

## SHORT REPORT

## HLA-G does not have a pathophysiological role in Graves' disease

E H Kemp, R A Metcalfe, P F Watson, A P Weetman

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**Aims:** It has been suggested that the non-classic HLA class I molecule HLA-G plays a role in autoimmune disease by protecting tissues from damage by infiltrating cytotoxic T cells. Such infiltration occurs in the thyroid of patients with Graves' disease (GD) and Hashimoto's thyroiditis (HT) and can eventually result in tissue destruction. The aim of the current study was to analyse thyroid tissue and thyrocytes obtained from individuals with autoimmune thyroid disease for the expression of HLA-G.

**Methods:** HLA-G expression was analysed in thyroid tissue taken from six patients with GD and one with HT by reverse transcriptase polymerase chain reaction. Thyroid tissue samples isolated from six patients with multinodular goitre (MNG) were used as non-autoimmune controls. HLA-G expression was also examined in cultured thyroid follicular cells (TFCs).

**Results:** The expression of HLA-G was not detected in the thyroid gland of patients with either GD, HT, or MNG. Furthermore, HLA-G expression could not be detected in cultured patient TFCs under basal conditions or after stimulation with the proinflammatory cytokines—interleukin 1 $\alpha$ , interferon  $\gamma$ , and tumour necrosis factor  $\alpha$ .

**Conclusions:** HLA-G expression does not occur in the thyroid of patients with GD, indicating that HLA-G does not play a pathophysiological role in this autoimmune disorder. Although the expression of HLA-G was not detected in the thyroid sample of the patient with HT, a greater sample size would be required to conclude that HLA-G does not have a part to play in this autoimmune thyroid disease.

Controversy and debate surround the role of the non-classic human lymphocyte antigen (HLA) class I molecule HLA-G.<sup>1,2</sup> Initially, the expression of HLA-G was reported to occur only in a limited subset of trophoblast cells at the fetal-maternal interface.<sup>3</sup> However, further studies have suggested that HLA-G is expressed in other tissues such as thymic epithelial cells,<sup>4</sup> in addition to tumours<sup>5</sup> and transplant biopsies.<sup>6</sup> With respect to activity, HLA-G molecules can inhibit natural killer cell mediated and antigen specific CD8<sup>+</sup> T cell mediated cytotoxicity,<sup>7</sup> induce apoptosis of activated CD8<sup>+</sup> T cells,<sup>8</sup> suppress the proliferation of allogeneic CD4<sup>+</sup> T cells,<sup>9</sup> and inhibit the transendothelial migration of natural killer cells.<sup>10</sup> Coupled with the reported tissue distribution, these inhibitory effects upon immune cells have led to the suggestion that HLA-G has a role in maintaining maternal-fetal tolerance,<sup>11</sup> reducing transplant rejection,<sup>12</sup> and in allowing the progression of tumours.<sup>13</sup> Furthermore, the expression of HLA-G has been reported in skin biopsies taken from patients with the chronic inflammatory diseases, psoriasis<sup>14</sup> and atopic dermatitis,<sup>15</sup> and in the muscle fibres of individuals with inflammatory myopathy.<sup>16</sup> Such observations have initiated the idea that HLA-G might protect tissues from dam-

age by infiltrating cytotoxic T cells during the process of inflammation<sup>2</sup> because HLA-G has been shown to modulate the T helper (Th) cytokine balance in favour of Th2 type anti-inflammatory cytokine responses.<sup>17</sup>

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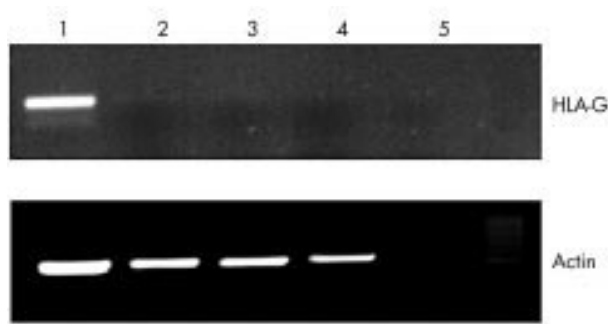
Autoimmune thyroid disease, as exemplified by Hashimoto's thyroiditis (HT) and Graves' disease (GD), is characterised by a lymphocytic infiltration of the thyroid gland. Proinflammatory cytokines such as interleukin 1 $\alpha$  (IL-1 $\alpha$ ) and interferon  $\gamma$  (IFN $\gamma$ ) are produced by the infiltrating T cells and macrophages,<sup>18</sup> and also by thyroid follicular cells (TFCs).<sup>19</sup> The reported detection of HLA-G transcripts in thyrocytes isolated from glands affected by GD,<sup>20</sup> together with the suggestion that HLA-G may have a tissue protective role in autoimmune disease,<sup>2</sup> prompted us to examine the expression of HLA-G in both thyroid tissue and TFCs taken from patients with autoimmune thyroid disease using reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, IFN $\gamma$  has been shown to induce HLA-G expression in some, but not all, normal and malignant cells.<sup>21-23</sup> Therefore, the expression of HLA-G in cultured thyrocytes after stimulation by the proinflammatory cytokines IFN $\gamma$ , IL-1 $\alpha$ , and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) was examined.

## MATERIALS AND METHODS

Disease diagnosis was confirmed in all patients with GD (n = 6), HT (n = 1), and multinodular goitre (MNG; n = 6) by biochemical testing and histological examination. Local ethical committee approval was obtained for our study and all thyroid tissue samples were taken after informed consent. Tissue samples were either immediately snap frozen in liquid nitrogen or processed to obtain primary TFC cultures as described below. Fetal chorionic membranes were isolated as described previously.<sup>24</sup>

Primary TFC cultures were prepared from GD thyroidectomy specimens using methods described elsewhere<sup>25</sup> and were grown to confluence in 25 cm<sup>2</sup> flasks before treatment for six, 12, and 24 hour periods with either 100 U/ml IL-1 $\alpha$  (Hoffman-La Roche, Nutley, New Jersey, USA), 100  $\mu$ U/ml bovine thyroid stimulating hormone (TSH; National Institute for Biological Standards and Control, Potters Bar, UK), 100

**Abbreviations:** GD, Graves' disease; HLA, human lymphocyte antigen; HT, Hashimoto's thyroiditis; IFN $\gamma$ , interferon  $\gamma$ ; IL-1 $\alpha$ , interleukin 1 $\alpha$ ; M-MLV, Moloney murine leukaemia virus; MNG, multinodular goitre; RT-PCR, reverse transcriptase polymerase chain reaction; s, soluble; TFC, thyroid follicular cell; Th, T helper; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; TSH, thyroid stimulating hormone



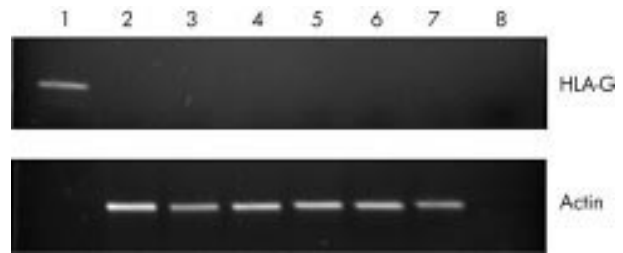
**Figure 1** RT-PCR analysis of HLA-G and  $\beta$  actin gene expression in thyroid tissue samples from patients with autoimmune thyroid disease. The cDNA samples were from: fetal chorionic membranes (lane 1); thyroid tissue from a patient with Graves' disease (lane 2); thyroid tissue from a patient with Hashimoto's thyroiditis (lane 3); thyroid tissue from a patient with multinodular disease (lane 4); and negative control without cDNA (lane 5).

U/ml recombinant IFN $\gamma$  (Roche Diagnostics GmbH, Mannheim, Germany), or 100 U/ml TNF $\alpha$  (Calbiochem, Nottingham, UK), as detailed previously.<sup>19</sup>

RNA was extracted from whole tissue (3 mm<sup>3</sup> pieces), fetal chorionic membranes, and cultured thyroid cells ( $5 \times 10^6$  cells) using TRIZOL<sup>®</sup> LS reagent (Life Technologies, Paisley, UK), according to the manufacturer's protocol. RNA was checked for quality by electrophoresis in a denaturing 1.5% agarose gel. Moloney murine leukaemia virus (M-MLV) RT (Promega, Southampton, UK) was used to synthesise cDNA, as described previously.<sup>19</sup> Briefly, 5  $\mu$ g of total RNA were reverse transcribed with 200 U of M-MLV RT, 500 ng random primers (Promega), and 1mM dNTPs (Promega) in RT buffer (Promega) with 10mM dithiothreitol in a final volume of 60  $\mu$ l for one hour at 37°C.

Two different oligonucleotide primer sets (Life Technologies) were designed to amplify HLA-G cDNA by PCR: DB-G1 (sense), 5'-CCGGAGTATTGGGAAG-3' and DB-G2 (antisense), 5'-CTCATAGTCAAAGACAG-3'<sup>24</sup>; G.257 (sense), 5'-GGAAGAGGAGACACGGAACA-3' and G.1004 (antisense), 5'-CCTTTTCAATCTG AGCTCTTCTT-3'.<sup>23</sup> Primers DB-G1 and DB-G2 detect the expression of membrane bound HLA-G isoforms HLA-G1 and HLA-G4 and soluble HLA-G isoform sHLA-G1 (or HLAG-5). Primers G.257 and G.1004 detect the expression of all membrane bound HLA-G isoforms HLA-G1, HLA-G2, HLAG-3, and HLA-G4 and soluble HLA-G isoforms sHLA-G1 (or HLAG-5) and sHLA-G2 (or HLA-G6). Expression of the  $\beta$  actin gene was used as a quantitative and qualitative control for the cDNA samples and the primer sequences for  $\beta$  actin amplification are detailed elsewhere.<sup>19</sup> To provide a positive control for HLA-G PCR amplifications, cloned HLA-G cDNA was used. Control PCR amplifications without cDNA were always carried out in parallel and were consistently negative. All PCR amplifications were performed in duplicate.

Hot start PCR amplifications (three minutes at 94°C as initial denaturation) were performed in 100  $\mu$ l reactions containing: 2.5 U of Taq DNA polymerase (Promega), 0.1 mM of each dATP, dGTP, dCTP, and dTTP (Promega), 2  $\mu$ l of RT mixture (cDNA), 1  $\mu$ M of each primer, 1.5mM MgCl<sub>2</sub>, 10mM Tris HCl (pH 9), 50mM KCl, and RNase free water to 100  $\mu$ l. Amplifications of 40 cycles were carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Connecticut, USA) using cycles of 94°C for one minute, 55°C for two minutes, and 72°C for two minutes, followed by a final extension of 10 minutes at 72°C. Amplification of  $\beta$  actin was performed using 25 cycles. PCR amplification products and 100 bp molecular weight markers (Promega) were separated on 1% agarose gels and stained with ethidium bromide. The primers for HLA-G produced PCR amplification products of 426 bp<sup>24</sup> and 770 bp,<sup>23</sup> whereas the  $\beta$  actin primers gave a product of 560 bp.



**Figure 2** Reverse transcription polymerase chain reaction analysis of HLA-G and  $\beta$  actin gene expression in thyroid cell culture. The cDNA samples were from: HLA-G cDNA in pcDNA3 (lane 1); unstimulated thyroid follicular cells (TFCs) (lane 2); TFCs stimulated with: interleukin 1 $\alpha$  (lane 3), interferon  $\gamma$  (IFN $\gamma$ ; lane 4), thyroid stimulating hormone (lane 5), tumour necrosis factor  $\alpha$  (TNF $\alpha$ ; lane 6) and TNF $\alpha$  plus IFN $\gamma$  (lane 7); and negative control without cDNA (lane 8).

## RESULTS

Thyroid tissue samples from patients with MNG (n = 6), GD (n = 6), and HT (n = 1) were analysed for HLA-G and  $\beta$  actin gene expression by RT-PCR. The results are illustrated in fig 1 using primers DB-G1 and DB-G2 for HLA-G.  $\beta$  Actin transcripts were demonstrated in all samples, confirming the integrity of the cDNA preparations. In contrast, no HLA-G mRNA was detected in the thyroid tissue from patients with GD, HT, or MNG, although it was clearly evident in the PCR amplifications with fetal chorionic membrane cDNA (fig 1) and with positive control HLA-G cDNA (fig 2). Primers G.257 and G.1004 also failed to amplify HLA-G from cDNA samples derived from thyroid tissue (data not shown).

Figure 2 shows the results of experiments to detect HLA-G mRNA in cultured thyroid cells by RT-PCR using primers DB-G1 and DB-G2. The  $\beta$  actin gene was detected in all the cDNA samples from primary thyroid cell cultures but the expression of HLA-G could not be demonstrated. The effects of TSH, IL-1 $\alpha$ , IFN $\gamma$ , TNF $\alpha$ , and TNF $\alpha$  plus IFN $\gamma$  upon HLA-G gene expression were analysed by RT-PCR following stimulation for six, 12, and 24 hour periods (fig 2). HLA-G mRNA was not detectable in the stimulated cultured thyroid cells. The same results were obtained for HLA-G expression in TFCs using primers G.257 and G.1004 (data not shown).

## DISCUSSION

According to previous reports,<sup>2 14-17</sup> HLA-G might act to protect tissues from damage by infiltrating cytotoxic T cells during the process of inflammation. The characteristic T cell infiltration of the thyroid in patients with autoimmune thyroid disease can eventually lead to tissue destruction, and HLA-G expression might reduce this effect. However, our findings suggest that HLA-G is not expressed in either thyroid tissue or in thyrocytes derived from patients with autoimmune thyroid disease: HLA-G transcripts could not be detected by PCR amplification using two different sets of HLA-G specific primers, even at a high cycle number. This is contrary to a recent study that detailed HLA-G expression in TFCs obtained from patients with GD.<sup>20</sup> Here, expression of full length membrane bound HLA-G1 and its membrane bound short forms—HLA-G2, HLA-G3, and HLA-G4—was demonstrated. The functions of the short forms of HLA-G have yet to be clarified, although their physiological role is thought to be extremely limited,<sup>1</sup> and how their expression might relate to thyroid autoimmunity is unknown. The study also reported that the soluble forms of HLA-G—sHLA-G1 and sHLA-G2—were not expressed in patient TFCs,<sup>20</sup> a finding that does reflect our results. Soluble HLA-G1 molecules have been proposed to suppress activated CD8<sup>+</sup> T cells<sup>8</sup> and help in the control of inflammation.<sup>17</sup> Absence of these sHLA-G1 functions may be responsible in part for the ongoing inflammation that is seen

**Take home messages**

- We did not detect HLA-G expression in the thyroids of patients with Graves' disease, suggesting that HLA-G does not play a pathophysiological role in this autoimmune disorder
- HLA-G expression was not detected in the thyroid sample of the patient with HT either, but more samples are needed to make any firm conclusions

in GD.<sup>20</sup> The differences seen in the expression of HLA-G in TFCs between our study and that of Castro and colleagues<sup>20</sup> may reflect variations in the groups of patients with GD analysed, particularly with respect to any drug treatments that they might have received during the course of the disease.

"Soluble HLA-G1 molecules have been proposed to suppress activated CD8<sup>+</sup> T cells and help in the control of inflammation"

Stimulation of primary thyrocytes with several proinflammatory cytokines, including IFN $\gamma$ , failed to induce HLA-G expression. These results contrast with several studies detailing IFN $\gamma$  stimulated expression of HLA-G in both normal and malignant cells,<sup>21 22</sup> but they do reflect the findings of Frumento *et al*,<sup>23</sup> who failed to stimulate HLA-G expression in melanoma cell lines using IFN $\gamma$ .

From our failure to detect HLA-G transcripts in either thyroid tissue or TFCs from patients with GD we conclude that HLA-G expression does not occur in the thyroid of patients with this autoimmune disorder and that HLA-G does not play a pathophysiological role in GD. Although the expression of HLA-G was not detected in the thyroid sample of the patient with HT, a greater sample size would be required to conclude that HLA-G does not have a part to play in this autoimmune thyroid disease.

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