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Interleukin 10 Induces the Expression of Membrane-Bound HLA-G and the Production of Soluble HLA-G on HeLa CCL-2 Cells

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Abstract

BACKGROUND: Interleukin-10 is a cytokine that has a pleiotropic effect on the immune system and inflammation. IL-10 can contribute to the anti-tumour immune response by increasing HLA-G expression.

AIM: This study aimed to determine the effect of IL-10 induction on membrane HLA-G expression and soluble HLA-G production of HeLa CCL-2 cells.

METHODS: HeLa CCL-2 cells were cultured in the well plate and divided into 4 groups consist of 1 control group that was not induced by IL-10 and 3 treatment groups that were induced by IL-10 500 ng/ml, 1000 ng/ml and 2000 ng/ml respectively. All groups were incubated for 48 hours in a 37°C incubator at 5% CO_2 atmospheric pressure. HLA-G measurements were carried out both in cell lysate and cell culture supernatant using ELISA and in membrane-bound using immunofluorescence method. The expression of HLA-G in membrane-bound calculated using the ImageJ application. Data obtained were analysed by ANOVA and LSD test.

RESULTS: In the control group, the HLA-G level in the culture supernatant was higher than in cell lysate ($p = 0.000$), as well as in all treatment groups ($p = 0.000$). There were significant differences between the treatment group ($p = 0.000$) and within the treatment group ($p = 0.000$) at HLA levels. The highest expression of HLA-G in HeLa cell membranes found in cell culture induced by IL-10 concentrations of 500 ng/ml, i.e., 59.28 AU in view. HLA-G membrane expression in the IL-10 1000 ng/ml induced group was significantly different from all treatment groups ($p = 0.000$).

CONCLUSION: HeLa CCL-2 cells express HLA-G on the membrane and release dissolved HLA-G without induction of IL-10 although IL-10 induction augments the presence and the production of HLA-G in HeLa CCl-2 cells.

Introduction

Interleukin-10, also called human cytokine synthesis inhibitory factor, is a cytokine secreted by monocytes which has a pleiotropic effect on the immune system and inflammation. Most leukocytes secrete IL-10 to a certain level. The highest secretion of IL-10 comes from monocytes and their mature forms: macrophages and dendritic cells. Specific granulocytes and agranulocytes: eosinophil and NK cells, and small lymphocytes: T and B cells, also release IL-10 but at lower levels [1].

Human leukocyte antigen (HLA)-G is HLA class 1 molecule consists of membrane-bound isoform (mHLA-G) namely HLA-G1, HLA-G2, HLA-G3 and HLA-G4 and 3 soluble HLA-G (sHLA-G) namely HLAG5, HLA-G6 and HLA-G7 [2]. HLA-G expression is initially observed in extra-villous cytotrophoblasts and is considered to play an important role in fetal-maternal immune tolerance [3]. In addition to extravillous cytotrophoblasts, HLA-G expression is limited to some healthy adult tissues, including the cornea, thymus medulla and Langerhans Island of pancreatic [4]. HLA-G expression can be activated in a variety of pathological conditions such as cancer,

viral infections, organ transplants, autoimmune diseases, and inflammation [5]. HLA-G has the effect of inhibiting the immune response and induces the production of regulator and suppressor cells so that tumour cells can escape immune surveillance [6].

It has been reported that IL-10 can contribute to anti-tumour immune responses by either decreasing regulation of HLA class I expression allowing tumours to exit lysis mediated by CTLs or by increasing HLA-G expression allowing cells to escape lysis by NK cells through interaction with inhibiting killer receptors in NK cells. In cervical cancer, HLA-G and IL-10 expression can be higher in tumour cells than in normal cervix and may be involved in early carcinogenesis. However, the relationship between IL10 and HLA-G in cervical cancer is not yet cleared [7].

Polakova & Russ, in 2000, informs that HeLa cells do not express HLA-G antigens by observing flowcytometry using 87G and 01G antibodies [8]. Flajollet et al., in 2009 using ras-responsive element-binding protein 1 (RREB-1) as an HLA-G transcription suppressor that has been known to suppress HLA-G promoters in HLA-G negative cells (HeLa cells) revealed that HeLa cells were referred to as negative cells HLA-G [9]. Therefore, in this study, we want to study the ability of IL-10 to induce HLA-G expression using the HeLa CCL-2 cells line.

Material and Methods

Chemicals and reagents

The research materials were HeLa CCL-2 cervical cancer cell line (ATCC), recombinant IL-10 (Elabscience), Alpha MEM (Gibco), 2% Penicillin Streptomycin, Fungison (Amphotericin B 0.5%), Fetal bovine serum (ATCC) 12.5% and Horse serum (ATCC) 12.5%, recombinant HLA-G (4H84) (Santa Cruze), and HLA-G Elisa kit (Elabscience).

The tools used laminar airflow Hood/tissue culture cabinet (Nuaire), CO_2 incubator, inverted microscope, centrifuge, 24-well plate, 96 well plates, micropipette, micro-tip, spectrophotometry, ELISA reader, Improved Neubauer Hemocytometer Chamber, fluorescent microscope (Olympus), round coverslip, object-glass, flask 25.

Human cell line culture

The HeLa CCL-2 cell lines were grown in Alpha MEM supplemented with Glutamax- I,4500 mg/L glucose (Invitrogen), 10% FCS (BioWhittaker), 1000 U of Penicillin, and 10 µg/ml Streptomycin. The cells were cultured in flask 25 and maintained at 37°C and 5% CO_2 until confluent. Trypsinization, the number of cells per ml, was counted with a hemocytometer.

HeLa CCL-2 Cell Induction with IL-10

A total of 105 cells/ml was seeded into the 24 wells plate, in which coverslips had been placed. IL-10 with several concentrations of 500 ng/ml, 1000 ng/ml, and 2000 ng/ml was added in HeLa cell culture in triplicate. For analysis of HLA-G secretion in the supernatant and cell lysate, 4 x 104 HeLa cells were also cultured into the 96 wells plate and were induced with IL-10. All plates were incubated for 48 hours in a 37°C incubator with CO_2 pressure 5 atmospheres.

Measurement of HLA-G membrane-bound expression with Double-label immunofluorescence

Cells on the coverslip were fixed for 10 min in absolute methanol at 4°C. Samples were then rehydrated in PBS tween 0.05% 3 times, and subsequently PBS Triton-X 1000 0.1% for 5 min. Cells were incubated for 60 min in blocking buffer (1%: BSA 0.1 g in PBS 10 ml). The HLA-G (4H84) mAbs 1:400 were applied and then incubated and were followed by incubations with biotinylated goat anti-mouse mAb and FITC-labeled streptavidin (1:1000) in the dark. After washing, goat anti-mouse labelled antibody was added for 60 min, followed by DAPI (1:1000) as counterstain. Samples were then mounted on object glass and observed by a fluorescence microscope.

Measurement of HLA-G supernatant and Celllysate with Elisa

Cell culture supernatant: The samples were centrifuged for 20 min at 1000 × g at 2 ~ 8°C and the supernatant were collected.

Cell lysates: The cells were washed gently with a moderate amount of pre-cooled PBS and dissociated the cells using trypsin. The cell suspension was collected into a centrifuge tube and centrifuged for 5 min at 1000 × g. The medium was discarded and washed 3 times with pre-cooled PBS. For each 1 × 10^6 cells, added 150-250 µL of pre-cooled PBS to keep the cells suspended. The freeze-thaw process was repeated several times until the cells are fully lysed and centrifuged for 10 min at 1500 × g at 4°C. The cell fragments were removed; the supernatant was collected to carry out the assay.

Elisa (HLA-G Elisa kit: Elabscience)

The standard working solution added to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 µL for each well). The samples were added to the other wells (100 µL for each well). The plate covered

with the sealer provided in the kit and incubated for 90 min at 37°C. The liquid was removed out of each well, do not wash. Immediately 100 µL of Biotinylated Detection Ab working solution added to each well. Cover with the Plate sealer and gently mix up and incubated for 1 hour at 37°C.

The solution was aspirate from each well, and 350 µL of wash buffer was added to each well. Soak for 1 ~ 2 min and the solution was aspirated from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. 100 µL of HRP Conjugate working solution was added to each well was covered with the Plate sealer and incubated for 30 min at 37°C. The solution was aspirate from each well, repeat the wash process for five times. 90 µL of Substrate Reagent was added to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. The plate was protected from light. 50 µL of Stop Solution was added to each well. The optical density (OD value) of each well was determined at once with a micro-plate reader set to 450 nm.

Statistical analysis

From the data obtained, a normality test was carried out using the Kolmogorov-Smirnov test. The data obtained will be tested by ANOVA ($p < 0.05$), and differences between groups were further tested by Least Significant Difference (LSD).

Results

In this study, HeLa CCL-2 cell cultures were divided into a control group and treatment groups. In the control group, it was found that supernatant HLA-G level was higher than HLA-G cell lysate level (65.50 ng/ml vs 21.59 ng/ml), as well as in all treatment groups as shown in Table 1. The mean HLA-G level in the supernatant (77.372 ng/ml) was higher than the mean HLA-G level in cell lysate (28.68 ng/ml).

Table 1: Comparison of HLA-G Supernatants with HLA-G CellLysate Level after IL-10 Induction

Sample	N	Mean of HLA-G (ng/ml)
Supernatants Control	3	65.50
IL-10 500 ng/ml	3	76.66
IL-10 1000 ng/ml	3	83.10
IL-10 2000 ng/ml	3	84.24
CellLysate Control	3	21.59
IL-10 500 ng/ml	3	24.32
IL-10 1000 ng/ml	3	32.67
IL-10 2000 ng/ml	3	36.16

There was a significant difference among treatment groups and within the treatment group on the levels of HLA-G secreted in the supernatant or on cell lysate ($p = 0.000$; $p = 0.000$). Comparison of HLA-G supernatant and cell lysate levels, as illustrated in Figure 1.

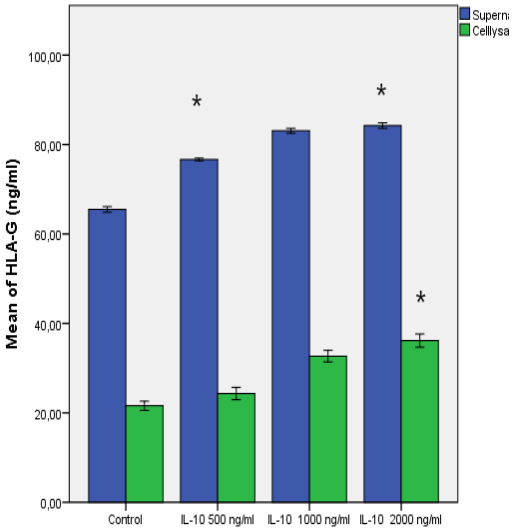

Figure 1: Comparison of HLA-G Supernatant Levels and Cell Lysate CCL-2 HeLa Cells After IL-10 Induction; The highest HLA-G secretion in the supernatant and cell lysate of HeLa cell culture were shown after induction of IL-10 concentration of 2000 ng/ml (84.24 ng/ml and (36.16 ng/ml)); Comparison the HLA-G supernatant and cell lysate on CCL-2 HeLa Cells culture differed significantly between control and treatment groups (sig < 0.05)

Membrane-bound HLA-G expression of HeLa CCL-2 cells was observed using a fluorescent microscope. Illustrations of HLA-G expression that were obtained in each treatment group is shown in Figure 2.

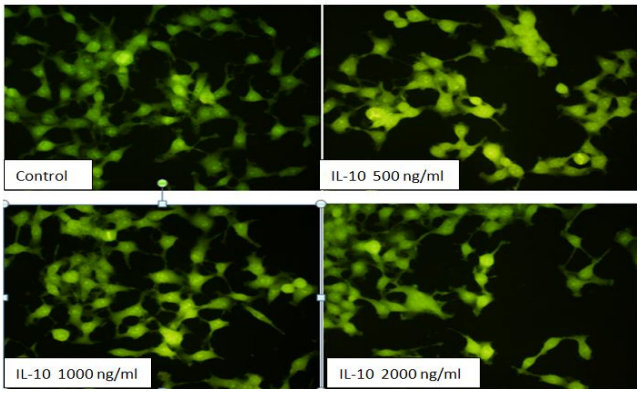

Figure 2: HLA-G Expression in HeLa CCL-2 Cells Culture; Fluorescent images in the treatment group induced by IL-10 500 ng/ml gives a stronger expression of HLA-G than the other treatment groups. By using ImageJ application, the mean HLA-G expression for the control and treatment fields were: 37.26; 59.28; 57.16 and 47.11 AU which were observed under a fluorescent microscope with a 400-x magnification

The averages of HLA-G expression were analysed using the ImageJ application (table 2). The highest expression of HLA-G (59.28 AU) was found on HeLa cell membranes IL-10 500 ng/ml-induced group. After being analysed with ANOVA, HLA-G membrane expression in the non-IL-10-induced group was significantly lower from the IL-10 induced groups, that were 500 ng/ml ($p = 0.014$) and 1000 ng/ml ($p = 0.020$). HLA-G membrane expression in the IL-10 500

ng/ml-induced group not significantly different from the IL-10-induced group 1000 ng/ml (p = 0.708) and 2000 ng/ml (p = 0.083). HLA-G membrane expression in the IL-10 1000 ng/ml-induced group not significantly different from the IL-10-induced group 2000 ng/ml (p = 0.130). Hence, HLA-G membrane expression in the IL-10 500 ng/ml and 1000 ng/ml-induced group was significantly different from non-IL-10 inducing, as shown in Figure 3 and Table 2.

Table 2: HLA-G Expression of CCL-2 HeLa Cell Membrane after IL-10 Induction

Treatment Group	N	Mean of HLA-G expression in view (AU)
Control	5	37.259
IL-10 500 ng/ml	5	59.279
IL-10 1000 ng/ml	5	57.156
IL-10 2000 ng/ml	5	47.108

In the control group, HeLa cells can express HLA-G on the membrane, although this group did not receive IL-10. HLA-G expression in the group treated with IL-10 500 ng/ml had the highest expression (59.279 AU), and significantly different from the control (p = 0.014).

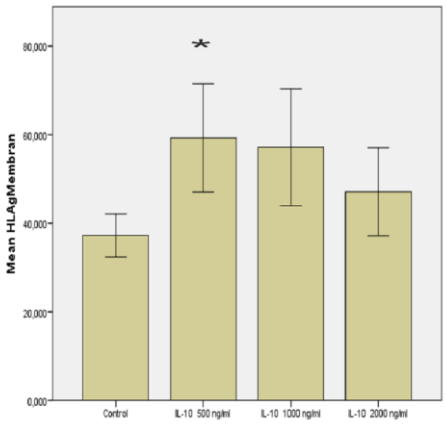

Figure 3: Comparison of HLA-G Expression of HeLa CCL-2 Cell Membrane

Discussion

Parameters of the tumour microenvironment that play a role in modulation of the HLA-G gene include stress conditions such as heat shock and hypoxia; cytokines such as IFNɣ, IL-10, leukaemia inhibitory factor (LIF), and GM-GSF; and hormones such as glucocorticoids and progesterone. The mechanism of action of all these parameters is not yet clear. In this study, IL-10 was used to induce the production of HLA-G protein on HeLa CCL 2 cells as a model of tumour cells. The results of this study demonstrated that the HeLa CCL-2 cells could express and secrete HLA-G with or without IL-10 treatment. The higher the concentration of IL-10 treatment, the higher the HLA-G expression in the membrane and HLA-G secretion to the supernatant, as shown in Table 1 and Figure 1. The previous study by Polakova & Russ, in 2000, Hela cells, along with 61 other cells line did not express HLA-G antigens as observed by flow cytometry using 87G and 01G antibodies. Research several years later, using the RT- PCR method revealed that stable levels of HLA-G mRNA in HeLa cells were very low(~1.5 x 10-5) compared to HLA-G levels in positive. JEG-3 choriocarcinoma cells with HLA-G (HLA-G mRNA level: 1). RREB-1 and DNA methylation at the HLA-G locus is complementary mechanisms [9]. However, this study proved that HeLa cells could express HLA-G before or after IL-10 induction.

The previous study of trophoblastic cells, IL-10 can activate HLA-G transcription in trophoblastic tissue. Northern blotting analysis and quantification of total RNA from trophoblasts showed that IL-10 induction increased the transcription level of HLA-G mRNA at least 7 times compared to the HLA- G mRNA of cells without IL-10. The RNase protection test also showed that HLA-G mRNA levels were stabilised 7.2-fold after induction of IL-10 in trophoblastic explants. To analyse IL-10-induced HLA-G transcription patterns, HLA-G-specific primers are used to strengthen the isolated HLA-G mRNA form. Southern blotting of HLA-G RT-PCR products reveals that induction of IL-10 enhances all transcripts, including G5 and G6, which encode dissolved HLA-G isoforms [11].

In this study, IL-10 treatment induces HLA-G expression on the HeLa cell membrane as well as HLA-G secretion into the HeLa cells culture supernatant. Possible underlying mechanisms can be explained based on previous studies on cervical cancer. It is well-known that progression is associated with a shift from Th1 to Th2 cytokine production. This shift toward a Th2 cytokine profile, characterised by IL-10 secretion, is associated with the progression of premalignant lesions to cancer [13]. Moreover, another study showed peripheral blood monocytes expressed classic HLA class I and proteins containing IL-10 receptors. HLA-G transcripts are analysed by RT- PCR, using primary HLA-G and HLA-G5-specific primers, which encode HLA-G5 soluble proteins. Moderate HLA-G gene transcripts in monocytes, weak HLA-G transcription signals enhanced after induction of IL-10. It is possible that IL-10 may contribute to the activation of HLA-G expression in melanoma cells, thus participating in the escape of tumours from immunosurveillance [11]. HLA-G expression at the surface of tumour cells can participate in the evaluation of anti-tumour immune responses and favour tumour progression [12].

In this study, the results of HLA-G production of cell lysate and HLA-G secretion in HeLa cell culture supernatant were confirmed by microscopic examination with HLA-G antibody (4H84) using a fluorescent microscope to determine the quantity of membrane HLA-G expression in each group

treatment. From the results of microscopic observations of the field of view, the highest results were obtained in the treatment group with IL-10 500 ng/ml induction with a mean of 59,28 AU, which was directly proportional to HLA-G levels in the supernatant and cell lysate. HLA-G expression of the membrane increases after IL-10 500 ng/ml treatment and decreases again at IL-10 2000 ng/ml. These results were consistent with previous studies which that analysed the expression of HLA-G proteins in induced and non-IL-10-induced monocytes using anti-HLA-G-specific 87G mAb. The study showed that the percentage of cells with positive HLA-G and HLA-G cell surface density increased after IL-10- induction [11].

In conclusion, HeLa CCL-2 cells expressed HLA-G on its membrane and release dissolved HLA-G without induction of IL-10, although IL-10 induction augments the presence and the production of HLA-G in HeLa CCl-2 cells.

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