Stem Cell Research 18 (2017) 5–13

Contents lists available at ScienceDirect

Stem Cell Research

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

# The administration of multipotent stromal cells at precancerous stage precludes tumor growth and epithelial dedifferentiation of oral squamous cell carcinoma

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ARTICLE INFO

*Article history:*
Received 6 July 2016
Received in revised form 1 November 2016
Accepted 22 November 2016
Available online 1 December 2016

*Keywords:*
Multipotent stromal cells
Mesenchymal stem cells
Precancerous lesion
Oral squamous cell carcinoma
Papilloma

ABSTRACT

Multipotent stromal cells (MSCs) are envisioned as a powerful therapeutic tool. As they home into tumors, secrete trophic and vasculogenic factors, and suppress immune response their role in carcinogenesis is a matter of controversy. Worldwide oral squamous cell carcinoma (OSCC) is the fifth most common epithelial cancer.
Our aim was to determine whether MSC administration at precancerous stage modifies the natural progression of OSCC.
OSCC was induced in Syrian hamsters by topical application of DMBA in the buccal pouch. At papilloma stage, the vehicle or $3 \times 10^6$ allogenic bone marrow-derived MSCs were locally administered. Four weeks later, the lesions were studied according to: volume, stratification (histology), proliferation (Ki-67), apoptosis (Caspase 3 cleaved), vasculature (ASMA), inflammation (Leukocyte infiltrate), differentiation (CK1 and CK4) and gene expression profile (mRNA).
Tumors found in individuals that received MSCs were smaller than those presented in the vehicle group ($87 \pm 80$ *versus* $54 \pm 62$ mm$^3$, $p < 0.05$). The rate of proliferation was two times lower and the apoptosis was 2.5 times higher in lesions treated with MSCs than in untreated ones. While the laters presented dedifferentiated cells, the former maintained differentiated cells (cytokeratin and gene expression profile similar to normal tissue).
Thus, MSC administration at papilloma stage precludes tumor growth and epithelial dedifferentiation of OSCC.


## 1. Introduction

Multipotent stromal cells, also referred to as mesenchymal stem cells (MSCs), are an heterogeneous subset of stromal cells present in several tissues including bone marrow, bone, adipose tissue, skin, kidney, umbilical cord and placenta (Friedenstein et al., 1968). The minimal criteria for defining MSCs are adherence to plastic surface; proliferation under the stimulus of fetal bovine serum; no expression of hematopoietic markers; expression of CD73, CD90, CD105, and differentiation into mesodermal cells (adipocytes, chondrocytes and osteocytes) (Dominici et al., 2006).

MSCs are envisioned as an ideal tool for cell therapy since they home into injured tissues whereas they could differentiate into tissue-specific cells (Ezquer et al., 2011), manage oxidative stress (Valle-Prieto and Conget, 2010), release trophic factors (Caplan and Dennis, 2006), promote neovascularization (Ball et al., 2007) or trigger an anti-inflammatory response (Uccelli and Prockop, 2010). It has been shown that donor MSCs also home into stablished tumors where they interact with cancer stem cells, regulate neovascularization and modulate the immune response (Al Moustafa et al., 2002; Bolontrade et al., 2012).

Currently, the role of MSCs in carcinogenesis is a matter of controversy. It has been reported that they favor tumor growth due to the immunosuppression (Djouad et al., 2003). Also, MSCs could enhance tumor metastatic potential since they can induce epithelia to mesenchyme transition (Huang et al., 2013). In contrast, it has been shown that MSCs inhibit tumorigenesis (Balasenthil et al., 2002a). The mechanisms apparently related to the antitumor effect could be: i) induction of cancer cell apoptosis (Ho et al., 2013), ii) avoidance of epithelium dedifferentiation (Ho et al., 2013), iii) inhibition of vascular network formation or apoptosis of vascular endothelial cells (Otsu et al., 2009; Secchiero et al., 2010), and iv) stimulation of anti-tumor immune response (Madrigal et al., 2014).

Worldwide oral squamous cell carcinoma (OSCC) is the fifth most common epithelial cancer, its annual incidence is over 300,000 diagnosed cases and its annual mortality is about 145,000 deaths (Rivera, 2015). Despite advances in its detection and treatment the mortality of OSCC remains high and its five-year survival rate is among the lowest of the major cancers (Rivera, 2015).

OSCC goes from normal keratinocyte transformation to random mutations linked to epigenetic processes that deregulate DNA repair

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http://dx.doi.org/10.1016/j.scr.2016.11.016

mechanisms, cell cycle, cell differentiation and apoptosis (Rivera, 2015). Thus, normal tissue evolves progressively through hyperplasia, dysplasia and carcinoma *in situ* until reaching the stage of invasive carcinoma (Rivera, 2015; Dvorak et al., 2011; Nishimura et al., 2012; Choi and Chen, 2005; Mendez et al., 2002; Arora et al., 2005).

The disease developed in the Syrian golden hamster using the mutagen 7.12-dimethylbenz-alpha-anthracene (DMBA) is a widely accepted animal model of OSCC (Chen et al., 2002; Nagini et al., 2009). It shares morphological, histological and molecular markers with human OSCC progression (Hasina et al., 2009; Ezquer et al., 2015; Aromando et al., 2014; Brandwein-Gensler et al., 2005).

The aim of this work was to determine whether MSC administration at precancerous stage modifies the natural progression of OSCC. For this, OSCC was induced in hamsters by topical application of DMBA in the buccal pouch. At papilloma stage, the vehicle or $3 \times 10^6$ allogenic bone marrow-derived MSCs were locally administered. Four weeks later, the lesions were studied according to their: volume (macroscopy), histology (H&E staining), rate of proliferation (immunohistofluorescence for Ki-67), rate of apoptosis (TUNEL), density of vasculature (immunohistofluorescence for ASMA), degree of inflammation (H&E and Toluidine blue staining), degree of differentiation (CK1 and CK4 immunohistofluorescence) and gene expression profile (RT-qPCR) (Supplementary Fig. 1).

## 2. Materials and methods

### 2.1. Animals

A total of 151 Syrian golden hamsters (*Mesocricetus auratus*) were used in this study. Nineteen were unmanipulated (5 used in OSCC natural progression study and 14 used in gene expression study), 56 served as MSC donors and 76 were OSCC induced (5 hyperplasia, 5 dysplasia, 5 papilloma and 5 carcinoma used in OSCC natural progression study; 14 OSCC + vehicle and 14 OSCC + MSC used for macroscopical and microscopical analysis, 14 OSCC + vehicle and 14 OSCC + MSC used for gene expression analysis).

Protocol was approved by the Ethic Committee of Facultad de Medicina Clinica Alemana-Universidad del Desarrollo (approval ID: 2011–14).

### 2.2. OSCC induction

Healthy male hamsters, eight weeks old, were painted thrice a week into their right buccal pouche with a N°4 camel-hair brush soaked with 50uL of mineral oil (Sigma-Aldrich, St. Louis, MO) or 50uL of 0.5% DMBA (Sigma-Aldrich) dissolved in mineral oil (Schwartz et al., 1988). Animals were housed at 22 °C, with a 12:12 h light-dark cycle, and water and food *ad libitum*.

### 2.3. Macroscopical analysis of OSCC progression

Nine and 13 weeks after the initiation of OSCC induction, hamsters were anesthetized by intraperitoneal injection of 20 mg/kg Xylazine (Centrovet, Santiago, Chile) and 20 mg/kg Ketamine (Ilium, Buenos Aires, Argentina). Buccal pouches were uncovered, and tumors were measured with a digital caliper (Mitutoyo Sul Americana LTDA, Brazil) and photographed using a digital camera (FUJIFILM-Finepi HS20 EXR). Two independent observers analyzed the photographs and described the lesions according to the presence of eritroplakia, leukoplakia, vascularization and exophytic or ulcerated rolled border nodules. The lesion volume was calculated using the formula: tumor volume ($mm^3$) = $0.52 \times [\text{width (mm)}]^2 \times \text{length (mm)}$ and the tumor increase was estimated as the ratio between final and initial volumes (Suzuki et al., 2011).

### 2.4. Microscopical analysis of OSCC progression

Four weeks after vehicle or MSC administration, hamsters were euthanized by an intraperitoneal injection of an overdose of Xylazine and Ketamine. Buccal pouches were procured and tumors were resected. Tumor specimens were fixed in 10% buffered formalin (Merck, USA), embedded in paraffin (Merck) and sectioned. Tissue sections of 4 μm were deparaffinized with Neoclear (Merck), rehydrated with graded alcohols, stained with H&E (Merck) and visualized with a light microscope (DM2000, Leica, Germany). Images were captured with a digital camera (DFC295, Leica). Tumor stage (hyperplasia, dysplasia, papilloma or carcinoma) was stated as previously described (Schwartz et al., 1988).

Histological analysis was performed in blind by three independent observers; one of them is a pathologist expert in oral diseases.

### 2.5. MSC isolation, ex vivo expansion and characterization

Healthy female hamsters, eight weeks old, were euthanized by an intraperitoneal injection of an overdose of Xylazine and Ketamine. Femurs and tibias were procured under sterile conditions. The epifisis were removed and bone marrow was collect by flushing bones with sterile phosphate buffer saline (PBS) (Gibco, Auckland, NZ). Recovered cells were resuspended in alpha-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 40 mg/mL gentamycin (Sanderson Laboratory, Chile), and plated at a density of $0.25 \times 10^6$ nucleated cells/$cm^2$. Every three days, culture medium was changed. When foci reached confluence, adherent cells were detached with 0.25% trypsin, 2.65 mM EDTA (Gibco). After one subculture, cells were characterized and transplanted.

Immunophenotyping was performed by flow cytometry after immunostaining with APC-conjugated mouse anti-CD45 (BD Pharmingen, San Diego, USA), FITC-conjugated mouse anti-ASMA (Sigma), FITC-conjugated mouse anti-vimentin (Oncogen, USA) (Bartholomew et al., 2002). To assess the differentiation potential, cells were incubated with adipogenic or osteogenic differentiation media (Contador et al., 2015; Conget and Minguell, 1999). Seven and 14 days later, they were stained with Oil Red O or Alizarin Red, respectively.

### 2.6. MSC administration

When lesions reached the stage of papilloma, animals were randomly distributed to experimental groups, anesthetized and their buccal pouchs were exposed using a surgical forceps. Using a 23-gauge needle (Terumo, Tokio, Japón), 400 μL of 5% hamster plasma (vehicle) or $3 \times 10^6$ MSCs resuspended in 5% hamster plasma were injected into the mucosa around the lesions (Cavalieri and Rogan, 1992).

### 2.7. Proliferation assessment

Tissue sections of 5 μm were deparaffinized and rehydrated. After blocking with 5% FBS, samples were incubated overnight at 4 °C with a dilution 1:50 of rabbit anti-K-i67 polyclonal serum (Abcam, USA). Then, samples were washed and incubated 2 h at room temperature with a dilution 1:400 of Alexa488-conjugated goat anti-rabbit IgG (Cell Signaling, Massachusetts, USA). Cross-reactivity of the secondary antibody was tested incubating tissue sections without the primary antibody. Nuclei were counterstained with a dilution 1:1500 of DAPI (Invitrogen, California, USA). Samples were observed in a fluorescence microscope. Images were captured with a digital camera and analyzed using Image J software (NIH Image J). The rate of proliferation was calculated as the quotient between Ki-67-positive cells and the total number of cells, counted in five representative optical sections (at least 1000 nuclei) using 40× magnification (Hasina et al., 2009).