Reconstruction of abdominal wall defects using small intestinal submucosa coated with gelatin hydrogel incorporating basic fibroblast growth factor[1]

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ABSTRACT

PURPOSE: To construct a new biomaterial-small intestinal submucosa coated with gelatin hydrogel incorporating basic fibroblast growth factor, and to evaluate the new biomaterials for the reconstruction of abdominal wall defects.

METHODS: Thirty six Sprague-Dawley rats were used in the animal experiments and randomly divided into three groups. The new biomaterial was constructed by combining small intestinal submucosa with gelatin hydrogel for basic fibroblast growth factor release. Abdominal wall defects were created in rats, and repaired using the new biomaterials (group B), compared with small intestinal submucosa (group S) and ULTRAPRO™ mesh (group P). Six rats in each group were sacrificed at three and eight weeks postoperatively to examine the gross effects, inflammatory responses, collagen deposition and neovascularization.

RESULTS: After implantation, mild adhesion was caused in groups B and S. Group B promoted more neovascularization than group S at three weeks after implantation, and induced significantly more amount of collagen deposition and better collagen organization than groups S and P at eight weeks after implantation.

CONCLUSION: Small intestinal submucosa coated with gelatin hydrogel incorporating basic fibroblast growth factor could promote better regeneration and remodeling of host tissues for the reconstruction of abdominal wall defects.

Key words: Abdominal Wall. Biocompatible Materials. Intestinal, Fibroblast Growth Factor 2. Rats.

Introduction

Reconstruction of the abdominal wall is important for extensive resections from surgery or large tissues that are lost during traumatic injuries[1]. Currently, there are many biological scaffold materials from human cadavers or other animal sources utilized for surgical treatment of abdominal wall defects, such as human acellular dermal matrix (HADM), small intestine submucosa (SIS) and so on. These biomaterials have been shown to facilitate the constructive remodeling of many different tissues in both preclinical animal studies and in human clinical applications, and they have been used in the field of abdominal wall reconstruction for many years. It has been reported that these biomaterials consist of a complex mixture of molecules that mediate structural and/or biological properties, and also could effectively promoted cellular infiltration, new extracellular matrix deposition and neovascularization[2,3]. In addition, these biomaterials could become indistinguishable from native tissues. These characteristics might provide distinct advantages over synthetic meshes, especially in overcoming the postoperative complications, such as stiffness of abdominal wall, adhesions, chronic inflammation, foreign body reaction, and so on. However, the biological and mechanical properties of these biological scaffold materials are variable and complications still occur occasionally. Thus, the research for better biomaterials remains necessary.

It has been widely recognized that growth factors could greatly contribute to tissue regeneration at different stages of cell proliferation and differentiation[4], and some members of the growth factor family have been tested in the prevention of wound failure and incisional hernia formation[5,6]. Among the growth factors essential for tissue regeneration is basic fibroblast growth factor (bFGF), which has been shown to be a potent stimulator of angiogenesis and cell migration and proliferation both *in vivo* and *in vitro*[7]. However, successful reconstruction of the abdominal wall induced by bFGF has not been always achieved. The most probable reason is the short half-life of bFGF *in vivo*. Therefore it is necessary to develop a drug-delivery system to gradually release bFGF *in vivo* and extend the biological activity. Recently, studies have shown that biodegradable gelatin hydrogels could incorporate bFGF and release it sustainedly *in vivo* to induce regeneration and reconstruction of host tissues[8,9]. This could be explained in terms of the basic–acidic interaction between the growth factor and gelatin hydrogels[10]. The acidic gelatin hydrogel could ionically immobilize in bFGF, and *in vivo* release of bFGF is mainly governed by hydrogel biodegradation[11]. That is, the growth factor release *in vivo* could be controlled by biodegradation of gelatin hydrogel itself.

In the present study, our aim was to combine small intestinal submucosa with a gelatin hydrogel incorporating basic fibroblast growth factor (bFGF-SIS) and to evaluate the effect of bFGF-SIS on the construction of abdominal wall defects in rats. We hypothesized that bFGF-SIS would enhance cellular and vascular infiltration and collagen formation from surrounding tissues and promote the regeneration and remodeling of host tissues for the reconstruction of abdominal wall defects after implantation.

Methods

All animal study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University (IACUC-2013-0101). Meanwhile, the experimental protocol was conformed to the guidelines of ethical use of animals, and suffering was made to minimize. Large swine (about 200kg) from a local slaughterhouse were used for the preparation of SIS. A total of 36 Sprague-Dawley rats (SD rats) weighing 240–280g, which were used for surgically created models of abdominal defects and histological and immunohistological analysis after the defect was repaired, were purchased from the Experimental Animal Center of Guangzhou Province, China. The rats were quarantined for one week prior to use and each rat was marked with an identification number. The rats were caged andW kept on a 12-hour light/12-hour dark schedule at 22ºC.

A gelatin sample with an isoelectic point (IEP) of 4.7-5.2, prepared from bovine bone through an alkaline process, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant bFGF with an IEP of 9.6 and a molecular weight of 16.0kDa was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). ULTRAPRO™ mesh, which is manufactured from approximately equal parts of absorbable poliglecaprone-25 monofilament fiber and non-absorbable polypropylene monofilament fiber, was obtained from Ethicon Inc. (Somerville, NJ, USA). Other chemicals were obtained from the Medical Research Center of Sun Yat-Sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China.

Preparation of gelatin hydrogels incorporating bFGF

Gelatin hydrogels were prepared through the chemical cross-linking of gelatin solution with glutaraldehyde according to the method described previously[12]. In brief, the glutaraldehyde solution and solid gelatin were immersed into the mixed solution

of gelatin (5.0%) and glutaraldehyde (0.1%), followed by leaving at 4ºC for 15 min to allow them to crosslink and solidify. The resulting hydrogel sheets were punched out to obtain gelatin hydrogel discs of 10 mm in diameter and 2 mm in thickness, then left at 4ºC for 12 h to allow the cross-linking reaction to proceed. After that, they were placed in 100 mM glycine aqueous solution at 37 ºC for 1 h to block the residual aldehyde groups of glutaraldehyde, and then washed three times with double distilled water at 37°C. To impregnate bFGF into the acidic gelatin hydrogels, 20μl of aqueous solution containing bFGF (0.01μg/μl) was dropped onto a freeze-dried hydrogel, and then left at 4°C for 12h. Since the volume of growth factor solution was much smaller than that theoretically impregnated into the hydrogel, 100% of the growth factor added could be entirely incorporated into the hydrogel[13].

bFGF in vitro release test

Release tests were conducted in 37°C incubator. The gelatin hydrogel disks impregnated with bFGF were put into a 6-well cell culture plate, and then 5mL sterile phosphate buffered saline (PBS) was added to each well. After that, the wells were covered with a parafilm sheet to avoid medium evaporation. Medium was changed at predetermined time points (1 day, 3 days, 7 days, 10 days, 14 days, 21 days and 28 days) and the collected medium was placed in a 15mL centrifuge tube and frozen at −20°C prior to quantification in quadruplex experiments. The amounts of bFGF released were determined using an enzyme-linked immuno-sorbent assay (EMD Biosciences, San Diego, USA). The optical density was determined at a wavelength of 450nm with reference of 570nm.

Preparation of SIS

SIS was obtained from the proximal jejunum of large swine and manufactured as previously described[14]. Briefly, after the small intestine was harvested, the mucosal, muscular layers and serosa were mechanically removed, leaving the submucosa and basilar layers of the tunica mucosa, which has been identified as SIS. The submucosa was then cut into 10 cm-long sections that were processed through a series of chemical cleaning steps, by incubation in ethylenediaminetetraacetic acid (EDTA), NaOH, HCl, and NaCl. Four layers of SIS were mechanically made by vacuum pressing to create a multilaminate device. The four layers SIS device was stored at –80ºC and thawed as needed. All SIS that were used for coating gelatin hydrogel incorporating bFGF and repairing the abdominal wall defects were four layers SIS.

Construction of bFGF-SIS

Firstly, the SIS was immersed into a mixed solution of gelatin (5.0%) and glutaraldehyde (0.1%), left at 4ºC for 15min (repeated three times), and then left at 4ºC for 12h. Secondly, gelatin-coated SIS was placed in 100mM glycine aqueous solution at 37 ºC for 1h, and then washed three times with double-distilled water. Finally, the gelatin-coated SIS was freeze-dried and sterilized with ethylene oxide gas, stored at -80ºC, and thawed as needed.

To impregnate bFGF with gelatin-coated SIS, 20μl of aqueous solution containing bFGF (0.25μg/μl) was dropped onto the freeze-dried gelatin-coated SIS at room temperature, and then left at 4ºC for 12h to obtain the gelatin hydrogel incorporating bFGF.

Surgical procedures and grouping

A total of 36 rats were randomly divided into three groups. Pentobarbital sodium (40mg/kg) was used for intraperitoneal anesthesia. After the rats were shaved, scrubbed, disinfected and covered with sterile draping, a 2.0×1.5cm full thickness defect in the anterior abdominal wall was surgically created, including subcutaneous tissue, fascia, muscle and the peritoneum. Then, the defects were primarily repaired with bFGF-SIS (group B), SIS (group S) or ULTRAPRO™ mesh (group P) respectively, and each implant oversized the defect by at least 0.25cm. The implant was used in the onlay position by fixing to the defect edge of abdominal wall with 4/0 monofilament polypropylene sutures (Prolene®, Ethicon). Finally, the skin was closed with 2/0 VWicryl sutures (Vicryl®, Ethicon). Following recovery, each rat was returned to its cage and free access to food and drinking.

Macroscopic examination of abdominal wall defect repairing

All animals were checked three days after the implantation, and then weekly checked for local and systemic complications, including: death, wound infection, dehiscence, bulging or abdominal wall hernia and fistula formation. Six rats in each group were randomly sacrificed at three and eight weeks after the implantation, and the gross repair effects were observed and recorded before the biomaterials were sampled.

Adhesion evaluation

For evaluation of the adhesion, six rats in each group were sacrificed at three and eight weeks postoperatively. A U-shaped incision was made in the abdominal wall, which included the biomaterial and its surrounding host tissue, without disturbing any adhesions. The degree of adhesions was assessed by two surgeons who were blinded to the study. The score of adhesions was obtained from each rat according to the criteria described previously[15]: 0, no adhesions; 1, thin and filmy adhesions easily separable by blunt dissection; 2, definite localized adhesions; 3, definite multiple visceral adhesions; and 4, dense adhesions extending abdominal wall.

Histology and immunohistochemistry

After the rats were sacrificed, the abdominal wall was removed *en bloc* for histology and immunohistochemistry examination. The specimens were fixed in 10% formaldehyde immediately after explantation, embedded in paraffin, and cut into 5μm sections, and each slide would contain the biomaterials, interface, as well as the surrounding native tissue. For the histological examination, the slides were stained with hematoxylin and eosin (HE) and examined under a light microscope (E600, Nikon, Japan) to quantitatively analyze the number of foreign body giant cells (FBGCs), polymorphonuclear cells (PMNCs) and mononuclear cells (MNCs) invading the implanted scaffolds. Fields were randomly selected at the interface between the implant and the surrounding tissue. Semi-qualitative assessment of collagen deposition was performed using Masson's Trichrome stain. The amount of inflammatory cell infiltration and the amount and organization of collagen deposition were scored using a method similar to that described by Badylak *et al*.[16]. and Konstantinovic *et al*.[17] (Table 1).

TABLE 1 - Histological scoring criteria used for microscopic examination.

Cellular infiltration	Score			
	0	1	2	3
FBGCs	0	1–5	6–10	>10
PMNCs	0	1–5	6–10	>10
MNCs	0	1–5	6–10	>10
Collagen Organization	Totally disorganized	Slightly organized	Moderately organized	Well organized
Amount	None	Mild	Moderate	Abundant
Vascularity	0	1–3	4–10	>10

FBGCs, Foreign body giant cells
PMNCs, Polymorphonuclear cells
MNCs, Mononuclear cells

Five non-overlapping fields per slide and two main slides were counted at ×400 magnification in a standardized and blinded fashion.

The neovascularizition were assessed using immunohistochemical staining of CD31 (neointimal cells) (Mouse -anti rat monoclonal antibody, 1:200; Abcam, Cambridge, USA). Sections incubated without the primary antibody served as negative controls. The mean percentage area of blood vessels (% Abv) was calculated for 10 randomly selected high-contrast fields (five non-overlapping fields per slide and two main slides) at ×200 magnification using Image-Pro Plus (version 6.0, Media Cybernetics Inc, Silver Spring, MD, USA). Percentage area of blood vessels = area of capillary vessels / total tissue area × 100%. All evaluations were performed by one pathologist who was blinded to the study design.

Statistical analysis

Experimental results were expressed as mean ± standard deviation. Statistical analysis was carried out using SPSS version 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). We performed statistical analyses using the one-way ANOVA, LSD-t test, Kruskal–Wallis test, and the Mann–Whitney test. Values of p below 0.05 were considered to represent statistically significant difference.

Results

bFGF in vitro release

In the present study, the hydrogels were swollen gradually within the well and bFGF was released into the surrounding medium. Figure 1 shows the *in vitro* time profiles of bFGF release from gelatin hydrogel.

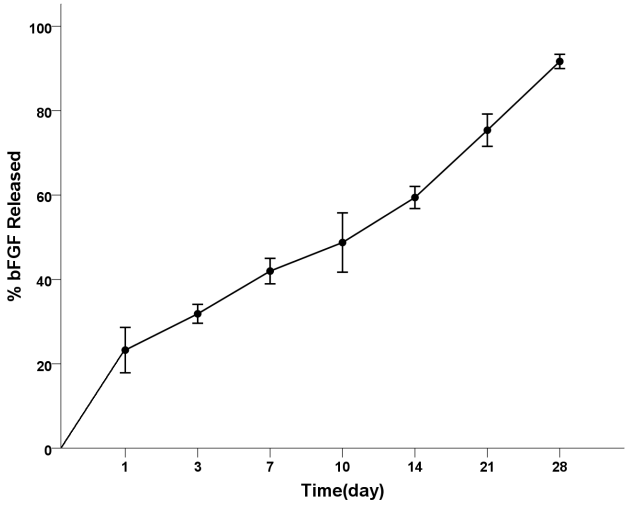

FIGURE 1 - Release profile of bFGF from gelatin hydrogels within 28 days.

At the first day, the gelatin released 23.3%±5.4% of bFGF, and the overall amount of bFGF released within 28 days was about 91.7%±1.7% of its initial loading concentration.

Macroscopic examination

All rats survived to the predetermined sacrifice date. All the defects were repaired successfully, and none of the rats showed any evidence of bulging or herniation. None of rats developed wound rupture, wound infection or fistula formation postoperatively. Wound dehiscence was noted in one case in group B, two cases in group S and two cases in group P at three days after implantation, all of which were disinfected and sutured then.

All the biomaterials used for the reconstruction of the abdominal wall defect remained visible three weeks after implantation (Figure 2A-C).

Eight weeks after implantation, bFGF-SIS and SIS were gradually integrated into the adjacent fascia tissues at the interface. Especially in group B, there was almost no distinction between bFGF-SIS and the surrounding fascia tissues. The ULTRAPRO™ mesh remained visible compared with bFGF-SIS and SIS (Figure 2D-F).

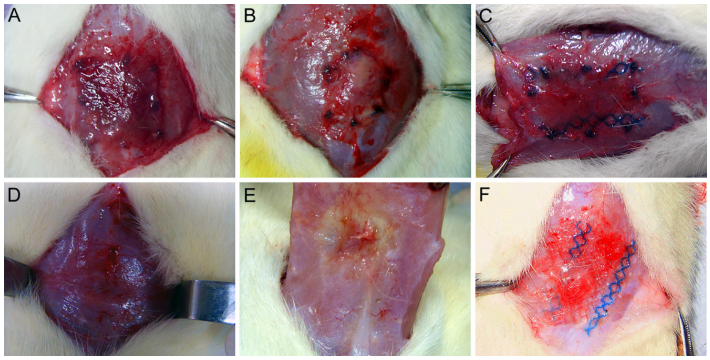

FIGURE 2 - Gross effects of three implants after implantation. (A) bFGF-SIS, three weeks; (B) SIS, three weeks; (C) ULTRAPRO™, three weeks; (D) bFGF-SIS, eight weeks; (E) SIS, eight weeks; (F) ULTRAPRO™, eight weeks.

Adhesion analysis

Three weeks after implantation, a lower degree of intra-abdominal adhesion was observed in groups B and S compared to group P ($p<0.01$), and there was no significant differences between groups B and S. Groups B and S showed thin and filmy adhesions, involving only omentum. However, adhesions seen in group P were firm and dense, which affected the omentum and bowel. Eight weeks after implantation, intra-abdominal adhesions in groups B and S were still slight and could be easily separated, whereas group P had more dense adhesions that needed a sharp dissection to separate. Differences in the adhesion scores between the three groups were significant (Figure 3).

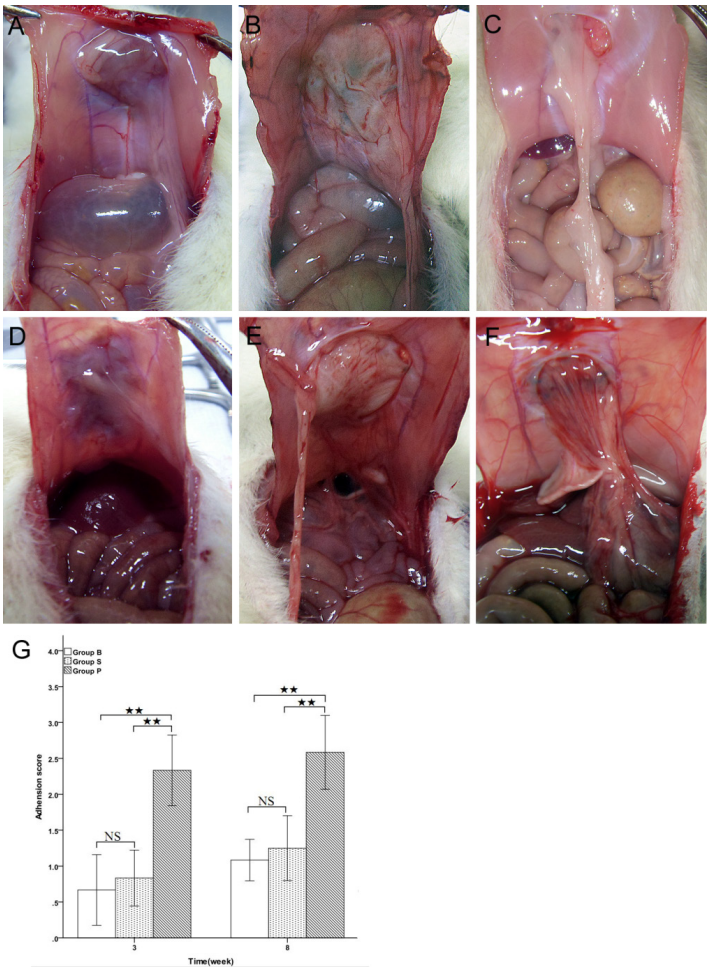

FIGURE 3 - Evaluation of intra-abdominal adhesion of three implants after implantation. (A) bFGF-SIS, three weeks; (B) SIS, three weeks; (C) ULTRAPRO™, three weeks; (D) bFGF-SIS, eight weeks; (E) SIS, eight weeks; (F) ULTRAPRO™, eight weeks; (G) adhesion scores in bFGF-SIS, SIS, and ULTRAPRO™ groups at three and eight weeks after implantation. *$p<0.05$, **$p<0.01$.

Microscopic evaluation

Three weeks after implantation, histological examination with HE staining showed that inflammatory cell infiltrations were detected in all three groups. Group P showed more pronounced inflammatory cell infiltration at the interface between the biomaterials and native tissues, which was significantly greater than that seen in groups B and S ($p<0.01$). The remodeling of the biomaterials was observed in groups B and S with newborn blood vessels formation and new collagen deposition, which was less organized around the biomaterials. Furthermore, partial degradation of bFGF-SIS and SIS was detected at three weeks after implantation. Much more newborn blood vessels and totally

disorganized collagen deposition were detected in group P (Figure 4A-C and Figure 5A-C).

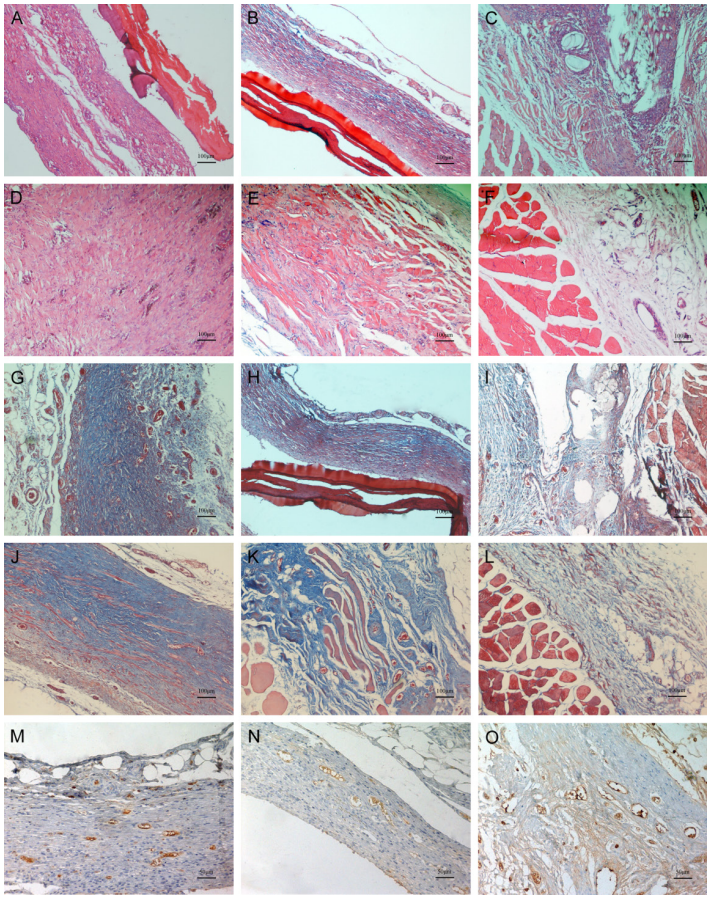

FIGURE 4 - Evaluation of histological appearance of three implants after implantation. (A-C) HE staining, three weeks, ×100. A, bFGF-SIS. B, SIS. C, ULTRAPRO™. (D-F) HE staining, eight weeks, ×100. A, bFGF-SIS. B, SIS. C, ULTRAPRO™. (G-I) Masson's Trichrome staining, three weeks, ×100. A, bFGF-SIS. B, SIS. C, ULTRAPRO™. (J-L) Masson's Trichrome staining, eight weeks, ×100. A, bFGF-SIS. B, SIS. C, ULTRAPRO™. (M-O) Immunostaining of anti CD31 antibody. Three weeks, ×200. A, bFGF-SIS. B, SIS. C, ULTRAPRO™.

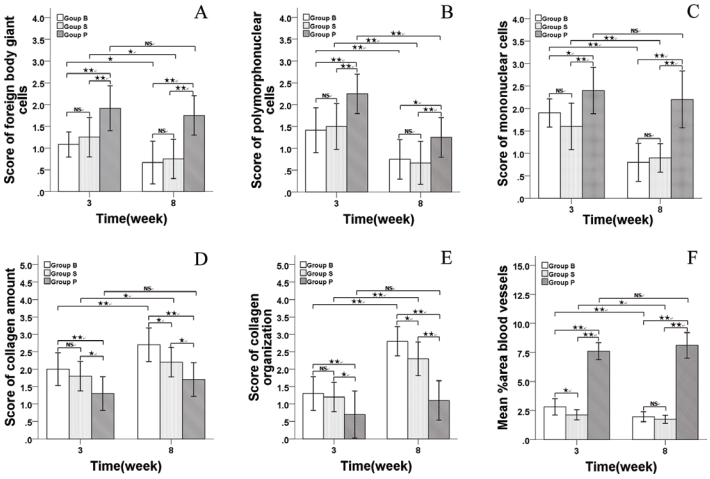

FIGURE 5 - Histological scores of three implants after implantation. (A) Statistical analysis of the scores for the number of FBGSs; (B) PMNs; (C) MNs; (D) amount of collagen; (E) organization of collagen; (F) amount of neovascularization. *p<0.05, **p<0.01.

At eight weeks after implantation, the inflammatory reactions gradually decreased in groups B and S, along with more collagen deposition around the biomaterials, both of which were organized orderly and incorporated into the host tissues. However, few blood vessels were observed in both groups, compared with that seen at three weeks after the implantation. Group P showed no sign of mesh degradation, and inflammatory cell infiltration were still obvious. In addition, there were more newborn blood vessels with no sign of regression in Group P, compared with groups B and S (Figure 4D-F and Figure 5A-C).

Masson's Trichrome staining showed that both groups B and S had a similar degree of collagen deposition and collagen organization over three weeks after implantation ($p>0.05$). However, the scores of both amount and organization of collagen deposition in group B were higher than that in group S at eight weeks after the implantation ($p<0.05$). Both groups B and S showed more amount and better organization of collagen deposition than group P from three to eight weeks after implantation ($p<0.01$, Figure 4G-L and Figure 5D-E).

Immunohistochemical staining with anti-CD31 showed that the density of newborn blood vessels in group B was significantly higher than that in group S at three weeks after implantation ($p<0.05$). Gradually, with the maturation of regenerated collagen fibers, spontaneous regression occurred in the newborn blood vessel, and there were decreased newborn blood vessels in both groups B and S at eight weeks after implantation. There were much more newborn capillaries in Group P than in both groups B and S from three to eight weeks postoperatively (Figure 4M-O and Figure 5F).

Discussion

The increasing use of biomaterials for tissue defects repair has driven its development. Among the biomaterials used for the reconstruction of the abdominal wall, synthetic biomaterials, such as polypropylene, polytetrafluoroethylene and polyester, only serve as a scaffold providing mechanical support. However, they could cause potential complications, such as infections, adhesions, bowel obstruction, chronic pain, and local stiffness of the abdominal wall. Biological meshes, also called tissue-derived biomaterials, are derived from humans or animals tissues. The predominant advantages of these biomaterials are that they could induce cellular infiltration, new extracellular matrix deposition and neovascularization and carry a lower risk of complications. However, problems are still unavoidable. In order to increase the effect of abdominal wall reconstruction, modifications and

innovations on biomaterials are needed. Nowadays, modification focusing on providing biomaterials with bioactive molecules (e.g. growth factors) is an important and effective approach, because these bioactive molecules could contribute to cell proliferation and differentiation during different periods of tissue regeneration[18].

Several studies have shown that bFGF could promote fibroblast proliferation, stimulate angiogenesis in the dermis, and healing of fascial tissues[5,18]. However, effective use of bFGF is not always achieved due to its rapid diffusion into body fluids and short half-life[19,20]. Furthermore, high doses of bFGF could cause severe side effects[21,22]. Therefore the need for a sustained exposure of growth factor at the defects site for better effect has led to the employment of delivery vehicles that facilitate a therapeutic, localized dose, provide a stabilizing matrix for the growth factor, and minimize toxic side effects usually associated with systemic administration. It is reported that bFGF molecules was found to be ionically complexed with the acidic gelatin[11], so that bFGF could be released by hydrogel degradation. In the present study, the release kinetics of bFGF was determined in temperature-controlled (37ºC) conditions. Within 28 days, more than 90% of the total amount of bFGF was released into the surrounding medium. Similar to previous study[11], the gelatin hydrogel tended to release bFGF in a burst manner at beginning[10], that is because the impregnation condition of bFGF (e.g. at 4°C for 12h) into the acidic gelatin hydrogel is not sufficient enough to achieve 100% ionic complexation[23]. Thereafter, no burst release was observed, and the gelatin hydrogel could sustainedly release bFGF in 28 days.

Intra-abdominal adhesion is an important evaluation criterion for the construction of the abdominal wall, which usually causes bowel obstruction, chronic pain and fistula formation after implantation. It has been reported that bleeding and inflammation are two major reasons for adhesion formation during the operation, because of trauma and foreign body reaction, the plasminogen activator, which directly increased the deposition of fibrin matrix was suppressed[24,25]. Therefore reduction of bleeding during the operation and subsequent inflammation could be a way to prevent adhesion formation. Our results showed that both bFGF-SIS and SIS caused minimal intra-abdominal adhesion compared with ULTRAPRO™ mesh. That is probably because with the degradation bFGF-SIS and SIS, both the biomaterials resulted in minimal inflammation response and foreign body reaction. Decreased inflammation could prevent severe adhesion formation. In addition, it revealed that SIS coated with gelatin hydrogel would not slow down its degradation.

The host response to biomaterials can be characterized by inflammation and foreign body reaction, the formation of collagen tissue, as well as neovascularization. In our study, histological observation showed that the host responses of bFGF-SIS and SIS were quite different from those observed with ULTRAPRO™ mesh. At three weeks after implantation, both bFGF-SIS and SIS caused obviously inflammatory response around the implants site. With time passed, inflammatory response decreased, and both bFGF-SIS and SIS degraded largely, both of which were replaced by a large amount of organized collagen tissue. Particularly for bFGF-SIS, there was an improved remodeling process with more amount and better organized collagen tissue deposition than SIS. Conversely, ULTRAPRO™ mesh caused a pronounced inflammatory response with obviously infiltration of PMNs, MNs or FBGCs at both three and eight weeks after implantation. The collagen tissues deposited around ULTRAPRO™ mesh were totally disorganized.

Angiogenesis—new blood vessel formation, accompanies fibroblast proliferation and allows nutrients and healing factors to enter the wound space[26]. It is also essential for the growth of granulation tissue and fibrous tissue, which could resist the distractive force of abdominal walls, and ischemic conditions around the biomaterial site often delays wound healing. In present research, quantitative immunohistochemistry showed that bFGF-SIS could promote more newborn blood vessels than SIS at early stage after implantation. More angiogenesis could make for more amount and better organization of collagen deposition. After that, with the maturation of regenerated collagen tissues, spontaneous regression occurred in newborn blood vessel, and there were few capillaries in group B and S, which was similar to normal facial tissues. These results indicated that bFGF-SIS could promote the regeneration of host facial tissues, because bFGF could facilitate the proliferation of dermal fibroblasts, vascular endothelial and smooth muscle cells[27-29].

Optimal doses of growth factors, which were used in wound healing to obtain maximum effect, are not definite for human beings or animals. Various doses from 1ng to 200μg have been tested in studies, however, not only the application ways and doses but also the experimental results are heterogenous[30-33]. In addition, it is not clear whether or not the effects of bFGF on wound healing are dose-dependent. In present study, 5μg bFGF was used, and results revealed that bFGF-SIS could promote better regeneration and remodeling of abdominal wall fascia tissues. It is surely possible that higher doses of bFGF could induce better results, however, it is necessary to test different doses in further studies.

One limitation of the present study which should be mentioned is that we only assessed the short-term effects of the bFGF-SIS for reconstruction of abdominal wall defects, and long-term studies are needed.

Conclusion

Small intestinal submucosa coated with gelatin hydrogel incorporating basic fibroblast growth factor could promote better regeneration and remodeling of host tissues for the reconstruction of abdominal wall defects.

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