Effects of human adipose-derived stem cells on the regeneration of damaged visceral pleural mesothelial cells: a morphological study in a rabbit model

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Abstract

OBJECTIVES: Although an alveolar air leak (AAL) after pulmonary resection is a troublesome complication that diminishes a patient’s quality of life and increases medical costs, current treatment and preventive methods for AAL are not effective. Therefore, we transplanted adipose-derived stem cells (ASCs) to the damaged visceral pleura to facilitate the regeneration of mesothelial cells and investigated the possibility of cell therapy as a treatment option for AAL.

METHODS: Stem cells were isolated and cultured from discarded fat tissues that were collected after liposuction procedures. Flow cytometry analysis was performed to evaluate their suitability as mesenchymal stem cells. Cultured stem cells were seeded onto polyglycolic acid (PGA) sheets and incubated for 5 days. Under general anaesthesia, 10 New Zealand rabbits underwent thoracotomies. After the visceral pleura was damaged, PGA sheets containing ASCs were transplanted into 5 rabbits (ASC group) and PGA sheets without cells were transplanted into the other 5 rabbits (control group). Rethoracotomies were performed after 4 weeks, and the transplanted areas in the visceral pleura were excised for analysis. Haematoxylin and eosin and Azan staining were performed. In addition, electron microscopic examinations were performed to investigate the ultrastructure of the regenerating mesothelium.

RESULTS: Cultured stem cells were positive for the surface proteins CD13, CD29, CD49d, CD90 and CD105, whereas they were negative for CD34, CD45 and human leukocyte antigen (HLA)-DR. The adhesions between the transplanted visceral pleura and parietal pleura were weaker in the ASC group than in the control group. On histological examination, the mesothelial cell monolayer of the visceral pleura was well preserved in the ASC group, whereas it was frequently lost in the control group. Electron microscopy demonstrated that the mesothelial cell monolayer and its abundant microvilli were well preserved in the ASC group, but were absent or disintegrated in the control group.

CONCLUSIONS: Transplantation of ASCs to the damaged visceral pleura can contribute to the treatment and prevention of AAL by improving the regeneration of mesothelial cells.

Keywords: Alveolar air leak • Mesothelial cell • Adipose-derived stem cell

INTRODUCTION

A prolonged alveolar air leak (AAL) that lasts for more than 5 post-operative days is a common complication that occurs in 8–26% of patients who have undergone pulmonary resection, and it leads to an increased chance of infection, pain and increased medical costs because of an extended hospital stay [1]. In particular, if an air leak originates from the exposed alveoli because of visceral pleural damage during pulmonary dissection, it is difficult to control because the leak can be from an extensive area, and surgical techniques, such as stapling or suturing, cannot easily fix the defect, resulting in injury to the surrounding normal tissue. Although synthetic sealants with fibrin glue are commonly used in clinical practice, they are not effective in closing a visceral pleural defect because they do not have enough adhesive strength. As a result, fibrous adhesions may be formed between the visceral and parietal pleura, and can cause decreased pulmonary function and loss of metabolic functions of the mesothelial cells.

Therefore, there is an emerging need for tissue-engineering methods that can restore the functions of the mesothelial cells, rather than providing simple physical closure. Although attempts have been made to transplant fibroblasts [2] or cultured autologous mesothelial cells [3, 4] to the damaged mesothelium, cell-based therapies have not yet been established. Therefore, we transplanted human adipose-derived stem cells (ASCs) into the damaged visceral pleura in a rabbit model and performed histological and ultrastructural analyses of the repaired visceral pleura to investigate the possibility of cell therapy as a treatment option for AAL.
MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Review Board of Bucheon St Mary’s Hospital, and informed consent was obtained from the patients in accordance with the Declaration of Helsinki. The animal study was performed after receiving the approval, and in accordance with the guidelines, of the International Animal Care and Use Committee of The Catholic University of Korea.

Isolation of adipose-derived stem cells and analysis of surface markers

ASCs were isolated and cultured from the abdominal subcutaneous adipose tissue of patients undergoing liposuction procedures according to the methods of Zuk et al. [5]. Briefly, the adipose tissue was rinsed with phosphate-buffered saline (PBS) and incubated in a stirred solution containing PBS and 0.075% collagenase for 30 min at 37°C; and it was then mixed with an equal volume of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and centrifuged at 1200 rpm for 10 min. The pellet was filtered through a nylon mesh (70 μm; nylon cell strainer, BD Falcon, Bedford, MA, USA) and seeded into a T75 flask; it was then incubated in a controlled humidified incubator with 5% CO₂ at 37°C.

For flow cytometry analysis, the ASCs were suspended in PBS containing 2% FBS at a density of 1 × 10⁶ cells/ml, and were incubated with mouse monoclonal antibodies (Serotec, Kidlington, Oxford, UK) directed against CD13, CD29, CD34, CD45, CD49d, CD90, CD105 and HLA-DR for 30 min at room temperature. The labelled cells were analysed using a FACSVantage SE cell sorter (Becton Dickinson, San Jose, CA, USA).

Transplantation of polyglycolic acid sheets

In the third passage, ASCs were seeded onto polyglycolic acid (PGA) sheets (3 × 3 cm; NeoVeil, Gunze, Kyoto, Japan) at a density of 1 × 10⁶ cells/ml, together with DMEM supplemented with 10% FBS, 1% penicillin-streptomycin and 10 ng/ml fibroblast growth factor-2 in a HydroCell flask (Nunc, Roskilde, Denmark). The medium was exchanged on the third day, and the ASCs that adhered to the PGA sheet were prepared for transplantation on the fifth day.

Ten New Zealand rabbits weighing 2.8–3.0 kg were used in this experiment after 4 weeks of adaptation. Animals were maintained on a 12-h light/12-h dark cycle, and had free access to commercial rabbit food and tap water; the room temperature (20–21°C) and relative humidity (50–60%) were constantly maintained. For the induction of anaesthesia, intramuscular tiletamine/zolazepam (1.5 mg/kg; Zoletil, Virbac, Carros, France), glycopyrrolate (0.2 mg/kg; Mobiniul, Myungmoon, Korea) and intravenous xylazine hydrochloride (2 mg/kg; Rompun, Bayer, Leverkusen, Germany) were injected. Tracheal intubation was performed using a #3 tracheal tube (Malinckrodt, Athlone, Ireland); the tube was connected to a respiratory machine (Dräger GmbH, Lubeck, Germany) and inspiration of isoflurane (1–3%; Forane, Choongwae, Korea) vaporized in 100% oxygen was performed. All of the animals were placed in the left lateral position and underwent a right thoracotomy via the fifth intercostal space. Lung parenchyma, including visceral pleura measuring an area of approximately 1.5 × 1.5 cm with a depth of 0.5 cm, was excised from the right lower lobe, and an air leak from the defect was confirmed. PGA sheets containing ASCs were transplanted into the damaged visceral pleura of 5 rabbits (the ASC group), and PGA sheets without cells were transplanted into the damaged visceral pleura of the other 5 rabbits (the control group); then, fibrin glue (0.5 ml; Greenplast, Greencross, Korea) was evenly instilled over the implanted PGA sheet. Each wound of the chest wall was closed layer by layer after a thoracic drainage catheter with a bag (100 ml; Barovac, Sewoon, Korea) was inserted. The animals were extubated once spontaneous breathing was restored, and the drainage catheters were removed when there were no more air leaks into the drainage bag.

Histological and ultrastructural analyses

Four weeks after transplantation, the animals were sacrificed with an intramuscular injection of tiletamine/zolazepam (Zoletil, 3 mg/kg) and intravenous injection of potassium chloride. A righthoracotomy was performed in each animal and the pleural space was examined grossly. The transplanted site of the right lower lobe was then resected for histological analysis. The resected specimens were fixed with 4% PFA and processed into 5-μm-thick paraaffin-embedded sections. Haematoxylin and eosin (H&E) and Azan staining were performed for microscopic examination.

For scanning electron microscopic (SEM) examination, the specimens were fixed in a solution of 2.5% glutaraldehyde buffered with 0.1 M phosphate for 24 h, and were then washed with 0.1 M phosphate buffer solution, dehydrated, critical point dried and coated with gold to a thickness of 10 nm. The visceral pleural surfaces were observed using SEM (JSM-5410LV, JEOL, Tokyo, Japan) at an acceleration voltage of 15 kV. For transmission electron microscopy (TEM), the specimens were fixed and dehydrated using the same method. The specimens were processed into 60-nm-thick Epon-embedded sections and stained with uranyl acetate and lead citrate. Cross sections of the visceral pleura were observed using TEM (JEM-1010, JEOL) at an acceleration voltage of 15 kV.

RESULTS

The cultured ASCs had a fibroblast-like appearance and were confirmed as mesenchymal stem cells (MSCs) by flow cytometry analysis in our previous study [6], which revealed that the isolated ASCs were positive for CD13 (90.3 ± 4.0%), CD29 (98.9 ± 0.7%), CD49d (13.6 ± 6.0%), CD90 (99.4 ± 0.1%) and CD105 (96.0 ± 2.8%), but were negative for CD34, CD45 and HLA-DR. On the third postoperative day, all of the animals were alive until sacrificed 4 weeks after the operation, and there was no evidence of infection or rejection response. All of the animals showed adhesions between the transplanted areas in the visceral pleura and the corresponding parietal pleura. Most of these adhesions were weak and could be separated by gentle traction; however, there...
were 2 cases of dense adhesions in the control group that required a sharp dissection.

The visceral pleura was thickened because of inflammation and foreign-body reactions in both groups. The mesothelial cells were well preserved in the ASC group (Fig. 1A); however, they were frequently lost in the control group (Fig. 1C). The thickness of the extracellular matrix in the connective tissue layer was greater in the control group (Fig. 1D,F).

Ultrastructural analysis showed that the mesothelial cell monolayer was well preserved in the ASC group (Fig. 2A,D), in which squamous or cuboidal cells with abundant microvilli were well integrated into the surface, as in the normal visceral pleura.

Figure 1: Light microscopic examination of the visceral pleura from the adipose-derived stem cells (ASCs) group (A,D), normal lungs (B,E) and the control group (C,F). (A,C) Haematoxylin and eosin staining of the visceral pleura demonstrated that the mesothelium was thickened in the ASC and control groups compared with the normal lung because of inflammation and fibrosis. However, the mesothelial cells were well preserved in the ASC group, but not in the control group. (D,F). Azan staining showed that the extracellular matrix in the connective tissue layer of the control group was thicker than that in the connective tissue layer of the ASC group or the normal lung.

Figure 2: Ultrastructure of the visceral pleural surface. (A,D) Electron microscopic examinations of the visceral pleura of the adipose-derived stem cells (ASCs) group demonstrated that the mesothelial cells were well preserved and had abundant microvilli. (B,E) Visceral pleura of the normal lung. (C,F) The visceral pleura of the control group showed disintegration of the mesothelial monolayer and exposure of collagen (arrowheads).
ASCs are free from ethical problems, can be harvested in abundant quantities, can be cultured rapidly and can be differentiated along multilineage pathways [11]. Furthermore, with the increased incidence of obesity in modern populations, subcutaneous adipose tissue is abundant and readily accessible [12]. ASCs are widely used in the field of regenerative medicine; however, they have rarely been used as a cell-based treatment for damaged pleura.

As stated earlier, the mesothelium has many important functions, but can be easily damaged during surgery; the results include pain, increased duration of hospital stay, decreased lung function, intestinal obstruction and infertility. Although the mechanism of regeneration of the mesothelium is not fully understood, Mutsaers [7] demonstrated that mesothelial repair occurs diffusely across the injured surface, which is different from that of other epithelial-like surfaces, where healing occurs solely from the wound edges as sheets of cells. This observation might be an evidence for the presence of sub-serosal mesenchymal precursor cells and their ability to migrate to the surface and differentiate into mesothelial cells when damaged [13]. Mutsaers [14] also stated that regenerating mesothelial cells are characteristically cuboidal in appearance rather than squamous in appearance and have prominent organelles, such as the Golgi apparatus. These findings were also observed in our study, in which the regenerating mesothelial cells were cuboidal or cobblestone in appearance and had a stratified structure in some areas (Fig. 3).Another characteristic finding of regenerating mesothelial cells that was observed in our study was the presence of abundant microvilli (Fig. 2D). Microvilli have a crucial role in mesothelial cell function by increasing surface area and trapping fluid. Although the distribution of microvilli is variable according to the site, it is known to be especially dense when cells are under a lot of stress or under repair [15].

Despite the recent progress in tissue engineering and biotechnology, there are no clinically applicable treatment methods using stem cells in the respiratory system as yet [16]. Rojas et al. [17] performed transplantation of bone marrow stem cells to promote repair of injured lung parenchyma, and Shigemura et al. [18] reported that the transplantation of ASCs in a lung volume reduction surgery model resulted in enhanced alveolar and vascular growth. However, there are no reports that can definitely elucidate the mechanisms by which the stem cells improve the regeneration of injured cells. Although we investigated only the morphological effects of ASCs on the regeneration of mesothelial cells in the present study, we hope to conduct further research to identify the mechanisms by which stem cells contribute to the repair of injured tissue.
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REFERENCES


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eComment. Experimental controversy regarding the role of adipose-derived stem cells in surgical oncology

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We read with great interest the article titled “Effects of human adipose-derived stem cells on the regeneration of damaged visceral pleural mesothelial cells: a morphological study in a rabbit model” by Kim et al. [1]. This well-designed study showed that adipose-derived stem cells (ASCs) can contribute to the treatment of alveolar air leak after pulmonary resection.

Cancer, however, is the first leading cause of lung surgery. Several reports have shown that the immunosuppressive capacity of ASCs may in some cases favour the growth of tumour cells, but contradictory results exist. Muehlberg et al. [2] reported that ASCs in a murine model promote tumour growth in vivo, not only when injected to the tumour site, but also when injected intravenously. Recent reports have documented the ability of ASCs to induce the proliferation of active breast cancer cells in vitro and in vivo via paracrine mechanisms [3]. Conversely, Kucerova et al. [4] showed that cytokine deaminase-expressing ASCs deliver the cytokine deaminase transgene to the site of tumour formation and mediate a strong anti-tumour effect in vivo. Cousin et al. [5] reported that ASCs strongly inhibit proliferation of pancreatic ductal adenocarcinoma cells, both in vitro and in vivo by interfering with the proliferation of tumour cells and altering cell cycle progression. These contradictory results indicate that the work is far from done, and further consensus protocols are necessary to fully elucidate the true effect of ASCs on tumour excision sites.

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References