Vet Res Commun (2010) 34 (Suppl 1):S25–S28
DOI 10.1007/s11259-010-9393-2

EXTENDED ABSTRACT

Isolation, characterization, and in vitro differentiation of ovine amniotic stem cells

A. Mauro · M. Turriani · A. Ioannoni · V. Russo · A. Martelli · O. Di Giacinto · D. Nardinocchi · P. Berardinelli

Published online: 1 May 2010

Abstract Stem cell (SC) regenerative therapy represents an emerging strategy for the treatment of human diseases. Since amniotic fluid-derived cells have been recently proposed as a promising source of human SCs, the present research aimed to amplify in vitro and characterize ovine amniotic fluid-derived SCs collected from the membranes (AMSCs) or fluid (AFSCs). These cells were found to proliferate, express the pluripotent SC markers OCT-4 and TERT, and differentiate in both osteogenic and smooth muscle lineages in vitro. However, AMSCs presented an earlier down-regulation of SC markers and a faster rate of differentiation. Thus, AMSCs and AFSCs may represent sources of characterized pluripotent SCs that can be easily collected and amplified in vitro. These ovine SCs may be used in preclinical studies on large animals to develop future human therapies.

Keywords Amniotic cells · Cell differentiation · Ovine · Stem cells

Abbreviations

AMSCs	Amniotic membrane stem cells
AFSCs	Amniotic fluid stem cells
FCS	Fetal calf serum
FGF	Fibroblast growth factor

Introduction

In recent years, several studies have demonstrated the ability to isolate amniotic membrane stem cells (AMSCs) and amniotic fluid stem cells (AFSCs) (In 't Anker et al. 2003; Tsai et

A. Mauro (✉) · V. Russo · A. Martelli · O. Di Giacinto · D. Nardinocchi · P. Berardinelli
Department of Comparative Biomedical Science, Normal Anatomy Section, University of Teramo,
Via Aldo Moro 45,
64100 Teramo, Italy
e-mail: amauro@unite.it

M. Turriani · A. Ioannoni
Department of Comparative Biomedical Science, Physiology Section, University of Teramo,
Via Aldo Moro 45,
64100 Teramo, Italy

al. 2004; Tsai et al. 2006). These cells have phenotypes attributable to pluripotent cells and can differentiate into different cell types due to the three different embryonic layers (De Coppi et al. 2007). This experimental evidence led to the classification of AMSCs and AFSCs as pluripotent stem cells, including them among stem cells for use in human regenerative therapy. Other biological characteristics of amniotic-derived cells have attracted the attention of researchers. In particular, their low immunogenicity, low tumorigenicity upon transplantation, widespread availability, and lack of ethical problems associated with their use, suggest they are ideal for wide use in regenerative medicine (Toda et al. 2007). However, despite their high potential, their use in the clinical field is still subject to finalization of important aspects such as: a) identification of protocols for in vitro amplification; b) molecular characterization by identification of specific markers that will allow monitoring of their in vitro performance during different processes starting from isolation to differentiation; c) definition of their in vitro cell plasticity; and d) in vivo analysis of their regenerative tissue capacity in preclinical models transferable to humans (Toda et al. 2007). On this basis, the purpose of this research was to develop a suitable and reproducible experimental protocol to isolate, culture, and amplify in vitro AMSCs and AFSCs derived from sheep fetuses. The sheep, in fact, represents a good animal model because its size and physiological conditions of development are very similar to those of humans. On the isolated and in vitro cultured cells, we also evaluated the expression of pluripotent stem cell-specific markers such as Oct-4 and TERT (In 't Anker et al. 2003; Toda et al. 2007). This characterization of their phenotype has allowed us to observe, during culture, the different behaviors of AMSCs and AFSCs, and to document their abilities to differentiate into osteogenic and smooth muscle lineages. The in vitro plasticity of AMSCs and AFSCs with respect to osteogenic and myogenic phenotypes was evaluated by hydroxyapatite deposition in the extracellular environment (staining of Alizarin Red and Von Kossa) and the expression of specific smooth muscle actin (α-SMA), respectively.

Materials and methods

Pregnancy induction Adult sheep were artificially inseminated by laparoscopy using validated protocols of oestrus synchronization (Lucidi et al. 2001).

AMSC and AFSC isolation and culture After 60–80 days of pregnancy confirmed by ultrasound, fetuses were isolated by caesarean section. AFSCs were obtained from amniotic fluid samples, while the amniotic membrane was separated under a stereo-microscope into two components: epithelial and connective tissue. AMSCs were obtained from epithelial tissue following 30 minutes of collagenease-hyaluronidase/trypsin enzymatic digestion (Sigma, St. Louis, MO). After centrifugation for 10 minutes at 500× *g*, isolated cells were resuspended in growth medium (GM) composed of DMEM plus 20% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomicin, 2 mM L-glutamine (Gibco, Grand Island, NY) and 5 ng/ml of fibroblast growth factor (FGF) (Sigma). The cells were cultured in Petri dishes and incubated at 38.5°C in 5% CO_2. After 7 days of culture, nonadherent cells were discarded and growth medium changed every 2 days up to confluence. Confluent cells were detached from the substrate by incubation in 0.25% Trypsin/EDTA for 10 minutes and then plated in the same growth conditions at a density of 2×10^4 cells/ml. The proliferative potential of the cultured cells was analyzed by bromodeoxyuridine incorporation (BrdU) using a BrdU Kit (Sigma).

Analysis of Oct-4 and TERT proteins Expression of Oct-4 and TERT, specific protein markers of stem cells, was examined during culture by immunofluorescence (IF) using specific primary antibodies including monoclonal anti-Oct-4 (Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal anti-hTERT (Calbiochem) detected with a specific Cy3-conjugated secondary antibody (Dako). The nuclei were stained with DAPI (Sigma).The qualitative and quantitative analyses were performed using a fluorescence microscope (AxioScope2) connected to semiautomatic software for morphometric image analysis, KS300 (Zeiss, Jena, Germany).

Induction of cell differentiation AMSCs and AFSCs at different passages were induced to differentiate by culture in osteogenic differentiation medium (DM-OST: 10% FCS, 150 μg/ml β-glycerophosphate, 50 μg/ml ascorbic acid, and 10^{-8}M dexamethasone) and smooth muscle medium (DM-SM: 2% FCS, 100 U/ml ITS) for a defined time period. Osteogenic differentiation was assessed by colorimetric visualization of calcium sediment (Alizarin Red S stain) and phosphate calcium (Von Kossa stain) in culture plates. Smooth muscle differentiation was assessed by analyzing the expression of α-SMA by immunohistochemistry using anti-α-SMA antibody (Oncogene) detected with a Cy3-conjugated secondary antibody (Sigma).

Results

Our results indicate that is possible to isolate a large number of cells ($1–2 \times 10^6$ cells total) from fetal sheep amniotic membrane and fluid. AMSCs and AFSCs showed a high proliferative potential in culture. Characterization by immunohistochemistry showed morphological and functional differences between the two amniotic-derived cell types. While AMSC cultures displayed homogeneous cells with a small size cuboid shape, central nuclei, and OCT-4 and TERT expression, instead AFSC cultures were characterized by a different cell morphology. In particular, AFSCs showed at least two different cell type populations depending on size and OCT-4 and TERT expression. The larger cells, in fact, showed no positive expression of the two stem cell markers, while nuclear OCT-4 and TERT expression was observed in the smaller cells. Although both amniotic cells populations were able to expand rapidly in vitro during different steps, the data obtained show different behaviors of AMSCs and AFSCs with respect to both the proliferation index and the phenotype maintenance in culture. In particular, the AMSCs presented a lower proliferative index than the AFSCs. Moreover, while almost all AMSCs at the initiation of culture showed nuclear TERT and OCT-4 expression, this aspect was progressively lost during culture and expression of these markers was downregulated significantly after the sixth passage. In contrast, although TERT and OCT-4 nuclear expression was limited to a smaller number of AFSCs, at the time of isolation this phenotype remained unchanged until the 12th passage. Finally, both the AMSCs and the AFSCs were able to differentiate into osteogenic and smooth muscle lineages when adequately stimulated in vitro as shown by the presence of calcium deposits and cytoplasmic smooth alpha actin (α-SMA) expression, respectively. However, while the process of cell differentiation was uniform in almost all AMSCs, in contrast in the AFSCs, the differentiation process was partial even though the cells maintained this capacity throughout the culture interval studied (12 passages).

Discussion

The data obtained in this study have demonstrated the ability to isolate a significant amount of stem cells (AMSCs and AFSCs) from the amniotic fluid and membranes of sheep

fetuses. Following isolation, these stem cells were able to proliferate and differentiate following osteogenic and smooth muscle lineages. It was also possible to identify two molecular markers to characterize the amniotic cells, and therefore, to monitor them from the isolation to in vitro differentiation. First, both ovine AMSCs and AFSCs demonstrated a high proliferative potential. In addition, AMSCs presented a higher cell homogeneity at the time of isolation combined with a higher incidence of the two pluripotency markers than observed in AFSCs. However, while AMSCs gradually lost OCT-4 and TERT nuclear expression after six passages, the AFSCs were able to maintain, outside of their natural environment, this phenotype until the 12th passage. This result shows for the first time the need to develop adequate AMSC culture conditions to optimize their in vitro amplification and to preserve their stemness over time. Both cell types were able to differentiate in vitro along osteogenic and smooth muscle lineages. Simultaneously, with the lost of the OCT-4 and TERT expression, osteogenic differentiation was demonstrated by crystals of calcium deposition in the culture plates, while smooth muscle differentiation was shown by the expression of smooth muscle actin in the cytoplasm of differentiated cells.

The greater but less prolonged in vitro differentiation capacity of AMSCs seems to confirm in culture their phenotypic instability as previously discussed for this cell type. Given that ovine amniotic stem cells have good plasticity in vitro, recently AMSCs and AFSCs have been used in preclinical studies to assess, after allograft, their ability to structurally and functionally integrate into host bone and cartilage tissue, and to further outline their possible application in regenerative medicine. In fact, the availability of large amounts of characterized pluripotent ovine stem cells offers the possibility to document with high experimental rigor the in vivo regenerative capacity of these cells as well as to study their short-term and long-term fate within the receiving tissue. These preclinical studies are needed to increase the safety of new regenerative therapeutic approaches. The relevance of the preclinical studies in medicine is also greater when the experimental species have a comparable size and physiology to humans (In ’t Anker et al. 2003; Toda et al. 2007). We must also consider the applicative advantage that can be offered, even in veterinary medicine, by the characterization of pluripotent stem cells. In particular, the availability of stem cells with reduced immunogenicity (Toda et al. 2007) open new possibilities in veterinary medicine to practice xeno-transplantation between animal species of considerable economic interest (e.g., horse) in which the degenerative articular ligaments are often the basis of a reduced quality of life and sports performance.

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