New advances in coeliac disease: serum and intestinal expression of HLA-G

M. I. Torres¹, M. A. López-Casado², J. Luque³, J. Peña³ and A. Ríos⁴

¹Department of Experimental Biology, Faculty of Sciences, University of Jaén, Paraje de las Lagunillas s/n, 23071 Jaén, Spain
²Department of Gastroenterology Pediatric, Hospital Virgen de las Nieves, Granada, Spain
³Department of Immunology, Hospital Universitario Reina Sofia, Córdoba, Spain
⁴Department of cell biology, University of Granada, Granada, Spain

Keywords: coeliac disease, gluten, HLA-G, IL-10, tolerance

Abstract

Coeliac disease (CD) is a common autoimmune disorder characterized by an immune response to ingested gluten and has a strong HLA association with HLA-DQ2 and HLA-DQ8, but as human HLA-DQ risk factors do not explain the entire genetic susceptibility to gluten intolerance. Our aim was to investigate whether HLA-G, a gene located in the MHC class I region, and with important role in the induction of immunotolerance, may contribute to CD susceptibility. We demonstrated the expression of soluble HLA-G (sHLA-G) forms in intestinal biopsy and in serum of patients with CD. Indeed, all patients tested showed a positive expression of HLA-G in intestinal mucosa with different grade of immunoreaction. The serum levels of sHLA-G found in coeliac patients depend on the association with other diseases of autoimmune nature or genetics, and also depend on the transgressions in the diet with gluten ingested. The enhancer expression of sHLA-G in CD could be due as part of a mechanism to try restore the tolerance process towards oral antigens in a disease caused by loss of tolerance to dietary antigens and counteract the inflammation. In summary, in this paper, we demonstrate the association of CD with sHLA-G expression.

Introduction

Coeliac disease (CD) is a common autoimmune disorder that has genetic, environmental and immunologic components (1–2). CD is characterized by an immune response to ingested wheat gluten and related proteins of rye and barley that leads to inflammation, villous atrophy and crypt hyperplasia in the intestine (2, 3). A defect in antigen processing by epithelial cells, together with the intrinsic properties of the gliadins, as well as the HLA-DQ haplotype of the individual are considered the principal factors involved in the pathogenesis of CD (3). Moreover, it has been shown that the disease is associated with the expression of HLA-DQ2 and HLA-DQ8 (4, 5). In addition, transglutaminase 2 (TTG2) may play an important role in CD development, acting as a deamidating enzyme and as a target auto-antigen in the immune response (6, 7). Exposure to gliadin and related prolamins in humans with an appropriate HLA-DQ haplotype is necessary but not sufficient for developing this disease. An additional risk factor for CD is an anomalous innate immune response. Induction of T cell activation by gluten probably constitutes a key event in the development of the disease (8). Concerning the innate immune response that preceded T cell activation, the resident professional antigen-presenting cells play an essential role for antigen recognition and T cell activation. Indeed, in coeliac patients the activation of the adaptive immune system likely occurs upon encounter of gliadin in the small intestine (9).

In addition to this hypersensitivity to gliadin mediated by T cells, cytokine secretion also contributes to the observed lesion in this pathology (10, 11). In fact, cytokines play a central role in the development of an immune response through their regulatory effect on immune cells, such as Tₚ, and monocytes. For example, IL-10 in conjunction with transforming growth factor-α lead to the enhanced secretion of IgA by activated B cells and inhibit the synthesis of pro-inflammatory cytokines tumour necrosis factor-β, IL-1β and IL-6 by monocytes (11). This immune-mediated pathology is predominantly characterized by a de-regulated immune response at the gut level dominated by T cells of the Tₚ,1 type, although humoral (Tₚ,2)
HLA-G expression in coeliac disease

Coeliac group (patients with irritable bowel syndrome (IBS)) in whom CD was excluded were included in the control non-IBS group. Twenty-four biopsies each patient were for histological analysis and one biopsy was employed by immunohistochemistry analysis. Two biopsies from the MHC class I region, may contribute to CD susceptibility. Because of its low polymorphism and restricted tissue distribution, HLA-G has been considered a non-classical gene or class Ib. HLA-G is highly expressed by human cytotoxic lymphocytes that constitute the maternal–foetal interface (16, 17). The HLA-G gene transcripts for at least seven different HLA-G mRNAs: four membrane-bound HLA-G isoforms, namely HLA-G1, -G2, -G3 and -G4, and three soluble proteins, namely HLA-G5, -G6 and -G7 (18). The presence of soluble HLA-G (sHLA-G) in the cerebrospinal fluid of multiple sclerosis (19) or class Ib. HLA-G is highly expressed by human cytotoxic lymphocytes that constitute the maternal–foetal interface (16, 17). The HLA-G gene transcripts for at least seven different HLA-G mRNAs: four membrane-bound HLA-G isoforms, namely HLA-G1, -G2, -G3 and -G4, and three soluble proteins, namely HLA-G5, -G6 and -G7 (18). The presence of soluble HLA-G (sHLA-G) in the cerebrospinal fluid of multiple sclerosis (19) or allograft acceptance after transplantation (20, 21) suggests a tolerogenic function for this molecule against innate and adaptive cellular immune responses. Interestingly, it has been suggested that HLA-G antigens may play a protective role in inflammation (14). Thus, sHLA-G molecules inhibit lytic activity of NK cells, induce apoptosis of CD8+ CTLs and affect CD4+ alloproliferation (22, 23). In this sense, the immune modulatory properties of sHLA-G explain its potential interest in CD. Our aim was to investigate whether HLA-G, a gene located in the MHC class I region, may contribute to CD susceptibility.

**Methods**

**Patients**

This study was performed after approval of the Ethics Committee. Biopsies of small intestine were obtained following gastrointestinal endoscopy on consenting patients being investigated for CD (the patients were followed at the Virgen de Las Nieves Hospital of Granada, Spain). Two biopsies from each patient were for histological analysis and one biopsy was employed by immunohistochemistry analysis. Twenty-four patients had features typical of active CD and nine patients in whom CD was excluded were included in the control non-coeliac group (patients with irritable bowel syndrome (n = 2), malabsorption syndrome (n = 2) and Crohn’s disease (n = 5)).

**Serological and histological analysis**

The diagnosis of CD is established by means of serologic screening tests accompanied by biopsy of the small intestine and confirmation of a clinical response to gluten elimination from the diet. Subjects were prospectively screened for CD using anti-endomysial antibodies (AEMAs), anti-gliadin antibodies (AAGs), tissue transglutaminase antibodies (TTGAs) and CD-specific HLA typing. The best single measure for screening is IgA anti-recombinant human tissue transglutaminase, measured by means of ELISA, which has high sensitivity and specificity for CD (24).

**Immunohistochemistry analysis**

Samples fixed in formalin and paraffin embedded were used for immunohistochemistry. HLA-G was detected using a mAb MEM-G/1 (Exbio, Prague, Czech Republic), which reacts with denatured HLA-G heavy chain, and 5A6G7 (Exbio) that recognized sHLA-G isoforms. An IgG1 isotypic mAb (Sigma) was used as negative control. Immunohistochemical staining was performed using the UltraTech HRP Streptavidin–Biotin Universal detection system (Immunotech, France). After deparaffinisation and rehydration, sections were microwaved in 10 mM citrate buffer (pH, 6.0) for antigen retrieval. Sections were rinsed in PBS, and the endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water. Sections were then treated with the protein-blocking agent, incubated sequentially with the primary antibody, and then with a biotinylated secondary antibody and with the streptavidin–peroxidase reagent. The immunoreaction was visualized with chromogen working solution AEC (3-amino-9-ethyl-carbazole) (Immunotech). Finally, the sections were counterstained with haematoxylin. A positive control and a negative control tissue were always considered.

**ELISA**

Microtiter plates were coated with the 5A6G7 mAb (10 µg ml⁻¹) as a capture antibody for the detection of sHLA-G in serum. The labelling was performed with the sHLA-G-Kit (Exbio). Optical densities were measured at 450 nm (Organon Teknika®, Turnhout, Belgium). The concentrations of sHLA-G were determined from the value of optical density according to the standard curves (Exbio).

**Statistical analysis**

All results are expressed as mean ± SEM. Data were evaluated for statistical significance using the STATGRAPHICS Plus 5 program. A non-parametric test (Kruskal–Wallis test) was used to determine the differences among groups. Kolmogorov-Smirnov test was used to compare the distribution between groups. P-values <0.05 were considered significant.

**Results**

Coeliac children presented impaired growth, chronic diarrhoea, abdominal distention, poor appetite and hypotonia. The mean weights and heights of the subjects with CD were not significantly different to the reference sex and age matched population (mean weight 13.43 ± 6.14, mean height 90.79 ± 20.05, mean age 3.74 ± 3.07), and the body mass index (BMI) in the coeliac patients was underweight for age with BMI less than fifth percentile (25). The diagnosis of CD is established by means of serologic screening tests accompanied by biopsy of the small intestine and confirmation of a clinical response to
Clinical data of coeliac patients

Table 1. Clinical data of coeliac patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Atrophy grade</th>
<th>AAG</th>
<th>AEMA</th>
<th>ATGA</th>
<th>HLA-DQB1</th>
<th>HLA-DR</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coeliac 1</td>
<td>1</td>
<td>F</td>
<td>7.50</td>
<td>66</td>
<td>3</td>
<td>60</td>
<td>1/320</td>
<td>27.7</td>
<td>0201,0501</td>
<td>1.3</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 2</td>
<td>2</td>
<td>F</td>
<td>9.70</td>
<td>80</td>
<td>3</td>
<td>30.8</td>
<td>1/160</td>
<td>28</td>
<td>0303,0601</td>
<td>7.15(2)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 3</td>
<td>2</td>
<td>F</td>
<td>9.67</td>
<td>79.50</td>
<td>3</td>
<td>68.40</td>
<td>1/160</td>
<td>38</td>
<td>0201,0202</td>
<td>3.7</td>
<td>A. Thyroiditis</td>
</tr>
<tr>
<td>Coeliac 4</td>
<td>6</td>
<td>F</td>
<td>12.30</td>
<td>88</td>
<td>3</td>
<td>200</td>
<td>1/320</td>
<td>90</td>
<td>0201,0501</td>
<td>1.3</td>
<td>Down s</td>
</tr>
<tr>
<td>Coeliac 5</td>
<td>1</td>
<td>M</td>
<td>7.8</td>
<td>75</td>
<td>2</td>
<td>12</td>
<td>1/160</td>
<td>10</td>
<td>0302,0301</td>
<td>4.4</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 6</td>
<td>12</td>
<td>F</td>
<td>21.5</td>
<td>118</td>
<td>3</td>
<td>19.2</td>
<td>1/160</td>
<td>28</td>
<td>0201,0301</td>
<td>3.11(5)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 7</td>
<td>2</td>
<td>F</td>
<td>9.7</td>
<td>74</td>
<td>3</td>
<td>199</td>
<td>1/160</td>
<td>18</td>
<td>0201,0202</td>
<td>3.7</td>
<td>Allergy</td>
</tr>
<tr>
<td>Coeliac 8</td>
<td>8</td>
<td>F</td>
<td>26.5</td>
<td>128</td>
<td>3</td>
<td>102</td>
<td>1/320</td>
<td>97</td>
<td>0202,0301</td>
<td>7.11(5)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 9</td>
<td>1</td>
<td>F</td>
<td>7.40</td>
<td>68.5</td>
<td>3</td>
<td>200</td>
<td>1/120</td>
<td>120</td>
<td>0201,0101</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 10</td>
<td>5</td>
<td>M</td>
<td>14.3</td>
<td>100.4</td>
<td>2</td>
<td>31.5</td>
<td>1/160</td>
<td>28</td>
<td>0301,0302</td>
<td>4.11</td>
<td>A. Thyroiditis</td>
</tr>
<tr>
<td>Coeliac 11</td>
<td>10</td>
<td>M</td>
<td>26</td>
<td>130</td>
<td>3</td>
<td>23.5</td>
<td>1/320</td>
<td>165</td>
<td>0202,0301</td>
<td>7.11(5)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 12</td>
<td>2</td>
<td>F</td>
<td>13</td>
<td>89</td>
<td>3</td>
<td>30</td>
<td>1/160</td>
<td>8</td>
<td>0201,0202</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 13</td>
<td>7</td>
<td>F</td>
<td>27</td>
<td>125</td>
<td>2</td>
<td>100</td>
<td>1/320</td>
<td>111</td>
<td>0201,0302</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 14</td>
<td>2</td>
<td>F</td>
<td>11.5</td>
<td>80.5</td>
<td>1</td>
<td>7.55</td>
<td>1/160</td>
<td>20</td>
<td>0201,0202</td>
<td>3.11</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 15</td>
<td>1</td>
<td>F</td>
<td>8</td>
<td>76.5</td>
<td>3</td>
<td>200</td>
<td>1/320</td>
<td>9.3</td>
<td>0201,0202</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 16</td>
<td>2</td>
<td>M</td>
<td>11.3</td>
<td>90</td>
<td>3</td>
<td>111</td>
<td>1/320</td>
<td>118</td>
<td>0201,0202</td>
<td>3.7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 17</td>
<td>2</td>
<td>F</td>
<td>14</td>
<td>92</td>
<td>3</td>
<td>49</td>
<td>1/5</td>
<td>70</td>
<td>0301,0202</td>
<td>11(5),7</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 18</td>
<td>3</td>
<td>M</td>
<td>11.5</td>
<td>89.5</td>
<td>3</td>
<td>144</td>
<td>1/5</td>
<td>0.7</td>
<td>0502,0602</td>
<td>15.16</td>
<td>Down s, allergy</td>
</tr>
<tr>
<td>Coeliac 19</td>
<td>1</td>
<td>M</td>
<td>6.6</td>
<td>65</td>
<td>3</td>
<td>2.23</td>
<td>1/5</td>
<td>0.4</td>
<td>0602,0604</td>
<td>13.15(2)</td>
<td>A. Thyroiditis, Down s</td>
</tr>
<tr>
<td>Coeliac 20</td>
<td>7</td>
<td>F</td>
<td>10.6</td>
<td>79</td>
<td>3</td>
<td>20.1</td>
<td>1/5</td>
<td>0.8</td>
<td>0201,0503</td>
<td>3.14(6)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 21</td>
<td>7</td>
<td>M</td>
<td>23</td>
<td>126</td>
<td>3</td>
<td>19.3</td>
<td>1/20</td>
<td>2.6</td>
<td>0201,0303</td>
<td>4.13(6)</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 22</td>
<td>2</td>
<td>F</td>
<td>12.8</td>
<td>89.5</td>
<td>3</td>
<td>36</td>
<td>1/360</td>
<td>95</td>
<td>0201,0301</td>
<td>7.11</td>
<td>No</td>
</tr>
<tr>
<td>Coeliac 23</td>
<td>2</td>
<td>F</td>
<td>11.5</td>
<td>83</td>
<td>1</td>
<td>55</td>
<td>1/320</td>
<td>90</td>
<td>0301,0501</td>
<td>11.10</td>
<td>Allergy</td>
</tr>
<tr>
<td>Coeliac 24</td>
<td>2</td>
<td>F</td>
<td>13</td>
<td>95</td>
<td>2</td>
<td>65</td>
<td>1/80</td>
<td>15</td>
<td>0302,0501</td>
<td>4.4</td>
<td>No</td>
</tr>
</tbody>
</table>

AAG, anti-gliadin antibody expressed as milligrams per litre; AEMA, anti-endomyosial antibody; ATGA, anti-transglutaminase antibody expressed as units per millilitre and Down s, down syndrome.
on a gluten-free diet. The C group presented very low levels of sHLA-G (1.5 U ml$^{-1} \pm 0.3$). There were significant differences between the patients of the group CD1 and the other two groups (CD2 and CD3), as well as between the patients of CD1 group and non-coeliac patients (C) (Fig. 2). Although not statistically significant, there were also differences between the patients of group CD2 and the non-coeliac patients.

It is interesting to underline the behaviour of one of the patients (JP), father of a coeliac child (MBP). This patient was considered as non-coeliac, presenting a normal biopsy and normal serological test values for endomysial antibodies (EMAs) and TTGAs. However, the patient presented low immune reactivity in the intestinal biopsy for sHLA-G and positive values of sHLA-G in serum (25 U ml$^{-1}$).

**Immunohistochemistry of IL-10**

Finally, we have determined IL-10 production in patients with CD. Immunohistochemistry for IL-10 was performed on formalin fixed tissues and the immune reactivity was semi-quantitatively scored. A strong IL-10 immunoreactivity was observed in the lamina propria in coeliac patients (Fig. 3). This cytokine was expressed mainly in areas infiltrated by inflammatory cells.

**Fig. 1.** Expresión de sHLA-G en pacientes coeliaques. Magnificación: $\times$200. (A) Expresión de sHLA-G en pacientes coeliaques activos con 5A6G7 (flecha). (B) Resultados negativos en el inmunostaining con el antígeno MEM/G1. (C) Expresión de sHLA-G en la superficie apical de la mucosa intestinal y criptas de Lieberkuhn (flechas).

**Fig. 2.** Niveles de sHLA-G en pacientes coeliaques ($n = 24$) y controles ($n = 5$) mediante ELISA. Expresado como unidades por mililitro. C, grupo control; CD1, grupo coeliaque 1; CD2, grupo coeliaque 2 y CD3, grupo coeliaque 3. a: versus grupo control, $P < 0.001$; b: diferencias entre grupos coeliaques, $P < 0.001$.

**Fig. 3.** Reactividad inmunológica de IL-10 en el tejido propio de los pacientes coeliaques (asterisco). Magnificación: $\times$200.
Discussion

In this study, we demonstrate an association of CD with HLA-G expression. To our knowledge, this is the first study to describe the expression of sHLA-G in biopsy samples and in serum from patients with CD. Conversely, membrane HLA-G molecules were not expressed in coeliac patients. The lack of membrane HLA-G expression may be linked to a specific regulatory process in the alternative splicing of the primary HLA-G transcript, which could favor selection of the soluble isoforms.

The immune modulatory properties of shLA-G justify its potential interest in the control of inflammatory diseases, such as CD. In this study, we have demonstrated that the plasmatic level of shLA-G was (i) more elevated in coeliac patients with other associated diseases, as Down syndrome or autoimmune thyroiditis, (ii) elevated or medium in coeliac patients with transgressions of the diet and (iii) low or even negative levels in free-gluten-diet coeliac patients for >5 years without transgression of the diet. So far, a gluten-free diet is the only therapy that can be provided to coeliac patients. Moreover, sometimes it is difficult to follow a completely gluten-free diet and some patients continue to include gluten in their diet. As a result, in some individuals, the recovery of the intestinal mucosa becomes extended and may take >18 months (27).

On the other hand, CD can be defined as ‘silent’ in an apparently healthy subject. Many of them have a normal or minimally abnormal intestinal mucosal architecture and no typical HLA predisposing genotype (DQ2 or DQ8) and they are negative to EMAs and/or anti-human TTGAs. Interestingly, in our study, one silent patient was positive for shLA-G expression at serum and mucosal level. Although this result is preliminary and a higher number of cases are required, it suggests that shLA-G expression can be considered as a useful marker in a silent coeliac patient.

The increased prevalence of autoimmune diseases in coeliac patients, including insulin dependent diabetes mellitus and autoimmune thyroid diseases, has been widely reported (27, 28). Our results showed a correlation between increased levels of shLA-G and CD associated with other autoimmune diseases. In agreement with that, the connection between CD and other autoimmune disorders might be dependent on a genetic linkage of these pathologies through HLA genes.

The soluble form of HLA-G is of special interest because its expression plays an important role in the induction of immune tolerance (29). An immune-suppressive function of HLA-G might thus contribute to control CD4+ and CD8+ activities, consequently, playing an important role in adaptive immunity (30, 31). In this sense, shLA-G has the function to inhibit the proliferation of activated T cells, and to induce apoptosis of T cells dose dependently, reinforcing the immune inhibitory role of shLA-G capable to be secreted during CD as part of a mechanism to restore the tolerance process towards oral antigens. Concerning the adaptive response in CD, a powerful anti-inflammatory response to gliadin might occur during the development of the disease. In coeliac patients, gluten intake seems to cause an overreaction in intra-epithelial T lymphocytes, with uncontrolled production of HLA-G-and IL-10. This may cause recruitment of intra-epithelial lymphocytes, leading to a vicious circle with amplified immune activity and maintenance of intestinal lesions.

Cytokines may play important roles in inducing HLA-G expression. In fact, it has been demonstrated that IL-10, up-regulate HLA-G expression (32). Therefore, we cannot exclude that in particular stimulatory situations, secreted cytokines induce HLA-G expression. Here, we consider the possibility that the production of sHLA-G is affected by macrophage secreted cytokines. Macrophages can control immune responses by secreting anti-inflammatory cytokines such as IL-10. The role of IL-10 in inducing HLA-G protein expression has been already demonstrated in monocytes and purified trophoblast cells (32, 33). Moreover, an association between IL-10 and HLA-G expression was demonstrated in cutaneous lymphomas (32) and in ulcerative colitis (15).

In conclusion, the enhancer expression of shLA-G in CD could be due as part of a mechanism to try restore the tolerance process towards oral antigens in a disease caused by loss of tolerance to dietary antigens. A powerful anti-inflammatory response to gliadin might occur during the development of the disease with uncontrolled production of HLA-G and IL-10 that counteract the inflammation or/and may cause recruitment of intra-epithelial lymphocytes, maintaining the intestinal lesions. Moreover, the expression of shLA-G may become an immunohistologic parameter for the diagnosis of CD. This has an important relevance in patients with typical or non-classical symptoms, even clinically silent, who remain undiagnosed and are exposed to the risk of long-term complications and do not display clinical manifestations of the CD.

Acknowledgements

The authors thank Dr. López Nevot MA for HLA typing and Dr. Fernández MI for English corrections.

Abbreviations

AAG anti-gliadin antibody
AEMA anti-endomysial antibody
BMI body mass index
CD coeliac disease
EMA endomysial antibody
shLA-G soluble HLA-G
TTGA tissue transglutaminase antibody
TTG2 transglutaminase 2

References

HLA-G expression in coeliac disease


