The Safety Property of β-D-Mannuronic Acid (M2000) as a Novel Immunosuppressive Agent on Differentiation, Maturation and Function of Human Dendritic Cells

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Abstract: The study’s background and aim: In this investigation, the safety property of M2000 (β-D-mannuronic acid) on differentiation, maturation and function of dendritic cells, was determined. β-D-mannuronic acid, as a novel immunosuppressive and anti-inflammatory agent, has been tested in various experimental models. In addition, DC-based immunosuppressive drugs can suppress the progression of autoimmune diseases, although, their notable side effects in increasing the risk of infectious diseases and cancers should be considered.

Material and methods: The effect of M2000 on differentiation, maturation and function of dendritic cells was examined. To investigate how M2000 affects human dendritic cells (DC) in a defined inflammatory environment, human peripheral blood mononuclear cells (PBMC) were isolated from healthy blood and monocytes were purified using anti-CD14 microbeads. Monocytes were incubated with M2000 in two different doses (6 and 12 μg/well) along with adding the granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 for inducing monocytes to immature DC and lipopolysaccharide for running DC maturation. The differentiation, maturation and function of dendritic cells were examined with flow cytometry and ELISA method.

Result: The results demonstrate that M2000 has no significant side on differentiation, maturation and function of dendritic cells in immature DC and mature DC process in vitro.

Conclusion: Our findings show that β-D-mannuronic acid (m2000) as a safe agent had no adverse effect on differentiation, maturation and function of dendritic cells which might be recommended as a novel immunosuppressive agent with no or fewer side effects in increasing the risk of infectious diseases and cancers.

Keywords: β-D mannuronic acid, M2000, Non-steroidal anti-inflammatory drugs, Dendritic cell, Flow cytometry, Elisa.

INTRODUCTION

M2000 (β-D-mannuronic acid) is a novel patented (DE-13 10247073) drug which could be classified as a Non-steroidal anti-inflammatory drug (NSAIDs). NSAIDs drugs play an important role in the management of inflammatory diseases [1]. Alginites consist of (1_4) linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) residues of widely varying in composition and sequence [2, 3]. Alginites are abundant in nature since they are seen in both capsular polysaccharides in some bacteria and as a structural element in marine brown algae. For several years,
researchers have tried to identify safer and more effective types of anti-inflammatory drugs. M2000 molecule is an agent with the lowest molecular weight and has less toxicity compared with other NSAIDs. Dendritic cells are highly specialized, professional antigen-presenting cells (APC) that orchestrate the immune response via integration of a variety of signals [4]. They can be found in an immature state in the blood. Immature DCs can uptake antigens and migrate to lymph nodes where they interact with T cells and B cells. Then, in the presence of endogenous or exogenous inflammatory signals, DCs undergo maturation and initiate the adaptive immune response [5]. DC maturation in response to TLR ligands and other pattern recognition receptors such as a CD14 receptor is marked by induction of CD83, CD86 and MHCII [6]. In other words, maturation of DCs is related to enhanced expression of co-stimulatory molecules such as CD80 and CD86 and MHC molecules [7].

In addition to expressing high levels of costimulatory molecules, mature DCs release large amounts of cytokines including IL-12, which can stimulate a Th1 immune response and IL-10 production. The release of IL-10 following up-regulation of costimulatory molecules and production of IL-12, blocks the DC maturation process, subsequently limiting the ability of DCs to initiate a Th1 response [8, 9].

DC-based immune-suppressive drugs can suppress the progression of autoimmune diseases, however, their notable side effects in increasing the risk of cancer and infectious diseases should be considered [10, 11]. In this study, we tried to investigate the safety effect of M2000 on the differentiation, maturation and function of dendritic cells (DCs).

MATERIALS AND METHODS

M2000 Production Method

Cultivation

Production of P. fluorescens alginate was performed in liquid PIA medium (shake flasks) containing bacteriological peptone (20 g/L), NaCl (5 g/L) MgCl2 (1.4 g/L), K2SO4 (10 g/L), and 20 mL of 87% glycerol/liter or in PM5 medium (fermentors) containing fructose (40 g/L), yeast extract (12 g/L), (NH4)2SO4 (0.6 g/L), Na2HPO4·2H2O (2.0 g/L), NaCl (11.7 g/L), and MgSO4·7H2O (0.3 g/L), and clerol FBA622 (antifoam, 0.5 g/L). The media were supplemented with proteases—Alkalase 2.4 L and Neutrase 0.5 L from Novo Nordisk (0.15 ml/L each in PIA and 0.25 ml/L each in PM5) in order to reduce extracellular alginate-lyase activity.

Alginate Isolation and Purification

Isolation of deacetylated alginate from culture supernatants was performed by adding an equal volume of isopropanol. The precipitate was collected by centrifugation and washed with both 70% and 96% ethanol. The dried precipitate was fully dissolved in a small volume H2O and DNase I and RNase A (10 μg/mL of each) were added and incubated at 37 °C for 4 h. The Pronase E (12.5 μg/mL) was then added and incubated overnight at 37 °C. Using dialysis tubing, one end was clamped and poured the solution into the tube using a glass funnel (the bag was 2/3 full, allowing room for expansion) and clamped another end. Then it was dialyzed against 2.5 L distilled H2O in a 4–5 L beaker, stirred very slowly using a magnetic stirrer. The water was changed after 2–3 days. The alginate solution was then lyophilized.

Deacetylation

Lyophilized alginate was dissolved in water to obtain a 0.5% (w/v) alginate solution and added 0.1 M NaOH and stirred 1 hour at RT and neutralized with HCl. Then it was dialyzed and lyophilized again.

Hydrolysis

Following, deacetylating alginate was dissolved in water at concentration 1 mg/mL and adjusted to pH 2 with 1 M HCl and incubated at 110 °C for 8 h in an autoclave. It was neutralized with 1M NaOH.

Collection of Monomer Fraction

Gel filtration chromatography (super peptide column from Amersham Biosciences) was used to remove salt. Uronic acid containing fractions were lyophilized and subjected to anionic exchange chromatography [12]. The monomer fraction was collected and confirmed by TLC.

Uronic acid determination Based on the method of Blumenkrantz and Asboe-Hansen (1973), which is a Galambos’ modified procedure [13], diphenyl
reagent and H$_2$SO$_4$-Tetraborat Solution was used to build a pink colored chromophore. Using neutral sugars in 10-fold excess gives a brown color which interferes with detection of the uronic acids. The absorbance was detected 1 at 520 nm in a spectrometer. There was a linear relationship between the absorbance and the amount of sugar that was present in the original sample. It was non-stoichiometric and therefore it was necessary to prepare a calibration curve using a series of standards of known uronic acid concentrations, pure alginate [14].

**DC Differentiation and Maturation**

In vitro human monocyte differentiation into DCs was performed as described with modifications [15]. Six human peripheral blood mononuclear cells (PBMC) were isolated through Ficoll-Hypaque (Mediatech Cellgro, USA) density gradient centrifugation from buffy coats provided from healthy blood donors (after informed consent protocol of the Declaration from Iranian Blood Transfusion Organization). Monocytes were purified from peripheral blood mononuclear cells using anti-CD14 microbeads (Miltenyi Biotec, Germany) and MACS column. The purified monocytes (> 95% purity) were cultured at 37°C in 24-well plates (700,000 cells per well) in 3 mL of serum-free AIM-V medium (Invitrogen) containing 100 ng/mL human granulocyte-macrophage-colony-stimulating factor (GM-CSF) (Peprotech, UK) and human interleukin-4 (IL-4, 20 ng/mL, R&D Systems, USA). Cells were cultured with two different doses of β-D-mannuronic acid 6 µg/well as the low dose and 12 µg/well as the high dose and 6 wells were selected as control (non-treated by M2000). A total of 0.5 mL of fresh medium with GM-CSF and IL-4 was added to the cell cultures on day 3. To induce DC maturation, lipopolysaccharide (LPS, 1 µg/mL, catalog no. L2654, Sigma-Aldrich, Germany) was added to cell cultures for 24 hours on day 5, and again two doses of β-D-mannuronic acid were added to the wells 4 hours before adding the LPS.

**Monoclonal Antibodies (mAbs) and Flow Cytometry**

Harvested cells were washed twice with PBS and resuspended in 100 µL PBS supplemented with 0.5% bovine albumin serum (BSA) and 0.1% sodium azide for staining. Cells were incubated for 10 min at room temperature with an Fc receptor blocking solution (BioLegend, San Diego, USA). Cells were then incubated at 4°C for 30 min with the following monoclonal mouse-anti-human antibodies (mAb): FITC-conjugated mAbs against cell surface molecules including CD83, CD14 and PE-conjugated mAbs against CD1a, CD86 and PE CY5-conjugated mAbs against MHCII (eBiosciences, USA). In all experiments, isotype controls were included using an appropriate mAb of the same Ig class or subclass. After staining, cells were washed twice in PBS supplemented with 0.5% BSA and 0.1% sodium azide and resuspended in PBS with 0.99% paraformaldehyde. Flow cytometry was performed using a Cytomics FC-500 flow cytometer (Beckman Coulter, India), and all subsequent analyses were made with FlowJo software (Tree Star, USA).

**Cytokine Measurement**

DC cytokines production was detected respectively in supernatants of DCs culture (IL-12p70 and IL-10). Supernatants were collected and kept frozen at -70°C until use. Cytokine concentration was measured by enzyme-linked immunosorbent assay (ELISA) Kit (BenderMed system, Austria) according to the manufacturer’s instructions.

**Statistical Analysis**

The results are expressed as means ± SEM. All data was analyzed using the SPSS 20 application. For non-normally distributed data, the nonparametric Friedman test was used in evaluating the statistical differences between three or four groups which were related to each other. Furthermore, the nonparametric Wilcoxon signed rank test was used to evaluate the differences between two groups with each other. $P$ value<0.05 was considered significant. All calculations were performed using GraphPad Prism software 5 (GraphPad Software Inc, USA).

**RESULT**

We previously reported that β-D-mannuronic acid is an anti-inflammatory agent which was tested in various experimental models. To extend this study, we tested whether it has any effect on differentiation, maturation and function of dendritic cells in vitro.
Differentiation

We examined the level expression of CD14 and CD1a in monocyte and immature Dendritic Cells with two different doses (6 and 12 μg/well) of M2000. Our findings showed that there was no significant difference between control group and the groups which were treated by β-D-mannuronic acid with 6μg/well and 12 μg /well in immature dendritic cells (Fig. 1).

Maturation

In order to determine the effects of two different doses (6 and 12 μg/well) of β-D-mannuronic acid on CD83, CD86 and MHCII

![Graph](image1)

**Fig. (1).** (a,b). Effect of M2000 with two doses, low (6 μg/well) and high (12 μg/well) on expression of CD14 and CD1a in monocyte and immature dendritic cell. There was no significant difference between control group and treated groups (P<0.05 was considered as significant).

![Graph](image2)

**Fig. (2).** Effect of M2000 with two doses, low (6 μg/well) and high (12 μg/well) on expression of CD83, CD86 and MHCII in immature and mature dendritic cell. There was no significant difference between control group and treated groups (P<0.05 was considered as significant).
expression, DCs were cultured in the presence of these two different doses (6 and 12 μg/well) of β-D-mannuronic acid. The expressions of MHC-II and co-stimulatory molecules were analyzed by flow cytometry. As shown in Fig. 2, co-stimulatory molecules and MHC-II expressions were not significantly different between the control group and the groups which were treated by β-D-mannuronic acid with 6 μg/well and 12 μg/well in immature and mature dendritic cells.

Function

Supernatants of cultured DCs were collected and cytokines concentration were measured by ELISA method. Our data showed that there was no significant difference between control group and the groups which were treated by β-D-mannuronic acid with 6 μg/well and 12 μg/well in mature dendritic cells (Fig. 3).

DISCUSSION

M2000 (β-D-mannuronic acid) has been tested as an anti-inflammatory and a novel immunosuppressive agent in various experimental models, such as animal models of multiple sclerosis, nephrotic syndrome, rheumatoid arthritis and immune complex glomerulonephritis [16-18]. The molecular mechanism of therapeutic efficacy of this novel patented (DE-13 10247073) drug is based on its inhibitory effects on matrix metalloproteinase2 activity, immune cells infiltration in inflammatory foci, reduction of the level of inflammatory cytokine IL-6, the decrease in antibody production, and induction of apoptosis using fibro sarcoma cell line [17]. Based on our research on an extensive scale, mannuronic acid is a very safe anti-inflammatory and immunosuppressive drug and in addition, it has the lowest molecular weight with no gastro-nephrotoxicity, in comparison with conventional NSAIDs such as diclofenac. This drug without having a ulcerogenic effect on stomach of animals revealed a potent therapeutic efficacy in disease control [19]. In the past few years, the role of antigen presenting cells (APC) has been shown as a central event for adaptive immune responses against extracellular bacteria, fungi, and other pathogens. DCs as an APC play a primary role in host defense and they are the only APCs capable of activating naive lymphocytes, which leads to initiate the protective immune responses against intracellular pathogens [20, 21]. In recent studies, it has been demonstrated that the degree of DC differentiation plays an important role in determining immune response [22]. In humans, CD83, as immunoglobulin superfamily member, has been shown to be upregulated after DC maturation [23]. In addition, the investigations showed that the CD86

![Fig. (3). (a,b). Effect of M2000 with two doses, low (6 μg/well) and high (12 μg/well) on expression of IL-10 and IL-12 in mature dendritic cell. There was no significant difference between control group and treated groups (P<0.05 was considered as significant).](image-url)
and MHCII levels usually increase significantly during DC maturation in humans and mice. In general, the expression of co-stimulatory molecules, CD80 and CD86, on DCs are known as a crucial secondary signal for the generation of effector T cells. Therefore, the up-regulation of CD80 and CD86 leads to enhance the activity of DCs. It is also shown that lipopolysaccharide (LPS) increases CD80 and CD86 expression on DCs at inflamed sites [24, 25]. It suggests that CD80 and CD86 play a crucial role in the initiation and maintenance of an immune response. In the other words, all surface antigens mentioned above usually increase significantly during DC maturation [26], moreover, it has been demonstrated that Th1 and Th2 CD4+ T cell polarization are controlled by DC factors, such as the cytokines which they produce [27]. Changes in the inflammatory environment can alter DC functions at all steps of their differentiation/maturation and effector functions [28]. In the recent years, one of the most important therapeutic targets of researchers is DC suppression in the control of autoimmune diseases; however, the notable and dangerous side effects in progression and development of cancer and infectious diseases could be considered. In the present study, we have demonstrated the safety of this drug on differentiation, maturation and function of dendritic cells.

Collectively, this investigation was aimed to determine the role of M2000 as a safe molecule on DC differentiation, maturation and function process under in vitro condition. However, the immunosuppressive effects of this drug have been proved in previous studies, our results showed that M2000 is a safe drug without any adverse effect on DC differentiation, maturation and function process in human immature and mature dendritic cells.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Declared None.

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