Effect of Selenium and Peroxynitrite on Immune Function of Immature Dendritic Cells in Humans

Background: Selenium and peroxynitrite are known to support the growth and activity of immune cells, including T cells, B cells and macrophages. However, the role of these factors in the immune function of human immature dendritic cells (imDCs) is not clear.

Material/Methods: Monocytes from a mixture of blood samples were isolated using Ficoll density gradient centrifugation and purified with immunomagnetic beads before being induced into imDCs. Cells then either received no treatment (control group), or treatment with sodium selenite (Na$_2$SeO$_3$, Se), 3-morpholinosydnonimine (SIN1, which decomposes into peroxynitrite), or Se+SIN1. Cell viability, migration, and antiphagocytic abilities, oxidative stress, and protein expression of extracellular signal-regulated kinases (ERK) and MMP2 were assessed using a CCK8 assay, cell counter and flow cytometry, microplate spectrophotometer, and Western blot analysis, respectively.

Results: Viability of imDCs was unaffected by 0.1 μmol/L of Na$_2$SeO$_3$, although 1 mmol/L of SIN1 decreased it significantly ($P<0.05$). Chemotactic migration and antiphagocytic abilities were inhibited and enhanced, respectively, by treatment with Na$_2$SeO$_3$ and SIN1 ($P<0.05$). Activities of superoxide dismutase and glutathione peroxidase were increased by Na$_2$SeO$_3$ and Se+SIN1 ($P<0.001$). Glutathione content decreased with exposure to Na$_2$SeO$_3$ and SIN1 ($P<0.05$), but increased after treatment with Se+SIN1 ($P<0.05$). Levels of reactive oxygen species only increased with SIN1 treatment ($P<0.05$). Treatment with Na$_2$SeO$_3$, SIN1 and Se+SIN1 increased ERK phosphorylation and decreased MMP2 protein expression ($P<0.05$).

Conclusions: Selenium and peroxynitrite can influence immune function in imDCs by regulating levels of reactive oxygen species or glutathione to activate ERK and promote antigen phagocytosis, as well as by decreasing MMP2 expression to inhibit chemotactic migration.

Keywords: Cell Migration Assays • Dendritic Cells • Oxidative Stress • Peroxynitrous Acid • Selenium
Background

Dendritic cells (DCs) serve as potent messengers between the innate and adaptive immune systems and can be classified as immature (imDCs) or mature (mDCs), depending on their stage of differentiation. Specifically, the former captures and processes antigens from the environment, while the latter presents them to T cells in the lymph nodes [1-3].

To date, studies have indicated that both mitochondrial reactive oxygen species (ROS) and superoxide anions (O$_2^-$) contribute to the development of DCs. For example, investigations in mice have reported that ROS activates DCs and promotes interaction between DCs and T cells during antigen presentation [4], as well as induces apoptosis or necrosis of DCs via Acinetobacter baumannii outer membrane protein A [5]. Among humans, ROS stimulates differentiation of mononuclear cells into DCs [6], and O$_2^-$ assists in the maturation of imDCs into mDCs [7]. Importantly, the reaction product of O$_2^-$ and nitric oxide (peroxynitrite) is required for cytokine production in human monocytes [8], as nitric oxide synthase inhibitors have been linked to a reduction in peroxynitrite formation and partial inhibition of O$_2^-$ induced maturation of DCs [7]. Previous studies showed that a high concentration of nitrite was found in LPS-activated mouse DCs [9]; however, the effect of nitrite in the tumor microenvironment [10] on DCs function remains unknown.

Interestingly, in recent years numerous investigations have also discovered that selenium exerts a substantial influence on the growth and function of immune cells by regulating their redox state through selenoproteins K, T, R, S, and 15 [11-18]. For mice, selenium can regulate the numbers of B cells and antibody production in their spleens [19-21], as well as the transformation of macrophages into different cell types [22]. Moreover, low levels of selenium (0.08 ppm) have been found to induce differentiation of T cells into Th2 cells, while high levels (1.0 ppm) trigger T cell differentiation into Th1 cells [19]. The effect of sodium selenite on the immune function of chicken DCs was recently studied [23,24]. In humans, however, studies have yet to clarify how physiological levels of selenium impact imDCs [4,23,24], especially under peroxynitrite conditions.

Previous studies suggested that mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERK), are involved in the maturation of DCs after various stimuli [24-28], and redox balance or oxidative stress may be the upstream signals of MAPKs [28,29]. It also demonstrated that matrix metalloproteinases (MMPs) play important roles in the migration of DCs [30]. However, the role of ERK and MMP2 in the function of human imDCs affected by selenium and peroxynitrite was unclear.

Against this background, the present study aimed to uncover the underlying mechanism by which selenium and peroxynitrite modulate the immune function of human imDCs, as well as to assess the migration and phagocytic capabilities, redox balance, and ERK and MMP2 signaling in these cells. We hypothesized that selenium and peroxynitrite would affect the activity of imDCs by oxidative stress, ERK, and MMP2.

Material and Methods

Preparation of imDCs from Human Peripheral Blood

Healthy individuals from Guizhou, China were enrolled and gave informed consent to participate. This study was approved by the Ethics Committee of Guizhou Medical University (Approval No. 2018 (15)). For each experiment, a mixture of blood from 10 donors was collected. Monocytes were first isolated from the fresh peripheral blood of our participants using Ficoll density gradient centrifugation and later purified with immunomagnetic beads. They were then cultured in an RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Hyclone, USA), 1% antibiotics (Ameresco, USA), 800 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech, Germany), and 500 U/mL recombinant human interleukin-4 (PeproTech, Germany) at 37°C in 5% CO$_2$ for 5 days, after which imDCs were obtained [31].

Cell Treatment and CCK8 Assay

imDCs were seeded into 96-well plates at a density of 1×10$^6$ cells/mL. Next, they were treated for 12 h in an RPMI 1640 medium supplemented with cytokines and 0.0001, 0.001, 0.01, 0.1, or 1 μmol/L of sodium selenite (Na$_2$SeO$_3$), Se, Sinopharm, China, 0.2, 0.5, or 1 mmol/L of 3-morpholinosydnonimine (SN1, Sigma, USA – this spontaneously decomposes to yield O$_2^-$ and nitric oxide before forming peroxynitrite). Cell viability was then assayed using a CCK8 kit (Beyotime, China), according to the manufacturer’s protocol. In subsequent experiments, imDCs were divided into 4 groups: control group (C, no treatment), Se group (treatment with Na$_2$SeO$_3$), SIN1 group (treatment with SIN1), and Se+Sin1 group (pretreatment with Se and challenge with SIN1).

Antiphagocytic Ability of imDCs

After imDCs were treated with Se (0.1 μmol/L) or SIN1 (1 mmol/L), imDCs at a density of 1×10$^6$ cells/mL were resuspended in phosphate-buffered saline (PBS), incubated with FITC-dextran (40KD, Sigma) at 37°C for 1.5 h, and then fixed with 3.7% paraformaldehyde at 4°C for 20 min. Next, they were washed twice with PBS and resuspended in it, and then 1×10$^4$ imDCs were subjected to a flow cytometry assay (NovoCyte,
The fluorescence intensity was analyzed and normalized to that of the control [32].

**Migration Ability of imDCs**

Cells at a density of $2.5 \times 10^6$ cells/mL were seeded into an upper transwell chamber with a 5 μm pore size (Millipore, Germany) containing an RPMI 1640 medium without FBS, and the lower chamber containing an RPMI 1640 medium with 10% FBS and either 100 ng/mL CCL2 (PeproTech, Germany) or not. After cells were incubated at 37°C for 36 h, they were counted by cell counter (Cellometer mini, Nexcelom, USA) and their migration rate was normalized to that of the control [33].

**Measurement of Oxidative Stress**

To assess superoxide dismutase (SOD) activity, imDCs at a density of $1 \times 10^6$ cells/mL were washed twice with cold PBS, homogenized in ice-cold PBS, subjected to a total SOD assay with WST-8 (Beyotime, China) at 37°C for 30 min, and then had their absorbance read by a microplate spectrophotometer (Cytoation5, Biotek) at 450 nm. To evaluate glutathione peroxidase (GPx) activity, imDCs at a density of $1 \times 10^6$ cells/mL were homogenized, subjected to a cellular glutathione peroxidase assay (Beyotime, China), and then had their absorbance read by a microplate spectrophotometer at 340 nm. To measure ROS levels, imDCs at a density of $1 \times 10^6$ cells/mL were washed twice with PBS, resuspended in PBS, incubated with 2', 7'-dichlorodihydro-fluorescein diacetate (Solarbio, China) for 20 min at 37°C, and then had their activity determined by flow cytometry assay [34]. To determine glutathione (GSH) content, imDCs at a density of $1 \times 10^6$ cells/mL were washed with PBS, repeatedly frozen and thawed with a protein removal reagent, and then had their absorbance read by a microplate spectrophotometer [35].

**Western Blotting**

Cell lysates were prepared using an RIPA buffer containing a protease inhibitor cocktail, and then antibodies purchased from Affinity for ERK (AF0155), pERK (AF1015), MMP2 (AF0577), and GAPDH (AF0911) were used for western blot analyses as previously described [36].

**Statistical Analysis**

Analysis of variance was applied to determine differences between groups, followed by Tukey’s test for multiple comparisons; P values <0.05 indicated statistical significance. The data are presented as mean±standard deviation (SD) of at least 3 independent experiments, and all analyses were performed using SPSS software (version 17.0, IBM, USA).

**Results**

**Cell Viability of imDCs**

Treatment with Na$_2$SeO$_3$ significantly increased viability of imDCs at concentrations of 0.0001, 0.001, and 0.01 μmol/L ($P<0.05$), but not at concentrations of 0.1 and 1 μmol/L ($P>0.05$) (Figure 1A). Treatment with SIN1 significantly decreased cell viability at a concentration of 1 mmol/L ($P<0.05$) (Figure 1B).

**Regulation of the Migration and Antiphagocytic Ability of imDCs**

Treatment with Se+SIN1 significantly increased the free migration ability of imDCs ($P<0.01$) (Figure 2A). The treatments of Se, SIN1, and Se+SIN1 all significantly decreased the chemotactic migration ability of imDCs ($P<0.001$), although exposure to SIN1 was found to partially restore chemotactic migration ability in imDCs first treated with Se ($P<0.001$) (Figure 2B).
Antiphagocytic ability of imDCs significantly increased following treatment with Se and SIN1 (P<0.05) (Figure 2C, 2D).

Redox Balance of imDCs

SOD activity in imDCs was significantly increased by treatment with Se, SIN1, and Se+SIN1 (P<0.001), although treatment with Se+SIN1 significantly decreased activity compared to that of SIN1 (P<0.001) (Figure 3A). GPx activity was significantly increased by treatment with Se and Se+SIN1 (P<0.001) (Figure 3B). GSH content significantly decreased following treatment with Se and SIN1 (P<0.05), but significantly increased with Se+SIN1 treatment compared to that of the control and SIN1 groups (P<0.05) (Figure 3C). ROS levels significantly increased upon treatment with SIN1 (P<0.05), but decreased after treatment with Se+SIN1 compared to that of SIN1 (P<0.05) (Figure 3D).

The Potential role of ERK and MMP2 in imDCs

Treatment with Se, SIN1, and Se+SIN1 significantly increased ERK phosphorylation in imDCs (P<0.05) (Figure 4A, 4B), but significantly decreased MMP2 levels (P<0.05) (Figure 4A, 4C).

Discussion

Previous studies have indicated that selenium influences production of B cells and their resident antibodies, as well as the differentiation of T cells and macrophages across multiple species [19-22]. It has been suggested that the differentiation and immune function of chicken DCs can also be influenced by selenium [23,24]; moreover, high levels of organoselenium drugs like ebselen have been shown to inhibit DC-induced proliferation of T cells and secretion of cytokines [4]. In this study, human imDCs were exposed to different dosages of Na$_2$SeO$_3$ and findings revealed that those below 0.1 μmol/L were capable of promoting proliferation. These results partially agree with data reported by Zhang et al [32], who stated that stromal cells enhanced the proliferation of mDCs and differentiation of mDCs into regulatory DCs (in a fibronectin-dependent manner). Since it is unknown why our imDCs proliferated, evaluation of the intake and baseline levels of selenium by our donors is necessary.

Several lines of research have also demonstrated that peroxynitrite is essential for immune function, including the release of proinflammatory cytokines from monocytes at a low concentration [8], and the regulation of inflammation in
immune cells [37,38]. A study showed that there were more than 20 μmol/L of nitrites in the LPS-activated mouse DCs [9], and higher nitrites levels (51.2 μmol/L) in tumor tissue were observed [10]. Here, treatment with 1 mmol/L of SIN1 reduced the viability and chemotactic migration of imDCs, but augmented antigen phagocytosis. In addition, administration of Na₂SeO₃ prior to SIN1 had no effect on the latter phenomenon. From this, we might predict that high levels of peroxynitrite produced in the tumor microenvironment will damage imDCs, but stimulate them to partake in antigen phagocytosis, and 0.1 μmol/L of selenium might be detrimental to the antigen uptake capacity of imDCs.

Redox balance is well known to play an important role in immunity, with research showing that selenium participates in the production of ROS in immune cells [18,28,29,39], peroxynitrite inhibits GPx activity in the absence of GSH [40], and GSH assists in the maturation of DCs [29]. The present experiments showed that treatment with Se, SIN1, and Se+SIN1 significantly increased SOD activity, with the highest amounts of ROS observed in imDCs treated with SIN1. Additionally, exposure to Se+SIN1 lowered ROS levels and SOD activity in imDCs when compared to those given SIN1, but increased GSH content and GPx activity. Among imDCs treated only with SIN1, no change in GPx activity was observed and it is likely this was due to the reduced amount of GSH. Lastly, administration of Se and SIN1 decreased levels of GSH in imDCs and this corresponded to enhanced antigen phagocytosis, although an increase in GSH content had no effect. Such outcomes may be attributed to the formation of superoxide anions [7] and peroxynitrite [8,37,38,41], and thus warrant further investigation.

Various stimuli have been found to contribute to the maturation of DCs by triggering phosphorylation of ERK [24-26]. In our study, results showed that treatment with Se and SIN1 increased ERK phosphorylation and led to enhanced phagocytosis, whereas exposure to Se+SIN1 inhibited ERK phosphorylation and resulted in decreased antigen phagocytosis when compared to imDCs given SIN1. Emerging evidence has demonstrated that ERK signaling is regulated by levels of ROS and GSH, as ERK phosphorylation induced by 2,4-dinitrofluorobenzene is inhibited by GSH [28,29]. Here, an inverse relationship was detected between the amount of ERK phosphorylation and GSH content, which is consistent with the aforementioned findings. In addition, given that phosphorylation levels of ERK in imDCs administered Se+SIN1 were higher than those in the control group and that phagocytosis was unaffected, it is possible that ROS and GSH were responsible.
Similar to ERK, increased MMP2 expression is critical for proper immune function, including being required for the migration of skin DCs [28], activation of DCs [42], and differentiation of monocytes to DCs [43]. In our analyses, low levels of MMP2 in imDCs corresponded to poor chemotactic migration. Curiously, however, we did not see activation of ERK heighten production of MMP2, as reported by others [44], so it is likely that the underlying mechanism is due to p38 [45,46] or other signaling pathways.

Conclusions

In summary, the results of this study suggest that selenium and peroxynitrite enhance levels of ROS or GSH to activate ERK and promote antigen phagocytosis in imDCs, as well as decrease MMP2 to inhibit chemotactic migration independent of ERK.

Conflict of Interest

None.
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