A Modified Aggregate Culture for Chondrogenesis of Human Adipose-Derived Stem Cells Genetically Modified with Growth and Differentiation Factor 5

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Abstract

Adipose-derived stem cells (ADSCs) are an attractive cell source for tissue engineering, and recently a modified aggregate culture of human ADSCs (hADSCs) was established based on preparation of three-dimensional (3D) cell aggregates in growth factor–enriched low serum medium using the hanging droplet method. Growth and differentiation factor 5 (GDF5) plays a critical role in chondrogenesis and cartilage development. In the present study, we examine (1) whether the modified aggregate culture is feasible for chondrogenic induction of hADSCs, (2) whether overexpressed GDF5 can promote chondrogenesis, and (3) the gene expression profile during chondrogenesis in this aggregate culture. hADSCs were infected with an adenovirus carrying the GDF5 gene (Ad-GDF5). Cells were cultured with chondrogenic media either in a modified aggregate culture or in an attached micromass culture that served as a control. The chondrogenic phenotype was assessed by morphology (n = 8), biochemistry (n = 3), and histology (n = 2). Expression of 12 genes was determined by quantitative real-time polymerase chain reaction (n = 3). We found that ADSCs cultured in the modified aggregates exhibited denser pellets and higher content of sulfated glycosaminoglycan (sGAG) compared with those cultured in the micromass. Infection of cells with Ad-GDF5 increased the aggregate size and sGAG content. It also up-regulated expression of GDF5, aggrecan, and leptin and down-regulated expression of COL I, while expression of COL II and COL 10 remained unchanged. We concluded that the modified aggregate culture is feasible for chondrogenic induction of human ADSCs. Infection with Ad-GDF5 appears to promote the chondrogenesis. These findings suggest that genetic modification of ADSCs with GDF5 in the modified aggregate culture could be useful for treating diseases with cartilage defects.

Key words: aggregate culture; cartilage regeneration; chondrogenesis; GDF5; genetic modification; human adipose-derived stem cell

Introduction

Degeneration of articular cartilage is commonly accompanied by trauma, malposition, and aging. It may cause severe clinical problems, such as substantial joint pain and degenerative arthritis due to the poor intrinsic capacity of injured cartilage for healing.1 Autologous chondrocyte implantation and matrix-associated chondrocyte implantation have proven to be effective surgical techniques for management of articular cartilage defects.2,3 However, both involve a minimum of two operations; the first is to harvest healthy cartilage biopsy for chondrocyte isolation and expansion, which imply donor site morbidity and chondrocyte dedifferentiation, respectively.1,2,4,5 Therefore, various groups have sought alternative cell sources such as mesenchymal stem cells (MSCs).6 Among the various sources, adipose tissue has been suggested to provide a superior stem cell source (adipose-derived stem cells [ADSCs]) due to their higher yield and the minimally invasive procedure to collect them.1,4,7

Chondrogenic induction of stem cells has been carried out in the presence of scaffolds8 or in their absence.8-11 In the case of scaffold-free chondrogenesis, cells are commonly cultured in either pellets8,9 or micromass10,11 to maintain a high density state. In an initial attempt to explore an optimal delivery method of human ADSCs (hADSCs) for therapeutic purpose, we established a technique for preparing three-dimensional (3D) cell aggregates in growth factor–enriched low serum medium with the hanging droplet method.12

Multiple growth factors have been explored to stimulate MSC chondrogenesis, such as transforming growth factor β1,13 insulin-like growth factor 1,13 fibroblast growth factor

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Among these growth factors, GDF5, also called bone morphogenetic protein 14,16 or cartilage-derived morphogenetic protein 1,8,15,17–19 regulates a variety of musculoskeletal processes, including chondrogenesis,8,15,16 joint formation,15,20 maintenance or repair of intervertebral disc,20–23 and tendon and ligament maintenance.24–27 Specifically, we demonstrated that overexpression of GDF5 via infection with an adenovirus carrying a human GDF5 gene fragment (Ad-GDF5) significantly augmented chondrogenesis of rat ADSCs.9

Based on our previous findings,9,12 we determined the following in the present study: (1) whether the reported 3D aggregates formulated as modified aggregates were able to induce chondrogenesis, (2) whether overexpressed GDF5 could enhance chondrogenesis of hADSCs with the modified aggregate culture, and (3) if changes in gene expression occurred with regard to this chondrogenesis.

Materials and Methods

Study design

Our study design with four experimental groups is depicted in Table 1. Variables included the culture method and cell type. Basic medium (BM) was Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 50 μg/mL ascorbic acid, while chondrogenic medium (CM) was DMEM with 1% FBS, 1% penicillin/streptomycin, 50 μg/mL ascorbic acid, 10 nmol/L dexamethasone, and 1% ITS-Premix.13

Experimental groups C and D were designed for the first purpose, and the cells were infected with Ad-GDF5 to prepare modified aggregates or micromass cultures.10,11 Micromass cultures were employed as control due to their simple preparation. Modified aggregates made from cells with or without Ad-GDF5 infection were employed in groups A–C sequentially denoted as non–Ad-GDF5/BM, non–Ad-GDF5/CM, and Ad-GDF5/CM for the other two purposes. Three replicates were performed for either proteoglycan or gene expression analysis, and each sample contained three aggregates or micromasses. An additional two aggregates or micromasses were applied to cryosectioning for histological and immunostaining analysis.

Cell isolation and treatment

We obtained human adipose tissue from surgical waste of a nondiabetic patient (29-year-old woman with body mass index of 45.4 Kg/m²) who underwent liposuction at University of Virginia Medical Center. The surgical waste usage protocol was reviewed and approved by the internal research board of University of Virginia.

ADSCs were isolated using previously described methods.28 Briefly, tissue was washed with complete Hanks buffer and then isolated with enzyme digestion and filtration. Pelleted stromal cells were filtered twice with 250-μm and 105-μm mesh, and the erythrocytes were removed with osmotic buffer. Cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12) with 10% FBS and 1% antibiotic-antimycotic at 37°C in a humidified incubator (Forma Scientific Inc., Marietta, OH). Nonadherent cells were removed 24–48 h after plating. Culture medium was changed every 2 days.

We generated an adenovirus carrying the GDF5 gene (Ad-GDF5) using the AdEasy™ adenoviral vector system (Stratagene, La Jolla, CA) as described previously.9 We infected the human ADSCs at passage 3 with Ad-GDF5 at a multiplicity of infection of 150 for 24 h.

For the modified cell aggregate culture, cells were suspended at a concentration of 125,000 cells/mL in growth factor-enriched low-serum medium (DMEM/F-12, 0.1 mmol/L L-glutamine, 10–8 mol/L dexamethasone, 100 μmol/L ascorbic acid 2-phosphate, 0.50% ITS+3, 0.05% fatty acid supplement, 1% nonessential amino acids, 10–6 mol/L estradiol, 10–8 mol/L progesterone, 500 ng/mL hydrocortisone, 10 ng/mL epidermal growth factor, 1 ng/mL platelet-derived growth factor, 1 ng/mL stem cell growth factor β, 1 ng/mL tumor necrosis factor α, 1 ng/mL interleukin 1β, 1% antibi-
ic/antimycotic) with 1% human serum.28 Briefly, tissue was washed with complete Hanks buffer and seated for 2 h before addition of extra medium. The aggregates were treated with either BM or CM.9 All cultures were maintained up to 3 weeks with medium changed every 2 days. Aggregates were harvested at 1, 2, or 3 weeks for morphology assessment. Microphotographs of aggregates were taken under an inverted light microscope, and the sizes of eight aggregates in each group were analyzed with the software ImageJ (National Institutes of Health, Bethesda, MD), expressed as fold of change and normalized to non-GDF5/BM group at 1 week.

| Group | Culture method | Cell/Ad-GDF5 | Medium |
|-------|----------------|--------------|--------|
| A     | Aggregate      | No           | BM     |
| B     | Aggregate      | No           | CM     |
| C     | Aggregate      | Yes          | CM     |
| D     | Micromass      | Yes          | CM     |

BM, basic medium; CM, chondrogenic medium.

Quantitative real-time polymerase chain reaction, biochemical, histological, and immunostaining analyses

For real-time reverse-transcription polymerase chain reaction (PCR), we isolated total RNA using the RNeasy® Mini kit (Qiagen, Valencia, CA) and synthesized cDNA with the Reverse Transcription System kit (Promega, Madison, WI), following the manufacturers’ instructions. Real-time PCR was performed with the Quantitect® SYBR Green PCR master mix (Qiagen). Gene expression was calculated using the delta delta Ct method31 and normalized to 18S, and expressed as fold of change. Expression of all genes except for leptin was expressed as fold of change over the non–Ad-GDF5/BM group. For leptin, since the expression in this group was not
detectable, gene expression was expressed as fold of change over the non–Ad-GDF5/C1 group. The primer sequences are listed in Table 2.

For biochemical analyses, cell aggregates or micromasses were digested in 200 μL of papain digestion buffer (United States Biological, Swampscott, MA; prepared 125 mg/mL in sterile phosphate-buffered saline, pH 6.0, with 5 mmol/L cysteine hydrochloride) for 18 h at 60°C. Sulfated glycosaminoglycan (sGAG) levels were measured with a spectrophotometer after incubation with 1,9-dimethylmethylene blue-chloride (Polysciences, Inc., Warrington, PA) dye and a spectrophotometer after incubation with 1,9-dimethylmethylene blue-chloride (Polysciences, Inc., Warrington, PA). DNA concentration was measured using a BCA protein assay (Pierce, Rockford, IL) and a spectrophotometer.

**Table 2. Primer Sequences Used for Real-Time Polymerase Chain Reaction**

| Molecule | Primer sequence | Size of product (bp) |
|----------|-----------------|---------------------|
| Growth and differentiation factor 5 | 5'-AGT AGA CTC CCC AAA CTC CTC AC-3' (sense) 5'-GCC TTC TCT TGC CTT GGC ATT-3' (antisense) | 237 |
| Aggrecan | 5'-CGC TAC TCG TCT ACC TTT-3' (sense) 5'-GCT CAT AGC CTT CGT-3' (antisense) | 106 |
| Collagen Ia1 | 5'-GCC ATC AAA GTC TTC TGC-3' (sense) 5'-ATC CAT CGG TCA TGC TCT-3' (antisense) | 145 |
| Collagen IIa1 | 5'-TCC CAG AAC ATC ACC TAC-3' (sense) 5'-AAC CTG CAT TTA CCC TCT-3' (antisense) | 131 |
| Collagen 10a1 | 5'-TGC TAG TAT CCT TGA ACT TGG TTC-3' (sense) 5'-CTG TGT TCT GGT GGT TAG-3' (antisense) | 98 |
| Collagen 15a1 | 5'-GTG GTC CAC CTA CCG AGC AT-3' (sense) 5'-TGT TCT GAC CAT CAA AGG AG-3' (antisense) | 197 |
| Glypican 3 | 5'-AAT GAA GGC CCC TGA GC-3' (sense) 5'-GCC AGT TCT GCC AGC AGT TCT-3' (antisense) | 228 |
| Collagen V | 5'-GCA CTG CTA TGC CTA TGA-3' (sense) 5'-GGA ATG AAG TCC AAA CCG GTG-3' (antisense) | 159 |
| Keratin 19 | 5'-TGA AGA GTC TTC CAA TCC TAC TGG-3' (sense) 5'-GAA TCG AAC CCT GAT TCC CCA-3' (antisense) | 113 |
| CD105 | 5'-GCC ACGGATT GTCCACACTCATG-3' (sense) 5'-GCCAAAGCTCTTCT TTAGTACCA-3' (antisense) | 176 |
| Hypoxia inducible factor 1α | 5'-GTC GCT TCG GCC AGT GTG-3' (sense) 5'-GGA AAG GCA AGT CCA GAG GTG-3' (antisense) | 152 |
| Leptin | 5'-GTG CGG ATT CCT GTG CCT TT-3' (sense) 5'-GGA ATG AAG TCC AAA CCG GTG-3' (antisense) | 174 |
| 18S | 5'-GTG ACC AGT TCA CTC TTG GT-3' (antisense) 5'-GAA TCG AAC CCT GAT TCC CCG TC-3' (antisense) | 99 |

Data analysis

Data from aggregate/micromass size, biochemical assay, and gene expression analysis were expressed as mean±SD. Statistical evaluation was performed with two-tailed Student’s t-test using Microsoft Excel® (Microsoft Corporation, Redmond, WA) to determine the difference between two groups.

Results

**Modified aggregate culture is superior to micromass culture for chondrogenesis**

After 3 weeks’ culture, the aggregates made from the hanging drop method were dense and spherical with few cells growing outward, whereas micromass cultures were irregular or sphere-like with cells growing outward. In addition, micromass cultures occasionally had aggregates attached to the side of the plate (Fig. 1A). Furthermore, biochemical analysis showed aggregates produced higher (150%; p<0.001) amounts of sGAG than micromass cultures produced (Fig. 1B).
Overexpressed GDF5 enhanced cell aggregate size and matrix production during chondrogenesis

The aggregate sizes were at their maximum at week 1 for all three groups (Ad-GDF5/CM = non-Ad-GDF5/CM > non-Ad-GDF5/BM; Fig. 2). At weeks 2 and 3, aggregates in the Ad-GDF5/CM group were larger (p < 0.01) than in the non-Ad-GDF5/CM group. The content of sGAG in the aggregates was significantly different among these three groups. Compared to non-Ad-GDF5/BM, non-Ad-GDF5/CM and Ad-GDF5/CM had an 80% and 160% increase, respectively (Fig. 3). Safranin O staining revealed a similar result, and strong staining was seen in every group (Fig. 4).

However, immunostaining analysis showed no apparent difference in COL II between non-Ad-GDF5/CM and Ad-GDF5/CM (Fig. 4).

Gene expression profiles during chondrogenesis

Twelve genes in total were investigated in aggregates cultured in different conditions for 3 weeks by real-time RT-PCR
analysis, including a growth factor, GDF5; matrix proteins, aggrecan (AGG), collagens type I (COL I), II (COL II), 10 (COL 10), and 15 (COL 15), glypican 3 (GPC3), and keratin 19 (KRT19); a matrix degrading enzyme, matrix metalloproteinase 3 (MMP3); an MSC surface marker, endoglin (CD105); a transcript factor, hypoxia inducible factor 1α (HIF1); and an adipose-derived hormonal protein, leptin [Ob(Lep)] (Fig. 5). Compared to the non–Ad-GDF5/CM group, the mRNA level of six genes was elevated in either the non–Ad-GDF5/CM group [GDF5, AGG, COL II, GPC3, KRT19, and Ob(Lep)] or the Ad-GDF5/CM group [GDF5, AGG, COL II, COL 15, GPC3, and Ob(Lep)]. Three genes (MMP3, CD105, and HIF1) in the non–Ad-GDF5/CM group and two (KRT19 and MMP3) in the Ad-GDF5/CM group decreased, while three (COL I, COL 10, and COL 15) in the non–Ad-GDF5/CM group and four (COL I, COL 10, CD105, and HIF1) in the Ad-GDF5/CM group remained unchanged. Compared with the non–Ad-GDF5/CM group, the differential pattern in gene expression in Ad-GDF5/CM group was as follows: seven genes up-regulated, GDF5, AGG, COL 15, MMP3, CD105, HIF1, and Ob(Lep); three down-regulated, COL I, GPC3, and KRT19; and two unchanged, COL II and COL 10.

Discussion

In this pilot study, we investigate for the first time chondrogenesis by hADSCs in modified aggregates and with overexpressed GDF5. Our data demonstrated that the modified aggregate culture produced better outcomes in both morphology and matrix production (Fig. 1). This observation is probably partly due to the cell outgrowth and subsequent dedifferentiation in the case of micromass cultures, but the
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evidence is obviously lacking at this point. Another possible
contributor might have been the specific medium used for
the aggregate preparation, which was enriched in growth fac-
tors with low serum. Indeed, the aggregates were found to
have high expression of extracellular matrix proteins such as
biglycan, various types of collagens, as well as growth fac-
tors beneficial to chondrogenesis, such as insulin-like growth
factor 1 and transforming growth factor β after 6 days’ culture
in the medium.12 In the present study, strong positive Safra-
min O staining was consistently seen in the non–Ad-GDF5/
BM group (Fig. 4A), and only a moderate increase (less than
twofold) in sGAG content was achieved in either non–
Ad-GDF5/CM or Ad-GDF5/CM group compared to the
non–Ad-GDF5/BM group (Fig. 3). These results imply that
the modified aggregates might undergo chondrogenesis
even in the absence of induction. As such, we provide herein
a novel 3D culture technique for chondrogenesis, in addi-
tion to those previously described, such as pellet8,9 and
hydrogel culture.4,23 Meanwhile, the modified aggregates
have been implicated to have the ability of accelerating dia-
betic wound healing.12 In order to broaden the scope of
their potential use, the impact of the specific medium on di-
differentiating ability (chondrogenesis, osteogenesis and adipo-
genesis, etc.) by the modified aggregates deserves further
investigation.

Improvement of chondrogenesis and cartilage develop-
ment by GDF5 has been well established during the past
two decades.8,9,14,15,19,23 In humans, it has been reported to
enhance the chondrogenic differentiation in bone marrow
MSCs from the fetus8 and adult,23,32 and rheumatoid fibro-
blast-like synovioocytes,33 as well as the chondrogenic trans-
differentiation in dermal fibroblasts.31 In the present study,
influence of GDF5 on hADSC chondrogenesis was investi-
gated by taking advantage of the modified aggregate culture
technique. Measurement of GDF5 at the protein level by
ELISA has previously demonstrated that Ad-GDF5 is an ef-
efective tool to mediate GDF5 overexpression in rat ADSCs.9
The Ad-GDF5 appears to work effectively in hADSCs as
well, since after infection the mRNA level of GDF5 robustly
increases >1000-fold, in terms of data from real-time RT-
PCR analysis (Fig. 5A). Furthermore, GDF5 gene overexpres-
sion could augment the chondrogenesis by hADSCs, revealed
by aggregate size (Fig. 2), proteoglycan production (Fig. 3),
and gene expression of chondrogenic markers (Fig. 5A).
These results are in accordance with our previous study re-
lated to rat ADSC chondrogenesis.9 Additionally, the aggre-
gate size became smaller over time for all three groups;
however, overexpressed GDF5 appears capable of slowing
the decrease in aggregate size (Fig. 2). More investigation is
necessary to clarify the related mechanisms.

Pilgaard et al.34 reported the transcriptional signature of
hADSCs preconditioned for chondrogenesis by hypoxia. It
is appealing to investigate the gene expression pattern during
chondrogenesis by hADSCs in the current aggregate culture.
As an initial attempt, the present experiment depicts expres-
sion of a total of 12 genes by real-time RT-PCR (Figs. 5A–
C). Obviously, further experiments are required to verify
the significance of gene expression pattern in this system
with regard to GDF5 regulation. However, some new find-
ings are of interest. First, we demonstrate that leptin expres-
sion is ultimately absent in human ADSC aggregates
cultured in basic medium, but markedly elevated after
3-week induction, in particular by >30-fold in the presence
of overexpressed GDF5 (Fig. 5C). Leptin is known mostly
for its role as a key regulator of body weight and food in-
take.35 More importantly, its pivotal modulation has also
been suggested in endochondral ossification during bone for-
formation, partly via prevention of premature mineralization
of the prehypertrophic chondrocytes at the growth plate.36 Sec-
ond, our present data show that GDF5 causes no increase in
type II collagen expression, as evidenced by both immuno-
sating (Fig. 4) and real-time PCR (Fig. 5A) analyses, which
seemed inconsistent with those we previously reported.9
The inconsistency might be attributed to the different culture
conditions (modified aggregate versus pellet; pretreatment
with growth factor–enriched medium versus without the pre-
treatment) and source of cells (human versus rat). Third,
overexpressed GDF5 inhibit expression of COL 1 (Fig. 5A)
but do not affect COL 10 (Fig. 5C). Combined with the fact
that leptin expression is robustly elevated by GDF5 overex-
pression, it seems that in our system (i.e., modified aggregates
and GDF5 genetic modification), chondrogenic hypertrophy
might be prevented. Lastly, we provide some new infor-
mation on the expression pattern of genes such as CD105, COL
15, GPC3, HIF1, KRT19, and MMP3, although to speculate
on the significance of these findings is a challenging task at
this stage. It is worth noting that the current modified aggre-
gate culture along with GDF5 genetic modification is quite
different from other culture systems previously reported for
chondrogenic induction. In addition, the exact roles of these
genes during chondrogenesis are not defined yet due to insuf-
icient experimental data. For example, CD105 is generally
characterized as a specific surface marker of MSCs, and a
CD105-enriched population within hADSCs has a stronger
capacity for chondrogenesis and osteogenesis as well as a
weaker capacity for adipogenesis.32 However, no data have
been published to date on the regulation of CD105 expression
itself during chondrogenesis.

Our study has several limitations. First, cells used in the ex-
periments were obtained from only one subject, and this may
cause uncertainty in the biological significance of the present
findings. Second, culture media with different compositions
were used for aggregate and micromass cultures in the
present study. This inconsistency might prevent an effective
comparison of the two methods. Additionally, adhesive
micromass cultures were not optimal controls for modified
degrees, due to their adhesive properties. In spite of
more complexity in preparation, nonadhesive pellets would
be a better control.7–9 Third, we did not compare the
ADSCs to chondrocytes. Since the ultimate purpose of the
research was to find an alternative source for autologous
chondrocytes, such a control would better define any limita-
tions of the ADSCs. Lastly, the present study provides exper-
imental data on gene expression only at the mRNA level and
lacks data at the protein level. In the future, Western blot or
ELISA should be conducted to confirm the findings by re-
time RT-PCR, especially for those genes of extreme signifi-
cance such as GDF5, leptin, COL I, and COL 10.

The merit of this study was in providing a putative cell
therapy in the absence of scaffolds. Although scaffolds can
help retain cells in the desired location and provide appro-
priate mechanical properties and/or biochemical signals in
tissue engineering, the long-term safety issues of the biomate-
rial components, such as the retention and degradation of
synthetic materials in situ and infectious risk and immunological reaction caused by biological materials, remains a big concern. Therefore, using a scaffold-free culture system has been proposed as an alternative to avoid those unknown risks. As such, further investigation deserves to be made to evaluate the in vivo efficacy of the modified aggregates of hADSCs with overexpressed GDF5 in treating cartilage defects. Furthermore, the aggregates might be engineered with different numbers of cells to form desirable-sized spheroids, providing flexibility of either filling defects of clinically relevant size or examining the dose-dependent effect of the cell transplantation treatment with an animal model.

Genetically modified stem cells have been proposed as a promising tool for cell therapy. They have been proven safe and effective in tissue repair or disease treatment by numerous animal tests and are now ready for human clinical trials.9 Our present data, together with those previously reported, support the notion that genetically engineered adipose stem cells with GDF5 might be applied for cartilage regeneration. It is worth noting that a variety of techniques for gene delivery that are based on viral and nonviral vectors have been extensively investigated from basic to translational research.

In conclusion, this is the first attempt to apply the specific aggregate culture for a chondrogenic induction, as well as to determine the impact of GDF5 on chondrogenesis by a primary human adipose stem cell culture. In spite of its preliminary nature, this study highlights the potential use of the ADSC aggregate with GDF5 genetic modification for cartilage repair.

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Author Disclosure Statement

No competing financial interests exist.

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