ORIGINAL ARTICLE

Expression of glucocorticoid receptors in non-neoplastic lymphoid follicles and B cell type malignant lymphomas

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Objective: To evaluate the expression of human glucocorticoid receptors (hGRs), such as hGR (4H2), hGR- α , and hGR- β , in non-neoplastic lymphoid follicles and B cell type malignant lymphomas.

Methods: The expression of hGRs in non-neoplastic lymphoid follicles and malignant lymphomas, including diffuse large cell lymphoma, mantle cell lymphoma, and follicular lymphoma, was examined immunohistochemically. HGR (4H2) expression was confirmed by double immunostaining of tissues and in isolated cells from tonsillar germinal centres, and by immunoelectronmicroscopy.

Results: In secondary lymphoid follicles of any non-neoplastic diseases—such as chronic tonsillitis, reactive lymphadenitis, and Kimura's disease—the germinal centre cells often expressed hGR (4H2) and hGR- α . Double immunocytochemical staining of isolated germinal centre cells showed that the majority of hGR (4H2) positive cells were CD20 positive B cells, and that follicular dendritic cells also expressed hGR. Immunoelectronmicroscopy revealed the presence of nuclear hGR (4H2) in the binucleated follicular dendritic cells and germinal centre cells. The frequency of hGR (4H2) expression in diffuse large B cell lymphoma was higher, that in mantle cell lymphoma was lower, and that in follicular lymphoma was intermediate among the types of malignant lymphoma. The hGR (4H2) expression was less frequent in cases of grade I follicular lymphoma.

Conclusions: There are differences in hGR expression between the germinal centre and the mantle zone in non-neoplastic lymphoid follicles, and differences of hGR (4H2) expression among the types of malignant lymphoma and grades of follicular lymphoma, which probably contribute to the different steroid sensitivities.

The administration of glucocorticoids inhibits lymphocyte migration, survival and pooling of lymphocytes in lymphoid tissues, and the production and secretion of inflammatory cytokines in many types of cell, resulting in significant effects for both cellular and humoral immunity.^{1–2} Glucocorticoids bind to receptors on target cells and regulate the complicated intracellular signalling pathway.^{3–5} There are two protein isoforms of the human glucocorticoid receptor (hGR), hGR- α and hGR- β , which arise from alternative splicing of the hGR pre-mRNA primary transcripts, and hGR is expressed on numerous types of cells in the brain, skeletal muscle, nasal mucosa, and lymphocytes,⁶ and their neoplastic counterparts.^{7–9} It is evident that the effect of steroid treatment depends principally on the expression level of hGRs in some human diseases.¹⁰

Secondary lymphoid follicles with a germinal centre are composed of five zones—the mantle zone (MZ), outer zone (OZ), dark zone (DZ), basal light zone (BLZ), and apical light zone (ALZ)—that each have distinctly different roles.¹¹ Follicular dendritic cells, which build a three dimensional reticular meshwork in whole lymphoid follicle, trap and retain antigens for a long period on their surface, and regulate the proliferation, selection, and differentiation of germinal centre B cells.¹¹ However, it remains questionable whether follicular cells in these five zones express hGR to the same extent and are equally sensitive to steroids. Furthermore, information about the expression and significance of hGR on neoplastic cells derived from the follicular compartments is limited.

The aim of the present study was to evaluate the expression of hGR in non-neoplastic lymphoid follicles in some lymphoid tissues and in B cell type malignant lymphomas. Our study provides information on the effectiveness of steroid treatment in non-neoplastic lymphoid diseases and B cell type malignant lymphomas.

METHODS

Tissue samples

Tissue specimens were obtained at the time of operation from 10 cases of chronic tonsillitis and reactive lymphadenitis without steroid administration. Lymph node and soft tissue specimens were also obtained from seven cases of Kimura's disease. Six of these patients were treated with oral prednisolone and one patient by desensitisation. Neoplastic lymph node specimens were obtained at first biopsy from 43 cases of malignant lymphoma without steroid treatment or chemotherapy. Histological diagnosis was made by WHO classification.¹² Fifteen cases of follicular lymphoma (seven grade I, six grade II, and two grade III), 11 cases of mantle cell lymphoma, and 17 cases of diffuse large B cell lymphoma were studied. Tissue specimens were routinely fixed in 10% formalin, embedded in paraffin, and cut into 5 μ m thick sections for immunohistochemistry. Fresh tissue specimens were obtained from another eight patients with chronic tonsillitis and used for cell isolation from germinal centres and immunoelectronmicroscopy.

Informed consent was obtained from all patients before biopsy and surgery, and the study was carried out in accordance with the ethics code for human experimentation, Yamagata University School of Medicine.

Immunohistochemistry for hGR

Immunostaining of hGR (4H2)

Paraffin sections were routinely immunostained using the labelled streptavidin-biotin peroxidase complex (LSAB) technique. After blocking endogenous peroxidase activity and after antigen retrieval, sections were incubated with a

Abbreviations: ALZ, apical light zone; BLZ, basal light zone; DZ, dark zone; HGR, human glucocorticoid receptor; MZ, mantle zone; OZ, outer zone

monoclonal antibody against hGR (4H2; mouse IgG2a, which is specific for human glucocorticoid receptor, N-terminal modulating region, Medac, West Hamburg, Germany)¹³ overnight at 4°C, and treated with the ultratech horseradish peroxidase (HRP) kit polyvalent biotinylated antibody (Immunoteck, Royalmount, Canada) for one hour, followed by the ultratech HRP kit streptavidin-peroxidase (Immunoteck) for 30 minutes. Positive cells were visualised with 3,3'-diaminobenzidine (DAB) (Dojin Chemicals, Kumamoto, Japan), and were counterstained with haematoxylin. The expression of hGR in non-neoplastic follicles was estimated in five zones—MZ, OZ, ALZ, BLZ, and DZ—with CD23 immunostaining (H107; mouse IgG2b, Nichirei, Tokyo, Japan).¹⁴ In non-neoplastic lymphoid follicles, at least 40 fields in each area of secondary lymphoid follicles were evaluated at high power ($\times 400$) under a microscope to determine the proportion of positive cells in each case. In case of malignant lymphomas, we assessed least 40 fields of neoplastic follicles of follicular lymphoma and of diffuse proliferating areas of other types of lymphoma. The average rate (per cent) of hGR (4H2) positive cells per total cells was calculated. The immunostaining was independently evaluated by two observers (SO and MY). Intraobserver and interobserver variations were calculated.

Immunostaining for hGR- α and hGR- β

The immunostaining was carried out by the LSAB technique using rabbit anti-hGR- α antibody (GR (P-20); sc-1002, which reacts with GR- α of human origin by western blotting; non-cross-reactive with hGR- β ; Santa Cruz Biotechnology, Santa Cruz, California, USA),¹⁵ followed by the LSAB2 kit/HRP (Dako, Glostrup, Denmark) and by the ABC technique, using rabbit anti-hGR- β antibody (PA3-514, which detects a 90 kDa protein representing GR- β from human cells by western blot; Affinity BioReagents, Golden, Colorado, USA),¹⁵ followed by biotin-SP conjugated AffiniPure F(ab')₂ donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and the ABC reagent (Vector Laboratories, Burlingame, California, USA). For hGR- β immunostaining, the sections were digested with 0.1% trypsin (Difco Laboratories, Detroit, Michigan, USA) for 30 minutes at 37°C for antigen retrieval. The sections were counterstained with methyl green. The average proportion (per cent) of hGR- α and hGR- β positive cells per total cells in tonsillar and lymphoma tissues was calculated, as for hGR (4H2).

Double immunohistochemical staining

Paraffin sections were firstly immunostained for hGR (4H2) using the LSAB method with 3-amino-9-ethyl carbazole (AEC; Dako) (red). The sections were then incubated with each antibody against CD4 (1F6; mouse IgG1, κ ; Novocastra, Newcastle upon Tyne, UK), CD8 (4B11; mouse IgG2b; Novocastra), CD20 (L26; mouse IgG2a, κ ; Dako), CD23 (1B12; mouse IgG1, κ ; Novocastra), and CD45RO (UCHL1; mouse IgG1, κ ; Dako) and were immunostained using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method with 5-bromo-4-chloro-3-indoxyl phosphatase and nitro-blue tetrazolium chloride (BCIP/NBT; Dako) (purple).

Isolation of single cells and cellular clusters from germinal centres and double immunocytochemical staining

Single cells and cellular clusters were isolated from tonsillar germinal centres with an enzyme cocktail consisting of collagenase IV (Sigma Chemical, St Louis, Missouri, USA) and deoxyribonuclease I (Sigma), according to a previously described method.¹⁴ Cytospin preparations were firstly immunostained for hGR (4H2) using the APAAP method

with BCIP/NBT (Dako) (purple). Sections were subsequently incubated with antibodies against S-100 protein (rabbit; Nichirei), CD3 (rabbit; Dako), CD20 (L26; Dako), CD21 (1F8; mouse IgG1, κ ; Novocastra), and CD57 (Leu7; mouse IgM; Becton-Dickinson, Franklin Lakes, New Jersey, USA) and were immunostained using the LSAB method with AEC (Dako) (red).

Immunoelectronmicroscopy of hGR (4H2) in the germinal centres

Immunoelectronmicroscopy for hGR (4H2) was carried out with tonsillar tissues fixed in periodate-lysine-paraformaldehyde, according to a previously described method.¹⁶

Controls for immunostaining

Negative controls for each immunostaining procedure were run in parallel by replacing the specific antibody with phosphate buffered saline, rabbit immunoglobulin, or mouse IgG1, IgG2a, or IgG2b (Dako).

Statistical analysis

Data are presented as the mean (SD). Statistical analyses were undertaken using the StatView 5.0 software package (Abacus Concepts, Berkeley, California, USA). Intraobserver reproducibility was evaluated by analysis of variance and interobserver reproducibility by Spearman's test. The Mann-Whitney non-parametric test was applied for the detection of significant differences ($p < 0.05$) in the incidence of occurrence of positive cells between zones of non-neoplastic lymphoid follicles, types of malignant lymphomas, and grades of follicular lymphoma.

RESULTS

HGR (4H2) immunostaining in non-neoplastic lymphoid follicles

The frequency of hGR expressing cells in the tonsillar MZ was significantly lower than in any other zone ($p < 0.01$) (fig 1A; table 1). There was a significant difference of hGR expression between the BLZ and the DZ ($p < 0.05$), but not among the OZ, ALZ, and BLZ. In cases of reactive lymphadenitis and Kimura's disease the OZ was not evident. In the two diseases the frequency of hGR expressing cells in the MZ was significantly lower than in any other zone ($p < 0.01$) (fig 1B; table 1). No significant differences were observed among the three zones of germinal centres in the reactive lymph nodes and tissues in Kimura's disease. In five cases with Kimura's disease that were followed up clinically to assess the responsiveness to steroid therapy, the four cases who were sensitive to steroids had a higher incidence of hGR positive cells in any of the four zones compared with the steroid resistant case (table 2). Evaluation of intraobserver reproducibility did not show statistically significant differences ($p = 0.978$ in tonsil and reactive lymphadenitis, and $p = 0.978$ in Kimura's disease), and a high grade of correlation was found between the two observers ($p = 0.925$ in tonsil and reactive lymphadenitis, and $p = 0.913$ in Kimura's disease).

Double immunohistochemical staining on paraffin sections indicated that the majority of hGR positive cells were CD20 positive B cells and the minority were CD4, CD8, or CD45RO positive T cells (fig 2, panels A to D). In addition, hGR and CD23 double positive follicular dendritic cells were evident in the germinal centres (fig 2E).

Double immunocytochemical staining on single cells and cellular clusters isolated from tonsillar germinal centres

The majority (about 60–70%) of hGR (4H2) positive cells were CD20 positive B cells (fig 3A) and the minority were

Figure 1 Immunohistochemical staining of human glucocorticoid receptor (hGR; 4H2) in secondary lymphoid follicles of a palatine tonsil with chronic tonsillitis (A) and a lymph node with reactive lymphadenitis (B). HGR was often expressed in the outer zone (OZ), apical light zone (ALZ), basal light zone (BLZ), and dark zone (DZ), but less often in the mantle zone (MZ). Counterstained with haematoxylin. Original magnification $\times 40$.

Table 1 The expression of human glucocorticoid receptor (4H2) in five zones of non-neoplastic lymphoid follicles

Lymphoid follicle zone	Chronic tonsillitis and reactive lymphadenitis (n = 20) (%) [*]	Kimura's disease (n = 7) (%) [*]
Mantle zone	25.18 (10.02) [†]	19.00 (10.14) [†]
Outer zone	66.40 (10.43)	
Apical light zone	72.12 (9.50)	67.31 (12.61)
Basal light zone	75.01 (8.20) [‡]	59.35 (13.23)
Dark zone	64.05 (9.60)	50.52 (12.12)

Values are mean (SD).

^{*}The incidence (%) of human glucocorticoid receptor positive cells was calculated in a high power field ($\times 400$).

[†] $p < 0.001$ (mantle zone v outer zone, apical light zone, basal light zone, and dark zone, respectively).

[‡] $p < 0.05$ (basal light zone v dark zone) by Mann-Whitney U test.

Table 2 Comparison of the incidence of human glucocorticoid receptor (4H2) positive cells between steroid sensitive and steroid resistant cases of Kimura's disease

Lymphoid follicle zone	Steroid sensitive cases (n = 4) (%) [*]	Steroid resistant cases (n = 1) (%) [*]
Mantle zone	34.72 (9.54)	12.84
Apical light zone	64.85 (11.28)	42.96
Basal light zone	59.22 (9.34)	35.85
Dark zone	49.45 (10.37)	43.16

Values are mean (SD) for steroid sensitive cases.

^{*}The incidence (%) of human glucocorticoid receptor positive cells was calculated in a high power field ($\times 400$).

CD3 positive T cells (10–20%) (fig 3B) and CD57 positive cells (10–20%) (fig 3C). Occasionally, S-100 protein or CD21 positive follicular dendritic cells simultaneously expressed hGR.

Immunoelectronmicroscopy of hGR (4H2) in germinal centres

HGR expression was confirmed in germinal centre lymphocytes and binucleate or multinucleate follicular dendritic cells, showing nucleosomal labelling in a dotted or coarse granular pattern (fig 4, panels A and B) compared with negative controls (fig 4C).

Immunohistochemistry of hGR- α and hGR- β in tonsillar tissues

As for hGR (4H2), hGR- α was expressed more frequently in germinal centres (about 80% of total cells) than in the MZ (about 40%) in non-neoplastic lymphoid follicles. Unlike hGR (4H2), about 70% cells of the LZ and MZ, respectively, expressed hGR- β with no significant differences between the two zones. Intraobserver and interobserver reproducibility achieved $p = 0.920$ and $p = 0.890$ in hGR- α , and $p = 0.908$ and $p = 0.873$ in hGR- β , respectively.

HGR (4H2) immunostaining in B cell type malignant lymphomas

In a comparison of the three types of malignant lymphoma, the incidence (%) of hGR expression was increased in diffuse large B cell lymphoma, decreased in mantle cell lymphoma, and intermediate in follicular lymphoma, respectively (fig 5, panels A to E; table 3); hGR expression in grade I follicular lymphoma was significantly less frequent than in grades II and III (fig 5, panels C to E). Intraobserver and interobserver reproducibility achieved $p = 0.966$ and $p = 0.916$, respectively.

Immunohistochemistry of hGR- α and hGR- β in B cell type malignant lymphomas

Tumour cells from malignant lymphomas often expressed both hGR- α and hGR- β , indicating no significant differences in the incidence of the two types of hGR positive cell between the types of malignant lymphoma and the grades of follicular lymphoma (fig 6, panels A to J; table 3). Intraobserver and interobserver reproducibility achieved $p = 0.933$ and $p = 0.893$ in hGR- α , and $p = 0.919$ and $p = 0.862$ in hGR- β , respectively.

DISCUSSION

In this study, B cells in the germinal centre of non-neoplastic lymphoid follicles, especially CD20 positive B cells, expressed hGR (4H2) more often than those in the MZ, indicating that in contrast to MZ B cells, germinal centre B cells with a high affinity for the antigen trapped and retained by follicular

Figure 2 Double immunostaining of human glucocorticoid receptor (hGR; 4H2) (red) and lymphoid cell and follicular dendritic cell markers (purple) on paraffin preparation of palatine tonsils with chronic tonsillitis. A majority of hGR positive cells were CD20 positive T cells (A) and the minority were CD4 positive (B), CD8 positive (C), or CD45RO positive (D). Note the hGR and CD23 double positive follicular dendritic cells in the germinal centre (E). LZ, light zone; MZ, mantle zone. Original magnification $\times 400$.

Figure 3 Double immunocytochemical staining of human glucocorticoid receptor (hGR; 4H2) (purple) and lymphoid cell and follicular dendritic cell markers (red) on cytopsin preparation of single cells and a cell cluster isolated from the tonsillar germinal centres. Note the simultaneous expression of hGR and CD20 (A), CD3 (B), or CD57 (C) on follicular lymphoid cells. Original magnification $\times 400$.

Figure 4 Immunoelectronmicroscopy showing the nuclear staining (arrows) of human glucocorticoid receptor (4H2) in a binucleated follicular dendritic cell (A) and lymphocytes (L) (B) in the tonsillar germinal centre. (C) Negative control lacking the specific primary antibody (4H2). Original magnification $\times 4800$, bar = 1 μm .

Figure 5 Immunostaining of human glucocorticoid receptor (hGR; 4H2) in B cell type malignant lymphomas. The expression of hGR was frequent in diffuse large B cell lymphoma (A) but less frequent in mantle cell lymphoma (B). The hGR expression in follicular lymphoma was significantly less frequent in grade I (C) compared with grades II (D) and III (E). Counterstained with haematoxylin. Original magnification $\times 400$.

dendritic cells may be highly sensitive to steroid therapy. Follicular dendritic cells play a very important role in the differentiation and maturation of B cells.^{17,18} The present study also confirmed hGR expression in follicular dendritic cells by double immunostaining, suggesting that steroids may also influence germinal centre immunity through modification of dendritic cell function.

Kimura's disease is an inflammatory disorder of unknown aetiology seen in endemic form in the Japanese but also in other parts of the world. It usually presents as a mass lesion in the subcutaneous tissue of the head and neck region or the major salivary glands, often associated with regional lymphadenopathy, usually in adolescents and young adult men. Microscopically, the tissues involved show dense lymphocytic

Figure 6 Immunostaining of human glucocorticoid receptor (hGR)- α (panels A to E) and hGR- β (panels F to J) in B cell type malignant lymphomas. Both hGR- α and hGR- β were often expressed in grades I (panels A and F), grade II (panels B and G), and grade III (panels C and H) of follicular lymphoma, in mantle cell lymphoma (panels D and I), and in diffuse large B cell lymphoma (panels E and J), with no significant differences among the grades of follicular lymphoma and types of B cell lymphoma. Counterstained with methyl green. Original magnification $\times 400$.

Table 3 Expression of human glucocorticoid receptors (hGRs) in B cell type malignant lymphomas

Histology	hGR (4H2) (%) [*]	hGR- α (%) [*]	hGR- β (%) [*]
Diffuse large cell lymphoma (n = 17)	80.36 (13.09)†‡	93.55 (5.99)	92.48 (6.72)
Mantle cell lymphoma (n = 11)	39.70 (20.01)†‡	88.22 (10.31)	83.76 (10.22)
Follicular lymphoma (all; n = 15)	51.08 (16.73)	89.76 (10.12)	82.18 (12.99)
Grade I (n = 7)	45.70 (17.74)‡	87.25 (10.03)	78.98 (10.78)
Grade II (n = 6)	55.53 (16.03)	90.23 (8.11)	84.55 (8.76)
Grade III (n = 2)	56.72 (7.19)	91.11 (7.19)	79.25 (9.71)

Values are mean (SD).

^{*}Incidence (%) of hGR positive cells calculated in a high power field ($\times 400$).

† $p < 0.001$, diffuse large cell lymphoma v mantle cell lymphoma and mantle cell lymphoma v follicular lymphoma, respectively (Mann-Whitney U test).

‡ $p < 0.05$, diffuse large cell lymphoma v follicular lymphoma and grade I follicular lymphoma v grades II and III, respectively (Mann-Whitney U test).

infiltration with lymphoid follicles, interstitial fibrosis, and hyalinised vessels. There is also extensive infiltration by eosinophils, with occasional formation of eosinophilic abscesses. An increase in the number of plasma cells and mast cells has been noted. Occasional lymphoid follicles show marked hyperplasia of germinal centres. In general, the mass lesion of Kimura's disease is removed by surgery. For subsequent treatment, steroids accompanied by antiallergic agents are the drugs of first choice. In the course of multiple courses of steroids, glucocorticoid resistance develops in some patients, particularly those with bronchial asthma,^{10, 19} because of downregulation of hGR expression. In this study, the cases of Kimura's disease that were sensitive to steroid treatment expressed hGR (4H2) more often than those that were resistant to steroids.

Significant differences were observed in the frequency of hGR (4H2) positive cells among the types of B cell lymphoma, and also between the grades of follicular lymphoma, reflecting the differential sensitivity to steroid therapy. It is important to be able to estimate steroid sensitivity in lymphoma patients because malignant lymphomas are treated with combined chemotherapy (CHOP, MACOP-B, or VACOP-B) which includes steroids such as prednisolone.²⁰⁻²²

HGR- α is expressed in most human tissues as a ligand dependent transcription factor.²³ HGR- β does not bind glucocorticoids and is an inhibitor of hGR- α activity.^{3, 24, 25} Relative expression of these two types of hGR may determine the level of glucocorticoid sensitivity.²⁶⁻²⁸ The incidence of hGR- α positive cells in tonsillar germinal centres was higher than that in MZ. However, hGR- β was expressed at almost equivalent frequencies in the germinal centre and MZ. In the light of the relative amounts of the two types of hGR, these data suggest a link between hGR- β expression and the frequent insensitivity to steroid administration in MZ cells compared with the germinal centre. However, steroid pulse therapy not only inhibits the migration of circulating lymphocytes into lymph nodes but also induces apoptotic cell death of germinal centre cells.²⁹ By contrast, there was no significant difference between the incidences of the two hGR isoform positive cells in malignant lymphomas. It is very difficult to estimate steroid sensitivity precisely in patients with malignant lymphomas, as almost all cases are treated by combined chemotherapy. The steroid sensitivity of malignant lymphomas may be regulated in a complex manner by many factors, including the expression level of hGRs in malignant cells, and the dose, timing, and duration of steroid administration.

Conclusions

This is the first report demonstrating the localisation of hGRs in non-neoplastic lymphoid follicles and malignant

lymphomas, and the different frequencies of hGR (4H2) positive cells between the MZ and germinal centre of non-neoplastic lymphoid follicles, among different types of B cell type lymphoma, and among different grades of follicular lymphoma.

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