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Review

Current status of bone regeneration using adipose-derived stem cells

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Summary. Many bone regeneration therapies have been developed for clinical use and have variable outcomes and serious limitations. The goal of bone regeneration is to repair a bone defect in a stable and durable manner. Cellular strategies play an important role in bone tissue engineering. Clinical factors important for successful bone regeneration are the recruitment of cells to the defect site and the production of a suitable extracellular matrix consistent with bone tissues. Adipose-derived stem cells (ASCs) can be obtained in large quantities with little donor site morbidity or patient discomfort. They are multipotent somatic stem cells and have a strong potential to differentiate and secrete growth factors. In this review, we discuss the osteogenic potential of ASCs with/without several types of scaffolds *in vivo* and their clinical application for bone regeneration.

Key words: Bone regeneration, Adipose-derived stem cells

Introduction

Bone regeneration after trauma, infection, and tumor resection is an important issue for reconstructive surgery. The process of bone regeneration involves a complex cascade of biological events controlled by many growth factors. These growth factors provide local signals to mediate the migration of osteoprogenitor cells and their subsequent differentiation, cell proliferation, vascularization, and production of extracellular matrix (Marquez et al., 2013).

Bone regeneration is performed with/without a scaffold. A scaffold is often used in clinical applications. Many surgical techniques have been developed to reconstruct bone using a scaffold, including autografts (Oikarinen and Korhonen, 1979), allografts (Salgado et al., 2004), xenografts (Goldstein, 2002), and alloplasts (Eppley and Sadove, 2000). Autografts are the ideal bone graft and are currently the therapeutic gold standard because they harbor all the components essential to induce bone regeneration (Oikarinen and Korhonen, 1979). However, the disadvantages associated with autografts are limited donor sources, donor site morbidities, and variable bone graft survival (Tadjoedin et al., 2002). Allografts, xenografts, and alloplasts are alternative treatments, each of which has distinct disadvantages that limit its clinical application. Allografts can be used to repair large defects; however, their use is limited by possible immune rejection, disease transmission, and their lower incorporation rate compared with that of autografts (Salgado et al., 2004). Xenografts have the same drawbacks as allografts, and their physiological structures and functions do not exactly match those of human tissue (Goldstein, 2002). Alloplasts have recently been used to regenerate bone. However, they typically lack osteoinductivity and may increase the risk of patients suffering foreign body reactions and infections (Eppley and Sadove, 2000). To improve the osteogenic properties of grafts, osteoprogenitor cells might need to be included because

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an ideal bone graft should provide osteogenic cells as well as osteoinductive factors for bone regeneration (Salgado et al., 2004).

An appropriate source of stem cells, together with an adequate scaffold and growth factors that speed up tissue regeneration, are crucial issues for successful clinical applications (Langer and Vacanti, 1993). A number of studies demonstrated that mesenchymal stem cells (MSCs) have high plasticity, with the ability to differentiate into cells of mesenchymal lineages, such as adipogenic, osteogenic, and chondrogenic cells (Saulnier et al., 2011). Bone marrow-derived mesenchymal stem cells (BMSCs) are the most well-known and well-characterized source of adult stem cells. Stem cells were first isolated from bone marrow (Friedenstein et al., 1968). The disadvantages associated with BMSCs are that the yield of stem cells from bone marrow aspirates is low and the procedure is painful. MSCs have the potential to directly differentiate into osteogenic cells and efficiently regenerate bone (Pittenger et al., 1999; Shayesteh et al., 2008). Additionally, they secrete a variety of growth factors and cytokines that promote angiogenesis and bone reconstruction (Chen et al., 2008; Osugi et al., 2012).

On the other hand, recent reports have demonstrated that MSCs can be isolated from adipose tissue after liposuction (Zuk et al., 2001). Adipose-derived stem cells (ASCs) can also differentiate into adipocytes, osteoblasts, chondrocytes, and myocytes. Several reports demonstrated that ASCs have the potential to differentiate into the osteogenic lineage as efficiently as MSCs (Dragoo et al., 2003; Mizuno et al., 2012). Moreover, the use of ASCs is an effective approach for bone regeneration *in vivo* (Parrilla et al., 2011; Pourebrahim et al., 2013).

The aim of this review is to describe the osteogenic capacity of ASCs and to discuss their use with/without a scaffold for bone regeneration. This review also highlights future studies that are needed to establish ASC therapy as a standard component of clinical care.

Characterization of ASCs

Adipose tissue is highly complex and comprises mature adipocytes (>90%) and stromal vascular fractions (SVFs), which include preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes, and ASCs (Yoshimura et al., 2009). Zuk et al. reported that donor age, adipose tissue type and anatomical location, type of surgical procedure, culture conditions, exposure to plastic, plating density, and medium formulations might influence the proliferation rate and differentiation capacity of these cells (Zuk et al., 2001).

SVFs isolated from adipose tissue contain a heterogeneous cell population. SVFs include putative ASCs (CD31-, CD34+/-, CD45-, CD90+, CD105-, and CD146-), endothelial (progenitor) cells (CD31+, CD34+, CD45-, CD90+, CD105-, and CD146+), vascular smooth muscle cells or pericytes (CD31-, CD34+/-, CD45-, CD90+, CD105-, and CD146+), and hematopoietic cells (CD45+) (Zimmerlin et al., 2010; Mizuno et al., 2012). ASCs share many surface markers with pericytes and BMSCs.

There are no significant differences in the yield of adherent stromal cells, growth kinetics, senescence, multilineage differentiation capacity, or gene transduction efficiency between BMSCs and ASCs isolated from the same patient (De Ugarte et al., 2003). Moreover, ASCs have the same differentiation potential as BMSCs.

Some studies have investigated the secretion profiles of ASCs (Rehman et al., 2004; Blaber et al., 2012). ASCs secrete angiogenic cytokines both *in vitro* and *in vivo*, which increases neovascularization and enhances wound healing in injured tissues (Nie et al., 2011; Nauta et al., 2013). Growth factors secreted by ASCs can elicit both paracrine and autocrine responses (Moutsatsos et al., 2001; Turgeman et al., 2001).

In vitro osteogenic potential of ASCs

ASCs are the one of the most promising types of stem cells for bone regeneration via cell-based approaches (Lindroos et al., 2011). Expanded ASCs improve bone regeneration *in vitro* and *in vivo* through their direct differentiation into mature osteoblasts and paracrine effects that facilitate the migration and differentiation of resident precursors.

In vitro studies have demonstrated that ASC osteogenesis can be enhanced by manipulating the concentrations of ascorbate and dexamethasone in the cell culture media (de Girolamo et al., 2007). The secretomes of SVFs (De Francesco et al., 2009) and ASCs (Kilroy et al., 2007; Kapur and Katz, 2013) contain different endocrine factors with bone-remodeling activity. ASCs secrete several growth factors (e.g., transforming growth factor (TGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF)) (Tajima et al., 2015) with angiogenic and antiapoptotic potentials, which makes them an interesting candidate for bone regeneration (Ikegame et al., 2011). In particular, VEGF is thought to be the main regulator of angiogenesis, enhances the survival and differentiation of endothelial cells, and contributes to osteogenesis (Kaigler et al., 2003). It can induce the formation of a new network of blood capillaries, which is required during bone regeneration (Colnot, 2005), and plays a major role in the recruitment of hematopoietic stem cells for bone regeneration (Ferrara, 2004).

Osteogenic differentiation-related growth factors

Several reports have indicated that a variety of growth factors, including platelet-derived growth factor (PDGF), TGF-β1, HGF, IGF, and VEGF, promote osteogenic differentiation (Landesberg et al., 2000;

Weibrich et al., 2002). Growth factors are mitogenic for osteoblasts and stimulate the migration of mesenchymal progenitor cells (Fiedler et al., 2002). These growth factors affect cellular proliferation and differentiation during bone repair (Huang et al., 2007). Moreover, angiogenesis is extremely important for bone regeneration. TGF-β, VEGF, HGF, and FGF are needed to induce and speed up angiogenesis in regenerating tissue and may reduce the healing time and enhance bone regeneration (Rubina et al., 2009). Bone morphogenetic protein (BMP)-2 is a potent osteoinductive molecule that increases and speeds up osteogenic differentiation and induces the healing of critical size defects in animals (Schofer et al., 2011). This molecule belongs to the TGF-β superfamily, promotes the differentiation of osteoprogenitor cells, and induces osteogenesis (Gautschi et al., 2007). Additionally, PDGF reportedly plays an important role in bone regeneration (Marx et al., 1998). The most important activities of PDGF include stimulation of mitogenesis and angiogenesis, activation of macrophages, and induction of paracrine signaling. These growth factors have different functions and cumulatively accelerate tissue and bone regeneration.

Furthermore, the fibrin matrix itself may also contribute to healing by providing a conductive scaffold for new matrix formation. Consequently, these growth factors are currently used in regenerative medicine to stimulate tissue regeneration, support bone reconstruction, and reduce the healing time (Marx et al., 1998). Platelet-rich plasma (PRP) contains several growth factors (e.g., PDGF, TGF, IGF, VEGF, and HGF) (Tozum and Demiralp, 2003; Blaber et al., 2012). Growth factors are endogenous proteins that direct the actions of a wide variety of cells by binding to and activating cell surface receptors (Varkey et al., 2004). Those growth factors that naturally occur within the healthy bone matrix or are expressed during fracture healing can be used to direct the development of structures, vascularization, and differentiation of bone cells (Janicki and Schmidmaier, 2011).

Scaffolds for bone regeneration

Scaffolds provide an osteoconductive space for bone tissue formation and are seeded with osteogenic progenitor cells. One of the most challenging goals for the development of bone graft substitutes is to produce a scaffold with osteoinductive potential, which may require biologically active molecules. A key issue in bone regeneration is that the scaffold should have an adequate pore size. The ideal scaffold has a high porosity and an interconnected pore network that is large enough to facilitate vascular invasion and bone development (Iezzi et al., 2012). Large pore sizes (>300 μm) promote neovascularization and favor mineralized bone ingrowth, whereas smaller pore sizes (90-120 μm) promote endochondral bone formation (Kuboki et al., 2001). For tissue engineering, the scaffold should ideally be biocompatible, have an appropriate porosity and pore size to facilitate neovascularization, and have a surface that allows cell adhesion, proliferation, and differentiation.

Allografts and xenografts are non-artificial scaffolds. An allograft is tissue transferred from a donor to a recipient of the same species but not of an identical genetic make-up. Representative examples are demineralized freeze-dried bone allografts (DFDBAs) and freeze-dried bone allografts (FDBAs). Several reports demonstrated that DFDBAs have osteoinductive ability in addition to osteoconductive ability (Reynolds and Bowers, 1996; Mott et al., 2002). FDBAs provide a source of type I collagen, which is the major organic component of bone, but do not produce inorganic calcium, which is a necessary scaffold for bone regeneration. DFDBAs are reportedly 3-fold more absorbable than FDBAs that are not decalcified (Wood and Mealey, 2012), and their absorption rate significantly differs depending on the supplier (Yang et al., 2015).

A xenograft is derived from a donor that is a different species to the recipient. A representative example is bovine-derived xenogenic bone grafts (BDXs). The implanted material usually serves as a scaffold for the ingrowth of capillaries, perivascular tissue, and osteoprogenitor cells from the recipient bed via osteoconduction. Organic components are completely removed from BDXs to avoid immunological reactions, and the remaining inorganic structure provides a natural architectural matrix and an excellent source of calcium (Richardson et al., 1999). BDXs undergo a low heat (300°C) chemical extraction process through which all organic components are removed, but the natural architecture of bone is maintained (Gross, 1997). Although BDXs are considered to be absorptive, there are reports that they remain in animal models for several years and that their absorbability is low (Piattelli et al., 1999; Yang et al., 2015). Significant improvements in bone fill and percentage gain are achieved using both DFDBAs and BDXs; however, there is no significant difference between them (Kothiwale et al., 2009).

Alloplasts used as scaffolds should mimic the morphology and structure of bone to optimize integration into the surrounding tissue and to provide a suitable microenvironment for adhesion and proliferation of MSCs (Tampieri et al., 2005). Hydroxyapatite (HA), Ca10(PO4)6(OH)2, one of the most well-characterized biomaterials, is non-bioresorbable and is currently used in clinical applications to replace damaged bone tissues. It resembles mineralized bone and supplies fundamental ions to newly forming bone (Schmitz et al., 1999). Calcium phosphate cement (CPC) consists of tetracalcium phosphate ($Ca_4(PO_4)_2O$: TTCP) and dicalcium phosphate anhydrous ($CaHPO_4$: DCPA), and is kept within a pH range of 7.4-9 until hardened. The Ca-to-P molar ratio is 1.67, and CPC is reportedly a

highly biocompatible and osteoconductive material (Sugawara et al., 2002, 2008). Beta-tricalcium phosphate (β-TCP), $Ca_3(PO_4)_2$, is bioresorbable and suitable for clinical use as a carrier of MSCs because of its chemical and crystallographic similarities to the inorganic phase of native bone (Liu et al., 2008). β-TCP supports cell ingrowth and promotes osteogenic differentiation of osteoprogenitor cells (E et al., 2010). As described above, there are many types of scaffolds, and it is important to understand the characteristics of each and to select an appropriate scaffold for each clinical case.

Preclinical evaluation of ASCs

ASCs possess an osteogenic potential *in vivo* (Zuk et al., 2001; Hicok et al., 2004; Tajima et al., 2015). These cells promote the healing of critical-sized cranial defects with/without a scaffold in mouse, rat, and rabbit models (Levi et al., 2010, 2011; Pieri et al., 2010; Wang et al., 2010; Kim et al., 2012). The effects of ASCs in segmental long bone defects resemble the fracture healing process (Kim et al., 2012; Arrigoni et al., 2013). Large animal models can be used to evaluate the bone-healing capacity. The porcine model has been used to assess the effect of direct or indirect delivery of ASCs to bone defects in the mandible (Wilson et al., 2012). It is noteworthy that successful bone regeneration has been achieved in different animal models using ASCs (Parrilla et al., 2011; Arrigoni et al., 2013; Choi et al., 2014) and SVFs (Rhee et al., 2011; Kim et al., 2012). Moreover, several studies suggested that engrafted stem cells have poor differentiation and survival rates; however, they mediate regeneration primarily through paracrine mechanisms (Kotobuki et al., 2008; Zimmermann et al., 2011; Ando et al., 2014).

The osteogenic capacity of ASCs is significantly lower than that of BMSCs (Im et al., 2005; Hayashi et al., 2008). On the other hand, the ease of accessibility and relative abundance of ASCs compared with BMSCs confer practical advantages and have contributed to continued interest in the clinical use of ASCs for bone regeneration (Lendeckel et al., 2004; Mesimaki et al., 2009).

Recent studies reported scaffolds for ASCs. Arrigoni et al. demonstrated that healing of tibial defects is improved by treatment with rabbit ASCs and HA compared with HA alone (Arrigoni et al., 2013). β-TCP matrix alone is sufficient to trigger the differentiation of ASCs toward an osteoblastic phenotype, regardless of whether cells are grown in proliferation or differentiation medium (Marino et al., 2010). Although each scaffold has advantages for bone repair, their use alone has not been shown to induce sufficient bone regeneration compared with that achieved by autologous bone grafts (Boeck-Neto et al., 2009). The utilization of ASCs in combination with a scaffold is an effective strategy to restore the function of damaged bone.

Meanwhile, the combination of growth factors and ASCs stimulates the healing of bone and soft tissue. Chou et al. showed that the combination of recombinant human BMP-2 and ASCs may increase the osteogenic potential *in vivo* (Chou et al., 2011). Our laboratory previously reported that an ASC/PRP admixture without a scaffold is effective for periodontal tissue engineering (Tobita et al., 2008) and cranial bone regeneration (Tajima et al., 2015) (Figs. 1, 2). Furthermore, many studies have reported that PRP supports bone regeneration with stem cells (Marx et al., 1998; Marx,

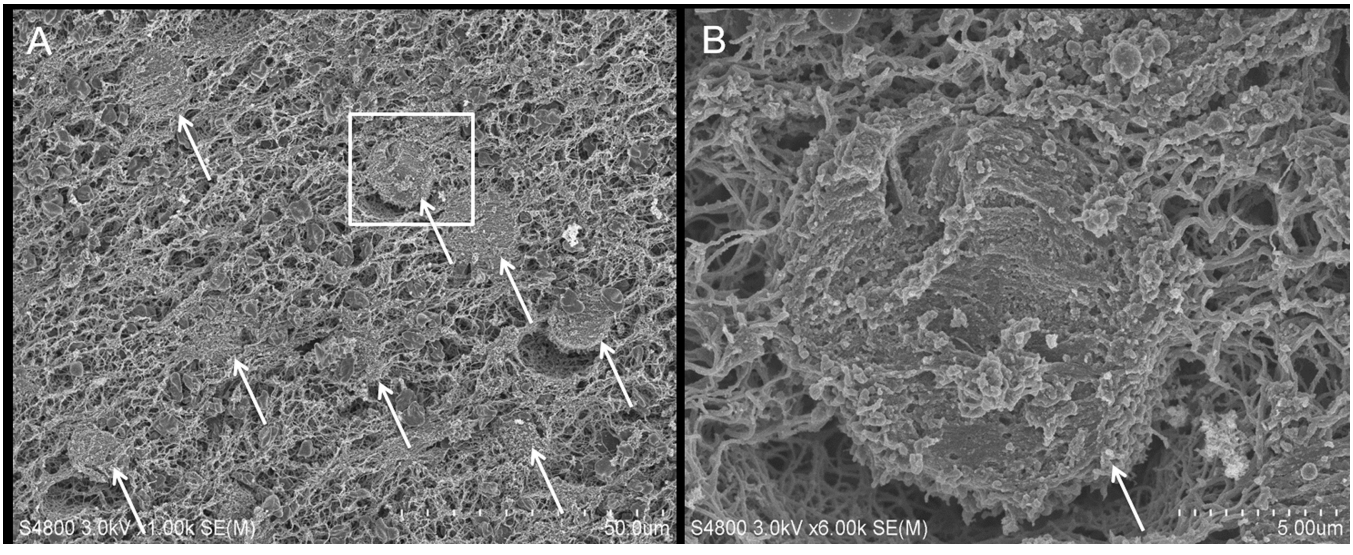

Fig. 1. Scanning electron microscopy images of an activated ASC/PRP admixture. **A.** Representative image of an activated ASC/PRP admixture. ASCs and dense fibrin fibers were observed. **B.** A higher magnification image of the boxed area shown in **A**. ASCs were encapsulated by a highly condensed fibrin fiber network that contained many platelets. Activated platelets were connected to the surface of ASCs. Arrow: ASCs.

2001; Yamada et al., 2004). These reports suggest that the growth factors in PRP are extremely useful for stem cell therapies to regenerate bone.

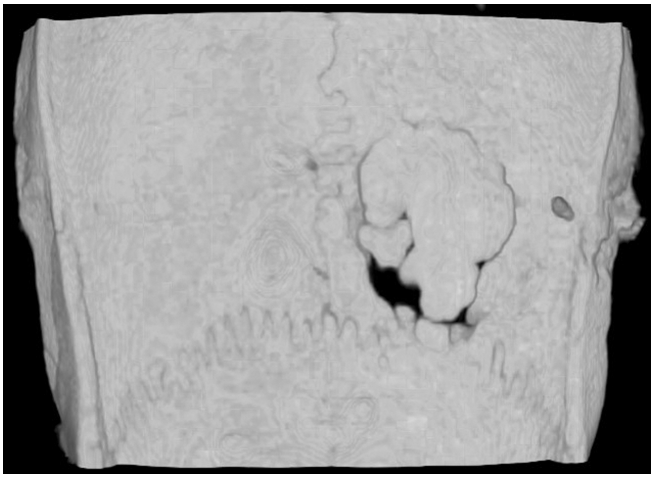

Fig. 2. Micro-computed tomography images of a rat cranial defect after transplantation of an ASC/PRP admixture. Most of the bone defect was covered with newly formed bone 8 weeks after transplantation.

Clinical use of ASCs for bone regeneration

On the basis of both *in vitro* experiments and preclinical studies, the ability of ASCs to facilitate bone regeneration has been tested in clinical studies. There are several reports concerning the utilization of ASCs for bone regeneration (Table 1). In the first clinical case, autologous SVFs were used to treat widespread traumatic calvarial bone defects (Lendeckel et al., 2004). A 7-year-old girl with post-traumatic calvarial defects was treated with autologous cancellous iliac bone in combination with autologous ASCs, fibrin glue, and a biodegradable scaffold. The patient exhibited continuous bone regeneration within 3 months of surgery. Mesimaki et al. have reported a novel method to treat a major maxillary defect resulting from benign tumor resection in an adult patient using autologous ASCs in combination with recombinant human BMP-2 and β-TCP granules (Mesimaki et al., 2009). The resulting bone had sufficient structural integrity to support dental implants 4 months after surgical reconstruction of the defect. They achieved satisfactory outcomes and obtained mature vascularized bone with good osteointegration and stability using autologous ASCs in a custom-made implant for reconstruction. In 2012, Sandor demonstrated the synergistic effect of autologous

Table 1. Clinical studies of ASCs for bone regeneration.

Type of cells	Scaffold	Number of patients	Repair site	Results	Ref.
Autologous SVFs	Autologous bone, fibrin glue, and biodegradable scaffold	1	Post-traumatic calvarial defect	New bone formation and almost complete calvarial continuity were achieved	Lendeckel et al., 2004
Autologous ASCs	β-TCP and rhBMP-2	1	Maxillary bone defect	Newly formed bone was obtained and dental implants were inserted 4 months after bone and soft tissue reconstruction	Mesimaki et al., 2009
Autologous ASCs	β-TCP and rhBMP-2	23	Craniofacial osseous defects	The jaw was successfully reconstructed in 20 of 23 patients who were followed closely postoperatively	Sandor, 2012
Autologous ASCs	β-TCP and resorbable mesh	4	Calvarial reconstruction	The tissue density of the defect site, as assessed in Hounsfield units measured by computed tomography, gradually increased to equal that of bone	Thesleff et al., 2011
Autologous ASCs	β-TCP and rhBMP-2	1	Ameloblastoma resection defect	Ten months after reconstruction, dental implants were inserted into the grafted site. Histologic and CD markers were examined and prosthodontic rehabilitation was completed	Sandor et al., 2013
Autologous ASCs	β-TCP and rhBMP-2	3	Ameloblastoma resection defects	In these three cases, the reconstruction successfully bridged large defects averaging 8.2 cm with uneventful healing	Wolff et al., 2013
Autologous ASCs	Bioactive glass or β-TCP and rhBMP-2	13	Cranio-maxillofacial hard-tissue defects in the frontal sinus (3 cases), cranial bone (5 cases), mandible (3 cases), and nasal septum (2 cases)	Successful integration of the construct into the surrounding skeleton was noted in 10 of the 13 cases	Sandor et al., 2014
Autologous ASCs	PRP	8	Alveolar bone defects	Three months after transplantation, healing of the operation wound was accelerated and a sufficient amount of bone was generated for the dental implants	Kulakov et al., 2008
Autologous SVFs	PRP and hyaluronic acid	3	Femoral head osteonecrosis	Magnetic resonance imaging scans were improved after the procedure, as evidenced by positive T1 signal changes consistent with medullary bone regeneration	Pak, 2012

ASC, adipose-derived stem cell; β-TCP, beta-tricalcium phosphate; PRP, platelet-rich plasma; rhBMP-2, recombinant human bone morphogenetic protein-2; SVF, stromal vascular fraction.

ASCs, resorbable scaffolds (β-TCP and bioactive glass), and BMP-2 in 23 patients with craniofacial osseous defects (Sandor, 2012). The use of autologous ASCs in combination with biomaterials led to bone reconstruction in 85% of cases, although the long-term success of this procedure needs to be verified using a large sample. Additionally, Thesleff et al. used autologous ASCs for calvarial reconstruction, tested alternative biomaterials (β-TCP and a resorbable mesh bilaminate scaffold), and obtained successful results in adult patients (Thesleff et al., 2011). Some reports have demonstrated successful bone regeneration using autologous ASCs and SVFs (Lendeckel et al., 2004; Kulakov et al., 2008; Mesimaki et al., 2009; Thesleff et al., 2011; Pak, 2012; Sandor, 2012; Sandor et al., 2013, 2014; Wolff et al., 2013), extending our limited knowledge regarding the potential of ASCs and SVFs for bone tissue repair.

As described above, these reports on clinical bone regeneration all used ASCs with a scaffold. For clinical applications, the scaffold must be safe and biocompatible. Moreover, a scaffold likely affects maintenance of the microenvironment and space making during the bone regeneration process. Furthermore, a scaffold needs to be used in combination with stem cells and growth factors for bone regeneration.

Conclusions

Bone regeneration is currently the most promising clinical application of ASCs. Stem cell-based bone regeneration has many potential therapeutic advantages over the use of autografts. It is necessary to establish a proof-of-concept and develop minimally invasive approaches and surgical processes in order to ensure the successful clinical application of ASCs. The use of ASCs without a scaffold can result in bone regeneration in animal models, but all clinical reports have used ASCs with scaffolds. It has been suggested that the use of an appropriate scaffold with ASCs is effective in clinical cases.

Cell-based constructs enhance bone formation in comparison with acellular constructs, showing more pronounced vessel and bone formation than that achieved with a scaffold alone. The field of scaffolds must continue to develop biocompatible materials that allow transplanted ASCs to grow, differentiate, and be exposed to growth factors.

Although clinical trials have demonstrated bone reconstruction using ASCs, many aspects need to be investigated and resolved. A cell-based study using ASCs in combination with an osteoinductive scaffold and osteogenic/angiogenic growth factors may help to optimize clinical procedures. Selecting an appropriate scaffold for ASCs is another critical issue. Further investigations are needed to standardize the procedures for using ASCs in clinical applications.

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