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Effect of Cytosolic Extract of Alternaria Alaternata Fungus on Monocyte-Derived Dendritic Cell Phagocytosis Ability and T-Lymphocyte Proliferation in the Presence of Myelin Basic Protein

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Abstract

Background: Multiple sclerosis (MS) is an autoimmune disease with impairment in function of CNS, meanwhile macrophages and dendritic cells (DC) can cause inflammation and damage to the myelin of nerve cells by releasing Reactive oxygen species (ROS) and other harmful substances when these cells get matured. We investigated the effect of *Alternaria alternata* (*A. alternata*) extract on phagocytic T cell stimulation activity of DC pulsed with Myelin Basic Protein (MBP) as a laboratory model of MS.

Materials and Methods: Plastic adherent monocytes were cultured with granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-4 for converting these cells to MoDc (Monocyte-Derived Dendritic Cell), pulsed with MBP, matured in the presence of monocyte-conditioned medium (MCM) in control group and MCM+Alternaria. *alternata* extract in treatment groups. Phagocytic activity of DC was evaluated and T cell responses were investigated by MTT test.

Results: Phagocytic activity in treatment groups decreased significantly in compare with control group. Meanwhile, DC couldn't stimulate T cell proliferation.

Conclusion: *A. alternata* extract decreased phagocytic activity of MoDc-pulsed with MBP and had no effect on T cell proliferation may provide a new strategy on immunotraphy of multiple sclerosis.

Introduction

Monocytes (MO) account for 3-7% of peripheral blood cells that are able to differentiate into denderitic cells (DC) or macrophage. It's main surface marker is CD14. MOs express high level of MHCII, and meanwhile produces large amount of TNF-α and ROS metabolite that both of them act as a damaging factor in multiple sclerosis (MS) [1].

MS is a progressive, neurodegenerative and autoimmune disease of central nervous system (CNS) that affected more than two million people all around the world. Since progressive lesions in central nervous system are irreversible, finding of approaches to reduce or impeding of emerging of these lesions must be beneficial [2]. Myelin basic protein (MBP) is the best known auto antigen in CNS [3]. *Alternaria alternata* (*A. alternata*) is a saprophytic fungus affecting plant tissue and can rarely cause some problems in human (ie; in mucous membrane, skin and derma) [4]. The antigen of *A. alternata* namely Alt-a1 has been used for immunotherapy and modulation of immune responses in asthma and allergic diseases [5].

In this study that has been carried out as a laboratory model of immune responses triggered against auto antigen involvement in MS, immature denderitic cells (ImDC) produced invitro pulsed by MBP (as a antigen) and induced to MS model of DCs and then at the present of maturation factor and the extract of *A. alternata* were cultured. Phagocytic activity of DCs was evaluated and then the capability of proliferation and differentiation autologous T cell co-cultured with these cells (MBP-pulsed DCs) were identified.

Materials and Methods

The aim of this study is production of DC in order to immunotherapy and alleviating of MS symptoms. This study has been conducted in cleaning room of research center of biotechnology at Uremia University from September 2011 till March 2012. The variety of tests have been used to evaluation of phagocytic activity of MBP-pulsed DCs. Stimulation of T cell induced by DC were examined by MTT test.

Preparation of Fungus extract:

A. alternata spore purchased from Irans industrial and scientific research organization (PTCC 5248) was cultured on Sabaroud dextrose agar at 25°C for 5 days. Mature fungi subculture on Czapek's agar to producing a large amount of spores.

The amount of 1×10^7 spores were collected and passed through Tampon transported on solution of Yeast nitrogen base (37ºC, 5% CO2, 5% humidity) for 48 hours in order to improvement of growth of mycelium. Then grown-up mycelium centrifuged (2000 g), were added to PBS buffer

containing 2 Mm Protease inhibitor (Sigma-USA); 50 Mm EDTA (Sigma-USA); 50 Mm Tris-Hcl and Sonicated on a sonicator (20,000 AMP), 10 sec interval (apparatus 10 sec is off and 10 sec is on) and duration 20 minutes. After sonication, the resulted Homogeny centrifuged (7000 g and 4ºC), the supernatant was dialyzed by a dialyzing bag (cut off: 10,000) full off.

Distilled water at 4ºC and lyophilized to reducing of volume of water. The resulted extract passed through a filter with fine pores (22 mμ in diameter). The protein of solution was measured by BRADFORD method [6], and the extract stored at -70ºC.

Preparation of peripheral Blood Mononuclear cell (PBMC):

Heparinized blood were obtained from volunteer donors (200 U/ml) in sterile condition and mixed with equal volume of culture medium RPMI-1640 (Gibco-UK). Diluents blood transported very gently on Fiqule (Sigma –USA) and centrifuged for 15 minutes in 800 g.The resulted PBMC located between Fiqule and diluents blood were collected, washed by RPMI-1640 and centrifuged for 10 minutes in 480 g in order to deleting of Fiqule. Cellular pellet washed again by RPMI-1640 for 10 minutes in 200 g. The number and viability of cells were assigned by Tripan blue.

The production of induced DCs at the present of MBP and its treatment with *A. alternata* extract:

The preparation of induced DC has been occurred at 3 stages; at first stage and 0th day, the adherent cells converted to IMDC by using GM-CSF and IL-4 (500 U/ml). At the second stage (3rd day), equal amount of GM-CSF and IL-4 (Sigma-USA) were added to culture to maturation of DC. At the third stage (4th day), MBP was added to culture to induction of auto reactive DC (In-vitro). Additionally, the amount of 50-100 μg/ml (MBP+Ext) of *A. alternata* extract and MCM (monocyte conditioned medium) 20 ng/ml were added in treatment group. But MCM has been used only in control group (MBP). All of these stages happened at sterile condition in incubation (37ºC; 5% CO_2). At 7th day, DCs were harvested, morphological changes of DC; stimulation of T cell proliferation and phagocytic capability were assigned.

Evaluation of DC-induced T cell proliferation by MTT test:

After harvesting of DCs, a cell suspension of DCs containing 1×10^5 cells from control group has been provided and co-cultured with autologous T lymphocyte at ratio 1/10 on 96 wells plate. This experiment conducted in 5 replicate, also the culture medium of RPMI-1640 was used as blank on 3 wells. After 72 hours incubation, 25 μl of MTT solution (5 mg/ml) in PBS were added on each well and incubated for 4 hours, during this time, living and proliferating cells in combine with MTT gave rise to furamazon. The furamazone could be dissolved by adding 100 μl of (Di Methyl sulfoxaid) DMSO. Finally, the color intensity at length wave of 490 nm was assigned and stimulation index was calculated by this equation:

$$\frac{OD\ Blank - OD\ control\ \ group}{OD\ Blabk - OD\ control\ \ group}\times 100$$

The evaluation of phagocytic activity:

20 μl latex bead florescent (FITC-conjugated) at concenteration $2/5\times10^5$ bead per ml, incubated on human serum AB^+ for 7 minutes. Then the amount of 25×10^5 MDC along with 60 μl phagocytic buffer reached to total volume 100 μl in 96-wells plate. After 48 hours incubation, the cells were harvested and by Quenching buffer the false florescent quenched.

At last the number of engulfed latex bead both in control group (MBP) and treatment group (MBP+Ext) were assigned by flow cytometery (DAKO-Germany). Pagocytic index was identified according to this equation:

$$Ph\% = \frac{GreenDC}{TotalDC}\times 100$$

All of the statistical analysis was calculated by SPSS-17, Tukey test has been used for data analysis. $p<0.05$ was considered significant. Microsoft excel has been used for drawing of figures. The data were presented as Mean±SEM.

Results

Morphological studies were conducted by invert microscope at different magnifications and the results showed that, after three days, the adherent cells (at the present of GM-SCF, IL-4) lost its adhesion and gave rise to treatment group of floating cells. These cells looked larger than monocytes containing large intracellular granules (Fig. 1).

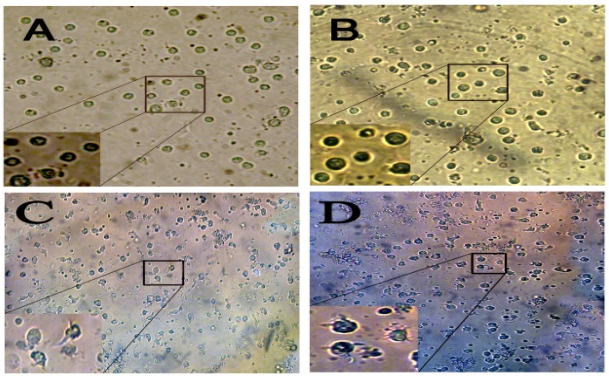

Figure 1. Photography of control and treat cells with invert microscope (200 x) A: peripheral blood monocyte separated, B: Dendritic cells matured in presence of MBP+MCM, C: Dendritic cells matured in presence of MBP+Ext 50 mg/ml, D: Mendritic cells matured in presence of MBP+Ext 100 mg/ml

The results of phagocytic evolution have been shown at two forms: A) the percentage of phagocytosis in each DC. B) phagocytosis of whole cells which have been showed at figure 3 and figure 4 respectively. Also this data has been showed graphical in figure 2 the results of fluorescence intensity and the percentage of phagocytosis

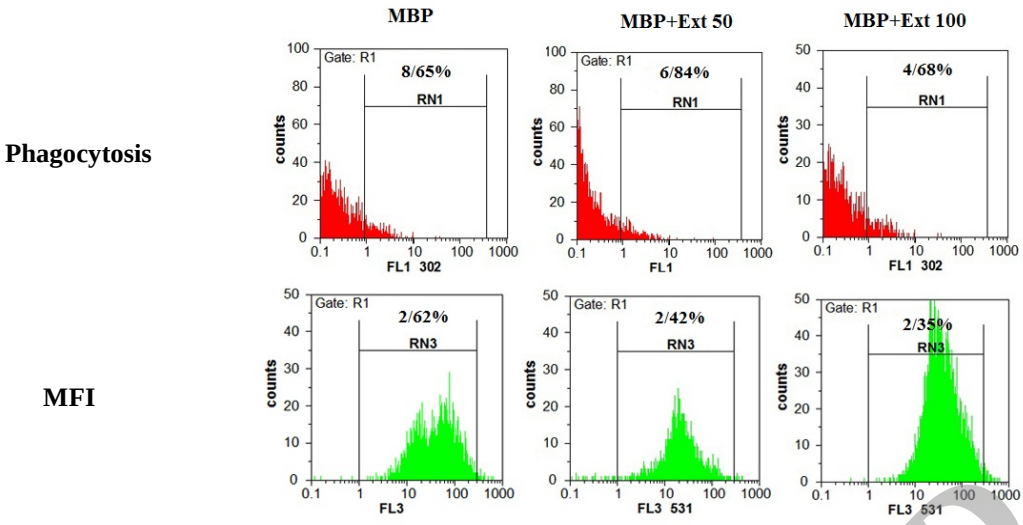

Figure 2. Examples graphical of flocytometry result of phagocytosis intreatment groups

(showed in figure 3) indicated that the rate of phagocytosis in both treatment groups decreased, significantly (p<0.05). Also, there is a significant increase in phagocytosis following using increasing amounts of fungi extract from 50 µl/ml up to 100 µl/ml. Difference between two treatment groups has been shown (p<0.05).

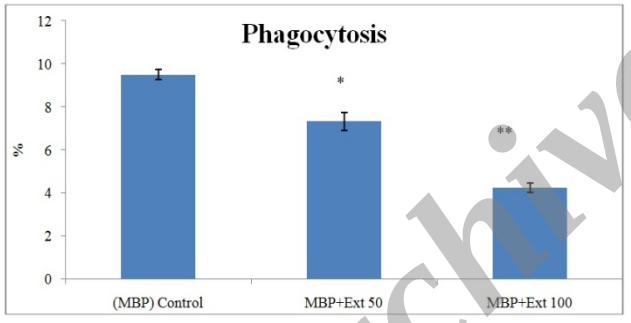

Figure 3. Percentage of total cell phagocytosis and antigen uptake [**: Indicate different of significant at level of p<0.001 and *: indicate different of significant al level of p<0.05 in analogy with control group (MBP)]

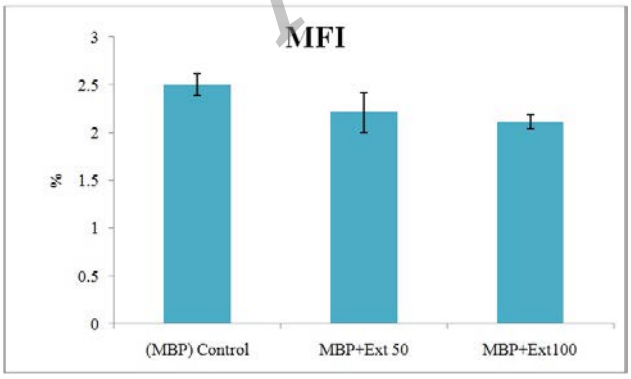

Figure 4. Percentage of any cell phagocytosis and antigen uptake

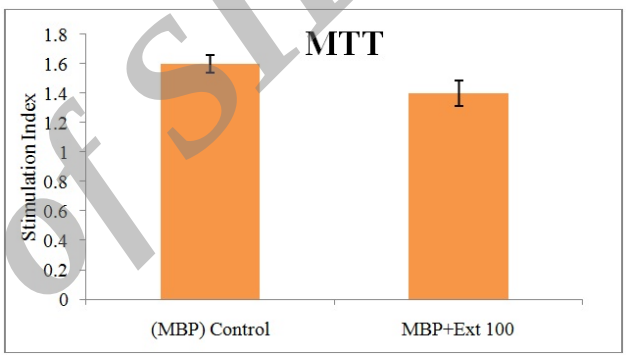

Figure 5. Index of lymphocyte proliferation stimulation by dendritic cells

Discussion

Morphological changes in three groups of DC indicated that a qualitative maturation in these cells occurred. These results showed that in control group (MBP), the maturation of cells happened relatively. However, in treatment group, the gross characterization cells indicated that fully maturation of DC tooke place, and these morphological changes expressed more apparently with increasing of extract doze from 50 up to 100 µl/ml. Additionally, phenotipical tests verified these results (Data have not been shown).

A. alternata antigen could be a potent antigen for immigration, maturation and presentation of antigen by DC In-vivo or In-vitro [4]. Gredler et al., showed that myelin-matured DC expressed costimulatory markers (such as CD80 and CD83) lower than control (DC matured by LPS). But, HLA-DR was higher than control. So, it can be suggested that myelin can make DC semi mature [7]. In CSF of individual that affected by MS and showed inflammatory signs, the number of plasmocytoid immature DC were higher than those without inflammatory sign [8]. ImDC were impotent in activation of T cell proliferation, so that it can be hypothesized that

these cells induce tolerance. Moreover, these cells can return to the normal situation by IFN-β treatment [9, 10].

Collectively, it can conclude that ImDCs play a key role in progression of MS in affected persons. Bartosike at al. showed that IFN-β, a standard drug for treatment of MS, acts surprisingly in maturation of DC in MS-affected persons, and suggested that the results of DC maturation by fungi extract (doze 50 µl/ml) were comparable with treatment group [11]. Another notable characterization of DC is phagocytic capabilities in such a way it can be expected that turning of ImDC into mDC can decreased the ability of DC to phagocytosis and also all needs to engulfing of antigen such as; surface receptor. In contrast, it increases the presentation of antigen which leads to escalating of DC ability in T cell stimulation [12].

The figure of phagocytic evolution in DC indicated that the fungi extract reduced the phagocytic capabilities and engulfing of antigen in DC compare with whole cells (p<0.05). This reduction became more apparent when fungi extract at doze of 100 µl/ml has been used (p<0.001). This effect was associated with plaque formation in CNS. So that, it was associated with increasing of ROS released from inflammatory cells. Meanwhile, when these cells become mature, it can cause decreasing of detrimental factors releasing [10, 13]. Treatment of murine DC involvement in EAE (Experimental Autoimmune Encephalomyelitis) by IL-10 & LPS, can lead to diminish of phagocytic ability of DC, Stienbrink at al. considered it as a promising therapeutic effect [14]. Thus, GA (Glatrimar astat) alleviate MS by impending of T cell proliferation [5, 9, 15]. However, in spite of decreasing of phagocytic activity, some of DCs continue to phagocytosis that showed no significant changes in phagosytic activity and antigen engulfment Showed in MFI (Mean Fluorescence Image) figure. Since the fungi extract has been added to culture one day after the onset of test, the figure of 3 showed that the fungi extract couldn't decrease the phagocytic activity MBP-pulsed DC. The triggering of autoreactive T cell proliferation would assign the severity of MS. Since the induction of autoreactive Th1 responses in susceptible individual can trigger MS [16]. *A. alternata* extract has no effect on increasing or decreasing of MS severity.

Finally, it has been suggested that the decreasing of MBP-pulsed DC phagocytosis along with ineffectiveness on triggering of Tcell responses implies that *A. alternata* might has beneficial therapeutic effect on MS. Surely, this effect needs more investigation. So that, the evolution of apopetic and cytotoxic effect of *A. alternata* extract on cells of immune system and also the amount of NO relesed from DC can provide a better understanding of this compound.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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References

1. Then Bergh F, Dayyani F, Ziegler-Heitbrock L. Impact of type-I-interferon on monocyte subsets and their differentiation to dendritic cells an in vivo and ex vivo study in multiple sclerosis patients treated with interferon-beta. J Neuroimmunol 2004; 146(1-2): 176-188.
2. Huang YM, Yang JS, Xu LY, et al. Autoantigen-pulsed dendrite cells induce tolerance to experimental allergic encephalomyelitis (EAE) in Lewis rats. Clin Exp Immunol 2000; 122(3): 437-44.
3. Lamers KJ, de Reus HP, Jongen PJ. Myelin basic protein in CSF as indicator of disease activity in multiple sclerosis. Mult Scler 1998; 4(3): 124-126.
4. Kobayashi T, Iijima K, Radhakrishnan S, et al. Asthma-related environmental fungus, Alternaria, activates dendritic cells and produces potent Th2 adjuvant activity. J Immunol 2009; 182(4): 2502-2510.
5. Viswanath P, Kurup B, Hari M, et al. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternate. Peptides 2003; 24(2): 179-185.
6. Bradford HF. Trends Biochem. Sci 1987; 12: 344-347.
7. Gredler V, Ebner S, Schanda K, et al. Impact of human myelin on the maturation and function of human monocyte-derived dendritic cells. Clin Immunol 2010; 134(3): 296-304.
8. Huang YM, Kouwenhoven M, Jin YP, et al. Dendritic cells derived from patients with multiple sclerosis show high CD1a and low CD86 expression. J Mult Scler 2001; 7(6): 95-99.
9. Sanna A, Fois ML, Arru G, et al. Glatiramer acetate reduces lymphocyte proliferation and enhances IL-5 and IL-13 production through modulation of monocyte-derived dendritic cells in multiple sclerosis. Am J Clin Exp Immunol 2005; 143(10): 357-362.
10. Comabella M, Lopez C, Alzayat H, et al. Altered maturation of circulating dendritic cells in primary progressive MS patients. J Neuroimmunol 2006; 175(8): 183-191.
11. Bartosik-Psujek H, Tabarkiewicz PJ, Pocinska K, et al. Immunomodulatory effects of IFN-b and lovastatin on immunophenotype of monocyte-derived dendritic cells in multiple sclerosis. Arch Immunol Ther Exp (Warsz) 2010; 58(4): 313-319.
12. GanjiBakhsh M, Nejati V, Delirezh N, et al. Mixture of fibroblast, epithelial and endothelial cells conditioned mediainduce monocyte-derived dendritic cell maturation. Cell Immunol 2011; 272(1): 18-24.
13. Beebe AM, Cua DJ, Malefyt R. The role of interleukin-10 in autoimmune disease: systemic lupus erythematosus (SLE) and multiple sclerosis (MS). Cytokine Growth Factor Rev 2002; 13(4-5): 403-412.
14. Steinbrink K, Wolfl M, Jonuleit H, et al. Induction of tolerance by IL-10-treated dendritic cells. J Immunol 1997; 159(13): 4772-81.

15. Abtahi MF, Delirezh N, Hobenaghi R and Mossayebi GH. Therapeutic effects of all-trans retinoic acid on experimental autoimmune encephalomyelitis and its role in T-helper lymphocyte responses. J Tehran Univ Med Sci 2012; 69(11): 710-717

16. Martin R, McFarland HF, McFarlin DE. Immunological aspects of demyelinating diseases. Annu Rev Immunol 1992; 10: 153187.

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