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Leukemia inhibitory factor (LIF) stimulates the human HLA-G promoter in JEG3 choriocarcinoma cells

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ABSTRACT

HLA-G is a non-classic class I MHC molecule specifically expressed by human invasive cytotrophoblast cells, which has been suggested to play a role in facilitating the immune tolerance of the conceptus. So far, very little is known about the regulation of the human HLA-G gene. The present study was, thus, designed to investigate the regulation of the human HLA-G promoter. JEG3 choriocarcinoma cells, which express HLA-G endogenously, were used as a model. A 890 bp fragment of the human HLA-G promoter was amplified by nested PCR from genomic DNA, cloned into pCR-Script and, after sequencing, subcloned into pGL3-Luc in front of the luciferase reporter gene. This vector was then used in transient transfection experiments in JEG3 cells. Parallel transfection experiments were performed using an α subunit (αSU)-Luc reporter plasmid as a control. Using this system, several potential modulating substances were tested in different concentrations and for different periods of time: phorbol ester (TPA), cAMP, IFNγ, IL-1, and leukemia inhibitory factor (LIF), with only LIF administration resulting in induction of the HLA-G promoter. LIF treatment also resulted in induction of HLA-G mRNA. JEG3 cells are shown to possess LIF receptors. LIF is a pleiotropic cytokine produced at the maternal-fetal interface which has been shown to play an essential role in implantation in mice. LIF is produced in high amounts by the human endometrium and the trophoblast itself, and LIF receptors are present on cytotrophoblast cells. LIF could, thus, play a role in modulating HLA-G production and immune tolerance at the maternal-fetal interface.

INTRODUCTION

Human Leukocyte Antigen Type G (HLA-G) is a non-classic class I MHC molecule specifically expressed by human cytotrophoblast cells that has been suggested to play a role in facilitating the immune tolerance of the conceptus (1-5). However, the regulation of the human HLA-G gene promoter in placental cells is still unknown. We, therefore, decided to investigate the regulation of human HLA-G promoter activity and HLA-G mRNA expression in JEG3 choriocarcinoma cells.

Among the potential regulators of HLA-G expression, we were especially interested in leukemia inhibitory factor (LIF). LIF is a pleiotropic cytokine that has been shown to be expressed at the maternal-fetal interface and to play an essential role in implantation in mice (6, 7). In the human endometrium, LIF is expressed in a cycle-dependent manner with a maximum at implantation time (8, 9). Furthermore, LIF has been shown to reduce hCG production of the trophoblast and to elevate the production of oncofetal fibronectin, indicating that it may be implicated in the differentiation process of the cytotrophoblast (10). All effects of LIF are mediated via the LIF receptor (LIF-R) (11), the expression of which in JEG3 cells has also not been studied so far.

To study the effect of LIF on the HLA-G promoter activity, we amplified a 890 bp fragment of the human HLA-G promoter by nested PCR from genomic DNA and subcloned it into a pGL3-basic vector in front of a luciferase reporter gene. This vector was used in transfection experiments in JEG3 cells. To quantify the HLA-G mRNA expression, RNA was isolated from JEG3 cells and Northern hybridization was performed using a HLA-G specific probe.

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METHODS

Cell culture

JEG3 choriocarcinoma cells were grown in DMEM medium (GIBCO, Eggenstein, Germany) with 10 % FCS and antibiotics supplemented. They were grown to confluence and splitted twice a week.

RNA Isolation and RT-PCR

RNA from unstimulated and LIF-stimulated cells was isolated at confluence by using the RNA-Clean System (AGS, Heidelberg, Germany), and cDNA was synthesized using Superscript RT (GIBCO, Eggenstein, Germany). RT-PCR was performed using cDNA from JEG3 cells and the following specific primers:

HLA-G (upper primer):
5′-CTGACCCTGACCGAGACCTGG-3′
HLA-G (lower primer):
5′-GTCGCAGCCAATCATCCACTGGAG-3′
LIF-R (upper primer):
5′-GAAAACTGTAAAGCATTACA-3′
LIF-R (lower primer):
5′-AGAGTCTGGAGACACTAA-3′

Northern Blot

RNA from cells treated with LIF for various time periods were run on a formaldehyde gel and blotted onto a Hybond-N membrane. The amplification product of the HLA-G RT-PCR was subcloned into a pCR-Script vector and the SstI/HinDIII restriction fragment of this plasmid was labeled with ^{32}P-dCTP and used as a probe for Northern hybridization. For quantitative analysis, the intensity of the hybridization bands was measured using a STORM-phosphoimager system.

Cloning of HLA-G-luc

890 bp of the 5' flanking region of the HLA-G gene were amplified by nested PCR from human genomic DNA and cloned into the luciferase reporter vector pGL3. The HLA-G primer sequences were:

Outer primers:
5′-GTG AGGGGCATTGTGACTG-3′
5′-GTCAGGGCCCCCGAGAGCA-3′
inner primers:
5′-AGTTTGTGCTGGCTCCTGGTTGC-3′
5′-GAGAATGAGTCCGGG TGGGTGAG-3′

Transfection experiments

For transfection experiments, JEG3 cells were plated in 12-well plates at 2×10^5 cells/well and transfected with HLA-G-luc following the protocol for DOTAP transfections (Boehringer, Mannheim, Germany, see also Ref. 12). Stimulation with the indicated amounts of LIF (Genzyme, Rüsselsheim, Germany), TPA (10^{-7} M), cAMP (0.05 mM), interferon (IFN) γ (100, 1000, 5000, 10000 U), or interleukin-1 (IL-1; 10, 50, 100, 1000 U) was performed for 24, 36, and 48 h.

RESULTS

Expression of HLA-G and LIF-R mRNA in JEG3 cells

After RNA isolation and reverse transcription, RT-PCR with HLA-G-specific primers and LIF-R-specific primers was performed to analyze whether JEG3 choriocarcinoma cells are a suitable model for analyzing the regulation of the HLA-G promoter. A specific band for both HLA-G (338 bp) and LIF-R (506 bp) was amplified in JEG3 cells (Fig. 1). These results indicate that JEG3 cells express HLA-G and LIF-R endogenously and may, thus, represent a good model for studying the regulation of HLA-G by LIF.

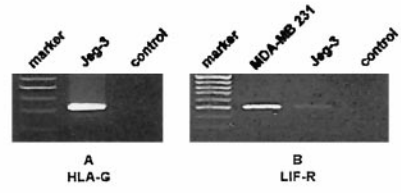

Fig. 1. Expression of HLA-G mRNA (A) and LIF-R mRNA (B) in JEG3 choriocarcinoma cells.

Stimulation of HLA-G promoter activity by LIF

For transcriptional studies, a 890 bp fragment of the HLA-G 5' flanking region was cloned into a pGL3 plasmid in front of a luciferase reporter gene (HLA-G-luc) and transfected in JEG3 cells. After transfection, cells were stimulated with various amounts of LIF for 24, 36, and 48 h. The promoter showed low basal activity which could be induced by treatment with LIF up to 2.8 fold (Fig. 2). Treatment with any of the other tested substances (TPA, cAMP, IFNγ, IL-1) showed no significant effect on the HLA-G promoter, while cAMP treatment resulted in the expected stimulation of a αSU-Luc control plasmid (data not shown).

Stimulation of HLA-G mRNA expression by LIF

To quantify the expression of HLA-G mRNA in JEG3 cells after stimulation with LIF, we isolated RNA of cells that were stimulated with 5000 U LIF for 24, 48, and 72 h and of unstimulated cells, and performed Northern blot analysis. Using a specific probe for HLA-G, we obtained one specific band of approximately 1.5 kb (Fig. 3A, upper panel).

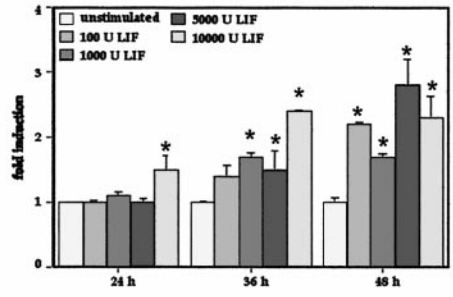

Fig. 2 Transient transfection of HLA-G-luc in JEG3 cells. Fold induction of luciferase activity after stimulation with various amounts of LIF for 24, 36 and 48 h. * $p < 0.02$ (student's t-test)

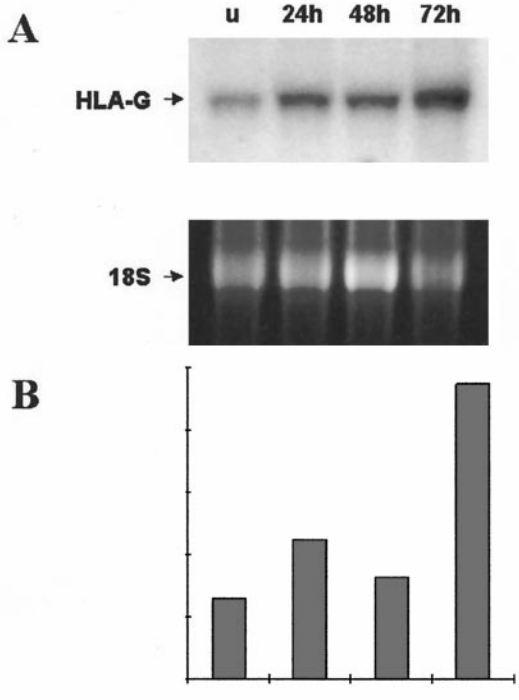

Fig. 3. HLA-G mRNA expression in JEG3 cells. Cells were stimulated with 5000 U LIF for 24, 48, and 72 h. A: Northern Blot (upper panel); 18S rRNA band of the formaldehyd gel (lower panel). B: Quantification of HLA-G mRNA amounts relative to the 18S rRNA bands.

Treatment with LIF resulted in stronger hybridization signals. As internal control the 18S rRNA band of the gel is shown (Fig. 3A, lower panel). A STORM phosphoimager system was used to quantify the HLA-G specific bands. The fluorescence units of the Northern blot were normalized with the intensity of the 18S rRNA bands. Figure 3B shows the normalized HLA-G mRNA expression in JEG3 cells upon stimulation with LIF. A significant elevation of HLA-G mRNA expression after treatment with LIF (up to 3.6 fold induction after 72 h) is observed.

DISCUSSION

In the present study we investigated the regulation of the human HLA-G promoter in transfection experiments using as a model JEG3 choriocarcinoma cells, which also express endogenous HLA-G. Using this system, several potential modulating substances were tested in different concentrations and for different periods of time. Significant induction of the HLA-G promoter was observed following LIF treatment for 24, 36, and 48 h.

LIF is a pleiotropic cytokine (6) produced at the maternal-fetal interface which has been shown to play an essential role in implantation in mice. In LIF k.o. mice, implantation does not occur (7) due to the absence of LIF at the maternal-fetal interface, while the same embryos do implant in wild-type mothers (7). At the human maternal-fetal interface, LIF is produced in high amounts by the human endometrium during the secretory phase (8, 9). LIF is also produced by the human placenta itself (13, and our own unpublished data), and LIF receptors are present on human trophoblast cells (13, and our own unpublished data) as well as on choriocarcinoma cells, as shown here. Thus, LIF from both endometrial and placental sources could be acting through LIF receptors on trophoblast cells in vivo to facilitate implantation. Despite its specific expression and its proven importance for implantation in mice, the exact roles of LIF in facilitating implantation are not known, but LIF has been shown to enhance the blastocyst formation rate (14) and to facilitate trophoblast differentiation (10). One of its functions, as shown here, is the regulation of the trophoblast-specific MHC class-I protein HLA-G.

HLA-G is a non-classic class I MHC molecule specifically expressed by human cytotrophoblast cells which invade the uterus (1, 15), and it has been

suggested to play a role in facilitating the immune tolerance of the conceptus (2-4). So far, very little had been known about the regulation of the human HLA-G gene. Northern blot experiments indicate that cytokines such as interferons and TNF-alpha are able to modestly enhance steady state levels of HLA-G mRNA (5), while no change was observed in protein expression. Other studies have shown stimulation in first trimester cytotrophoblasts and JEG3 choriocarcinoma cells with interferon type γ (IFNγ), while IFNα was inactive (16). Recently, Moreau et al. showed induction of HLA-G mRNA by IL-10, a cytokine produced by the placenta (17). Little is known about the regulation of HLA-G at the promoter level. Experiments in transgenic mice showed more efficient transcription of a transgene containing 1.5 kB of the 5'-region compared to 1.2 kB of the 5'-region, indicating that regulatory elements might be present in 250 bp at the 5'-end of this region (18). Potential regulatory elements in the HLA-G promoter which are present in related genes are found to be probably inactive in the HLA-G gene (19, 20). In cells which do not express HLA-G, protein complexes with potential inhibitory effect have been described on the EcoRI/HindIII 244bp fragment which directs HLA-G expression in mouse placenta (21). Also, methylation has been implicated as a silencing factor, but its role is far from clear (22). The results presented in the present study, thus, show for the first time induction of HLA-G promoter activity by a cytokine, LIF, which is present at the maternal-fetal interface.

An interesting question to be answered by future experiments regards potential transcription factors involved in the regulation of the HLA-G promoter. A computer search (TFSEARCH ver.1.3) indicated the presence in the investigated fragment of several potential binding sites for different transcription factors such as GATA-proteins which have been shown to be expressed in the human placenta (23), as well as factors such as AP-1 (activating protein-1) and interferon regulatory factor-1 (IRF-1) and IRF-2. IRF-1 has been shown to be induced by LIF (24). Also, LIF has been shown to induce c-fos, a member of the AP-1 transcription factor family (25). The regulatory involvement of these transcription factors in the invasive cytotrophoblast is currently under investigation.

In conclusion, we could demonstrate that the cytokine LIF, which was previously shown to be essential for implantation in mice, induces transcription of the human HLA-G promoter in JEG3 choriocarcinoa cells and could, thus, play a role in modulating HLA-G production and immune tolerance at the human maternal-fetal interface.

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REFERENCES

1. **McMaster MT, Librach CL, Zhou Y, Lim K-H, Janatpour MJ, DeMars R, Kovats S, Damsky C, Fisher SJ.** 1994 Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. J Immunol 154:3771-3778.
2. **Chumbley G, King A, Robertson K, Holmes N, Loke YW.** 1994 Resistance of HLA-G and HLA-A2 transfectants to lysis by decidual NK cells. Cell Immunol 155:312-322.
3. **Carosella ED, Dausset J, Kirszenbaum M.** 1996 HLA-G revisited. Immunol Today 7:407-409.
4. **Moreau P, Paul P, Rouas-Freiss N, Kirszenbaum M, Dausset J, Carosella ED.** 1998 Molecular and immunologic aspects of the non-classical HLA class I andtigen HLA-G: evidence for an important role in the maternal tolerance of the fetal allograft. Am J Reprod Immunol 40:136-144.
5. **Yang Y, Geraghty DE, Hunt JS.** 1995 Cytokine regulation of HLA-G expression in human trophoblast cells. J Reprod Immunol 29:179-195.
6. **Gough NM, Williams RL.** 1989 The pleiotropic actions of leukemia inhbitory factor. Cancer Cells 1:77-80.
7. **Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ.** 1992 Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. Nature 359:76-9.
8. **Chen D-B, Hilsenrath R, Yang Z-M, Le S-P, Kim S-R, Chuong CJ, Poindexter III AN, Harper MJK.** 1995 Leukemia inhibitory factor in human endometrium during the menstrual cycle: cellular origin and action on production of glandular epithelial cell prostaglandin in vitro. Hum Reprod 10:911-918.
9. **Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Steward CL.** 1996 Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci USA 93:3115-3120.
10. **Nachtigall MJ, Kliman HJ, Feinberg RF, Olive DL, Engin O, Arici A.** 1996 The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. J Clin Endocrinol Metab 81:801-806.
11. **Gearing DP.** 1993 The leukemia inhibitory factor and its receptor. Advances Immunol 53:31-58.
12. **Bamberger CM, Bamberger AM, Wald M, Chrousos GP, Schulte HM. 1997 Inhibition of mineralocorticoid activity by the ß-isoform of the human glucocorticoid receptor. J Steroid Biochem Mol Biol 60:43-50.**
13. **Kojima K, Kanzaki H, Iwai M, Hatayama H, Fujimoto M, Narukawa S, Higuchi T, Kaneko Y, Mori T, Fujita J.** 1995 Expression of leukemia inhibitory factor (LIF) receptor in human placenta: a possible role for LIF in the growth and differentiation of trophoblasts. Human Reprod 10:1907-1911.

14. **Dunglison GF, Barlow DH, Sargent IL.** 1996 Leukemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. Hum Reprod 11:191-196.
15. **Chumbley G, King A, Holmes N, Loke YW.** 1993 In situ hybridization and Northern blot demonstration of HLA-G mRNA in human trophoblast populations by locus-specific oligonucleotide. Hum Immunol 37:17-22.
16. **Grabowska A, Chumbley G, Carter N, Loke YW.** 1990 Interferon-gamma enhances mRNA and surface expression of class I-like antigen on human extravillous trophoblast. Placenta 11:301-308.
17. **Moreau P, Adrian-Cabestre F, Menier C, Guiard V, Gourand L, Dausset J, Carosella ED, Paul P.** 1999 IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. Int Immunol 11:803-811.
18. **Schmidt CM, Ehlenfeldt RG, Athanasiou MC, Duvick LA, Heinrichs H, David CS, Orr HT.** 1993 Extraembryonic expression of the human MHC class I gene HLA-G in transgenic mice. J Immunol 151:2633-45.
19. **Gobin SJ, van Zutphen M, Woltman AM, van den Elsen PJ.** 1999 Transactivation of classical and non-classical HLA class I genes through the IFN-stimulated response element. J Immunol 163:1428-1434.
20. **Gobin SJ, van den Elsen PJ.** 1999 The regulation of HLA class I expression: is HLA-G the odd one out? Semin Cancer Biol 9:55-59.
21. **Moreau P, Lefebre S, Gourrand L, Dausset J, Carosella ED, Paul P.** 1998 Specific binding of nuclear factors to the HLA-G promoter correlates with a lack of HLA-G trascripts in first trimester human fetal liver. Hum Immunol 59:751-757.
22. **Onno M, Amiot L, Bertho N, Drenou B, Faucket R.** 1997 CpG methylation patterns in the 5' part of the nonclassical HLA-G gene in peripheral blood CD34+ cells and CD2+ lymphocytes. Tissue Antigens 49:356-364.
23. **Steger DJ, Hecht JH, Mellon PL.** 1994 GATA-binding proteins regulate the human gonadotropin alpha-subunit gene in the placenta and pituitary gland. Mol Cell Biol 14:5592-602.
24. **Abdollahi A, Lord KA, Hoffmann-Liebermann B, Liebermann DA.** 1991 Interferon regulatory factor 1 is a myeloid differentiation primary response gene induced by interleukin 6 and leukemia inhibitory factor: role in growth inhibition. Cell Growth Diff 2:401-407.
25. **Jenab S, Morris PL.** 1998 Testicular leukemia inhibitory factor (LIF) and LIF receptor mediate phosphorylation of signal transducers and activators of transcription (STAT)-3 and STAT-1 and induce c-fos transcription and activator protein-1 activation in rat Sertoli but not germ cells. Endocrinology 139:1883-1890.