Optimization of human mesenchymal stem cell isolation from synovial membrane: Implications for subsequent tissue engineering effectiveness

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Abstract
Synovium-derived mesenchymal stem cells (SDMSCs) are one of the most suitable sources for cartilage repair because of their chondrogenic and proliferative capacity. However, the isolation methods for SDMSCs have not been extensively characterized. Thus, our aim in this study was to optimize the processes of enzymatic isolation followed by culture expansion in order to increase the number of SDMSCs obtained from the original tissue. Human synovium obtained from 18 donors (1.5 g/donor) was divided into three aliquots. The samples were minced and subjected to collagenase digestion, followed by different procedures: Group 1, Tissue fragments were removed by filtering followed by removing floating tissue; Group 2, No filtering. Only floating fragments were removed; Group 3, No fragments were removed. Subsequently, each aliquot was sub-divided into two density subgroups with half. In Group 1, the cell-containing media was plated either at high (5000 cells/cm²) or low density (1000 cells/cm²). In Groups 2 and 3, the media containing cells and tissue was plated onto the same number of culture dishes as used in Group 1, either at high or low density. At every passage, the cells plated at high density were consistently re-plated at high and those plated at low density were likewise. The expanded cell yields at day 21 following cell isolation were calculated. These cell populations were then evaluated for their osteogenic, adipogenic, and chondrogenic differentiation capabilities. The final cell yields per 0.25 g tissue in Group 1 were similar at high and low density, while those in Groups 2 and 3 exhibited higher when cultured at low density. The cell yields at low density were 0.7 ± 1.2 × 10⁷ in Group 1, 5.7 ± 1.1 × 10⁷ in Group 2, and 4.3 ± 1.2 × 10⁷ in Group 3 (Group 1 vs Groups 2 and 3, p < 0.05). In addition, the cells obtained in each low density subgroup exhibited equivalent osteogenic, adipogenic, and chondrogenic differentiation. Thus, it was evident that filtering leads to a loss of cells and does not affect the differentiation capacities. In conclusion, exclusion of a filtering procedure could contribute to obtain higher number of SDMSCs from synovial membrane without losing differentiation capacities.

1. Introduction
Mesenchymal stem cells (MSCs) can be isolated from various tissues and have the potential to self-renew and differentiate into multiple lineages such as osteogenic [1], chondrogenic [2], adipogenic [3,4], myogenic [5] and neurogenic [6] specificities. Among the MSC sources, synovium-derived mesenchymal stem cells (SDMSCs) have been demonstrated to exhibit superior chondrogenic and proliferation potentials compared to MSCs derived from other tissue [7–9]. A considerable number of studies of cartilage repair have been conducted using SDMSCs with promising results [10–13]. For successful cell-based therapy, securing a sufficient number of cells is critical. It depends on the delivery method, specific to our “scaffold-
free tissue engineered construct” procedure [14], we need in average 1.3 \times 10^6 cells for treatment cartilage defect and quality inspection in our clinical trial (UMIN000008266), [15]. However, optimized methods for culturing SDMSCs, including the isolation from synovial membranes have not been fully characterized. Most of the previous studies on the culture of SDMSCs reported the isolation of cells using a standard collagenase digestion followed by filtering to remove debris [7–13,16–23] before starting the primary culture. However, there may be a potential loss of additional MSCs in the filter-trapped undigested tissue fragments. We hypothesized that the use of undigested tissue fragments would lead to increases in the number of SDMSCs available from the original synovial tissue. Moreover, it was previously reported that plating density can influence the proliferation of MSCs [8,24]. Taken together, it was important to quantify how the filtering process, as well as the plating density of cells thereafter could affect the number of SDMSCs obtained within a clinically relevant duration of cell culture.

In the present study we aimed to maximize the yield of cultured human SDMSCs starting from equivalent weights of synovial membrane material. The results presented indicate that both the MSC isolation method, as well as the propagation density, significantly influences the assessed outcomes.

2. Materials and methods

2.1. Harvest of synovial membrane and isolation of cells

Our study protocol was approved by the institutional committee for medical ethics. Written informed consent was obtained from all patients. Human synovial membranes were obtained (1.5 g per patient) from 18 patients (10 male and 8 female donors; mean age, 25.5; range 16–48 years: Table 1) during arthroscopic surgery. Synovial tissues from each donor were divided into three aliquots (0.5 g each) and meticulously minced using surgical scissors. The minced tissues were then digested in a collagenase solution [440 U/ml collagenase A, Type AFA (Worthington Biochemical Corporation, Lakewood, NJ, USA)] in growth medium containing high-glucose Dulbecco’s Modified Eagle Medium (DMEM, Wako Chemical Corp., Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO, USA) and 1% antibiotic–antimycotic solution (Sigma–Aldrich) according to the previously established protocol [12,16,25]. Specifically, we used the same, animal origin free, collagenase at the same concentration as previously established protocol [12,16,25].

Table 1
Synovial samples used in this study.

| Sample number | Age | Sex | Diagnosis                        |
|---------------|-----|-----|----------------------------------|
| 1             | 16  | Male| Anterior cruciate ligament injury|
| 2             | 30  | Male| Anterior cruciate ligament injury|
| 3             | 23  | Male| Synovitis (After anterior cruciate ligament reconstruction) |
| 4             | 35  | Female| Synovitis (After anterior cruciate ligament reconstruction) |
| 5             | 30  | Male| Anterior cruciate ligament injury |
| 6             | 23  | Male| Anterior cruciate ligament injury |
| 7             | 44  | Female| Anterior cruciate ligament injury |
| 8             | 16  | Male| Osteochondromatosis              |
| 9             | 20  | Female| Anterior cruciate ligament injury |
| 10            | 18  | Female| Anterior cruciate ligament injury |
| 11            | 44  | Male| Anterior cruciate ligament injury |
| 12            | 19  | Male| Anterior cruciate ligament injury |
| 13            | 23  | Female| Anterior cruciate ligament injury |
| 14            | 16  | Male| Anterior cruciate ligament injury |
| 15            | 17  | Male| Meniscal injury                  |
| 16            | 18  | Female| Anterior cruciate ligament injury |
| 17            | 19  | Female| Meniscal injury                  |
| 18            | 48  | Female| Anterior cruciate ligament injury |

The cultured cells were subjected to passaging when reaching 80% confluency. Cells were harvested by treatment with trypsin–EDTA (0.25% trypsin and 1 mM EDTA; Gibco BRL, Life manufacturer’s instruction. Following 3 or 16 h of incubation, undigested tissues were removed from the cell-containing liquid with a 70-μm nylon filter (BD Falcon, Franklin Lakes, NJ, USA) followed by centrifugation (1500 rpm for 5 min). Subsequently, the floating undigested tissue fragments were removed (Group 1). In Group 2, No filtering was performed but floating undigested tissue fragments were removed after centrifugation. In Group 3, No filtering and the floating undigested tissue fragments were not removed, and all components used for the subsequent cell culture. Therefore, Group 1 contained only cells; Group 2 contained cells and the precipitated undigested tissue fragments; while Group 3 contained cells plus both the precipitated and floating undigested tissue fragments (Fig. 1). In each group, all the contents were resuspended in 10 ml of complete media.

For microscopic observation, cell-containing media (10 μl) from Groups 1–3 were applied onto cell counter plates for analysis.

DNA was extracted from the cell-containing liquid (100 μl) after collagenase digestion in all groups with a DNeasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, cells and small tissues were digested with lysis buffer and proteinase K. DNA was purified, and the DNA content was quantified in a spectrophotometer. Total DNA content per 0.5 g tissue was calculated according to the ratio of sampling volume (100 μl) to the total volume (10 ml) of the cell-containing suspension.

2.2. Plating and subsequent primary cell culture

Each aliquot (Groups 1–3) from individual donors was further divided into two subgroups (5 ml each) for subsequent plating at two different cell densities.

For Group 1, the cells were plated at high (5000 cells/cm²) or low density (1000 cells/cm²). For Groups 2 and 3, media containing cells and undigested tissues was plated onto the same number of culture dishes used in Group 1, either at high or low density. Cells were cultured in the growth medium containing 10% FBS at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The medium was replaced every 4 days.

2.3. In vitro expansion of cell populations

The cultured cells were subjected to passaging when reaching 80% confluency. Cells were harvested by treatment with trypsin–EDTA (0.25% trypsin and 1 mM EDTA; Gibco BRL, Life
times with PBS, fixed in 4% paraformaldehyde, and then stained with 0.5% Alizarin Red S.

2.4.2. Adipogenic differentiation

2 × 10^6 cells were plated in 12-well plates and cultured in complete medium for 2 days. The medium was then changed to adipogenesis medium (STEMPRO® Adipogenesis Differentiation Kit, Life Technologies) and cultured for another 7 days. The medium was replaced two times a week. After induction for 7 days, wells were washed two times with PBS and stained with Oil Red O.

2.4.3. Chondrogenic differentiation

2 × 10^5 cells were placed in 15 ml polypropylene tubes and centrifuged at 1500 rpm for 10 min. The cell pellets were cultured in complete medium for 2 days. The medium was then switched to chondrogenesis medium, comprised of DMEM supplemented with 1% insulin–transferrin–selenium supplement (Corning® ITS Premix, Corning Life Sciences, Bedford, MA, USA), 0.2 mM Asc-2P (Sigma–Aldrich), and 200 ng/ml recombinant human BMP2 (OsteoPharma, Osaka, Japan) for another 21 days. The medium was replaced two times per week. For histological analysis, two pellets were fixed with 4% paraformaldehyde, embedded in paraffin, cut into 5-μm sections, and stained with Safranin O. Nuclei were counterstained with hematoxylin. For quantifying the glycosaminoglycan (GAG) content, three pellets were digested with 0.4 M papain extraction reagent overnight at 65 °C, and the GAG content was measured by Blyscan sulfated GAG assay kit (Biocolor, Carrickfergus, Ireland).

2.5. Statistical analysis

Data was presented as the mean ± one standard error of the mean. Data input and calculations were performed with IBM SPSS Statistics 22 (IBM, Armonk, NY, USA). In all analyses, we used a linear mixed model. We included the combination of groups and densities as a fixed effect and a repeated effect. A value of p < 0.05 was considered statistically significant.

3. Results

3.1. The effect of filtering following collagenase digestion

In Group 1, cell density appeared to be lower than in Groups 2 and 3, in which more cells along with cell aggregates and undigested tissues were observed (Fig. 2a). The total DNA content was 3.9 ± 7.2 μg in Group 1, 42.0 ± 7.2 μg in Group 2, 42.3 ± 10.0 μg in Group 3. There were significant differences between Groups 1 and 2 (p < 0.01) and between Groups 1 and 3 (p < 0.05). The differences for each comparison were approximately 10 fold. No significant differences between Groups 2 and 3 were detected (Fig. 2b).

Extending the initial collagenase digestion time to 16 h did not lead to any significant increases in cell numbers for Group 1 (data not shown) and therefore, it was likely that the cells populated within the undigested tissues were not released even by longer exposure to collagenase.

3.2. Comparison of cell numbers obtained after 21-day culturing of each sub-group

The cell number after 21 days of culture per 0.25 g tissue was calculated for each subgroup. There were no significant differences between the high and low density subgroups (0.6 ± 0.9 × 10^7 cells vs 0.7 ± 1.2 × 10^7 cells) in Group 1, whereas more cells were obtained for the low density subgroups than the high density subgroups for Groups 2 (5.7 ± 1.1 × 10^7 cells vs 3.1 ± 1.07^7 cells, p < 0.05) and 3 (4.4 ± 1.2 × 10^7 cells vs 1.5 ± 1.5 × 10^7 cells, p < 0.05) (Fig. 3).
The yields from the low density subgroups in Groups 2 and 3 were significantly greater than the values for Group 1 (Group 2 vs 1, >8-fold; \( p < 0.01 \), Group 3 vs 1, >6-fold; \( p < 0.05 \)). The cell viability was over 90% after all the different protocols (data was not shown).

3.3. In vitro differentiation and histological analyses of cells

Since cell expansion in the low density culture subgroups showed enhanced results compared to the findings obtained at high density for all of the groups (1, 2 and 3), we focused on comparing the differentiation capacity of the expanded cells only among the lower density culture subgroups.

3.3.1. Osteogenesis

After 21 days of osteogenic induction, mineralization was similarly observed for all groups, with calcium deposition stained with Alizarin Red S (Fig. 4a).

3.3.2. Adipogenesis

Following 7 days of adipogenic induction, under light microscopy, Oil Red O-stained sections revealed similar formation of lipid vacuoles for cells of each group (Fig. 4b).

3.3.3. Chondrogenesis

After 21 days of chondrogenic induction, the cell pellets cultured in chondrogenic media showed intense staining for Safranin O in each group (Fig. 5b). Regarding GAG quantity, there was no significant statistical difference among the three groups (1, 2, 3) (Fig. 5c).

4. Discussion

In most of the previous studies used for the preparation of SDMSCs, a filtering process was included following collagenase digestion to remove undigested tissue fragments [7–13,16–23]. The present study has revealed that such filtering procedures result in a significant loss (approximately 90% by DNA content measurement) of the number of the cells obtained when compared with cultures not subjected to filtering. It was likely that not all of the cells contained in the collagenase-treated media adhered to the culture dish and participate in subsequent proliferation. Therefore, loss of 90% of cells by the comparison of DNA content may be an overestimation. However, it is reasonable to presume that the majority of the SDMSCs are still within the undigested tissues after collagenase digestion for 3 or 16 h.

Final cell yields after 21 days of culture in the non-filtered groups (i.e. Group 2 and 3) were higher than those of the filtered group (i.e., Group 1). As previously reported [8,24], the proliferative efficiency was higher at low density culture than at high density for the non-filtered groups (Group 2 and 3), and the final cell yield in Group 2 (low density culture subgroup) which exhibited the best yields in the non-filtered groups yielded 5-fold more cells than those in Group 1. Conversely, such differences based on culture cell density were not detected for the filtered group (Group 1). All these results very clearly indicate the negative effect of the filtering process.

The differences between Groups 2 and 3 were based on whether the floating undigested tissue fragments separated after centrifugation were utilized for the subsequent culture. Microscopic
observation further suggested that these floating tissue fragments are comprised mainly of adipose synovium, a finding consistent with the floating properties of the fragments, and whose strong chondrogenic differentiation capacity has been reported previously [28,29]. Based on these reports, we assessed whether retention of such floating tissue fragments plus the precipitated undigested tissue fragments at the subsequent culture stage would be beneficial on subsequent culture to obtaining better yields of SDMSCs. However, the results showed no such significant differences between Groups 2 and 3. It could be speculated that the floating undigested tissue fragments may not have become well adhered to the culture dish and thus, might have missed the opportunity for outgrowth of the SDMSCs. The preparation method for Group 2 is simpler than that for Group 3 because there is no need to carefully preserve the floating tissue fragments at the collagenase washing out process. Thus, both in terms of efficiency of cell expansion and simplicity in preparation method, we recommend the Group 2 method for the isolation of SDMSCs.

There was a possibility that isolating methods affect cell differentiation capacity, however three groups showed similar stain in
osteogenic and adipogenic differentiation and there was no significant statistical difference among the three groups in GAG quantity. So we conclude that the cells in non-filter groups have equivalent differentiation capacities to those in filter group.

As another method of cell isolation from tissues, explant culture has been initially reported as adipose tissues [30], Wharton's jelly [31], and synovium [23]. In all these papers, explant culture was compared with an enzymatic method employing filtering; in the report about synovial tissues, the cell yield from explant culture was reported to be equal to that from the enzymatic method. Conversely, in the present study, cell yield from the enzymatic method without filtering was superior to that with filtering, and the cell differentiation capacity was not affected by the use of filtering or not. Although we did not directly compare our methods tested with explant culture, these findings suggest that the enzymatic method without filtering (enzymatic explant method) provides more cells than explant culture. The enzymatic digestion of collagenous matrix might have facilitated cellular expansion out of the matrix as compared with traditional explant culture.

One issue which still remains unclear was whether the MSCs readily released from the synovial membrane tissue by the collagenase treatment represent a unique subpopulation when compared to those that are retained in the undigested tissue fragments. As the number of cells released from the synovial membrane tissue was not increased when the collagenase treatment was extended to 16 h (data not shown), the digestion time more than 3 h was not a limiting factor and the cells released appeared to be a subset of the total cells available.

As a potential concern, the use of serum in our collagenase-based digestion media might have reduced the action of collagenase, leading to the presence of undigested tissue debris after digestion process. We included serum according to the protocols previously published [12,16,25], and did not investigate the experiments with serum-free digestion media. In the literature, there have been several reports of synovial digestion by collagenase without the use of serum [7,19,32]. In these studies, the duration of digestion was 3 h to overnight, and notably, all the reports included filtering process after collagenase digestion. This suggests the presence of residual debris (undigested tissue) after collagenase digestion regardless of the presence of serum. Thus, the presence of serum or not in the collagenase solution does not likely affect the major conclusion of the present study.

Recent study reported that more primitive progenitors are included in non-adherent cells [33,34], so there may be potential improvement of the quantity and quality of stem cell population if we follow the procedures. In the present study, we discarded medium that could potentially contain non-adherent cells at the time of medium change and passage and thus could not confirm the improvement. This issue needs to be clarified in the future study.

Some other limitations of the present study include its relatively small sample size, no comparison of gender, and individual differences. More donor samples are needed to be assessed to further clarify these issues, as well as perhaps inclusion of clonal, genetic, and epigenetic analysis of the different cell populations.

5. Conclusions

We have developed a simpler and significantly more efficient method for SDMSCs isolation than the conventional isolation method which employs a filtering process. Without the use of any special equipment such as a bioreactor or the addition of biological reagents or growth factors, we succeeded in increasing the cell yield over 5-fold, a finding which is very relevant when considering future clinical applications to repair cartilage defects. In addition, this methodology may be applicable to MSC isolation from other tissue cell sources, but future research to examine the feasibility of applying this method to other MSC sources will be required.

Conflict of interest

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