Toxicology Letters 218 (2013) 207–214

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

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

Effects of salinomycin on human bone marrow-derived mesenchymal stem cells *in vitro*

A. Scherzed[a,1], S. Hackenberg[a,1], K. Froelich[a,1], K. Rak[a,1], A. Technau[a,1], A. Radeloff[a,1], U. Nöth[b,2], C. Koehler[a,1], R. Hagen[a,3], N. Kleinsasser[a,*]

[a] *Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, University of Wuerzburg, Josef-Schneider Str. 11, 97080 Würzburg, Germany*
[b] *Department of Orthopaedic Surgery, Koenig-Ludwig-Haus, Julius Maximilians University of Wuerzburg, Brettreichstraße 11, 97074 Würzburg, Germany*

HIGHLIGHTS

- We investigated the functional impairment of hBMSC by salinomycin *in vitro*.
- There were no differences in the immunophenotype and multi-differentiation capacity of hBMSC induced by salinomycin treatment.
- Cytotoxic effects were observed at concentrations of 30 μM and above.
- Essential functional properties of hBMSC were unaffected by salinomycin.

ARTICLE INFO

Article history:
Received 25 October 2012
Received in revised form 29 January 2013
Accepted 1 February 2013
Available online 12 February 2013

Keywords:
Salinomycin
Human bone marrow mesenchymal stem cells
Toxicity

ABSTRACT

Various hypotheses on the origin of cancer stem cells (CSCs) exist, including that CSCs develop from transformed human bone marrow mesenchymal stem cells (hBMSC). Since the polyether antibiotic salinomycin selectively kills CSCs, the present study aims to elucidate the effects of salinomycin on normal hBMSC.

The immunophenotype of hBMSC after salinomycin exposure was observed by flow cytometry. The multi-differentiation capacity of hBMSC was evaluated by Oil Red O and van Kossa staining. Cytotoxic effects of salinomycin were monitored by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay. Furthermore, spheroid formation and migration capacity were assessed.

There were no differences in the immunophenotype and multi-differentiation capacity of hBMSC induced by salinomycin treatment. Cytotoxic effects were observed at concentrations of 30 μM and above. Neither the migration capability nor the ability to form spheroids was affected.

Essential functional properties of hBMSC were unaffected by salinomycin. However, dose-dependent cytotoxicity effects could be observed. Overall, low dose salinomycin showed no negative effects on hBMSC. Since mesenchymal stem cells from various sources respond differently, further *in vitro* studies are needed to clarify the effect of salinomycin on tissue-specific stem cells.

1. Introduction

Despite all advances in treatment of patients suffering from head and neck squamous cell cancer (HNSCC), survival rates have not improved over 30 years. The American Cancer Society estimated 36,540 new oral cavity and pharynx, and 12,720 larynx cancer cases in 2010 (Jemal et al., 2011). Surgery, chemotherapy, radiation and the combination of these modalities are all accepted treatment options. Nevertheless, 40–60% of patients develop loco-regional failure and distant metastasis (Prestwich et al., 2011). One cause for cancer treatment failure may be the presence of a certain subpopulation, the so-called cancer stem-like cells or cancer-initiating cells (CSCs) (Boman and Wicha, 2008; Davis et al., 2010). There are several hypotheses regarding the origin of CSCs. One theory suggests that these cancer-initiating pluripotent cells may originate from human bone marrow-derived mesenchymal stem cells (hBMSC) (Liu et al., 2011).

hBMSC are undifferentiated cells capable of self-renewal and proliferation (Pittenger et al., 1999). Under certain conditions, hBMSC are able to differentiate into different mesenchymal cell types, *e.g.*, osteocytes, adipocytes, and chondrocytes (Bianco et al.,

* Corresponding author at: Department of Oto-Rhino-Laryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery, University of Wuerzburg, Josef-Schneider Str. 11, 97080 Würzburg, Germany. Tel.: +49 93120121323; fax: +49 931 201 21322.
E-mail address: kleinsasser_n@klinik.uni-wuerzburg.de (N. Kleinsasser).
[1] Tel.: +49 9312010; fax: +49 931 201 21322.
[2] Tel.: +49 9318033128; fax: +49 9318031109.
[3] Tel.: +49 93120121701; fax: +49 931 201 21248.

0378-4274/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxlet.2013.02.001

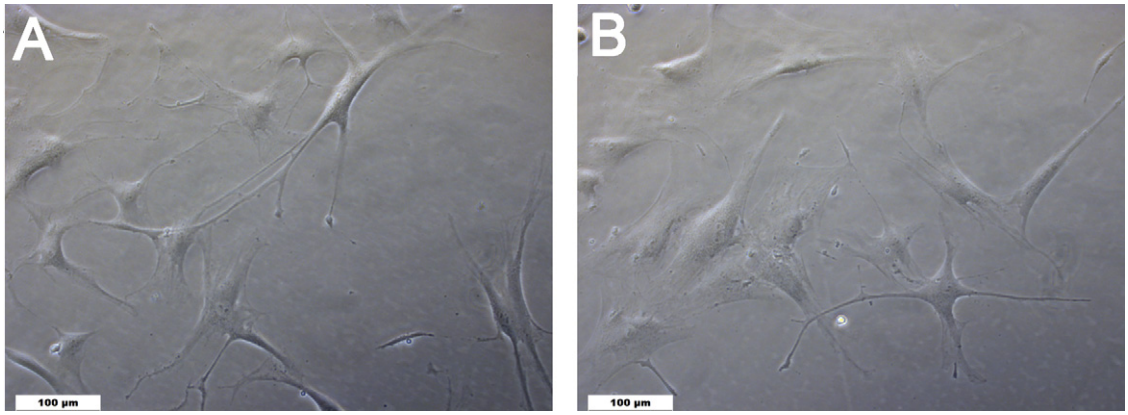

Fig. 1. Inverse light microscopy presenting cellular morphology of hBMSC treated with DMEM-EM (A) and with 10 μM salinomycin for 24 h. The spindle-shaped morphology of hBMSC was preserved after salinomycin treatment. Scale bar represents 100 μm.

2001). Differentiation into other cell types, including epithelial cells, has been shown in several studies (Charbord, 2010; Ferrand et al., 2011). In a study conducted by Liu et al., transformed hBMSC induced various tumor types, including epithelial, neural, muscular, fibroblast, blood vessel endothelial, and tumors of poor differentiation *in vivo* (Liu et al., 2006).

In a high-throughput screening Gupta and colleagues presented salinomycin as a selective inhibitor of CSCs (Gupta et al., 2009). They could show that salinomycin could kill all CSCs at very low concentrations (0.1–10 μM). Salinomycin as a polyether antibiotic acts as an ionophore. It interacts selectively with alkali ions and has a preference for potassium ions (Riccioni et al., 2010). It is widely used in veterinary medicine as an antiprotozoal agent against coccidial parasites (Boehmerle and Endres, 2011), and is more than 100-fold more effective than paclitaxel in killing CSCs. However, the complex mechanisms involved are not yet completely understood.

Based on the hypotheses that CSCs may originate from transformed hBMSC, gathering information regarding the effects of salinomycin on normal hBMSC is warranted. The present study investigates the functional impairment of hBMSC by salinomycin, such as alterations in cell surface markers, differentiation capacity, cytotoxic effects, migration capability as well as spheroid formation ability *in vitro*. According to the publication of Gupta et al. all CSCs were killed at concentrations ranging from 1 to 10 μM of salinomycin. Therefore, we used 10 μM salinomycin since this was the concentration most reliably (Gupta et al., 2009).

2. Materials and methods

2.1. hBMSC isolation and culture

Human bone marrow was obtained from seven voluntary patients undergoing surgery in the Department of Orthopedics. The study was approved by the Ethics Committee of the Medical Faculty, University of Wuerzburg (12/06) and informed consent was obtained from all of the individuals included. hBMSC were isolated as described by Lee et al. from fresh bone marrow aspirates using Ficoll density gradient centrifugation (30 minutes [min], 1300 rpm, density = 1077 g/ml, Biochrom AG, Germany) (Lee et al., 2004; Scherzed et al., 2011). The cells in the interphase were collected and washed twice using phosphate buffered saline (PBS) (Roche Diagnostics GmbH, Mannheim, Germany) containing 2% fetal calf serum (FCS) (Linaris, Wertheim - Bettingen, Germany). The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Invitrogen, Karlsruhe, Germany)-expansion medium (DMEM-EM) with 10% FCS, 1% penicillin/streptomycin (Sigma–Aldrich, Schnelldorf, Germany) and were counted using a Neubauer chamber. The cells were incubated overnight at 37 °C and 5% CO_2 in DMEM-EM. Following incubation, the tissue culture plates were washed to remove residual non-adherent cells. Medium was changed every 2nd day. When cells reached > 70% confluence, they were trypsinized with 0.25% trypsin (Gibco Invitrogen), resuspended in DMEM-EM and subcultured at a concentration of 2000 cells/cm^2. Morphology was analyzed by inverted microscopy (Leica DMI 4000B Inverted Microscope, Leica Microsystems, Wetzlar, Germany).

2.2. Expression of cell surface markers

hBMSC surface profiling after isolation and treatment with salinomycin 1 μM, 5 μM, and 10 μM was performed according to the following procedure. hBMSC were trypsinized, washed in PBS and incubated with 5% FCS for 1 h on ice. After another washing step with PBS, cells were incubated with anti CD90 (Cat: 559869), anti CD73 (Cat: 550257), anti CD44 (Cat: 555478), and anti CD31 (Cat: 550761) (all antibodies were obtained from BD Bioscience, Heidelberg, Germany). Cell surface analysis was performed by flow cytometry (BD FACSCantoTM, BD Bioscience). hBMSC cultivated in DMEM-EM served as control.

2.3. Multi-differentiation capacity

Adipogenic differentiation was performed in a 24-well plate (BD Falcon, Heidelberg, Germany) as described by Pittenger et al. (1999). hBMSC were plated at a density of 1–2 × 10^4 cells/cm^2 and treated with salinomycin. Adipogenic differentiation was induced by DMEM-EM, 10^{-7} M dexamethasone (Sigma–Aldrich), and 10 ng/ml recombinant human insulin. Staining with Oil Red O confirmed the presence of intracellular lipid droplets. Osteogenic differentiation was carried out in a 24-well plate (BD Falcon). The osteogenic induction medium was prepared as described by Pittenger et al. and was composed of DMEM-EM, 10^{-7} M dexamethasone, 10^{-3} M β-glycerophosphate, 2^{-4} M ascorbate-2-phosphate (Pittenger et al., 1999) (all Sigma–Aldrich). Every third day the medium was changed. As negative control, cells were maintained in DMEM-EM. The cells were stained according to the von Kossa method to show the presence of calcium mineral components.

2.4. HLaC 79 clone 1

The head and neck squamous carcinoma cell line HLaC 79 had been established from a lymph node metastasis of a laryngeal squamous cell carcinoma (Zenner et al., 1979). A multi resistant clone of HLaC 79 (Schmidt et al., 2009) was used as positive control cells.

2.5. Cytotoxicity

The MTT-assay as well as the Annexin V-propidium iodide test was performed in order to evaluate the cytotoxic effect of salinomycin on hBMSC. HLaC 79 clone 1 a multidrug resistant HNSCC cell line (Schmidt et al., 2009) was used as positive control.

2.5.1. MTT-assay

The mitochondrial activity of hBMSC treated with salinomycin was investigated using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, Sigma–Aldrich) colorimetric staining method according to Mosmann (1983). Cells were seeded at a density of 1 × 10^4 in a 96-well rounded bottom plate. Salinomycin at various concentrations was added to the cells for 24 h. After 24 h cells were washed with PBS. Then all plates were incubated with 100 μl of MTT solution (1 mg/ml) followed by 5 h incubation at 37 °C with 5% CO_2. After removal of MTT, 100 μl of isopropanol was added for 1 h at 37 °C with 5% CO_2. The color conversion of the blue formazan dye was measured with the multi-plate reader (Titertek Multiskan PLUS MK II, Labsystems, Helsinki, Finland) at a wavelength of 570 nm. All experiments were performed in triplicate using hBMSC of all patients (n = 7).

2.5.2. Annexin V-propidium iodide test

Apoptosis was evaluated using the Annexin V-APC kit of BD Pharmingen (BD Biosience) according to the kit manual. In brief, hBMSC in suspension and adherent cells were harvested and washed twice with cold PBS. Cells were resuspended in