriented to visualize membrane sides that are intended to guide bone regeneration as opposed to epithelial tissue.

Osteoblast cell isolation and differentiation

Human bone chips were cultured according to an explant model [53] under signed informed consent approved by the ethics committee of the Canton of Berne, Switzerland as previously described [54]. Primary human alveolar osteoblasts from three donors not demonstrating any signs of periodontal disease were detached from the tissue culture plastic using trypsin solution (Invitrogen, Basel, Switzerland). Cells used for experimental seeding were from passages 4–6. During cell seeding, α-MEM medium was supplemented with 50 μg/ml ascorbic acid and 2 mM β-glycerophosphate to promote osteoblast differentiation. Primary osteoblasts were seeded on membranes at a density of 10,000 cells in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) for cell attachment, cell proliferation, and scanning electron microscopy (SEM) experiments and 50,000 cells for alizarin red experiments. For PCR experiments, cells were seeded at 100,000 cells on membranes in six-well culture plates. For experiments lasting longer than 5 days, medium was replaced twice weekly.

Adhesion and proliferation assays

Primary osteoblasts were seeded on the growth factor soak-loaded and non-loaded bioresorbable collagen membranes in 24-well plates at a density of 10,000 cells per well. Cells were quantified using measurement of DNA at 4 and 8 h for cell adhesion and 1, 2, 3, and 5 days for cell proliferation. At desired time points, the cells were washed with PBS and lysed by ultrasonic homogenization in 400 μl of 0.1 % (v/v) Triton X-100 (Sigma Aldrich, Basel, Switzerland). The DNA contents of the cell extracts were determined using a commercial kit including the fluorescent dye Picogreen (QuantIT, Invitrogen) under standard protocol. Fluorescent readings were performed on an Infinite 200 microplate reader (Tecan Group Ltd. Männedorf, Switzerland) at an

Fig. 1 SEM analysis of collagen barrier membrane. **a, b** Membrane surface reveals many collagen fibrils that are intertwined with one another with various diameters and directions (magnification $A=\times 50$, $B=\times 200$). **c** High-resolution SEM demonstrates collagen fibrils ranging in diameter between 1 and 5 μm (magnification$=\times 1,600$). **d** Cross-sectional view of collagen barrier membrane of approximately 300 μm (magnification$=\times 100$)

excitation wavelength of 480 nm and an emission reading of 520 nm. Experiments were performed in triplicate with three independent experiments for each condition. Data were analyzed for statistical significance using two-way analysis of variance with Bonferroni test.

Scanning electron microscopy

For SEM, bioresorbable membranes were fixed in 1 % glutaraldehyde and 1 % formaldehyde with or without osteoblasts seeded at a density of 10,000 cells per 24-well culture. Following dehydration with ethanol, samples were dried to a critical point (Type M.9202 Critical Point Dryer, Roth & Co. Hatfield, PA, USA). Next, the samples were sputtered (DCM-010, Balzers, Liechtenstein) with a 10-nm layer of gold and analyzed microscopically using a scanning electron microscope (XL30 FEG, Philips, Netherlands) to determine micro- and nano-topographies of bioresorbable membrane as well as cell shape of osteoblasts attached to membrane surfaces.

Real-time PCR

Total RNA was isolated using TRIZOL reagent and RNAeasy Mini kit (QIAGEN, Basel, Switzerland) at time points 3 and 10 days for osteoblast differentiation markers. Primer and probe sequences for genes encoding alkaline phosphatase (ALP, Hs01029144_m1), runt-related transcription factor 2 (Runx2, Hs00231692_m1), collagen1α1 (COL1A1, Hs01028970_m1), osteocalcin (OC, Hs01587814_g1), and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH, Hs03929097_g1) were purchased as pre-designed gene expression assays (Applied Biosystems, Basel, Switzerland). Real-time (RT)-PCR was performed using 20 μl final reaction volume of TaqMan® One-Step Master Mix kit. RNA quantification was performed using a Nanodrop 2000c with 100 ng of total RNA was used per sample well. All samples were assayed in triplicate and three independent experiments were performed. The $\Delta\Delta$Ct method was used to calculate gene expression levels normalized to GAPDH values and calibrated to control membranes without additional growth factors at 3 days. Data were log-transformed prior to analysis by two-way ANOVA with Bonferroni test using GraphPad Software v. 4 (GraphPad Software, La Jolla, CA, USA).
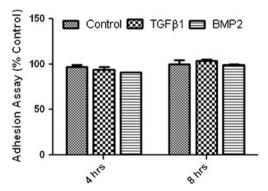

Fig. 2 Attachment assay of 10^4 primary human osteoblasts seeded on control, TGFβ1 soak-loaded, or BMP2 soak-loaded barrier membranes as assessed by total dsDNA. No significant difference in cell attachment could be observed for all groups at all time points
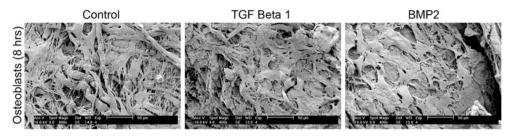
Fig. 3 SEM analysis of primary human osteoblasts seeded on control (**a**), TGFβ1 soak-loaded (**b**), and BMP2 soak-loaded (**c**) collagen barrier membranes at 8 h post-seeding. Osteoblasts attach and spread well on all surfaces demonstrating the excellent biocompatibility of collagen barrier membranes with or without growth factor incorporation

Alizarin red quantification

Alizarin red staining was performed to determine the presence of extracellular matrix mineralization after 14 days. Osteoblasts were seeded at a density of 50,000 cells per 24-well culture dish onto bioresorbable collagen membranes. After 14 days, cells were fixed in 96 % ethanol for 15 min and stained with 0.2 % alizarin red solution in water (pH 6.4) at room temperature for 1 h. Alizarin red was dissolved using a solution of 20 % methanol and 10 % acetic acid in water for 15 min. Liquid was then transferred to cuvettes and read on a spectrophotometer at a wavelength of 450 nm. After subtraction of background, absorbance values were normalized to DNA content. Data were analyzed for statistical significance using one-way analysis of variance with Tukey's test.

Results

Barrier membrane visualization

Collagen barrier membranes developed from porcine origin are viewed in Fig. 1 by SEM. Membrane sides intended to guide bone regeneration show a high composition of collagen in fibrillar form (Fig. 1a, b). High-resolution SEM demonstrates various diameter sizes of collagen fibrils ranging in size from 1 to 5 μm (Fig. 1c). The cross-sectional view of collagen barrier membranes is presented in Fig. 1d.

Adhesion of osteoblasts to barrier membranes

At 4 and 8 h post-seeding, primary human osteoblasts adhered to collagen membranes irrespective of coating with TGFβ1 or BMP2 displaying its excellent biocompatibility (Fig. 2). In 4 h, osteoblasts seeded on barrier membranes displayed near 100 % adhesion. SEM analysis of primary osteoblasts seeded on barrier membranes was visualized at 8 h post-seeding (Fig. 3). All osteoblasts attached well on all surfaces and displayed excellent cell spreading on each surface (Fig. 3).

Osteoblast proliferation on barrier membranes

Osteoblast numbers were quantified at 1, 3, and 5 days post-seeding (Fig. 4). At 1 day, no significant difference was observed between all groups (Fig. 4). At 3 days post-seeding, barrier membranes soak-loaded with TGFβ1 and BMP2 displayed significant increases in cell numbers when compared to control non-soaked barrier membranes (Fig. 4). Similar patterns were also observed at 5 days post-seeding with barrier membranes soak-loaded with BMP2 showing the highest rate of proliferation at both 3 and 5 days post-seeding (Fig. 4).

Osteoblast differentiation on barrier membranes

Osteoblasts were assessed for Runx2, OC, COL1α1, and OSX at 3 and 14 days post-seeding. Analysis of the transcription factor Runx2 gene expression showed no significant differences in mRNA levels at 3 and 14 days for all groups (Fig. 5a). COL1α1 mRNA levels showed significantly higher values of mRNA at 3 and 14 days post-seeding
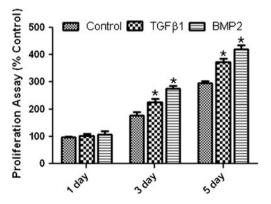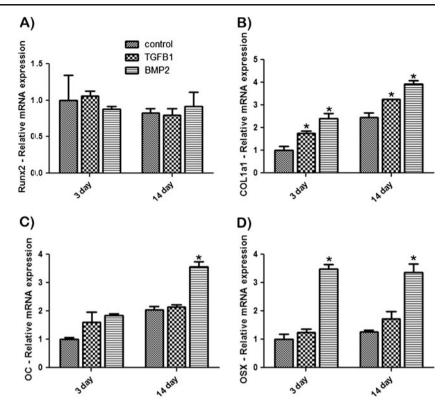

Fig. 4 Proliferation assay of 10^4 primary osteoblasts seeded on control, TGFβ1 soak-loaded, or BMP2 soak-loaded barrier membranes as assessed by total dsDNA. (*Asterisk* denotes significant difference, $p < 0.05$)

Fig. 5 Real-time PCR of osteoblasts seeded on control, TGFβ1 soak-loaded, and BMP2 soak-loaded barrier membranes for genes encoding **a** Runx2, **b** Col1α1, **c** OC, and **d** OSX. (*Asterisk* denotes significant difference, *p*<0.05)

for osteoblasts seeded on barrier membranes containing either TGFβ1 or BMP2 (Fig. 5b). Up to a twofold increase in COL1α1 mRNA levels was observed for BMP2 soak-loaded barrier membranes. At 14 days post-seeding, a significant increase in mRNA expression of OC was observed for osteoblasts seeded on BMP2-treated barrier membranes when compared to control and TGFβ1 samples (Fig. 5c). OSX also displayed up to a threefold increase in mRNA expression in BMP2 soak-loaded membranes when compared to control and TGβF1 samples (Fig. 5d). Alizarin red staining at 14 days demonstrated significantly increased mineralization for osteoblasts seeded on BMP2 soak-loaded barrier membranes when compared to control and TGFβ1 samples (Fig. 6).

Discussion

Over the past 20 years, the use of barrier membranes has dramatically improved clinical outcomes in patients with significant bone loss [3–6]. Furthermore, the combination of barrier membranes and different bone grafts has been shown to additionally ameliorate the results [55–57]. In the present study, the hypothesis that additional growth factors such as TGFβ1 and BMP2 would additionally improve the outcomes generated from resorbable collagen barrier membranes was tested on osteoblast adhesion, proliferation, and differentiation. Since the majority of our understanding for the TGFβ superfamily comes from loss-of-function studies which result in embryonic

lethality, much of our understanding of the regulatory mechanisms of action for either growth factor is determined using in vitro studies [33]. Results from the present study would support the use of either growth factor in GTR or GBR procedures.

Previously, it has been shown that TGFβ1 enhances osteoblast proliferation and differentiation by stimulating expression of ALP, BSP, and osteonectin as determined by immunohistochemistry, RT-PCR analysis, and in vitro mineralization [58, 59]. In the present study, it was found that TGFβ1 had an effect on osteoblast proliferation. Still, only limited additional benefits were observed during differentiation of osteoblasts as assessed by real-time PCR experiments and alizarin red staining. Interestingly, it has been
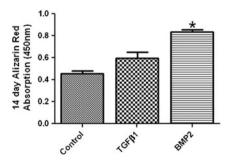

Fig. 6 Normalized alizarin red staining absorbance values at 14 days post-seeding. BMP2 soak-loaded barrier membranes displayed significantly higher mineralization when compared to control and TGFβ1 soak-loaded barrier membranes. (*Asterisk* denotes significant difference, *p*<0.05)

reported that primary human osteoblasts harvested from different age groups showed that TGFβ1 had a more pronounced effect on cells harvested from old patients as opposed to young and middle-aged patients [60].

The other growth factor tested in this study, BMP2, seems to be the growth factor of choice for clinical application in a variety of surgical procedures including dentistry, orthopedics, fracture healings, and spinal fusions [61–63]. In three studies comparing BMP2 and TGFβ1, BMP2 was able to stimulate osteoblast proliferation and/or differentiation such as ALP and OC in murine cell lines MC3T3-E1 and C3H10T1/2, while TGFβ1 had a less pronounced effect [37, 64, 65]. Intriguingly, neither BMP2 nor TGFβ1 had an effect on Runx2 in the current study, suggesting actions independent or downstream of this osteoblast-specific transcription factor. BMP2 induced the expression of OSX while TGFβ1 had only moderate effects (Fig. 5). Previously, it has been demonstrated that BMP2 induces OSX expression through upregulation of Dlx5 and its phosphorylation by p38 [66].

Previously, the combination of BMP2 for clinical application has been investigated both in vivo and in clinical trials [47–50]. Cochran et al. tested rhBMP-2 using a collagen sponge carrier to stimulate bone formation in defects in the canine mandible around endosseous dental implants. The addition of rhBMP-2 significantly enhanced new bone area and percentage of bone-to-implant contact after 4 and 12 weeks of healing [47]. In a subsequent study, the use of BMP2 for patients receiving rhBMP-2 loaded in an absorbable collagen sponge (ACS) in human extraction sites or in sites that required alveolar ridge augmentation demonstrated safety 2 years following surgical implantation of rhBMP-2/ ACS (0.43 mg/ml) [48].

More recently, Sawyer et al. demonstrated in a rat model that the release of rhBMP2 from a collagen scaffold is a clinically applicable approach for the repair and regeneration of critically sized craniofacial bone defects [49]. However, a randomized controlled clinical trial evaluating the long-term outcome of implants placed in bone augmented with a xenogenic bone substitute material and a collagen membrane with or without the addition of rhBMP-2 demonstrated no statistically significant differences between test and control sites after 3 and 5 years posttreatment [50].

Results from previous animal and human studies combining BMPs with collagen membranes have demonstrated mixed outcomes [47–50, 67]. One question that remains unresolved is the effect/use of high doses of rhBMP2 for single application procedures. In the present in vitro study, the local use of BMP at a concentration of 10 ng/ml was able to stimulate an effect in primary human osteoblasts. Previously, lower concentrations of BMP2 combined to 3D poly(lactic-co-glycolic acid) scaffolds ranging in doses from 30 to 240 ng/mm^3 were able to increase bone regeneration in a 5-mm critical sized rat calvarial defect in a dose-

dependent manner [67]. Clinically, the use of doses ranging from 4 to 8 mg has been reported, an increase of almost 1,000 times the concentration used in the present study. Since collagen barrier membranes are able to adsorb growth factor proteins, it is plausible that a lower concentration may be required. These observations further indicate the necessity to accurately administer the appropriate doses of growth factors for specific clinical procedures. The use of an animal model to test the effect of both TGFβ1 and BMP2 in combination with a collagen barrier membrane is, however, mandatory before clinical application.

In conclusion, the combination of a collagen barrier membrane with growth factors TGFβ1 and BMP2 significantly influenced adhesion, proliferation, and differentiation of primary human osteoblast. All osteoblasts attached well to membranes irrespective of additional growth factors. Both TGFβ1 and BMP2 significantly enhanced osteoblast proliferation while BMP2 additionally increased osteoblast differentiation. The combination of collagen membranes with soak-loaded growth factors may bear clinical relevance by additionally improving healing following GBR. Further in vivo studies are thus warranted to support the clinical relevance of this treatment approach.

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