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Dendritic Cells Bearing HLA-G Inhibit T-Cell Activation in Type 1 Diabetes

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ABSTRACT

HLA-G is normally expressed on human trophoblast cells. It is a non-classical MHC molecule class I b. The role of HLA-G in diabetic type 1 is not known.

We investigated the role of IFN-β in induction HLA-G expression on the monocyte derived dendritic cells (DC) in diabetes type 1.

Treatment of dendritic cell with IFN-β in vitro from diabetic patients (n=20) and normal subjects (n=20) resulted to the production and expression of HLA-G on these cells from both groups. However, comparison of DC from the diabetic patients with DC from the controls revealed lower levels of HLA-G molecules in DC from diabetic patients. Using mixed lymphocyte reaction (MLR), it was found that DC expressing HLA-G mediated the inhibition of autologous T cell activation.

It is concluded that IFN-β can increase HLA-G in DC from diabetic patients; subsequently it may prevent the immune regularly pathway in the diabetic pathogenesis.

Key words: Autoimmunity; Diabetes type 1; Dendritic cell; HLA-G; IFN-β (Interferon β)

INTRODUCTION

Diabetes type 1 is an autoimmune disease in which the patients produce autoantibodies against antigens of islet beta cells of pancreatic gland, however T-cells play a vital role in the inflammatory disorder of the islet cells and in modulating self reactive immune response.[1,2] The T cells which recognize self-antigens are normally under strict physiological control for activation.

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The exact mechanisms of how and under what circumstances the autoreactive T cells are activated and how the tolerance to the antigens of β-cells is diminished remain to be elucidated. However, an important event in the cascade of antigen recognition and activation is presentation of the antigen to the T-cells by antigen-presenting cells (APC). Variety of positive as well as negative regulatory signals are provided by APC in particular by dendritic cells.[1-3]

Peripheral activation of auto reactive T cells, which eventually enter in the islet cells in pancreatic gland, is considered an important event in initiating the immune-pathogenesis cascade in diabetes type 1.[4] Prevention of activating T cells in the islet cells is very important in the diabetes type 1. It is likely that the cytokines as

soluble mediators of the immune system may play a part in this modulation.

Interferon-beta (IFN-β), a type 1 Interferon, is normally induced by viruses or ds RNA[5] which is considered an immunomodulatory molecule for dendritic cells.[6]

Moreover IL-12, a key cytokine produced by dendritic cell, promotes inflammatory processes and skews T cell response to Th1 that are likely to be associated with disease activity in type 1 diabetes.[7] The immune response is naturally regulated by various mechanisms aimed at controlling hyperactivity and preventing self destruction . An association between HLA DR3 and DR4 and type 1 diabetes is generally accepted.[1, 2]

HLA-G a non-classical MHC class I antigen is characterized by a limited polymorphism and is encoded by an altered native transcription of spliced mRNAs resulting in at least seven different isoforms, including membrane bound HLA-G1, G2, G3,G4 and HLA-G5 (formerly HLAG15), G6 (formerly HLA-G25), and G7 proteins.[8] These were first detected on extravillus cytotrophoblast cells.[9] These antigens play an important role in cancer, pregnancy and transplantation .Moreover functional studies have identified HLA-G as a key mediator in immune tolerance.[10-12]

The presence of HLA-G and its ligands have also been demonstrated in autoimmune tissue lesion,[13] suggesting that the immune biological role of HLA-G is broader than previously assumed.

In this study we analyzed the effect of IFN-β in inducing HLA-G expression on dendritic cells in type 1 diabetes. We also investigated the role of DC bearing HLA-G in inhibition of autologous T- cell activation.

MATERIALS AND METHODS

Patients

The patients were those admitted in the endocrinology department of Shariati hospital, a teaching hospital affiliated to Tehran University of Medical Sciences. The diagnosis of illness was made, according to clinical and laboratory findings, by consultant endocrinologist. The patients who took part in this study were informed clearly of the nature of this investigation and they gave the consultant endocrinologist a written consent for taking blood sample for measuring certain variables as well as using the blood cells for the present study .The blood samples which served as normal control were obtained from Iranian blood transfusion services.

To study the immune phenotype and cell surface expression of monocyte derived DC bearing HLA-G , 50ml blood samples were collected from 20 patients with diabetes type 1 , with median age 29.3 and 20 normal controls with median age 30.76. All diabetic patients were under treatment with insulin and showed a mean fasting blood sugar of 162 mg/dl (Table 1).

Table 1. Features of diabetic patients and normal subjects who participated in the study.

Total numbers	Patients (n=20)	Controls (n=20)
Gender	Male: 11, Female: 9	Male: 16, Female: 4
Mean age (range) years	29.3 (13-49)	30.76 (22-44)
Insulin dependent	All	None
Family history of Diabetes	All	None
Fasting blood sugar (F.B.S) mg/dl	165.5±62.6	90.3±10.4

Generation of Dendrite cells from Adherent Blood Mononuclear Cells

Blood mononuclear cells (MNC) were isolated from heparinzed blood by centrifugation on a Ficoll histipaque 1.077 (sigma, U.S.A). The cells from the interphase were collected and washed three times with RPMI 1640 medium (sigma).

Cell viability was determined by trypan blue exclusion. Monocytes 95% isolated from MNC by plastic flasks adherence. 5-8 $\times 10^6$ /ml of MNC were then cultured in 5ml RPMI 1640 medium supplemented with 10% fetal calf serum, 50units/ml penicillin and 50µg/ml streptomycin. After 2h of incubation at 37℃ in an atmosphere of 5% CO2, the adherent cells were cultured in complete medium containing 1000 units /ml rhGM-CSF (Serotec, UK) and 500unit/ml rh IL-4 (Serotec) to generate DC.

Non adherent cells were removed for T cell isolation.

The T cells (>85%) were isolated by nylon wool. These cells were counted using monoclonal antibody CD3 (DAKO) and flowcytometry and were then frozen in 10% dimetylsulfoxide containing a mixture of 60% RPMI and 30% FCS.

Isolation of β Islet Cells of Insulinoma

A 49 year old male patient was diagnosed to suffer from hyper insulinoma. The insulinoma was surgically removed from the lateral part of the pancreas corpus. The tumor was found to be non-malignant. Islet – β cells were isolated from the insulinoma tissue by treatment with collagenase digestion method.[14,15]

Having discarded tissue culture medium, the beta cells were suspended in RPMI 1640 medium; cells were washed twice, with PBS buffer pH 7.4 and then were incubated with 0.05% trypsin and 0.053mM EDTA at 37°Ċ for 7 min.

When all cells were detached from the plastic dish, the cell suspension was washed twice with PBS buffer. The identification of Islet β-cells were determined by the technique of immunohistochemistry using monoclonal antibody against insulin (Dako).

Insulinoma cells were lysed to obtain the soluble antigens of β-cells. This was achieved by adding 100 µl lysis buffer containing 100 mM NaCl, 50 mM Tris-HCL, pH 7.4, 0.5 mM EDTA, 0.1%SDS, 100 µM phenylmethylsulphonylfluoride and 1mM isopropyl fluorophosphates.[16] 3000×10^3 cells were immediately snap-frozen in liquid nitrogen and then they were thawed to lyse the cells and to obtain a molecular solution. The suspension was centrifuged at 12000 g for 5 min at 4°C.The concentration of protein in an aliquot of the clarified supernatant was determined by Bradford method.[17] An aliquot of protein antigen solution (50 µg/ml) was added on day 4 to dendritic cells culture for antigen processing by these cells.

Flowcytometry

Surface expressions of immune cells were determined by flowcytometry using a fluorescent activated cell sorter (Partec Pas).

Cultured DC cells were washed once with PBS, pH 7.4, and centrifuged at 1800×g for 8 min at 25°C. To each 100 micro liters of cell suspension FITC conjugated monoclonal antibodies against CD83 or CD14 (DAKO) were added. Samples were incubated for 15 min at 4°C in the dark. The cells were washed twice with PBS and resuspended in 200 µl of PBS. Finally in order to identity and enumerate the cells, the cell suspensions were analyzed by flowcytometry.

DCs were gated. Background staining with prevalent mouse IgG was subtracted from each specific staining. The results were expressed as percentage of cells staining positively with CD83 or CD14.

Maturation of Monocyte-Derived DC and Phenotype Analysis of DC

The immature monocyte-derived DCs were then activated by adding TNF-α (10ng/ml) and IFN-β (1000units/ml, Avonex; Biogen, Cambridge) into the culture to promote maturation of these cells and HLA-G expression, which was detected by anty HLA-G monoclonal antibody conjugated to Fluorecien isothyocinate (FITC).

Phenotype analysis of monocyte-derived DCs was carried out using flowcytometry.

Expression of the CD83 marker indicating the maturation of DC was detected using anti CD83 monoclonal antibody. 76.7±18.4 of cell population showed expression of CD83.

RNA Extraction, cDNA Synthesis and Specific PCR

Total RNA was extracted from the DCs treated with IFNβ and from jeg-3 coriocarcinoma cell line (National cell bank of Iran). This cell line was used as a positive control for HLA-G expression, using RNAzol Bee reagent (Biosite, TABY, Sweden) based on the manufacture instructions. RNA was checked for quality by electrophoresis using 1.5% agarose gel. The transcriptional levels of HLA-G were evaluated by reverse transcriptase PCR in comparison with those of jeg-3 cells. Strand cDNA was first synthesized using 3µg of total RNA in 20 µl reaction mixture consisting of 5X reaction buffer 4µl ; 10mM dNTPs 2µl; 100µM dithiotheritol (DTT) 1.5 µl; 10 pMol/ml random hexamer (N6)1 µl, and M-MLV reverse transcriptase 200units(Metabion). Thereafter, the mixture was incubated at 42°C for 45 min.

The following oiligonucleotides were used in this study

HLA-G1/2: HLA-G1/2for: 5-TCATGCTGAGATG GAAGCAG; HLA-G1/2-REV: 5-TCTCCACAGCAC AGCCAGC AND β actin: for: 5-TGGCCACGGCTG CTTCCAGC-3; rev: 5-CAGGAGGAGCAATGATCT TGAT-3

The specific PCR was carried out according to the following procedure, briefly, 25 µl reaction mixture of PCR was prepared using 2.5 µl of 10x buffer , 3µl of 25mM mgcl2 , 1.5µl dNTPs (10Mm), 10pmol of each primer and 1 unit of amplify Tag gold DNA polymerase (perkin Elmer Biosystems, USA)). PCR was performed in 35 cycles, initiated by 1 cycle at 95°C for activating the tag Gold's DNA polymerase followed by 94°C; 30s, 60°C; 30s, 72°C; 30s and final

extension of 10minutes at 72°C. Amplification of β actin was performed using 26 cycles. PCR amplification products and 100bp molecular weight markers VIII (Roche) were separated on 1% agarose gels containing etidium bromide. The primers for HLA-G yielded PCR amplification products of 118bp, whereas the β actin primers gave a product of 321bp.Control PCR amplifications without cDNA were always carried out in parallels provided consistently negative results. All PCR amplification was performed in duplicate.

Co-culture Experiments with T cells

Isolated T cells and autolagous DC were co cultured. In 96 U-shaped–bottomed plates, 10×10^4 T-cell were cultured in each well with 2×10^4 DC and a final volume of 200 µl per well.

All samples were run in triplicates. Cultures were incubated at 37°C in a humidified 5% CO2 atmosphere for 5 days and then pulsed with 200 µl 3-[4,5-dimethylthiazolyl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) as a color indicator of metabolic activity, denoting cell proliferation(18). Supernatant harvested at 4h later, and then dimethylsulfoxide (DMSO) was added (200 µl). The color change was read in an Elisa reader at 550nm wave length.

Statistical Analysis

For statistical analysis, we used paired *t*-tests. Each paired *t*-test was calculated for diabetic patients as well as the normal donors. The *P* values were determined in all cases and they were considered statistically significant at $P< 0.05$.

RESULTS

***In vitro* Up Regulation of HLA-G Expression on DC by IFNβ**

IFNβ substantially raised the density of HLA-G molecules on DC in vitro in comparison with the control devoid of IFN-β, as observed by flowcytometry (Figure 1). Unstimulated purified PBMC from the patients and normal controls were also tested for expression of HLA-G molecule. Differences were not significant before IFN-β stimulation. However, treatment of cells with IFN-β resulted in an increased production of HLA-G molecule in dendritic cells from the normal subjects as well as diabetic patients ($p<0.05$) (Figure 1; Table 1).

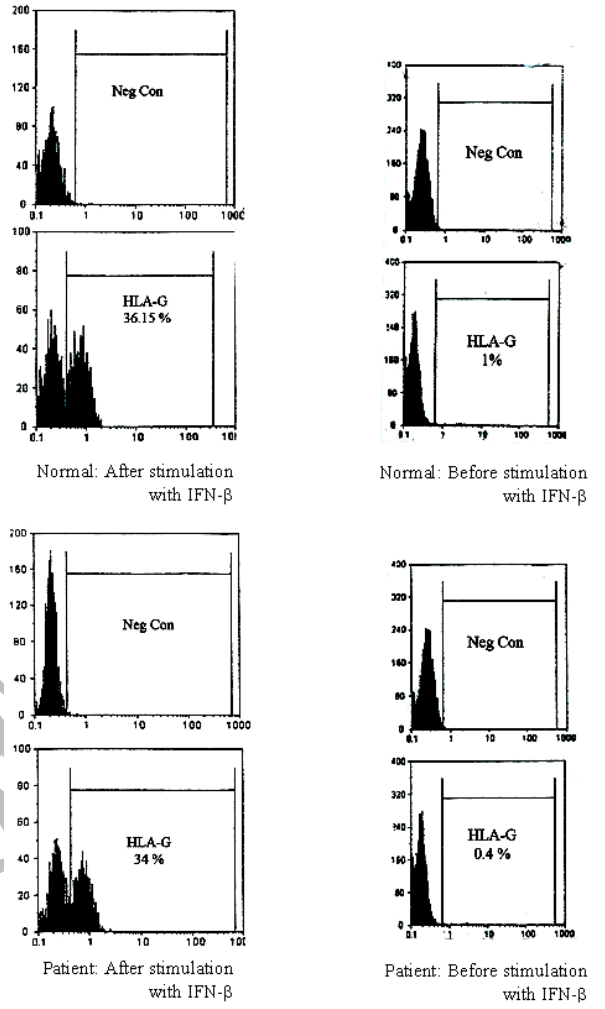

Figure 1. Flowcytometric analysis of DC from patients and normal controls before and after in vitro treatment of these cells with IFN-β (Vertical line: Cells Counts; Horizontal line: Fluorescent1)

IFN-β Enhances the Level of HLA-G Transcripts in Dendritic cell

Dendritic cells from patients with diabetes type 1 and controls were analyzed for expression of HLA-G and β actins genes by RT-PCR after treatment with IFN-β. The results are illustrated in figure 2.

Inhibition of Autolougous T-Cell Activation by DC Bearing HLA-G

The functional potential of mature monocyte-derived DC to induce proliferation of T lymphocytes was investigated by MLR in presence or absence of IFNβ. Proliferation of T lymphocytes co-cultured with mature DC was found to decrease by about 40% in presence of DC bearing HLA-G. (Table 3)

Table 2. Effect of IFN-β on expression of HLA-G on DCs in vitro as evaluated by flowcytometry. The results are percent of DC bearing HLA-G.

Cells	Total samples	DC without IFN-β		DC+1000 Units IFN-β		P value
		Mean	SD	Mean	SD	
Diabetic DC	20	0.2	0.06	34.35	13.15	<0.05
Normal DC	20	0.5	0.15	36.15	11.09	

DC bearing HLA-G in MLR inhibited T-cell proliferation in the normal subjects as well as diabetic patients. Since we compared the T cell proliferation responses in DC expressing HLA-G and in DC lacking HLA-G, upon addition of MTT, T- Cell proliferation was inhibited in co-culture system containing DC bearing HLA-G in both diabetic as well as normal subjects as indicated by metabolic activity assay (MTT) (Table 3). RT-PCR analysis of HLA-G gene expression also revealed higher expression of HLA-G gene in the cells from diabetic Patients after treatment with IFN-ß (Figure 2).

DISCUSSION

An important step in the present study was to induce HLA-G on DC derived from monocytes. This was achieved by treatment of DC in vitro with IFN-β. This cytokine has been shown to promote the differentiation of blood monocytes to DC[19] and to contribute to DC maturation. It was also demonstrated in this study that IFN-β up regulated considerably HLA-G expression on dendritic cells (Figure 1, Table 2) and gene expression in cells from diabetic patients(figure 2).

In a variety of experimental systems it has been demonstrated that DC bearing HLA-G acquire the potential to down regulate and to inhibit cellular activities.[20] DC bearing HLA-G induce inhibition of activity in NK, CD4 and CD8 lymphocytes.[21-24]

The cells which are inhibited by HLA-G bearing DC carry specific receptors for HLA-G. There are, for example, receptors on NK cells for HLA-G namely KIR2DL4, ILT2 and ILT4 that down regulate cell proliferation upon interaction with HLA-G.[25,26] The presence of HLA-G receptors on CD4 T cells has also been shown for immunoglobulin like transcript 2 (ILT-2) indicating this molecule a possible candidate mediating inhibitory effects.[27] In the present study, the inhibition of T cells proliferation was observed only when cells were treated with IFN-β (Table 3). Thus IFN-β and HLA-G molecules on the dendritic cells are two components contributing to this phenomenon, limiting cells proliferation, thereby reducing the potential of T-cells. It is quite evident that the cells bearing HLA-G are an important negative immune-regulatory constituent for this suppressing effect. It is speculated that IFN-β by increasing HLA-G on dendritic cells can also down regulate inflammatory response in vivo mediated by T cells in islet tissues of pancreatic gland. It has been reported that higher HLA-G levels correlated with a better outcome of heart transplantation.[28] Levels of HLA-G on dendritic cells may also be correlated to disease activity in diabetic patients and it could also serve as a useful marker for disease progress and treatment.

We would like to propose that HLA-G may be a factor limiting autoimmune response, thus potentially constituting a new component for controlling and suppressing the detrimental role of T-cells in the pathogenesis of diabetes type 1.

Table 3. Effect of DC-bearing HLA-G on $CD3^+$T-Cell proliferation in MLR, evaluated by MTT assay. The data are OD of MTT at 550 nm read in Elisa reader

MLR	Total samples	Without IFN-β		1000 Units IFN-β		P value
		Mean	SD	Mean	SD	
Diabetic (MLR; LYM+DC)	20	417.7	37.4	199.6	36.9	<0.05
Normal (MLR; LYM+DC)	20	365.3	96.34	192.45	67.35	

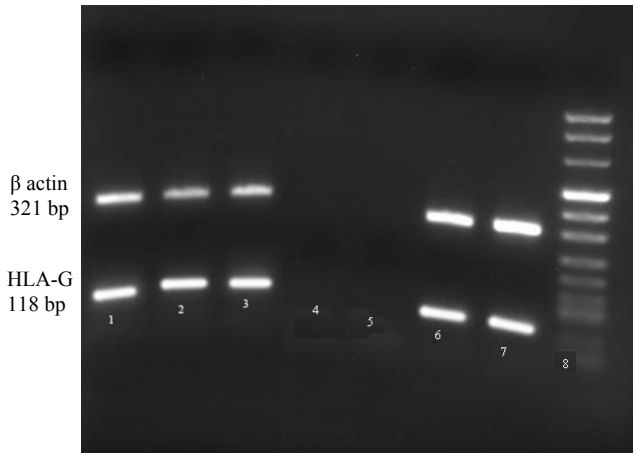

Figure 2. RT-PCR analysis of HLA-G and β actin as a house keeping gene expression in diabetes type 1 and controls after treatment with IFN-β. The cDNA samples consisted of: jeg-3 cell line as positive control (lanes 6, 7); dendritic cell from patients with diabetes type 1 (Lane 1, 3) DCs from a normal (Lane 2); negative control with out cDNA (lane 4, 5); DNA weight marker VIII (lane 8).

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