Intra-articular adipose-derived mesenchymal stem cells from rheumatoid arthritis patients maintain the function of chondrogenic differentiation

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Abstract

Objectives. To evaluate the chondrogenic potential, phenotype and percentage of IA adipose-derived mesenchymal stem cells (ADSCs) from RA patients in comparison with OA patients. The effect of TNF treatment on ADSC differentiation was also examined.

Methods. Adipose tissue was obtained from RA and OA patients. ADSCs were isolated and cultured until passage 4. After that period, the phenotype and percentage of these cells were analysed by flow cytometry. Passage 4 cells were cultured in chondrogenic medium with or without TNF. After 3 weeks of differentiation the expression of Sox9, aggrecan (Acan) and collagen 2a (Col2a) mRNA was assessed by RT-PCR and GAG deposition by alcian blue staining.

Results. The phenotype and percentage of ADSCs were similar in both RA and OA. The results of alcian blue staining showed effective chondrogenesis in RA and OA ADSCs. TNF inhibited GAG deposition in both RA and OA samples similarly. Sox9, Acan and Col2a mRNA expression was significantly increased in chondrogenic-medium-treated cells ($P < 0.05$) and decreased after TNF exposure ($P < 0.01$). No statistically significant differences between RA and OA were observed.

Conclusion. ADSCs from RA and OA patients are similar with regard to their phenotype, percentage in IA tissue and chondrogenic potential, which is reduced after exposure to TNF.

Key words: adipose-derived mesenchymal stem cells, chondrogenesis, rheumatoid arthritis, osteoarthritis, TNF.

Introduction

Adipose-derived mesenchymal stem cells (ADSCs) have the ability to differentiate into many cell types including chondrocytes and osteoblasts [1], which make them especially promising in RA treatment. ADSCs also have strong immunosuppressive properties [2, 3] and their therapeutic effect has been proved in an experimental animal model of RA [4].

RA is characterized by excessive immune response accompanied by progressive joint tissue destruction. In the rheumatic joint there is an imbalance between cartilage regeneration and degradation. Fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes, as well as chondrocytes, secrete aggrecanases and MMPs—enzymes capable to cleave cartilage constituents. Local synthesis of tissue inhibitors of MMPs is down-regulated and the expression of genes supporting chondrogenesis is also decreased [5].

One of the candidates for the treatment of damaged articular cartilage is mesenchymal stem cells (MSCs). In case of injuries of articular cartilage, mesenchymal chondroprogenitor stem cells must enter the damaged region and initiate the repair response [6]. ADSCs residing in rheumatoid IA adipose tissue are particularly interesting because of their location in the place where inflammatory response persists and where the damaged cartilage is situated. These cells could contribute to the repair of damaged cartilage but there is insufficient data concerning their regenerative potential in an inflammatory environment.
were used for differentiation assay and cytometric analysis. Consequently, it is worthwhile to verify whether regenerative potential of ADSCs might be altered in the rheumatoid joint environment. TNF, present in excessive amounts in RA and crucial for its pathogenesis, is known to inhibit chondrogenesis [8]. Thus the influence of this cytokine on ADSCs might be of great importance in cartilage repair and degradation in the rheumatoid joint.

IA adipose tissue in healthy knee joint is composed of four fat pads. The biggest and closest to the synovial membrane fat pad is the Hoffa’s infrapatellar fat pad (IPFP). During total knee joint replacement surgery the Hoffa’s fat pad is the main part of isolated IA adipose tissue and the ADSCs examined in this study are derived mainly from this structure.

The objective of this study was to answer the question of whether the chondrogenic function of ADSCs from rheumatoid joint is maintained and whether TNF, the key cytokine in RA pathogenesis, may have specific influence on this type of cell. We compared the chondrogenic potential of RA ADSC with that of ADSC from OA patients and determined whether the effect of TNF is distinct in these two diseases. We have also compared the number and phenotype of ADSCs from RA and OA patients.

Materials and methods

Patients

Twenty-four patients with RA and 18 with OA were selected from the Rheumoortopaedic Clinic of the Institute of Rheumatology in Warsaw, Poland. All patients gave their written informed consent according to the Declaration of Helsinki and the study was approved by the Institute of Rheumatology Ethics Committee.

Cell culture and differentiation

ADSCs were isolated from IA adipose tissue obtained from RA and OA patients during total knee joint replacement surgery. Adipose tissues were cut and digested in 0.25% trypsin solution (20 min, 37°C, agitation). After that, tissue was filtered, washed in PBS and centrifuged 3 min at 1200 g. The lower stromal vascular fraction (SVF) was harvested and a further two centrifugations in PBS were performed. If necessary, the red blood cells were lysed. Cells were seeded onto 25 cm² culture flasks in DMEM/F12/10% fetal calf serum (FCS)/penicillin/streptomycin/plasmocin medium (GIBCO, Grand Island, NY, USA) and 100 nM (Sigma-Aldrich, St Louis, MO, USA)/penicillin 100 U/ml (Polfa Tarchomin S.A., Warsaw, Poland)/streptomycin 100 μg/ml (Polfa Tarchomin S.A., Warsaw, Poland).

Flow cytometric analysis

After four passages, ADSCs were treated with non-enzymatic cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) and washed with FACS buffer (phosphate-buffered saline, 0.1% Na₂SO₃, 1% FCS). Cells (5 × 10⁵) were resuspended in 50 μl of FACS buffer and stained for 30 min on ice with fluorochrome conjugated murine anti-human mAbs against following surface markers: CD90-FITC, CD105-PE, CD73-APC (eBioscience, San Diego, CA, USA), CD34-PE, CD45-PE, CD19-PE and CD14-APC (BD Pharmingen, San Diego, CA, USA). Appropriate isotype controls were used. Cells were analysed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA).

Alcian blue staining

After differentiation, cell pellets were fixed for 60 min in 10% buffered formaldehyde, routinely processed and embedded in paraffin wax. Sections of 2 μm thickness were cut, mounted onto glass slides, deparaffinized with three xylene washes (10 min each), dehydrated in graded alcohol, rinsed three times in xylene, embedded in paraffin wax. Sections of 2 μm thickness were cut, mounted onto glass slides, deparaffinized with three xylene washes (10 min each), dehydrated in graded alcohol and stained with 1% alcian blue (Alcian blue 8 Gx from Merck KGaA, Darmstadt, Germany), pH 2.5 for 30 min. Slides were rinsed in deionized water, dehydrated in graded alcohol, rinsed three times in xylene and cover-slipped with Entellan (Merck KGaA, Darmstadt, Germany).

RNA isolation, reverse transcription and semi-quantitative PCR

After 21 days of differentiation, cells were washed and total cellular RNA was isolated (RNA II Isolation Kit; Macherey-Nagel, Düren, Germany). cDNA synthesis was performed using 200 U of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified with 1.25 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA) and 0.25–0.5 μM of sense and antisense primers as follows: Sox9 (304 bp): sense 5’-TCTCTGGTTTGGCATTT-3’; antisense 5’-TGACTGGAATCT-3’; Acan (501 bp): sense 5’-ATGGCCAAGACATTAGTG-3’; antisense 5’-TCTCTGGAAAGCTTCTTCTAAGCT-3’; Col2a (432 bp): sense 5’-TTCAAGCTTAGATGACAT3’; antisense 5’-AGACTCTAGTGACTGAGG-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 200 bp): sense 5’-TGATCTGGAAAGGACTCA-3’; antisense 5’-GTGGTCTAGTGAGGACTCA-3’.
The thermal cycle profile, volume and composition of the reaction mixture differ for every gene (details in Table 1). Each PCR was carried out for 30 cycles, except for GAPDH, which was carried out for 23 cycles. PCR products were size fractionated on 1.5% agarose gel with the addition of ethidium bromide and photographed under UV transillumination. Quantitative analysis of mRNA expression was assessed relative to GAPDH expression using gel analysis software (Kodak Digital Science 1D, New Haven, CT, USA).

Statistical analysis

All statistical analyses were made using STATISTICA 6.0 software (StatSoft Inc., Tulsa, OK, USA). Data are expressed as median and percentiles (25th–75th percentile) unless otherwise noted. The normality of data distribution was assessed by Shapiro–Wilks test. All data except patient age were not normally distributed. The Mann–Whitney U-test was used for comparison between RA and OA groups (except difference in patient age, which was assessed by Student’s t-test). The differences between gene expression in control, differentiated and TNF-treated samples within the limits of one group (RA or OA) were evaluated using the Wilcoxon signed-rank test. Differences were considered significant for *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

Patients

Demographic and clinical characteristics of the study patients are presented in Table 2. Patients were properly age matched and the difference in mean age was not statistically significant (P = 0.42). CRP concentration was significantly higher in the RA group (P < 0.00001). All rheumatoid patients received glucocorticoids and MTX. None of them were treated with biologic therapy.

Flow cytometry

In order to evaluate the percentage and phenotype of ADSCs residing in IA adipose tissue from RA and OA patients, cells were analysed by flow cytometry. Cells in both patient groups were almost completely positive for CD105, CD90 and CD73 expression (Fig. 1A and B). The mean percentage of cells expressing CD105, CD90 and CD73 exceeded the 95% recommended by the International Society for Cellular Therapy [9] for every marker. Less than 2% of analysed cells expressed CD45, CD19 and CD14; however, CD34 was present by ~10% of cells (Fig. 1A and B). The number of cells co-expressing CD105, CD90 and CD73 was 78.2% for RA and 77% for OA, which was not significantly different (Fig. 1C).

Alcian blue staining

Alcian blue is one of the most widely used dyes for GAGs. We examined GAG deposition in ADSC pellets cultured in control, chondrogenic and chondrogenic TNF-supplemented medium. Staining by alcian blue shows strong GAG deposition in both RA and OA ADSC pellets cultured in chondrogenic medium. In control medium, the blue-stained regions are absent or hardly visible (due to non-specific dye binding). Pellets cultured with the addition of TNF show weak blue staining, which indicates that chondrogenesis was induced but simultaneously inhibited by TNF action (Fig. 2).

RT-PCR analysis

We performed an analysis of chondrogenic marker mRNA expression. The relative differences in Sox9, Acan and Col2a mRNA expression between untreated, chondrogenic-medium- and TNF-treated ADSCs, as well as between cells from RA and OA patients were evaluated by RT-PCR. In OA and RA ADSCs, there was a statistically significant increase in expression of each marker in chondrogenic-medium compared with untreated control cells but no significant differences were observed between RA and OA groups (Fig. 3). Similarly, there was a statistically significant decrease in TNF-supplemented medium

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**Table 1** Thermal cycle profile, volume and composition of reaction mixture for each gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Volume, µl</th>
<th>[MgCl2], mM</th>
<th>[dNTPs], mM</th>
<th>Thermal profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox9</td>
<td>25</td>
<td>2</td>
<td>0.2</td>
<td>95°C 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>94°C 30 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52°C 45 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72°C 10 min</td>
</tr>
<tr>
<td>Acan</td>
<td>25</td>
<td>2</td>
<td>0.1</td>
<td>95°C 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>95°C 30 s</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>55°C 30 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72°C 10 min</td>
</tr>
<tr>
<td>Col2a</td>
<td>50</td>
<td>2.5</td>
<td>0.08</td>
<td>95°C 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>95°C 30 s</td>
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<td></td>
<td></td>
<td>58°C 30 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68°C 45 s</td>
</tr>
<tr>
<td>GAPDH</td>
<td>50</td>
<td>2.5</td>
<td>0.1</td>
<td>95°C 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>63°C 30 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72°C 10 min</td>
</tr>
</tbody>
</table>

dNTPs: deoxynucleotide triphosphates.

**Table 2** Demographic and clinical characteristics of the study patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA patients (n = 24)</th>
<th>OA patients (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (s.d.)</td>
<td>58.5 (6) (42–67)</td>
<td>60.4 (5) (50–69)</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>24/0</td>
<td>15/3</td>
</tr>
<tr>
<td>Duration of RA, mean (range), years</td>
<td>20 (5–40)</td>
<td></td>
</tr>
<tr>
<td>CRP, median (range), mg/l</td>
<td>18.5 (5–84)</td>
<td>4.5 (1–12)</td>
</tr>
</tbody>
</table>
compared with the chondrogenic medium alone but no significant difference between RA and OA ADSC response to TNF supplementation (Fig. 4).

**Discussion**

This study is the first report evaluating the percentage, phenotype and chondrogenic potential of ADSCs isolated from rheumatoid IA adipose tissue. There is controversy concerning the ADSC phenotype, and data about surface marker expression are not coherent. In the majority of published data, CD105 (endoglin) and CD90 (Thy-1) are reported to be the most characteristic markers of these cells [10–12], but without consensus concerning the level of their expression. Other molecules, e.g. CD73, CD29, CD51 and CD49e, are also stated to be present on these cells [13]. The data concerning CD34, which is the marker of haematopoietic and endothelial cells, are also conflicting. SVF isolated from adipose tissue contains CD34+ cells, and several publications state...
**Fig. 2** Alcian blue staining.

Chondrogenesis of ADSCs is efficient in both RA and OA samples. Pellets were stained with alcian blue after 21 days of culture in indicated media. Cells in chondrogenic medium are surrounded by GAG-rich extracellular matrix (blue colour) in contrast to control and TNF-supplemented samples.

**Fig. 3** Induction of Sox9, Acan and Col2a mRNA expression in ADSCs from RA and OA patients.

Relative gene quantities are expressed after normalization to GAPDH. Data are expressed as median and percentiles of 24 (RA) and 18 (OA) independent experiments. Statistical analysis was performed as described in the Materials and methods section. Asterisks indicate statistically significant differences between control and chondrogenic medium; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. N.S.: non-significant; C: control medium; CH: chondrogenic medium.

**Fig. 4** Inhibition of Sox9, Acan and Col2a mRNA expression in TNF-treated (10 ng/ml medium) ADSCs from RA and OA patients.

Relative gene quantities are expressed after normalization to GAPDH. Data are expressed as median and percentiles of 24 (RA) and 18 (OA) independent experiments. Statistical analysis was performed as described in the Materials and methods section. Asterisks indicate statistically significant differences between chondrogenic and TNF-supplemented medium; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. N.S.: non-significant; CH: chondrogenic medium; TNF: chondrogenic medium + TNF.
that ADSCs express CD34 on their surface [14–16]. Nevertheless, the recommendation of the International Society for Cellular Therapy concerning identification of MSCs indicates that CD105, CD90 and CD73 molecules must be expressed by >95% of cells and CD45, CD34, CD14 and CD19 by <2% [9].

We compared the expression of surface markers on ADSCs isolated from RA and OA patients and failed to observe any differences between phenotype and percentage of these cells in tested groups. In both OA and RA almost all ADSCs expressed CD90, CD105 and CD73 (<97%) while they did not express CD45, CD14 or CD19 (Fig. 1), which is in accordance with the above-mentioned criteria for defining multipotent mesenchymal stromal cells. However, CD34 was expressed on ~10% of analysed cells, which may indicate that ADSCs from IA tissue are either slightly contaminated with haematopoietic and endothelial cells or represent subsets with distinct surface marker profiles, but with similar characteristics with regard to morphology and capacity for differentiation.

The regenerative potential of ADSCs is well documented [1, 17]. ADSCs cultured in special chondrogenic medium with addition of TGF-β1 were reported to express specific chondrogenesis markers: Sox9, aggrecan, collagen 2a and GAGs [18]. Sox9 is the transcription factor that acts as a master regulator of chondrocyte differentiation and is necessary for commitment to the chondrocyte fate at the time the chondrocyte and osteoblast lineages segregate from a common progenitor [19]. Similarly, collagen 2a expression, regulated by Sox9, is initiated in cells undergoing chondrogenesis [20]. In contrast, cells cultured in standard conditions and dedifferentiated cells do not express collagen 2a or express it in minimal amounts [21]. Aggrecan is the most important proteoglycan of cartilage, which aggregates with HA [22]. We found that the expression of all these molecules was significantly increased in ADSCs cultured in chondrogenic medium, which indicates an effective chondrogenesis process (Fig. 3). Moreover, we confirmed that by alcian blue staining, which detects GAGs. The control ADSCs remained GAG negative whereas cells cultured in chondrogenic medium were strongly GAG positive (Fig. 2). This effect was similar in both patient groups. Also, the expression of mRNA encoding other chondrogenic markers did not differ between RA and OA groups. Comparable literature data concern bone marrow-derived MSCs (BMSCs). Dudics et al. [23] reported that BMSCs from RA and OA patients possessed similar chondrogenic potential in vitro and therefore may be used in cartilage replacement therapy. Lian et al. [24] presented data showing that ADSCs from knee joint fat pad of OA patients had the ability to differentiate into chondrocytes, reduce cartilage degradation and modulate the inflammatory response, and hence exhibited the potential to be used for therapeutic applications in OA. Thus, our data are in accordance with previous reports. Interestingly, the osteoblastic potential of BMSCs from RA and OA patients was reported to be equivalent [25] and our preliminary results (not shown) suggest similar osteoblastic potential of ADSCs.

Therefore this information indicates the same in vitro regenerative properties of MSCs from RA and OA patients.

To mimic the in vivo conditions, we supplemented chondrogenic ADSC cultures with TNF, the cytokine of inflammatory and regulatory potential. Among other activities TNF is a well-known inhibitor of chondrogenesis [26, 27], and its excessive level in RA contributes to progressive cartilage degradation [28]. We were interested in verifying whether there are any differences in OA and RA ADSC differentiation response in vitro in the presence of this cytokine. In our experiments, TNF strongly inhibited expression of all chondrogenic markers by ADSCs, having the most potent effect on Col2a expression (Fig. 4). Importantly, upon TNF exposure the chondrogenesis of RA and OA ADSCs was reduced to a similar degree. Therefore we confirm that ADSCs are another cell subset in which chondrogenic properties are suppressed by TNF. The chondrogenesis of BMSCs is inhibited by TNF in NF-κB-dependent manner [27] and the chondrogenic capacity of FLS is inhibited by TNF through p38 mitogen activating kinase pathways [29]. In the rheumatoid synovium, MSCs have impaired chondrogenic potential due to the inflammatory environment (partially composed of TNF), and a negative relationship between synovial MSC chondrogenic capacity and magnitude of synovitis has been reported [30]. Inflammatory SF also affects chondrogenesis in the joint: RA SF was reported to reduce cartilage matrix formation by subchondral progenitor cells, whereas OA SF failed to exert any negative effect [31]. Thus, it is likely that the in vivo regenerative potential of RA and OA ADSCs is differentially modified by the distinct local cytokine environment present in each disease, while under artificially established in vitro conditions it is hard to discern these differences. However, the conditioned media derived from osteoarthritic synovium were recently reported to inhibit chondrogenesis of human MSCs [32], suggesting a similar anti-chondrogenic environment in both OA and RA joints. On the other hand, MSCs have well-documented immunosuppressive properties [33] and TNF has been shown to reverse in vitro the immunoinhibitory effect of MSCs on T-cell proliferation isolated from mice with CIA [34]. Knowing that adipose tissue from rheumatoid joints is a potent source of TNF and that TNF can act on rheumatoid adipose tissue in both an autocrine and a paracrine manner [7, 35] we can expect that the regenerative capacity of RA ADSCs may be also reversed by this cytokine in vivo.

Our study has several limitations. We were unable to obtain articular adipose tissue samples from healthy donors, so the OA samples served as a control. The RA patient group was quite heterogeneous regarding the duration of disease and CRP concentration. However, patient groups were properly age matched (58.5 vs 60.4). The other aspect that should be taken into consideration in our further studies is the TNF concentration. Knowing that SF TNF concentration is significantly higher in RA than in OA patients [36], the applied TNF dose might be adjusted in order to mimic the in vivo conditions. The
applied semi-quantitative RT-PCR method may also be considered a weakness of this study; however, it clearly confirms the results obtained by alcian blue staining.

In conclusion, we evaluated the percentage and the chondrogenic potential of ADSCs from RA and OA patients as well as the effect of TNF on this differentiation process in vitro. We have demonstrated that ADSCs from RA and OA patients do not differ regarding their phenotype and percentage in IA adipose tissue and maintain similar chondrogenic potential in vitro, which is strongly reduced upon exposure to TNF. All these results show that ADSCs from rheumatoid adipose tissue display the same characteristics and function as OA ADSCs in vitro and therefore both may be used in cell therapy for cartilage repair.

Rheumatology key messages
- The chondrogenic potential, percentage and phenotype of IA ADSCs from RA and OA patients are equivalent.
- TNF inhibits hondrogenesis from RA and OA ADSCs similarly.

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