Optimal Condition of Isolation from an Adipose Tissue-Derived Stromal Vascular Fraction for the Development of Automated Systems

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Abstract

BACKGROUND: The stromal vascular fraction (SVF) isolated from adipose tissue, which contains stem cells as well as other cell types, has been applied in various research fields. Although different enzymatic concentrations and treatment durations have been applied to isolate the SVF, optimal conditions have not been established. Thus, we aimed to establish the optimal conditions for isolation of the SVF from adipose tissue by automated systems.

METHODS: The SVF was collected from removed adipose tissues of five donors during surgery. The SVF was treated with 0.1% or 0.2% collagenase type I for 20, 40, or 60 min. Then, colony forming unit (CFU) assays and flow cytometry were performed to characterize the adipose stem cells (ASCs). A cytokine array was used to investigate the correlation between colony-formation ability and the secretion of isolated ASCs.

RESULTS: Treatment with 0.1% collagenase type I for 60 min resulted in a higher SVF yield, whereas treatment with 0.1% collagenase for 40 min resulted in higher CFU values. In addition, expression of interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1 in the SVF was higher in the high-CFU group than in the low-CFU group.

CONCLUSION: The optimal conditions for isolation of the SVF from adipose tissue were treatment with 0.1% collagenase type I for 40 min. We identified the conditions required for efficient SVF isolation based on high CFU values, and our results will facilitate the development of automated systems.

Keywords Adipose tissue · Stromal vascular fraction · Isolation · Automated systems

1 Introduction

Human mesenchymal stem cells (MSCs) have the capacity to self-replicate, proliferate and differentiate into several mesodermal lineages, such as osteocytes, adipocytes, and chondrocytes [1]. MSCs have been isolated from bone

marrow, adipose tissue, dermis, muscle, heart, tendon, spleen, embryonic tissues. Human adipose tissue is an abundant source of mesenchymal stem cells, which show therapeutic potential in esthetic and regenerative medicine [2, 3]. Adipose tissue is found throughout the human body. It contains adipocytes, as well as the SVF, which comprises adipose stem cells (ASCs), preadipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cells [4, 5]. The use of human adipose-derived stromal vascular fraction (SVF) in therapy has increased in recent years [6–8].

In this study, we aimed to identify the optimal conditions for isolation of the SVF by automated systems. Oberbauer et al. [9] reported that automated systems yield greater numbers of cells compared with manual isolation.

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Accordingly, we used an automated system to minimize contamination of the SVF. In the evaluation of automated cell-isolation systems, several standardized factors are taken into consideration, such as the process, enzyme type, reagents, and donor characteristics [9]. To develop an automated cell-isolation system, the appropriate enzyme concentration and treatment duration must first be determined. There are several different methods to separate the SVF, including at least one that is not based on enzymatic activity [10]. Busser et al. [11] evaluated both non-enzymatic and enzymatic systems for isolating SVF cells. Among many enzymes, collagenase type I is typically employed for its ability to degrade collagen in the extracellular matrix, facilitating separation of the SVF. However, prolonged exposure of collagen to type I collagenase can disrupt the cell surface, leading to the death of SVF cells [12]. We hypothesized that enzyme concentrations and treatment duration would affect the yield and viability of the SVF, as well as the functionality of the isolated ASCs. Although the optimal conditions for SVF separation have yet to be established [13–15], we were able to identify enzyme separation conditions that preserved the stability and functionality of ASCs. In this study, we compared the yield and viability of the SVF isolated using different enzyme concentrations and treatment durations. We used flow cytometry to confirm the stem cell function of the isolated cells by evaluating the expression of positive and negative markers in the SVF as well as the ability of the SVF to form colonies.

2 Materials and methods

2.1 Isolation of SVF

The SVF was collected from adipose tissue removed from five donors during surgery. The adipose tissue was washed three times with phosphate-buffered saline (PBS; Wisent Inc., Quebec, Canada) and chopped into small pieces. The pieces were treated with 0.1% or 0.2% type I collagenase for 20, 40, or 60 min at 37 °C in a Cellticator (Medican, Seoul, Korea). The digested adipose tissue was washed three times in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Wisent Inc.) and 1% antibiotic–antimycotic solution (Gibco-BRL) and centrifuged for 3 min at 1300 rpm. The supernatants were discarded; the cell pellets were suspended in DMEM and passed through a 100- μ m strainer (Becton–Dickinson, San Jose, CA, USA). The filtered cells were centrifuged for 3 min at 1300 rpm and the supernatants were discarded. The cell pellets were suspended in DMEM and counted using a hemocytometer. ASCs were obtained by plating the

SVF cells, thereby enriching the adherent ASCs. The SVF was then seeded in culture dishes at a density of 7000/cm² and incubated at 37 °C in 5% CO₂. Upon reaching 80–90% confluence, the cells were treated with trypsin–ethylene-diaminetetraacetic acid (Gibco BRL) and subcultured. The culture medium was replaced every 3 days.

2.2 Flow cytometry

ASCs (passage 3) were trypsinized and resuspended in Hanks' balanced salt solution (Wisent Inc.) containing 2% FBS. The cells were then incubated with monoclonal fluorescein isothiocyanate-conjugated antibodies against CD90, CD13, human lymphocyte antigen class II (HLA-dr; Becton–Dickinson), and CD105 (AbD Serotec, Oxford, UK) and PE-conjugated antibodies against CD34, CD73, CD45, and CD146 (Becton–Dickinson) for 30 min on ice in the dark. After washing with Hanks' balanced salt solution, cell-surface markers were detected and quantitated using Summit software, version 5.2 (Beckman Coulter, Brea, CA, USA).

2.3 Colony formation assay

To assay colony formation, 1000 ASCs (passage 3) were seeded into 100-mm culture dishes and incubated for 3 weeks at 37 °C in 5% CO₂ in the appropriate culture medium. Next, the culture medium was discarded and the cells were washed three times with PBS. The cells were fixed in methanol (Duksan, Ansan, South Korea) for 5 min and the methanol was removed. The fixed ASCs were stained in 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, and then washed with PBS and distilled water. The stained cells were dried at room temperature and colonies (defined as a cluster of at least 40 cells) were counted. The culture medium was replaced every 3 days.

2.4 Cytokine array

To analyze cytokine secretion, ASCs (passage 3) were seeded in 100-mm culture dishes at a density of 6000/cm². The medium was exchanged the following day for fresh DMEM containing 1% antibiotic–antimycotic. After incubation for 72 h, the supernatant was collected and subjected to analysis using a RayBio Human Angiogenesis Antibody Array C1 (RayBiotech, Norcross, GA, USA) that had been pretreated according to the manufacturer's instructions. The membrane of the cytokine array was analyzed using an LAS 4000 (Fujifilm, Tokyo, Japan), and the relative ratios were quantified using Multi Gauge software (Fujifilm).

2.5 Statistical analysis

Statistics were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The results are presented as mean $\pm$ standard deviation. Statistical significance was determined using *t*-tests with a significance level of $p < 0.1$ or $p < 0.01$.

3 Results

3.1 Yield changes according to enzyme concentration and treatment duration of SVF

Stem cell yields increased with enzyme treatment duration and enzyme concentration. Treatment for 20 and 40 min with 0.2% collagenase yielded a greater number of cells than did treatment for the same times with 0.1% collagenase. Treatment with 0.1% collagenase for 60 min yielded the greatest number of cells (Fig. 1). The yields obtained using 0.1% collagenase increased with the treatment time, whereas the yields obtained using 0.2% collagenase did not differ significantly with longer treatment.

3.2 Viability according to enzyme concentration and treatment duration of SVF

Cell viability decreased as the treatment duration increased, with a significant decrease at 60 min (Fig. 2). At 60 min, 90% of the cells incubated with 0.1% collagenase were viable, compared to less than 80% of the cells incubated with 0.2%.

Fig. 1 Yield of stromal vascular fraction (SVF). Adipose tissue was incubated for 20, 40, or 60 min with 0.1% or 0.2% collagenase type I. SVF yields increased in a time-dependent manner (* $p > 0.1$, ** $p > 0.01$)

Fig. 2 Survival of the stromal vascular fraction (SVF). Adipose tissue was incubated for 20, 40, or 60 min with 0.1% or 0.2% collagenase type I. The survival of SVF decreased in a time-dependent manner (* $p > 0.1$), and the magnitude of the decrease was greater after treatment for 60 min than after treatment for 40 min

3.3 Flow cytometry analysis according to the difference in enzyme concentration and treatment duration of SVF

All cells were separated and analyzed by flow cytometry. More than 80% of the population in each treatment group expressed the stem-cell-positive markers CD13, CD90, CD73, and CD105 (Fig. 3). The stem-cell-negative markers CD34, CD45, CD146, and HLA-dr were present in less than 20% of the population. There was no significant difference in the antigen–antibody response on the cell surface with different treatment times.

Fig. 3 Results of flow cytometry. CD13, CD90, CD73, and CD105 were used as positive markers and CD34, CD45, CD146, and HLA-dr as negative markers. More than 80% of the ASCs were positive and less than 20% were negative, based on their marker-expression patterns

3.4 Colony-forming unit of SVF according to the enzyme concentration and treatment duration

The lowest CFU values were observed in the groups treated with 0.1% and 0.2% collagenase for 20 min. Treatment for 40 min resulted in significantly more CFUs compared with treatment for 20 min. CFU values were significantly lower after 60 min of treatment than after 40 min of treatment. After 40 min of treatment, cells treated with 0.1% collagenase showed more colony formation than did cells treated with 0.2% collagenase (Fig. 4).

3.5 Growth factor cytokine array according to colony-forming unit ability of treated SVF

Cytokine profiles were evaluated in the high-CFU group (treated with 0.1% collagenase for 40 min) and the low-CFU group (treated with 0.1% collagenase for 20 min). Cells in both the high- and low-CFU groups expressed angiogenin, GRO α/β , interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T cell-expressed and secreted (RANTES), tissue inhibitor of metalloproteinase (TIMP)-1 and -2, and vascular endothelial growth factor (VEGF)-A (Table 1, Fig. 5A). The expression levels of IL-6, IL-8, and MCP-1 were significantly higher in the high-CFU group than in the low-CFU group (Fig. 5B).

4 Discussion

Adipose tissue is composed of mature adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, monocytes, and lymphocytes; it is a rich source of

pluripotent ASCs. Although isolation of the SVF from adipose tissue is a simple process that is performed in biological laboratories, for immediate clinical application of this method there are limitations that must be overcome. To prevent contamination, ensure the integrity of clinical research, and comply with regulatory requirements, an optimized automated system that provides consistent output is needed. In this study, collagenase type I was employed to obtain ASCs, although use of collagenase type II or trypsin has also been reported [16]. Both the concentration and duration of enzyme treatment vary among studies; therefore, we performed a controlled study comparing different collagenase type I concentrations (0.1% and 0.2%) and treatment durations (20, 40, and 60 min) to determine the optimal conditions. The replication and self-renewal abilities of cells are indicative of their stem-cell potential but these abilities are easily impaired by prolonged exposure to enzymes [11, 17]. In the present study, using 0.1% collagenase, a treatment time of 60 min resulted in lower cell viability than did a treatment time of 40 min. This demonstrated that a high collagenase concentration induced cell damage and death. Additionally, prolonged exposure to the enzyme increased cell damage and death. Thus, to determine the optimal conditions for SVF isolation, we explored different combinations of enzyme concentrations and exposure times. Our results indicate that to obtain healthy cells the optimal conditions for SVF isolation are 0.1% collagenase with a relatively short-term treatment of 40 min.

The SVF groups were cultured up to passage 3 under various conditions to characterize the isolated SVF. By flow cytometry, CD105, CD90, CD13, and HLA-dr (markers of mesenchymal stem cells) were expressed in more than 80% of cells, whereas the hematopoietic markers CD34, CD31, and CD45 were not expressed [9, 16, 18]. There was no significant difference in the antigen–antibody response on the cell surface. A CFU assay was performed to evaluate self-renewal, a unique characteristic of stem cells [19]. The CFU assay is one of several methods used to determine stem cell activity. In this study, using the CFU assay, various combinations of enzyme concentrations and exposure times were classified into high and low groups; 0.1% collagenase type I for 40 min produced relatively high CFU values, whereas 0.1% collagenase type I for 20 min resulted in lower CFU values. Then, we subjected each group to a cytokine array to confirm the correlation between CFUs and cytokine expression. The cytokine array was performed to determine whether the colony-forming ability affected the secretion of cells [20]. Cells in the high- and low-CFU groups were analyzed using an angiogenesis array, because angiogenesis contributes to wound healing and tissue regeneration. Cells in both the high- and low-CFU groups expressed angiogenin, GRO α/β , IL-6, IL-8,

Fig. 4 Colony-formation ability. The number of colonies was greater after treatment for 40 min than after treatment for 20 or 60 min (* $p > 0.1$)

Table 1 The cytokine array used in this study

POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78(CXCL5)	bFGF
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78(CXCL5)	bFGF
GRO a/b/g	IFN-gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
GRO a/b/g	IFN-gamma	IGF-1	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
PLGF	RANTES	TGF beta1	TIMP-1	TIMP-2	TPO	VEGF-A	VEGF-D
PLGF	RANTES	TGF beta1	TIMP-1	TIMP-2	TPO	VEGF-A	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

Fig. 5 Cytokine levels. **A** Adipose stem cells were incubated in conditioned medium for 72 h. **B** Cytokine levels compared with the positive control. IL-6, IL-8, and MCP-1 levels were higher in the high-CFU group (* $p < 0.1$)

MCP-1, RANTES, TIMP-1 and -2, and VEGF-A [21, 22]. IL-6 and IL-8 are paracrine factors that contribute to wound healing, and their expression increases during an inflammatory response [23]. Lin et al. reported that IL-6-deficient mice experienced delayed wound healing compared with normal mice, demonstrating that IL-6 plays a critical role in wound healing due to the regulation of

leukocyte infiltration, angiogenesis, and collagen accumulation [24]. MCP-1 reportedly plays an important role in angiogenesis. Cells with a strong colony-formation ability stimulate wound healing by upregulating their production and secretion of IL-6, IL-8, and MCP-1 [25, 26]. The expression of IL-6, IL-8, and MCP-1 was highest in SVF cells treated with 0.1% collagenase type I for 40 min. These isolated SVF cells, therefore, are expected to be the most suitable for clinical applications. The effects of the SVF on wound healing and its use in other clinical research fields have been reported in several studies. After 60 min, cell yields were greater after treatment with 0.1% collagenase type I than after treatment with 0.2% collagenase type I; however, the viability and colony formation ability of the cells were significantly reduced. The SVF isolated by treatment with 0.1% collagenase type I for 40 min had the greatest colony-formation ability. Therefore, the optimal conditions appear to be a 0.1% concentration of collagenase and a relatively short treatment time (40 min). These conditions allow automated isolation of SVF cells while maintaining their stem-cell properties, making the cells suitable for clinical applications.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

Ethical statement The study protocol was approved by the institutional review board of Seoul St. Mary's Hospital of Korea (No. KC12TNMI0367).

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