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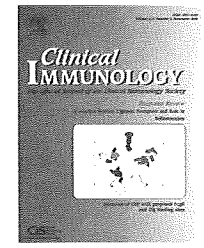

The relationship between the expression of the glucocorticoid receptor in biopsied colonic mucosa and the glucocorticoid responsiveness of ulcerative colitis patients

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Glucocorticoid receptor;
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Regulatory T cell;
Foxp3

Abstract The objective of this study was to clarify the relationship between the frequency of infiltrating cells expressing the glucocorticoid receptors (GR) α and β in biopsied colonic mucosa and the glucocorticoid (GC) responsiveness of ulcerative colitis (UC) patients. Active UC patients ($n=38$) were divided into GC-sensitive and GC-resistant groups. GRβ$^+$ cells were significantly higher in the GC-resistant group than in the GC-sensitive and control groups. GRα mRNA was expressed in all UC patients, while GRβ mRNA was expressed in only 1 patient in the GC-sensitive group ($n=6$) and 7 patients in the GC-resistant group ($n=8$). Double-positive cells for GRβ and CD4 or CD19 were frequently observed. The Foxp3$^+$ cell count was significantly higher in the GC-sensitive group than in the GC-resistant group, but double Foxp3$^+$GRβ$^+$ cells were not observed. These results indicated that the sensitivity of GC therapy could probably be predicted by immunostaining biopsy specimens for GRβ and Foxp3.

Introduction

Glucocorticoid (GC) therapy is recognized as an effective therapy for inflammatory bowel disease, autoimmune disease, and allergic disorders; furthermore, GCs are widely used as the primary drug of choice for the active stage of ulcerative colitis (UC) [1]. However, the mechanism of action of GCs has not yet been completely elucidated, and the reasons why they have different effects on individual patients in different clinical settings also remain a mystery.

The expression of glucocorticoid receptors (GRs) in target cells is an essential component of the pharmacological action of GCs: GCs bind to GRs and result in the effect. The GR has 2 isoforms, GRα and GRβ, which are produced by alternate splicing of mRNA from the same gene [2]. GRα binds to GCs, translocates from the cytoplasm to the nucleus, and forms homodimers [3]. Thereafter, it binds to the GC response elements present in the promoter region of the target gene

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and regulates the transcriptional activity of the various genes involved in inflammation, producing an anti-inflammatory response [4,5]. In contrast, GRβ is considered to be a possible inhibitor of GRα. This may be explained by the fact that GRβ cannot bind to GCs [6,7] or that it forms heterodimers with GRα and thus cannot bind with the GC response elements of the target gene [8,9].

In the peripheral blood mononuclear cells of UC patients, GRβ mRNA has been reported to be highly expressed in groups with a poor response to GC therapy and minimally expressed in groups with a good response to GC therapy [10]. In addition, there have been similar reports for bronchial asthma and rheumatoid arthritis [11,12]. In contrast, GRβ has been reported to be unable to control the action of GRα [13], while the expression of GRβ does not cause a poor GC response in UC [14,15]. Clearly, the role of GRβ remains controversial. Previous reports have examined the expression of GRα and GRβ in peripheral blood mononuclear cells and cultured cells like HeLaS3 and COS7, but no reports have extensively examined the expression of GRα and GRβ in the inflammatory cells of colonic mucosal tissue, a locus of inflammation.

$CD4^+$ T cells play a crucial role in eliciting an immune reaction to foreign antigens. Regulatory T cells (Tregs) account for 5–10% of the fraction of $CD4^+$ T cells and express CD25. Tregs are involved in maintaining immunological self-tolerance by suppressing autoreactive lymphocytes [16]. The transcription factor Foxp3 is specifically expressed in Tregs, and acts as the master control gene for the development of Tregs [17]. Tregs are known to exist at various sites, including the intestinal mucosa [18,19]. Patients with active UC have decreased numbers of peripheral Tregs and increased numbers of lamina propria Tregs, suggesting a correlation between increased Treg numbers and decreased severity, and supporting the hypothesis that Tregs traffic to sites of inflammation in an attempt to restore immune homeostasis [20,21]. Therefore, if Tregs express GRβ, they would be closely associated with GC resistance/refractoriness. However, to our knowledge, there have been no reports on the correlation between $Foxp3^+$ Treg localization in the lamina propria, the GC responsiveness of patients with active UC, or GRβ expression on these Tregs. The current study was designed to assess these issues.

The aim of this study was to clarify the relationship between the frequency and type of infiltrating cells expressing GRα, GRβ, and Foxp3 in biopsied colonic mucosa, and the GC responsiveness of UC patients.

Materials and methods

Patients and materials

The subjects were patients with initial onset or relapse of UC. Specimens were taken via colonoscopic biopsy from 38 active UC patients with a clinical activity index (CAI) [22] of 5 or higher. Patients with a CAI of 4 or lower were omitted from the present study, because they were clinically in remission and therefore did not require GC therapy. All patients had previously received a 5-aminosalicylic acid preparation but no immunomodulators like azathioprine, methotrexate, or Remicade. A GC (20 mg or more) was administered to all patients, and a biopsy was performed within a week of the start of treatment. The patients were divided into 2 groups, GC-sensitive and GC-resistant in accordance with a previous report [10], and GC was administered to active UC patients with a CAI of 5 or higher. Subjects with a CAI of 4 or lower and to whom 20 mg or less of GC was administered within 4 weeks were defined as sensitive, while those with a CAI of 5 or higher after 4 weeks or those who required surgery were defined as resistant. The GC-sensitive group consisted of 18 patients with a mean age of 41.5±13.7 years (19–67 years), and a mean CAI of 7.1±1.9 (5–13) before GC administration (Table 1). The GC-resistant group consisted of 20 patients with a mean age of 38.3±13.8 years (15–57 years), and a mean CAI of 10.1±3.4 (5–15) before GC administration. Specimens that were taken via a rectal mucosal biopsy from 10 patients who underwent a colonoscopic polypectomy and who had a mean age of 47.3±8.7 years (39–64 years) were used as control. Informed consent was obtained from all patients. The tissue specimens were fixed in 10% buffered formalin for 12 h at room temperature, and were then embedded in paraffin for immunohistochemistry of GRα, GRβ, and Foxp3; immunodouble staining for GRβ and CD4, CD8, CD20, or CD68; and fluorescence immunodouble staining for GRβ and Foxp3. Some parts of the tissue specimens were fixed in 4% paraformaldehyde (PFA) for 6 h at 4 °C, immersed in sucrose gradient buffers, and frozen in a Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) for immunodouble staining of GRβ and CD19. The frozen specimens were kept at −80 °C until cryostat sectioning. Some parts of the tissue specimens were stored at 4 °C for reverse transcription-polymerase chain reaction (RT-PCR) of GRα and GRβ mRNAs.

Table 1 Cases used in this study.

Clinical data	Disease/control		
	Ulcerative colitis		Control (n=10)
	GC-sensitive (n=18)	GC-resistant (n=20)	
Male/female	12/6	15/5	6/4
Age (years)	41.5±13.7	38.3±13.8	47.3±8.7
Clinical activity index	7.1±1.9	10.1±3.4	–
Disease duration (years)	7.5±7.1	6.1±4.5	–

GC; glucocorticoid. Data of age, clinical activity index, and disease duration are represented as mean±SD.

Immunohistochemistry

Immunostaining for GRα, GRβ, and Foxp3

The formalin-fixed and paraffin-embedded sections of the colonic tissues (from 18 GC-sensitive patients with UC, 20 GC-resistant patients with UC, and 10 controls) were immunostained for GRα, GRβ, and Foxp3. Five-μm-thick paraffin sections were cut. After deparaffinization, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide. The slides were immersed in 0.01 M of phosphate-buffered saline (PBS; pH 7.4) with 0.01 M citrate buffer (pH 6.0; Muto Pure Chemicals, Tokyo, Japan) and microwaved (400 W) for 25 min at 90 °C. The primary antibodies used in this study were anti-GRα (rabbit; SCB, Santa Cruz, CA), anti-GRβ (rabbit; ABR, Golden, CO), and anti-Foxp3 (236A/E7, mouse IgG1; Abcam, Cambridge, UK). The slides were then incubated at 4 °C overnight with the relevant primary antibodies. The labeled streptavidin–biotin peroxidase method (Ultratech HRP Streptavidin–biotin Universal Detection System, DAKO, Glostrup, Denmark) was used for the immunohistochemistry of GRα and Foxp3. GRβ immunostaining was performed using the avidin–biotin peroxidase complex (ABC) as a secondary antibody. A positive reaction was detected as a brown coloration with 3,3′-diaminobenzidine (DAB; Dojin Chemicals, Kumamoto, Japan). The sections were counterstained with hematoxylin.

Immunodouble staining for GRβ with lymphocyte and macrophage markers in GC-resistant patients with UC

Immunodouble staining for GRβ by using lymphocyte and macrophage markers (CD4, CD8, CD19, CD20, and CD68) was performed to identify the $GR\beta^+$ cell type in the GC-resistant group. The paraffin sections obtained from 10 GC-resistant patients with UC were used for immunodouble staining for GRβ with CD4, CD8, CD20, and CD68. Briefly, the deparaffinized tissue sections were immersed in either 0.1% trypsin (Difco, Franklin Lakes, NJ) in PBS with 0.15% $CaCl_2$ for 30 min at 37 °C or in 0.01 M citrate buffer (pH 6.0) and microwaved (400 W) for 25 min at 90 °C. First, CD4, CD8, CD20, and CD68 were immunostained. The primary antibodies used in this study were anti-CD4 (OPD4, mouse IgG1κ; DAKO), anti-CD8 (C8/144B, mouse IgG2bκ; DAKO), anti-CD20 (L26, mouse IgG2aκ; DAKO), and anti-CD68 (KP1, mouse IgG1κ; DAKO). The slides were incubated at 4 °C overnight with the relevant primary antibodies. The alkaline phosphatase labeled-dextran polymer (Envision/AP; DAKO) was used as a secondary antibody. A positive reaction was detected as a pink coloration with the Fuchsin Substrate System (DAKO). The slides were immersed in 0.01 M citrate buffer (pH 6.0) and microwaved (400 W) for 10 min at 90 °C. Next, the endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide, and the slides were incubated at 4 °C overnight with the anti-GRβ antibody. Immunostaining for GRβ was done using the ABC technique as a secondary antibody. A positive reaction was detected as a brown coloration with DAB. The sections were counterstained with hematoxylin.

The PFA-fixed, frozen cryostat sections, which were obtained from 7 GC-resistant patients, were used for immunodouble staining for GRβ and CD19. First, CD19 immunostaining was performed. The primary antibodies used in this study were anti-CD19 (HD37, mouse IgG1κ; DAKO). The slides were incubated at 4 °C overnight with the relevant primary antibodies. Envision/AP was used as a secondary antibody. A positive reaction was detected as a pink coloration with the Fuchsin Substrate System. Next, the slides were incubated at 4 °C overnight with the anti-GRβ antibody. The endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide. Immunostaining for GRβ was performed using ABC as a secondary antibody. A positive reaction was detected as a brown coloration with DAB. The sections were counterstained with hematoxylin.

Fluorescence immunodouble staining for GRβ and Foxp3

The formalin-fixed and paraffin-embedded sections of the colonic tissues (from 3 GC-sensitive and 3 GC-resistant patients with UC) were fluorescence immunodouble stained for GRβ and Foxp3. After deparaffinization, the slides were immersed in PBS with 0.01 M citrate buffer (pH 6.0), microwaved (400 W) for 25 min at 90 °C, and incubated at 4 °C overnight with the anti-GRβ antibody. The Alexa 568-conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR) was used as a secondary antibody. Next, the slides were incubated at 4 °C overnight with the anti-Foxp3 antibody. The Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) was used as a secondary antibody. The fluorescent signals were visualized using a fluorescence microscope (BX60; Olympus, Tokyo, Japan) equipped with a mercury light and appropriate filters.

Control immunostaining study

The formalin-fixed and paraffin-embedded sections and fresh-frozen cryostat sections of the lymph nodes from patients with reactive lymphadenitis were similarly immunostained to be used as positive controls. In addition, the sections were incubated with 0.01 M PBS and non-immune rabbit or mouse immunoglobulin (DAKO), respectively, instead of the primary antibody.

Immunohistochemical evaluation of positive cells

At random, 8–13 fields of view were chosen from the lamina propria with no lymph follicle. Immunopositive cells for GRα, GRβ, and Foxp3 were counted under light microscopy (×400) independently by 2 observers, and the average number of positive cells in 1 view was calculated. Immunodouble staining for GRβ with lymphocyte and macrophage markers (CD4, CD8, CD19, CD20, and CD68) was performed in the GC-resistant group. The proportion of each type of double-positive cells to all GRβ-positive cells was calculated. Values were represented as mean ± standard deviation.

RT-PCR of GRα and GRβ mRNAs

Total RNA was isolated from the tissue specimens (from 6 GC-sensitive and 8 GC-resistant UC patients) by using EASYPrep RNA (Takara Bio, Tokyo, Japan). The RNA was dissolved in RNase-free water and stored at −80 °C.

One μg of total RNA was used directly for the one-step RT-PCR reaction with a total volume of 50 μl, including 2 μl of QIAGEN OneStep RT-PCR Enzyme Mix (Qiagen), 0.6 μM of specific primers, 400 μM each of dNTPs, and 10 μl of QIAGEN OneStep RT-PCR Buffer (Qiagen). The primers used for PCR were as follows: GRα, 5′-CCTAAGGACGGTCTGAAGAGC-3′ and 5′-GCCAAGTCTTGGCCCTCTAT-3′, amplifying a

477-bp product; and GRβ, 5′-CCTAAGGACGGTCTGAAGAGC-3′ and 5′-CCACGTATCCTAAAAGGGCAC-3′, amplifying a 366-bp product [6]. The samples were heated for 30 min at 50 °C and for 15 min at 95 °C before 35 (for GRα) or 40 cycles (for GRβ) of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min. β-actin was amplified as a housekeeping gene by using the human β-actin RT-PCR Primer Set (Toyobo, Osaka, Japan). The PCR products and a 100-bp DNA ladder (Takara Bio, Shiga, Japan) were separated on 2% agarose gel and were then incubated with 10 ng/ml ethidium bromide for 30 min. The signals were detected with UV (260 nm) excitation and the use of a trans-illuminator, and the images of the gels were captured with a CCD camera.

Statistical analysis

Statistical analysis was performed using the Statview-J software program (Abacus Concepts, Berkeley, CA). Inter-observer reproducibility was evaluated by Spearman's test. The ANOVA test followed by Scheffé test was used for the analysis of the number of GRα$^+$ and GRβ$^+$ cells, and for that of immunodouble staining for GRβ with lymphocyte and macrophage markers. Fisher's exact test was used for the analysis of GRβ mRNA. The Mann–Whitney *U* test was used for the analysis of the number of Foxp3$^+$ cells. The Spearman rank test was used for the analysis of the correlation between GRβ and Foxp3. Differences with a value of $p<0.05$ were considered to be statistically significant.

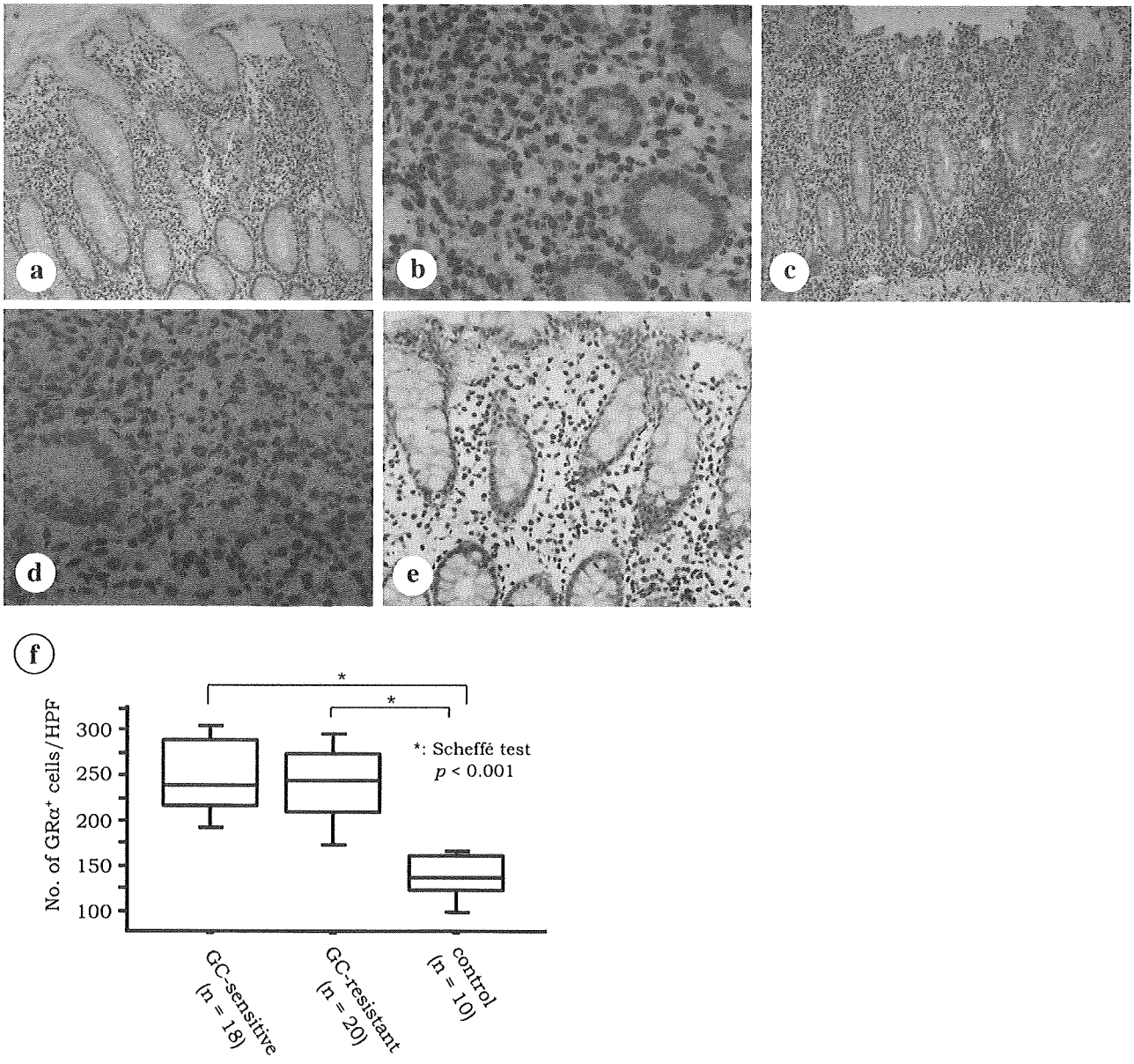

Figure 1 The distribution of glucocorticoid receptor-α (GRα)$^+$ cells in the biopsied colonic lamina propria of the glucocorticoid (GC)-sensitive group (a, b), GC-resistant group (c, d), and control group (e). Cells were counterstained with hematoxylin (original magnification: a and c, ×100; b and d, ×400; e, ×200). The number of GRα$^+$ cells per field in the GC-sensitive and GC-resistant groups was significantly higher than that in the control group (Scheffé test, $p<0.001$) (f). No significant difference was observed between the GC-sensitive and GC-resistant groups (Scheffé test, $p=0.905$).

Results

Relationship between GRα$^+$ and GRβ$^+$ cell count and GC responsiveness in the colonic mucosa of UC patients

The GRα$^+$ cell count per field in the colonic lamina propria was significantly higher (Scheffé test, $p<0.001$) in both the GC-sensitive (245.0±45.9 cells) and GC-resistant groups (238.4±48.1 cells) than in the control group (135.7±26.5 cells) (Figs. 1a–e). A significant difference was not noted between the GC-sensitive and GC-resistant groups (Scheffé test, $p=0.905$; Fig. 1f).

Most of the GRβ$^+$ cells present in the colonic lamina propria of UC patients were mononuclear cells with stained nuclei (Figs. 2a–e). Unlike GRα, the neutrophils were negative for GRβ. The GRβ$^+$ cell count per field was significantly higher (Scheffé test, $p<0.001$) in the GC-resistant group (66.5±16.1 cells) than in the GC-sensitive (11.3±5.1 cells) and the control group (4.2±1.6 cells). In addition, a significant difference between the GC-sensitive group and control group was not noted (Scheffé test, $p=0.474$; Fig. 2f).

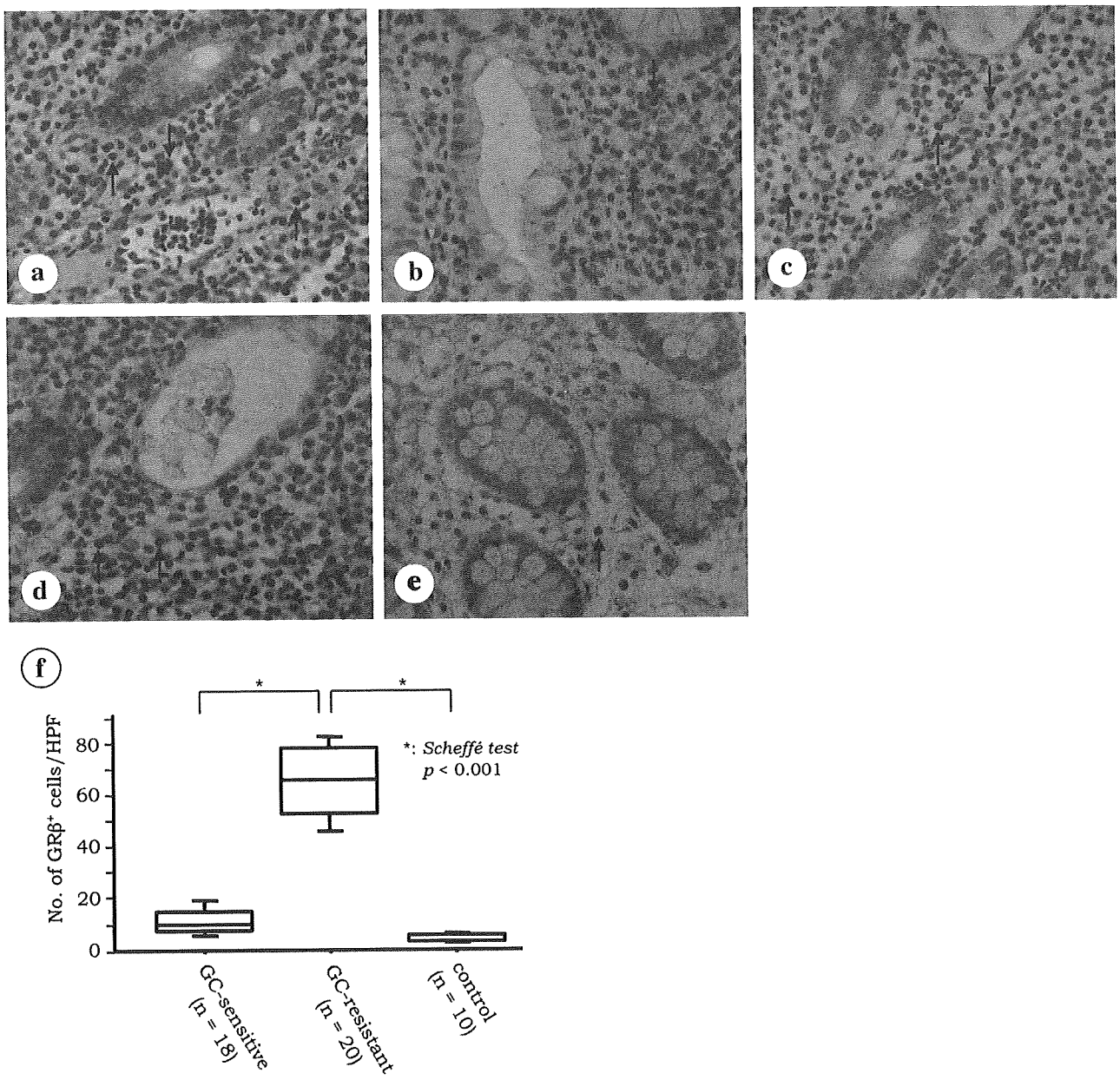

Figure 2 The distribution of glucocorticoid receptor-β (GRβ)$^+$ cells in the biopsied colonic lamina propria of the glucocorticoid (GC)-sensitive group (a, b), GC-resistant group (c, d), and control group (e). GRβ$^+$ cells are indicated by arrows. Cells were counterstained with hematoxylin (original magnification: a–e, ×400). The GRβ$^+$ cell count per field was significantly higher in the GC-resistant group than the GC-sensitive and control groups (Scheffé test, $p<0.001$) (f).

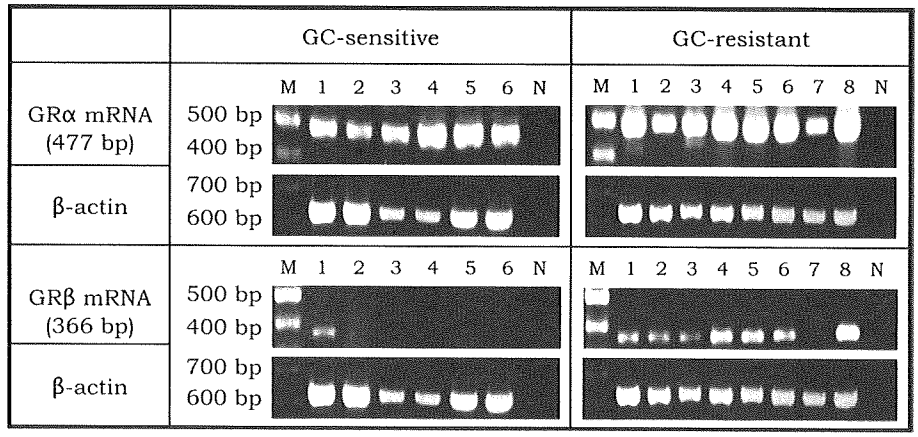

Figure 3 The expression of glucocorticoid receptor-α (GRα) and GRβ mRNA in biopsied colonic mucosa of the GC-sensitive group ($n=6$) and the GC-resistant group ($n=8$). β-actin (645 bp) was used as a housekeeping gene. GRα mRNA (477 bp) was expressed in all cases (upper column). On the other hand, GRβ mRNA (366 bp) was expressed in only 1 of 6 patients in the GC-sensitive group, and 7 of 8 patients in the GC-resistant group (lower column). M; molecular marker, N; negative control.

Association between the expression of GRα and GRβ mRNA and GC responsiveness in the colonic mucosa of UC patients

RT-PCR was performed for 6 patients in the GC-sensitive group and 8 patients in the GC-resistant group. GRα mRNA was expressed in all patients in both the GC-sensitive and GC-resistant groups (Fig. 3). In contrast, GRβ mRNA was expressed in 1 of 6 patients in the GC-sensitive group and 7 of 8 patients in the GC-resistant group, and a significant statistical difference was observed (Fisher's exact test, $p=0.016$).

Type of GRβ$^+$ cells and immunodouble staining with lymphocyte and macrophage markers in the GC-resistant group

The immunodouble staining for GRβ with lymphocyte and macrophage markers (CD4, CD8, CD19, CD20, and CD68) was performed in order to identify the GRβ$^+$ cell type in the GC-resistant group (Figs. 4a–f). The proportion of each type of double-positive cell with respect to all the GRβ$^+$ cells was then calculated. CD4$^+$GRβ$^+$ cells ($n=10$) accounted for 25.8% ±6.9%, CD8$^+$GRβ$^+$ cells ($n=10$) for 12.2%±2.4%, CD19$^+$GRβ$^+$ cells ($n=7$) for 23.9%±6.3%, CD20$^+$GRβ$^+$ cells ($n=10$) for 6.1% ±4.9%, and CD68$^+$GRβ$^+$ cells ($n=10$) for 8.0%±2.5%. Of all the GRβ$^+$ cells, double-positive cells for GRβ and CD4 or CD19 were significantly more numerous than double-positive cells for GRβ and CD8, CD20, or CD68 (Scheffé test, $p<0.01$) (Table 2). The remaining GRβ$^+$ cells were other types of mononuclear cells and stromal cells, including vascular endothelial cells and fibroblasts. Very few epithelial cells in the crypt base were weakly positive, contrasting with the stronger staining of intraepithelial lymphocytes.

Association between Foxp3$^+$ cell count and GC responsiveness in the colonic mucosa of UC patients

Immunostaining for Foxp3 was performed using cells from the GC-sensitive ($n=15$) and GC-resistant groups ($n=19$) (Figs. 5a–d). The mean Foxp3$^+$ cell count per field was 14.4± 6.8 cells in the GC-sensitive group and 8.3±2.7 cells in the GC-resistant group. The Foxp3$^+$ cell count per field was significantly higher in the GC-sensitive group than the GC-resistant group (Mann–Whitney U test, $p=0.011$; Fig. 5e).

Relationship between GRβ$^+$ cells and Foxp3$^+$ cells in the colonic mucosa of UC patients

Fluorescence immunodouble staining for GRβ and Foxp3 performed using cells from the GC-sensitive ($n=3$) and GC-resistant groups ($n=3$). In these 6 patients, double-positive cells for GRβ and Foxp3 were not noted (Figs. 5f–g).

Reproducibility analysis

A high grade of correlation was found between the 2 observers (Table 3).

Discussion

Endoscopic findings of the colonic mucosa and peripheral blood findings such as counts of immune cells and their precursors, cytokines, and other inflammatory mediators are essential to diagnose UC. However, the pathological findings of biopsied colonic mucosa are indispensable for making a definite diagnosis and for evaluating pathobiology, including GR expression and responsiveness to GC therapy, because UC is characteristically a mucosal and submucosal disease [23]. Therefore, although the expression of GRα and GRβ mRNA has been evaluated in peripheral blood mononuclear cells in UC patients [10,14,15], it is better to analyze colonic tissue [14,15] and in particular, colonic mucosa–a locus of inflammation–to evaluate the mRNA expression. GR may be expressed not only in immune cells but also ubiquitously in some types of cells in whole colonic tissue from UC patients. Until now, only one study [14] has reported the expression of GR, but not that of GRα and GRβ in the colonic mucosa of UC patients in a few cases ($n=4$), showing nuclear localization of GR in immune cells and crypt epithelial cells. The design of the current study differed from previous studies focusing on

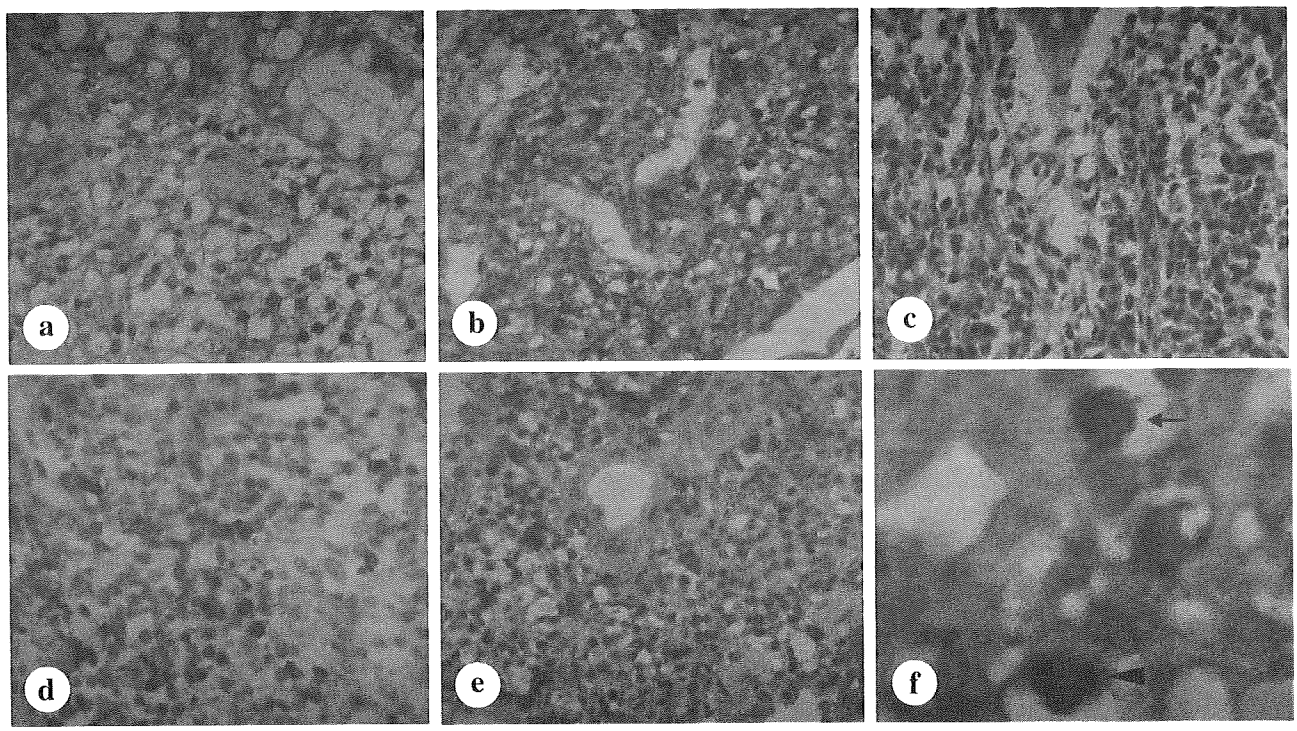

Figure 4 The type of cells expressing glucocorticoid receptor-β (GRβ) in the glucocorticoid (GC)-resistant group. The positive reaction for GRβ and lymphocyte and macrophage markers such as CD4 (a, f), CD8 (b), CD19 (c), CD20 (d), and CD68 (e) was detected as pink and brown coloration, respectively. Panel f demonstrated a high-power view of the $CD4^+GR\beta^+$ cells (arrowhead) and $CD4^+GR\beta^+$ cells (arrow). Cells were counterstained with hematoxylin (original magnification: a–e, ×400; f, ×1000).

mucosal immune cells; our study demonstrated the expression of both GRα and GRβ in immune cells in the colonic lamina propria of UC patients and the correlation of the frequency of positive cells with GC responsiveness.

In the present study, most $GR\beta^+$ cells were mononuclear. The $GR\beta^+$ cell count was minimal in the control group. This correlates with a report [24] that GRβ mRNA is not expressed in normal colonic mucosa. The $GR\beta^+$ cell count was significantly higher in the GC-resistant group than the GC-sensitive group (Scheffé test, $p<0.001$). In addition, GRβ mRNA was also noted at higher levels in the GC-resistant group than the GC-sensitive group. An increasing $GR\beta^+$ cell count and mRNA expression in the colonic mucosa is associated with a resistance to GC therapy. This result is similar to the results from studies in which GRβ mRNA was expressed at high levels in the peripheral blood mononuclear cells of UC patients in a group that responded poorly to GC therapy [10,25]. The default mRNA splicing pathway for GR is one that produces GRα, but in contrast to the GC-sensitive group, it alternates to the pathway producing GRβ in the GC-

Table 2 The type of cells expressing glucocorticoid receptor-β (GRβ) in colonic mucosa of the glucocorticoid-resistant ulcerative colitis patients.

Immunodouble staining	Number of cases	Percentage of the double positive cells among $GR\beta^+$cells*	
$CD4^+GR\beta^+$	10	25.8±6.9 (12.4, 35.4)	b
$CD8^+GR\beta^+$	10	12.2±2.4 (8.9, 15.6)	a
$CD19^+GR\beta^+$	7	23.9±6.3 (15.4, 32.1)	b
$CD20^+GR\beta^+$	10	6.1±4.9 (1.2, 16.7)	b
$CD68^+GR\beta^+$	10	8.0±2.5 (4.2, 12.5)	b

*Data are represented as mean±SD (min, max). *F*-value and *p*-value were 31.963 and <0.0001, respectively, by ANOVA test. [a]$p<0.007$ and [b]$p=0.001$ by Scheffé test.

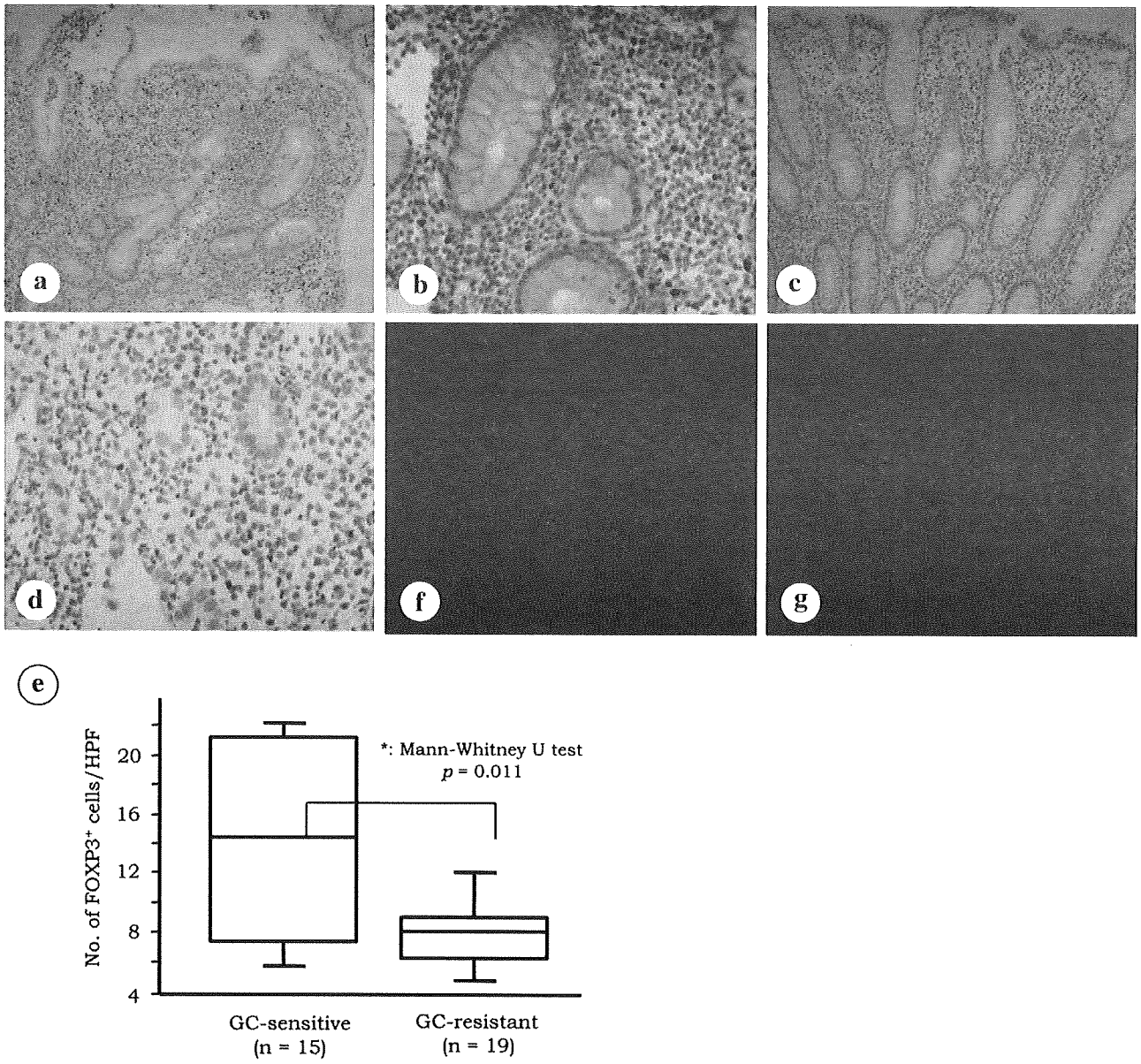

Figure 5 The distribution of Foxp3$^+$ cells in the glucocorticoid (GC)-sensitive (a, b) and GC-resistant groups (c, d). Cells were counterstained with hematoxylin (original magnification: a and c, ×100; b and d, ×200). The number of Foxp3$^+$ cells was significantly higher in the GC-sensitive group than the GC-resistant group (Mann–Whitney *U* test, p=0.011) (e). Fluorescence immunodouble staining for glucocorticoid receptor-β (GRβ) (red) and Foxp3 (green) demonstrated that the GC-sensitive group (f) had relatively more Foxp3$^+$ cells and less GRβ$^+$ cells than the GC-resistant group (g) (original magnification: f and g, ×400).

resistant group. A report described that GRβ was not an inhibiting factor for GRα because the amount of expression of GRβ is remarkably lower than that of GRα [14]. Webster et al. [26] reported that the half-life of GRβ was twice as long as that of GRα; GRα is down-regulated as a result of binding GCs, but GRβ is not down-regulated, suggesting that at low levels of expression, GRβ could function as an inhibitor of GRα.

CD4$^+$ and CD19$^+$ cells accounted for a large proportion of the GRβ$^+$ cells present in the colonic mucosa of the GC-resistant group, suggesting that CD4$^+$ and CD19$^+$ lymphocytes may, as a result of the GC response, be closely related. CD19 is expressed on pro-B/pre-B cells, which are the initial stages of B lymphocyte differentiation, to mature B cells, and the expression of CD19 is considered necessary for the development and maturation of normal B lymphocytes in the bone marrow. Jinno et al. [27] reported that UC involves abnormalities in the mechanism of differentiation and maturation of B lymphocytes and is characterized by a heavy proliferation of abnormal plasma cells expressing CD19, and that CD19$^+$ plasma cells are often observed in groups of patients with resistance to GC therapy and are, therefore, associated with GC resistance. Our study did not examine whether the CD19$^+$GRβ$^+$ cells present in the colonic mucosa of the group with a poor GC response were CD19$^+$ plasma cells, but the finding that CD19$^+$ lymphocytes are associated with GC resistance was similar to Jinno et al.

Table 3 Interobserver variation.

Immunostaining	Number of cases	Interobserver variation	
		r	*p*
GRα	48	0.908	<0.0001
GRβ	48	0.885	<0.0001
CD4/GRβ	10	0.875	<0.0001
CD8/GRβ	10	0.896	<0.0001
CD19/GRβ	7	0.862	<0.0001
CD20/GRβ	10	0.890	<0.0001
CD68/GRβ	10	0.902	<0.0001
Foxp3	34	0.887	<0.0001

GR; glucocorticoid receptor, *r*: Spearman's coefficient of correlation.

$CD4^+$ T cells are known to be closely associated with the pathology of UC. In the active stage of UC, $CD4^+$ T cells increase and are activated [28]. Treg cells may be related to the onset and restoration of active UC [21,29,30]. In this study, $CD4^+GR\beta^+$ cells were numerous in the GC-resistant group and $Foxp3^+$ Tregs were significantly more numerous in the GC-sensitive group than the GC-resistant group, suggesting that increasing Treg counts in active UC lamina propria may be indicative of a good GC response. However, double-positive cells for GRβ and Foxp3 were not observed in the current study. On the basis of the above findings, Treg and $GR\beta^+$ cells are considered to be separate cells.

In the present study, $GR\beta^+$ was expressed not only in T and B cells and macrophages but also in other types of mononuclear cells and stromal cells. Dendritic cells often accumulate beneath the crypt epithelium and adjacent to crypt abscesses in active UC [31], and these cells are positive for GR [14]. Very few epithelial cells in the crypt base were weakly positive and the vast majority were negative; this is consistent with previous reports [14,32] revealing that GR expression was strong in esophageal squamous epithelia, pancreatic islet cells, and hepatocytes, but generally weak or negative in gastric and colonic epithelia.

In conclusion, the present study firstly demonstrated the frequency and type of cells expressing GRα and GRβ in the biopsied colonic mucosa, and evaluated the relationship between the frequency of $GR\alpha^+$, $GR\beta^+$, and $Foxp3^+$ cells and GC responsiveness in UC patients. In this study, $GR\beta^+$ cells in the biopsied colonic mucosa were primarily lymphocytes and some neutrophils were also present. $CD4^+$ and $CD19^+$ cells accounted for a large proportion of the $GR\beta^+$ cells present in the colonic mucosa of the GC-resistant group, suggesting that both $CD4^+$ and $CD19^+$ lymphocytes may be related to the GC response. $Foxp3^+$ cells were significantly more numerous in the GC-sensitive group than the GC-resistant group. The sensitivity of GC therapy could probably be predicted by immunostaining the biopsy specimens for GRβ and Foxp3.

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Epidemiology of hepatocellular carcinoma in Japan

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Primary liver cancer, 95% of which is hepatocellular carcinoma (HCC), is ranked third in men and fifth in women as a cause of death from malignant neoplasms in Japan. The number of deaths and death rate of HCC began to increase sharply in 1975. These numbers peaked at 34510 and 27.4/100000, respectively, in 2004, but decreased to 33662 annual deaths and a 26.7/100000 death rate in 2006. Although hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are both major causes of HCC, HCV-related HCC represents 70% of all cases. The incidence of HCC without hepatitis B surface antigen (HBsAg) or antibodies to HCV (anti-HCV) accounts for 8%–15% of HCC patients nationwide. Geographically, HCC is more frequent in western than eastern Japan, and death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. Interferon therapy for chronic hepatitis C reduces the risk of development of HCC, especially among patients with sustained virological response. Further research should focus on the mechanisms of carcinogenesis by HCV and HBV, development of more effective treatments, and establishment of early detection and preventative approaches. Better understanding of HCC unrelated to HCV and HBV, possibly caused by steatohepatitis and diabetes, should also be a major concern in future studies.

Key words: HCC, HCV, HBV, nonalcoholic steatohepatitis (NASH), interferon

Introduction

The three leading causes of death in Japan since 1981 are malignant neoplasms, cardiovascular diseases, and cerebrovascular diseases. For the past 30 years, liver cancer has been the third leading cause of death from malignant neoplasms in men, following lung and stomach cancer. In women, liver cancer has ranked fifth as a cause of death during the past decade, following colon, stomach, lung, and breast cancer. Primary liver cancer can be classified into three types according to the cell from which the cancer originated, namely, hepatocellular carcinoma (HCC), cholangiocellular carcinoma, and other. As HCC accounts for up 95% of all primary cancer cases, the term "liver cancer" usually means HCC.[1]

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two major causes of HCC in Japan.[2,3] The increase in incidence of HCC in Japan, however, is largely attributable to the increase of HCV infection in the general population during the past 50 to 60 years.[2]

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Changes in deaths and death rates of primary liver cancer

Changes in annual deaths from primary liver cancer among different age groups between 1958 and 2006 are shown in Fig. 1. The total number of deaths from HCC was stable at fewer than 10000 persons/year until 1975 before showing a sharp increase. The spike in 1995 resulted from a change in the International Classification of Disease (ICD) code from ICD 9 to ICD 10, which included intrahepatic bile duct cancer, accounting for approximately 5% of HCC deaths.

The majority of HCC mortalities were in patients below the age of 69 until 1999, when this age reached 70 years. In 2006, 66% of patients with HCC were over 70. The number of deaths from HCC reached 34510 in 2004, but decreased to 33662 in 2006.

The death rates of liver cancer by sex (Fig. 2) are consistently higher in men than in women. A sharp rise in the death rate of primary liver cancer in men began

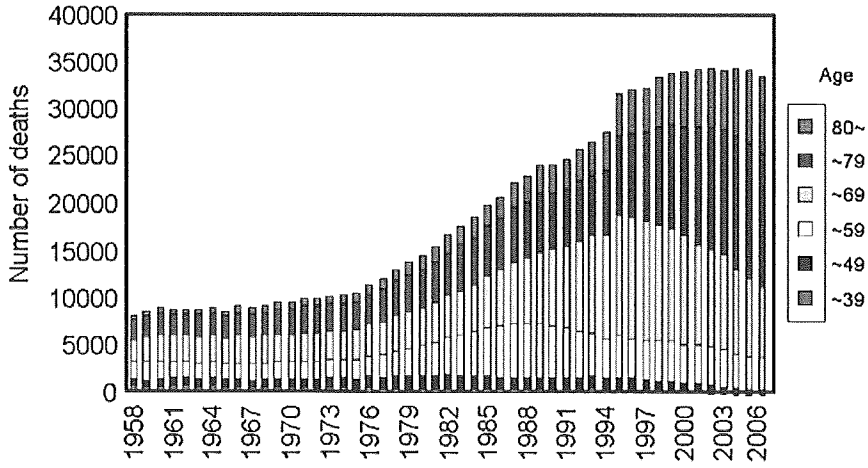

Fig. 1. Changes in annual deaths of patients (by age, in years) with primary liver cancer between 1958 and 2006. (Taken from the Vital Statistics of Japan, released every year by the Ministry of Health, Labour, and Welfare)

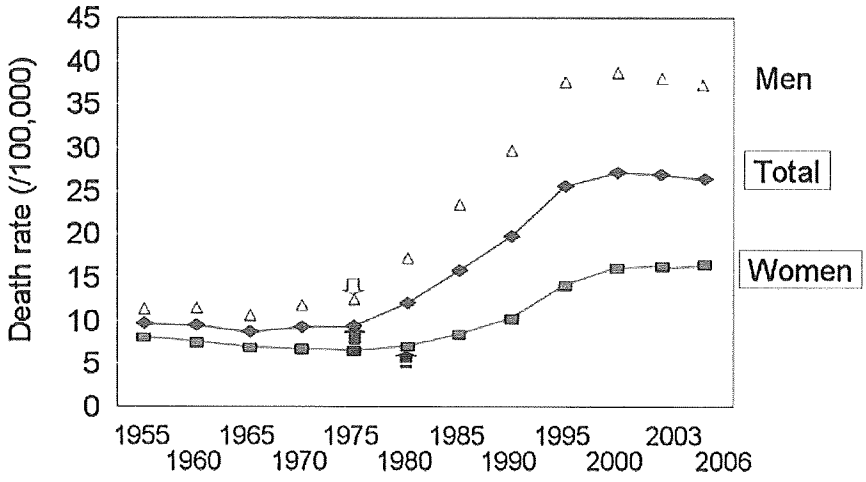

Fig. 2. Changes in the death rate of primary liver cancer in men (*triangles, yellow*), women (*rectangles, pink*), and in total (*diamonds, blue*)

in 1975, and a more gradual rise in women commenced in 1980. The total age-adjusted death rate peaked in 2002 (27.5/100000 persons in 2002), and decreased to 27.0 in 2003. In 2006, the total age-adjusted death rate stood at 26.7/100000, which is caused by a decrease in death rate (36.7) in men, but offset by an increase in women to 17.2.

Age and sex in HCC

Changes in the mean age of HCC patients and male/female ratio every 2 years between 1984 and 2003 are shown in Fig. 3. In that period, the mean age of female HCC patients was higher than that of males, and the mean ages of both sexes progressively increased. As reported previously, however, HBV-related HCC was stable from 1982 to 2003, implying that this change originated from HCV-related HCC patients. The male/female ratio was 4.5 in 1984–1985 and 2.5 in 2002–2003 (see Fig. 3), showing that the proportion of female patients with HCC had increased. This increase in female patients is also considered as a consequence of increased HCV-related HCC.

Changes in etiology of HCC in Japan

A nationwide survey on primary liver cancer has been conducted every 2 years since 1968 by the Liver Cancer Study Group of Japan.[1,4–9] Five serological surveys performed between 1990 and 2001 have documented that most patients with HCC are positive for either HBsAg or antibodies to HCV (anti-HCV). Tests for HBsAg became available in 1975 and those for anti-HCV in 1990. HBsAg-positive cases of HCC constituted 42% of patients in 1977–1978, but only 15.5% in 2002–2003 (Fig. 4). In contrast, anti-HCV-positive cases of HCC accounted for more than 70% of cases diagnosed until 2000–2001. However, this number dipped to 69.6% in 2002–2003, and has since remained at less than 70%. In contrast, HCV of unknown origin and other cases of HCC have been increasing gradually, and constituted 14.9% of all cases in 2002–2003.

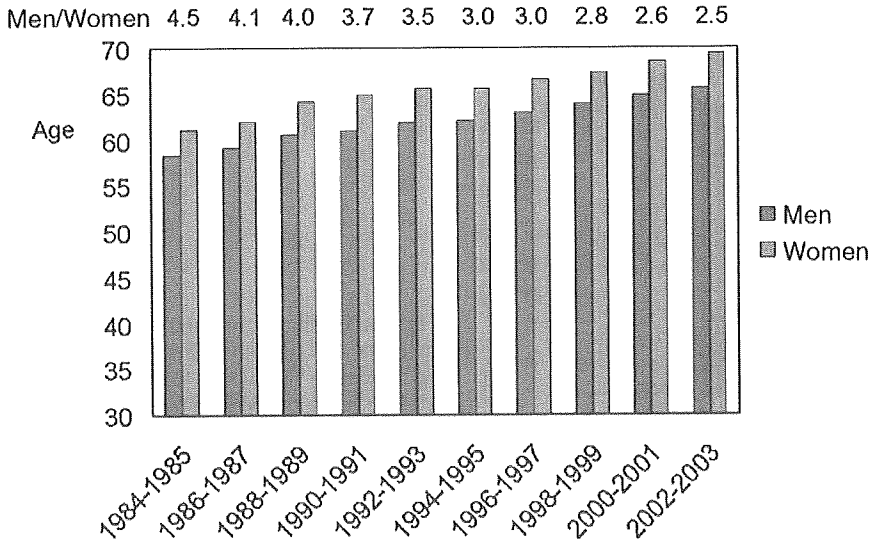

Fig. 3. Changes in the mean age (in years) of men (*blue bars*) and women (*pink bars*) patients with hepatocellular carcinoma (HCC) between 1984 and 2003

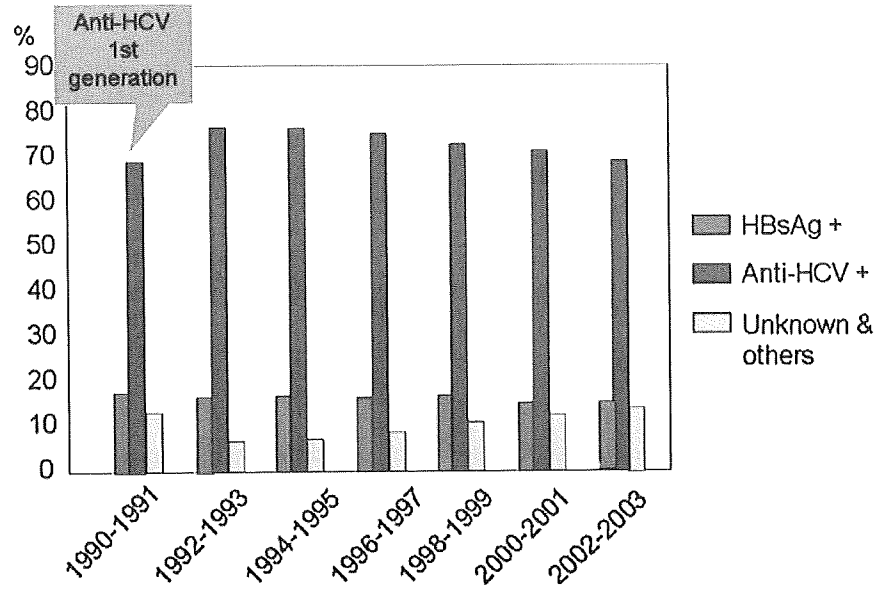

Fig. 4. Changes in the etiology of HCC between 1990 and 2003: hepatitis B surface antigen (*HBsAg+, pink*), antihepatitis C virus (*anti-HCV+, blue*), and *unknown* and *others* (*green*)

In cross-sectional studies conducted at Shinshu University Hospital, HCV-related HCC was found in the majority of cases (72%) (Fig. 5). Non-B non-C HCC (NBNC-HCC) accounted for 10% of cases in 2002–2007. In these 28 patients, nonalcoholic steatohepatitis (NASH) accounted for 14%.

Geographic variation of liver cancer and HBV/HCV infection

Although Japan is a relatively small country with a homogeneous population, the incidence of HCC varies greatly among different regions. The Vital Statistics of Japan for 2005 published in 2007 by the Japanese Ministry of Health, Labour, and Welfare on the incidence of deaths as a result of HCC in its 48 prefectures shows a steady increase in death rates of HCC from east to west in Japan. The average age-adjusted death rate of HCC among 48 prefectures was 27.2 per 100000 persons in 2005 (Fig. 6). Furthermore, nationwide health screening for HBsAg and anti-HCV in citizens over 40 years of age has been performed since 2002, and the prevalence rates of these markers have been analyzed for each prefecture in Japan. In 2006, the average HBsAg and anti-HCV prevalences were 1.0% and 0.7%, respectively, in this group (see Fig. 6). There was a highly significant association between the death rate of HCC and prevalence of anti-HCV in each prefecture (Fig. 7; correlation coefficient = 0.66; $P < 0.001$,

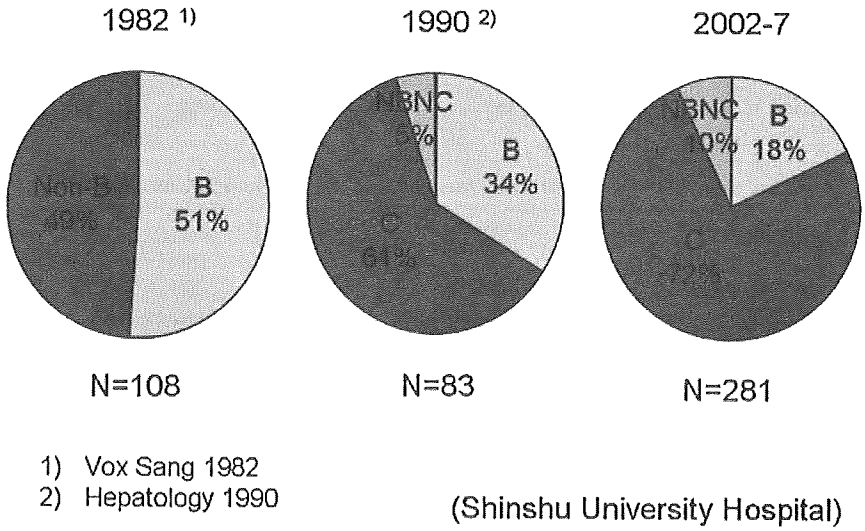

Fig. 5. Clinical features of hepatitis B (*B*) virus (HBV)- and hepatitis C (*C*) virus (HCV)-related HCC in 1982, 1990, and 2002–2005 at Shinshu University Hospital. *NBNC*, non-B non-C

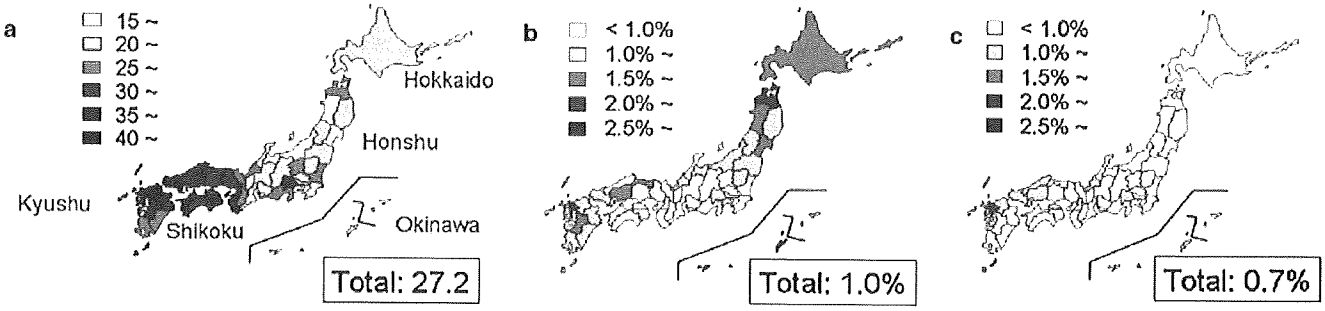

Fig. 6. **a** Death rate of primary liver cancer was 27.2 per 100000 in 2005 among people over 40 years of age in 48 prefectures in Japan. In the same group in 2006, HBsAg prevalence was 1.0% (**b**) and anti-HCV prevalence was 0.7% (**c**)

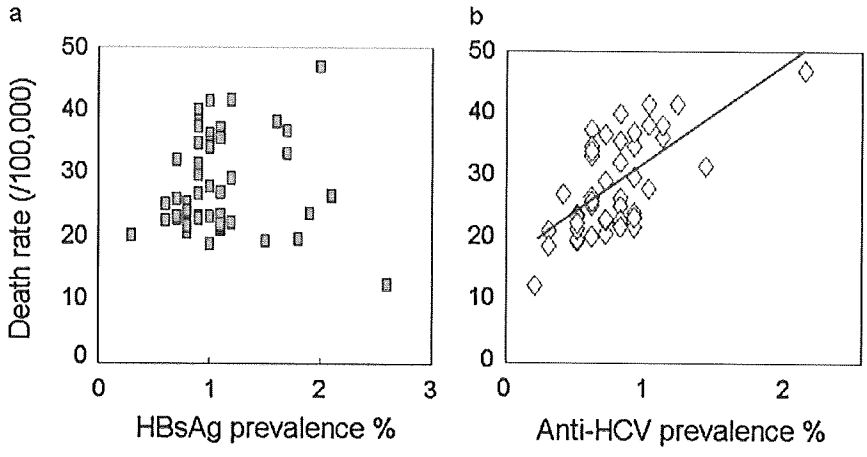

Fig. 7. Relationship between the death rate of primary liver cancer and prevalence of (**a**) HBsAg ($r = 0.02$, P = NS) and (**b**) anti-HCV ($r = 0.66$, $P < 0.001$, $y = 16.3x + 16.1$) among the general population over 40 years of age in 2006

$y = 16.3x + 16.1$), but no correlation with the prevalence of HBsAg was seen (Fig. 6). For instance, although Okinawa Prefecture had the highest prevalence of HBsAg (2.6%), its HCC death rate was the lowest (12.5/100000 persons). A possible explanation for this discrepancy is that the HBV genotype Bj, which shows good clinical prognosis,[10,11] is the dominant HBV genotype in Okinawa. In contrast, areas with high rates of anti-HCV, especially in western Japan, had high death rates from HCC. HCV appears to be the major contributor to primary liver cancer in these regions; Saga Prefecture shows both the highest HCC death rate (46.9/100000) and highest prevalence rate of anti-HCV (2.1%) in Japan.

Table 1. Summary of findings in representative studies on the incidence of hepatocellular carcinoma (HCC) among patients with chronic hepatitis C virus (HCV) infection treated with interferon alone in Japan

	Untreated		Treated					
			Non-SVR		SVR		Total	
Author	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%	No. HCC/no. cases	%
Kasahara[12]			41/709	5.8	5/313	1.6	46/1022	4.5
Imai[13]	19/140	13					18/419	4.3
Ikeda[14]	67/452	15	23/730	3.2	5/461	1.1	28/1191	2.4
Yoshida[15]	67/395	17	214/1556	13.8	27/836	3.2	241/2392	10.1
Okanoue[16]			119/849	14.0	8/397	2.0	127/1246	10.2
Ikeda[17]	59/352	17	34/171	19.9	1/53	1.9	94/576	16.3
Total	212/1339	16	432/4015	10.8	46/2060	2.2	554/6846	8.1

SVR: sustained virological response

Antiviral therapy suppresses the incidence of HCC

As described in prior sections, HCV infection is the major cause of HCC in Japan, suggesting that eradication of HCV may decrease the incidence of HCC. A summary of different studies on the incidence of HCC among patients with chronic hepatitis C who were treated with interferon in Japan can be found in Table 1.[12–17] These studies show a moderate decrease in the risk of HCC in patients with chronic hepatitis C treated with interferon, especially in patients with sustained virological response as compared with nonresponders and nontreated patients.

Recently, Ikeda et al. prospectively studied patients with chronic HCV infection and evidence of occult HBV infection [negative results for HBsAg and HBV DNA but positive results for antibodies to hepatitis B core antigen (anti-HBc) in serological testing].[17] Patients with HCV-related cirrhosis and positive results for anti-HBc were at high risk for HCC, even in patients with a sustained virological response to interferon (IFN) therapy. Thus, anti-HBc positivity is a marker of high risk for HCC among patients with HCV-related cirrhosis.

Between 1992 and 2001, approximately 300000 patients with chronic hepatitis C received IFN monotherapy in Japan. As shown in Fig. 1, it is remarkable that the number of deaths and the death rate of HCC began to decrease in 2005. These phenomena suggest that antiviral treatment indeed reduces the risk of HCC in patients with HCV infection.

Conclusion

The number of deaths and death rate of HCC showed a sharp increase from 1975 onward but had begun to decrease in 2006. Although both HBV and HCV infection play a major role in HCC in Japan, HCV-related HCC represents 70% of all cases. The incidence of HCC without HBsAg or anti-HCV accounts for 7%–15% in Japan, and half of NBNC-HCC cases are of unknown origin. Geographically, HCC is more frequent in western than eastern Japan, and the death rates of HCC in each prefecture correlate with anti-HCV, but not HBsAg, prevalence. IFN therapy for chronic hepatitis C reduces the risk of development of HCC, especially in patients with sustained viral response.

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＜特別寄稿＞

免疫抑制・化学療法により発症するB型肝炎対策 —厚生労働省「難治性の肝・胆道疾患に関する調査研究」班劇症肝炎分科会および「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」班合同報告—

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索引用語：劇症肝炎　HBV再活性化　*de novo* B型肝炎　核酸アナログ製剤　リツキシマブ

近年，化学療法，免疫療法，移植療法の進歩に伴い，多様な抗癌剤や免疫抑制剤を使用する機会が増加している．以前よりB型肝炎ウイルス（HBV）キャリアに合併した悪性腫瘍患者に対し，ステロイドを併用した化学療法を施行した場合，HBVの急激な増殖すなわちHBVの再活性化（reactivation）により致死的な重症肝炎が発症することが知られていた[1)2)]．HBV遺伝子にはglucocorticoid enhancement elementが存在するため[3)]，ステロイドにより直接的にウイルス複製が助長されるだけでなく，化学療法による免疫抑制や治療終了後に生じる免疫学的な均衡の破綻により，HBVの増殖とともに広範な感染肝細胞の破壊を伴う重症肝炎が惹起される．このようなHBVキャリアに対する化学療法時にはラミブジンなどの核酸アナログを予防投与してHBV再活性化を避けることが必要である[4)]．

一方，HBs抗原陰性でHBc抗体ないしHBs抗体陽性例は従来HBV既往感染とされ，臨床的には治癒の状態と考えられてきた．しかしこのような既往感染例でもHBV-DNAの複製が長期間持続することが明らかになっている[5)~7)]．最近，移植後やB細胞表面抗原CD20に対する抗体であるリツキシマブなど強力な免疫抑制剤の使用により，このような既往感染例からもHBV再活性化により重症肝炎が発症することが報告され，de novo B型肝炎と呼ばれている[8)~10)]．厚生労働省「肝硬変を含めたウイルス性肝疾患の治療の標準化に関する研究」班の全国調査によりこのようなde novo B型肝炎は通常のB型肝炎

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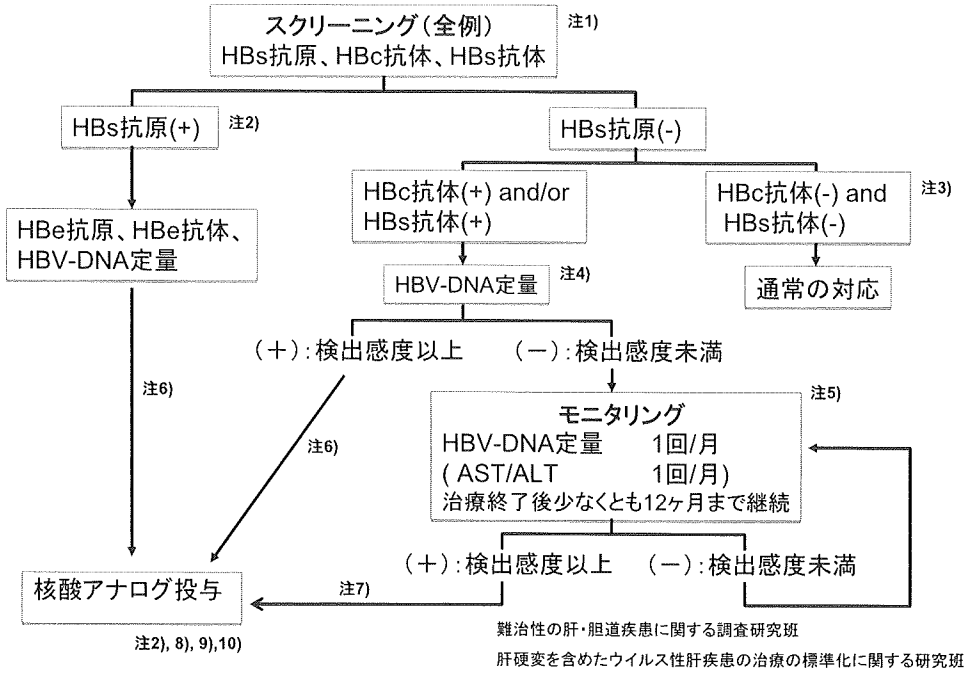

Fig. 1　免疫抑制・化学療法により発症するB型肝炎対策ガイドライン*

補足

*血液悪性疾患に対する強力な免疫・抑制化学療法中あるいは終了後にHBs抗原陽性あるいはHBs抗原陰性例の一部にHBV再活性化によりB型肝炎が発症し，その中には劇症化する症例があり，注意が必要である．その他の疾患においても治療によるHBV再活性化のリスクを考慮して対応する必要がある．また，ここで推奨する核酸アナログ予防投与のエビデンスはなく，劇症化予防効果を完全に保証するものではない．

注1）CLIA法で測定することが望ましい．

注2）HBs抗原陽性例は肝臓専門医にコンサルトすること．全ての症例で核酸アナログ投与にあたっては肝臓専門医にコンサルトするのが望ましい．

注3）初回治療時にHBc抗体，HBs抗体未測定の再治療例では抗体価が低下している場合があり，HBV-DNA定量検査などによる精査が望ましい．

注4）PCR法およびリアルタイムPCR法により実施する．より検出感度の高いリアルタイムPCR法が望ましい．

注5）リツキシマブ・ステロイド使用例，造血細胞移植例はHBV再活性化の高リスクであり，注意が必要である．フルダラビンは強力な免疫抑制作用を有するが，HBV再活性化のリスクは不明であり，今後注意が必要である．

注6）免疫抑制・化学療法を開始する前，できるだけ早期に投与を開始するのが望ましい．

注7）免疫抑制・化学療法中はHBV-DNA定量検査が検出感度以上になった時点で直ちに投与を開始する．

注8）核酸アナログはエンテカビルの使用を推奨する．

注9）下記の条件を満たす場合には核酸アナログ投与の終了を検討して良い．
スクリーニング時にHBs抗原（＋）例ではB型慢性肝炎における核酸アナログ投与終了基準を満たす場合．スクリーニング時にHBc抗体（＋）and/or HBs抗体（＋）例では，(1) 免疫抑制・化学療法終了後，少なくとも12カ月間は投与を継続すること．(2) この継続期間中にALT（GPT）が正常化していること．（但しHBV以外にALT異常の原因がある場合は除く）(3) この継続期間中にHBV-DNAが持続陰性化していること．

注10）核酸アナログ投与終了後12カ月間は厳重に経過観察する．経過観察方法は各核酸アナログの使用上の注意に基づく．経過観察中にHBV-DNA定量検査が検出感度以上になった時点で直ちに投与を再開する．

に比して劇症化する頻度が高率で，死亡率も高いことが明らかになった[11)~13)]．また，厚生労働省「難治性の肝・胆道疾患に関する調査研究」班で実施している劇症肝炎・遅発性肝不全（LOHF）の全国調査でもここ数年，特に悪性リンパ腫に対しリツキシマブとステロイドを併用したR-CHOP治療例からの劇症化やde novo B型肝炎が増加傾向にあり，予後不良であった[14)15)]．以上のような経緯から，早急なHBV再活性化対策が必要となり，両研究班が合同でワーキンググループを立ち上げ，Fig. 1に示すガイドラインを作成した．

ガイドラインの要旨は以下のとおりである．まずHBV再活性化リスク群の同定を目的にスクリーニング検査として，全ての症例にHBs抗原およびHBc抗体，HBs抗体を測定する．HBs抗原が陽性の場合はさらにHBe抗原，HBe抗体，HBV-DNA定量検査を実施する．HBs抗原陽性例では，無症候性キャリアだけではなく，慢

性肝炎，肝硬変例が含まれる可能性があるので肝臓専門医にコンサルトする必要がある．HBs抗原陽性例での再活性化のリスクは大きいので，基本的に核酸アナログの予防投与を実施する．但し，HBV再活性化のリスクが少ない悪性疾患以外の若年HBe抗原陽性無症候性キャリアに対するステロイド治療例などでは，核酸アナログ予防投与の有効性に関するエビデンスはなく経過観察など他の選択肢があり，適応は慎重に判断する必要がある．HBs抗原陰性でHBc抗体，HBs抗体いずれも陰性の場合は通常の対応とする．HBs抗原陰性でHBc抗体ないしHBs抗体が陽性，すなわち感染既往例と判断される場合は更にHBV-DNA定量検査を実施し，HBV-DNAが陽性の場合は核酸アナログの予防投与を行う．一方，HBV-DNAが陰性の場合はHBV-DNAを毎月モニタリングしながら，陽性化した時点で直ちに核酸アナログを投与する．特にリツキシマブ・ステロイド使用例，造血細胞移植例は再活性化のリスクが高いので慎重な対応が必要である．核酸アナログ予防投与例の投与中止時期に関する明確なエビデンスはないが，HBs抗原陰性，HBc抗体ないしHBs抗体陽性例では免疫抑制・化学療法終了後も12カ月間は投与を継続し，この継続期間中に一定の基準を満たせば投与終了も可能とした．以下にガイドライン作成にあたり論点になった事項を補足する．①スクリーニングにあたってはHBs抗原だけではなくHBc抗体，HBs抗体をできるだけ感度の高い検査法で実施する必要がある．HBs抗原陰性でHBc抗体，HBs抗体いずれも陰性の場合でも，患者が既に免疫抑制状態にある場合には抗体が検出されないことがあり，HBV-DNA定量検査まで測定することが望ましい．②B型キャリア例の急性増悪では発症後早期の核酸アナログ治療が有効であるが，HBV再活性化による劇症化例は発症後の核酸アナログ治療では予後不良であり，発症前の予防投与が必要である．しかし既往感染例でのHBV再活性化率は明らかでなく，また本邦におけるHBc抗体ないしHBs抗体陽性の既往感染例の頻度は高率であることより，全ての症例に核酸アナログの予防投与を実施するのは医療経済的にも困難である．Huiらの報告[16]ではHBs抗原陰性例のHBV再活性化では，HBV-DNAが陽性化し，肝炎が発症するまでに12～28週（平均18.5週）を要しており，したがってHBV-DNAをPCR法またはリアルタイムPCR法で毎月モニタリングし，検出感度以上になった時点で直ちに核酸アナログを投与しても肝炎の重症化は予防可能と推測される．③核酸アナログ製剤はB型慢性肝炎の治療ガイドライン[17]に準拠して，エンテカビル投与を推奨している．しかし，投与期間が長期に及ばない場合など，より安価なラミブジンへの代用も検討の余地がある．④核酸アナログ投与終了に関する明確な基準はない．HBs抗原陽性例では使用する各核酸アナログの投与終了基準に準ずる．HBs抗原陰性，HBc抗体ないしHBs抗体陽性例では免疫抑制・化学療法終了後も12カ月間は投与を継続し，この継続期間中にALTの正常化とHBV-DNAの持続陰性化が見られる場合は投与終了の検討も可能である．但し，HBV以外にALT異常の原因がある場合はALTの正常化は必須ではない．また，核酸アナログ予防投与終了後のHBV再活性化例の報告もあり，投与終了後も更に12カ月間は厳重な経過観察が必要である[18]．

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