HMGB1 has been shown to be elevated in the blood of patients with septic shock [8] or hemorrhagic shock [13]. High levels of HMGB1 were also detected in the synovial fluid of rheumatoid arthritis patients [14]. Moreover, Hatada et al. [15] reported that patients with disseminated intravascular coagulation showed high plasma levels of HMGB1. They also demonstrated that HMGB1 plasma levels correlated well with the severity of the disease. Experiments have revealed that the extracellular form of HMGB1 mediates the induction of delayed endotoxin lethality [8], acute lung injury [16], joint inflammation in rheumatoid arthritis [17], and ischemic liver damage [18, 19].

It has also been shown that anti-HMGB1 antibodies are elevated in some patients with rheumatoid arthritis [20], ulcerative colitis [21], or autoimmune hepatitis [22]. The pathological role of these antibodies is unclear. However, since HMGB1 exists only in the intracellular space under normal conditions, the presence of anti-HMGB1 antibodies may suggest that HMGB1 has at some time existed in the extracellular space.

To date, extensive studies have been performed on the role of HMGB1. However, no concrete information has been available concerning the relationship between HMGB1 (or anti-HMGB1 antibodies) and renal diseases. In our unpublished preliminary study, patients with antineutrophil cytoplasmic antibody-related glomerulonephritis (ANCA-GN), Henoch-Schönlein purpura nephritis (HSPN), and IgA nephropathy (IgAN) tended to be positive for serum HMGB1, while patients with diabetic nephropathy (2 out of 38 were positive) or chronic renal failure (1 out of 9 was positive) did not. Therefore, in the present study, we focused on patients who underwent renal biopsy and tested for the presence of serum HMGB1 as well as for the antibodies that accompanies it.

Patients and Methods

Patients and Biopsy Samples

Patients who underwent renal biopsy at Nagoya University Hospital and affiliated hospitals between June 2004 and March 2006 were eligible for the study, and those who agreed to participate in the present study were registered. As controls, 49 healthy volunteers were also enrolled.

In patients with a normal renal function, the primary indications for renal biopsy were urinary protein levels >100 mg/dl or >1 g/day and microhematuria associated with proteinuria (>0.5 g/day). In patients with rapidly progressive renal insufficiency, the indication for renal biopsy was no obvious renal atrophy. In diabetic patients, renal biopsy was performed especially when the patient was suspected of having other renal diseases in addition to diabetic nephropathy. Such conditions included hematuria, proteinuria (>1 g/day) without diabetic retinopathy, and rapid onset of nephritic syndrome. In certain situations, patients who did not meet the aforementioned criteria underwent renal biopsy, but these patients were not eligible for this study.

The following hospitals participated in this study: Anjou Kosei Hospital, Kainan Hospital, Kakegawa City Hospital, Nishio City Hospital, Masuko Memorial Hospital, Fukuroi City Hospital, Nagoya Daiichi Red Cross Hospital, Nakatsugawa City Hospital, Chuburosai Hospital, Tokai-Chuo Hospital, Tousei Hospital, Handa City Hospital, Nagoya Kyoritsu Hospital, Nagoya Medical Center, and Nagoya University Hospital. Patients with other major diseases, including liver cirrhosis, shock, inflammatory bowel diseases, and malignancy, were excluded. Controls were those who showed normal serum creatinine levels and were negative for urinary protein. Peripheral blood samples and 24-hour urine samples were taken at the time of renal biopsies after obtaining informed consent. Blood was centrifuged at 1,500 *g* for 10 min, and the supernatant was used as serum.

Study Protocols

The levels of HMGB1 were measured in serum of all study participants. The patients who underwent renal biopsies were divided into nine groups according to their diagnosis, as shown in table 1. The ratios of patients who were positive for serum HMGB1 were determined for each group, and the prevalence was compared with that of the controls. The levels of serum HMGB1 in each group of patients were also compared with those of the controls. The relationship between the expression of HMGB1 and the pathological or clinical parameters was assessed. The parameters investigated were pathological diagnosis, age, gender, serum creatinine, CRP, IL-1β, IL-6, TNF-α, urinary protein, and the presence or absence of anti-HMGB1 antibodies in the sera. IL-1β, IL-6, and TNF-α were measured using ELISA kits (Quantikine HS human IL-1β immunoassay, Quanti Glo human IL-6 Immunoassay 2nd generation, and Quanti Glo human TNF-α immunoassay 2nd generation, respectively; R & D Systems, Minneapolis, Minn., USA). Anti-HMGB1 antibody was detected by Western blotting. Other clinical data were gathered from our patient and pathology databases and reviews of medical records. The protocols were approved by the Nagoya University Ethical Committee.

Renal Histology

All renal biopsies were processed for light microscopy, immunofluorescence, and electron microscopy according to standard techniques. For each case, glass slides stained with hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, and periodic acid-methenamine-silver were reviewed. Immunofluorescence was performed on 2-μm cryostat sections by use of a panel of FITC-conjugated rabbit anti-human antibodies to IgG, IgM, IgA, C3, C1q, and fibrinogen (Dako Corp., Carpinteria, Calif., USA).

Determination of Serum HMGB1 Levels

The HMGB1 concentrations in sera were measured by ELISA at Shino-Test Corp. (Tokyo, Japan) as described [23]. Validated interassay and intra-assay coefficients of variation were <10%, and the limit of detection of this ELISA system was 0.3 ng/ml [24]. Therefore, a level of 0.3 ng/ml or more was defined as positive HMGB1.

Table 1. Prevalence and levels of serum HMGB1

Diagnosis	Number of patients	Number of patients with positive HMGB1 (%)	p vs. controls	average	HMGB1, ng/ml	
					min./median/max.	p vs. controls
ANCA-GN	22	13 (59.1)	<0.001	2.741	0/0.6/17.5	<0.001
HSPN	8	6 (75.0)	0.006	3.213	0/0.8/15.9	<0.001
IgAN	62	22 (35.5)	0.076	2.152	0/0/66.1	0.054
MN	41	14 (34.1)	0.207	1.610	0/0/19	0.100
FSGS	16	6 (37.5)	0.569	0.994	0/0/4.8	0.251
DN	9	3 (33.3)	1.355	2.400	0/0/19.5	0.214
SLE	24	5 (20.8)	4.870	3.383	0/0/39.6	2.780
MCD	27	4 (14.8)	7.368	1.833	0/0/42.2	7.256
Others	49	11 (22.4)	2.859	0.580	0/0/8.7	1.393
Total	258	85 (32.9)	0.034	1.645	0/0/66.1	0.034
Controls	49	6 (12.2)		0.208	0/0/3.2	

ANCA-GN = Anti-neutrophil cytoplasmic antibody-related glomerulonephritis; HSPN = Henoch-Schönlein purpura nephritis; IgAN = IgA nephritis; MN = membranous nephritis; FSGS = focal segmental glomerulosclerosis; DN = diabetic nephropathy; SLE = systemic lupus erythematosus nephritis; MCD = minimal change disease; others = other diseases (crescentic glomerulonephritis, mesangial proliferative glomerulonephritis, minor glomerular abnormality, benign glomerulosclerosis, tubulointerstitial nephritis, endocapillary proliferative glomerulonephritis).

Differences were considered to be significant at $p < 0.05$ (after Bonferroni correction).

Detection of Antibodies against HMGB1

The presence or absence of antibodies against HMGB1 in the sera was determined by Western blotting as described [24]. The serum samples, diluted 50-fold in PBS, were added to each lane of the membrane. Sera which were known to be anti-HMGB1 positive or anti-HMGB1 negative were used as controls.

Immunohistochemistry

Paraffin-embedded tissues were serially sectioned at 6 μm and soaked in a 0.3% H_2O_2 solution for 30 min. To study the localization of HMGB1, the sections were incubated with affinity-purified anti-HMGB1 polyclonal antibody (anti-peptide sequence KPDAAKKGVVKAEK) [24]. To visualize the presence of macrophages, the sections were boiled in sodium citrate buffer (10 mM, pH 6.0) for 30 min and incubated with a mouse anti-human CD68 monoclonal antibody (Dako). After washing, the slides were incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Histofine; Nichirei Corp., Tokyo, Japan) and were then further incubated with peroxidase-conjugated streptavidin (Histofine). The slides were incubated with diaminobenzidine substrate (Vector Laboratories, Burlingame, Calif., USA) and counterstained with methyl green solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan).

Statistical Analyses

StatView version 5.0 (SAS Institute, Cary, N.C., USA) was used. For comparison of the data between two groups, the χ^2 test for categorical variables and the Mann-Whitney U test for quantitative variables were used. The patients were categorized into nine different groups according to their pathological diagnosis. The Bonferroni correction was applied for multiple comparisons of HMGB1 in serum. In the present study, HMGB1 in the serum of the control group was compared with that of the nine different groups and with that of all patients as one group. Since multiple ($n = 10$) comparisons were done, the Bonferroni correction was applied to each p value [25]. For comparison of clinical data among multiple groups, ANOVA and Fisher's PLSD (protected least significant difference) method were used. The associations of the presence of HMGB1 in the sera and the pathological or clinical parameters were analyzed by the univariate logistic regression method. Data are expressed as mean ± SD. Differences were considered significant at $p < 0.05$.

Results

Serum HMGB1 and Renal Diseases

During the study period, 321 patients underwent renal biopsy at Nagoya University Hospital and affiliated hospitals, and 298 were eligible for the study. Out of the 298 patients, 258 (86.6%) were included in the study after consenting. The distribution of the patients with each pathological diagnosis is shown in table 1. Forty-nine healthy volunteers served as controls after written informed consent was obtained.

Out of the 258 patients, 85 (32.9%) were positive for serum HMGB1. In contrast, only 4 out of the 49 controls (12.2%) were positive. The levels of HMGB1 were also

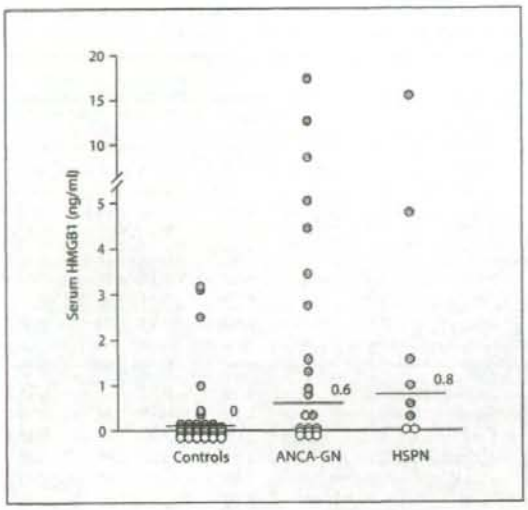

Fig. 1. Serum HMGB1 levels in controls and in patients with ANCA-GN and HSPN. Patients with ANCA-GN or those with HSPN showed significantly higher levels of HMGB1 in the sera than the controls ($p < 0.0001$). Horizontal bars indicate the median; open circles represent patients with negative serum HMGB1 and closed circles those with positive serum HMGB1.

Fig. 2. Detection of anti-HMGB1 antibodies in serum. The presence or absence of anti-HMGB1 antibodies in serum was determined by Western immunoblotting. Lane 1: positive control serum; lane 2: negative control serum; lanes 3 and 4: sera from ANCA-GN patients with positive serum HMGB1; lane 5: serum from a membranous nephritis patient with positive serum HMGB1; lane 6: serum from a focal segmental glomerulosclerosis patient with negative serum HMGB1; lane 7: serum from a systemic lupus erythematosus patient with negative serum HMGB1, and lane 8: serum from an IgAN patient with positive serum HMGB1.

higher among those who underwent renal biopsies than among the controls ($p = 0.0034$; table 1). The ratios of the patients who were positive for serum HMGB1 and the levels of serum HMGB1 were determined for each kidney disease, and these were compared with those of the controls. Only among the patients with ANCA-GN or HSPN was there a >50% prevalence of HMGB1-positive serum. Patients with ANCA-GN and those with HSPN showed a significantly higher prevalence of positive HMGB1 as compared with the controls ($p < 0.0001$ and $p = 0.0006$, respectively, vs. controls). The serum levels of HMGB1 were significantly higher in the patients with ANCA-GN or HSPN in comparison with the controls (minimum/median/maximum in ng/ml 0/0.6/17.5, 0/0.8/15.9, and 0/0/3.2, respectively, $p < 0.0001$ vs. controls; table 1, fig. 1). The patients with other diseases (including other types of primary glomerulonephritis, diabetic nephropathy, and lupus nephritis) did not show a significantly higher incidence of the presence of serum HMGB1 when compared to the controls (table 1).

When serum HMGB1 positivity was used as an indicator of a diagnosis for patients who underwent renal biopsies, the sensitivity was 63.3% (95% confidence interval CI 43.9–80.1%), and the specificity was 71.1% (95% CI 64.7–76.9%) for diagnosing patients with either ANCA-GN or HSPN.

Presence or Absence of Anti-HMGB1 Antibodies in Sera

Anti-HMGB1 antibody was detected in 4 out of 85 patients (4.71%) who showed negative serum HMGB1 and in 10 out of 173 patients (5.7%) who showed positive serum HMGB1. Anti-HMGB1 antibody was not detected in any of the patients with ANCA-GN, HSPN, or IgAN. A representative result of the Western blot is shown in figure 2.

Association of Serum HMGB1 with Pathological and Clinical Parameters

The factors which were associated with positive serum HMGB1 were determined by logistic regression analysis. As shown in table 2, among the patients who underwent renal biopsies, ANCA-GN (odds ratio OR 3.29, 95% CI 1.34–8.04, $p = 0.009$) and HSPN (OR 6.49, 95% CI 1.28–23.89, $p = 0.023$) were associated with positive serum HMGB1. Minimal change disease (MCD) was negatively

associated with positive serum HMGB1 (OR 0.32, 95% CI 0.11–0.96, p = 0.042). None of the other clinical parameters, including proinflammatory cytokines such as CRP, IL-1β, IL-6, and TNF-α, or the presence of anti-HMGB1 antibody were associated with positive serum HMGB1. The presence of glomerular crescents showed a tendency to be associated with positive serum HMGB1, which however was not statistically significant (OR 1.71, 95% CI 0.96–3.04, p = 0.066).

Subanalysis of Patients with IgAN

As there were 62 IgAN patients participating in this study, this group was selected for subanalysis. As shown in table 1, patients with IgAN tended to be positive for HMGB1 (35.5%) compared to controls (12.2%), although the difference was not statistically significant. It is well known that IgAN is a heterogeneous disease, and we speculated that there would be some patients more likely to be positive for serum HMGB1 among this group. Therefore, we studied the association of the various clinical parameters with positive serum HMGB1. Table 3 shows that glomerular crescent formation (which suggests that the disease is in an active phase) was significantly associated with positive serum HMGB1 (OR 3.8, 95% CI 1.27–11.41, p = 0.016). In contrast, other pathological parameters, including glomerular hypercellularity, interstitial infiltration, and tubular atrophy, did not show a significant association with positive serum HMGB1. Further analysis revealed that out of 22 IgAN patients who were positive for serum HMGB1, 13 (59.1%) had glomerular crescents, while 11 (27.5%) out of 40 IgAN patients who were negative for HMGB1 showed crescent formation (p = 0.029). The levels of serum HMGB1 were significantly higher in IgAN patients with glomerular crescents as compared with IgAN patients without (minimum/median/maximum in ng/ml 0/1.4/66.1 and 0/0/5.7, respectively, p = 0.0067).

Localization of HMGB1 in the Kidney

Renal biopsy specimens from 11 patients with ANCA-GN (5 serum HMGB1 positive and 6 negative), from 2 patients with HSPN (both HMGB1 positive), and from 12 patients with IgAN (5 serum HMGB1 positive and 7 negative) were available for inclusion in our immunohistochemical studies. Three patients with MCD served as controls. Among the 11 patients with ANCA-GN, strong staining for HMGB1 was observed in the mononuclear cells of the interstitium in 1 out of the 5 patients who were serum HMGB1 positive and in 3 out of the 6 patients who were serum HMGB1 negative. Concerning the 12 patients with IgAN, HMGB1 was strongly stained in the interstitial mononuclear cells in 3 out of the 5 patients who were serum HMGB1 positive and in 2 out of the 7 patients who were serum HMGB1 negative. Among those who showed positive HMGB1 staining in the interstitium, 2 with ANCA-GN and 3 with IgAN also showed positive staining for HMGB1 in some of the mononucle-

Table 2. OR for positive serum HMGB1 by logistic regression analysis in patients who underwent renal biopsies

	OR	95% CI	p
Diagnosis			
ANCA-GN	3.29	1.34–8.04	0.009
HSPN	6.49	1.28–23.89	0.023
IgAN	1.16	0.63–2.11	0.62
MN	1.21	0.60–2.43	0.58
FSGS	1.23	0.43–3.52	0.68
DN	1.01	0.24–4.17	0.97
SLE	0.51	0.18–1.41	0.19
MCD	0.32	0.11–0.96	0.042
Others	0.52	0.25–1.09	0.085
Clinical parameters			
Age	1.01	0.99–1.02	0.11
Gender (male)	1.56	0.91–2.71	0.11
Serum creatinine	1.03	0.79–1.34	0.79
CRP	1.08	0.97–1.21	0.14
Urinary protein	1.01	0.88–1.15	0.88
IL-1β	1.35	0.63–2.88	0.43
IL-6	0.99	0.99–1.01	0.48
TNF-α	0.98	0.83–1.17	0.88
Serum Anti-HMGB1 Ab	1.77	0.41–7.54	0.43
Glomerular crescents	1.71	0.96–3.04	0.066

Diagnosis: For explanation of abbreviations see footnote to table 1.

Table 3. OR for positive serum HMGB1 by logistic regression analysis in patients with IgAN

	OR	95% CI	p
Age	1.01	0.98–1.04	0.39
Gender (male)	1.75	0.58–5.22	0.31
Serum creatinine	0.95	0.31–2.95	0.93
CRP	0.89	0.07–10.30	0.92
Urinary protein	0.85	0.52–1.39	0.53
IL-1β	2.57	0.14–47.03	0.52
IL-6	5.1	0.65–39.55	0.11
TNF-α	2.57	0.14–47.03	0.52
Glomerular crescents	3.8	1.27–11.41	0.016

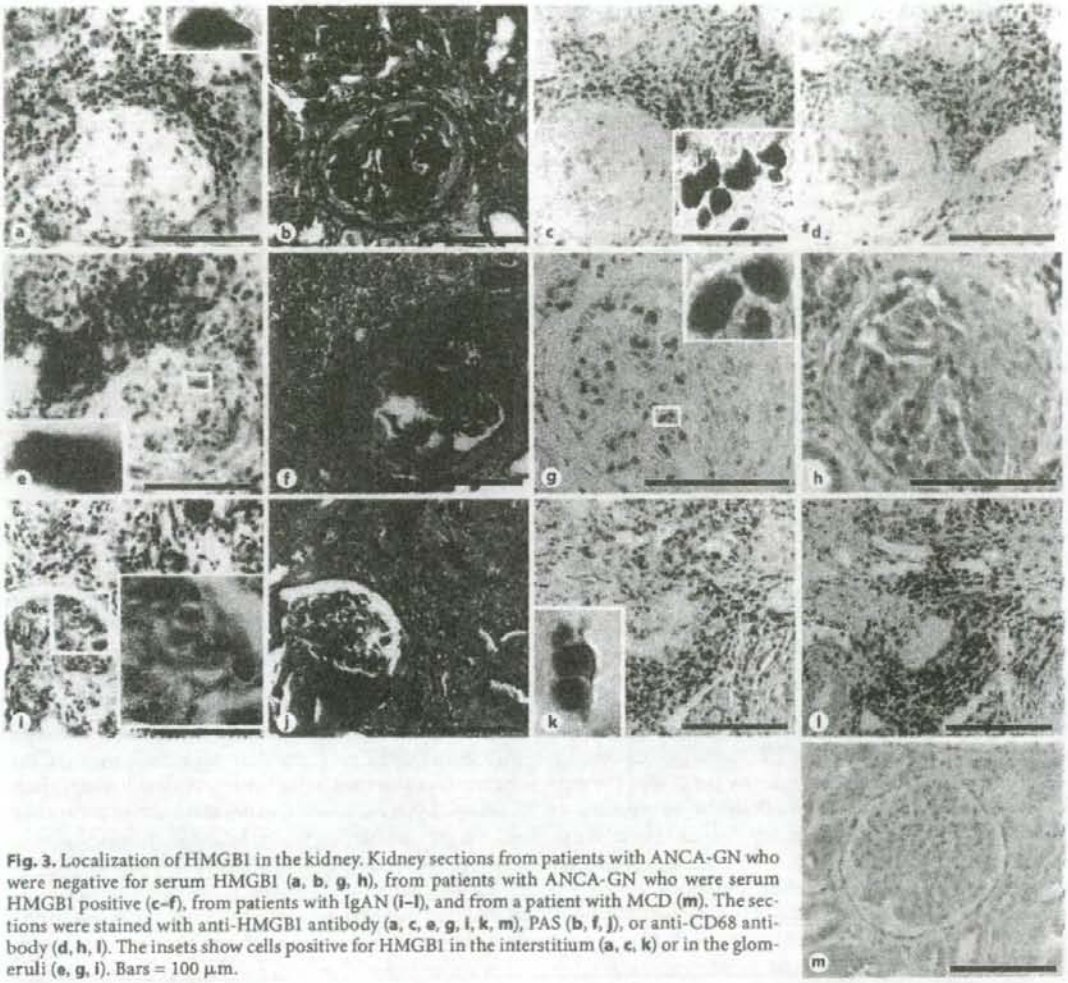

Fig. 3. Localization of HMGB1 in the kidney. Kidney sections from patients with ANCA-GN who were negative for serum HMGB1 (**a**, **b**, **g**, **h**), from patients with ANCA-GN who were serum HMGB1 positive (**c**–**f**), from patients with IgAN (**i**–**l**), and from a patient with MCD (**m**). The sections were stained with anti-HMGB1 antibody (**a**, **c**, **e**, **g**, **i**, **k**, **m**), PAS (**b**, **f**, **j**), or anti-CD68 antibody (**d**, **h**, **l**). The insets show cells positive for HMGB1 in the interstitium (**a**, **c**, **k**) or in the glomeruli (**e**, **g**, **i**). Bars = 100 μm.

ar cells of the glomeruli. In both the interstitium and the glomeruli, HMGB1 was localized in the nuclei as well as in the cytoplasm of the mononuclear cells, which were most likely to be macrophages (fig. 3). Only weak staining was observed in the mononuclear cells of the interstitium in the other 7 patients with ANCA-GN, in the other 7 patients with IgAN, and in the 2 patients with HSPN. The localization was similar regardless of the presence or absence of serum HMGB1. No staining was observed in the patients with MCD (fig. 3).

Discussion

This is the first study showing the presence of HMGB1 in the sera of patients with renal diseases. The results showed that serum HMGB1 was more likely to be positive in patients who underwent renal biopsies than in controls. The criteria used in the present study may suggest that the patients in the biopsy group would have some renal damage, leading to a higher incidence of positive HMGB1. The results of the present study showed that

among patients with various renal diseases, those with ANCA-GN or HSPN showed a significantly higher tendency to be positive for HMGB1. In contrast, no significant relationship was found for the presence of anti-HMGB1 antibodies in the sera among patients with biopsy-proven renal diseases.

Logistic regression analysis showed that pathological diagnoses of ANCA-GN and HSPN were the factors associated with positive serum HMGB1. Even among patients undergoing renal biopsies – the population more likely to be serum HMGB1 positive than controls –, patients with ANCA-GN or HSPN are more likely to show positive serum HMGB1. In contrast, lupus nephritis did not show a tendency to be positive for serum HMGB1. Of interest was the fact that no significant association was observed between the presence of serum HMGB1 and other inflammatory parameters, including CRP, IL-1β, IL-6, and TNF-α [24]. The data in the present study suggest that in patients with renal disease HMGB1 is not simply a marker of inflammation in general but rather a marker of a more specific condition, such as vasculitis.

When patients with IgAN were further analyzed, glomerular crescent formation was associated with positive serum HMGB1. Since crescent formation is a well-known indicator of disease activity, HMGB1 may be related to the activity of IgAN. In addition, because IgAN is considered to be one form of vasculitis [26], the results also suggest that those who showed positive serum HMGB1 were more likely to have renal vasculitis, including ANCA-GN, HSPN, or active IgAN. For the purpose of comparison, we studied the expression of HMGB1 in the sera of patients with other nonrenal diseases. Four out of 5 patients with nonrenal vasculitis showed positive serum HMGB1 [unpubl. data]. Although the number of patients examined was too small to show any definite relationship, these data may support our current hypothesis that the presence or absence of serum HMGB1 is a key factor in understanding the pathology of vasculitis.

In our study, immunostaining for HMGB1 was performed on the kidney sections obtained from patients with ANCA-GN, HSPN, IgAN, or MCD. No positive staining was observed in the kidney specimens from MCD patients who were diagnosed with minor glomerular abnormalities. By contrast, in patients with ANCA-GN or IgAN, HMGB1 was strongly stained mainly in the macrophages of the interstitium, and staining was infrequently observed in the mononuclear cells in the glomeruli. The positive HMGB1 staining was observed regardless of the presence or absence of serum HMGB1. In other words, a discrepancy was noticed between the HMGB1 expression in the kidney and the presence or absence of HMGB1 in the sera. Therefore, it seems unlikely that the HMGB1 in the sera came mainly from the cells in the kidney. Rather, we assume that in patients with active vasculitis, including ANCA-GN, HSPN, or IgAN, serum HMGB1 was secreted from inflammatory cells [11, 12] or endothelial cells [27–30].

Concerning the role of HMGB1, relatively recent studies [31] have shown that HMGB1 released from the damaged organ acts to mobilize inflammatory cells and/or stem cells to the injured site, where they work to mediate tissue repair. In contrast, HMGB1 has also been shown to be harmful to organs when it is overly released [9–12]. Animal experiments revealed that HMGB1 can induce tissue damage in the lung [16], the joints [17], and the liver [18, 19]. We found that when purified HMGB1 protein was administered to the rat kidney, strong macrophage inflammation was observed in the interstitial area [unpubl. data]. Taking all these animal experiments together, we are considering the possibility that HMGB1 is expressed in sera-activated immune cells and accelerates renal diseases.

In summary, the present study demonstrated that HMGB1, a proinflammatory cytokine, is likely to be detected in sera of patients with biopsy-proven renal vasculitis, including ANCA-GN, HSPN, and IgAN with crescent formation. Further studies are needed to determine the pathological roles of HMGB1 in the kidney.

References

1 Paonessa G, Frank R, Cortese R: Nucleotide sequence of rat liver HMG1 cDNA. Nucleic Acids Res 1987;15:9077.

2 Ferrari S, Ronfani L, Calogero S, Bianchi ME: The mouse gene coding for high mobility group 1 protein (HMG1). J Biol Chem 1994;269:28803–28808.

3 Wen L, Huang JK, Johnson BH, Reeck GR: A human placental cDNA clone that encodes nonhistone chromosomal protein HMG-1. Nucleic Acids Res 1989;17:1197–1214.

4 Bustin M: Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol 1999;19:5237–5246.

5 Hori O, Brett J, Slattery T, et al: The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of RAGE and amphoterin in the developing nervous system. J Biol Chem 1995;270:25752–25761.

6 Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, Ingram M, Lu A, Tanaka H, Hori O, Ogawa S, Stern DM, Schmidt AM: Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature 2000;405:354–360.

7 Fages C, Nolo R, Huttunen HJ, Eskelinen E, Rauvala H: Regulation of cell migration by amphoterin. J Cell Sci 2000;113:611–620.

8 Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ: HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248–251.

9 Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ: HMGB1 as a DNA-binding cytokine. J Leukoc Biol 2002;72:1084–1091.

10 Yang H, Wang H, Tracey KJ: HMG-1 rediscovered as a cytokine. Shock 2001;15:247–253.

11 Erlandsson Harris H, Andersson U: Mini-review: the nuclear protein HMGB1 as a proinflammatory mediator. Eur J Immunol 2004; 34:1503–1512.

12 Wang H, Yang H, Tracey KJ: Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med 2004;255:320–331.

13 Ombrellino M, Wang H, Ajemian MS, Talhouk A, Scher LA, Friedman SG, Tracey KJ: Increased serum concentrations of high-mobility-group protein 1 in haemorrhagic shock. Lancet 1999;354:1446–1447.

14 Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, Inoue K, Yamada S, Ijiri K, Matsunaga S, Nakajima T, Komiya S, Maruyama I: High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. Arthritis Rheum 2003;48: 971–981.

15 Hatada T, Wada H, Nobori T, Okabayashi K, Maruyama K, Abe Y, Uemoto S, Yamada S, Maruyama I: Plasma concentrations and importance of high mobility group box protein in the prognosis of organ failure in patients with disseminated intravascular coagulation. Thromb Haemost 2005;94:975–979.

16 Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ: HMG-1 as a mediator of acute lung inflammation. J Immunol 2000;165: 2950–2954.

17 Kokkola R, Sundberg E, Ulfgren AK, Palmblad K, Li J, Wang H, Ulloa L, Yang H, Yan XJ, Furie R, Chiorazzi N, Tracey KJ, Andersson U, Harris HE: High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. Arthritis Rheum 2002;46:2598–2603.

18 Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR: The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005;201: 1135–1143.

19 Watanabe T, Kubota S, Nagaya M, Ozaki S, Nagafuchi H, Akashi K, Taira Y, Tsukikawa S, Oowada S, Nakano S: The role of HMGB-1 on the development of necrosis during hepatic ischemia and hepatic ischemia/reperfusion injury in mice. J Surg Res 2005;124: 59–66.

20 Uesugi H, Ozaki S, Sobajima J, Osakada F, Shirakawa H, Yoshida M, Nakao K: Prevalence and characterization of novel pANCA, antibodies to the high mobility group nonhistone chromosomal proteins HMG1 and HMG2, in systemic rheumatic diseases. J Rheumatol 1998;25:703–709.

21 Sobajima J, Ozaki S, Uesugi H, Osakada F, Shirakawa H, Yoshida M, Nakao K: Prevalence and characterization of perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) directed against HMG1 and HMG2 in ulcerative colitis (UC). Clin Exp Immunol 1998;111:402–407.

22 Sobajima J, Ozaki S, Uesugi H, Osakada F, Inoue M, Fukuda Y, Shirakawa H, Yoshida M, Rokuhara A, Imai H, Kiyosawa K, Nakao K: High mobility group (HMG) non-histone chromosomal proteins HMG1 and HMG2 are significant target antigens of perinuclear anti-neutrophil cytoplasmic antibodies in autoimmune hepatitis. Gut 1999;44:867–873.

23 Yamada S, Inoue K, Yakabe K, Imaizumi H, Maruyama I: High mobility group protein 1 (HMGB1) quantified by ELISA with a monoclonal antibody that does not cross-react with HMGB2. Clin Chem 2003;49:1535–1537.

24 Yamada S, Yakabe K, Ishii J, Imaizumi H, Maruyama I: New high mobility group box 1 assay system. Clin Chim Acta 2006;372:173–178.

25 Taylor WE, Bhasin S, Artaza J, Byhower F, Azam M, Willard DH Jr, Kull FC Jr, Gonzalez-Cadavid N: Myostatin inhibits cell proliferation and protein synthesis in C2C12 muscle cells. Am J Physiol Endocrinol Metab 2001;280:E221–E228.

26 Davin JC, Ten Berge IJ, Weening JJ: What is the difference between IgA nephropathy and Henoch-Schönlein purpura nephritis? Kidney Int 2001;59:823–834.

27 Degryse B, Bonaldi T, Scaffidi P, Müller S, Resnati M, Sanvito F, Arrigoni G, Bianchi ME: The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol 2001;152: 1197–1206.

28 Mullins GE, Sunden-Cullberg J, Johansson AS, Rouhiainen A, Erlandsson-Harris H, Yang H, Tracey KJ, Rauvala H, Palmblad J, Andersson J, Treutiger CJ: Activation of human umbilical vein endothelial cells leads to relocation and release of high-mobility group box chromosomal protein 1. Scand J Immunol 2004;60:566–573.

29 Reinhart K, Bayer O, Brunkhorst F, Meisner M: Markers of endothelial damage in organ dysfunction and sepsis. Crit Care Med 2002; 30(5 Suppl):S302–S312.

30 Bradley JR, Lockwood CM, Thiru S: Endothelial cell activation in patients with systemic vasculitis. QJM 1994;87:741–745.

31 Palumbo R, Sampaolesi M, De Marchis F, Tonlorenzi R, Colombetti S, Mondino A, Cossu G, Bianchi ME: Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J Cell Biol 2004;164:441–449.

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A Novel Function of the Receptor for Advanced Glycation End-Products (RAGE) in Association with Tumorigenesis and Tumor Differentiation of HCC

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Background: The expression of the receptor for advanced glycation end products (RAGE) has an impact on the mechanisms giving rise to characteristic features of various cancer cells. The purpose of this study was to elucidate the clinicopathological relevance of the level of RAGE expression in patients with hepatocellular carcinoma (HCC) and to explore the effect of RAGE expression on the characteristic features of HCC.

Methods: The expression of RAGE was assessed in paired cancer and noncancerous tissues with HCC, using reverse-transcription polymerase chain reaction (RT-PCR), and immunohistochemistry. The quantitative RT-PCR data were analyzed in association with the clinicopathological factors of the patients with HCC. In in vitro experiments, the survival of RAGE-transfected Cos7 and mock-transfected Cos7 cells was compared under hypoxic conditions. In addition, after reducing RAGE levels in RAGE-transfected Cos7 cells by siRNA, similar experiments were performed.

Results: The expression of RAGE mRNA was lower in normal liver than in hepatitis and highest in HCC. Furthermore, in HCC, it was high in well- and moderately differentiated tumors but declined as tumors dedifferentiated to poorly differentiated HCC. Furthermore, HCC lines resistant to hypoxia were found to have higher levels of RAGE expression, and RAGE transfectant also showed significantly prolonged survival under hypoxia.

Conclusions: Our results suggest that HCC during the early stage of tumorigenesis with less blood supply may acquire resistance to stringent hypoxic milieu by hypoxia-induced RAGE expression.

Key Words: Hepatocellular carcinoma (HCC)—Receptor for advanced glycation end-products (RAGE)—Hypoxia-Reverse-transcription polymerase chain reaction (RT-PCR)—Immunohistochemistry.

Although many cancers arise from chronic inflammation, the relationships between carcinogenesis, cancer promotion, and its molecular characteristics remain poorly understood. Hepatocellular carcinoma (HCC), which typifies an inflammation-

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related tumor, is one of the most common malignancies in the world, especially in Asia and Africa. Japan has a high incidence of chronic viral hepatitis, cirrhosis, and HCC. The resolution of inflammatory activity at the molecular level may correlate with prevention of hepatocarcinogenesis and cancer promotion.

The receptor for advanced glycation end-products (RAGE) is a multiligand receptor classified as an immunoglobulin superfamily cell surface molecule and acts as a counter-receptor for high-mobility group box 1 (HMGB1),[1] advanced glycation end-products (AGEs), S100/calgranulins, and amyloid-β peptides. These interactions trigger the activation of key cell signaling pathways (e.g., p38 and p44/42 MAP kinase,[2] NF-κB, cdc42/rac,[3] and the generation of reactive oxygen species, and result in the production of proinflammatory cytokines.[5] RAGE-mediated proinflammatory processes are now considered to contribute to the progression of many chronic diseases, such as neuropathy, nephropathy,[6] macrovascular disease, amyloidoses, inflammatory conditions (e.g., rheumatoid arthritis and inflammatory bowel disease) and sepsis.[5,7] In addition to RAGE-mediated proinflammatory events, recent studies have revealed that the interaction of RAGE and its ligands and the resultant signaling play a causative role in the characteristic modulation of cancer cell functions, i.e., increasing tumor invasion and metastasis.[8] Furthermore, several clinical studies have demonstrated the (strong) association of RAGE expression with the malignant potential of various cancers such as gastric cancer,[9] colon cancer,[10,11] common bile duct cancer,[12] pancreatic cancer,[13] and prostate cancer,[14] although one report showed a reverse correlation between RAGE expression and tumor progression.[15] Thus, RAGE expression may be expected play have a significant role in the development of HCC, although no data have been reported for this tumor. In terms of HCC, it is known that the pathophysiological conditions or circumstances surrounding tumorigenesis are quite different from those reported for other cancers. For example, small HCC tumors at an early stage are continuously exposed to low oxygen and high glucose as well as the noncancerous hepatic tissues with cirrhosis in which tissue derangement occurs.[15] In addition, RAGE is the receptor for AGEs produced from excessive glucose metabolism,[16] extracellular HMGB1 released from necrotic cells which could be induced by hypoxia and inflammation, and serum amyloid A (SAA) produced in response to the proinflammatory cytokine IL-6,[17] and these RAGE ligands are possibly generated and/or released from inflamed hepatic tissues. Our central hypothesis is thus that RAGE expression may play a particular role in tumorigenesis of HCC, in addition to the role in the invasive and/or metastatic potential of cancer cells. With this background, the objectives of this study were: (1) to clarify the relationship between RAGE expression and the clinico-pathological features of HCC and (2) to investigate the functional role of RAGE expression in HCC development.

MATERIAL AND METHODS

Human samples

From March 2000 to September 2005, 65 patients with primary HCC were treated surgically in the Department of Surgical Oncology and Digestive Surgery, Kagoshima University School of Medicine. Of these 65 patients, 12 who had diabetes mellitus and 6 who underwent preoperative therapy were excluded from the study. RAGE expression for Diabetes patients upregulates at various tissues.[18,19,20] A further 11 patients were excluded because their RNA samples were degraded. Samples from the remaining 36 patients (30 men and 6 women with a mean age of 67.1 years) were included in the study. Seven patients (19.4%) were positive for hepatitis B surface antigen and 21 (58.3%) were positive for the antibody to hepatitis C virus. Eight patients (22.2%) were negative for both of these viruses. Twenty-four patients had chronic hepatitis and four had liver cirrhosis. The mean tumor size was 49.7 mm (range, 16–150 mm). The histological grade of each tumor was determined according to the general rules for the clinical and pathological study of primary liver cancer (The Liver Cancer Study Group of Japan, 2000).[21] Four tumors (11.1%) were well-differentiated HCC, 28 (77.8%) moderately differentiated HCC, and 4 (11.1%) poorly differentiated HCC. Postoperative tumor recurrence was observed for 11 patients (30.6%) (Table 1). For immunohistochemical study, a further 12 HCC samples (six well- and six poorly differentiated HCC), obtained surgically during the same period at JA Kagoshima Kouseiren Hospital, were added and used to verify the correlation of RAGE expression with tumor differentiation. Finally, a total of 48 HCC nodules (10 well-differentiated HCC, 28 moderately differentiated HCC, and 10 poorly differentiated HCC) were studied. As a control study, six normal liver samples were collected from patients with benign or metastatic liver tumors. Written informed consent, recognized by the ethical committees of Kagoshima

TABLE 1. *Background of patients*

Gender	
Male	30 (83%)
Female	6 (17%)
Mean age	67.2 years
Virus type	
B	7 (19.4%)
C	21 (58.3%)
None	8 (22.2%)
Background of livers	
Chronic hepatitis	24 (60.7%)
Liver cirrhosis	4 (11.1%)
Normal liver	8 (22.2%)
Mean tumor size	49.7mm
Histological grade	
Well differentiated	4 (11.1%)
Moderately differentiated	28 (77.8%)
Poorly differentiated	4 (11.1%)

Postoperative tumor recurrence 11 (30.6%).

University School of Medicine and JA Kagoshima Kouseiren Hospital, was obtained from each patient before tissue acquisition.

Immunohistochemistry

Consecutive 4-μm sections were cut from each paraffin-embedding block. Sections were immunostained by anti-RAGE antibody (Santa-Cruz, CA, USA) according to the conventional immunoperoxidase technique. Briefly, after peroxidase blocking with 3% H_2O_2/methanol for 10 min, specimens were blocked with phosphate buffered saline (PBS) containing 5% normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). Anti-RAGE antibody was used at 1/200. After overnight incubation at 4°C with the primary antibody, specimens were briefly washed in PBS and incubated at room temperature with the secondary antibody conjugated with peroxidase. The specimens were then washed in PBS and color-developed by diaminobenzidine solution (DAKO). After washing with water, specimens were counterstained with Meyer's hematoxylin (Sigma Chemical Co., St Louis, MO, USA). Immunostaining of all cases was performed at one time to ensure the same conditions of antibody reaction and DAB exposure. A total of 48 HCC nodules (10 well-differentiated HCC, 28 moderately differentiated HCC, and 10 poorly differentiated HCC) were studied. To evaluate the immunohistochemical staining, ten fields were selected and expression in 1000 tumor cells (100 cell/fields) was evaluated with high-power (×200) microscopy. The immunohistochemical expression of RAGE was defined as positive if distinct staining of the cell membrane was observed in at least 10% of tumor cells.

Cell and cell culture

Hepatoma cell lines HepG2, HuH7, HT17, and Li7 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University and Hep3B was obtained from the European Collection of Cell Cultures. HepG2, Hep3B, and HT17 were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (1000 mg/l) and HuH7 and Li7 were cultured with RPMI. RAGE-transfected Cos7 and its mock-transfectant were kindly provided by Drs. Yamamoto, Department of Biochemistry and Molecular Vascular Biology, Kanazawa University. These transfectants were maintained with DMEM supplemented with 10% Fetal Calf Serum (FCS) in the presence of 650 μg/ml G418. A hypoxic environment was created by placing a cell culture dish with one pouch of Anaero Pack into an airtight jar. (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). This created nearly 0% O_2 conditions.

Gene silencing of RAGE with specific siRNA

Cells were seeded in 12-well plates at a density of 1×10^5 cells per well and allowed to adhere overnight. Then siRNAs for the target gene or its control oligoribonucleotide mixed with Dharma FECT2™ transfection reagent (Dharmacon Inc. Chicago, USA) was added to the cells and incubated for 48h at 37°C The efficacy of gene silencing was evaluated using immunoblot analysis.

MTT assay

Cell viability was monitored after incubation for 24, 36, and 48 hours by MTT assay. Briefly, 0.5 mg/mL 3-[4,5]-2,5-diphenyltetrazolium bromide (MTT) in fresh medium was added to each well and the cells were incubated for an additional 3 hours. Afterwards, the blue formazan crystals were dissolved in 1 mL isopropanol and measured spectrophotometrically at 570 nm.

Immunoblot analysis

Whole cell lysates were prepared as per the Santa Cruz protocol. One milliliter of Radio-Immunoprecipitation Assay (RIPA) buffer was added to a 100 mm cell culture plate. The plates were gently rocked for 15 min at 4°C. Adherent cells were scrpaed with a cell scraper, followed by incubation for 30–60 min on ice. The cell lysate was microcentrifuged at 10,000g

for 10 min at 4°C. The supernatant fluid was the total cell lysate. The supernatant was transferred to a new microfuge tube and the pellet was discarded. Twelve microgram lysates were subjected to immunoblot analysis using a 12.5% sodium dodecyl sulfate (SDS) -polyacrylamide gel followed by electrotransfer onto nitrocellulose filters. The filters were immunoreacted with anti-RAGE antibody (a gift from TORAY Research Institute, Sagamihara, Japan) or with anti-HMGB1 antibody (BD Biosciences, Tokyo, Japan) and then incubated with peroxidase-conjugated anti-goat IgG (Medical and Biological Laboratories, Nagoya, Japan). The immune complex was visualized using the Enhanced Chemiluminescence (ECL) Western blot detection system (PIERCE, Rockford, IL, USA). The amount of B-actin as an internal control was also examined using a specific antibody (Cytoskelton Inc., Denver, CO, USA and Santa Cruz, CA, USA). At least three independent experiments were performed.

Quantitative RT-PCR

For reverse-transcription PCR (RT-PCR) and real-time quantitative PCR, total RNA was extracted from 30 mg frozen tissue using Total RNA Mini (VIOGENE, CA, USA). For cDNA synthesis, the RNA samples (1 μg) were converted into cDNA by reverse transcription (RT) using random primers (TAKARA, Siga, Japan) according to the manufacturer's instructions. To estimate the mRNA expression levels of several genes quantitatively, polymerase chain reaction (PCR) amplification was performed using a Light-Cycler system (Roche, Mannheim, Germany) and the Light-Cycler Fast Start DNA Master SYBER green I kit (Roche). Primers were as follows: RAGE: 5′-AAA CAT CAC AGC CCG GAT TG-3′ and 5′-TCC GGC CTG TGT TCA GTT TC-3′, HMGB1: 5′-GCT CAG AGA GGT GGA AGA CCA-3′ and 5′-GGT GCA TTG GGA TCC TTG AA-3′ (14), GAPDH: 5′-TTG GTA TCG TGG AAG GAC TCA-3′ and 5′-TGT CAT CAT ATT TGG CAG GTT T-3′. Amplification was carried out in 20 μL reactions containing 4 mM $MgCl_2$, 2 μL of primer, 2 μL of Light-Cycler-FastStart DNA Master SYBR green I reagent, and 2 μL of cDNA. Reaction conditions were an initial incubation at 95°C for 10 min, followed by 50 cycles at 95°C for 10 s for denaturation, 64°C for 10 s for annealing of the RAGE primers, 54°C for 10 s for annealing of the HMGB1 primers and 60°C for 10 s for annealing of the GAPDH primers and 72°C for 10 s for extension. Melting curves were obtained according to the protocol under the following conditions: 0 s denaturation period at 95°C, starting temperature of 65°C, end temperatures of 95°C, and rate of temperatures increase of 0.1°C/s. The quantitative value of the target gene (RAGE mRNA) in each sample was normalized using GAPDH expression as an internal control. The quantitative RT-PCR assay was carried out twice and the mean value was calculated. Finally, the mRNA expression ratio of cancerous (C) to noncancerous (N) tissues was calculated using the following formula: $R = \log\{\text{target gene (C)/GAPDH (C)}\}$, $R = \log\{\text{target gene (N)/GAPDH (N)}\}$. These experiments were carried out twice to confirm reproducibility.

Statistical analysis

Statistical analysis was performed using the JMP IN version 5.1.2 software system (SAS institute Inc., Cary, NC, USA). Each value of mRNA expression was log transformed before statistical analysis. Gene expression was compared among normal liver, hepatitis, and HCC using Student's *t*-test. The relationships between RAGE-, HMGB1-mRNA expression levels and clinicopathological features were evaluated using Student's *t*-test and the Mann–Whitney *U* test, as appropriate. Immunohistochemical study of RAGE in HCC tissues was evaluated using the χ^2 test. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

RAGE and HMGB1 expression in normal liver, hepatitis, and HCC

RAGE antibody yielded a strong band compared to control mouse lung extracts. Using Western blotting and RT-PCR, protein and mRNA expression of RAGE and HMGB1 were examined in both cancer and noncancerous tissues from three cases (Fig. 1a, b). All 3 cases showed co-expression of RAGE and HMGB1 protein and mRNA in these tissues.

Quantitative RAGE mRNA expression in HCC and noncancerous lesions

Comparing the quantitative expression of RAGE mRNA in paired cancer and noncancerous tissues (i.e., chronic hepatitis or liver cirrhosis) of 36 cases, HCC tissues showed significantly higher expression than noncancerous tissues ($p < 0.01$, Fig. 2a).

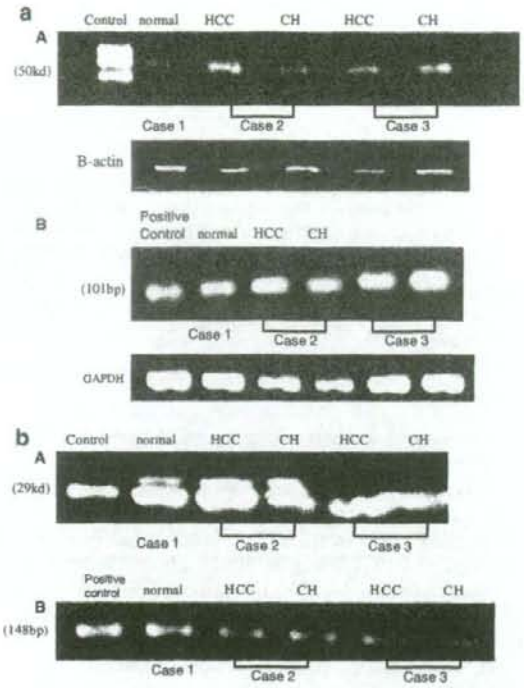


FIG. 1. (a) RAGE expression by Western blotting (A) and RT-PCR (B). (b) HMGB1 expression by Western blotting (A) and RT-PCR (B). Abbreviations: normal, normal liver; CH, chronic hepatitis.

Moreover, the mean values of RAGE mRNA expression in cancer and noncancerous tissues were higher ($p < 0.01$ and $p = 0.08$) than that in normal liver tissues (i.e., non-inflamed liver from benign or metastatic liver tumor patients) (Fig. 2b).

Relationship between RAGE mRNA expression and clinicopathological features

To elucidate the biological significance of RAGE expression in HCC, we compared the levels of RAGE mRNA expression with the clinico-pathological features of 36 patients. As shown in Table 2, we noted significant differences in RAGE mRNA expression in association with gender, age, the levels of protein induced by vitamin K absence or antagonist (PIVKA-II), and postoperative recurrence. In terms of gender and age, the results are in accordance with a previous report.[23] The expression levels of RAGE mRNA tended to be lower in poorly differentiated tumors compared to well- or moderately differentiated tumors ($p = 0.06$). Moreover, the levels of RAGE mRNA showed a negative correlation with PIVKA-II levels and the presence or absence of recurrence. There were no significant differences regarding tumor size, intrahepatic metastasis, and vascular invasion. On the other hand, it should be noted that a significant difference in the levels of RAGE expression was observed between hepatitis virus-positive and virus--egative tissues ($p < 0.01$) in the study of noncancerous inflammatory liver tissues (i.e., chronic hepatitis or liver cirrhosis) (data not shown).

RAGE expression and tumor differentiation in HCC

To verify the correlation of RAGE expression with HCC differentiation, we carried out an immunohistochemical examination. (Fig. 3a–c) Well- and moderately differentiated HCC showed a high percentage of RAGE positivity (70% and 64%, respectively); only 2 of 10 (20%) were positive in poorly differentiated HCC (Fig. 3d). Statistically, there were significant differences in the rates of positivity between well- and moderately differentiated HCC and poorly differentiated HCC.

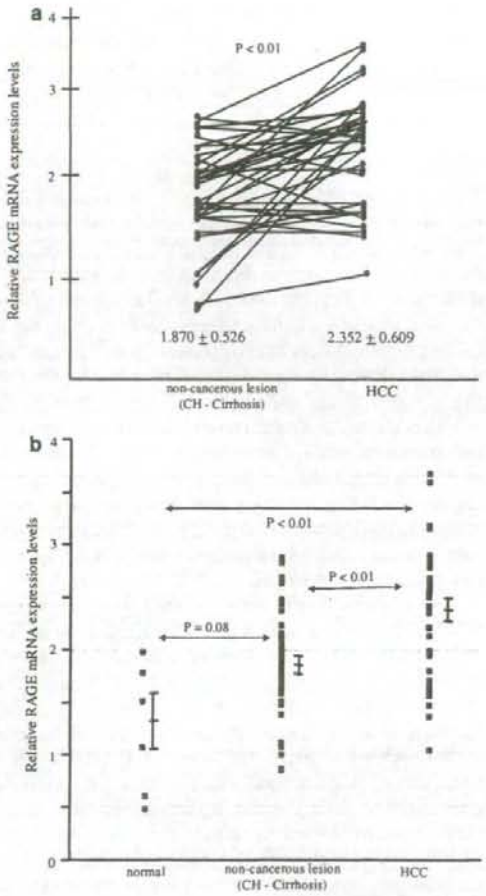


FIG. 2. (a) Quantitative RAGE mRNA expression in paired cancerous (HCC) and noncancerous tissues from 36 cases. (b) Quantitative RAGE mRNA expression in normal liver ($n = 6$) and noncancerous and cancerous tissues ($n = 36$).

RAGE expression in hepatoma cell lines

RAGE antibody yielded a strong band on Western blot analysis of the control RAGE transfected Cos7 cells (Fig. 4a, lane 1). In this experimental setting, the well-differentiated HCC cell line HuH7 (lane 6) expressed RAGE protein at a modest level. The poorly differentiated HCC cell line HT17 (lane 7) expressed RAGE protein at a low level. Hepatoma cell lines Hep3B and Li7 (lane 4 and 5), derived from tumors of unknown state of differentiation, expressed RAGE protein at a modest level. HepG2 (lane 3) did not express RAGE protein at all. These results were very similar to RAGE mRNA expression and depend on the pathological differentiation of HCC.

Enhanced RAGE expression under hypoxic conditions

Early carcinogenesis occurs in an avascular environment and under conditions of hypoxia.[15,23,24] In order to clarify the role of RAGE expression under hypoxic conditions, all hepatoma cell lines were cultured in an anaerobic environment. Li7 could survive for 48 h, Hep3B for 12 h, and HuH7 for 24 h under such conditions, up to the appearance of signs of cell death. HepG2 and HT17 rapidly died under anaerobic conditions. The RAGE-positive cell lines (Li7, Hep3B and HuH7) were more tolerant of anaerobic conditions than RAGE-negative or weakly expressing cell lines (HepG2 and HT17). Moreover, RAGE expression in the cell lines that survived was clearly upregulated in response to the hypoxic conditions (Fig. 4b).

Enhancement of cell survival in anaerobic conditions following RAGE transfection

These findings led to the hypothesis that RAGE expression could confer tolerance to hypoxia in HCC. Therefore, we investigated the survival of RAGE-transfected and mock-transfected cells under hypoxic conditions. In continuous incubation under hypoxic conditions, as expected, RAGE-transfected Cos7 cells clearly survived longer than mock-transfected Cos7 cells (observed at 36 h and 48 h) (Fig. 5).

Decline of cell survival in hypoxic conditions following RAGE reduced by siRNA

Consistent with the previous result, reduction of RAGE expression with anti-RAGE siRNA re-increased susceptibility to hypoxia-induced injury in RAGE-overexpressed Cos7 transfectant (Fig. 6a,b), suggesting that RAGE expression might play an important role in the acquirement of hypoxia-resistant cellular phenotype.

DISCUSSION

Hepatocellular carcinogenesis and associated tumorigenesis occur in a stringent and restrictive environment, such as the cirrhotic liver in which tissue oxygen supply is insufficient. Moreover, in the liver, with a dual blood supply from the hepatic ar-

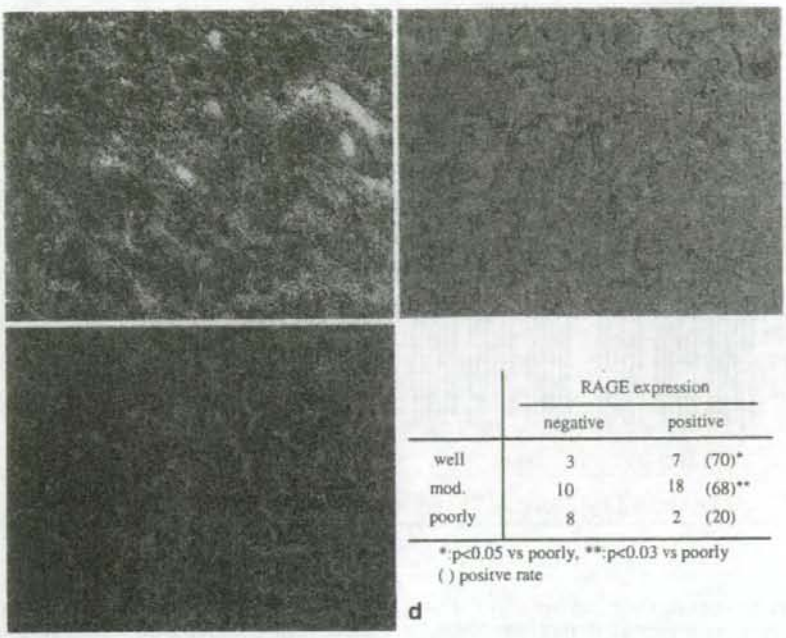
TABLE 2. *Relationship between tumor RAGE expression and clinicopathological features*

Factors	n	Tumor RAGE mRNA expression Mean ± SD	*p* value
Gender			
Male	30	2.267 ± 0.341	0.03
female	6	2.777 ± 0.619	
Age			
≥65 years	26	2.468 ± 0.568	0.03
<65 years	10	2.052 ± 0.638	
Tumor size (mm)			
≥30	11	2.395 ± 0.613	0.39
<30	25	2.333 ± 0.620	
Portal invasion			
Absent	22	2.358 ± 0.655	0.52
Present	14	2.348 ± 0.594	
Venous invasion			
Absent	26	2.339 ± 0.577	0.47
Present	10	2.357 ± 0.633	
Vascular invasion			
Absent	19	2.362 ± 0.633	0.46
Present	17	2.340 ± 0.601	
Intrahepatic metastasis			
Absent	27	2.378 ± 0.662	0.33
Present	9	2.273 ± 0.435	
Gross classification			
Localized type	21	2.448 ± 0.636	0.13
Invasive type	15	2.217 ± 0.564	
Differentiation			
Well	4	2.365 ± 0.566	0.43
Moderately	28	2.420 ± 0.580	0.08
Poorly	4	1.860 ± 0.783	
Stage			
I,II	16	2.367 ± 0.643	0.55
III,IV	20	2.340 ± 0.597	
PIVKA†U			
Normal	9	2.704 ± 0.385	
High	25	2.186 ± 0.630	0.03
AFP			
Normal	12	2.557 ± 0.586	0.16
High	24	2.250 ± 0.607	
Virus			
B	7	2.237 ± 0.606	
C	21	2.382 ± 0.569	
None	8	2.374 ± 0.779	
Recurrence			
Absent	25	2.485 ± 0.628	0.04
Present	11	2.050 ± 0.456	

tery and portal vein, liver-specific oxygen and glucose supply are available dependent upon the degree of liver disorder. The multiligand receptor RAGE is particularly relevant in this context. The ligands for RAGE may be produced abundantly during HCC development. For example, HMGB1 released from necrotic cells and AGE generated in the process of higher hepatic glucose metabolism are considered to be major candidates (i.e., the presence of HMGB1 and carboxymethyl lysine in HCC; data not shown). Hepatic SAA production in response to IL-6 may also be included.[19] In addition, RAGE expression and its downstream signaling are now considered to play a significant role in the progression of various cancers as well as the development of inflammatory lesions.

Another aspect of the biology of RAGE was suggested by the features of retinoic acid-induced neuroblastoma differentiation, in which RAGE expression played a more important role in cellular survival than in neurite outgrowth.[25] In this model, inhibition of RAGE function partially blocked the increase in levels of the anti-apoptotic protein Bcl-2 in the process of neuronal differentiation,[25] indicating that RAGE and its signaling also might contribute to the survival of certain cancer cell types undergoing "differentiation". Furthermore, although indirectly, our previous study demonstrated that NF-κB, currently considered as a causative transcriptional factor for various inflammatory events and also known as a major RAGE-mediated signal, played an important role in the survival of neuronal tumor cells[26,27] and in experimentally developed HCC.[28]

The present study demonstrated that increased RAGE expression was highly associated with the status of pathological "differentiation" in HCC, which played a significant role in acquisition of the hypoxia-resistant phenotype of tumor cells. This conclusion is supported by several lines of experimental evidence. First, the level of RAGE expression was higher in well- and moderately differentiated HCC, while it diminished as the tumors dedifferentiated to poorly differentiated HCC. This was consistent with the evidence that a negative correlation was observed between the level of RAGE mRNA expression and either the level of PIVKA II or the incidence of postoperative recurrence. Second, the analysis of five HCC lines revealed that three of these (Li7, Hep3B, and HuH7) that are resistant to hypoxic stress characteristically showed higher levels of RAGE expression compared to the two hypoxia-intolerant cell lines HepG2 and HT17. Third, sublethal hypoxia exposure induced significantly increased RAGE expression in hypoxia-resistant HCC lines. In the analysis of the association between the level of RAGE expression and the "differentiation status" of HCC lines, the level of RAGE expression was higher in "possibly differentiated" lines (i.e., HCC with low Alpha-Fet protein (AFP) production), consistent with the results from clinical samples. Finally, cells overexpressing RAGE exogenously showed prolonged survival under hypoxic conditions compared to control mock-



	RAGE expression	
	negative	positive
well	3	7 (70)*
mod.	10	18 (68)**
poorly	8	2 (20)

*:p<0.05 vs poorly, **:p<0.03 vs poorly
( ) positve rate

d

FIG. 3. RAGE expression by immunohistochemical staining: (a) well-differentiated HCC, (b) moderately (mod.) differentiated HCC, (c) poorly differentiated HCC, (e) the number of RAGE positive or negative cases according to tumor differentiation.

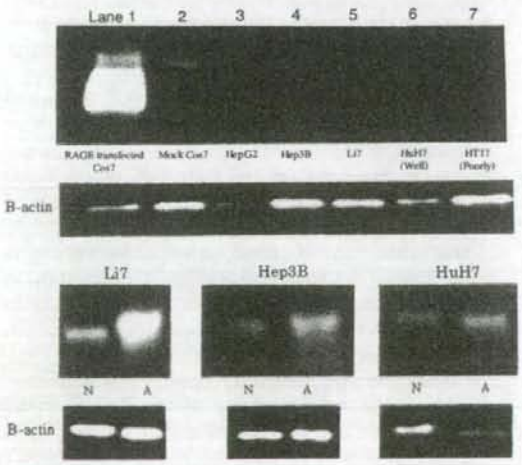


FIG. 4. (a) RAGE expression in hepatoma cell lines by Western blotting. (b) RAGE expression in hepatoma cell lines under hypoxic conditions by Western blotting. N, normoxic conditions; A, anaerobic conditions.

transfected cells, and siRNA experiments demonstrated similar results.

Our conclusion also provides a new hypothetical concept that hepatic RAGE expression may be relevant to the stage or severity of inflammation and the incidence of carcinogenesis and early tumorigenesis of HCC (Fig. 7). In the process of the development of an HCC lesion, increased RAGE expression

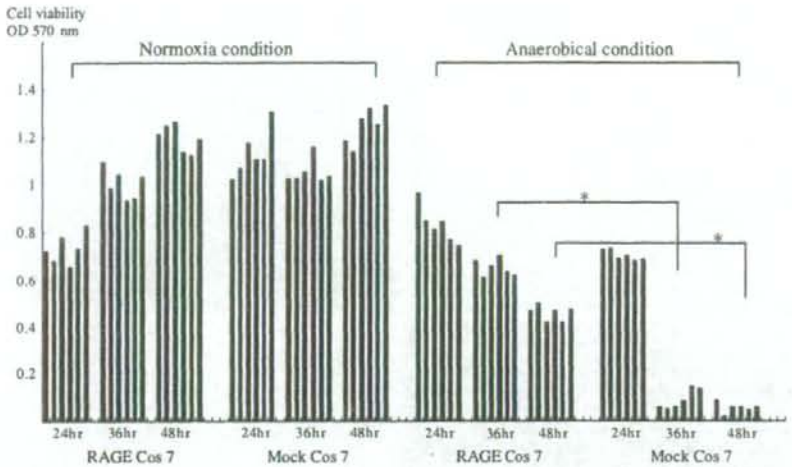


FIG. 5. Comparison of cell survival of RAGE-transfected Cos7 and mock-transfected Cos7 cells under anaerobic conditions. Cell survival of both groups was estimated from six dishes at each time point using the MTT assay. *p < 0.01 by Student's t-test.

by inflamed hepatocytes may confer adaptation to an advanced hypoxic environment during remodeling and carcinogenesis with accelerated cell proliferation. In this context, an HCC lesion which is highly associated with inflammation caused by either hepatitis viruses or drugs is developed through a process of multistage carcinogenesis, i.e., from inflammatory lesions (e.g., hepatitis, cirrhosis and precancerous lesion) to adenomatous hyperplasia, and eventually into HCC. Once cancer is established, HCC dedifferentiates step-by-step to a more malignant histology, from well- and moderately to poorly differentiated HCC. It has also been pointed out that the vascular supply changes significantly between each stage of tumorigenesis. According to the evidence from several clinical studies,[15,29,30] both adenomatous hyperplasia and well-differentiated HCC are hypovascular tumors primarily fed by the portal vein system, while moderately and poorly differentiated HCC are hypervascular tumors primarily fed by arterial blood. In addition, early carcinogenesis and the development of the malignant phenotype generally occur in an avascular environment.[31-33] Increased RAGE expression, at least in part, may thus play an important role in the mechanism of early HCC development from precancerous inflammatory lesions. In addition, about the expression of RAGE being lower in poorly differentiated HCC when compared to well and moderately differentiated HCC, gastric and colon cancers have been showed that RAGE and other ligands interacts act on tumor promotion in clinical materials.[9,11] However, prostate cancer was demonstrated to show no correlation between RAGE expression and tumor differentiation and depth.[14] Moreover, non-small-cell lung cancers have been showed a reverse correlation between RAGE expression and tumor stage.[34] This result is partially similar to our results. RAGE expression of each clinical samples is various.

Some reports have shown that RAGE-expressed cells have invasion and migration potential. Our data from clinical samples did no' relate to the potential. In vivo, invasive and metastatic potential are reflected by many factors, which may have caused our results.

Our results raise many questions concerning mechanistic and practical processes. It is important to know whether RAGE can bind HMGB1 secreted from activated macrophages in hepatic inflammation, or if occupancy of RAGE by inhibitors would obviate binding of the stimulatory ligands. The functional role of downstream signaling of the RAGE would also be important to determine the cytoprotective mechanism under hypoxia.

Our findings have provided the first evidence of the clinical relevance and function of RAGE in HCC, namely differentiation-associated RAGE expression that confers a hypoxia-resistant phenotype. Although other mechanisms may also be important, our data also introduce the concept that RAGE and its

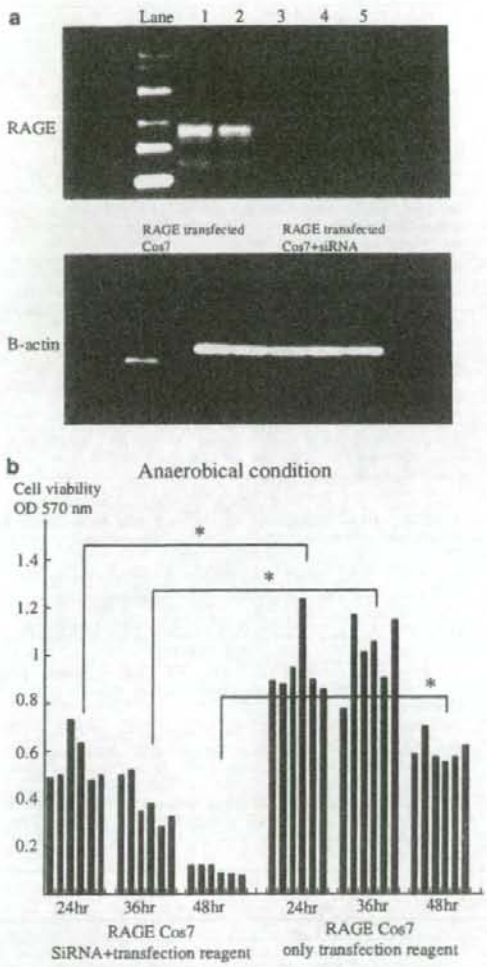


FIG. 6. RAGE level in RAGE-transfected Cos7 cells were reduced by siRNA RAGE-transfected Cos7 cells reduced RAGE level by siRNA decreased more than the cells mixed with only transfection reagent. *p < 0.01 by Student's t-test.

functions may be possible candidates for therapeutic targets in the treatment of HCC.

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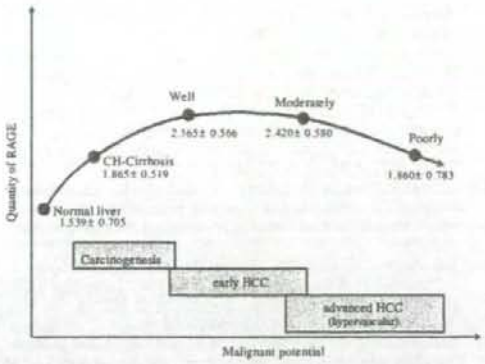

FIG. 7 Scheme of the change of RAGE expression according to the sequential change of liver tissue: normal → chronic hepatitis (CH) → cirrhosis → HCC. The value indicates the quantitative RAGE mRNA expression.

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REFERENCES

1. Rauvala H, Pihlaskari R. Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *J Biol Chem* 1987; 262:16625–35.
2. Yeh CH, Sturgis L, Haidacher J, et al. Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-kappaB transcriptional activation and cytokine secretion. *Diabetes* 2001; 50:1495–504.
3. Huttunen HJ, Fages C, Rauvala H. Receptor for advanced glycation end products (RAGE)-mediated neurite outgrowth and activation of NF-kappaB require the cytoplasmic domain of the receptor but different downstream signaling pathways. *J Biol Chem* 1999; 274:19919–24.
4. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab* 2001; 280:E685–94.
5. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285:248–51.
6. Yamamoto Y, Kato I, Doi T, et al. Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* 2001; 108:261–8.
7. Liliensiek B, Weigand MA, Bierhaus A, et al. Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. *J Clin Invest* 2004; 113:1641–50.
8. Taguchi A, Blood DC, del Toro G, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. *Nature* 2000; 405:354–60.
9. Kuniyasu H, Oue N, Wakikawa A, et al. Expression of receptors for advanced glycation end-products (RAGE) is

closely associated with the invasive and metastatic activity of gastric cancer. *J Pathol* 2002; 196:163–70.
10. Kuniyasu H, Chihara Y, Kondo H. Differential effects between amphoterin and advanced glycation end products on colon cancer cells. *Int J Cancer* 2003; 104:722–7.
11. Kuniyasu H, Chihara Y, Takahashi T. Co-expression of receptor for advanced glycation end products and the ligand amphoterin associates closely with metastasis of colorectal cancer. *Oncol Rep* 2003; 10:445–8.
12. Hirata K, Takada M, Suzuki Y, Kuroda Y. Expression of receptor for advanced glycation end products (RAGE) in human biliary cancer cells. *Hepatogastroenterology* 2003; 50:1205–7.
13. Takada M, Koizumi T, Toyama H, Suzuki Y, Kuroda Y. Differential expression of RAGE in human pancreatic carcinoma cells. *Hepatogastroenterology* 2001; 48:1577–8.
14. Ishiguro H, Nakaigawa N, Miyoshi Y, Fujinami K, Kubota Y, Uemura H. Receptor for advanced glycation end products (RAGE) and its ligand, amphoterin are overexpressed and associated with prostate cancer development. *Prostate* 2005; 64:92–100.
15. Hayashi M, Matsui O, Ueda K, et al. Correlation between the blood supply and grade of malignancy of hepatocellular nodules associated with liver cirrhosis: evaluation by CT during intraarterial injection of contrast medium. *AJR* 1999; 172:969–76.
16. Cooper ME. Importance of advanced glycation end products in diabetes-associated cardiovascular and renal disease. *Am J Hypertens* 2004; 17:31–8.
17. Yan SD, Zhu H, Zhu A, et al. Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis. *Nat Med* 2000; 6:633–4.
18. Chen Y, Yan SS, Colgan J, et al. Blockade of late stages of autoimmune diabetes by inhibition of the Receptor for Advanced Glycation End Products. *J Immunol* 2004; 173:1399–405.
19. Bierhaus A, Haslbeck KM, Humpert PM, et al. Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J Clin Invest* 2004; 114:1741–51.
20. Wendt TM, Tanji N, Guo J, et al. RAGE Drives the development of glomerulosclerosis and implicates Podocyte activation in the pathogenesis of diabetic nephropathy. *Am J Pathol* 2003; 162:1123–37.
21. The general rules for the clinical and pathological study of primary liver cancer. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989;19:98–129.
22. Bierhaus A, Humpert PM, Morcos M, et al. Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005.
23. Kudo M. Morphological diagnosis of hepatocellular carcinoma: special emphasis on intranodular hemodynamic imaging. *Hepatogastroenterology* 1998; 45(Suppl 3):1226–31.
24. Kudo M. Imaging blood flow characteristics of hepatocellular carcinoma. *Oncology* 2002; 62(Suppl 1):48–56.
25. Sajithlal G, Huttunen H, Rauvala H, Munch G. Receptor for advanced end products plays a more important role in cellular survival than in neurite outgrowth during retinoic acid-induced differentiation of neuroblastoma. *J Biol Chem* 2002; 277:6888–97.
26. Abeyama K, Stern DM, Ito Y, et al. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. *J Clin Invest* 2005; 115:1267–74.
27. Abeyama K, Kawano K, Nakajima T, Takasaki I, Kitajima I, Maruyama I. Interleukin 6 mediated differentiation and rescue of cell redox in PC12 cells exposed to ionizing radiation. *FEBS Lett* 1995; 364:298–300.
28. Ueno S, Aoki D, Kubo F, et al. Roxithromycin inhibits constitutive activation of nuclear factor {kappa}B by diminishing oxidative stress in a rat model of hepatocellular carcinoma. *Clin Cancer Res* 2005; 11:5645–50.
29. Ueda K, Terada T, Nakanuma Y, Matsui O. Vascular supply in adenomatous hyperplasia of the liver and hepatocellular carcinoma: a morphometric study. *Hum Pathol* 1992; 23:619–26.
30. Matsui O, Kadoya M, Kameyama T, et al. Benign and malignant nodules in cirrhotic livers: distinction based on blood supply. *Radiology* 1991; 178:493–7.
31. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis?. *Nat Rev Cancer* 2004; 4:891–9.
32. Dewhirst MW, Secomb TW, Ong ET, Hsu R, Gross JF. Determination of local oxygen consumption rates in tumors. *Cancer Res* 1994; 54:3333–6.
33. Helmlinger G, Yuan F, Dellian M, Jain RK. Interstitial pH and pO_2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med* 1997; 3:177–82.
34. Bartling B, Hofmann HS, Weigle B, Simm A. Down-regulation of the receptor for advanced glycation end-products(RAGE) supports non-small cell lung carcinoma. *Carcinogenesis* 2005; 26:293–301.

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Tumor necrosis factor-α stimulates gingival epithelial cells to release high mobility-group box 1

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Morimoto Y, Kawahara K-I, Tancharoen S, Kikuchi K, Matsuyama T, Hashiguchi T, Izumi Y, Maruyama I. Tumor necrosis factor-α stimulates gingival epithelial cells to release high mobility-group box 1. J Periodont Res 2008; 43: 76–83.

Background and Objective: High-mobility-group box 1 functions as a late-phase inflammatory mediator. It can be released extracellularly by macrophages and necrotic cells through lipopolysaccharide and tumor necrosis factor-α. The objective of this study was to clarify the source of high-mobility-group box 1 in chronic periodontitis tissues and tumor necrosis factor-α-stimulated gingival epithelial cells, and subsequently elucidate its inducible inflammatory pathway.

Material and Methods: Chronic periodontitis and healthy gingival sections were stained for high-mobility-group box 1 by immunohistochemistry and immunofluorescence. The amounts of high-mobility-group box 1 released into the gingival crevicular fluid and supernatants from gingival epithelial cells stimulated by tumor necrosis factor-α were examined by western blot. The phosphorylation of mitogen-activated protein kinases (MAPKs) in gingival epithelial cells was also examined.

Results: High-mobility-group box 1 was detected in the cytoplasm and nucleus of gingival epithelial cells with periodontitis. Western blotting revealed a significant increase in high-mobility-group box 1 expression in the gingival crevicular fluid from periodontitis patients. High-mobility-group box 1 production in gingival epithelial cells was increased following stimulation with tumor necrosis factor-α. The molecular dialogue between tumor necrosis factor-α and gingival epithelial cells involved modulation of the activities of p38MAPK, Jun N-terminal kinase and p44/42. Interestingly, only phosphorylation of p38MAPK contributed to more than half of the signaling initiated by tumor necrosis factor-α-elicited high-mobility-group box 1 release.

Conclusion: High-mobility-group box 1 is continuously released from the gingival epithelial cells modulated by tumor necrosis factor-α. These findings imply that high-mobility-group box 1 expression and possibly p38MAPK constitute important features in periodontitis.

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Key words: gingival epithelial cell; high mobility-group box 1; periodontitis; tumor necrosis factor-α

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High-mobility-group box 1, a primarily nuclear protein, is present in many eukaryotic cells (1) and has a highly conserved sequence among species. It consists of two tandem domains, designated high-mobility-group boxes A and B, each of which is ≈75 amino acids in length, and a highly acidic carboxyl terminus of 30 amino acids in length. High-mobility-group box 1 appears to have distinct functions in cellular systems. It acts as an

intracellular regulator of transcription and plays a crucial role in the maintenance of DNA functions (2). Extracellular high-mobility-group box 1 released from various cells (i.e. macrophages/monocytes, endothelial cells and pituicytes) or necrotic cells (3–6) and stimulated by lipopolysaccharide or tumor necrosis factor-α acts as a pro-inflammatory cytokine through the multiligand receptor for advanced glycation end-products (7,8) and toll-like receptors 2 and 4 (9). Extracellular high-mobility-group box 1 has been found to play critical roles in the progression of chronic inflammatory diseases, such as septic shock, rheumatoid arthritis and atherosclerotic lesions (3,8,10,11).

Periodontitis is a chronic inflammatory disease in which the production of numerous pro-inflammatory cytokines (i.e. interleukin-1β, interferon-γ and tumor necrosis factor-α) is amplified by several bacteria-derived virulence factors (12), thereby leading to the destruction of soft tissues and bone (13). Among the pro-inflammatory cytokines, tumor necrosis factor-α plays important roles in various inflammatory conditions (14) and has recently gained attention in periodontal diseases as a result of its effects on bone and soft tissue metabolism (15). For example, tumor necrosis factor-α has been reported to have a strong potential for increasing bone resorption (16,17) and to be involved in the degradation of connective tissues (18).

The receptor for advanced glycation end-product expression has been detected in human gingival tissues from subjects with chronic periodontitis, with or without type 2 diabetes (19), and can be induced by advanced glycation end-products and tumor necrosis factor-α (20). Therefore, the pro-inflammatory effects of extracellular high-mobility-group box 1 acting through the receptor for advanced glycation end-products may be involved in the pathogenesis of periodontitis. However, the involvement of extracellular high-mobility-group box 1 in periodontitis remains unknown. In our preliminary study, high-mobility-group box 1 expression was detected in gingival tissues with chronic periodontitis. Interestingly, its expression was also observed in gingival epithelium with periodontitis.

The purpose of the present study was to determine whether gingival epithelial cells express high-mobility-group box 1 with or without tumor necrosis factor-α and release high-mobility-group box 1 into the local microenvironment. Furthermore, the mechanism for high-mobility-group box 1 expression in response to tumor necrosis factor-α stimulation, including the activation of mitogen-activated protein kinase (MAPK) signaling pathways, was also investigated.

Material and methods

Chemicals

Human recombinant tumor necrosis factor-α was purchased from PeproTech EC (London, UK). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA, USA). U0126 was purchased from Promega (Madison, WI, USA). All other reagents were supplied by Sigma-Aldrich Inc. (St Louis, MO, USA).

Antibodies

A high-mobility-group box 1 antibody was obtained from Shino-Test (Tokyo, Japan). A tumor necrosis factor receptor 1 and 2 neutralizing antibody was purchased from R & D Systems Inc. (Minneapolis, MN, USA). A CD68 antibody was purchased from DakoCytomation (Glostrup, Denmark). MAPK assay kits (containing polyclonal antibodies against p38, Jun N-terminal kinase/stress-activated protein kinase and p44/42, phospho-p38, phospho-Jun N-terminal kinase/stress-activated protein kinase and phospho p44/42) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Gingival crevicular fluid sampling

Gingival crevicular fluid samples were obtained from three patients with chronic periodontitis (two men, one woman; average age 63.7 years; probing depth 7–10 mm; alveolar bone loss 24–30%) and from three healthy controls after informed consent was obtained according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences. Gingival crevicular fluid was collected using periopaper (Proflow Inc., Amityville, NY, USA), as previously described (21). Briefly, periopaper was placed into the periodontal pocket for 30 s and then transferred to 50 μL of sodium dodecyl sulfate sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol). Following an interval of 60 s, the procedure was repeated twice. The high-mobility-group box 1 protein levels in the gingival crevicular fluid samples were analyzed by western blotting.

Preparation of gingival tissues

Both healthy ($n = 5$) and chronic periodontitis ($n = 10$; six men/four women; average age 59.2 years; probing depth 7–10 mm; alveolar bone loss 24–30%) tissues were obtained from patients after informed consent was obtained according to guidelines approved by the Ethical Committee at Kagoshima University Graduate School of Medical and Dental Sciences. Diseased sites that exhibited severe periodontal attachment loss were selected at random. The excised tissues were immediately fixed in 4% paraformaldehyde and then embedded in paraffin.

Immunohistochemistry

Paraffin-embedded sections (5 μm) of gingival tissues were deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. After blocking endogenous peroxidase activity by 3% H_2O_2 for 15 min, the sections were processed for immunostaining using a Histofine simple stain kit (Nichirei Bioscience Inc., Tokyo, Japan). Sections were incubated in each primary antibody (anti-high-mobility-group box 1 rabbit immunoglobulin, 2 μg/mL; and anti-CD68 mouse immunoglobulin, 1 μg/mL) overnight at 4°C. After washing, the sections were incubated in Histo-