Comparison of *in vitro* chondrogenic potential of human mesenchymal stem cells derived from bone marrow and adipose tissue

Ľuboš Danišovič[1], Ivan Varga[2,3], Štefan Polák[2], Marcela Uličná[1], Lívia Hlavačková[4], Daniel Böhmer[1] and Ján Vojtaššák[1]

[1] *Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 811 08 Bratislava, Slovakia*

[2] *Institute of Histology and Embryology, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 811 08 Bratislava, Slovakia*

[3] *Institute of Histology and Embryology, Faculty of Medical Speciality Studies, Slovak Medical University, Limbová 12, 833 03 Bratislava, Slovakia*

[4] *Institute of Pathology, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 811 08 Bratislava, Slovakia*

Abstract. In the present work, the human bone marrow and adipose tissue-derived mesenchymal stem cells (MSCs) were isolated and expanded under *in vitro* condition. After their phenotypic analysis, the chondrogenic differentiation was induced by using of the three-dimensional culture system without supplementation of growth factors, and their chondrogenic potential was compared. Obtained results proved that both types of MSCs undergo the process of chondrogenic differentiation. Comparative analysis showed that chondrogenic potential of adipose tissue-derived MSCs was slightly decreased in comparison with bone marrow-derived MSCs. However, both evaluated MSCs may play important role in the cartilage tissue engineering.

Key words: Chondrogenic potential — Mesenchymal stem cells — Bone marrow — Adipose tissue — *In vitro* study

Introduction

Inborn anomalies, pathological processes and injuries may lead in the degeneration of hyaline cartilage. It is difficult to treat these patients, because mature hyaline cartilage has a very limited self-healing potential (Strehl et al. 2005), and mitotic activity of chondrocytes is very low (Tew et al. 2000). Small defects are regenerated by the migration of chondrocytes (Hunziker and Rosenberg 1996). Full-thickness damages are healed by the formation of inferior fibrocartilage, but in many cases osteoarthritis develops and surgical intervention is the only option (Tuli et al. 2003).

Brittberg et al. (1994) has presented first important attempt for repair of hyaline cartilage defects by utilization of autologous chondrocytes. Unfortunately, this technique has some disadvantages, such as the reacquisition of phenotype of chondrocytes expanded *in vitro* (Schnabel et al. 2002) and non-uniform distribution of cells (Sohn et al. 2002).

Several studies focused on the utilisation of adult mesenchymal stem cells (MSCs) isolated from bone marrow as an alternative to chondrocytes were performed (Lee et al. 2004a; Bosnakovski et al. 2005). MSCs are characterized by its self-renewal ability. Moreover, they are capable of differentiating into different cell lineages, including chondrocytes under proper *in vitro* conditions (Pittenger et al. 1999). The *in vitro* differentiation of MSCs requires specific cell-cell interactions and additions of growth factors such as transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs) and insulin growth factor-1 (IGF-1) (Schmitt et al. 2003; Indrawattana et al. 2004). Bosnakovski et al. (2004) reported spontaneous chondrogenic differentiation of MSCs in three-dimensional culture system, without growth factors. However, the use of bone marrow-derived MSCs has some limitations like a discomfort and pain associated with bone marrow sampling, low cell quantity after isolation

Correspondence to: Ľuboš Danišovič, Institute of Medical Biology and Genetics, Faculty of Medicine, Comenius University in Bratislava, Sasinkova 4, 811 08 Bratislava, Slovakia
E-mail: lubos.danisovic@fmed.uniba.sk

and decreased differentiation potential in older individuals (Banfi et al. 2002).

More recently, it has been reported that adipose tissue-derived MSCs obtained from liposuction can differentiate into various types of cells (Zuk et al. 2001; Gimble and Guilak 2003; Huang et al. 2004). The potential of utilization of these cells is attractive for the cartilage tissue engineering because subcutaneous fat is abundant in human body and liposuction procedure is minimally invasive for patient. Furthermore, yield of adipose-derived tissue MSCs and their proliferation rates is higher in comparison to bone marrow (Strem and Hedrick 2005).

In the present study, we isolated human bone marrow and adipose tissue-derived MSCs and compared their chondrogenic potential under *in vitro* conditions.

Materials and Methods

Sampling procedure

Bone marrow, adipose tissue and hyaline cartilage were collected from male donors ($n = 2$; 27 and 33-year-old) during planed orthopaedic surgeries, following patient's informed consent. All samplings were performed in accordance with The Helsinki Declaration and were approved by the local ethical committee.

Isolation and cell culture of MSCs

Bone marrow-derived MSCs were isolated from fresh samples, which were supplemented with 20 U·ml^{-1} heparin in sterile physiologic solution as described previously (Pittenger at al. 1999; Bosnakovski et al. 2004). Nucleated cells were separated by the gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Sweden) at 1800 rpm for 40 min. Obtained cells were washed twice in phosphate buffered solution (PBS; Oxoid, GB) and were centrifuged at 1200 rpm for 5 min. Pellets were resuspended in complete culture medium consisted of α-MEM (PAA, Austria), 20% foetal bovine serum (FBS; PAA, Austria) and gentamicin in the final concentration of 160 µg·ml^{-1} (Lek, Slovenia). The obtained cells (2.5×10^5 cells/ml) were plated in 100 mm Petri dishes (TPP, Switzerland) and were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in the air. After 24 h, non-adherent cells were removed by changing the culture medium. During the following cultivation the medium was refreshed every three days. After 7–10 days, cells were detached by 0.25% trypsin (PAA, Austra) and sub-cultured up to third passage.

Adipose tissue-derived MSCs were obtained from fresh subcutaneous fat as described before (Gimble and Guilak 2003). Briefly, lipospirates were carefully rinsed with PBS and mechanically disaggregated by scalpel. Subsequently, adipose specimens were digested by 0.1% collagenase (Serva, Germany) in PBS for 60 min at 37°C with agitation at 50 rpm. Released cells and residual adipose tissues were centrifuged at 1200 rpm for 5 min. The supernatant layer of oil, fat and primary adipocytes, and the underlying layer of collagenase were aspirated. The pellet was resuspended in PBS with following centrifugation at 1200 rpm for 10 min. The final pellet was resuspended in complete culture medium consisted of DMEM/F12 1 : 1 (PAA, Austria) with 10% FBS and gentamicin in the final concentration of 160 µg·ml^{-1}. Cells were seeded at density of 2.5×10^5 cells/ml in 100 mm Petri dishes and were incubated in a humidified atmosphere of 5% CO_2 in the air at 37°C. Non-adherent cells were aspirated with medium after 24 h of cultivation and fresh medium was added. Culture medium was changed every three days. To obtain sufficient number of adipose-derived MSCs they were sub-cultured three times.

Chondrocytes were obtained from articular cartilage according protocols published previously (Brittberg et al. 1994). Briefly, cartilages were washed with PBS and mechanically disaggregated using scalpel. Obtained specimens were digested with 0.25% trypsin for 1 h, followed by digestion with 0.1% collagenase for 12 h at 37°C. DMEM/F12 1 : 1 with 10% FBS was added to released cells and they were centrifuged at 1200 rpm for 10 min. The pellet was resuspended in DMEM/F12 1 : 1 with 10% FBS and gentamicin in the final concentration of 160 µg·ml^{-1}. Cells were seeded at density of 2.5×10^5 cells/ml in 100 mm Petri dishes and were incubated in a humidified atmosphere of 5% CO_2 in the air at 37°C. Chondrocytes were sub-cultured two times and culture medium was refreshed in three-day interval.

Phenotypic analysis of MSCs

Bone marrow-derived MSCs and adipose tissue-derived MSCs from the third passage were analyzed by direct and indirect immunofluorescence, according to protocols specific for each antibody. In each case 10,000 events were acquired and analyzed by a Coulter Epics ALTRA flow cytometer. The following antibodies (Table 1) were used for cell staining: anti-CD29-PE, anti-CD44-FITC, anti-CD90-FITC, anti-CD105-FITC (BD Pharmingen, USA); anti-CD31-FITC (Chemicon, USA); anti-CD45-PE-Cy5 (DakoCytomation, Denmark); anti-CD34-FITC, anti-HLA class I-FITC, anti-HLA class II-FITC (Exbio, Czech Republic); anti-human fibroblast surface protein (Sigma Aldrich, USA) with a secondary FITC-conjugated donkey anti-mouse IgG antibody (Chemicon, USA).

Chondrogenic differentiation of MSCs

For chondrogenic differentiation, a three-dimensional pellet culture system was used. Pellets were formed by centrifugation of 1×10^6 bone marrow-derived MSCs and adipose tissue-derived MSCs (both from third passage) at 1500 rpm

Table 1. Description of analysed markers

Marker	Description
CD29	fibronectin receptor; general marker of MSCs
CD31	platelet endothelial cell adhesion molecule; typical for endothelial cells, lymphocytes, platelets, macrophages and fibroblasts
CD34	cell surface glycoprotein, typical for haematopoietic progenitor stem cells
CD44	receptor for hyaluronic acid; typical for specific types of MSCs
CD45	leukocyte common antigen; expressed in haematopoietic stem cells
D90	thymocyte differentiation antigen 1; general marker of stem cells
CD105	regulatory component of the TGF-β receptor-cell complex; general marker of human MSCs
HLA class I	component of human leukocyte antigen system; expressed by undifferentiated cells, including MSCs
HLA class II	component of human leukocyte antigen system; predominantly expressed on haematopoietic cells but not in MSCs
Human fibroblast surface protein	expressed by fibroblasts and macrophages

for 10 min in 15 ml polypropylene tubes (TPP, Switzerland). The chondrogenic medium consisted of DMEM/F12 1 : 1, 10% FBS and gentamicin in the final concentration of 80 $\mu g \cdot ml^{-1}$. Chondrocytes from the second passage cultured as pellets were used as a control. All tubes were maintained in the incubator at 37°C with humidified atmosphere of 5% CO_2. The culture medium was carefully refreshed every third day during 21 days.

Histological and immunohistochemical analysis

After 21 days, the pellets were fixed in 4% paraformaldehyde for 4 h, then dehydrated in ethanol and embedded in paraffin. Sections with a thickness of 5 μm were cut from paraffin blocks, deparaffinized with xylene and rehydrated with distilled water. The obtained sections were stained with hematoxylin and eosin (Sigma Aldrich, USA) to visualize the morphology of the cells,

with Masson's trichrome (Sigma Aldrich, USA) to visualize the collagenous matrix, and with alcian blue (Sigma Aldrich, USA) to visualize the acid mucopolysaccharides.

The presence of collagen type II was detected by immunohistochemistry. A monoclonal antibody directed against collagen type II (Chemicon, USA) was used in accordance with the manufacturer's recommendations.

Results

Bone marrow-derived MSCs and adipose tissue-derived MSCs grew as isolated colonies when primary plated. After 7–10 days they were sub-cultured and grew in confluent monolayer. Both types of cultured cells had a bipolar to polygonal fibroblast-like shape (Fig. 1). During further sub-cultivation (up to third passage) they maintained this
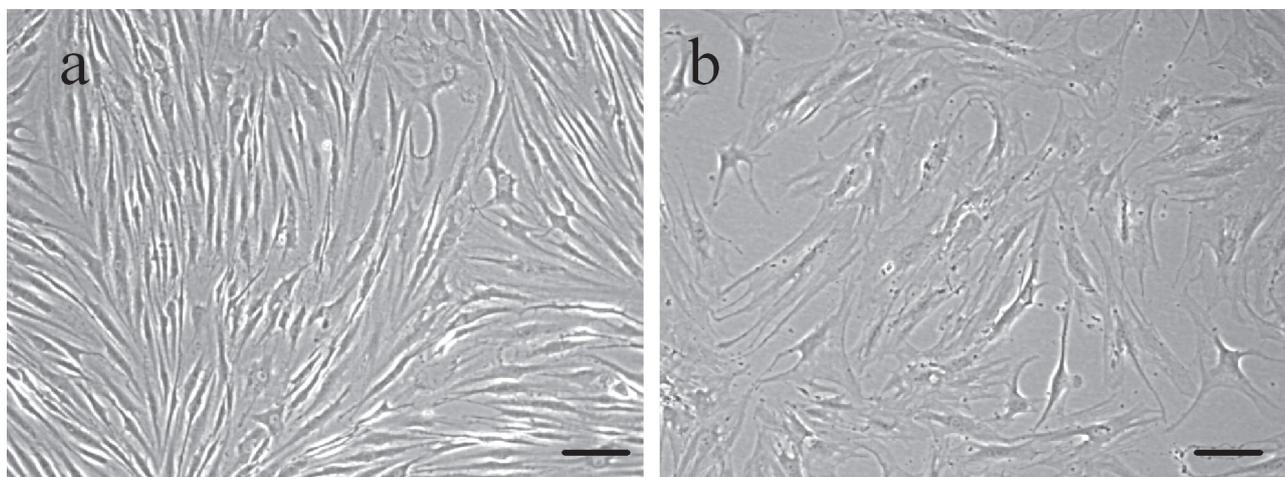

Figure 1. Human bone marrow-derived MSCs (a) and adipose tissue-derived MSCs (b), third passage of *in vitro* culture. Scalebar = 100 μm.
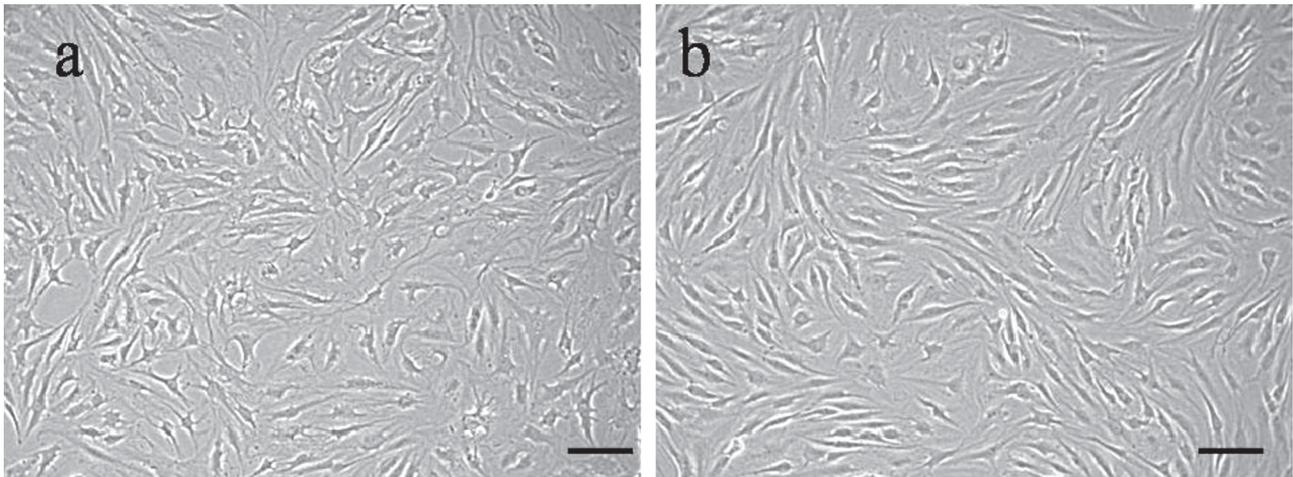
Figure 2. *In vitro* culture of human articular chondrocytes – primary culture (a) and third passage (b). Scalebar = 100 μm.

morphology. Moreover, any changes in their proliferation activity were not observed. Primary isolated chondrocytes had typical polygonal appearance. During following passages they gradually lost their original shape and showed fibroblast-like morphology (Fig. 2).

The results obtained from cytometric analysis (Table 2) showed that bone marrow-derived MSCs and adipose tissue-derived MSCs from third passage were positive for MSCs surface markers: CD29, CD44, CD90 and CD105. They were also HLA class I positive and HLA class II negative. The expression of haematopoietic and endothelial lineage markers CD31, CD34 and CD45 was not detected. Moreover, analysed cells did not express anti-human fibroblast surface protein.

When bone marrow-derived MSCs and adipose tissue-derived MSCs were centrifuged into micromasses, condensation of the pellets into single aggregates was observed after

Table 2. Phenotypic analysis of bone marrow-derived and adipose tissue-derived MSCs

Marker	Bone marrow--derived MSCs	Adipose tissue--derived MSCs
CD29	+	+
CD31	–	–
CD34	–	–
CD44	+	+
CD45	–	–
CD90	++	++
CD105	++	++
HLA class I	++	++
HLA class II	–	–
Human fibroblast		
surface protein	–	–

– negative; + positive (≤50%); ++ positive (≥80%).

24 h. Any significant differences were not observed when compared with pellets formed by chondrocytes maintained in same culture system. The condensed pellets grew continually during further 21 days. They gradually became white and opaque. After termination of experiment, differences in size of agregates were not observed.

Photomicrographs of hematoxilin and eosin stained samples (Fig. 3) showed a homogenous cell distribution in all samples. Cells were mostly round with darkly stained nuclei what indicate their excessive proliferation. Pellets of bone marrow-derived MSCs and chondrocytes contained more actively synthesizing cells than did pellets of adipose tissue-derived MSCs. Moreover, the density of cells in adipose tissue-derived MSCs aggregates was lower when compared with bone marrow-derived MSCs and chondrocytes. Masson's trichrome staining showed that cells in each group were separated by extensive regions of diffuse extracellular matrix, which was predominantly collagenous (Fig. 4). Alcian blue staining visualized deposits of acid mucopolysaccharides (Fig. 5). The highest intensity of this staining was recorded in samples of chondrocytes, while the intensity in adipose tissue-derived MSCs samples was lowest.

Immunohistochemical labelling against collagen type II proved the presence of this chondrogenic marker in all samples after 21 days of cultivation (Fig. 6). Stronger staining was recorded in the pellets of chondrocytes and bone marrow-derived MSCs when compared with those of adipose tissue-derived MSCs.

Discussion

Recently, adult MSCs seem to be promising tool for cartilage tissue engineering (Salgado et al. 2006; Jorgensen
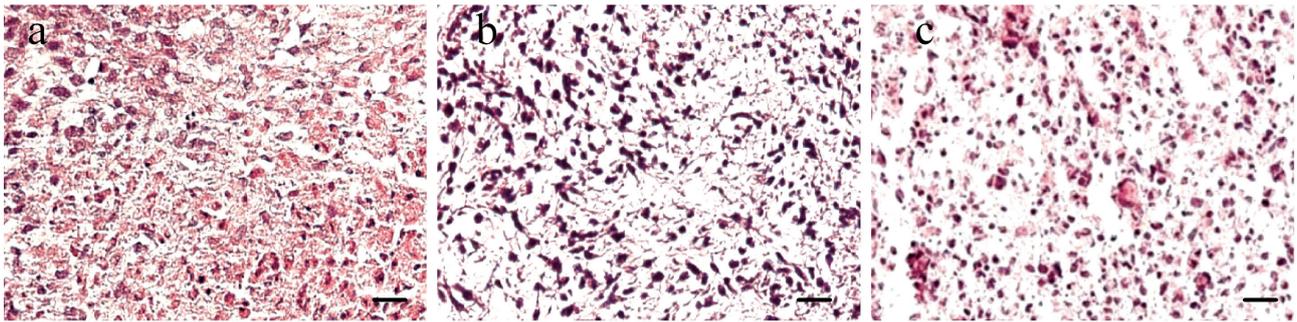
Figure 3. Hematoxylin and eosin staining of human bone marrov-derived MSCs (a), adipose tissue-derived MSCs (b) and chondrocytes (c).
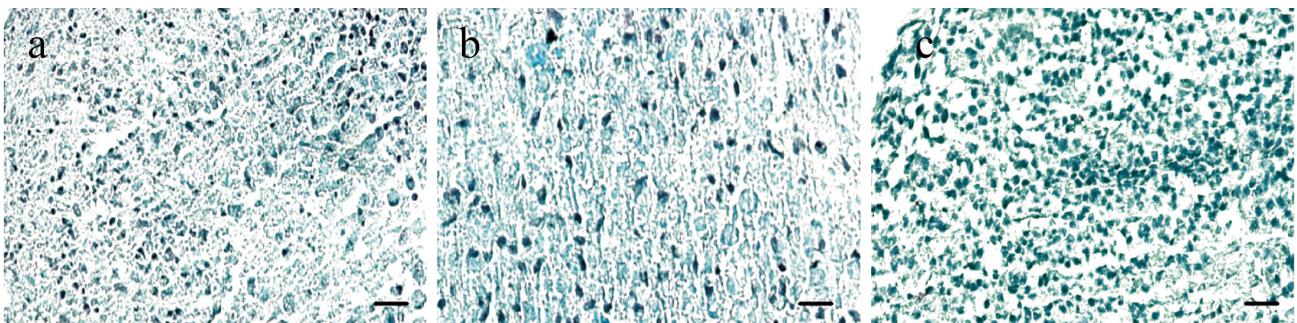

Figure 4. Masson's trichrome staining of human bone marrov-derived MSCs (a), adipose tissue-derived MSCs (b) and chondrocytes (c).
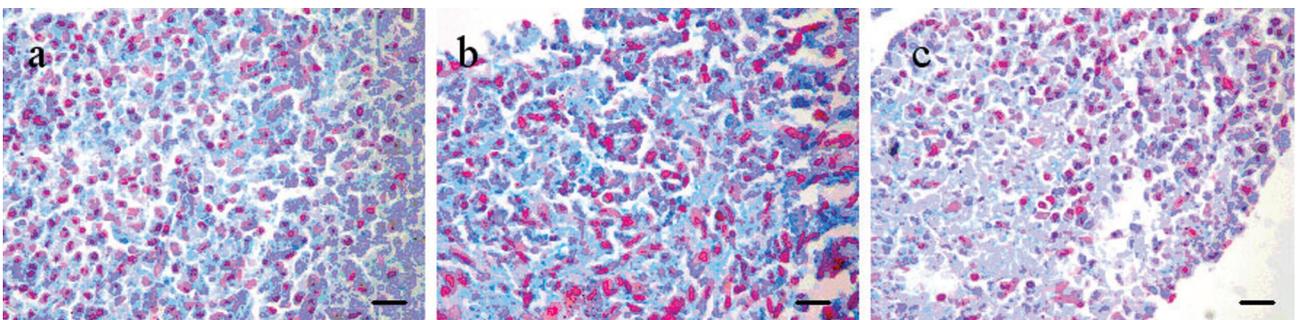

Figure 5. Alcian blue staining of human bone marrov-derived MSCs (a), adipose tissue-derived MSCs (b) and chondrocytes (c). The nuclei are stained by nuclear red.
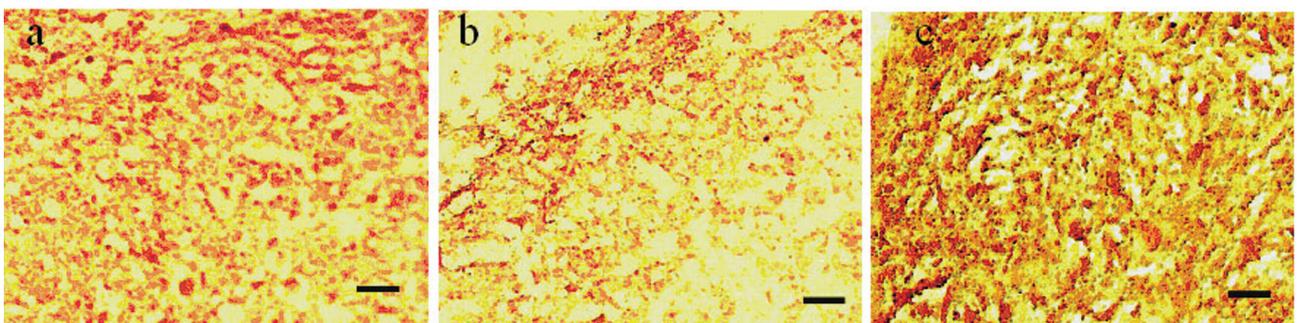

Figure 6. Immunohistochemical stainig of collagen type II in human bone marrov-derived MSCs (a), adipose tissue-derived MSCs (b) and chondrocytes (c). (Scalebar = 100 μm for Figures 3, 4, 5, 6).

et al. 2008). In our study, we compared *in vitro* chondrogenic potential of MSCs from bone marrow and adipose tissue. Biological material was obtained from two male donors who underwent planed surgeries. All sampling procedures were performed in agreement with the ethical principles.

Microscopical observation during *in vitro* cultivation showed that, bone marrow-derived MSCs and adipose tissue-derived MSCs displayed distinctive characteristics of stem cells (e.g. fibroblast-like morphology and intensive proliferation activity). Similar observations were published by other authors (Lin et al. 2005; Kern et al. 2006).

We found a similar phenotype of bone marrow-derived MSCs and adipose tissue- derived MSCs when examining cell surface markers. They were CD29, CD44, CD90 and CD105 positive, which are considered as general surface antigens of MSCs (Barry and Murphy 2004). On the other hand, they did not express CD31, CD34 and CD45, typical surface markers of haematopoietic stem cells (Pittenger et al. 1999; Baddoo et al. 2003). Moreover, they expressed HLA class I but not HLA class II. A similar phenomenon was observed in other types of undifferentiated cells, including MSCs (Le Blanc et al. 2003; Götherström et al. 2004).

Previous studies have reported the chondrogenic potential of human bone marrow-derived MSCs and adipose tissue-derived MSCs (Guilak et al. 2004; Lee et al. 2004b; Pansky et al. 2007). We decided to compare if these potentials are identical. To induce chondrogenic differentiation we used three-dimensional culture system without supplementation of growth factors (e.g. TGF-β1, TGF-β3, BMP-6, IGF-1) which were used in other works (Pittenger et al. 1999; Schmitt et al. 2003; Indrawattana et al. 2004). This system is appropriate to start chondrogenesis *in vitro* because of high density of cells that provide not only cell-to-cell contact but also contact of cells and their microenvironment, which contains variety of signal molecules. Moreover, it mimics cellular condensation process and hypoxic environment occurring during chondrogenic differentiation *in vivo* (Denker et al. 1999; Stott et al. 1999).

Our results showed chondrogenic differentiation of both bone marrow-derived MSCs and adipose tissue-derived MSCs, which is in accordance with the findings of Bosnakovski et al. (2004), who reported the spontaneous chondrogenic differentiation of bovine MSCs in three-dimensional pellet culture. The chondrogenic nature of the differentiated MSCs in our aggregates was supported by the expression of a cartilaginous extracellular matrix, containing collagens and acid mucopolysaccharides as demonstrated by histological staining and immunohistochemical analysis. Comparison of the collagen type II production showed stronger staining in the samples of chondrocytes and bone marrow-derived MSCs when compared with those of adipose tissue-derived MSCs. This proved lowered chondrogenic potential

of adipose tissue-derived MSCs. In contrast, Winter et al. (2003) demonstrated that bone marrow-derived MSCs and adipose tissue-derived MSCs are not significantly different in their ability to undergo chondrogenic differentiation. So the question of which MSCs are better replacements for chondrocytes in the cartilage tissue engineering is still under dispute (Lee et al. 2004b; Sakaguchi et al. 2005; Strem and Hedrick 2005).

In conclusion, we confirmed that both human bone marrow-derived MSCs and adipose tissue-derived MSCs underwent the process of chondrogenic differentiation. Comparative analysis shoved lowered chondrogenic potential of adipose tissue-derived MSCs in comparison with bone marrow-derived MSCs and chondrocytes. However, both evaluated MSCs may play important role in the cartilage tissue engineering.

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