Regenerative Therapy 7 (2017) 24–33

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Autologous adipose-derived stem cell sheets enhance the strength of intestinal anastomosis

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ARTICLE INFO

Article history:
Received 17 April 2017
Received in revised form
14 June 2017
Accepted 26 June 2017

Keywords:
Adipose-derived stem cells
Prevent anastomotic leakage

ABSTRACT

Objective: Adipose-derived stem cells (ASCs) are capable of multiple differentiation pathways, imparting immunomodulatory effects, and secreting factors that are important for wound healing. These characteristics can be exploited to decrease the incidence of anastomotic leakage.
Methods: In order to delay local wound healing at the anastomotic site, we induced ischemia in a portion of porcine small intestine by ligating vessels. Then, we injected mitomycin C into the serosa of the small intestine above the ligated vessels. Anastomotic sites were created by 2 cm incisions made in the opposite mesenteric area. ASCs were isolated from the porcine subcutaneous fat tissues and expanded under culture conditions. ASCs were trypsinized and seeded on temperature-responsive dishes and cultured to form confluent sheets. Three ASC sheets were transplanted onto the serous membrane after suturing. The extent of anastomotic wound healing was evaluated by bursting pressure, hydroxyproline content, and mRNA expression of collagen-1 alpha1 and collagen-3 alpha1.
Results: We found that transplantation of ASC sheets increased anastomotic site bursting pressure. Additionally, transplantation of ASC sheets increased the hydroxyproline content of the anastomoses. Furthermore, transplantation of ASC sheets increased mRNA expression of collagen-1 alpha1 and collagen-3 alpha1.
Conclusions: Our findings showed that transplantation of autologous ASC sheets enhanced collagen synthesis and anastomotic strength. Further studies are necessary to identify substances that, in combination with ASC sheets, might enhance collagen synthesis and healing in sites of anastomosis.

1. Introduction

Anastomotic leakage is a feared complication of colorectal surgery. Wound dehiscence of the anastomotic surgical site leads to not only postoperative morbidity and mortality but also cancer recurrence and poor patient prognosis [1,2]. Although anastomotic devices, surgical techniques, and preoperative care have improved, the rate of anastomotic leakage after colorectal surgery remains high at 3–14% [3,4].

Preoperative chemoradiotherapy for rectal cancer improves local disease control, but can also induce inadequate anastomotic healing and may sometimes lead to severe anastomotic leakage. Previous studies have reported that preoperative chemoradiotherapy influenced the risk of anastomotic leakage postoperatively [5–8].

Generally, intestinal wound healing after anastomosis is divided into three phases: the inflammation phase, proliferation phase, and remodeling phase [9,10]. The strength of the fused intestinal anastomotic site declines in the early postoperative period, and collagen metabolism is necessary in order to provide compensatory strength [10]. In the inflammation phase, collagenase and other matrix metalloproteinases break down the mature collagen at the anastomotic site, resulting in a reduction in the intestinal anastomosis breaking strength because of the decreased concentration of existing collagen [11,12]. In the proliferation phase, fibroblasts are

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

http://dx.doi.org/10.1016/j.reth.2017.06.004

attracted into the anastomotic site to synthesize a collagen-rich granulation tissue [10,12]. The anastomotic strength depends on the presence of collagenous networks produced by fibroblasts that infiltrate the anastomotic site during the proliferation phase [13–15]. Preoperative chemoradiotherapy can disturb these normal wound healing processes in anastomosis sites, and may sometimes lead to severe anastomotic leakage. Thus, methods to enhance wound healing are needed to reduce the risk of anastomotic leakage in delayed wound healing conditions, such as preoperative chemoradiotherapy.

Regenerative medicine and the tissue engineering field have provided new therapeutic options for preventing anastomotic leakage. Adipose-derived stem cells (ASCs) are capable of multiple differentiation pathways, imparting immunomodulatory effects, and secreting factors that are important for wound healing [16,17]. Because of these characteristics, ASCs have recently become a prime candidate for the repair of damaged tissue and other regenerative medicine applications [18–20]. Several experimental studies have reported that local injections of homogenous ASC suspensions improve the healing of intestinal anastomotic sites [21,22].

In this study, we evaluated the ability of transplanted ASC sheets to improve anastomotic strength and wound healing at intestinal anastomotic sites under delayed wound healing conditions, representative of conditions such as preoperative chemoradiotherapy.

2. Materials and methods

2.1. Animal care

All animals were treated with experimental procedures approved by the Committee for Animal Research of Tokyo Women's Medical University (approval number 15–21 2015). Eleven miniature pigs (6 months of age, 17–20 kg, females, obtained from the Nippon Institute for Biological Science) were used in this study. Four pigs were used to develop delayed wound healing model. Another seven pigs were used to evaluate efficacy of transplanted ASC sheets at intestinal anastomotic sites under delayed wound healing conditions. The pigs were injected intramuscularly with 0.04 mg/kg atropine and 15 mg/kg ketamine as premedication. Then, they received 2.5 mg/kg propofol intravenously. An endotracheal tube was inserted and anesthesia was maintained using sevoflurane and nitrous oxide inhalation with mechanical ventilation.

2.2. Experimental design

The experimental design and procedure for evaluating the transplantation of autologous ASC sheets onto the serous membrane after anastomosis in the pig's small intestine are shown in Fig. 1. Two weeks prior the operation, ASCs were isolated from the subcutaneous fat of pigs and expanded under the culture conditions described later. ASCs were seeded on temperature-responsive dishes and cultured for five days. Eight anastomotic sites and an anastomosis bypass were created under same conditions of the delayed wound healing model by the method described later. The eight sites were divided randomly into ASC sheet transplantation (anastomotic site wrapped with three ASC sheets) and untreated groups (no additional treatment). One pig was euthanized on postoperative day 1 for evaluating mRNA expressions of FGF2, TGF-β1, collagen-1 alpha1, and collagen-3 alpha1 in tissue obtained from the anastomotic site surroundings, and two pigs were euthanized on postoperative day 5. The final four pigs were euthanized on postoperative day 7 for evaluating the strength of and collagen formation in the anastomotic sites.

2.3. Isolation of the ASCs

The lower abdominal subcutaneous adipose tissue was surgically excised from the miniature pigs. The ASCs were isolated from the lower abdominal subcutaneous adipose tissue (20 g). After mincing the tissue into small pieces, the adipose tissue was enzymatically digested with 0.1% Collagenase (Collagenase NB 4G Proved Grade, SERVA Electrophoresis, Heidelberg, Germany) at 37 °C for 1 h. Then, the tissue was placed in a shaker for high-speed shaking at 150 rpm. After centrifugation, the floating adipose tissue was discarded. After filtration and centrifugation at 500 g for 5 min, cells were collected in a pellet. The cells in the pellet were resuspended and cultured in a complete culture medium (Dulbecco's modified Eagle's medium; DMEM, Invitrogen, CA, USA) and supplemented with 10% fetal bovine serum (Japan Bio Serum, Hiroshima, Japan) and with 1% penicillin/streptomycin (Gribco, CA, USA) at 37 °C at 5% CO_2. ASCs were passaged every 3 days at a density of 1.7×10^3 cells/cm^3 until the third passage was achieved.

2.4. Characterization of the ASCs

2.4.1. Flow Cytometry Assay

The isolated ASCs' surface antigen expression was analyzed by flow cytometry according to a previously reported method [23]. One million passage 3 ASCs were suspended in 100 μL PBS containing 10 μg/ml fluorescein isothiocyanate-conjugated primary antibodies specific to mesenchymal stromal cells (MSCs) (CD29, CD44, CD90, and CD105) and hematopoietic cells (CD31 and CD45) ($n = 3$). The following expression markers reactive with the porcine antigen isoforms were used: Alexa Fluor 647 Mouse Anti-Pig CD29 (BD Bioscience, New Jersey, USA), Anti-CD44 antibody [IM7] (Abcam plc, Cambridge, UK), APC Mouse Anti-Human CD90 (BD-Biosciences), Anti-CD105 antibody [MEM-229] (Abcam plc), PE Mouse Anti-Rat CD31 (BD Biosciences), and Monoclonal Antibody to CD45/LCA (CD45R)-PE (Acris Antibodies, Inc. CA, USA) [24–28]. Cell fluorescence was evaluated with a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan) and the data were analyzed using Karuza for Gallios software (Beckman Coulter).

2.4.2. Differentiation and proliferation of ASCs in vitro

The capacities of passage 3 ASCs to differentiate into adipogenic lineages and osteogenic lineages were evaluated using a previously reported method [23]. For adipogenesis, the medium was switched to an adipogenic medium consisting of a complete medium supplemented with 0.5 μmol/L isobutyl-1-methyl xanthine (Sigma–Aldrich, St. Louis, USA), 0.5 μmol/L dexamethasone (Fuji Pharma, Tokyo, Japan), and 50 μmol/L indomethacin (Wako Pure Chemical Industries, Osaka, Japan). After 14 days, the cells were fixed with 4% PFA and stained with fresh Oil Red O solution (Wako Pure Chemical Industries). For osteogenesis, the medium was switched to a calcification medium consisting of a complete medium supplemented with 50 μmol/L ascorbic acid (Wako Pure Chemical Industries), 10 mmol/L β-glycerophosphate (Sigma–Aldrich), and 100 nmol/L dexamethasone. The cells were incubated for 21 days, and then stained with 1% alizarin red S solution. The proliferation capacities of passage 3 ASCs were evaluated according to the previously reported colony-forming unit assay method [23]. Briefly, 100 cells were cultured in 60-cm^2 dishes for 9 days and stained with crystal violet. Then, proliferation capacity was measured.

2.5. Preparation of ASC sheets

Passage 3 ASCs were seeded on 35-mm-diameter temperature-responsive culture dishes (UpCell; CellSeed, Tokyo, Japan) at a density of 2.4×10^6 cells/dish and cultured in complete culture