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OMENTAL FLAP CLOSURE OF REFRACTORY WOUNDS: RAT MODEL

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ABSTRACT

Omental flaps, with their associated rich and pliable vascular arcades, are frequently used in clinical practice for the reconstruction of complex and irregular defects. There is little experimental evidence, however, to prove that omental flaps can be a useful tool for the defects. Using a gastric-wall defect model, we performed histological and immunocytochemical examinations. We created an omental flap lining a 2.0-mm defect perforating the center of the anterior wall of a rat stomach. We examined the tissue response during gastric wall regeneration by H&E and Masson trichrome stains. We also performed immunocytochemical studies for the detection of proliferating cell nuclear antigen (PCNA), factor VIII-related antigen, fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). One day after the operation, the omental flap was found to firmly adhere to the gastric serosa surrounding the defect. An extensive inflammatory response occurred from Day 1 to 3 with dilated vessels in the omentum. From Days 3 to 7, a significant number of PCNA-positive cells, FGF-2-positive cells and VEGF-positive cells were observed at the edge of the mucosa and within the granulation tissue. On Day 4, in place of extensive inflammation, an exuberant granulation tissue response was observed from the omentum. The defect had been covered by stratified villi by Day 7. This study demonstrated that an omental flap came to rapidly adhere to the defect serving as a source of extensive inflammation and granulation for the rich and pliable vascular arcades.

Key Words: Adhesion, Gastric wall defect, Granulation, Inflammation, Omental flap

INTRODUCTION

Omental flaps can easily fill the irregular cavities left by debridement of the sternum or other bones affected by osteomyelitis[1-3] or by removal from the skull base of a tumor with intracranial extension.[4] Omental flaps have also been used in the reconstruction of complex defects of the head and neck[5-7] and other contour deformities,[8,9] since the omental flaps are well-vascularized with a long vascular pedicle. There is little experimental evidence, however, to prove that omental flaps are a useful tool for complex wounds due to their pliability and good vascularization.[10]

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We used a gastric-wall defect model and performed histological examinations to investigate the processes involved in omental flap coverage of complex everted wounds.

MATERIALS AND METHODS

Experimental animals and maintenance

Animals received humane care in compliance with the Nagoya University Guidelines for Animal Care and Use, which are based on the National Research Council's criteria outlined in the Guide for the Care and Use of Animals (USA, 1985). Eight-week-old male Sprague-Dawley rats (Chubu Kagaku Shizai, Nagoya, Japan), weighing 250–300 g at surgery (n=33), were housed three animals per cage in an air-conditioned room maintained at 23°C with 60% humidity on an alternating 12-h light/12-h dark cycle. Rats were given chow and running water *ad libitum*, with the exception of fasting periods before and after surgery.

Surgical procedure

Animals initially received inhalation anesthesia with diethyl ether, followed by additional anesthesia with an intraperitoneal injection of pentobarbital sodium (25 mg/kg body weight; Nenbutal®, Abott, USA). A laparotomy was then performed to expose the anterior portion of the stomach. We perforated the central anterior wall of the stomach between the fundus and the corpus with a needle (2.0-mm diameter; KAI, Seki, Japan), as shown in Fig. 1A. We initially investigated the possibility of the optimal single-needle puncture to produce an adequately sized complex defect. When the stomach wall was perforated with a 1.0-mm needle, the defect produced was simple, neither complex nor irregular. Larger-diameter defects produced a refractory wound accompanied by an extensive inflammatory reaction. When the diameter of the defect produced exceeded 3 mm, however, the defect could not be reliably patched with an omental flap. Thus, a 2.0-mm defect was chosen as the optimal defect size. An omental flap with a pedicle, elevated and isolated on the right gastroepiploic vessels, was used to patch the stomach defect. The flap was affixed to the stomach in a perpendicular direction along the long axis from the fundus to the corpus, as shown in Fig. 1B, using five to six sutures placed with 10-0 nylon threads. The abdominal incision was closed primarily using 4-0 nylon sutures. The animals were refed on the day of the operation.

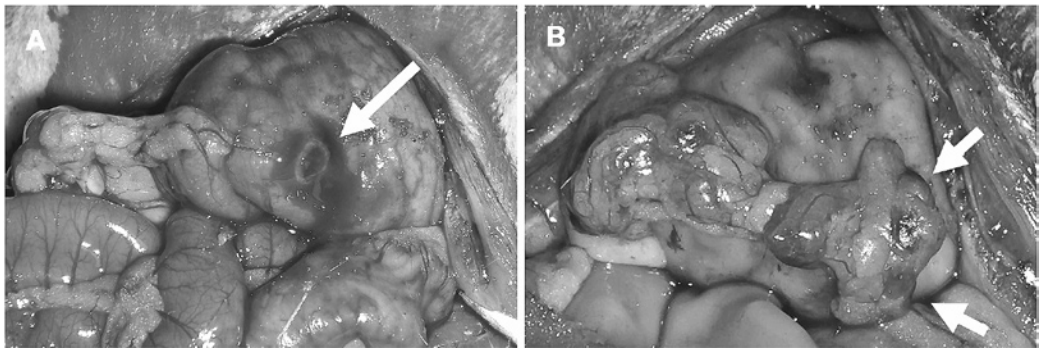

Fig. 1 Surgical technique. (A) The center of the anterior wall of the stomach was perforated using a needle between the fundus and corpus to generate the defect (arrow). (B) The omental flap was fixed to the stomach perpendicular to the wall in a long axial direction (arrows).

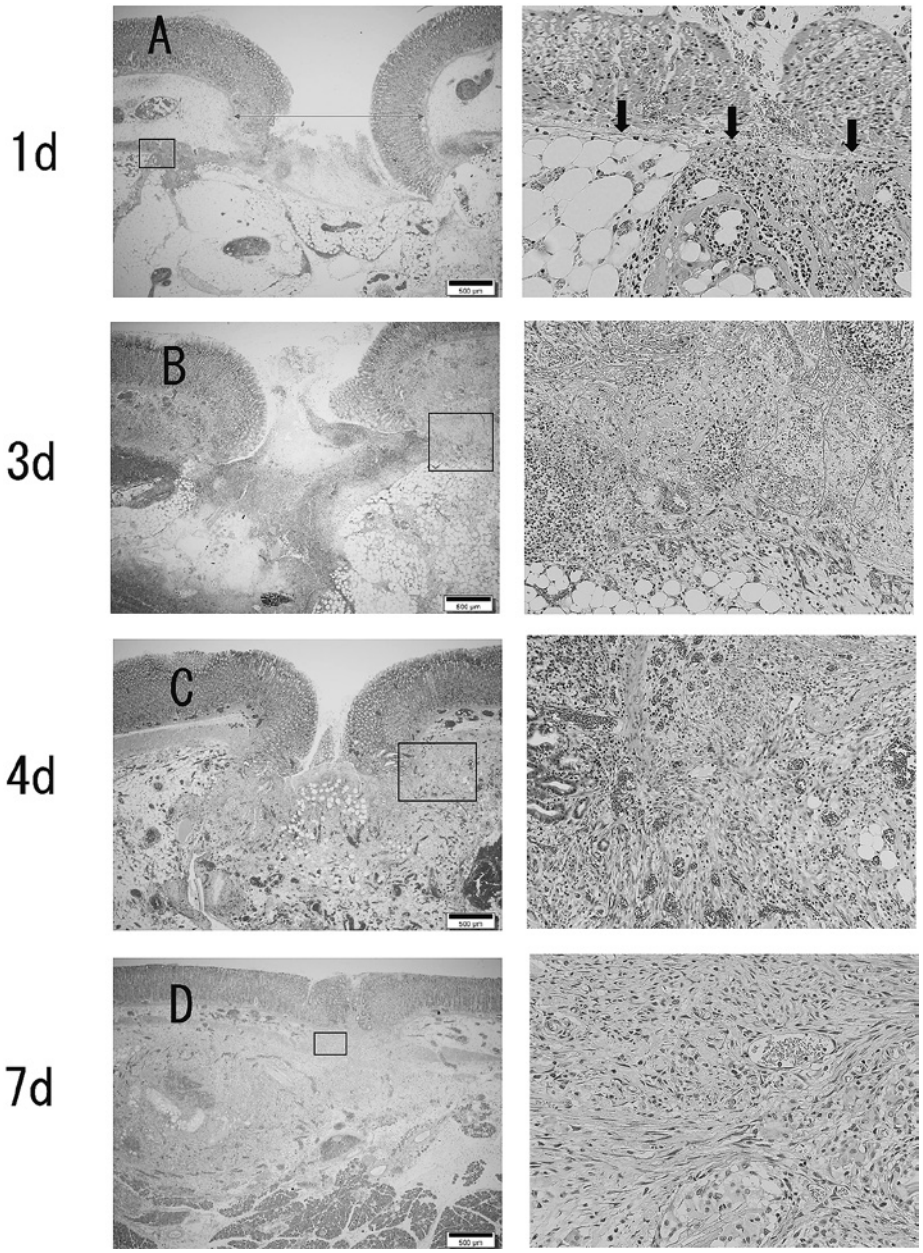

Fig. 2 H&E stained histological sections. Boxes in the low magnification images (left panel) indicate the location of the higher magnification images (right panel). Scale bar = 500 μm. (A) Day 1. The everted mucosal edges were edematous. Fibrin matrix filled the ulcerated defect. An arrow denotes the distance between the opposing edges of the mucosal muscular layer in each sagittal wound section. The surface of the omental flap adhered tightly to the gastric serosa (inset, arrows). (B) Day 3. Extensive inflammatory cell infiltration was clearly observed at the defect. Granulation tissue was first seen in the area of adhesion between the gastric serosa and the omental flap (inset). (C) Day 4. Granulation tissue filled and proliferated within the defect. The plane between the omental flap and the gastric wall at the adhesion site was difficult to distinguish (inset). (D) Day 7. Stratified villi had completely covered the defect. Scar formation was evident, containing densely packed, disorganized collagen fiber bundles (inset).

Gross examination

A laparatomy was performed on three rats on postoperative Days 1, 2, 3, 4, 5, 6, 7, 10, 21, 30, and 60. They were then sacrificed by a diethyl ether overdose. We incised the stomach along the greater curvature and removed specimens containing the omental flaps and stomach contents. After gross examination of the specimens, photographs of each wound were taken with an EX-Z57® camera (CASIO, Tokyo, Japan).

Histology

After fastening the tissue adequately with pins to a corkboard to display, specimens were incubated in 4% paraformaldhyde for 24 h at room temperature. Samples were then embedded in paraffin blocks, from which 5-μm serial sections were prepared along the sagittal plane through the widest margin of the wound. After staining deparaffinized sections with hematoxylin-eosin, photomicrographs of each defect were taken with an OLYMPUS BX50® stereoscopic microscope (OLYMPUS, Tokyo, Japan). Sections were also stained using Masson trichrome to evaluate the degree of collagen organization within the granulation and the scar. We assessed tissue reactivity to the omental flap by histologic evaluations, including assessments of the degree of inflammatory cell infiltration, blood vessel development, and formation of granulation tissue.

Quantitative analysis of inflammatory cells and vascular openings

On each postoperative day, under 600 × magnification we quantitatively assessed the number of inflammatory cells present, expressing the results as the mean inflammatory cells per microscopic field in a central area of the defect (approximately 2500 μm^2). The quantitative assessment of vascular openings[11] was evaluated under 120 × magnification by staining the deparaffinized sections with a rabbit polyclonal antibody against rat factor VIII-related antigen (DakoCytomation, Denmark).[12] Findings were expressed as the mean number of vascular openings per microscopic field in a central area of the defect (approximately 0.64 mm^2). Mean values were calculated for each of the three rats on each operative day.

Immunohistochemical observation

For immunostaining by the indirect immunohistochemistry technique, we used a rabbit anti-fibroblast growth factor-2 (FGF-2) polyclonal antibody (Calbiochem Inc, Gibbstown, USA), a rabbit anti-Vascular Endothelial Growth Factor (VEGF) Ab-1 polyclonal antibody (Thermo Fisher Scientific Anatomical Pathology, CA, USA) and a Proliferation Cell Nuclear Antigen (PCNA) monoclonal antibody (Invitrogen PCNA Staining Kit, Invitrogen Ltd, UK). PCNA, a known marker of cell proliferation, reaches a peak in mid-S phase. We attempted to identify possible effector cells by analyzing the distribution of PCNA-positive cells, which likely play an important role in wound healing. The specificity of the immunohistochemical procedures was confirmed by the incubation of sections with non-immune serum instead of the primary antibody.

RESULTS

Gross observation

On postoperative Days 1 and 2, we confirmed a deep defect resembling a small dimple in the mucosa. After postoperative Day 3, due to contraction, the defects appeared oval or linear in shape, with diameters less than 2 mm. The contraction direction was unrelated to the direction of the rolling omental flap. By postoperative Day 6, the depth of the defect had decreased significantly, becoming a shallow lesion filled with mucosal tissue. No apparent differences were

observed on postoperative Days 7, 10, 21, 30, and 60.

Histological observations and qualitative analysis

On Day 1, fibrin matrix was observed to fill the defect. Although the mucosal edges were edematous and everted vertically (Fig. 2A), the surface of the omental flap adhered tightly to the gastric serosa around the defect (Fig. 2A, inset). Inflammatory cells had infiltrated the superficial surface of the omentum at the defect. Simultaneously, vessels in the omentum were dilated, including red blood cells and polymorphonuclear leukocytes. An acute inflammatory reaction was also observed surrounding the defect at the submucosal level. By Day 3, extensive inflammatory cell infiltration was dominated by polymorphonuclear leukocytes and macrophages; this reaction was more intense at the center of the defect (Fig. 2B). Granulation tissue was first seen within the area of adhesion of the gastric serosa to the omental flap (Fig. 2B, inset). New blood vessels originated from the surface of the omentum at the adhesion site. Some thickening of the mucosal membrane was visible. Proliferation started from the edge of the defect, with irregular and poorly developed villi observed at the margins.

By Day 4, granulation tissue dramatically proliferated within the lesion to fill the defect after the regression of inflammation (Fig. 2C). The plane between the omental flap and the gastric wall at the adhesion site had become difficult to distinguish due to the proliferation of granulation tissue (Fig. 2C, inset). The irregular villus layer was elongated at the lesion margin. There was an obvious increase in fibroblasts and vascular opens on the surface of the omental flap (Fig. 2C and Fig. 3A). Some dilated vessels could be observed on the periphery of the omental flap. The number of inflammatory cells on the defect had decreased significantly (Fig. 4). By Day 6, the granulation was significantly thickened despite decreased cellularity. Collagen fibers became bolder with increased densities, and cells had significantly decreased (Fig. 3B, inset).

By Day 7, we observed significant reductions in mucosal edema. Stratified villi had completely covered the defect, and granulation tissue was gradually being replaced by scar tissue (Fig. 2D, inset). A mild inflammatory reaction continued throughout the defect. The number of inflammatory cells peaked on day 3, decreasing thereafter to reach a constant level after Day 21 (Fig. 4). The number of vascular openings increased over time, peaking on Day 5, then decreasing again until Day 10 (Fig. 4). While the number of vascular openings remained constant, the diameters of these vessels increased with time.

The muscular layer of the mucosa, however, had not regenerated by Day 21 (Fig. 3C). The sizes and structures of the overlying gastric glands were irregular and distributed in a random pattern. Below the epithelium, the density of collagen in the scar tissue had diminished from the densest layer seen on Day 10. The collagen patterns were distinctly different from the regular patterns seen in normal submucosal tissue (Fig. 3C, inset). The irregularity of the collagen tissue persisted as late as Day 60, with a predominance of delicate collagen fibers.

PCNA findings

From Days 3 to 7, large numbers of PCNA-positive cells could be observed, primarily located on both sides of the adhesion (Fig. 5, “lateral” panel). PCNA-positive cells were seen with fibroblasts and endothelial cells predominating. From Days 4 to 10, significant numbers of PCNA-positive cells were observed at the center of granulation (Fig. 5, “center” panel). Approximately 50% population of the cells in the center of granulation showed positive to PCNA immunostaining. By Day 7, once the defect had been completely covered, the number of PCNA-positive cells decreased significantly at the center of the granulation tissue. PCNA-positive cells were detected from Days 7 to 10 in the bottom layer of the newly formed mucosal epithelium.

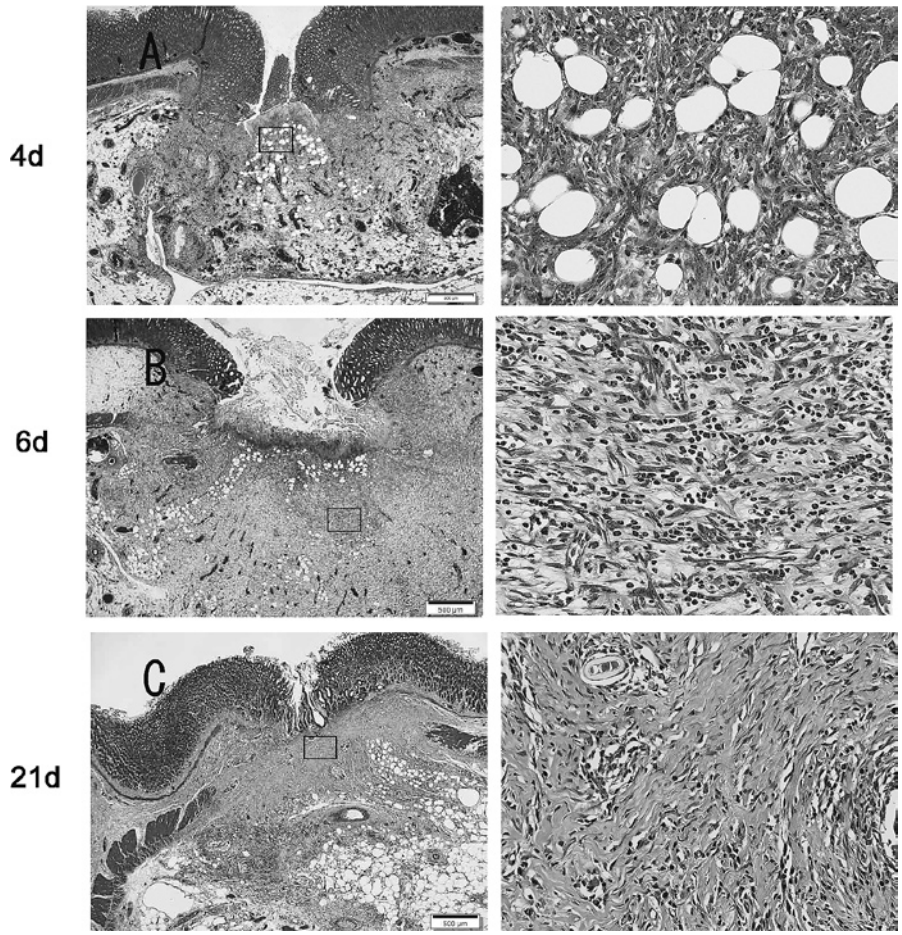

Fig. 3 Masson trichrome stained histological sections. Boxes in low magnification images (left panel) indicate the location of the higher magnification images (right panel). Scale bar = 500 μm. (A) Day 4. Much of the adjoining omental flap and damaged gastric wall was replaced by granulation tissue. There was an obvious increase in fibroblasts and vascular opens on the surface of omental flap (inset). (B) Day 6. Granulation significantly thickened. Collagen fibers became bolder with increased densities, and cells significantly decreased (inset). (C) Day 21. The muscular layer of the mucosa did not regenerate. The collagen patterns were distinctly different from the regular pattern typically seen in normal submucosal tissue (inset).

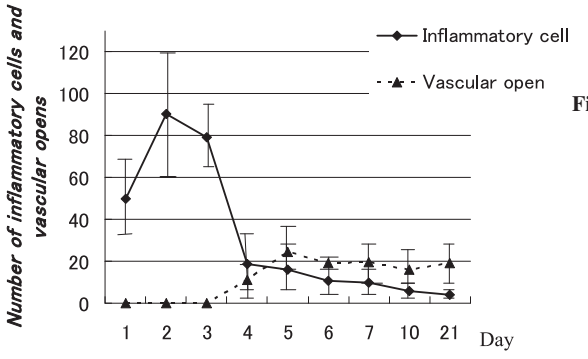

Fig. 4 Number of inflammatory cells per microscopic field under 600 × magnification. Data are expressed as the mean ±SD (n=3 in each group). The number of inflammatory cells reached a peak on Day 3, decreasing gradually thereafter, but remaining constant after Day 21. The number of vascular openings increased over time, peaking on Day 5, then decreasing until Day 10.

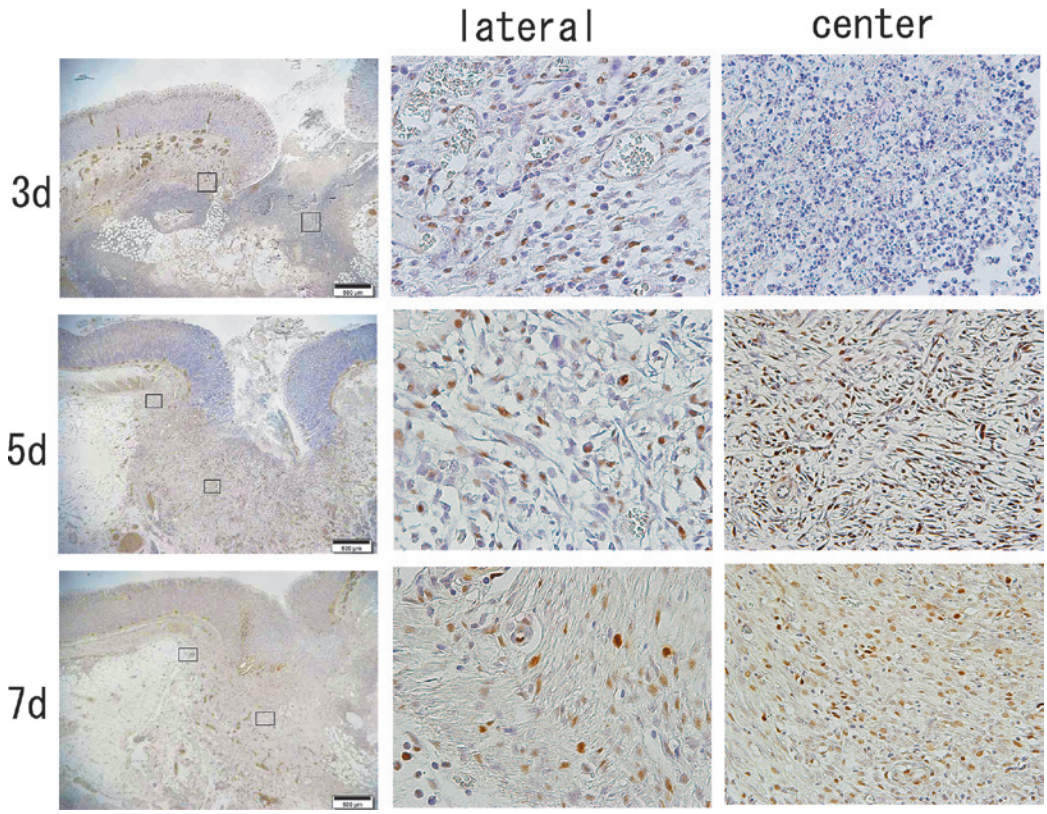

Fig. 5 Immunohistochemical staining of PCNA. Boxes in the low magnification images (left panel) indicate the location of the higher magnification images middle and right panel). Scale bar = 500 μm. Day 3. Large numbers of PCNA-positive cells could be observed, primarily on both sides of the adhesion ("lateral" panel). PCNA-positive cells were seen with predominating fibroblasts and endothelial cells. Day 5. Significant numbers of PCNA-positive cells were observed at the center of granulation. Approximately 50% of the cell population in the center of granulation was positive to PCNA immunostaining ("center" panel). Day 7. Once the defect had been completely covered, the number of PCNA-positive cells decreased significantly at the center of the granulation tissue.

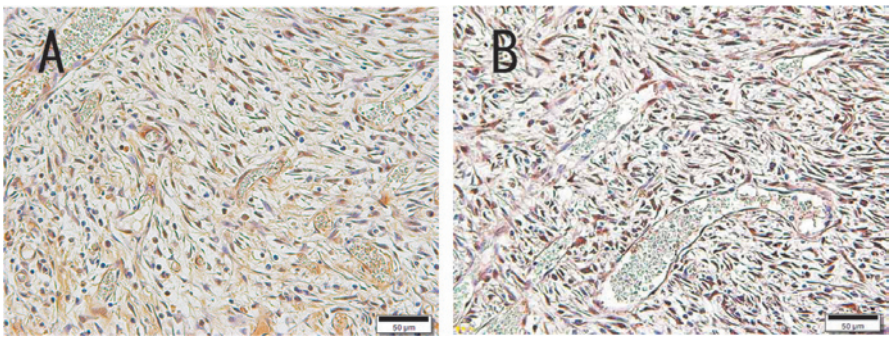

Fig. 6 Immnunohistochemical staining of FGF-2 and VEGF. (A)FGF-2 and (B) VEGF stains. Scale bar = 50 μm. From Day 4 to Day 7, FGF-2-positive cells and the VEGF-positive cells were seen with predominating endothelial cells and fibroblasts.

FGF-2 and VEGF findings

On operative Day 3, the FGF-2 and the VEGF-positive cells could only be observed at the edge of the submucosa. From Days 4 to 7, the number of FGF-2 and the VEGF-positive cells increased at the center of the granulation tissue as well as on both sides of the basal mucosal layer (Fig. 6). Both FGF-2 and the VEGF-positive cells were seen with endothelial cells and fibroblasts predominating. Several glandular cysts on Day 5 were positive for FGF-2 immunostaining.

DISCUSSION

Omental flaps have been used in the reconstruction of complex defects since the omentum contains a large amount of pliable, well-vascularized tissue. In general, the requirements for confirming the closures of complex wounds are (1) a firm fit and tight adhesion to the irregular cavity, (2) immediate recruitment of inflammatory cells and proliferation of granulation tissue from blood vessels. The omentum, being comprised of pliable tissue, fits the complex cavity. However, there is little experimental evidence to suggest a tight adhesion to an irregular cavity. Our observations revealed that the omentum had firmly adhered to the gastric serosa surrounding the defect on Day 1. Wilkosz *et al.*[13] also reported adhesion of the omentum to a peritoneal trauma site one day after surgery in a murine surgical model, demonstrating that adhesion develops as early as one day after surgery. Moreover, a PCNA study showed a proliferation of fibroblasts and the formation of new vessels at the adhesion site on Day 3, eventually to be converted into fibrous tissue (fibrous adhesion).

Inflammation occurred from Day 1 and reached a peak on Day 3 at the defect site and its surroundings. The abundant vessels in the omentum may dilate and contribute to the rapid supply of inflammatory cells. The number of inflammatory cells then dramatically decreased after Day 3, with granulation tissue rapidly filling and proliferating within the defect. This occurred coincidentally with the rapid development of neovasculature from preexisting vessels in the omental flap. These findings demonstrated that the abundant vessels in the omental flap played a role as a source of new vessels.

Immunolocalization of the PCNA study confirmed cell proliferation in this granulation tissue on both sides of the adhesion. FGF-2-positive cells and VEGF-positive cells were co-distributed in granulation tissue during Days 4 to 7. Our group has reported a lipid fraction from the omentum that contained angiogenic factors.[14] Litbarg and colleagues reported that when activated by a foreign body, the omentum showed increases in VEGF and blood density. Granulation tissue may arise from the induction and formation of fibroblasts and endothelial cells from these growth factors produced by the flap.[15]

The defects, which were initially left open as a control, resulted in random adhesions accidentally occurring around them within a few days. However, since such adhesions do not completely form to cover wounds, we do not present our data here.

In conclusion, omental flap coverage can promote the healing of refractory defects, acting as a source of inflammation and granulation as well as a pliable and well-vascularized tissue to fit and adhere to refractory defects.

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