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Original Research Article

Nicotine can modulate the effects of the mesenchymal stem cells on neutrophils

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ABSTRACT

Purpose: It has been revealed that mesenchymal stem cells (MSCs) express some of the nicotinic receptor subunits. Moreover, the crosstalk between MSCs and neutrophils is not far-fetched. Therefore, the aim of the present study is to determine the role of nicotine on the effects of MSCs on neutrophils.
Methods: After the isolation of mesenchymal stem cells from the bone marrow of rats, these cells were pulsed with different concentrations of nicotine (0, 0.1, 0.5, and 1 μM) for different periods (24, 48, and 72 h). Then, the neutrophils were co-cultured with MSCs for 4 h and the functions of neutrophils were evaluated.
Results: The obtained findings showed that MSCs pulsed with nicotine significantly enhanced the viability and the phagocytic activity of co-cultured neutrophils and simultaneously, decreased the production of reactive oxygen substances (ROS), induced by f-MLP in neutrophils, more profound than MSCs without treatment. Moreover, MSCs, pulsed with nicotine at low to moderate concentrations, preserved the respiratory burst, triggered by opsonized yeast in neutrophils. Nevertheless, a high concentration of nicotine can interfere with the latter aspect of the crosstalk between MSCs and neutrophils.
Conclusion: The obtained data can offer a new insight into the potential mechanisms, underlying the immunomodulatory effects of nicotine.

1. Introduction

Mesenchymal stem cells (MSCs) are adherent, fibroblast-like, and multipotent non-hematopoietic progenitor stromal cells, which can be found in the bone marrow as well as many other tissues, such as adipose tissue, umbilical cord blood [1], dermal tissue [2], and peri-endothelial areas [3]. According to the surrounding microenvironment, they can differentiate into various mesodermal cell lineages, including adipocytes, osteocytes, chondrocytes, and myocytes [4,5]. Numerous documents have demonstrated that MSCs possess a potent immunoregulatory function, both in vitro and in vivo [5,6]. In this domain, the preliminary and clinical studies, alongside multiple animal models, have suggested that MSC therapy can be a worthwhile strategy to ameliorate acute graft-versus-host disease (GVHD) and autoimmune conditions [7].

In general, nicotine is a potent parasympathomimetic alkaloid, which is typically found at a high level in tobacco leaves and at a more limited level, in other Solanaceae (nightshade) families [8]. It also exists in some pesticide formulations [9]. Moreover, nicotine is a major compound, effective on a smoker's dependence on cigarettes. On average, each cigarette contains 10–14 mg of nicotine [10].

Different studies have exhibited that the MSCs' functions are under the control of a large number of signaling systems [11–13]. Interestingly, it has been demonstrated that MSCs express the nicotinic acetylcholine receptor subunits α_3, α_5 [12], β_2, β_4 [14], and α_7 [12,14]. It has been discovered that the stimulation of MSCs, with nicotine or nicotinic receptor-agonist, has promoted immediate and transient increases in intracellular Ca^{2+} concentration, via the nicotinic acetylcholine receptor α_7 subunit and has induced the phosphorylation of extracellular signal-regulated kinases 1 and 2 [12]. Ca^{2+} can initiate a variety of signaling pathways that affect MSC proliferation, cytoskeletal rearrangement, and differentiation [15]. Therefore, nicotine, as an environmental factor and/or a therapeutic agent, may interfere with some functions of MSCs.

The crosstalk between MSCs and immunocytes (such as neutrophils) has been documented in some recent investigations

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[16,17]. Nevertheless, there is no information about the role of nicotine in the crosstalk between MSCs and neutrophils. Therefore, the current survey was designed to investigate the effects of nicotine on the interaction of the bone marrow-derived mesenchymal stem cells and the neutrophils.

2. Materials and methods

2.1. Regents

Nicotine, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), nitroblue tetrazolium (NBT), Ficoll–Hypaque, dioxane, dimethyl sulfoxide (DMSO), trypsin, and phosphate-buffered saline (PBS) were procured from Sigma–Aldrich (St. Louis, MO). In addition, ethylenediaminetetraacetic acid (EDTA) and May–Grunwald–Giemsa stain were provided from Merck (Darmstadt, Germany). Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD). Lastly, dextran was obtained from Fresenius Kabi (Verona, Italy).

2.2. Isolation and treatment of MSCs

MSCs can be obtained from diverse tissues based on their ability to adhere to the culture plates [1]. However, the isolation of MSCs from the bone marrow is the simplest way to expand MSCs ex vivo. In brief, the bone marrow cells of deeply anesthetized Wistar rats were collected by flushing femurs and tibias. They were, then, rinsed twice by centrifugation at 1200 rpm for 5 min in PBS. Afterward, the isolated cells were plated in 75 cm^2 tissue-culture flasks, with concentrations of 0.3–0.4 $\times$ 10^6 cells/cm^2, in a DMEM medium supplemented with 15% fetal calf serum. Thereafter, the cells were incubated in a humidified 5% CO_2 at 37 °C. Four days after the primary culture initiation, non-adherent cells were removed and adherent cells were fed every other day. Then, the MSCs were removed by trypsin/EDTA, when the cultures reached 80% confluence. Thereafter, the cells were counted and passed at 1:3 ratios (about 1.5 $\times$ 10^6 cell/75-cm^2 flask). Cell passage was done up to subculture 3. Afterward, MSCs were incubated with different concentrations of nicotine (0, 0.1, 0.5, and 1 μM) for different periods (24, 48, and 72 h).

2.3. Characterization of MSCs

Immunophenotyping of rat's MSCs was done with antibodies against rat antigens CD29 (Integrin b chain; Ha2/5; FITC), CD45, and CD90 (Thy-1/Thy-1.1-FITC), and their isotype controls (IgG2a; FITC) [18]. In brief, ells (5 $\times$ 10^5 in 100 μl PBS/0.5% BSA/2 mmol/EDTA) were mixed with 10 μl of the fluorescently labeled monoclonal antibody (anti-rat CD45-FITC, CD29-PE and CD90-PCY5) and incubated in the dark at 2–8 °C for 30 min. The monoclonal antibodies were all purchased from Becton Dickinson. The cells were washed twice with PBS containing 2% BSA and the pellet was re-suspended in PBS and analyzed immediately on DAKO flow cytometer (Partec, Germany), using Cyflogic software (version: 1.2.1).

2.4. Neutrophil isolation and incubation with MSCs

Neutrophils were isolated from citrate-supplemented cardiac puncture blood of deeply anesthetized Wistar rats, as described previously [11]. In brief, the blood was centrifuged and the buffy coat was subjected to dextran sedimentation (1%, w/v), which was then followed by centrifugation on a Ficoll–Hypaque density gradient. The plasma and the mononuclear cell layer were removed, and the erythrocytes were eliminated using hypotonic lysis. Afterward, the neutrophils were washed and suspended in DMEM.

For co-culture experiments, 2 $\times$ 10^6 neutrophils were added to each well of the 24-well flat-bottomed plates, containing 2 $\times$ 10^5 MSCs, and incubated for 4 h at 37 °C in a moist atmosphere of 5% CO_2. Afterward, the neutrophils were isolated and used for the next experiments.

2.5. Evaluation of neutrophil viability

Neutrophil viability was assessed by fluorescence microscopy, similar to the procedure described earlier [19]. In brief, 100 μl of 1 mg/ml propidium iodide and 100 μl of 1 mg/ml acridine orange were mixed in PBS in order to produce 10 ml of staining solution. Then, neutrophils (2 $\times$ 10^6 cell/ml) were mixed as 1:1 with the staining solution, in microtiter wells. The percentage of live cells was evaluated in an improved Neubauer rhodium hemocytometer under fluorescence microscopy.

2.6. Phagocytosis assay

This assay was performed as described above, but with some modifications [20]. After stationary incubation of the neutrophils with the opsonized heat-inactivated yeast at 37 °C for 1 h, the cells were washed, cytocentrifuged onto glass slides, and fixed in methanol. The slides were stained with May–Grunwald–Giemsa. Then, yeast ingestion was assayed by light microscopy under oil immersion. Finally, the phagocytosis activities of neutrophils were expressed as the percentage of neutrophils, which internalized, at least, one yeast.

2.7. Respiratory burst of activated neutrophils

Neutrophils were incubated for 30 min with f-MLP (1 μM) and 0.1% NBT. The unused NBT was removed through washing, and the reduced dye was extracted in dioxane and quantitated at 520 nm [21].

2.8. Respiratory burst after challenge with opsonized yeast

The intracellular generation of reactive oxygen species (ROS), after challenge with opsonized yeast, was measured by NBT reduction, the same as previously described but with some modifications [22,23]. The neutrophils (2 $\times$ 10^6 cell/ml) were mixed with 0.1% NBT in PBS (pH 7.4) and heat-killed opsonized Baker's yeast (10^8 cells/ml) and/or non-opsonized Baker's yeast in the same volumes. The mixture was incubated for 1 h at 37 °C. The unused NBT was, then, removed through washing and the reduced dye was extracted in dioxane and quantitated at 520 nm. The final results were presented as the proportion of the respiratory burst induced by opsonized yeast, to the respiratory burst induced by non-opsonized yeast.

2.9. Statistical analysis

The data were analyzed, using the one-way ANOVA, plus Dunnett's post hoc test and presented as means ± S.D. The *P* values of less than 0.05 were considered statistically significant.

3. Results

The adherent cells, isolated from the bone marrow, gradually grew into small colonies. During the first subculture, MSCs exhibited diverse morphologies, including ovoid, bipolar and large, and flattened morphologies. The subculture 3 of adherent cells showed a homogeneous fibroblast-like, spindle-shaped