Stem Cell Research (2013) 11, 540–551

Available online at www.sciencedirect.com

SciVerse ScienceDirect

www.elsevier.com/locate/scr

Amniotic fluid stem cells in a bone microenvironment: Driving host angiogenic response

Teodelinda Mirabella [a,b,c,*], Chiara Gentili [a,b], Antonio Daga [b], Ranieri Cancedda [a,b,**]

[a] Department of Experimental Medicine (DIMES), University of Genova, 16132 Genova, Italy
[b] IRCCS AOU San Martino — IST National Institute for Cancer Research, 16132 Genova, Italy
[c] Cardiovascular Research Center, Yale Medical School, 06511, New Haven, CT, USA

Received 17 July 2012; received in revised form 10 February 2013; accepted 11 February 2013
Available online 24 February 2013

Abstract The repair of skeletal defects remains a substantial economic and biomedical burden. The extra-embryonic fetal stem cells derived from amniotic fluid (AFSCs) have been used for the treatment of large bone defects, but mechanisms of repair are not clear. Here we studied the potential contribution of human AFSCs to the modeling of an ectopic bone.

We found that AFSCs are not osteogenic in vivo, and, compared to bone marrow-derived stromal cells, recruit more host CD31 and VEGF-R_2 positive cells. Finally, when AFSCs were co-implanted with human-bone forming cells, a normo-osteosynthesis occurred, the engineered ossicle was hyper-vascularized, but AFSCs were not retrieved in the implant within 2 weeks. We concluded that AFSCs do not contribute to the deposition of new bone, but act as a powerful proinflammatory/proangiogenic boost, driving a host response, ending in AFSC clearance and vascularization of the bone environment.

In our model, a source of osteocommitted cells, capable to engraft and proliferate in vivo, is needed in order to engineer bone. The angio-attractant properties of AFSCs could be exploited in strategies of endogenous cell homing to actively recruit host progenitors into a predefined anatomic location for in situ bone tissue regeneration.

Introduction

Although bone tissue engineering offers a valid alternative to current bone autograft and allograft surgeries, the repair of skeletal defects still represents a substantial economic and biomedical burden (Conrad and Huss, 2005). Tissue engineering strategies for the replacement of load-bearing tissues require a combination of a scaffold, a source of bone forming cells, and growth factors (Langer and Vacanti, 1993). Native bone forming cells, osteoblasts, are typically difficult to

Abbreviations: AFSC, amniotic fluid derived stem cells; OB, osteoblasts; BMSC, bone marrow stromal cells.

** Correspondence to: T. Mirabella, Cardiovascular Research Center, Yale University School of Medicine, 300 George St, 06511 New Haven, CT, USA.

* Correspondence to: R. Cancedda, IRCCS AOU San Martino-IST National Institute for Cancer Research, Largo Rosanna Benzi 10, 16132 Genova, Italy. Fax: +39 010 5737257.

E-mail addresses: teodelinda.mirabella@yale.edu (T. Mirabella), ranieri.cancedda@unige.it (R. Cancedda).

1873-5061/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.scr.2013.02.006

isolate and to expand in vitro (Declercq et al., 2004). A stem cell population with an osteogenic potential would be preferred. Bone marrow-derived stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells), which have been long used for bone tissue engineering applications, require specialized harvesting and purification procedures and their presence is also reduced in aged bone marrow (Caplan, 2004).

Amniotic fluid was described by De Coppi et al. (2007) as a new source of stem cells, which can be directed into the three primary embryonic lineages of mesoderm, ectoderm and definitive endoderm. Amniotic fluid-derived stem cells (AFSCs) are easily accessible and readily available from amniocentesis samples that would be otherwise discarded. AFSCs may approach the plasticity degree of embryonic stem cells, but their use in regenerative medicine does not present disadvantages such as ethic controversies, the risk of teratoma formation and the need of a feeder layer to grow.

Guldberg's group (Peister et al., 2009) demonstrated the potential of AFSCs to produce 3D mineralized bioengineered constructs in vitro and in vivo, suggesting that AFSCs may be an effective cell source for functional repair of large bone defects. In particular, it was shown that: *i)* in vitro pre-differentiated AFSCs produced more mineralized matrix when implanted subcutaneously (Peister et al., 2009); *ii)* in 2D and 3D cultures AFSCs have a delayed but more robust mineralization rate compared to the BMSCs (Peister et al., 2011); and *iii)* AFSC and BMSC-seeded polymer scaffolds, orthotopically implanted, repair rat critically sized femoral defects with no significant differences between the two sources and with significantly higher bone in-growth compared to acellular scaffolds (Dupont et al., 2010).

Finally, Rodrigues et al. further pointed out the concept that the culture environment can pre-commit AFSCs toward an osteogenic phenotype, and that AFSCs differentiation stage has a significant impact on the outcome of the in vivo healing of large bone defects (Rodrigues et al., 2012).

All together these observations suggest that: *i)* AFSCs, although expressing mesenchymal markers, are quite uncommitted compared to adult and more osteogenic-directed BMSCs; and *ii)* in spite of their not being osteogenically committed, AFSC can drive an in vivo bone tissue repair comparable to bone marrow MSC.

Our group has previously demonstrated that undifferentiated AFSCs, when implanted in vivo, are not retrieved in the site of implantation, but they play a pivotal role in mounting a reparative response through the recruitment of host progenitor cells (Mirabella et al., 2011a). In particular, the AFSC secretome, which is rich in chemokines and growth factors, is responsible for a cell paracrine effect on vessel growth and possibly local niche activation (Mirabella et al., 2011b, 2012).

The induction of endogenous cell homing; i.e., the recruitment of host stem cells into a predefined anatomic location for in situ tissue regeneration, represents an alternative to the traditional tissue engineering approach. Biomaterials, cells, drugs, and biological molecules can act as "navigation cues and signals" stimulating endogenous cell homing (Chen et al., 2011).

Here, using bone forming cells and a ceramic scaffold, we engineered a microenvironment, which gives rise to bone tissue after 1–2 months in vivo. We studied how the presence of human amniotic fluid-derived stem cells contribute to the in vivo modeling of the formed ectopic bone and we found that the recruitment of vessels and inflammatory cells can: *i)* drive a host's response which determines AFSC fate, and *ii)* induces the vascularization of the engineered bone, without affecting the normal osteogenesis.

Materials and methods

Cell isolation and culture

Human AFSCs were supplied by the Cytogenetic Laboratory of Galliera Hospital (Genoa, Italy) as normal karyotyped amniocentesis samples from twenty 15–17 weeks pregnant women, after obtaining written informed consent. AFSCs were isolated as previously described (De Coppi et al., 2007; Mirabella et al., 2011b; 2012). Briefly, cells were harvested by trypsinization from confluent back-up human amniocentesis cultures, cKit immunoselected on a Mini-MACS apparatus (Milteyi Biotec, Bergisch Gladbach, Germany) and expanded in αMEM medium (Gibco, Milan, Italy) containing 15% ES cell FBS (Gibco), 2 mM L-glutamine and 100 U penicillin, 100 μg streptomycin (Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA, USA), at 37 °C within a 5% CO_2 atmosphere. Cultured cells between passage 6 and 10 were tested for the expression of lineage specific surface molecules in a Cyan ADP Cytofluorimeter (Beckman Coulter) and the absence of hematopoietic markers together with the presence of mesenchymal markers was confirmed (Mirabella et al., 2012; Arnhold et al., 2011). Luciferase-transduced AFSCs were checked for the same markers and for proliferation rate and used within passage 10.

Samples of human bone were obtained from six informed consent-giving patients undergoing hip joint replacement surgery at the Orthopaedic Surgery Unit of San Martino Hospital (Genoa, Italy). We report as osteoblasts (OBs) the heterogeneous population of bone forming cells isolated from the trabecular bone with a specific protocol (Meikle et al., 1992) which collects only the collagenase digested fractions. Briefly, the bone was cut into small pieces (2 mm × 2 mm), washed in Ringer solution (10 min, 10 times), and then sequentially digested in 1 mg/ml Trypsin (Gibco, Milan, Italy), 10 min; 2 mg/ml Dispase (Sigma, Milan, Italy), 20 min; and 800 U/ml Collagenase type 1 (Biochrom, AG Berlin, Germany), 1 h, 2 times in Ringer solution at 37 °C. Only cells released by the collagenase digestions were collected, washed, plated at a density of 10.000 cells/cm^2, and grown to 95% confluence in Iscove's Modified Dulbecco's Medium (Euroclone, Milan, Italy) supplemented with 10% FBS (Lonza, Verviers, Belgium), at 37 °C in an humidified atmosphere of 5% CO_2; OB were tested for alkaline phosphatase activity (ALP Detection Kit from Sigma, St. Louis, MO, USA) and exclusively used at passage 1 or 2. Each time these OB are ectopically implanted in vivo, they form bone (100% of success rate) without supporting hematopoiesis. For experiments requiring cell cycle inactivation, osteoblasts were pretreated