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ORIGINAL PAPER

Effectiveness of autologous serum as an alternative to fetal bovine serum in adipose-derived stem cell engineering

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Abstract In cell culture, medium supplemented with fetal bovine serum is commonly used, and it is widely known that fetal bovine serum supplies an adequate environment for culture and differentiation of stem cells. Nevertheless, the use of xenogeneic serum can cause several problems. We compared the effects of four different concentrations of autologous serum (1, 2, 5, and 10 %) on expansion and adipogenic differentiation of adipose-derived stem cells using 10 % fetal bovine serum as a control. The stem cells were grafted on nude mice and the in vivo differentiation capacity was evaluated. The isolation of adipose-derived stem cells was successful irrespective of the culture medium. The proliferation potential was statistically significant at passage 2, as follows: 10 % autologous serum >10 % fetal bovine serum = 5 % autologous serum >2 % autologous serum = 1 % autologous serum. The differentiation capacity appeared statistically significant at passage 4, as follows: 10 % fetal bovine serum >10 % autologous serum = 5 % autologous serum >2 % autologous serum = 1 % autologous serum. Ten percent autologous serum and 10 % fetal bovine serum had greater differentiation capacity than 1 and 2 % autologous serum in vivo, and no significant difference was observed between the groups at ≥5 % concentration at 14 weeks. In conclusion, 10 % autologous serum was at least as effective as 10 % fetal bovine serum with respect to the number of adipose-derived stem cells at the end of both isolation and expansion, whereas 1 and 2 % autologous serum was inferior.

Keywords Fetal bovine serum alternative · Autologous serum · Adipose-derived stem cell · Adipogenic differentiation

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Introduction

Adipose-derived stem cells (ASCs) are easy to harvest because of their abundant availability, excellent expansion capacity, and their differentiation potential

(Aust et al. 2004; De Ugarte et al. 2003; Zuk et al. 2002). Human adipose tissue in particular is easily obtained from patients undergoing plastic surgery. Therefore, ASCs are considered to be an attractive alternative to bone marrow-derived mesenchymal stem cells (BMSCs) (De Ugarte et al. 2003).

For application of stem cells to clinical studies, such as cell therapy and tissue engineering, a large number of stem cells are needed (Baksh et al. 2004). Most isolation and expansion protocols for clinical-scale production of mesenchymal stem cells use a medium supplemented with fetal bovine serum (FBS). However, use of xenogeneic serum can cause several problems. Viral or bacterial infections and prion transmission can arise (Dedrick 1997; Will et al. 1996; Klein and Dumble 1993). The immunologic reactions to fetal serum albumin have been reported (Kievits et al. 1988; Mackensen et al. 2000).

A chemically-defined, standardized, xenogeneic antigen- and serum-free media is ideal. An alternate formula to FBS with artificially created growth factors and substances has been developed (Brunner et al. 2010; Falkner et al. 2006). Moreover, a serum-free 3D culture system that allows the expansion of ASCs as floating spheres in a defined medium was introduced (Dromard et al. 2011). However, the switch to serum-free media still demands a time-consuming literature survey and a manufacturer search for appropriate medium formulations, because of production problems, formulation upgrades or simply the associated costs (Dromard et al. 2011; Brunner et al. 2010; Falkner et al. 2006). Therefore, considerable effort has been directed toward searching for possible FBS alternatives. Recently, human blood supplements have been identified as promising substitutes, and include autologous (Honmou et al. 2011; Pérez-Ilzarbe et al. 2009; Nimura et al. 2008; Kobayashi et al. 2005; Shahdadfar et al. 2005; Stute et al. 2004; Koller et al. 1998) and allogeneic serum (Kocaoemer et al. 2007; Shahdadfar et al. 2005; Kuznetsov et al. 2000; Koller et al. 1998), and platelet derivatives (Pérez-Ilzarbe et al. 2009; von Bonin et al. 2009; Kocaoemer et al. 2007).

There have been studies comparing expansion and osteogenic differentiation of BMSCs with autologous serum (AS) to FBS, and studies which have determined the best concentration of AS as the medium for expanding BMSCs (Pérez-Ilzarbe et al. 2009; Kobayashi et al. 2005; Shahdadfar et al. 2005; Stute et al. 2004; Koller et al. 1998). There have been few studies comparing AS to FBS as the culture medium for ASCs or demonstrating a difference in vivo. Therefore, we compared the effects of four different concentrations of AS (1, 2, 5, and 10 %) on the expansion and adipogenic differentiation of ASCs using 10 % FBS as the control, and ASCs which were cultivated and differentiated in each media were grafted on nude mice, and the in vivo differentiation capacity was evaluated.

Methods

Donor

Of patients who underwent liposuction or transverse rectus abdominis myocutaneous flaps at Seoul National University Hospital, 10 patients with a voluntary agreement for written informed consent were included in this study. All were females under 60 years of age, with a body mass index less than 30 kg/m^2. Discarded adipose tissues or lipoaspirates were harvested from the abdomen, flank and thigh regions, and 100 ml of blood were obtained from the each patient. This study was conducted after obtaining approval by the Institutional Review Board of Seoul National University (H-0605-032-174).

Autologous serum

Venous whole blood was drawn into blood bags without anticoagulant and stored at 4 °C overnight. The blood was centrifuged at 3,000 rpm for 10 min, the serum was aliquoted into Eppendorf tubes (Eppendorf, Hamburg, Germany), and stored at −20 °C until use. Heat inactivation of the serum was not performed because growth factors and nutrients necessary for cell culture may be temperature-sensitive. Four concentrations of AS were tested (1, 2, 5, and 10 %).

Isolation and expansion of ASCs

Minced adipose tissues or lipoaspirates were washed with phosphate buffer solution (Gibco-BRL, Grand Island, NY, USA), and digested with 0.5 % collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) under gentle agitation for 60 min at 37 °C. The digested tissues were centrifuged at 470 g for 5 min to obtain a pellet. The supernatant discarded and the cell pellet resuspended in 160 mM $ClNH_4$ to eliminate red blood

cells. After 10 min at 37° C, the cells were again centrifuged, resuspended in Dulbecco's modified Eagle's media containing 0.2 mM ascorbic acid and 10 % FBS, and were filtered through a 100-μm nylon cell strainer (BD Biosciences, Bedford, MA, USA). The cells were counted on a hemocytometer (Superior, Marienfeld, Germany), and 3×10^4 cells per well were seeded in 24-well plates (Nunc, Roskilde, Denmark) with Dulbecco's modified Eagle's media with 10 % FBS, and 1, 2, 5, and 10 % AS at 37 °C in a humidified atmosphere containing 5 % CO2. After 3 days, the non-adherent cells were discarded and the plastic adherent cells were further expanded with the medium described above and fed every 3 days. At 70–80 % confluency, cells were trypsinized and reseeded in other wells. At passage 2, the cells were counted on a hemocytometer and the number of cells between each group was compared.

Adipogenic differentiation

ASCs were plated at a concentration of 3×10^4 cells per well. Adipogenic differentiation medium was added after 1–2 days following confluence for 48 h, as follows: 0.5 mM isobutyl-methylxanthine (Sigma, St. Louis, MO, USA), 0.1 μM dexamethason (Sigma), 0.1 % insulin transferrin selenium (ITS), and 0.1 mM indomethacin (BD Biosciences, Bedford, MA, USA). Then, the same medium with only insulin added was used for 1–2 days. The differentiation was conducted for 14 days. The differentiation was assessed at passage 4 using Oil Red O stain as an indicator of intracellular lipid accumulation. The cells were washed with phosphate buffered solution, then fixed in 10 % formalin for 10 min and stained with 0.3 % Oil Red O (Sigma) in a mixture of isopropanol and water (3:2) and extracted with 4 % Nonidet (Sigma)/isopropanol for quantification. Absorbance was measured using a spectrophotometer (Molecular Device Co., Sunnyvale, CA, USA) at 490 nm. The mean ± SD of the absorbance of five groups (1, 2, 5, and 10 % AS, and 10 % FBS) was calculated.

In vivo transplants

Absorbable gelatin sponge (Gelform; Pfizer, New York, NY, USA) was used to deliver cells (McCarty et al. 2010). The carrier was cut with a 7-mm punch into uniform cylinder blocks. The cells of each group were suspended in culture medium at a concentration of 5×10^5 cells in 25 μl, and seeded in the carrier. A total of 10 BALB/c nude mice (weight 20–25 g) were used. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (Seoul, Korea). Briefly, animals were anesthetized using inhaled isofluorane. Six small incisions were performed on the back. The surrounding soft tissues between the pannuculus carnosus and deep fascia of the muscles were dissected using small Metzembaum scissors. After pocketing, the absorbable gelatin sponges containing the cells of each group (1, 2, 5, and 10 % AS, and 10 % FBS) and the control group were transplanted into each pocket. The control group was defined as having no cell but the absorbable gelatin sponge. Skin was then closed and sutured with 6-0 blue nylon. Fourteen weeks after transplantation, biopsies of the grafts were performed, and the volumes were measured by the graduated ruler under the operating microscope. The greatest longitudinal diameter (length) and the greatest transverse diameter (width) were determined. Graft volume was calculated by the modified ellipsoidal formula: $\textit{Tumor volume} = 1/2(\textit{length} \times \textit{width}^2)$ (Euhus et al. 1986; Tomayko and Reynolds 1989). The specimens were formalin-fixed and paraffin-embedded. Four-micrometer sections were deparaffinized and stained with hematoxylin and eosin. To identify the engraftment of ASCs, the monoclonal mouse antihuman nuclei (1:100; Chemicon) was used as a marker.

Statistical analysis

All data were used to calculate the group means ± standard deviation. The Kruskal–Wallis test and the Mann–Whitney U test for post hoc test were used to compare the values of cell numbers after expansion, absorbance after adipogenic differentiation, and graft volume among groups. The non-parametric methods were used to compare the whole of the group means. Differences were considered to be significant at a $p < 0.05$. All statistical tests were performed using the SAS software package (version 9; SAS Institute, Cary, NC, USA).

Results

Isolation and expansion potential

The isolation of ASCs was successful irrespective of the culture medium. For comparing the effect of

different serum conditions on the proliferative capacity of ASCs, the number of cells was counted. The cells were observed grossly by light microscopy on day 5 of primary culture. At this time, the highest cell number was generated with 10 % AS (Fig. 1). The difference between each group was more remarkable at passage 2. The number of cells in each group at passage 2 was an average of 219,000 ± 60,452 with 1 % AS, 264,000 ± 47,187.57 with 2 % AS, 374,000 ± 64,325.56 with 5 % AS, 538,000 ± 76,274.36 with 10 % AS, and 428,000 ± 26,677.55 with 10 % FBS (Fig. 2). The proliferation potential was statistically significant, as follows: 10 % AS >10 % FBS = 5 % AS >2 % AS = 1 % AS. The connected single-donor data points were shown in Fig. 3.

Adipogenic differentiation capacity

We observed the formation of lipid vacuoles in all groups. The mean absorbance at each group on passage 4 was 0.027097 ± 0.008717 with 1 % AS, 0.034835 ± 0.014123 with 2 % AS, 0.0433 ± 0.019894 with 5 % AS, 0.255267 ± 0.086207 with 10 % AS, and 0.5086 ± 0.126216 with 10 % FBS (Fig. 4). The differentiation capacity appeared statistically significant, as follows: 10 % FBS >10 % AS = 5 % AS >2 % AS = 1 % AS. The differentiation capacity depended on the serum concentration between the AS groups. We observed that 10 % FBS had a significantly higher differentiation capacity than 10 % AS when comparing 10 % FBS with 10 % AS ($p = 0.0004$). The connected single-donor data points were shown in Fig. 5.

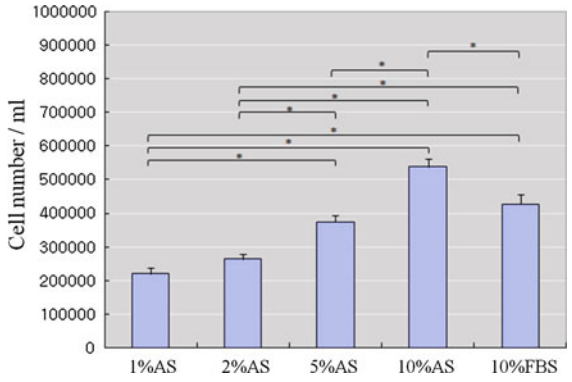

Fig. 2 Expansion potential of ASCs cultured in 1, 2, 5, and 10 % AS and 10 % FBS at passage 2. There were significant differences among groups by Kruskal–Wallis test ($p < 0.001$). There was also a significant difference between each of the 2 groups ($*p < 0.05$). No significant differences were observed between 1 % AS and 2 % AS, and 5 % AS and 10 % FBS. 10 % AS showed the most prominent expansion characteristics. *T-bars* represent SD from the mean. *AS* autologous serum, *FBS* fetal bovine serum

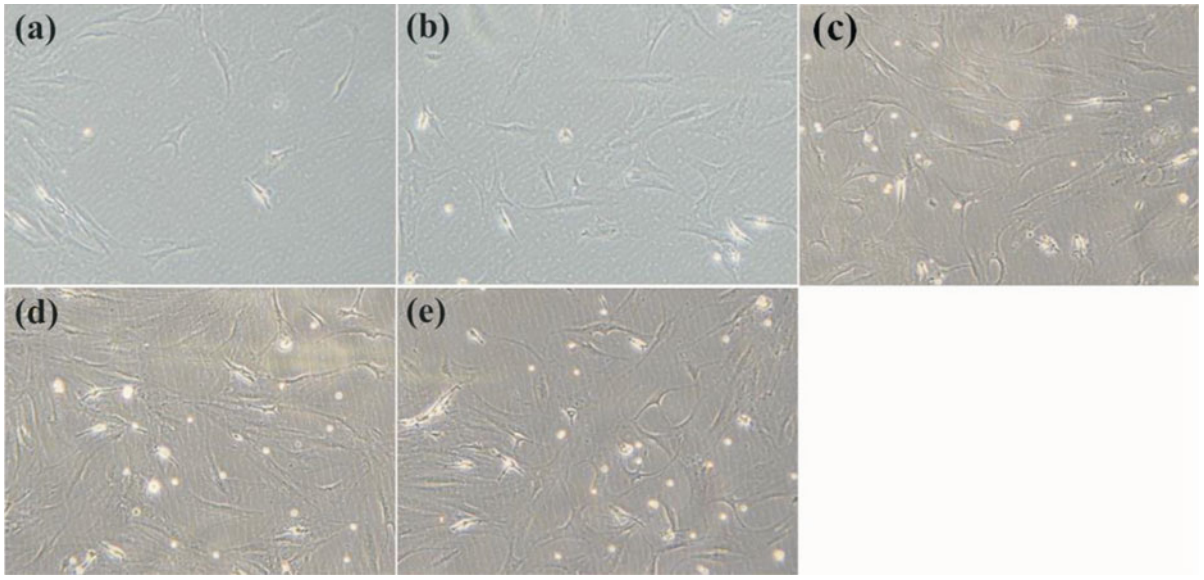

Fig. 1 Expansion of ASCs on day 5 of primary culture. **a** 1 % AS; **b** 2 % AS; **c** 5 % AS; **d** 10 % AS; **e** 10 % FBS. Ten percent AS had dominant proliferative potential under light microscopy (Magnification ×100). *AS* autologous serum, *FBS* fetal bovine serum

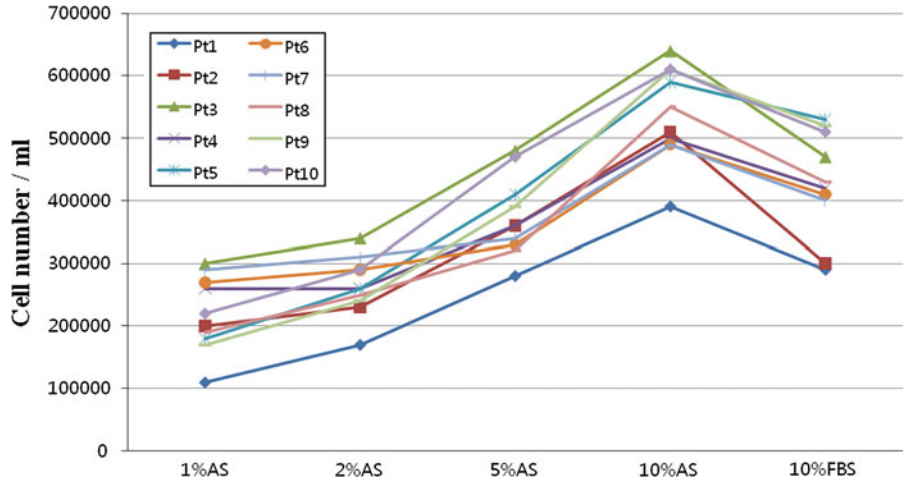

Fig. 3 Expansion potential according to each donor of ASCs cultured in 1, 2, 5, and 10 % AS and 10 % FBS at passage 2. *AS* autologous serum, *FBS* fetal bovine serum

Gross and histologic measurement after in vivo transplants

At 14 weeks, the transplanted gelatin sponge had disappeared and the light yellow soft mass-like adipose tissue was found (Fig. 6). Because there was little subcutaneous tissue in the backs of the nude mice, the graft was clearly distinguished from the surrounding subcutaneous tissue and easily separated. The biopsies with hematoxylin and eosin stain showed typical adipose cells (Fig. 7, left). Through the use of the anti-human antibodies, persistent transplanted human cells could be detected in sections at the time of sacrifice (Fig. 7, right). The volume of grafts at each group was an average of 0.005 ± 0.002236 mm^3 with 1 % AS, 0.0045 ± 0.0035 mm^3 with 2 % AS, 0.0085 ± 0.006344 mm^3 with 5 % AS, 0.012 ± 0.006782 mm^3 with 10 % AS, and 0.0115 ± 0.007089 with 10 % FBS (Fig. 8). Ten percent AS and 10 % FBS had greater differentiation capacity than 1 and 2 % AS in vivo, and no significant difference was observed between the groups at a concentration of ≥5 %. The connected single-donor data points were shown in Fig. 9.

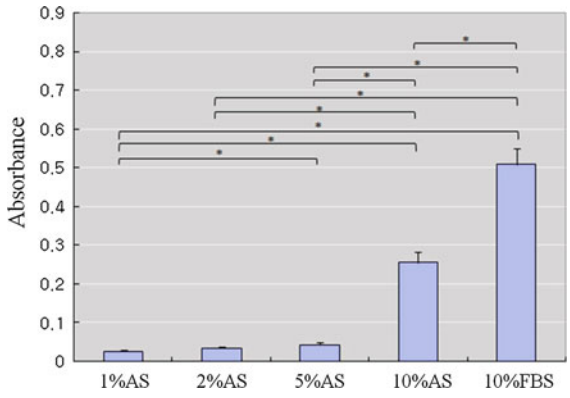

Fig. 4 Adipogenic differentiation of ASCs cultured in 1, 2, 5, and 10 % AS and 10 % FBS at passage 4. There were significant differences among groups by Kruskal–Wallis test ($p < 0.001$). There was also a significant difference between each of the 2 groups (*$p < 0.05$). No significant differences were observed between 1 % AS and 2 % AS, and 2 % AS and 5 % AS. The differentiation capacity depended on the serum concentration between AS groups. Ten percent FBS had a significantly higher differentiation capacity than 10 % AS when compared with 10 % FBS with 10 % AS ($p = 0.0004$). *AS* autologous serum, *FBS* fetal bovine serum

Discussion

Considerable efforts have been directed toward searching for possible FBS alternatives. Human blood supplements have been identified as promising substitutes, which have a similar effect on cell culture compared with FBS and simultaneously are free from ethical concerns and infection (Kocaoemer et al. 2007; Kobayashi et al. 2005; Shahdadfar et al. 2005; Stute et al. 2004). Human blood supplements include autologous and allogeneic serum, and platelet derivatives. The effectiveness of human serum was based mainly on studies using AS and most studies have suggested that AS is similar or better than FBS with respect to the isolation, expansion, and differentiation of stem cells. Shahdadfar et al. (2005) reported that BMSCs were expanded rapidly and with stable gene

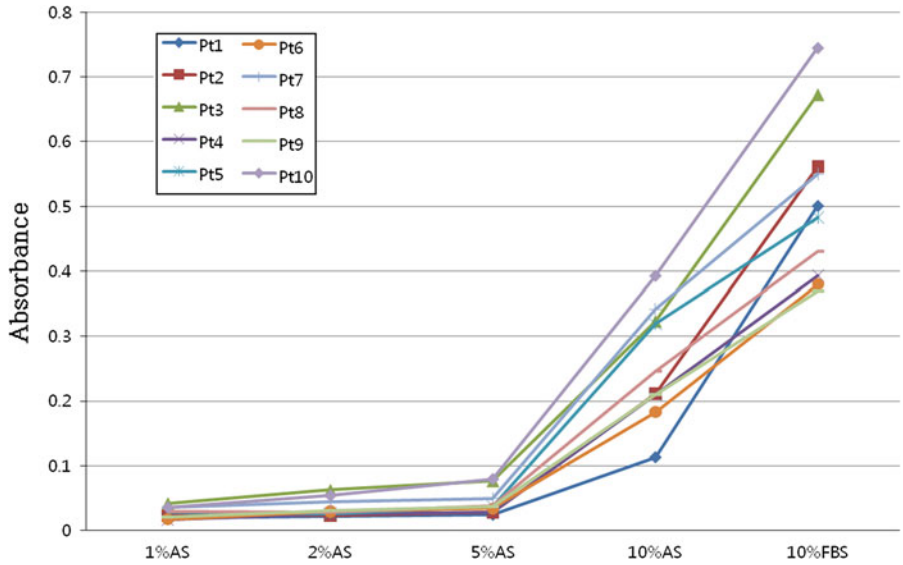

Fig. 5 Adipogenic differentiation according to each donor of ASCs cultured in 1, 2, 5, and 10 % AS and 10 % FBS at passage 4. *AS* autologous serum, *FBS* fetal bovine serum

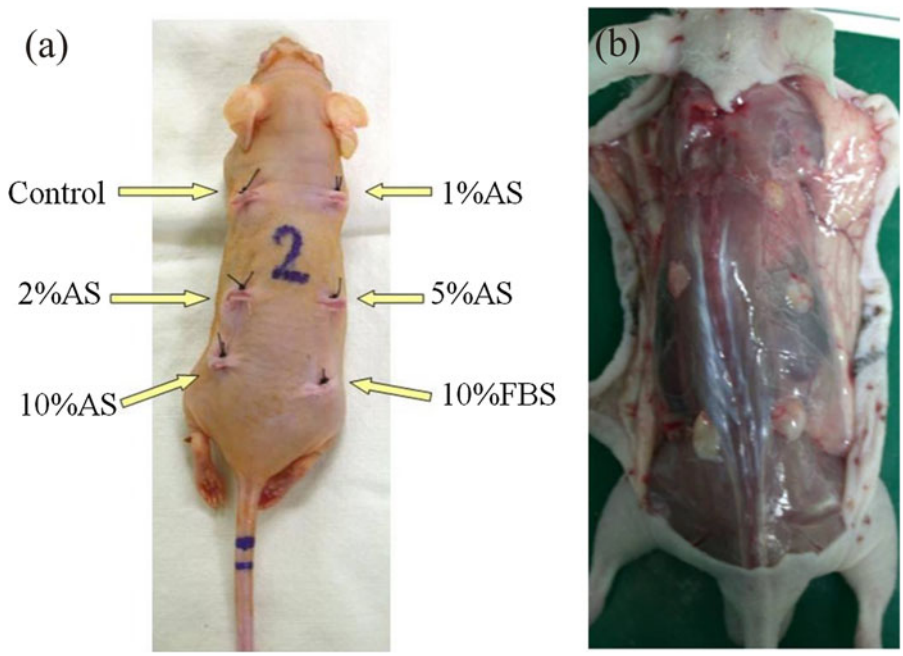

Fig. 6 In vivo transplantation of adipogenic differentiated stem cells. **a** Immediate transplantation. **b** After 14 weeks of the transplantation. *Light yellow colored* fat-like tissues were clearly distinguished from the surrounding subcutaneous tissue. *AS* autologous serum, *FBS* fetal bovine serum. (Color figure online)

expression in AS than FBS. Kobayashi et al. (2005) noted that AS might provide sufficient ex vivo expansion of human BMSCs possessing multi-differentiation potential than FBS. Stute et al. (2004) noted that 10 % AS appeared at least as good as 10 % FBS with respect to both isolation and expansion of human mesenchymal stem cells, while 1 % and 3 % AS was inferior. Because the amount of AS is limited, further research of allogeneic serum is being conducted. Thus far, allogeneic serum has been insufficient to substitute for FBS because of inconsistent results, which have been effective in expanding ASCs (Kocaoemer et al. 2007), but not in expanding BMSCs (Shahdadfar et al. 2005; Koller et al. 1998; Kuznetsov et al. 2000). Koller et al. (1998) described more favorable effects of animal sera (20 % FBS, 20 % horse serum, or a mixture of both) on colony-forming unit fibroblast (CFU-F) formation in comparison with 20 %

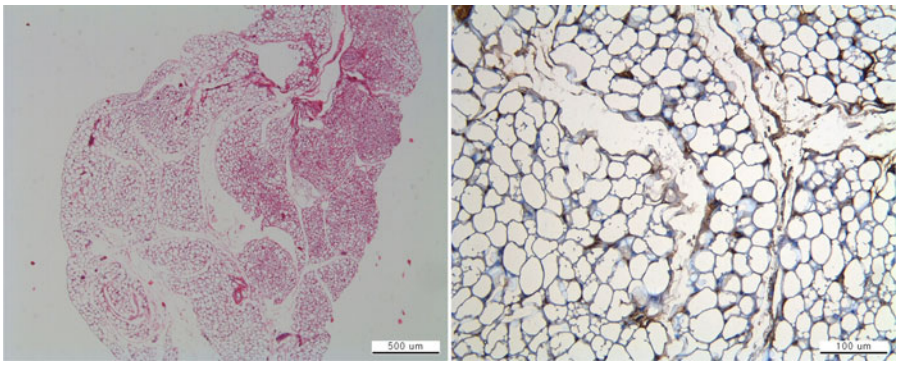

Fig. 7 Histologic results after 14 weeks of in vivo transplantation. (*left*) Hematoxylin and eosin staining shows droplets of fat and signet ring sign, which are distinctive findings of adipose tissue. One *bar* means 500 μm. (*right*) After treatment with anti-human nuclei antibodies, intense brown labelling of the cell nuclei was detected, indicating that the biopsy tissue is of human origin. One *bar* means 100 μm

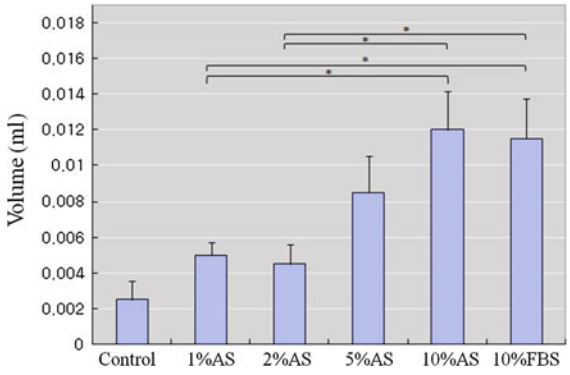

Fig. 8 Volume assessment after 14 weeks of in vivo transplantation. Ten percent AS and FBS had significantly larger volumes than 1 or 2 % AS, ($*p < 0.05$), while no significant difference was observed among other groups. *AS* autologous serum, *FBS* fetal bovine serum

allogeneic human plasma. Kuznetsov et al. (2000) tested the proliferation and colony-forming efficiency of human BMSCs in α-MEM until two passages in four donors and bone formation in vivo with 20 % FBS, which were better than culture in 20 % human AB-serum.

There have been several studies comparing the expansion and osteogenic differentiation of BMSCs with AS to FBS, or which concentration of AS was the best medium for expanding BMSCs (Pérez-Ilzarbe et al. 2009; Kobayashi et al. 2005; Shahdadfar et al. 2005; Stute et al. 2004; Koller et al. 1998). However, the effectiveness of AS as the culture medium for mesenchymal stem cells may depend on the origin of the mesenchymal stem cells, and there has been little research involving ASCs. Therefore, we compared the effects of four different concentrations of AS (1, 2, 5, and 10 %) on the expansion and adipogenic differentiation of ASCs using 10 % FBS as the control. Because in vitro expansion and differentiation which were controlled under an independent environment to the exclusion of several interferences and changes were different from in vivo, ASCs which were cultivated and differentiated in each media were grafted on nude mice, and the in vivo differentiation capacity was evaluated. Our study did not include concentrations >10 %. Because the amount of AS is limited, and Spees et al. (2004) reported that calf serum protein was detected in cultures of mesenchymal stem cells with 20 % FBS and caused strong immunologic reactions. We evaluated the cell expansion potential at passage 2 and differentiation capacity at passage 4. Because there was a significant difference between the groups on day 11 in the expansion of BMSCs using human serum, platelet-rich plasma, and FBS (Kocaoemer et al. 2007), and the difference in adipogenic differentiation potential at passage 4 was significant (Stute et al. 2004). In vitro 10 % AS appeared to be at least as good as 10 % FBS with respect to expansion of ASCs, while 1, 2, and 5 % AS were inferior. It can be safely assumed that higher concentrations of AS contain higher amounts of mitogenic growth factors. For adipogenic differentiation, 10 % FBS was more effective than AS regardless of the concentration. It appeared identical to the results reported by Stute et al. (2004) and Oreffo et al. (1997). This may be because 10 % FBS has a higher degree of adipogenic factors and lipid content (Stute et al. 2004). In in vivo transplants, no significant

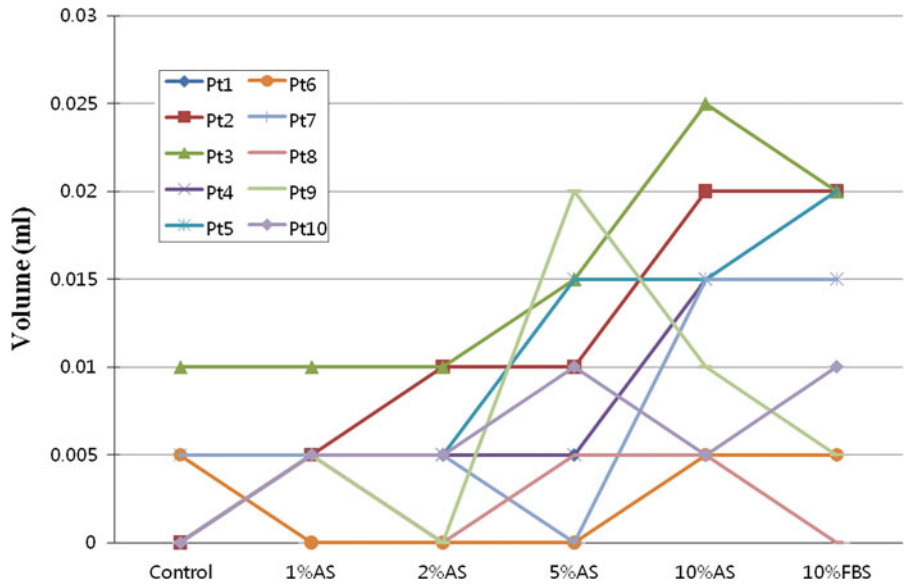

Fig. 9 Volume assessment according to each donor after 14 weeks of in vivo transplantation. *AS* autologous serum, *FBS* fetal bovine serum

difference in adipogenic differentiation capacity was observed between 10 % AS and 10 % FBS. We grafted ASCs into nude mice on day 6 of adipogenic differentiation. At the timing of grafting there were some differences in adipogenic differentiation according to each concentration or material. However, except for initial days on the process of in vitro differentiation before grafting on nude mice, the rest of the conditions were similar. Therefore, it cannot help being affected insufficiently from each concentration or material, which might have resulted in a difference of differentiation capacity between in vitro and in vivo. Just like our results, Nimura et al. (2008) noted that the in vitro chondrogenic potential of human synovial MSCs was higher with 20 % FBS than with 10 % AS, but the in vivo chondrogenic potential of rabbit synovial MSCs was similar between two groups following the grafting of undifferentiated rabbit synovial MSCs into rabbits.

With respect to the number of ASCs at the end of both isolation and expansion, 10 % AS appeared at least as effective as a 10 % FBS, whereas 1 and 2 % AS were inferior. Therefore, it can be safely assumed that 10 % AS contains a sufficient amount of the growth factors and substances that are necessary for the isolation and expansion of ASCs, and the cellular yield is high enough to generate ASCs on a clinical scale without the addition of growth factors. However, AS has limited availability. Kobayashi et al. (2005) noted that the addition of basic fibroblast growth factor (bFGF) to AS enhanced the proliferation rate of BMSCs significantly. It can be safely assumed that the amount of AS necessary for cell culture may be reduced by the addition of bFGF. However, Kobayashi et al. (2005) did not compare AS with bFGF to that without bFGF, and in the results from a study by Pérez-Ilzarbe et al. (2009), the addition of bFGF to 10 % AS do not cause a significant difference in the expansion of BMSCs when compared to 10 % FBS. Therefore, further studies will be necessary to confirm the effect of bFGF.

Up until just a few years ago, there had been a number of clinical trials exploring the use of mesenchymal stem cells for treatment of various diseases, most of which used FBS as the culture medium (Quarto et al. 2001; Vacanti et al. 2001; Wakitami et al., 2002; Le Blanc et al. 2004; Bang et al. 2005). However, most recently, clinical trials of mesenchymal stem cells using human blood supplements as the culture medium have been conducted. von Bonin et al. (2009) evaluated BMSCs expanded in human platelet lysates in patients with refractory graft versus host disease. Honmou et al. (2011) introduced the BMSCs, expanded in AS, into stroke patients. However, there has been few clinical trials involving ASCs expanded in human blood supplements. To elucidate the effectiveness of other human blood supplements as well as AS as a culture medium for ASCs, further clinical trials of ASCs need to be performed with a supplement free of xenogeneic proteins to ensure adherence to

GMP and the delivery of mesenchymal stem cells as a clinically safe cell product (Dimarakis and Levicar 2006).

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