

Immunohistochemistry

Immunostaining for GR α , GR β , 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 GR α , GR β , and Foxp3. Five- μ m-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-GR α (rabbit; SCB, Santa Cruz, CA), anti-GR β (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 GR α and Foxp3. GR β 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 GR β with lymphocyte and macrophage markers in GC-resistant patients with UC

Immunodouble staining for GR β by using lymphocyte and macrophage markers (CD4, CD8, CD19, CD20, and CD68) was performed to identify the GR β ⁺ cell type in the GC-resistant group. The paraffin sections obtained from 10 GC-resistant patients with UC were used for immunodouble staining for GR β 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% CaCl₂ 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 IgG1 κ ; DAKO), anti-CD8 (C8/144B, mouse IgG2b κ ; DAKO), anti-CD20 (L26, mouse IgG2a κ ; DAKO), and anti-CD68 (KP1, mouse IgG1 κ ; 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-GR β antibody. Immunostaining for GR β 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 GR β and CD19. First, CD19 immunostaining was performed. The primary antibodies used in this study were anti-CD19 (HD37, mouse IgG1 κ ; 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-GR β antibody. The endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide. Immunostaining for GR β 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 GR β 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 GR β 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-GR β 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 GR α , GR β , and Foxp3 were counted under light microscopy ($\times 400$) independently by 2 observers, and the average number of positive cells in 1 view was calculated. Immunodouble staining for GR β 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 GR β -positive cells was calculated. Values were represented as mean \pm standard deviation.

RT-PCR of GR α and GR β 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 μ g of total RNA was used directly for the one-step RT-PCR reaction with a total volume of 50 μ l, including 2 μ l of QIAGEN OneStep RT-PCR Enzyme Mix (Qiagen), 0.6 μ M of specific primers, 400 μ M each of dNTPs, and 10 μ l of QIAGEN OneStep RT-PCR Buffer (Qiagen). The primers used for PCR were as follows: GR α , 5'-CCTAAGGACGGTCTGAA-GAGC-3' and 5'-GCCAAGTCTTGGCCCTCTAT-3', amplifying a

477-bp product; and GR β , 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 GR α) or 40 cycles (for GR β) 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 transilluminator, 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 GR α ⁺ and GR β ⁺ cells, and for that of immunodouble staining for GR β with lymphocyte and macrophage markers. Fisher's exact test was used for the analysis of GR β 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 GR β and Foxp3. Differences with a value of *p* < 0.05 were considered to be statistically significant.

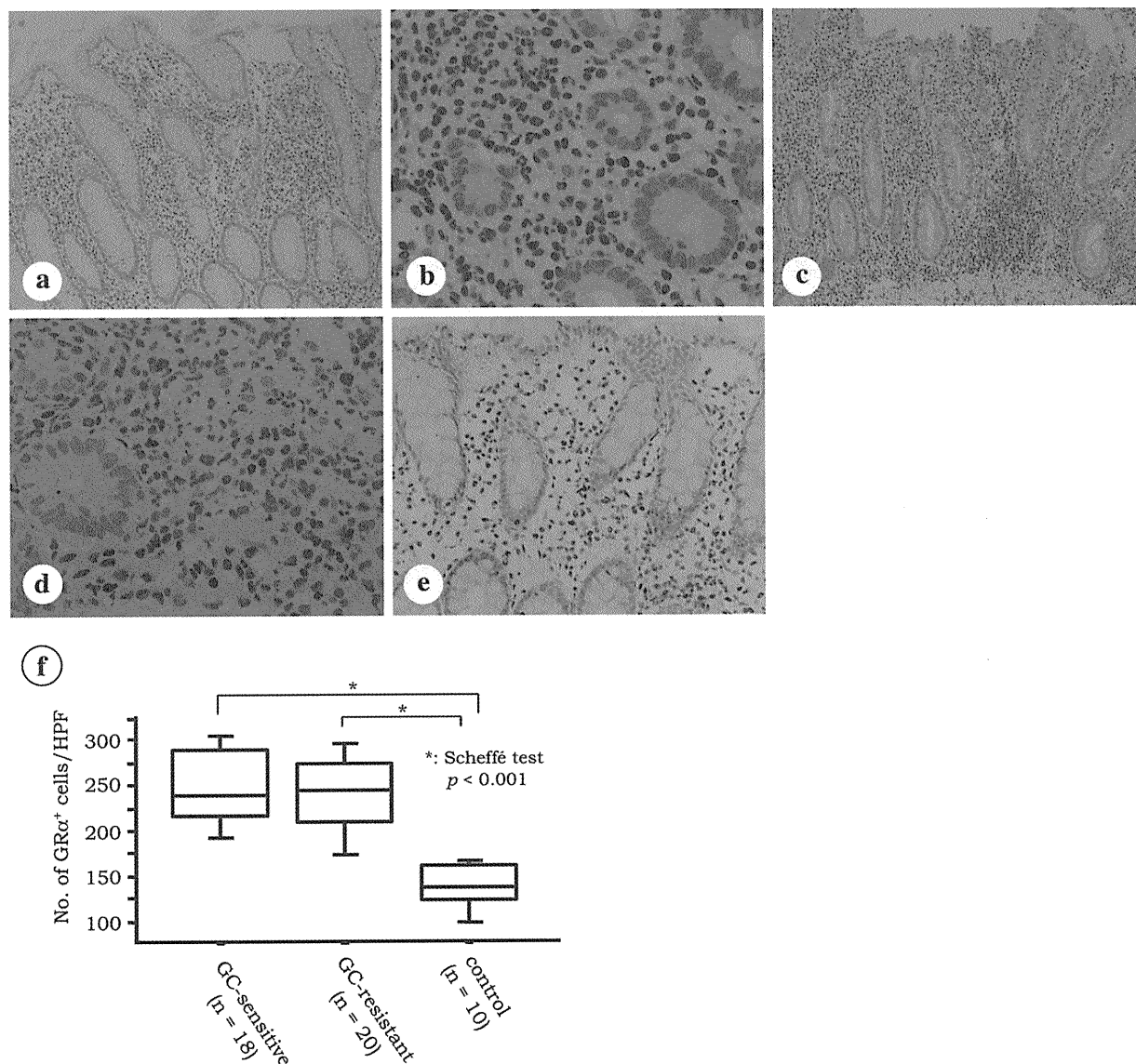


Figure 1 The distribution of glucocorticoid receptor- α (GR α)⁺ cells in the biopsied colonic lamina propria of the glucocorticoid (GC)-sensitive group (a, b), GC-resistant group (c, d), and control group (e). Cells were counterstained with hematoxylin (original magnification: a and c, $\times 100$; b and d, $\times 400$; e, $\times 200$). The number of GR α ⁺ 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 $GR\alpha^+$ and $GR\beta^+$ cell count and GC responsiveness in the colonic mucosa of UC patients

The $GR\alpha^+$ 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 $GR\beta^+$ cells present in the colonic lamina propria of UC patients were mononuclear cells with stained nuclei (Figs. 2a–e). Unlike $GR\alpha$, the neutrophils were negative for $GR\beta$. The $GR\beta^+$ 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).

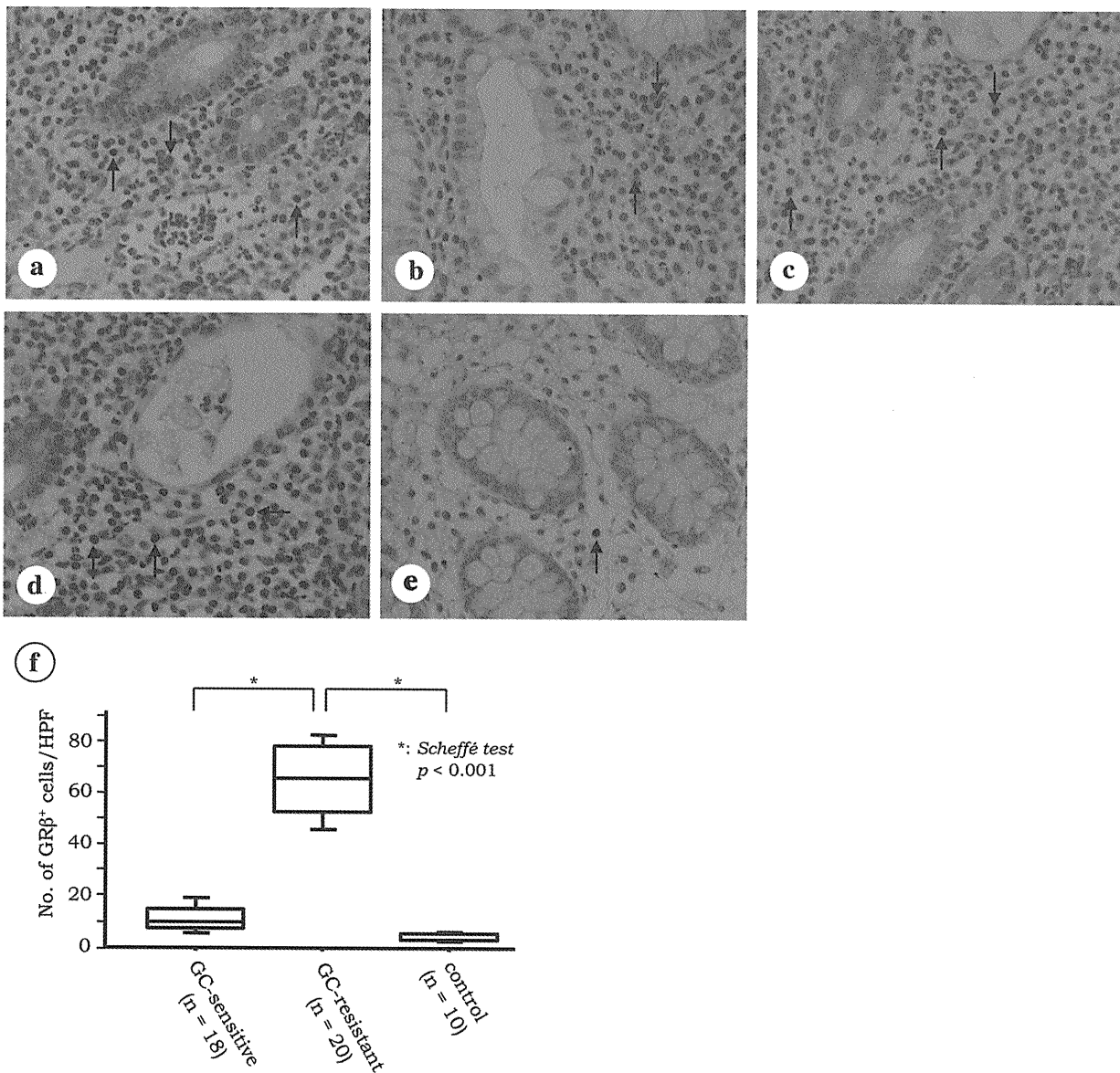


Figure 2 The distribution of glucocorticoid receptor- β ($GR\beta$)⁺ cells in the biopsied colonic lamina propria of the glucocorticoid (GC)-sensitive group (a, b), GC-resistant group (c, d), and control group (e). $GR\beta^+$ cells are indicated by arrows. Cells were counterstained with hematoxylin (original magnification: a–e, $\times 400$). The $GR\beta^+$ 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).

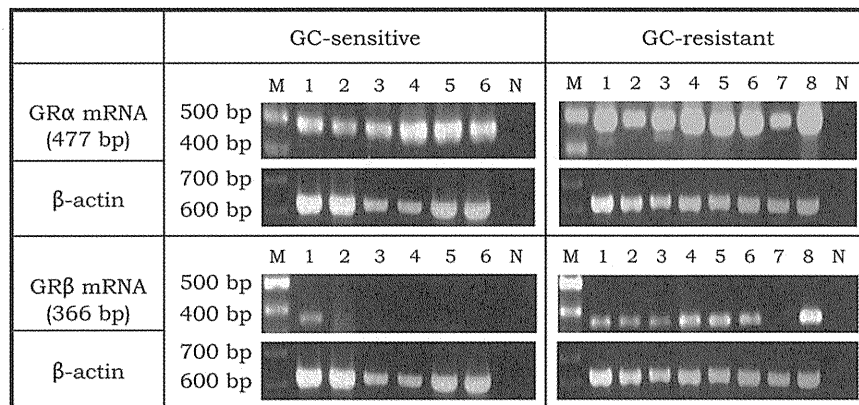


Figure 3 The expression of glucocorticoid receptor- α (GR α) and GR β mRNA in biopsied colonic mucosa of the GC-sensitive group ($n=6$) and the GC-resistant group ($n=8$). β -actin (645 bp) was used as a housekeeping gene. GR α mRNA (477 bp) was expressed in all cases (upper column). On the other hand, GR β 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 GR α and GR β 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. GR α mRNA was expressed in all patients in both the GC-sensitive and GC-resistant groups (Fig. 3). In contrast, GR β 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 GR β ⁺ cells and immunodouble staining with lymphocyte and macrophage markers in the GC-resistant group

The immunodouble staining for GR β with lymphocyte and macrophage markers (CD4, CD8, CD19, CD20, and CD68) was performed in order to identify the GR β ⁺ cell type in the GC-resistant group (Figs. 4a–f). The proportion of each type of double-positive cell with respect to all the GR β ⁺ cells was then calculated. CD4⁺GR β ⁺ cells ($n=10$) accounted for 25.8% \pm 6.9%, CD8⁺GR β ⁺ cells ($n=10$) for 12.2% \pm 2.4%, CD19⁺GR β ⁺ cells ($n=7$) for 23.9% \pm 6.3%, CD20⁺GR β ⁺ cells ($n=10$) for 6.1% \pm 4.9%, and CD68⁺GR β ⁺ cells ($n=10$) for 8.0% \pm 2.5%. Of all the GR β ⁺ cells, double-positive cells for GR β and CD4 or CD19 were significantly more numerous than double-positive cells for GR β and CD8, CD20, or CD68 (Scheffé test, $p<0.01$) (Table 2). The remaining GR β ⁺ 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 \pm

6.8 cells in the GC-sensitive group and 8.3 \pm 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 GR β ⁺ cells and Foxp3⁺ cells in the colonic mucosa of UC patients

Fluorescence immunodouble staining for GR β 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 GR β 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 GR α and GR β 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 GR α and GR β 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

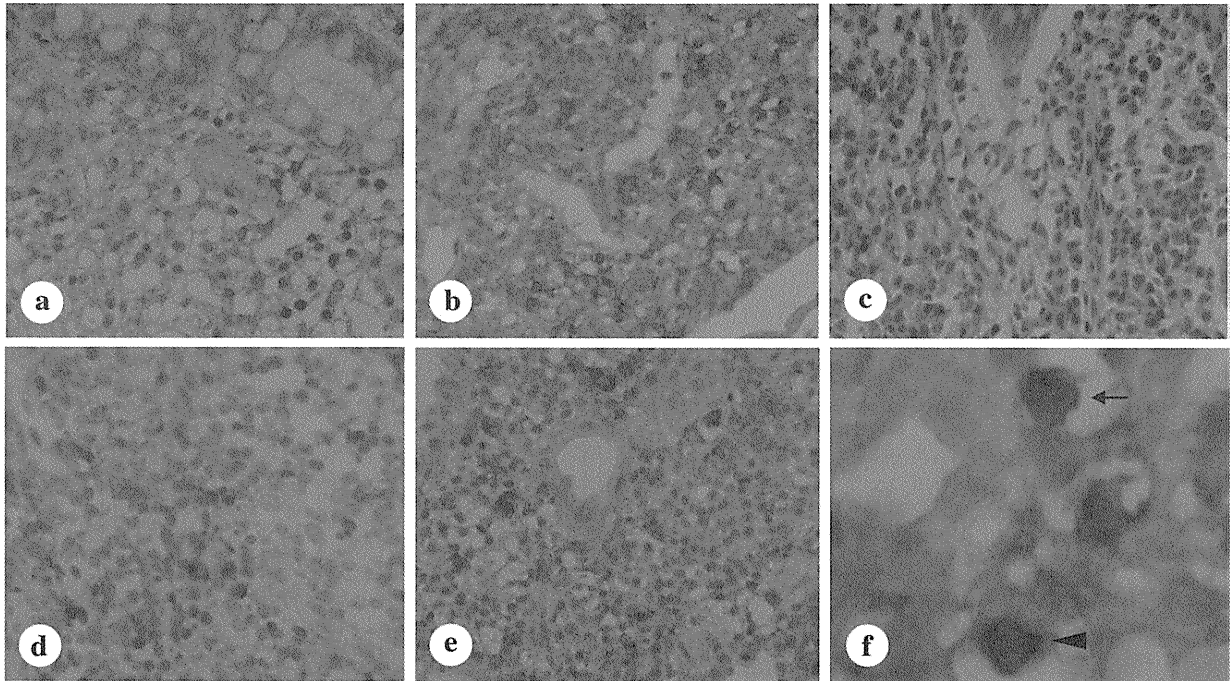


Figure 4 The type of cells expressing glucocorticoid receptor-β (GRβ) in the glucocorticoid (GC)-resistant group. The positive reaction for GRβ and lymphocyte and macrophage markers such as CD4 (a, f), CD8 (b), CD19 (c), CD20 (d), and CD68 (e) was detected as pink and brown coloration, respectively. Panel f demonstrated a high-power view of the CD4⁺GRβ⁺ cells (arrowhead) and CD4⁺GRβ⁺ 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 GRβ⁺ cells were mononuclear. The GRβ⁺ 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 GRβ⁺ 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 GRβ⁺ 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 GRβ ⁺ cells*
CD4 ⁺ GRβ ⁺	10	25.8±6.9 (12.4, 35.4)
CD8 ⁺ GRβ ⁺	10	12.2±2.4 (8.9, 15.6)
CD19 ⁺ GRβ ⁺	7	23.9±6.3 (15.4, 32.1)
CD20 ⁺ GRβ ⁺	10	6.1±4.9 (1.2, 16.7)
CD68 ⁺ GRβ ⁺	10	8.0±2.5 (4.2, 12.5)

*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.

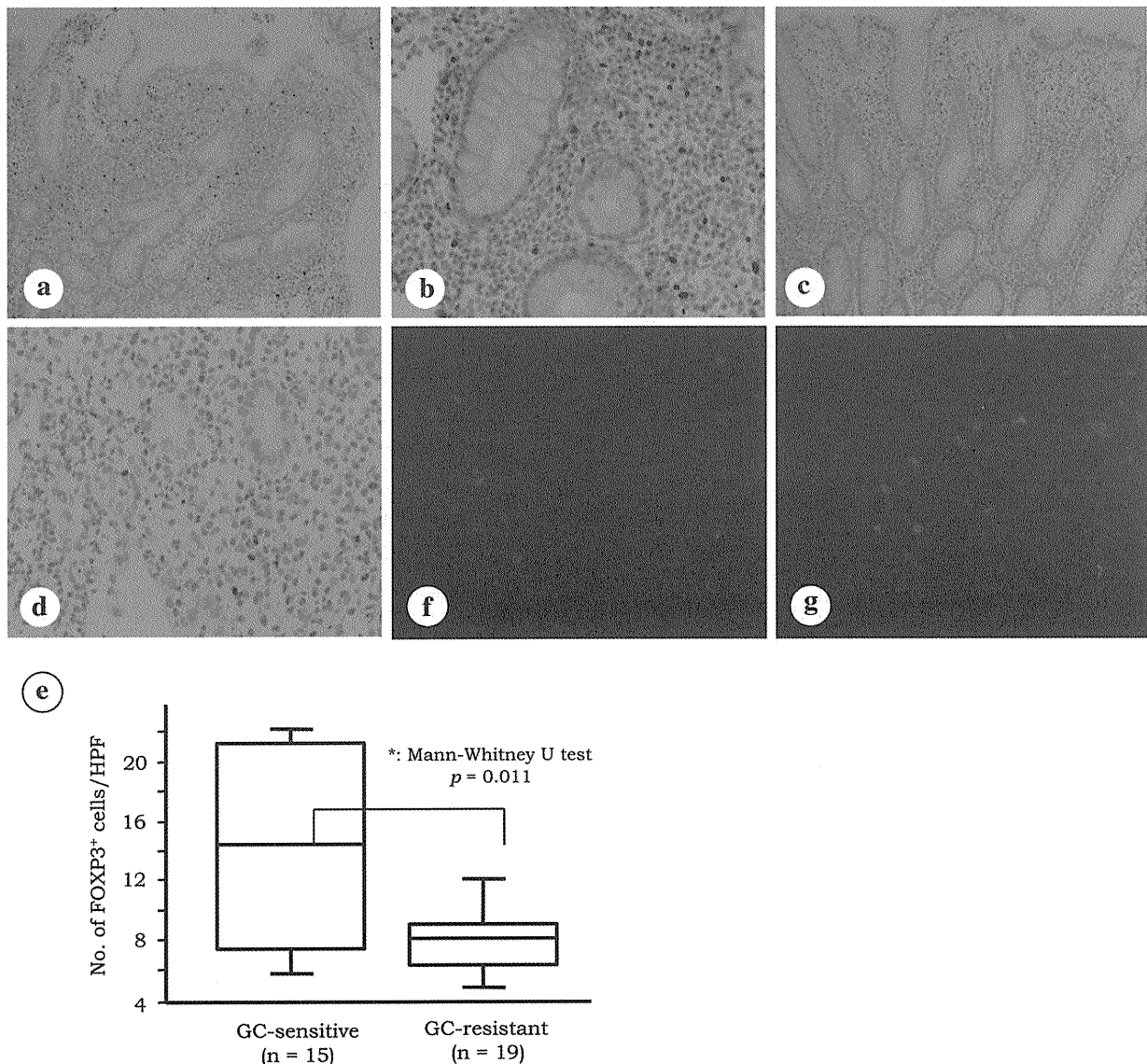


Figure 5 The distribution of Fcpx3⁺ cells in the glucocorticoid (GC)-sensitive (a, b) and GC-resistant groups (c, d). Cells were counterstained with hematoxylin (original magnification: a and c, ×100; b and d, ×200). The number of Fcpx3⁺ cells was significantly higher in the GC-sensitive group than the GC-resistant group (Mann–Whitney U test, p=0.011) (e). Fluorescence immunodouble staining for glucocorticoid receptor-β (GRβ) (red) and Fcpx3 (green) demonstrated that the GC-sensitive group (f) had relatively more Fcpx3⁺ 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	
		<i>r</i>	<i>p</i>
GR α	48	0.908	<0.0001
GR β	48	0.885	<0.0001
CD4/GR β	10	0.875	<0.0001
CD8/GR β	10	0.896	<0.0001
CD19/GR β	7	0.862	<0.0001
CD20/GR β	10	0.890	<0.0001
CD68/GR β	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 β ⁺ 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 GR β and Foxp3 were not observed in the current study. On the basis of the above findings, Treg and GR β ⁺ cells are considered to be separate cells.

In the present study, GR β ⁺ 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 GR α and GR β in the biopsied colonic mucosa, and evaluated the relationship between the frequency of GR α ⁺, GR β ⁺, and Foxp3⁺ cells and GC responsiveness in UC patients. In this study, GR β ⁺ 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 β ⁺ 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 GR β 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

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

Received: June 10, 2008 / Accepted: July 3, 2008

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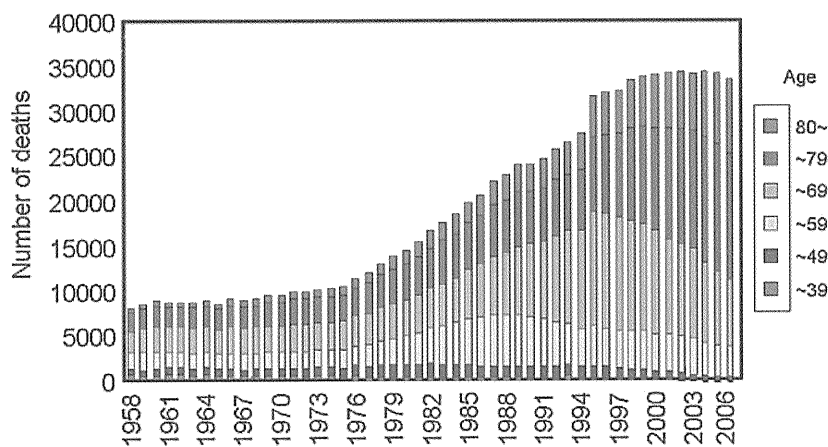


Fig. 1. Changes in annual deaths of patients (by age, in years) with primary liver cancer between 1958 and 2006. (Taken from the Vital Statistics of Japan, released every year by the Ministry of Health, Labour, and Welfare)

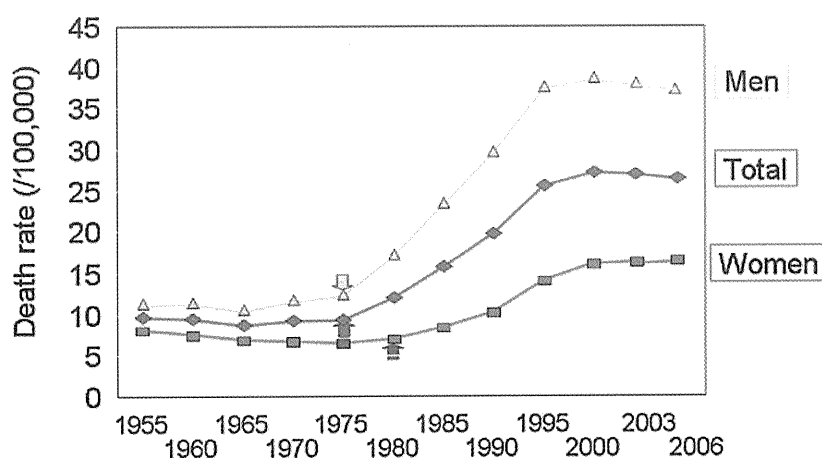


Fig. 2. Changes in the death rate of primary liver cancer in men (triangles, yellow), women (rectangles, pink), and in total (diamonds, blue)

in 1975, and a more gradual rise in women commenced in 1980. The total age-adjusted death rate peaked in 2002 (27.5/100,000 persons in 2002), and decreased to 27.0 in 2003. In 2006, the total age-adjusted death rate stood at 26.7/100,000, 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.

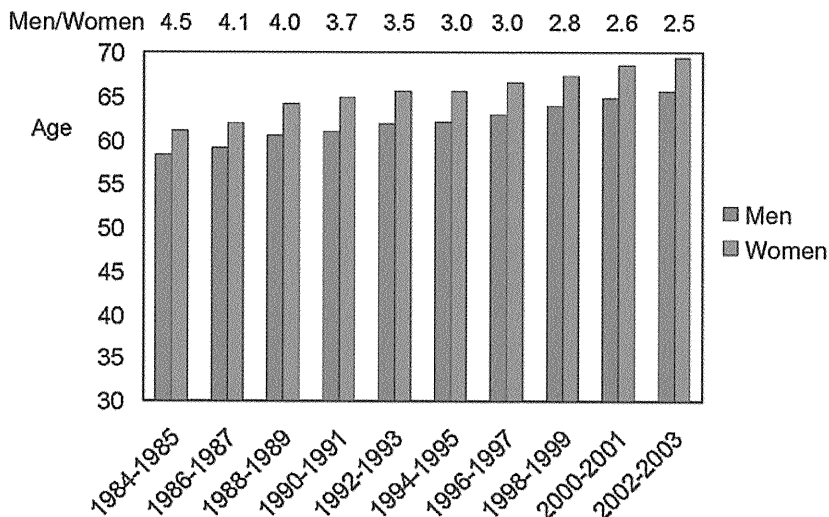


Fig. 3. Changes in the mean age (in years) of men (blue bars) and women (pink bars) patients with hepatocellular carcinoma (HCC) between 1984 and 2003

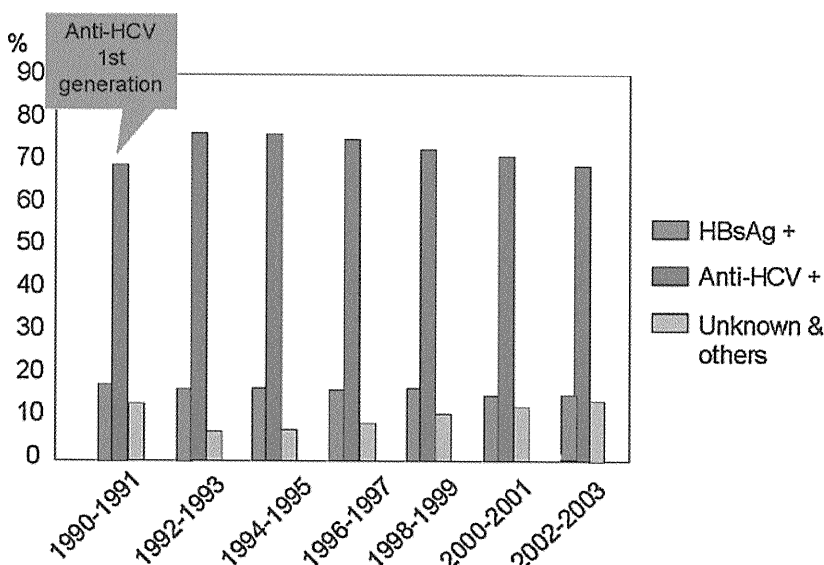


Fig. 4. Changes in the etiology of HCC between 1990 and 2003: hepatitis B surface antigen (HBsAg+, pink), antihepatitis C virus (anti-HCV+, blue), and unknown and others (green)

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 Min-

istry 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; $P < 0.001$,

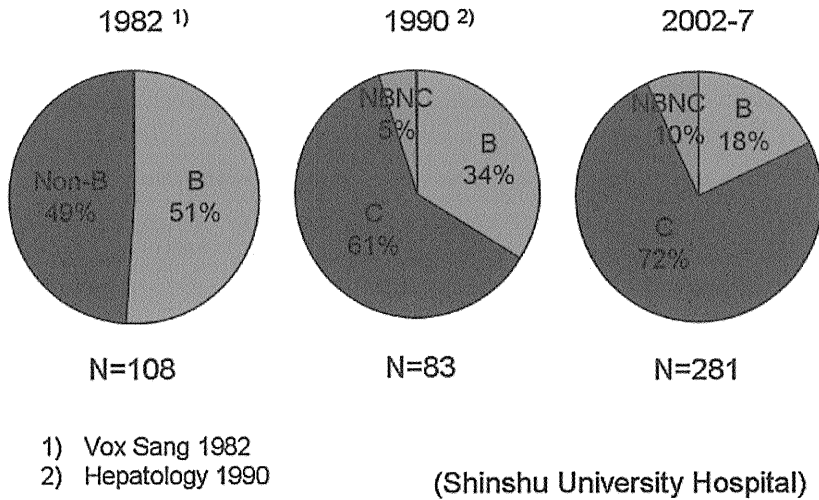


Fig. 5. Clinical features of hepatitis B (B) virus (HBV)- and hepatitis C (C) virus (HCV)-related HCC in 1982, 1990, and 2002–2005 at Shinshu University Hospital. NBNC, non-B non-C

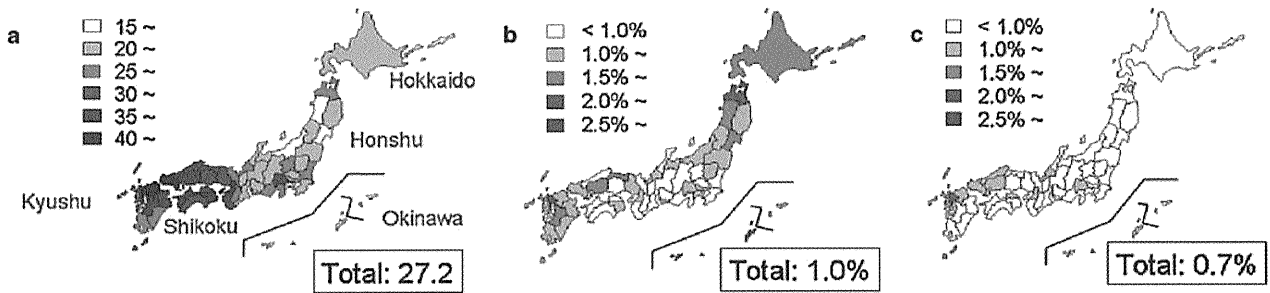


Fig. 6. a Death rate of primary liver cancer was 27.2 per 100 000 in 2005 among people over 40 years of age in 48 prefectures in Japan. In the same group in 2006, HBsAg prevalence was 1.0% (b) and anti-HCV prevalence was 0.7% (c)

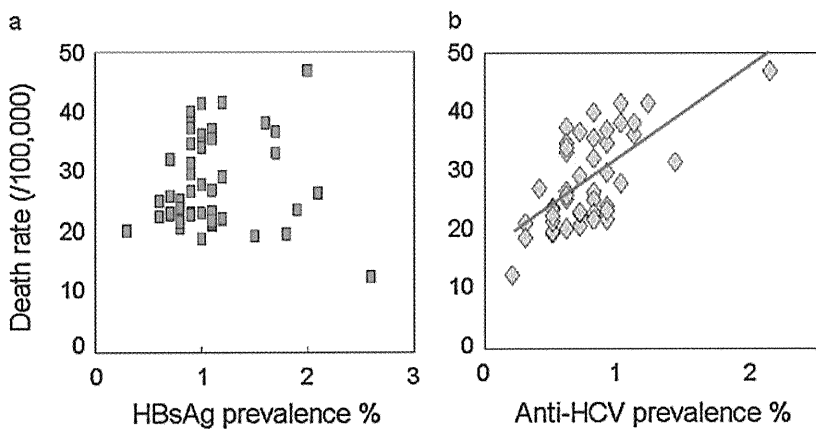


Fig. 7. Relationship between the death rate of primary liver cancer and prevalence of (a) HBsAg ($r = 0.02$, $P = \text{NS}$) and (b) anti-HCV ($r = 0.66$, $P < 0.001$, $y = 16.3x + 16.1$) among the general population over 40 years of age in 2006

$y = 16.3x + 16.1$), 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 (12.5/100 000 persons). A possible explanation for this discrepancy is that the HBV genotype Bj, which shows good clinical prognosis,^{10,11} is the dominant HBV geno-

type 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 (46.9/100 000) 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

Author	Treated							
	Untreated		Non-SVR		SVR		Total	
	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 300 000 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.

Acknowledgments. This study was supported by Grants-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Science, and Sports of Japan (18790456).

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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 HBe 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肝臓や末梢血単核球中では低レベルながら 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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<受付日2008年10月23日><採択日2008年12月8日>

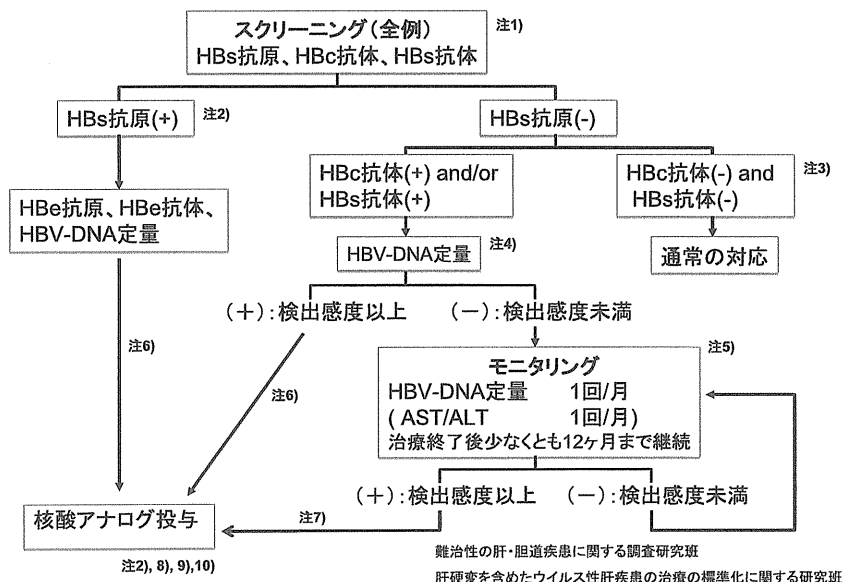


Fig. 1 免疫抑制・化学療法により発症する B 型肝炎対策ガイドライン*

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、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 抗体未測定の場合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 抗原が陽性の場合にはさらに 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ため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 HBe 抗体, HBs 抗体いずれも陰性の場合には通常の対応とする。HBs 抗原陰性で HBe 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 が陽性の場合には核酸アナログの予防投与を行う。一方, HBV-DNA が陰性の場合には 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, HBe 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 抗原だけでなく HBe 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 HBe 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。② B 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 HBe 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, HBe 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⁾。

本ガイドライン作成にあたってはワーキンググループ委員の他, 名古屋市立大学腫瘍・免疫内科学および鹿児島大学大学院消化器疾患・生活習慣病学血液内科グループの協力および助言を得た。今後は本ガイドラインを血液内科をはじめとする関係領域に周知させていくとともに, 各分野と協力して本ガイドラインの有効性を検証していくことが重要である。

謝辞: 本研究は厚生労働省科学研究費難治性疾患克服研究事業および肝炎等克服緊急対策研究事業からの助成金によって支援された。

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Prevention of immunosuppressive therapy or chemotherapy-induced
 reactivation of hepatitis B virus infection
 —Joint report of the Intractable Liver Diseases Study Group of
 Japan and the Japanese Study Group of the Standard Antiviral
 Therapy for Viral Hepatitis—

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Key words: fulminant hepatitis HBV reactivation *de novo* hepatitis B nucleoside analog
 rituximab

Kanzo 2009; 50: 38—42

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HEPATOLOGY

Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure

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Key words

adipose, differentiation, hepatocyte, liver regeneration, mesenchymal stem cell.

Accepted for publication 13 April 2008.

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Abstract

Background and Aim: Multipotential mesenchymal stem cells (MSC), present in many organs and tissues, represent an attractive tool for the establishment of a successful stem cell-based therapy in the field of regeneration medicine. Adipose tissue mesenchymal stem cells (AT-MSC), known as adipose-derived stem cells (ASC) are especially attractive in the context of future clinical applications because of their high accessibility and minimal invasiveness during the procedure to obtain them. The goal of the present study was to induce human ASC into functional hepatocytes *in vitro* within a very short period of time and to check their therapeutic potential *in vivo*.

Methods: *In vitro* generated ASC-derived hepatocytes were checked for hepatocyte-specific markers and functions. Afterwards, they were transplanted into nude mice with liver injury. Twenty-four hours after transplantation, biochemical parameters were evaluated in blood serum.

Results: We have shown here that ASC can be differentiated into hepatocytes within 13 days and can reach the functional properties of primary human hepatocytes. After transplantation into mice with acute liver failure, ASC-derived hepatocytes can restore such liver functions as ammonia and purine metabolism. Markers of liver injury, alanine aminotransferase, aspartate aminotransferase, as well as ammonia, were decreased after ASC-derived hepatocyte transplantation.

Conclusions: Our data highlight the properties of ASC as having a special affinity for hepatocyte differentiation *in vitro* and liver regeneration *in vivo*. Thus, ASC may be a superior choice for the establishment of a therapy for injured liver.

Introduction

The liver is exposed to many factors such as drugs, xenobiotics and viruses, which cause chronic hepatitis and liver cirrhosis. In most cases these lead to hepatocellular carcinoma and finally to organ failure, where there is chronic inflammation, fibrosis and no longer any regeneration ability.¹

At present, liver transplantation is the only effective treatment for severe liver injuries. However, because of organ rejection and lack of donors, alternative strategies are urgently needed.

Human primary hepatocytes are commercially available; however, maintaining them in *in vitro* culture is very difficult, if not nearly impossible. After a few days of *in vitro* culturing they lose their functions. Additionally, their usage does not solve the problem of rejection. These factors limit their experimental applications and exclude their clinical usage.

In the last few years, extrahepatic cell populations with the potential to impact liver diseases have been discovered. The poten-

tial candidate stem cells for therapy of an injured liver are mesenchymal stem cells (MSC), which can be obtained from different sources such as bone marrow (BM),² umbilical cord blood (UCB),³ amniotic fluid (AF),⁴ scalp tissue,⁵ placenta,⁶ or adipose tissue (AT)^{7,8} of the human body. These cells reveal a multipotentiality and semi-infinite proliferation ability. The hepatogenic differentiation capacity of MSC has been confirmed in many independent studies on BM-MSC,^{9–14} UCB-MSC,^{15–16} and adipose-derived stem cells (ASC).^{17–19} The possibility for their future application in the therapy of liver diseases is very promising. MSC can easily be obtained from a patient's own tissues, isolated *ex vivo*, expanded, differentiated toward hepatocytes, and transplanted back into the patient in the form of either undifferentiated MSC or MSC-derived hepatocytes. Such a possibility sidesteps the limits regarding ethical issues and immunocompatibility problems. Importantly, MSC represent an advantageous cell type for allogenic transplantation as well, because they are immuno-privileged with low major histocompatibility complex (MHC) I (histocompatibility