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ORIGINAL ARTICLE

Activity of Dipeptidyl Peptidase-IV/CD26 and Aminopeptidase N/CD13 in Secretome of Mesenchymal Stem Cells after Treatment with LPS and PMA

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ABSTRACT

Background: Emerging evidence suggests that secretome of mesenchymal stem cells has many anti-inflammatory and regenerative properties, which makes it a suitable candidate for the treatment of autoimmune and degenerative diseases. Dipeptidyl Peptidase-IV (DPP-IV)/CD26 and Aminopeptidase N (APN)/CD13 are ubiquitous ecto-enzymes which can digest various substrates including some chemokines and neuropeptides that are involved in inflammatory conditions. **Objective:** To evaluate the enzymatic activity of DPP-IV/CD26 and APN/CD13 in MSC conditioned media (MSC-CM). **Methods:** The MSCs were isolated from the mouse's abdominal adipose tissues and were cultured without or with preconditioning by adding 2 µg/mL phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS). The levels of interleukin-10 (IL-10), nitric oxide (NO), as well as the enzymatic activities of DPP-IV/CD26 and APN/CD13 were measured in MSC-CM. **Results:** The level of IL-10 and the enzyme activity of APN/CD13 did not show any changes in the MSC-CM of stimulated and non-stimulated cells. However, NO production was increased after treatment by LPS or PMA; nevertheless, the DPP-IV/CD26 activity was decreased in MSC-CM merely following the stimulation of cells with LPS. **Conclusion:** Our results indicated that MSC-secretome had DPP-IV/CD26 and APN/CD13 activity. The DPP-IV/CD26 activity was decreased following stimulation of MSCs by toll-like receptor 4 agonist. Further studies are needed to reveal the possible contribution of DPP-IV/CD26 and APN/CD13 in the anti-inflammatory functions of MSC-CM.

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INTRODUCTION

Mesenchymal stem cells (MSCs), comprising a small number of multipotent adult stem cells, exist in almost all tissues. They exhibit substantial capacities in regenerative medicine owing to their prominent capability to give rise to specialized cells following migration to the sites of damage, ability to suppress immune responses, and availability in large quantities in the patients' own adipose tissue or bone marrow (1). A large number of clinical trials (964 as of June 2019 registered at ClinicalTrials.gov) have been conducted to examine MSCs as a potential therapy for different diseases, including malignancies, graft versus host disease, diabetes, and hematological and neurological diseases. Despite the previous attention to the differentiation potential of these cells, in many therapeutic contexts, MSCs exert their healing effects not solely through engraftment and differentiation but rather through providing secretory factors (secretome) with myriads of beneficial effects (2). Secretory derivatives such as conditional media or exosomes can have significant advantages over MSCs in medical applications and efficacy.(3). The MSCs-secretome induces regenerative processes in the injured tissue, protects cells from apoptotic cell death, down-modulates the immune responses, and assists in angiogenesis (4). The MSC-secretome contains growth factors, cytokines, and micro-RNAs (3). Enzymes are another remarkable component of the MSC-secretome which are yet to be fully investigated. Aminopeptidase N (APN/CD13, EC 3.4.11.2) and Dipeptidyl Peptidase-IV (DPP-IV/CD26, EC 3.4.14.5) are ubiquitously expressed cell-surface proteases (ecto-enzymes) whose shedding products are known as soluble APN (sAPN) and soluble DPP-IV (sDPP-IV) (5,6). CD26 has a variety of substrates, including immunomodulating peptides such as lymphotoxin, exotoxin, RANTES, MCP, IP-10, and nutritional and metabolic peptides such as GLP-1, GLP-2, GIP, GHRF, GRP, NPY, and VIP. DPP-IV plays a principal role in glucose metabolism through the degradation of incretins. Therefore, DPP-IV inhibitors are used in the treatment of type 2 diabetes (6). Furthermore, stromal cell-derived factor-1 (SDF-1) is a chemokine considered as another important substrate of DPP-IV. It has been revealed that the inhibition of DPP-IV potentiates the actions of SDF-1 and enhances cardiac recovery and neovascularization (6). Similarly, APN has been proven to be associated with the degradation of angiotensins, several neuropeptides, immunomodulatory peptides, and cytokines by removing the neutral amino acids from the N-terminus of substrates (5). Previous studies have demonstrated that DPP-IV/CD26 is expressed on hematopoietic stem cells, adipose-derived mesenchymal stem cells, and embryonic stem cells. Likewise, APN/CD13 expression on the adipose-derived mesenchymal stem cells also has been reported (7-10). A previous functional analysis showed that CD13 is essential for optimal MSC adhesion, vascular network formation, and migration, thereby contributing to MSC-mediated tissue repair (8). Despite the previous findings regarding the expression of CD26/DPP-IV and CD13/APN on the cell surface of MSCs, the concentrations and activities of these ecto-enzymes in MSC-secretome have not been reported yet. Given the widespread and growing use of MSC-based materials to treat diseases such as diabetes (11), rheumatoid arthritis (RA) (12), cerebral injury (13), and myocardial infarction (14) and based on the findings underscoring the pathological roles of DPP-IV/CD26 and APN/CD13 in auto-inflammatory and autoimmune diseases, it was deemed important to investigate the activities of these enzymes in MSC-secretome. The current study was conducted to analyze APN/CD13 and DPP-IV/CD26 activities in the conditioned media of the MSCs

of adipose tissues isolated from BALB/c mice. The results revealed that MSC-CM had DPP-IV/CD26 and APN/CD13 activity, which changed after preconditioning by use of lipopolysaccharide (LPS).

MATERIALS AND METHODS

Animals. To isolate MSCs, female inbred 6-8-week-old BALB/C mice (weighing18-20 g) were obtained from the Pasteur Institute of Tehran. Prior to the experiments, the mice (n=6) were retained in the animal house under controlled conditions for at least 1 week. The animal standard condition was provided according to the Ethics and Research Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1397.712).

MSCS Isolation and Culture. To isolate MSCs, the abdominal adipose tissues were initially treated with 0.1% collagenase type IV (Gibco; Thermo Fisher Scientific, Inc.). The cells were then cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) media (Gibco, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, USA) and 100 U/mL Penicillin/Streptomycin (Invitrogen, USA). The fresh medium was replaced every 24 h. The viability of the MSCs was monitored using Trypan blue. For preconditioning, MSCs culture medium was supplemented with 2 ng/mL of phorbol 12-myristate 13-acetate (PMA) or (LPS). The proliferation and viability of the cells were assessed through the use of invert light microscope (Nikon, Japan). The levels of IL-10 and NO and the activity of DPP-IV and APN were measured at the second passage, 24 h after treatment with LPS or PMA.

Flow cytometry. To analyze MSC-associated surface antigens by flow cytometry, the MSCs at the second passage (P2) were primarily detached using 0.125% trypsin solution and washed with phosphate-buffered saline (PBS); afterwards, they were re-suspended in PBS containing 5% FBS. The following monoclonal antibodies (BD, USA) were utilized: CD29-PE, CD44-PE, CD90-PE, CD11b-PE, and CD45-PE; additionally, in order to define the cut-off values and determine the non-specific binding, the isotype controls were included in the analyses. Totally, 5×10^4 MSCs were harvested, incubated with an appropriate amount of antibodies at 4°C for 15 min, and washed with PBS. A BD FACSCalibur® flow cytometer was used to analyze the samples, and FlowJo™ 7.6.1 software was employed to analyze the results.

Analysis of the Osteogenic and Adipogenic Differentiation Potential. MSCs at the second passage and at 80% confluence were analyzed to determine their capability to differentiate toward adipocytes and osteocytes. Firstly, MSCs were cultured in 5% CO_2 at 37°C for 21 days in DMEM/F12 medium supplemented with 10% FBS, glycerol phosphate (10 mM), dexamethasone (10 mM), ascorbic acid-2 phosphate (5 mg/mL), penicillin (100 U/mL), and 100 mg/mL streptomycin (all obtained from Sigma-Aldrich, Germany). The osteogenic medium was exchanged every 3 to 4 days. To evaluate mineralization, the cells were stained after three weeks with 2% Alizarin Red S solution (Sigma Aldrich, Germany). In order to analyze the potential of MSCs towards adipocyte lineage differentiation, confluent cells were incubated with the adipogenic induction medium, which included indomethacin (100 mM), insulin (5 mM), 3-isobutyl-methylxanthine (0.5 mM), and dexamethasone (250 mM) (all achieved from Sigma-Aldrich, Germany). After 21 days, the intracellular oil droplets in the cultured cells were

stained by oil red-O. An inverted microscope (Nikon, Japan) was utilized to capture the images.

Cytokine Assay. MSCs (P2) were grown in six-well plates. PMA or LPS was used to stimulate the cultured cells for 24 h. The level of interleukin-10 (IL-10) was analyzed in the conditioned media by use of enzyme-linked immunosorbent assay (ELISA) kits (Mabtech, Sweden) based on the manufacturer's instructions.

Nitric Oxide Assay. The concentration of nitric oxide (NO) in the conditioned media was examined according to a previously described method (15). The sample was mixed with equal amounts of Sulfanilamide 1% and 0.1% NED (N-(1-naphthyl) ethylenediamine). Promptly, the plate was incubated for 10 minutes at room temperature in dark. Subsequently, the optical density (OD) of the developed color was measured at 520-550 nm, and the NO concentration was calculated in accordance with the OD of standards.

Enzyme Activity of DPP-IV in MSC-CM. The chromomeric approach was employed to measure DPP-IV activity (16). A solution of 1 mM Gly-Pro-p-nitroanilide hydrochloride (Sigma, USA) in a buffer of 100 mM Tris-HCL at a pH value of 8.0 was used as the chromogenic substrate. To hydrolyze the substrate and production of p-nitroaniline, 100 µL of the substrate solution was initially added to 10 µL of MSC-CM diluted with 90 µL of 100 mM Tris-HCL. The mixture incubation was performed at 37°C for 20 min. Secondly, a buffer of 1 M sodium acetate at pH=4.4 was applied to stop the enzyme activity. The microplate reader was utilized for the quantification of p-nitroanilide concentration at 405 nm. A p-nitroaniline range of 10-100 µM was considered as standard. Sodium acetate buffer was added to prepare a negative control for the reaction prior to the substrate addition. All assays were carried out in triplicate.

Enzymatic Activity Assay of APN. To assess the enzymatic activity of CD13/APN, L-leucine-p-nitroanilide (Sigma, USA) was utilized as the substrate and the release of p-nitroaniline was evaluated spectrophotometrically at 405 nm (17). In the first step, 90 µL of the substrate mixture (1 mM L-Leucine p-nitroanilide in methanol) and 10 µL of MSC-CM were mixed. Next, an Anthos microplate absorbance reader 2020 (Biochrom Ltd., Cambridge, UK) was employed to measure the production of p-nitroaniline after 60 min of incubation. Considering a millimolar extinction coefficient value of 10.8 for p-nitroaniline at a wavelength of 405 nm, the enzymatic activity of APN was calculated according to the following equation:

$$\text{Unit/ml} = \frac{\frac{\Delta A450nm}{\min test} - \frac{\Delta A450nm}{\min blank}}{(10.8)(0.1)} \times (dilluation\ factor)$$

Statistical Analysis. The GraphPad Prism 5.01 software (GraphPad Software Inc., USA) was utilized to conduct the statistical analysis. To provide the non-parametric distributions, all the studied parameters were expressed as the median value and the 25th and 75th percentiles. The Kruskal–Wallis tests were used to compare the non-parametric data. p-value<0.05 was considered as statistically significant.

RESULTS

MSCs Morphology, Immunophenotyping, and Differentiation.

MSCs were isolated from the abdominal fat layer obtained from the female BALB/c mice. The typical morphology of the MSCs attached to the tissue culture plastic surfaces displayed small, spindle-shaped cells (Figure 1A). Moreover, the potential of MSCs to differentiate into osteocytes (Figure 1B) and adipocytes (Figure 1C) was examined through the functional differentiation assay.

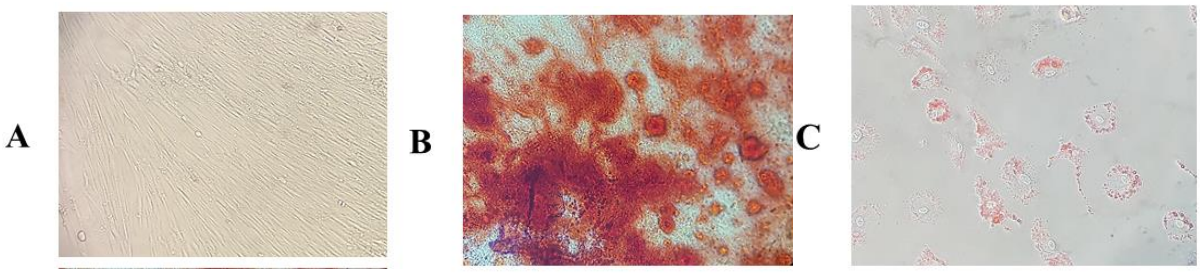

Figure 1. Microscopic characteristics of adipose tissue-derived mesenchymal stem cells. (A) Expanded MSCs had the uniform appearance of fibroblast-like cells. (B) Adipogenic differentiation potential of MSCs was assessed by Oil-red staining. Functional differentiation of MSCs to osteocytes was analyzed by Alizarin S staining of the calcium nodules. MSCs: mesenchymal stem cells.

For further characterization, the MSCs were harvested at the second passage for immunophenotyping via flow cytometry. The cell type of MSCs was confirmed by the presence of CD29, CD44, and CD90 and the absence of CD11b and CD45 (Figure 2).

Nitric oxide production by MSCs increased following LPS and PMA treatment.

The NO production is considered as a potent immunomodulatory mechanism used by MSCs. The median of NO concentration in the supernatant of the non-stimulated MSCs was 3.24 ± 0.93 µM/L. The results of the current study showed that more than 48 h exposure to PMA or LPS significantly increased NO production by MSCs in comparison with non-stimulated cells (for PMA: 4.26 ± 0.21 µM/L; p=0.003; for LPS: 6.10 ± 0.48 µM/L, p=0.004, Figure 3A).

Interleukin-10 production by MCS did not change following stimulation by PMA and LPS.

IL-10 is one of the most important anti-inflammatory cytokines generated by MSCs. So as to compare the anti-inflammatory capability of stimulated MSCs with non-stimulated cells, the level of IL-10 was measured in MSC-CM. Supernatants of MSC cultures were collected two days after the initial culture. In the supernatants of non-stimulated cells, the median of IL-10 was 137.52 ± 9.06 pg/mL; however, the co-incubation of cells with PMA or LPS did not change the levels of IL-10 (PMA: 144.70 ± 4.54 and LPS: 141.20 ± 7.96, p=0.07) (Figure 3B).

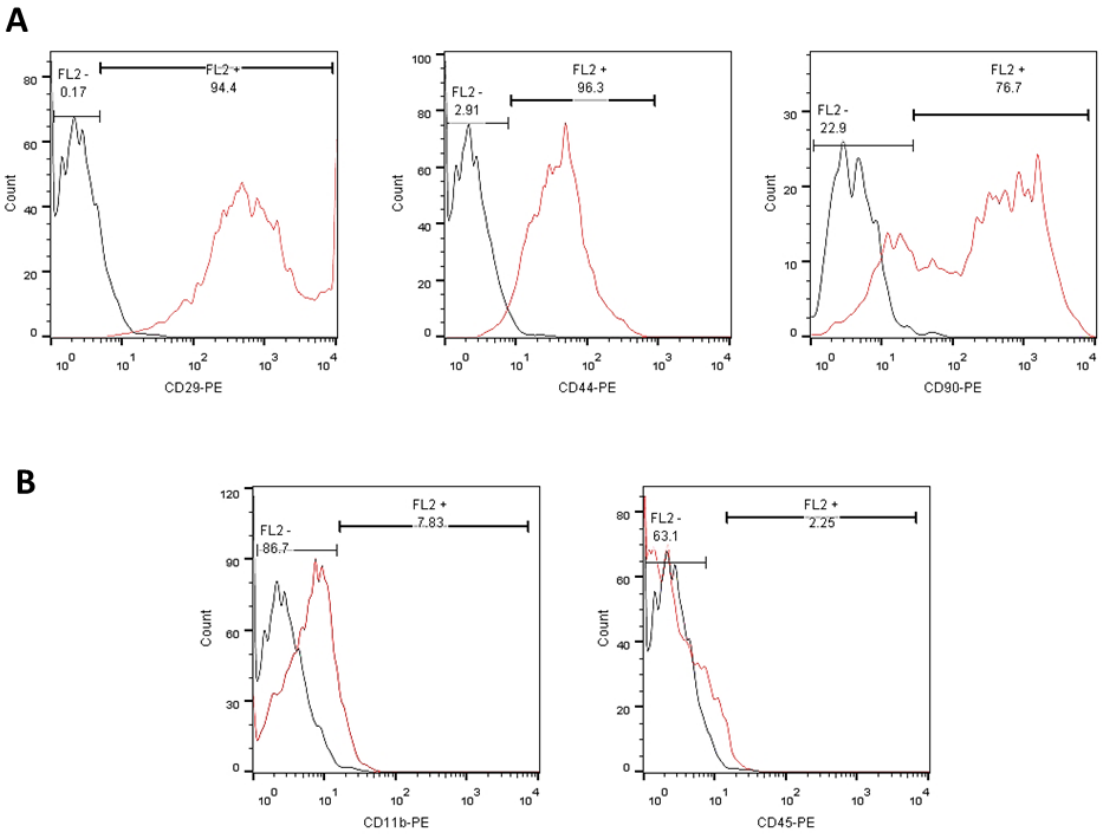

Figure 2. Phenotypic characteristics of mesenchymal stem cells by flow cytometry. Representative histograms of MSCs phenotype assessed by flow cytometry display the expression profile of (A) the positive (CD29, CD44, and CD90) and (B) the negative markers (CD11b and CD45). MSCs: mesenchymal stem cells.

MSC-CM showed CD26/DPP-IV and CD13/APN activity.

Primarily, the baseline enzymatic activity of APN/CD13 and DPP-IV/CD26 was measured in the MSC-CM of non-stimulated cells. The results showed that the medians of APN/CD13 and DPP-IV/CD26 activity were 1.35 ± 0.17 U/mL and 34.82 ± 6.59 pmol/mL, respectively (Figure 3C and 3D). Secondly, MSCs were cultured in the cell culture medium supplemented with LPS or PMA for 48 h to investigate whether MSCs stimulation by either LPS or PMA could influence the DPP-IV/CD26 and APN/CD13 activities. MSCs treated with both stimulators exhibited APN enzyme activity rates similar to those of the non-stimulated cells (p=0.078, p=0.078). A similar finding was obtained when the DPP-IV activity in the MSC-CM of PMA-stimulated cells (33.73 ± 7.61 pmol/mL) was compared to the enzymatic activity in the supernatant of non-stimulated cells (p=0.132, Figure 3C). However, the enzymatic activity of DPP-IV/CD26 in MSCs, which was stimulated by LPS, was 20.63 ± 3.87 pmol/mL, indicating a significant reduction compared to the non-stimulated MSCs (p=0.004).

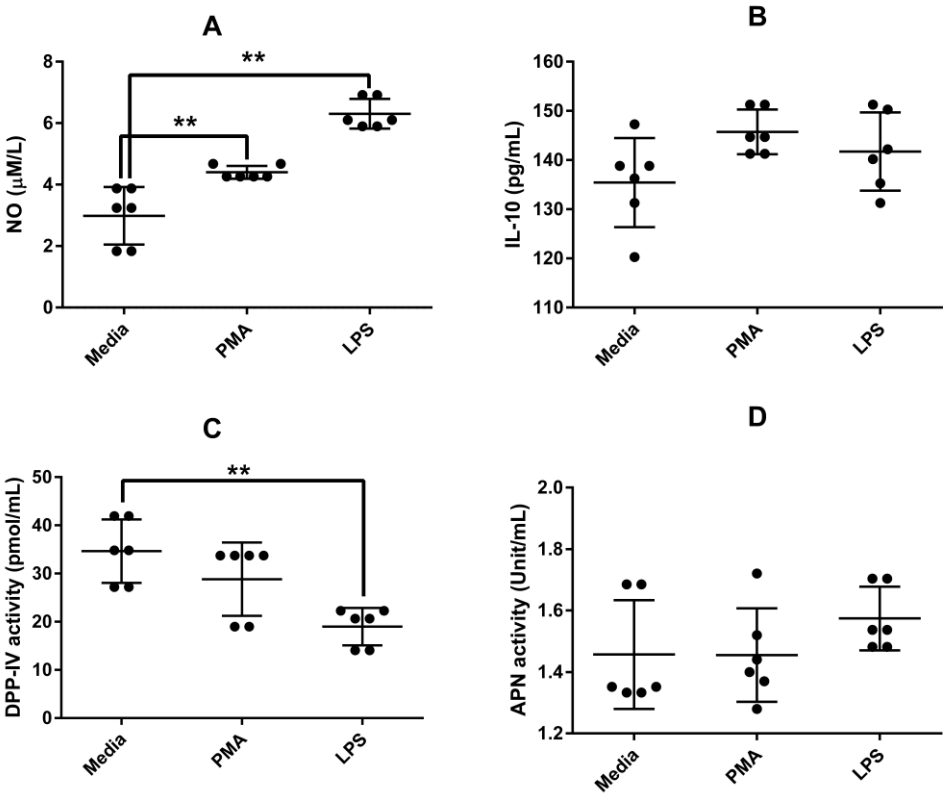

Figure 3. Production of nitric oxide and interleukin-10 and the enzymatic activity of DPP-IV and APN by mesenchymal stem cells before and after treatment by LPS and PMA. (A and B) the NO and IL-10 concentrations were measured in the supernatant of 1×10^6 MSCs prior to and following treatment by LPS and PMA. (C and D) the activity of DPP-IV and APN was measured in the conditioned media of MSCs with or without stimulation by LPS and PMA. MSC: IL-10: interleukin-10. LPS: lipopolysaccharide. MSCs: mesenchymal stem cells. NO: nitric oxide. PMA: phorbol 12-myristate 13-acetate. Results are median ± SE; n = 6, **p≤ 0.01 vs. control.

DISCUSSION

The composition of MSC-secretome is considered to be critical in translating MSC-derived products to clinical applications. Recent studies have shown that MSCs secrete dozens of pro-regenerative and anti-inflammatory mediators, including growth factors and cytokines and enzymes such as IDO (3). In keeping with the prominent and pervasive expression of DPP-IV/CD26 and APN/CD13 by embryonic and adult stem cells (7-10), we assessed DPP-IV/CD26 and APN/CD13 activities in MSC-CM in the current experiment. The means of DPP-IV/CD26 and APN/CD13 activity in MSC-CM were 27.97 ± 6.48 U/L and 1.46 U/ml ± 0.19, respectively. Based on the previous findings, there is clear evidence that the level or activity of soluble DPP-IV or APN is positively associated with various inflammatory disorders, including RA (18), SLE (17), obesity (19), diabetes, and cardiovascular diseases (20). The similarity in the change pattern of DPP-IV/CD26 and APN/CD13, observed in many diseases, and the common substrate digested by both, indicate a co-operative mode of action between APN and DPIV. Indeed, APN hydrolyzes the substrates as far as proline appears in the second

position of the N-terminal sequence, thereby generating DPP-IV-susceptible substrates (21). This mechanism vindicates the superiority of simultaneous inhibition of DPP-IV and APN enzymatic activity over the anti-inflammatory effect of single DPP-IV/CD26 or APN/CD13 inhibitors (22). Given these findings, the impacts of co-operative DPPIV/CD26 and APN/CD13 activity on the outcome of MSCs or MSC-secretome therapy should be considered in inflammatory diseases, particularly diabetes and cardiovascular disorders. Apparently, a reduction in DPP-IV or APN activity is not always a therapeutic goal, rather augmenting the activity might have some beneficial effects. For instance, the APN activity has been proven to decrease in the serum and brain of patients affected by Alzheimer's disease (AD) (23). Given the neuroregenerative capability and APN activity of MSCs/MSC-secretome, the MSC-based therapy may be conducive to compensating for the APN deficiency, facilitating neuronal functioning, and augmenting neuroregeneration in AD patients. Evidence suggests that MSCs are susceptible to environmental changes, and their regenerative potential and immunosuppressive functions could increase when exposed to an inflammatory milieu (24). In the current study, treatments by LPS or PMA were considered as preconditioning. Preconditioning by use of soluble stimulators in the culture medium was recognized as a potent strategy to increasing MSC immunomodulatory functions. PMA is a well-known protein kinase C activator, and LPS is a potent toll-like receptor 4 (TLR4) agonist, both of them have significant effects on the MSCs activity (25,26). It has been reported that the addition of LPS and PMA to the culture could not change the expression of stemness markers (27). On the other hand, there is growing evidence that LPS preconditioning or MSC exposure to other TLR4 agonists enhances the paracrine protective, regenerative, and repairing properties of MSCs (26,28). Likewise, the data of the current study showed that LPS and PMA increased NO production while only LPS decreased the DPP-IV activity in the secretome of MSCs. NO has several immune-regulatory effects such as inhibiting T cell proliferation and activity, suppressing the production of IL-12, and negatively regulating T helper 17 differentiation (29). DPP-IV inhibitors enhanced NO production (30), but the mechanism is yet to be investigated. The increase in NO production and the decrease in DPP-IV activity, observed following stimulation with LPS, clearly underline the fact that stimulating factors changed the secretome composition of MSCs . Additionally, the findings propose that the stimulation of MSCs by LPS could enhance the immunoregulatory properties through enhancing the NO production and reducing DPP-IV activity. Another remarkable finding is that the effect of preconditioning, including LPS and PMA treatment, on the composition of MSC-secretome was diverse since neither treatments could change the APN's activity whereas DPP-IV activity was diminished following the exposure to LPS. Indeed, under defined and controlled conditions, activating MSCs by pro-inflammatory agents can improve their immunosuppressive effects; however, this is may not occur *in vivo*. During pathologic inflammatory conditions in disease settings, MSCs encounter a wide range of inflammatory agents. The outcome of the effects of various factors on MSCs might be the functional disability of MSCs similar to the systemic lupus erythematosus (18), multiple sclerosis, and diabetes. Therefore, inflammatory conditions with any extent and duration could not necessarily have the same impacts on the therapeutic effects of MSCs. To the best of our knowledge, the enzymatic activities of DPP-IV/CD26 and APN/CD13 in MSC have not been explored; however, functional studies have shown that these activities participate in the homing of stem cells. The transplantation of CD13

knock-out MSCs in a model of severe ischemic limb injury showed that their adhesion and migration were significantly impaired (8). Likewise, Singh *et al*. reported that mice lacking CD26 exhibited impaired hematopoietic stem cells (HSCs) trafficking (19). In contrast, Khurana *et al.* observed that the inhibition of DPP-IV enzymatic activity in HSPCs isolated from murine or human led to higher *in vitro* migration and bone marrow homing (20). Similarly, deletion or inhibition of CD26 on HSCs augmented homing and engraftment through a SDF-1/CXCL12 mediated manner, suggesting that CD26 is a novel target for elevating the transplantation efficiency (31). In the same way, by acting on the homing of vascular regenerative cells, DPP-4 inhibition might also broadly endorse vascular repair in diabetes patients (32). In addition to the roles of these molecules in migration and homing, it seems that CD13 plays indispensable roles as a positive regulator in adipogenic (9) and angiogenic (33) differentiation. Altogether, these findings highlight the role of CD26 and CD13 in the migration and differential potency of stem cells. In conclusion, this study is the first to report DPP-IV/CD26 and APN/CD13 activities in the MSC-secretome. The role these ecto-peptidases play in MSC-secretome therapy might be quite convoluted due to their potential to digest several cytokines and hormones and exert some impacts on several signaling pathways in the immune system. More complicating, under certain pathologic conditions, the increase in the levels of these molecules can be considered as a therapy goal. Therefore, more studies are required to shed light on the effects of these molecules during inflammation treatment via MSC-based therapy and define the effects of the disease-related factors on the enzymatic activity of MSC-secretome.

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