FGF-2-loaded collagen scaffolds attract cells and blood vessels in rat oral mucosa

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BACKGROUND: Wound contraction and scar formation after cleft palate repair impair the growth of the maxilla. The implantation of a growth factor-loaded scaffold might solve these problems.

METHODS: The tissue response to fibroblast growth factor (FGF)-2 loaded collagen scaffolds was evaluated after implantation in the palate of rats. Scaffolds, with and without FGF-2, were implanted submucoperiosteally in the palate of 25 rats and evaluated after up to 16 weeks. On hematoxylin and eosin (H&E)-stained sections, the cell density and the number of giant cells within the scaffolds were quantified. Infiltration of inflammatory cells, myofibroblasts, and the number of blood vessels were quantified after immunohistochemistry.

RESULTS: The cell density was significantly higher in the FGF-2 group up to 4 weeks after implantation (102% at 2 weeks, *P* < 0.001). The number of blood vessels was also significantly higher in the FGF-2 group at 1 and 2 weeks (316% at 1 week, *P* = 0.003), but the myofibroblast score was lower (100% at 2 weeks, *P* = 0.008). A comparable mild and rapidly subsiding inflammatory response and foreign body reaction were found in both groups.

CONCLUSION: FGF-2-loaded scaffolds displayed a faster influx of host cells, an increased rate of vascularization, and a reduced differentiation of myofibroblasts. These scaffolds might therefore be highly suitable for intra-oral reconstructions, such as cleft palate repair.

Keywords: FGF-2; in vivo test; oral; scaffold; wound healing

Introduction

Wound contraction and scar formation impair the growth of the maxilla and the development of the dento-alveolar complex after cleft palate repair (1). Commonly used approaches for repairing soft tissue defects involve autologous grafts taken from a different part of the oral cavity (2). However, often too little tissue is available and donor site morbidity can be a problem (3). Another solution for the lack of tissue is the use of biomaterials as a scaffold for a tissue substitute. Collagen is a promising material and has found diverse applications in tissue engineering because of its biocompatibility and biodegradability (4–6). Cross-linking of the collagen scaffolds is an effective method to improve the biostability and the mechanical properties (7). Vascularization is a prerequisite for appropriate tissue regeneration and function. Angiogenic factors like fibroblast growth factor 2 (FGF-2) have been studied extensively *in vitro* (8) and *in vivo* (9, 10). In addition to its ability to promote vascularization, FGF-2 is known to inhibit the differentiation of fibroblasts into myofibroblasts, thus diminishing wound contraction (11). Collagen scaffolds alone already reduce wound contraction, but loading with FGF-2 has an additional effect in skin (12). FGF-2-loaded collagen scaffolds also show a faster vascularization in skin (9). However, wound healing in skin and oral mucosa is quite different because of environmental differences, differences in tissue architecture, and differences in fibroblast properties (13–15). FGF-2 injected in the palatal mucosa of rats accelerated wound healing (16) and vascularization (17). However, the effect on myofibroblasts was not studied. So far, no experimental data are available on FGF-2-loaded collagen scaffolds implanted in an oral environment.

In this study, collagen scaffolds with and without FGF-2 were implanted submucoperiosteally in the palate of rats. The tissue response to these scaffolds was evaluated and quantified. Our hypothesis was that scaffolds loaded with FGF-2 reduce the number of myofibroblasts and increase the rate of vascularization.

Materials and methods

Animals

Fifty 5-week-old male Wistar rats (Harlan, Zeist, The Netherlands), weighing between 106 and 166 g, were
used. All animals were kept under normal laboratory conditions and were fed standard rat chow and water *ad libitum*. The rats had been acclimatized to the animal housing facility for 1 week before the start of the experiment. The experiment was approved by the Board for Animal Experiments of Radboud University Nijmegen.

**Collagen scaffolds**

Type I collagen was purified from bovine Achilles tendon, and a chemically cross-linked scaffold was prepared (18). Briefly, a 0.8% (w/v) type I collagen suspension in diluted acetic acid was shaken overnight at 4°C and homogenized on ice using a Potter-Elvehjem homogenizer. Air bubbles were removed by centrifugation at 250 g for 10 min at 4°C. The suspension was then slowly poured into a plastic mould (10 ml/25 cm²), frozen in a bath of ethanol and solid CO₂ (-80°C), and lyophilized in a lyophilizer (Zirbus, Bad Grund, Germany). Scaffolds were cross-linked using 33 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 6 mM N-hydroxysuccinimide (NHS) in 50 mM 2-morpholinoethane sulphonic acid (MES) pH 5.5 containing 40% ethanol for 4 h at 22°C. Scaffolds were then washed with 0.1 M Na₂HPO₄, 1 M NaCl, 2 M NaCl, and MilliQ water, frozen in ethanol/CO₂ again and lyophilized.

**FGF-2-loaded scaffolds**

Recombinant rat FGF-2 was produced in *Escherichia coli* and purified with a heparin-sepharose column. FGF-2 was loaded onto the EDC/NHS-cross-linked collagen scaffolds by incubating them in 7 µg/ml growth factor in phosphate-buffered saline (PBS) (pH 7.2) per 8 mg scaffold for 30 min, followed by washings with PBS. About 372 ± 75 µg FGF-2/g scaffolds is bound to the scaffolds (9).

**Surgical procedures**

The rats were anesthetized with an intraperitoneal injection of 1 ml/kg body weight of ketamine (Nimatek, Eurovet, Bladel, The Netherlands) combined with an intraperitoneal injection of 0.25 ml/kg body weight of xylazine (Sedamun; Eurovet, Bladel, The Netherlands). A standardized transversal incision of 3 mm was made to the palatal mucoperiosteum at the level of the contact points between the first and second molar. The mucoperiosteum was then elevated caudally for three mm with a periodontal probe to create a submucoperiosteal envelope. A circular collagen scaffold with a diameter of 3 mm was placed in the envelope and a 10 x 0 vicryl suture was used to close it. Twenty-five rats received a cross-linked scaffold without FGF-2. The other twenty-five rats received a FGF-2-loaded collagen scaffold. The rats were medicated post-operatively with 0.02 mg/kg body weight buprenorfine (Temgesic®; Schering Plough, Brussels, Belgium) subcutaneously as an analgesic.

**Study design**

Groups of five rats were killed at 1, 2, 4, 8 and 16 weeks post-implantation and processed for histological analyses of the wound tissue.

**Histology**

The rats were perfused using freshly prepared 4% paraformaldehyde in PBS. The rats were then decapitated, and the palate was dissected from the skull. The palate samples were fixed in 4% paraformaldehyde solution for 24 h. Hereafter, the samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) in PBS at 4°C. Decalcification was checked with radiographs. All samples were embedded in paraffin. Serial paraffin sections of 6 µm were cut in the transversal plane and stained with hematoxylin and eosin (H&E, according to Delafield). Histomorphometric analyses were performed blindly using an ocular micrometer. The total number of giant cells and the cell density in the scaffolds were determined for every sample. The cell density in the scaffolds was determined with a microscopic grid and expressed as the number of cells/mm².

**Immunohistochemistry**

Paraffin sections were collected on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized, and rehydrated. Before staining, the slides were rinsed in PBS for 10 min. α-Smooth muscle actin staining was performed to detect myofibroblasts. ED-1 staining to detect inflammatory cells, and collagen type IV staining to detect blood vessels.

**α-Smooth muscle actin staining**

Sections were treated with 3% H₂O₂ in methanol for 10 min to block endogenous peroxidase and rinsed in PBS. Then, the sections were pre-incubated in 10% normal donkey serum (Chemicon Europe, Hampshire, UK) in PBS. After pre-incubation, the sections were incubated with monoclonal mouse anti-α-smooth muscle actin (Sigma Chemical Co., St Louis, MO, USA) 1:6400 for 60 min. After washing with PBS, detection was carried out using biotinylated donkey anti-mouse IgG (Jackson Labs, West Grove, PA, USA) 1:100 for 60 min, and the avidin-biotin complex method (Vectastain ABC-Elite kit; Vector Laboratories, Burlingame, CA, USA). The presence of myofibroblasts was scored on a scale from 0 to 3.

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<th>0</th>
<th>No or only a few myofibroblasts present</th>
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<td>1</td>
<td>Groups of myofibroblasts around the scaffold; no or only a few myofibroblasts inside the scaffold</td>
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<td>2</td>
<td>Groups of myofibroblasts around and inside the scaffold</td>
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<td>3</td>
<td>Myofibroblasts throughout the scaffolds and the surrounding tissue</td>
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**ED-1 staining**

The ED-1 staining was performed similar to the α-smooth muscle actin staining, but the sections were incubated with monoclonal mouse-anti rat ED-1 (Serotec, DPC, Breda, the Netherlands) 1:400 for 60 min. This antibody recognizes a single-chain glycoprotein of 90–110 kDa that is expressed predominantly on the lysosomal membrane of myeloid cells (19). It mainly stains macrophages and monocytes.
The infiltration of inflammatory cells was scored on a scale from 0 to 3.

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<td>0</td>
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<td>Groups of inflammatory cells around the scaffold; no or only a few cells inside the scaffold</td>
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<td>3</td>
<td>Inflammatory cells throughout the scaffold and the surrounding tissue</td>
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**Type IV collagen staining**  
The type IV collagen staining was performed similar to the α-smooth muscle actin staining, but the sections were incubated with rabbit anti-collagen type IV (Eurodiagnostica B.V., Arnhem, The Netherlands) 1:200 for 60 min. The total number of blood vessels present in the scaffolds was counted.

**Statistics**  
All measurements were performed on triplicate sections from each sample. The data for cell density, the number of blood vessels, and the number of giant cells were compared between the two groups at each time point by a t-test. Differences in the scores for myofibroblasts and the degree of inflammation were compared at each time point by a Mann-Whitney rank sum test. In all tests, a Bonferroni correction was applied, hence a \( P \)-value of \( <0.01 \) was considered significant. Differences in time were compared with a one-way analysis of variance for the number of blood vessels, the number of giant cells, and for cell density. Significant differences were further analyzed by the Holm-Sidak method. Differences in time for myofibroblasts and the degree of inflammation were compared at each time point by a Kruskal–Wallis one-way ANOVA on ranks. Significant differences were further analyzed by the Dunn’s method. A \( P < 0.025 \)

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**Figure 1**  
Cell density. Representative H&E-stained sections of 1-week samples are shown. (A) Collagen sample 1 week after implantation. Indicated are the nasal cavity (N), palatal bone (B), and molars (M). (B) Collagen sample 1 week after implantation. (C) Collagen-FGF-2 sample 1 week after implantation (bar = 100 \( \mu \)m). The scaffolds (\#) are indicated. (D) Quantification of the cell density within the scaffolds. *Significantly higher cell density in the FGF-2 group compared with the collagen group at 1, 2 and 4 weeks.
Figure 2  Inflammation. Inflammatory cells were detected with an ED-1 staining. Representative ED-1-stained sections of 1- and 4-week samples are shown. No significant differences were found between the collagen group and the FGF-2 group. (A) Collagen sample 1 week after implantation. (B) Collagen sample 4 weeks after implantation (bar = 100 μm). The scaffolds (#) are indicated. (C) Inflammation scores. The box plots display the 25th, 50th, and 75th percentiles, if available.

Figure 3  Giant cells. The total number of giant cells in the scaffolds was determined on H&E-stained sections. Representative 1- and 4-week samples are shown. No significant differences were found between the collagen group and the FGF-2 group. (A) FGF-2 sample 1 week after implantation. (B) FGF-2 sample 4 weeks after implantation (bar = 100 μm). Scaffolds (#) and giant cells (arrows) are indicated. (C) Number of giant cells within the scaffolds.
was considered significant, as a Bonferroni correction was applied.

Results

The collagen scaffolds were clearly visible in the sections of both groups at 1 and 2 weeks. They are located on the palatal bone between the second molars and are covered with the palatal mucoperiosteum (Fig. 1A). At 4 weeks, two scaffolds in the collagen group were lost. In time, the scaffolds in the FGF-2 group seemed to decrease in size more than the scaffolds in the collagen group. At 8 weeks, only two scaffolds were visible in the FGF-2 group, while at 16 weeks all FGF-2-loaded scaffolds had completely degraded. In the collagen group, all scaffolds were still visible at eight and 16 weeks. In the next sections, some histological and immunohistochemical aspects are shown and quantified.

Cell density

The cell density within the scaffolds was determined on H&E-stained sections (Fig. 1). At 1 week, cells had already invaded the scaffolds. A significantly higher cell density was observed in the FGF-2 group compared with the collagen group from 1 to 4 weeks (Fig. 1B–D). At 8 weeks, no differences in cell density were observed anymore (Fig. 1D). Within the collagen group, a gradual increase in cell density occurred in time until 8 weeks. Cell density was significantly higher at 4 weeks compared with the first week. At 8 and 16 weeks, the cell density was significantly higher compared with the first and second week. Within the FGF-2 group, cell density increased

Figure 4  Blood vessels. Blood vessels are stained with an antibody against collagen type IV, which stains all basement membranes. Representative collagen type IV-stained sections of 1- and 4-week samples are shown. (A) Collagen sample 1 week after implantation. (B) FGF-2 sample 1 week after implantation. (C) Collagen sample 4 weeks after implantation. (D) FGF-2 sample 4 weeks after implantation (bar = 100 μm). Scaffolds (#) and blood vessels within the scaffolds (arrows) are indicated. (E) Number of blood vessels within the scaffolds. *Significantly higher number of blood vessels in the FGF-2 group compared with the collagen group at 1 and 2 weeks.
in time until 4 weeks. A significant difference was found between the first week and all other time points.

Infiltration of inflammatory cells was scored on a scale from 0 to 3 on ED-1-stained sections (Fig. 2). At 1 week, inflammatory cells were mainly found at the edges of the scaffolds (Fig. 2A). At 2 and 4 weeks, inflammatory cells were observed in and around the scaffolds (Fig. 2B). At 16 weeks, almost all inflammatory cells had disappeared from the collagen group. No significant differences were found between the collagen group and the FGF-2 group (Fig. 2C). A significant time difference was only found between two and 16 weeks within the collagen group, although there was a general significant effect of time for both groups.

**Giant cells**

The total number of giant cells in the scaffolds was counted on H&E-stained sections (Fig. 3). Their number increased from 1 to 4 weeks (Fig. 3A,B), while after 16 weeks almost no giant cells remained in the collagen group. No significant differences were found between the collagen group and the FGF-2 group (Fig. 3C). There was a general significant effect of time within both groups, but not for isolated time points.

**Vascularization**

The number of newly formed blood vessels within the scaffolds was counted on sections stained for type IV collagen, which is present in all basement membranes (Fig. 4). A gradual increase in the number of blood vessels was found from 1 (Fig. 4A,B) to 4 weeks.
(Fig. 4C,D). After 4 weeks, the number of blood vessels gradually declined. At 1 and 2 weeks, a significantly higher number of blood vessels were found in the FGF-2 group compared with the collagen group (Fig. 4E). Within the collagen group, the number of blood vessels was significantly higher at 4 weeks compared with all other time points. A significant difference was also found between 1 and 8 and 16 weeks. Within the FGF-2 group, no significant effect of time was noted.

**Myofibroblasts**

α-Smooth muscle actin was detected in blood vessels and in myofibroblasts (Fig. 5). At 1 week, groups of myofibroblasts were observed at the borders and inside the scaffolds in the collagen group (Fig. 5A), while in the FGF-2 group only a few myofibroblasts were present at the borders (Fig. 5B). At 1 and 2 weeks, significantly less myofibroblasts were present in the FGF-2 group compared with the collagen group (Fig. 5E). At 4 weeks, some myofibroblasts were still present in the collagen group, while in the FGF-2 group no myofibroblasts remained (Fig. 5C,D). At later time points, myofibroblasts were no longer present in both groups. Within the collagen group, a significant difference was found between 2 and 8 and 16 weeks, while in the FGF-2 group only a general significant effect of time was noted.

**Discussion**

In this study, the tissue reaction to cross-linked collagen scaffolds with and without FGF-2 was compared following submucoperiosteal implantation in the palate of rats. A sham group without scaffolds was not included, as significant changes were not expected after 1 week, if only the envelope for implantation is created.

The inflammatory response and the foreign body reaction after implantation of both the collagen and the FGF-2-loaded scaffolds were only mild and subsided rapidly. Therefore, we concluded that the scaffolds are biocompatible in the oral tissue environment. Although the exact effects of FGF-2 on inflammation are still unclear, some studies describe a stimulatory effect (20). In our study, however, no difference in inflammatory response was found with and without FGF-2.

FGF-2 has a short biological half-life in vivo after intravenous administration (21), but collagen scaffolds are able to bind FGF-2 and show a biphasic release profile in vitro; an initial burst followed by a gradual and sustained release. After 4 weeks of incubation in vitro, the cumulative release of FGF-2 from our collagen scaffolds was only 64% (9). However, the release profile in vivo might be different due to the presence of binding proteins and proteolytic enzymes.

FGF-2 clearly enhanced the ingrowth of cells into the scaffolds, consistent with the results in skin (22). These cells are probably fibroblasts and also endothelial cells, as angiogenesis is stimulated. FGF-2 is chemotactic for various cell types including endothelial cells, fibroblasts, and macrophages (23). In addition, FGF-2 stimulates the proliferation of fibroblasts (24). Following invasion of the scaffolds, the host cells release additional growth factors (25), which may further enhance cellular influx. The higher cell density in the FGF-2-loaded scaffolds might also cause the faster degradation, although similar scaffolds implanted subcutaneously showed little degradation up to 10 weeks (9). This might be explained by the difference in implantation site, as oral wound healing is generally believed to proceed faster than in skin. This includes a faster re-epithelialization, less contraction, and less scar formation. The cause of these differences is not completely understood, but saliva, leukocytes, growth factors, and specific fibroblast subpopulations seem to play a role (13–15, 26, 27). However, most studies on intra-oral wound healing were performed in buccal mucosa instead of the palatal mucosa. A study performed on the palatal mucosa showed a delayed wound healing compared with skin (28). In a comparative study between implantation of collagen scaffolds without growth factor in the palate and in the skin, we found no differences in the rate of cell ingrowth or degradation (29). Therefore, the response to FGF-2 is probably different in the palate and the skin, which leads to the faster degradation. FGF-2 is known to enhance matrix metalloproteinase-1 (MMP-1) expression in dermal fibroblasts, but the response in oral fibroblasts might be stronger (30).

Angiogenic properties of FGF-2 injected in the skin and in the palate have been reported earlier (17, 31, 32). It is also known that the cross-linking of collagen scaffolds already increases their angiogenic properties (33), and that FGF-2-loaded collagen scaffolds have angiogenic effects after subcutaneous implantation (9). However, ours is the first study on implanted FGF-2-loaded collagen scaffolds in the oral mucosa. In the buccal mucosa, a slower vascularization was observed than in skin (34), but in our previous study a faster rate of vascularization was observed in the palatal mucosa (29). In our study, vascularization in the palate occurred even faster in the presence of FGF-2. It is also shown by others that after subcutaneous implantation, the combination of VEGF with FGF-2 further increases vascularization (35).

Our study showed significantly less myofibroblasts in the FGF-2-loaded scaffolds in the first two weeks. As myofibroblasts cause wound contraction (36), FGF-2 might reduce this process. The reduction in myofibroblast number is probably a combination of reduced differentiation and an increased rate of apoptosis (8, 10, 12, 37). Myofibroblasts are induced by transforming growth factor-β1 (TGF-β1) (38), but FGF-2 seems to down-regulate the number of TGF-β1 receptors, thereby decreasing the differentiation to myofibroblasts (39). FGF-2 induced apoptosis might be affected through a caspase-dependent and p53-independent mechanism as is shown for tumor cells (37, 40). Poor vascularization of scaffolds is considered to be one of the main problems in tissue engineering. Another major problem in the field of wound healing is wound contraction caused by myofibroblasts. Our results indicate that the loading of collagen scaffolds with FGF-2 leads to a faster influx of host cells, an increased rate of vascularization and a reduced differentiation of myofibroblasts in palatal mucosa.
Therefore, FGF-2-loaded scaffolds might be suitable for tissue engineering in cleft palate repair and other intra-oral reconstructions.

References


