Journal of Bone Oncology 5 (2016) 5–14

Contents lists available at ScienceDirect

Journal of Bone Oncology

journal homepage: www.elsevier.com/locate/jbo

Research Paper

Mesenchymal stem cells increase proliferation but do not change quiescent state of osteosarcoma cells: Potential implications according to the tumor resection status

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ARTICLE INFO

Article history:
Received 7 October 2015
Received in revised form
13 November 2015
Accepted 30 November 2015
Available online 12 December 2015

Keywords:
Mesenchymal stem cell
Osteosarcoma
Adipose tissue transfer
Cell cycle
Quiescence

ABSTRACT

Conventional therapy of primary bone tumors includes surgical excision with wide resection, which leads to physical and aesthetic defects. For reconstruction of bone and joints, allografts can be supplemented with mesenchymal stem cells (MSCs). Similarly, adipose tissue transfer (ATT) is supplemented with adipose-derived stem cells (ADSCs) to improve the efficient grafting in the correction of soft tissue defects. MSC-like cells may also be used in tumor-targeted cell therapy. However, MSC may have adverse effects on sarcoma development. In the present study, human ADSCs, MSCs and pre-osteoclasts were co-injected with human MNNG-HOS osteosarcoma cells in immunodeficient mice. ADSCs and MSCs, but not the osteoclast precursors, accelerated the local proliferation of MNNG-HOS osteosarcoma cells. However, the osteolysis and the metastasis process were not exacerbated by ADSCs, MSCs, or pre-osteoclasts. *In vitro* proliferation of MNNG-HOS and Saos-2 osteosarcoma cells was increased up to 2-fold in the presence of ADSC-conditioned medium. In contrast, ADSC-conditioned medium did not change the dormant, quiescent state of osteosarcoma cells cultured in oncospheres. Due to the enhancing effect of ADSCs/MSCs on *in vivo*/*in vitro* proliferation of osteosarcoma cells, MSCs may not be good candidates for osteosarcoma-targeted cell therapy. Although conditioned medium of ADSCs accelerated the cell cycle of proliferating osteosarcoma cells, it did not change the quiescent state of dormant osteosarcoma cells, indicating that ADSC-secreted factors may not be involved in the risk of local recurrence.

1. Introduction

High-grade osteosarcoma is an aggressive primary malignant bone tumor that is associated with a relatively good outcome; since pre-operative and adjuvant combination chemotherapy has been introduced the survival rate at 5 years for the non-metastatic form at diagnosis has been 50–70% [1,2]. Osteosarcoma treatment comprises surgical excision with wide resection of the tumor after neo adjuvant chemotherapy. Adjuvant chemotherapy is then adapted to histological response [3]. Concerning surgical techniques, limb sparing is currently the preferred option. Reconstruction is dependent on resection site: if epiphysis cannot be preserved, mega prosthesis sometimes associated with an allograft is typically used [4]. Otherwise, different conservative techniques are described: massive autograft [5], vascularized autograft sometimes associated with allograft or isolated allograft [6–8].

A permanent remission from osteosarcoma can be anticipated after 10 years of event free survival [9–13], where after the primary challenge is to ameliorate the quality of life of patients suffering from physical and aesthetic defects caused by tumor resection. For recovery of damaged soft tissues, plastic and reconstructive surgery includes autologous grafts of adipose tissue. Regenerative medicine promises new alternatives through the use

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http://dx.doi.org/10.1016/j.jbo.2015.11.002

of mesenchymal stem cells (MSCs), which are bone marrow-resident and multipotent cells. They have been originally identified as a source of bone progenitor cells, but they also differentiate into adipocytes, chondrocytes and myoblasts. Human MSCs may be combined with scaffolds to increase bone healing as reported previously [10,12]. Moreover, the use of MSCs cultured from bone marrow to supplement an osteoarticular allograft in patients treated after bone tumor resection did not increase the risk of local tumor recurrence compared to control populations. Additionally, MSCs can be modified to express tumor-targeted agents [14,15] and used as "mesenkillers" [16].

Adipose tissue represents an alternative source of MSC-like cells, avoiding the problems of pain, morbidity and low cell number associated with bone marrow harvest [17]. In plastic and reconstructive surgery, adipose tissue-derived stromal cells (ADSCs) may be used to increase *in situ* survival of the autologous adipose-tissue graft [18–23]. ADSC have also been utilized as cellular delivery vehicles in bone reconstruction [24].

The use of adjuvant MSC-like cells in the treatment of osteosarcoma may be an important therapeutic issue for patients with lung metastasis associated with poor outcome (30% survival rate at 5 years) [25]. However, the impact of unmodified MSCs on tumor progression remains unpredictable [26]. For instance, it has been observed that rat and human MSCs can promote tumor growth and metastasis in osteosarcoma models [27–30].

Facing a unique clinical case of osteosarcoma recurrence following autologous adipose-tissue transfer [30], we started to investigate the interactions between osteosarcoma and adipose tissue by using pre-clinical experiments [30,64]. In the present report, we compared the interactions of MNNG-HOS cells-induced osteosarcoma with human ADSCs/MSCs and with human pre-osteoclasts. It is established that osteoclasts are involved in osteosarcoma progression and are believed to either enhance or suppress metastases [31–33]. In this study, pre-osteoclasts did not increase the tumor size and the lung metastasis. In contrast, ADSCs and MSCs increased the size of MNNG-HOS-induced tumors, but the metastasis process and rate of osteolysis were not exacerbated. Paracrine effects of ADSCs were investigated on osteosarcoma cells after culture in monolayer or oncospheres in order to observe the effects on proliferative or quiescent cell stages. The addition of 50% ADSC-conditioned medium significantly increased the *in vitro* proliferation of two osteosarcoma cell lines (MNNG-HOS and Saos-2), whereas it did not decrease the proportion of cells in G_0 phase. These results suggest that ADSCs/MSCs may be safe in reconstructive surgery after bone tumor resection and not involved in the risk of local recurrence. However, ADSCs/MSCs do not appear to be good candidates for tumor-targeted cell therapy in osteosarcoma, given their enhancing effects on tumor progression.

2. Materials and methods

2.1. Ethics statement

Adipose tissue samples were obtained from patients who underwent abdominal liposuction in the plastic surgery department of Nantes University Hospital (France). Bone marrow aspirates were obtained from patients during orthopaedic surgical procedures in Tours University Hospital (France). Blood samples were obtained from the "Etablissement Français du Sang" in Nantes. Oral consent was obtained from informed patients in accordance with French law (Art. L. 1245-2 of the French public health code, Law no. 2004-800 of 6 August 2004, Official Journal of 7 August 2004). The donors had no significant medical history.

Experiments involving animals were conducted in accordance with French guidelines (named "Charte nationale portant sur l'éthique de l'expérimentation animale" by the French ethics committee) and were approved by the regional committee on animal ethics named CEEA.PdL.06, with project authorization number 2013.4.

2.2. Cell lines and culture conditions

2.2.1. Osteosarcoma cell lines

MNNG-HOS and Saos-2 cells were purchased from the American Type Culture Collection (ATCC numbers CRL-1547 and HTB-85 respectively, Manassas, VA, USA). The cells were cultured in Minimum Essential Medium alpha with nucleosides and 1 g/L D-Glucose (Gibco® MEM α; Life technologies, Saint Aubin, France) and supplemented with 10% fetal bovine serum (FBS, GE Healthcare, Vélizy-Villacoublay, France), at 37 °C in a humidified atmosphere (5% CO_2/95% air). For culture under anchorage-independent conditions, medium was supplemented with 1.05% of methylcellulose (R&D Systems, Lille, France) and 2.5% FBS. MNNG-HOS cells were named LucF-HOS cells when they were modified to express the Enhanced Fluorescent Green Protein (EGFP) and firefly luciferase (LucF) genes as previously described [34].

2.2.2. Adipose- or bone marrow-derived stem cells

ADSCs were obtained from human fat samples which were removed using the Coleman's procedure [30,35–37] and MSCs were obtained from human bone marrow aspirates [38]. From human fat or bone marrow samples, adherent cells were obtained and at passage 3, they were characterized. As previously described, [30,39] flow cytometry analysis was performed to detect surface markers (CD105, CD90, CD75, CD45, CD34 and CD3) and their differentiation capacity towards osteogenic, adipogenic, chondrogenic or leiomyocyte lineages was assessed. ADSCs were transduced using EGFP-expressing lentiviral particles [34].

2.2.3. CD14 cells

They were obtained from human peripheral blood mononuclear cells and selected with CD14 Microbeads by MACS technology (Miltenyi Biotec, Paris, France) [40]. To induce pre-osteoclast differentiation, $CD14^+$ monocytes were cultured in alpha-MEM containing 10% FBS, 25 ng/mL^{-1} of human macrophage colony stimulating factor (MCSF; from R&D Systems) and 100 ng/mL^{-1} of human RANKL (kindly provided by Amgen Inc., Thousand Oaks, USA) for 7 days.

2.2.4. Conditioned media

ADSCs and MSCs were cultured to near confluence with MEM α medium supplemented with 10% FBS, washed twice and cultured overnight in serum-free medium which was then collected and frozen (−20 °C), constituting ADSC- or MSC-conditioned medium (CM).

2.3. Osteosarcoma model

2.3.1. Tumor induction

Four-week-old female athymic mice (NMRI nu/nu; Elevages Janvier, Le Genest St Isle, France) were housed under pathogen-free conditions at the Experimental Therapy Unit (Faculty of Medicine, Nantes, France). The mice were anaesthetized by inhalation of an isoflurane-air mix (2% for induction and 0.5% for maintenance, 1 L/min) before any surgical manipulation and by intraperitoneal injection of a ketamine-xylazine mix (16 mg/kg and 66 mg/kg respectively) for bioluminescence measurements. Osteosarcoma development was induced into the tibial anterior muscle, by injection of 2×10^6 MNNG-HOS cells, alone or with ADSCs, MSCs or pre-osteoclasts at indicated ratios. Tumor volume was calculated with the formula (l^2xL)/2 where l and L represent