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REVIEW ARTICLE

Mesenchymal stem cells from human adipose tissue and bone repair: a literature review

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Abstract Mesenchymal stem cells (MSCs) emerge as potential tools for treatment of various diseases. Isolation methods and tissue of origin are important factors that determine the amount of obtained cells and their ability to differentiate. MSCs can be isolated from adipose tissue (ADSCs), bone marrow (BMSCs) or umbilical cord (UC-MSCs), and its characterization must follow the criteria required by the International Society for Cellular Therapy. Osteogenic differentiation capacity of ADSCs can still vary according to the culture medium used, as well as by adding factors that can alter signaling pathways and enhance bone differentiation. In addition, nanotechnology has also been used to increase osteoblastic induction and differentiation. ADSCs enhanced the prospect of treatment in different diseases, and in regenerative medicine, these cells can also be associated with different biomaterials. There is a great progress in studies with ADSCs, mainly because it is easy to access, which makes bioengineering techniques for bone tissue feasible.

Introduction

By definition, stem cell is an undifferentiated cell, without tissue-specific markers, and capable of proliferation, self-renewal and plasticity. The description of multipotent mesenchymal stem cells was first made in the 70s, where Friedenstein and colleagues showed bone marrow cells were able to differentiate into osteoblasts (Afanasyev, Elstner, & Zander, 2009). The term mesenchymal stem cell was introduced by Caplan (1991) to demonstrate that such cells, isolated from adult or embryonic tissue, were able to differentiate into chondrocytes and osteoblasts (Pittenger et al., 1999).

According to the International Society for Cellular Therapy (ISCT), there are three minimum requirements for a population of cells to be classified as Mesenchymal Stem

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Cells (MSCs). The first is that MSCs must be adherents to plastic, when grown under standard culture conditions, using cell culture bottles. The second request is to follow the criteria of presence and absence of specific markers that should be in over 98% of cells in culture. Finally, the third criterion is that cells must be able to differentiate into osteocytes, chondrocytes and adipocytes, in standard in vitro differentiation conditions (Dominici et al., 2006).

MSCs have great ability to modulate immune responses and alter the progression of several inflammatory diseases. Tissue lesions are often accompanied by inflammation whose mediators can act as chemotactic roads, mobilizing and directing the MSCs to damaged sites. Before the tissue repair itself, MSCs prepare a microenvironment through its ability to modulate inflammatory process by release of growth factors. Such a process named crosstalk between MSCs and immune responses has shown potential clinical applications, as for example, in treatment of bone and cartilage defects, myocardial infarction and autoimmune diseases (Ma et al., 2014). The interest and expectations in MSCs-based therapies for inflammatory and autoimmune diseases have grown remarkably over the past few years, supported by encouraging data that demonstrate MSCs immunomodulatory properties and therapeutic effects in experimental animals (DelaRosa, Dalemans, & Lombardo, 2012).

In recent years, researchers found MSC secrete soluble factors that exert immuno-suppressive effects by modulating both innate immune responses (macrophages, dendritic cells and NK), and adaptive immune responses (B cells and T cells CD4+ and CD8+) (Bassi et al., 2012). MSCs present low immunogenicity, and are considered ''immunologically privileged'' because they express a relatively low level of major histocompatibility complex (MHCs) of class I and II, and have no surface expression of costimulatory molecules (CD80, CD86 and CD40), that are necessary to fully enable T cells. More importantly, certain active immunoregulatory factors secreted by MSCs can regulate immune reactions in cells and surrounding tissues (Yang et al., 2017).

There are three main sources of MSCs for human studies and therapies: bone marrow, umbilical cord and adipose tissue. The stem cells derived from human adipose tissue are similar to bone marrow-derived MSCs in morphology and phenotype, but show important differences in regard to harvest and cell yield (Markarian et al., 2014). MSCs can also be isolated from fetal liver (Zhang et al., 2005), fetal lung (Fukuchi et al., 2004; Schwab, Hutchinson, & Gargett, 2008), endometrium and periodontal ligament (Park et al., 2011), dental pulp (Zuk et al., 2002), synovial membrane (Hermida-Gomez et al., 2011) and compact trabecular bone (Sakaguchi et al., 2004).

The increase in obesity triggered changes in medical practices, such as tummy tucks and liposuction. Despite the adipose tissue be routinely discarded after surgery, there are researchers who have documented its use as an abundant resource of multipotent stromal cells for regenerative medicine (Zuk et al., 2002), thanks to its easy isolation, expansion of culture, ability to differentiate and immunomodulatory properties. Collection of this tissue is performed by less invasive methods, with more amount of material collected than in bone marrow collection (Heo, Choi, Kim, & Kim, 2016).

This literature review describes the necessary steps for the use of MSCs to bone repair such as isolation, characterization and differentiation.

Isolation of mesenchymal stem cells

Comparative analysis between MSCs isolation demonstrated adipose derived stem cells (ADSCs) do not present differences in morphology and immune phenotype when compared with bone marrow stem cells (BMSCs) and umbilical cord mesenchymal stem cells (UC-MSCs). However, the frequency of ADSCs in adipose tissue exceeds the frequency of BMSCs in Medullary stroma. Through liposuction, it is possible to get about 2–6 $\times$ 10 cells in 300 mL of adipose tissue, which represents a number approximately 500 times more than the number of cells obtained from bone marrow isolation. This number of ADSCs, however, can vary, depending on the method of isolation (Mosna, Sensebe, & Krampera, 2010; Yarak & Okamoto, 2010; Zuk et al., 2002).

There are many processes for digestion and isolation of adipose tissue, as those using collagenase enzymes, trypsin, dispase, or related enzymes. Despite these processes, there is a consensus in relation to temperature (37 °C), length of digestion (30 min to 1:00) and weight/volume ratios of tissue (Gimble & Guilak, 2003).

The conventional enzymatic method for insulation is widely used. Nevertheless, methodologies with lower costs and more effective are being proposed by different research groups. Ghorbani, Jalali, and Varedi (2014) proposed a non-enzymatic isolation method with use of fetal bovine serum (FBS) in the early 24 h of adipose tissue culture, and after this time non-adherent cells are discarded and adherent cells are covered with Dulbecco's modified eagle medium (DMEM) containing 20% FBS.

Comparing different methods of isolation, variable cellular yield and cell viability were observed, according to Table 1.

In addition to enzymatic and non-enzymatic processes, companies and laboratories had developed total or partial automated systems, in order to standardize and to ensure sterility in the isolation process. These types of systems had arisen for enrichment of adipose tissue for autologous transplantation in plastic surgery, and then were adapted for isolation of cell fractions. Some are based on use of enzymes, others in non-enzymatic processes, but for the most part, resolved issues such as standardization of operating system, sterility and absence of toxins and xenobiotics, normally required by the regulatory authorities of this kind of therapy. Although many are not closed, high cost and difficult handling, generally these systems have been optimized and are promising for therapy, due to demand of a large yield in isolation for clinical application (Minonzio et al., 2014).

Mesenchymal stem cells characterization

After the process of isolation and cultivation of cells, intrinsic features of MSCs must be confirmed. Although expression of cell surface markers is used to define MSCs, its characterization is functionally relevant and provides criteria for quality control.

Table 1 Comparison of yield and cell viability of different isolation methods.

Enzyme	Yield	Viability (%)	Author
Collagenase	701,000 cells/mL	82.4	Doi et al. (2013)
	160,000 cells/mL	90.0	Guven et al. (2012)
	368,000 cells/mL	74.5	Landerholm and Chapman (2014)
	404,000 cells/mL	93.9	Aust et al. (2004)
Trypsin	75,000 viable cells/cc	80.0	Markarian et al. (2014)
Liberase	350,000 cells/mL	95.0	Bobis-Wozowicz et al. (2014)
	180,000 cells/g	85.0	Minonzio et al. (2014)
Non-enzymatic	140,666 cells/mL	87.3	Landerholm and Chapman (2014)
	25,000 viable cells/cc	65.0	Markarian et al. (2014)
	23,000 cells/mL	85.0	Condé-Green et al. (2014)

Table 2 Positive and negative markers to characterize isolated MSCs.

Positive	Negative	Author
CD105	CD45	Dominici et al. (2006)
CD73	CD34	Dominici et al. (2006)
CD90	CD14 or CD11b	Dominici et al. (2006)
	CD79α or CD19	Dominici et al. (2006)
	HLA-DR	Dominici et al. (2006)
	CD38	Samsonraj et al. (2015)
	CD19	Samsonraj et al. (2015)
	CD31	Samsonraj et al. (2015)

Analyses are performed according to ISCT criteria, among them, analysis of cell surface markers by flow cytometry. A specific marker of MSCs has not yet been identified, so ISCT as well as various authors have described a series of positive and negative markers that should be evaluated for phenotyping of isolated cells (Li et al., 2014; Peng et al., 2009).

Mafi, Hindocha, Mafi, Griffin, and Khan (2011) showed many authors have related a variety of cell surface markers to adult MSCs, despite some conflicting results about existence and expression of them. CD105, CD73 and CD90 are the most commonly cell surface markers reported for MSCs. The release criteria used to characterize MSCs according to The International Society for Cellular Therapy are CD105, CD73 and CD90 positive cells, and negative for HLA-DR, CD14, CD34, CD19 and CD45 (Table 2) (Dominici et al., 2006). Samsonraj et al. (2015) showed the absence of CD19, CD38 and CD31 markers in MSCs. It was found that expression of these markers was homogeneous and uniform. All positive markers were consistently above 90%, and negative markers were expressed in <5% (Kargozar et al., 2016).

The main difference between stromal vascular fraction cells (SVF) and suspensions ADSCs is a high level of $CD45^{+}$ cells in the first and the low or undetectable in the second. Bourin et al. (2013) showed ADSC should be negative (90%) for stromal markers such as CD13, CD73 and CD90 (Table 3). $CD271^{+}$ marker is associated with increased cell proliferation and clonogenic capacity in liposuction samples (Yoshimura et al., 2006). Expression of CD34 greatly depends on culture condition, being expressed during initial phase (between 8 and 12 passages), and then lower expression with continuous cell division, until it disappears (Bourin et al., 2013). After third passage, isolated cells appeared as relatively homogeneous populations. Presented analysis results by flow cytometry showed positive marking for CD105, CD90, CD29 and CD73, and negative for CD45 and CD34 (Kargozar et al., 2016).

Table 3 Positive and negative markers to characterize isolated ADSCs.

Positive	Negative	Author
CD105	CD45	Bourin et al. (2013) and Dominici et al. (2006)
CD73	CD34	Bourin et al. (2013) and Dominici et al. (2006)
CD90	CD14 or CD11b	Bourin et al. (2013) and Dominici et al. (2006)
CD36	CD79α or CD19	Bourin et al. (2013) and Dominici et al. (2006)
CD29	HLA-DR	Yoshimura et al. (2006)
CD44	CD34	Yoshimura et al. (2006)
CD49d	CD38	Yoshimura et al. (2006)
CD151	CD19	Yoshimura et al. (2006)
	CD31	Yoshimura et al. (2006)
	CD106	Yoshimura et al. (2006)

Osteogenic differentiation ability of mesenchymal stem cells

Osteogenic differentiation capacity of MSCs can be influenced by different factors, one of which is the methodology used for isolation. Markarian et al. (2014) compared isolation methods of ADSCs using four different strategies: collagenase, red blood cell lysis buffer, trypsin and centrifugation. They realized the use of trypsin originated a cell population with a bone differentiation capacity seven times greater than cells population isolated using a conventional methodology with collagenase.

The culture medium used is also a determining factor in osteogenic differentiation ability. ADSCs from two different donors were cultured on three types of basal medium: DMEM + Low [Glucose] (LG), DMEM + High [Glucose] (HG), DMEM + F12, and compared advantages and limitations of each type. Addition of Fibroblast Growth Factors (FGF) in

each basal media studied was also analyzed. With regard to the osteogenic differentiation capacity, medium with high concentration of glucose plus FGF presents better results. On the other hand, cells grown in medium with low concentration of glucose, have advantage in relation to the ability of cell proliferation (Ahearne, Lysaght, & Lynch, 2014).

A mixture containing Dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly) is routinely used to induce osteogenic differentiation in a treatment of at least 3 weeks. This set of inducers elicits a series of regulatory processes to induce in vitro mineralization (Langenbach et al., 2013; Song, Caplan, & Dennis, 2009). An initial study showed the optimum concentration of Dex for osteogenesis in chicken cells was 100 nM (Tenenbaum & Heersche, 1985). This concentration was widely used in experiments, but some researchers reached an optimum concentration for mineralization nodules formation of 10 nM (Walsh, Jordan, Jefferiss, Stewart, & Beresford, 2001). Dex induces expression of Runx2 by FHL2/transcription activated by β-catenin mediation. In addition, Dex increases Runx2 activity by upregulation of TAZ and MKP1 (Hong et al., 2009).

Asc is required as a cofactor for enzymes that hydroxylate proline and lysine in procollagen, inducing an increase in Type I collagen secretion. This results in an increased binding of α2, β1 to Col1 integrins, phosphorylating ERK1/2 in MAPK signaling pathway and subsequent translocation of PERK1/2 to the nucleus, where it binds to Runx2 and induces gene expression of osteogenic proteins. β-Glycerophosphate serves as a phosphate source for bone mineralization and induces osteogenic genes expression from ERK1/2 phosphorylation (Langenbach & Handschel, 2013).

Some signaling pathways that influence osteogenic differentiation have also been studied. Gu et al. (2015) showed activation of JNK/MAPK signaling pathways is involved in BMP-2 induced osteogenic differentiation in BMSC. Blockade of the JNK signaling pathway by SP600125 was responsible for decreased extracellular calcium deposition, increased lipid droplet accumulation, decreased alkaline phosphatase (ALP) production and decreased expression of osteogenic genes as Runx2, ALP and osteocalcin (OCN), always dose-dependent (Fig. 1).

According to Peng et al. (2009), strontium can promote osteogenic differentiation in mesenchymal stem cell lines by increasing Runx2 activity through activation of Ras/MAPK signaling pathways. ERK1/2 was the main kinase identified in this pathway because it participated in Runx2 activation after treatment. Osteogenic differentiation was significantly inhibited after ERK1/2 blockade by PD98059.

In addition to signaling pathways, microRNAs with a role in bone differentiation, such as miR-22 and its HDAC6 receptor (downstream), have been identified as relevant regulators of the balance between adipogenic and osteogenic differentiation in human ADSCs. These results indicated that miR-22 and HDAC6 could be potential targets in therapy for bone diseases (Baer et al., 2013).

Adiponectin is one of the most abundant adipocytokines released by adipocytes, and it plays an important role in glucose metabolism and energy homeostasis. Increasing evidence has shown a positive association between adiponectin and bone formation. Researchers demonstrated adiponectin increased the expression of osteoblast-related genes in human ADSCs. Activation of the AMP – protein kinase (AMPK) pathway has been related to osteogenic induction medium, regulating the perpetuation of human ADSCs in osteogenic lines. Once adiponectin has been shown to positively regulate AMPK phosphorylation, this indicates its involvement in increased osteogenic differentiation (Chen, Wu, Lu, Guo, & Tang, 2015).

Nanotechnology has also been used to increase the ability of cellular bone differentiation. Gold nanoparticles have been suggested as useful tools for bone regeneration due to their non-toxic and stimulating effects on MSC differentiation. Effects of chitosan scaffolds conjugated with gold nanoparticles were investigated as a determinant inductor of MSCs in osteoblasts, relating mechanical stimulation as a regulator for catenin/Wnt signaling pathway activation in mineralization (Choi et al., 2015).

Rozila et al. (2016) did a study to evaluate the osteogenic potential of ADSCs co-cultured with osteoblasts in polymeric scaffolds of PHB–BHA (poly beta-hydroxybutyrate–poly beta hydroxybutyrate-co-valerate). Only stem-cell cultures were tested on the scaffold as a control, and different proportions of stem cells and osteoblasts on the scaffold were tested. This type of polymeric skeleton was previously indicated as a positive influencing factor in bone differentiation being confirmed with these experiments. Both scaffold with stem cells and co-culture showed good results in bone differentiation, but the ratio of 1:1 (ADSCs:osteoblast) obtained better results.

The choice of cryopreservation methodology is fundamental for adipose tissue-derived MSCs to maintain their differentiation potential and thus be successfully employed in regenerative medicine. González-Fernández et al. (2015) evaluated six different cryosensor protocols for ADSCs. The results showed cryopreserved cells composed of 10% dimethylsulfoxide (DMSO) and 90% of SFB had a viability rate higher than 90% after thawing. In addition, the authors compared the potential for osteogenic, adipogenic and chondrogenic differentiation, and the same presented unchanged between frozen and unfrozen cells. This study agrees with Liu et al. (2008) that evaluated human ADSCs, showing cryopreservation does not affect growth and differentiation potential, confirming that even after freezing cryopreserved MSCs can be used as a source of cells for regenerative medicine.

Applications

The use of mesenchymal stem cells allows a new therapeutic modality for treatment of different diseases such as: cardiac dysfunction, cancer, neurological diseases, tissue lesions and diabetes. Cell therapy, based on stem cells usage, is mainly aimed at repairing and regenerating tissue. In a study by Chen et al. (2015) it was found injured tissue expresses chemotactic factors to help recruit stem cells to lesion sites and repair it. Results of Tobita, Uysal, Ogawa, Hyakusoku, and Mizuno (2008) after using ADSCs have suggested these cells can promote regeneration of periodontal tissue.

Some studies combine stem cells with biomaterials to enhance the use of these cells in vivo. Recently, Song et al. (2016) demonstrated ADSCs can differentiate into osteoblasts when encapsulated in calcium alginate,

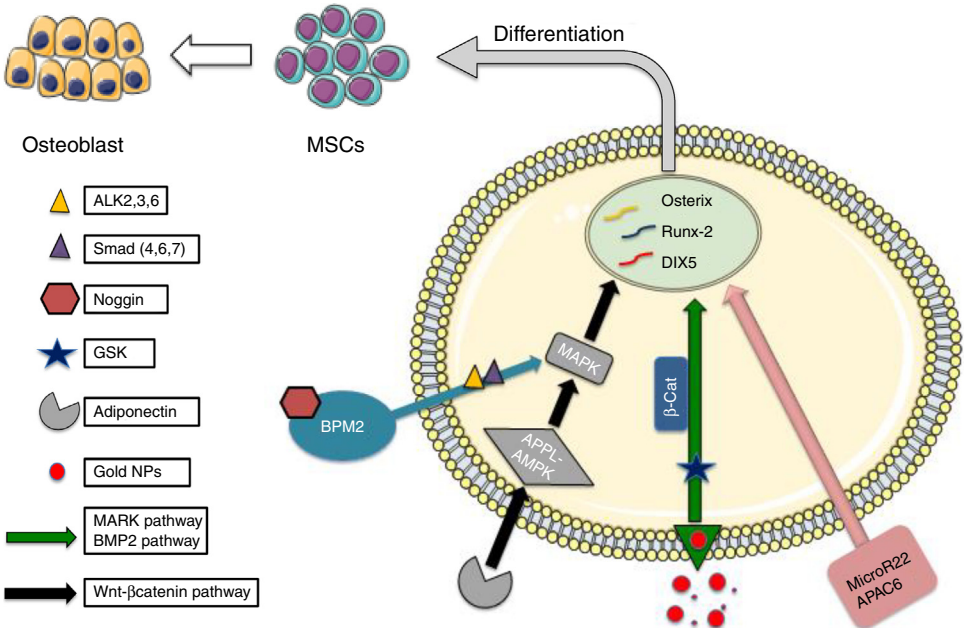

Figure 1 Illustration of the reciprocal regulation between adipocytes and osteoblasts during MSC differentiation and the ultimate control of the osteo-adipogenic balance factors which affect the decision of mesenchymal stem cells: adipocytes or osteoblasts. MSC are balanced by this showed differentiation commitment. The fat-induction factors inhibit osteogenesis, and, conversely, bone-induction factors hinder adipogenesis. The genes and molecules trigger different signaling pathways and activate various transcription that guides MSCs to commit to either lineage.

contributing efficiently to treating bone damage. Banas et al. (2007) have used nanoparticle-associated ADSCs for evaluation in upper limb ischemia models. Godoy Zanicotti, Coates, and Duncan (2016) investigated the osteogenic capacity of ADSCs, cultured under serum-free conditions, on different titanium surfaces, but did not find significant differences in bone formation.

Sándor et al. (2014) evaluated 13 cases of skull-maxillofacial bone defects in frontal sinus, cranial bone, mandible and nasal septum. Three patients with ameloblastoma required mandibular resection, with an average size defect of 8.2 cm. Adipose tissue was collected and a titanium mesh was used with βTCP and 12 mg rhBMP2. Reconstruction of the three mandibular defects with autologous ADSCs was successful, with cicatrization without intercurrences. For frontal sinus reconstruction, three patients were used, all with chronic infections that were not successful in conventional surgical and non-surgical treatment. The three patients were followed up with clinical evaluations performed at 1 week, 1, 6 and 12 months after surgery and thereafter annually. Computed tomography and magnetic resonance imaging were performed 1 and 12 months postoperatively. After 1 month of follow-up, patient 2 presented new bone formation in more than 95% of the frontal sinus region, which remained stable throughout the follow-up.

Patients who underwent resection of hemangioma and meningioma, bone cranial defects were filled with βTCP granules along with autologous ADSC. These patients presented clinical and radiographic evidence of bone defect healing, although one patient presented a meningioma recurrence, but after a second resection, the patient had positive evidence on healing the cranial defects.

Mesimaki et al. (2009) describe a new method for reconstructing a major maxillary defect in an adult patient using autologous ASCs, combined with human rhBMP-2 recombinants and βTCP granules. The patient was a 65-year-old man who underwent hemimaxillectomy 28 months earlier due to a large recurrent keratocyst. The postoperative period was uneventful; in the biopsy it was verified that bone neoplasm was vital and there was vascularization.

In this way, it is possible to observe a great number of advantages in using ADSCs, due to its ease collection and a large number of cells when compared to BMSCs. In addition, the potential for osteogenic differentiation allows the application of these cells with great perspectives in regenerative medicine.

Conflicts of interest

The authors declare no conflicts of interest.

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