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Adenosine metabolism of human mesenchymal stromal cells isolated from patients with head and neck squamous cell carcinoma

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ABSTRACT

Background: Mesenchymal stromal cells (MSC) are a major component of the tumor microenvironment in patients with head and neck squamous cell carcinoma (HNSCC). MSC display innate and regulatory immunologic functions, very similar to many hematopoietic 'classical' immune cells. Conversion of ATP to immunosuppressive adenosine is an immunosuppressive mechanism utilized by other hematopoietic immune cells. The present study explores the adenosine metabolism of tumor derived MSC in comparison to autologous MSC from non-malignant tissue.
Methods: From HNSCC patients ($n = 10$), paired MSC were generated from tumor tissue (tMSC) and autologous healthy control tissue (cMSC). Differentiation properties and phenotype (CD105, CD73, CD39, CD90, CD26, CD29) were compared by flow cytometry. Production of immunosuppressive adenosine (ADO) by functionally active ectonucleotidases, CD39 and CD73, was determined by luminescence and mass spectrometry. Suppressive activity of ADO was tested in CFSE proliferation assays of isolated T-cells. Plasticity of cMSC was explored after incubation with tumor-cell conditioned media.
Results: Differentiation into osteogenic, chondrogenic and adipogenic directions was comparable in tMSC and cMSC. Expression of ectonucleotidases, CD39 and CD73, was decreased in tMSC as compared to corresponding cMSC, which correlated with decreased ATP metabolism in mass spectrometry. Proliferation of $CD4^+$ T-cells was significantly suppressed by exogenous ADO. Tumor-conditioned medium was unable to down-regulate ADO production in cMSC.
Conclusion: We identified MSC of the oropharyngeal mucosa as an important producer of exogenous ADO. In patients with HNSCC, reduced expression of ADO may contribute to excessive inflammation and tumor growth.

1. Introduction

It is now well established that extracellular adenosine (ADO) regulates the human immune system (Saze et al., 2013; Schuler et al., 2012) and is highly immunosuppressive for the majority of immune cells. Extracellular ADO is produced primarily by the ectonucleotidases CD39 and CD73; ATP is hydrolyzed to 5′-AMP

Abbreviations: ADO, adenosine; CFSE, carboxyfluorescein succinimidyl ester; HNSCC, head and neck squamous cell cancer; HO-1, heme oxygenase 1; IDO, indoleamine 2′3 dioxygenase; MSC, mesenchymal stromal cells; PBMC, peripheral blood mononuclear cells; PGE_2, prostaglandin E2; TCMcMSC, tumor conditioned medium control MSC; Treg, regulatory T-cells.

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by CD39, and 5′-AMP is further hydrolyzed to ADO by CD73 (Fig. S1). Finally, ADO is deaminased to inosine by adenosine deaminase, which in humans is associated with the expression of CD26 (Kameoka et al., 1993).

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Both CD39 and CD73 are expressed on a variety of immune cells. While murine regulatory T-cells (Treg) express both ectonucleotidases on their surface, this is not true for human Treg, which only express CD39 (Schuler et al., 2012). Therefore, human Treg are dependent on other sources of CD73 for the production of ADO (Dwyer et al., 2010; Schuler et al., 2014). In contrast, human $CD73^+$ T-cells hydrolyze 5′-AMP to ADO and are more susceptible to HIV infection (Schuler et al., 2013). The highest potential for extracellular ADO production, however, is found in human B-cells and exosomes (Clayton et al., 2011; Saze et al., 2013), although the significance of this is presently unclear.

Mesenchymal stromal cells (MSC) are fibroblast-like multipotent progenitor cells with the potential to differentiate into osteogenic, chondrogenic and adipogenic cell types and may express phenotype-specific surface biomarkers including CD73, CD90 and CD105 (Dominici et al., 2006). Although MSC can be isolated from a variety of fetal and adult tissues, the possible influence of the source tissue on the function of MSC is presently unknown. Accumulating evidence suggests that MSC have innate and regulatory immune activity. For example, immune activating properties, namely the attraction of neutrophil leukocytes by expression of pro-inflammatory cytokines have been described for human MSC (Brandau et al., 2010). Moreover, MSC can variably affect the growth of head and neck squamous cell cancer (HNSCC) (Kansy et al., 2014; Lim et al., 2012). In contrast, emerging evidence supports the concept that MSC possess an immunosuppressive function, which allows them to regulate other cell populations including antigen presenting cells, T-cells, B-cells and natural killer cells (Abumaree et al., 2012; Dokic et al., 2013). Importantly, cross-talk between MSC and Treg results in additive suppressive capacity of both cell populations (Engela et al., 2013). Not surprisingly, ADO also influences the function of MSC, and several reports show that stimulation of ADO receptors enhances osteogenic differentiation in MSC (Carroll et al., 2012; D'Alimonte et al., 2013). Similar enhancing effects on MSC differentiation have been shown for exogenous ATP (Ciciarello et al., 2013). Surface expression of the ectonucleotidase CD39 is correlated with improved differentiation (Gullo and De Bari, 2013), while the expression of CD73 is associated with decreased differentiation of MSC towards a chondro-osteogenic cell type (Ode et al., 2013).

Of note, extracellular ATP and ADO may have differential effects on the mobility of MSC. While ATP promotes the migration of human MSC (Ferrari et al., 2011), ADO inhibits chemotaxis in murine MSC (Mohamadnejad et al., 2010). Also, murine MSC co-express CD39 and CD73, and production of exogenous ADO contributes significantly to suppression of murine T-cell proliferation in vitro (Sattler et al., 2011).

Although, several immune regulatory cell populations have been identified in the tumor microenvironment, it is not clear at all which effect an increased immune suppression has on the tumor progression. One the one hand, a fully functional immune system can have the capacity to kill vital tumor cells. On the other hand, many tumor entities arise from chronic inflammation and may profit from excessive inflammation. These inflammation-induced tumors include hepatic cancer based on chronic hepatitis, gastrointestinal tumors from colitis and also HNSCC induced by chronic infection with high risk human papilloma virus (HPV). In order to better understand the key players of immune suppression in the tumor microenvironment, we investigated in detail, the ADO metabolism in human MSC derived from head and neck cancer patients in comparison to healthy controls.

2. Material and methods

2.1. Isolation of MSC

Tissue was obtained during HNSCC surgery in the pharynx and larynx ($n = 10$). Paired tissue samples were taken from the tumor site and from surrounding healthy tissue in the same patient. The specific patients' characteristics are listed in Table 1. Patients' written informed consent was obtained for the study as approved by the local ethics committee. Paired MSC were generated from tumor tissue (tMSC) and autologous healthy tissue (cMSC) as follows. Tissue samples were collected aseptically in NaCl 0.9% (Fresenius Kabi, Germany) and washed several times with Ringer solution for removal of erythrocytes (Braun, Germany). Tissues were then cut into 1–2 mm pieces, washed extensively and digested for 40 min at 37 °C in Ringer solution containing collagenase type II (5 mg/mL, Cell Systems, Germany). The supernatant was discarded after centrifugation ($300 \times g$). Tissues were further treated for 60 min at 37 °C with Ringer solution containing dispase (33 mg/mL, Roche Applied Science, Germany). After additional centrifugation, the cell pellet was resuspended and cultured in tissue culture flasks containing DMEM medium (Invitrogen, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom, Germany), 1% penicillin/streptomycin (Invitrogen) and 1% sodium-pyruvate (Invitrogen) at 37 °C and 5% CO_2. Non-adherent cells were removed by washing with phosphate buffered saline (PBS) after 48 h. Cells were continuously passaged by Accutase (Invitrogen) treatment after reaching subconfluence.

2.2. Tri-lineage in vitro differentiation

Differentiation towards osteogenic, adipogenic and chondrogenic lineage was induced as previously described (Jakob et al., 2010). In brief, cells were seeded at a density of 3000 cells/cm^2 on round glass slides in 12-well culture dishes (Greiner Bio-One, Germany). For osteogenic differentiation cells were cultured for 21 days in MSC Osteogenic Differentiation Medium (PromoCell, Germany). Medium change was performed every 3–4 days. For verification, cells were stained for 2 min with alizarin red S solution to confirm the calcium phosphate salts. For adipogenic differentiation, we used MSC Adipogenic Differentiation Medium (PromoCell) for 14 days. To examine the generation of oil droplets in the cytoplasm after differentiation, cells were fixed with 10% formalin and stained with Sudan-III (Sigma–Aldrich, Germany) for 20 min at room temperature. Hematoxylin (Thermo Scientific, Germany) was used to visualize nuclei. Chondrogenic differentiation was induced after 48 h of culture in standard medium supplemented with dexamethasone, 1 μmol/L of L-proline (Sigma–Aldrich), 10 ng/mL of TGF-β3 (PeproTech, Germany) and 1% BD ITS Culture supplement (BD Bioscience). A chondropellet was used for staining and analysis. The presence of glycosaminoglycans was confirmed by Alcian blue staining for 60 min (8GX, Roth, Germany) on dried, formalin-fixed cryosections (5 μm). cMSC and tMSC were tested for their chondrogenic, osteogenic and adipogenic multipotency. Both, cMSC and tMSC showed comparable tri-lineage differentiation after qualitative assessment.

2.3. Flow cytometry

Immunophenotyping of MSC (passage 5–6) was performed by flow cytometry using the following specific monoclonal antibodies: CD29 PE (clone MAR4), CD45 V500 (clone HI30, both BD Bioscience), CD105 FITC (clone 166707, R&D Systems, Germany), CD26 APC