

Anti-tumor effects of cimetidine on hepatocellular carcinomas in diethylnitrosamine-treated rats

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Abstract. Cimetidine is known to have an anti-tumor effect on certain types of malignancies, though on hepatocellular carcinomas (HCCs), its effect remains unclear. We studied the anti-tumor effects of cimetidine on chemically-induced HCCs in rats. Four-week-old male Wistar rats (n=105) were divided into 4 groups. Those in groups A and B were administered diethylnitrosamine (DEN) intraperitoneally at 100 mg/kg body weight every week for 6 weeks, during which rats in group A were given tap water and those in group B received cimetidine (100 mg/kg/day) in their drinking water. Rats in groups C and D were administered saline instead of DEN and given tap water with 100 mg/kg/day of cimetidine, respectively. The animals were sacrificed at 7, 12, 22 and 32 weeks after the first administration of drugs and examined. Liver nodules were observed only in groups A and B, with the number of nodules, maximum diameter of the largest nodule, and liver weight significantly lower in group B. Immunohistochemistry findings showed that glutathione S-transferase placental-positive preneoplastic foci were significantly decreased in group B. Cimetidine treatment decreased the number of proliferating cell nuclear antigen-positive hepatocytes and tended to enhance natural killer (NK) cell activity in splenic lymphocytes. In addition, flow cytometry revealed that the proportion of NK cells among total splenic lymphocytes was not affected by cimetidine treatment. Our results showed that cimetidine has an inhibiting effect on hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is currently the fifth most commonly occurring type of cancer and the third leading cause of death from cancer worldwide (1). Most patients with

HCC also suffer from liver cirrhosis, which is the major risk factor for HCC, mainly caused by infection with either the hepatitis B virus (HBV) or the hepatitis C virus (HCV) (2). Ursodeoxycholic acid (3) and glycyrrhizin (4) have been reported to be effective in preventing liver carcinogenesis, while recently the preventive effects of vitamin K2 (5) and acyclic retinoid (6) towards recurrence in patients with HCC have been reported. Nevertheless, HCCs frequently occur in patients with HCV cirrhosis at an annual rate of ~8% in Japan, with recurrence within 5 years seen in 70% of cases (7). Interferon therapy prevents the incidence of HCC in patients with HCV-related cirrhosis (8), however, it is somewhat difficult to use in cases of uncompensated cirrhosis or serious complications, because of its many adverse effects. It is important to develop safe and more effective chemopreventive drugs for treatment of hepatocarcinogenesis.

Cimetidine was first developed in 1974 as a histamine H₂ receptor antagonist and its anti-tumor effect was initially reported in 1979 by Armitage and Sidner, who reported 2 cases of metastatic cancer with complete remission following administrations of cimetidine (9). In 1988, it was reported that post-operative treatment with cimetidine improved survival in a gastric cancer patient (10). Since that time, several studies have reported survival advantages with cimetidine treatment, mainly in patients with gastrointestinal cancer (11-14). Further, positive effects have also been demonstrated in non-gastrointestinal cancer patients, such as those with renal cell carcinoma (15), malignant melanoma (16) and glioblastoma (17).

In contrast, there has been no clinical study of the anti-tumor effects of cimetidine in patients with HCC, though Fujikawa *et al* recently reported results of an *in vitro* study. Their findings indicated that cimetidine inhibited cell proliferation and migration induced by an epidermal growth factor (EGF) in HCC cell lines (18). In the present study, we studied the anti-tumor effects of cimetidine on chemically induced HCCs in rats.

Materials and methods

Animals. A total of 105 four-week-old male Wistar rats were obtained from Charles River Japan (Yokohama, Japan) and maintained at a controlled room temperature with a 12-h light/dark illumination cycle. All rats were housed in wire

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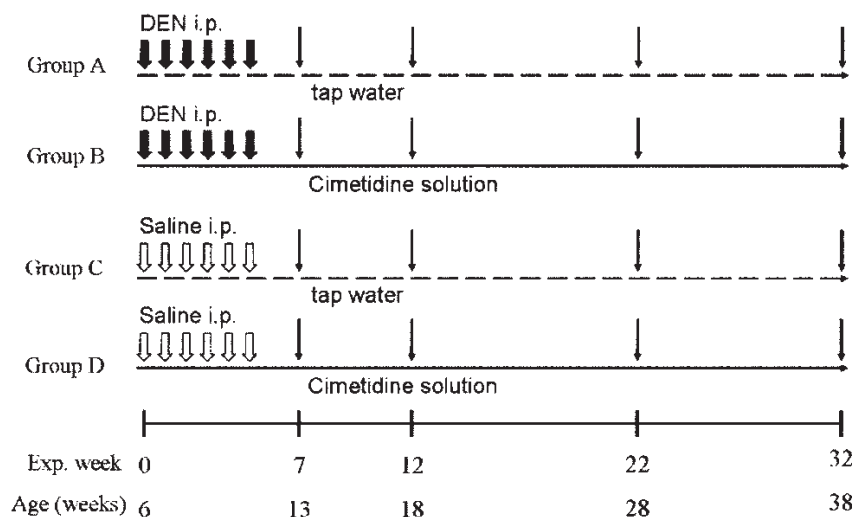


Figure 1. Experimental design. DEN (100 mg/kg body weight) was administered to rats in groups A (n=25) and B (n=27), while saline was administered to rats in groups C (n=27) and D (n=26) intraperitoneally once a week for 6 weeks. In addition, rats in groups B and D received cimetidine (100 mg/kg/day) in their drinking water, while those in groups A and C received tap water alone for drinking. DEN and cimetidine treatment were started at the same time. The rats were sacrificed (↓) at 7, 12, 22 and 32 weeks after the first administration of drugs.

cages (3-5 rats/cage), with MF (Oriental Yeast, Tokyo, Japan) given as a basal diet. The animal experimental protocol was approved by the Institute for Animal Experimentation of Shimane University and conducted in accordance with its guidelines.

Experimental schedules. The experimental design is shown in Fig. 1, which was a modification of a protocol for diethylnitrosamine (DEN)-induced chemical hepatocarcinogenesis, as described previously (19). On arrival, the rats were quarantined and, after a 2-week adaptation period, divided into 4 groups for the experiment. Rats in group A (n=25) were allowed free access to tap water, while those in group B (n=27) received cimetidine (Dainippon Sumitomo Pharma, Osaka, Japan) (100 mg/kg/day) in their drinking water during the experimental period. In order to provide 100 mg/kg/day of cimetidine to the group B rats, drinking water volume and body weight were monitored twice a week from the beginning of the adaptation periods to the end of the experiment, with the appropriate concentration of cimetidine in drinking water calculated each time. In both groups, DEN (Nakarai Tesque, Kyoto, Japan) at 100 mg/kg of body weight was administered intraperitoneally once a week for 6 weeks from the start of the experiment. Rats in group C (n=27) were given tap water and those in group D (n=26) were given cimetidine in their drinking water, with saline administered intraperitoneally once a week for 6 weeks to both groups. In rats that received them, DEN and cimetidine treatments were started at the same time. The rats were sacrificed under ether anesthesia at 7, 12, 22, and 32 weeks after beginning the experiment. A blood sample was taken from the right atrium and the livers were removed, weighed and fixed in 10% buffered formalin. Fixed livers were sliced into 3-mm sections and the numbers and sizes of liver nodules were determined, with nodules >3 mm in diameter and counted for later analysis. The liver slices were embedded in paraffin blocks, stained with hematoxylin and eosin, and used for immunohistochemical studies. Doses of

100 mg/kg/day of cimetidine have been reported to produce serum levels of 0.16-0.35 $\mu\text{g/ml}$ and inhibit the growth of tumor xenografts in experimental mice (20). This serum level range is comparable with the level in a human receiving 300 mg orally (21).

Laboratory tests. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured by a consensus method recommended by the Japanese Society of Clinical Chemistry (JSCC). The assay was conducted by SRL Inc. (Tokyo, Japan).

Immunohistochemistry. Liver tissues from a portion of the left lobe (22) were used for immunohistochemical studies. Fixed sections were deparaffinized with xylene and incubated with 3% H_2O_2 . Then, the sections were incubated with rabbit anti-glutathione S-transferase placenta (GST-P) polyclonal antibody (MBL, Nagoya, Japan) diluted at 1:1000 for 30 min, followed by incubation with the secondary antibody using a Dako ChemMate EnVision kit/HRP (Dako Japan, Kyoto, Japan). The bound antibody was detected by incubating the slides with AEC (Dako Cytomation, Kyoto, Japan). Then, double-staining for proliferating cell nuclear antigen (PCNA) and GST-P was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The sections were subjected to heat-induced antigen retrieval in distilled water at 121°C for 30 sec. After incubation with normal horse serum for 30 min, the sections were incubated with mouse anti-PCNA monoclonal antibody (Dako Cytomation) diluted at 1:500 for 30 min. They were then incubated with secondary biotinylated anti-mouse immunoglobulin G for 20 min, followed by incubation with alkaline phosphatase-labeled avidin-biotin complex solution for 20 min. The bound antibodies were detected by incubating the slides with BCIP/NBT (Dako Cytomation), then the sections were counter-stained with hematoxylin. The total amount of pre-

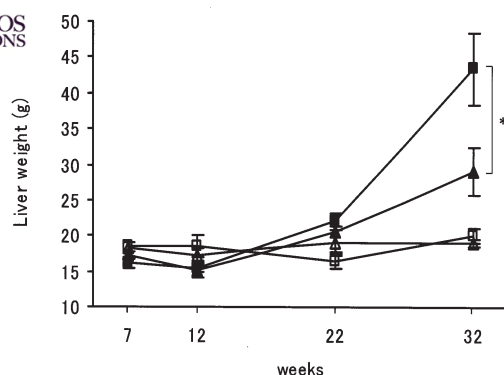


Figure 2. Changes in liver weights. The liver weight in group B was significantly lower than in group A at week 32. ■, group A; ▲, group B; □, group C; △, group D; * $p < 0.05$.

neoplastic foci was expressed as the area of GST-P-positive cells in the histological sections, calculated with image analyzing software (NIH image; Wayne Rasband, National Institutes of Health, USA). The number of PCNA-positive cells in each section was evaluated by determining the percentage of positive nuclei in at least 1,000 cells.

Preparation of splenic lymphocytes. Splenic lymphocytes were isolated using a density gradient centrifugation method. The spleens were removed and excised, then finely minced in Hanks' Balanced Salt Solution (HBSS) (Gibco-Invitrogen, Tokyo, Japan). Then, the suspension was passed through a 40- μ m cell strainer (Becton Dickinson, Tokyo, Japan) and the cell concentration adjusted to $\sim 2 \times 10^7$ cells per ml. Five milliliters of the cell suspension was carefully layered over the top of 5 ml of Lympholate-Rat (Cedarlane Labs, Hornby, Ontario, Canada) in 15-ml centrifuge tubes. The tubes were centrifuged for 20 min at 1500 \times g at room temperature, and the white cell layer was carefully removed using a Pasteur pipette and transferred to a new tube. These cells were washed with phosphate-buffered saline (PBS) (Nissui, Tokyo, Japan) and cultured in a complete medium consisting of RPMI-1640 (Gibco-Invitrogen), 10% heat-inactivated fetal bovine serum (ICN Biomedicals Inc., OH, USA), and a 1% antibiotic/antimycotic solution (Gibco-Invitrogen).

Cytotoxicity test. Cytotoxicity tests were performed using splenic lymphocytes collected on week 28 ($n=4$ rats in each group) with a ^{51}Cr releasing assay. YAC-1 cells, kindly provided by the Cell Resource Center for Biochemical Research, Institute of Development, Aging and Cancer, Tohoku University, were used as target cells. YAC-1 cells (2×10^6) were labeled with 3.7 MBq of $\text{Na}_2^{51}\text{CrO}_5$ (MP Biomedicals, Irvine, CA) at 37°C for 1 h in a shaking water bath. Labeled target cells were washed with PBS and adjusted to $1 \times 10^5/\text{ml}$ in a complete medium, then seeded at a concentration of 1×10^4 cells/well in micro-well plates. The effector cells (lymphocytes) were added to the wells at effector to target (E:T) ratios of 100:1, 50:1, and 25:1 in a total volume of 200 μl . Spontaneous release was determined from wells containing labeled target cells and 100 μl of complete medium. Maximum release was obtained by treating the target cells

with 100 μl of 1 N HCl. All assays were performed in triplicate. After incubation at 37°C for 4 h, the plate was centrifuged. Subsequently, 100 μl of supernatant was aspirated from each well and ^{51}Cr release was determined in a γ counter (Aloka ARC-2000, Tokyo, Japan). The results are expressed as percent lysis according to the following formula: percent lysis = [(sample release - complement control release)/(maximum release - complement control release)] $\times 100\%$.

Flow cytometry. The proportion of natural killer (NK) cells among splenic lymphocytes was examined by flow cytometry ($n=4$ rats in each group). Isolated splenic lymphocytes were stained with the monoclonal antibodies fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD161 (Immunotech, Marseilles, France) and phycoerythrin (PE)-conjugated mouse anti-rat CD45 (Immunotech), diluted appropriately and according to the manufacturer's instructions. Cells were gated as determined by forward- and side-scatter criteria to avoid potential contamination by dead cells or debris. Analysis of stained cells was performed using a Beckman Coulter Epics XL, with data analyzed using Expo32 software.

Statistical analysis. All data are represented as the mean \pm SE. Statistical comparisons between two groups were conducted with a Mann-Whitney U-test. Multiple comparisons were conducted with one-way factorial ANOVA, followed by a *post-hoc* multiple comparison analysis. $P < 0.05$ was considered statistically significant.

Results

Liver weight. Liver weights were gradually increased in the DEN-treated groups (A and B), though those in group B were significantly lighter at week 32 (43.5 ± 5.0 g in group A, 29.1 ± 3.4 g in group B, $p < 0.05$; Fig. 2). The average ratio of the liver weight to total body weight showed a similar tendency as the liver weights (data not shown).

Laboratory tests. Serum ALT levels in the DEN-treated groups (A and B) were higher than those in the non-treated groups (B and C) throughout the experiment. However, serum ALT levels in group B were significantly lower than those in group A at week 32 (81.3 ± 4.0 IU/l in group A, 66.3 ± 4.3 IU/l in group B, $p < 0.05$). A similar tendency was also found for serum AST, LDH, and ALP, though there were no statistically significant differences between groups A and B (data not shown).

Macroscopic findings for liver nodules. Liver nodules were observed in the DEN-treated groups (A and B) at week 12, 22, and 32 (Fig. 3A and B). Histological diagnoses of the liver nodules were made according to criteria reported by Squire and Levitt (23), which showed the present nodules to be neoplastic or hepatocellular carcinoma. No liver nodules were observed in groups C and D. The number of liver nodules in group B was less as compared to group A, with a significant difference at week 22 (26.8 ± 7.1 nodules/liver and 5.4 ± 2.2 nodules/liver, respectively, $p < 0.05$; Fig. 3C). Further, the maximum diameter of the largest nodule in

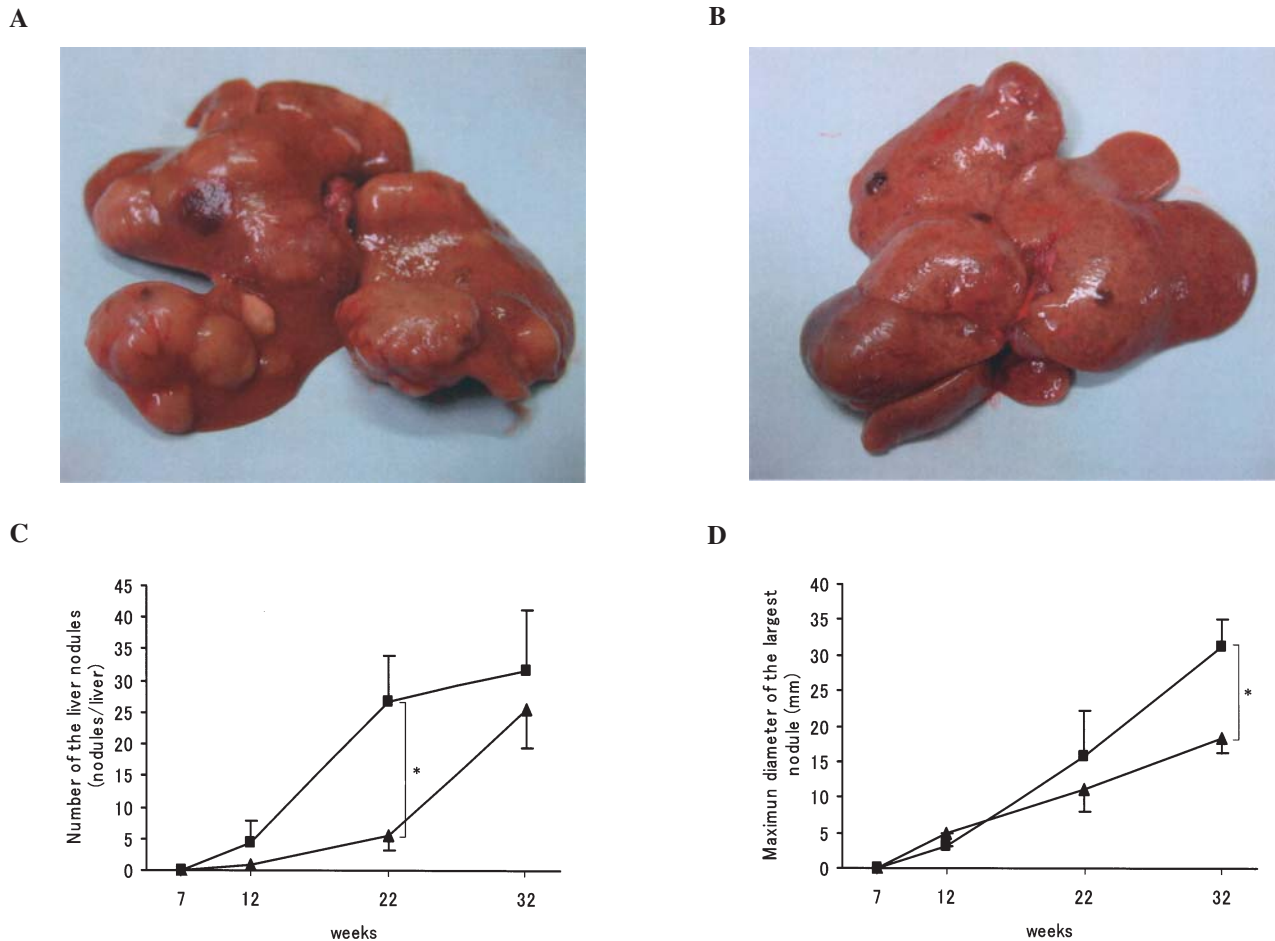


Figure 3. Macroscopic appearance. Liver nodules at week 32 in groups A (A) and B (B). (C) The number of liver nodules in group B was lower. (D) The maximum diameter of the largest nodule in the liver was also reduced in group B. ■, group A; ▲, group B; * $p < 0.05$.

group B was also smaller than that in group A, with a significant difference found at week 32 (31.3 ± 3.9 mm and 18.4 ± 2.1 mm, respectively, $p < 0.05$; Fig. 3D).

Immunohistochemical analysis. GST-P-positive hepatocytes were observed from week 7 in groups A and B, and the areas with GST-P-positive hepatocytes gradually expanded over time (Fig. 4A and B). In contrast, GST-P-positive hepatocytes were few in groups C and D, and only seen at week 32 ($0.03 \pm 0.02\%$ and $0.05 \pm 0.04\%$, respectively). The GST-P-positive area was significantly smaller in group B as compared with group A at week 22 ($14.1 \pm 3.4\%$ and $27.2 \pm 3.9\%$, respectively, $p < 0.05$) and week 32 ($15.1 \pm 3.3\%$ and $30.8 \pm 3.9\%$, respectively, $p < 0.05$) (Fig. 4E). Further, PCNA-positive hepatocytes were observed in both GST-P-positive and negative areas (Fig. 4C and D), though the percentage of PCNA-positive hepatocytes was higher in the GST-P-positive areas. In addition, the percentage of PCNA-positive hepatocytes in both GST-P-positive and negative areas in each period tended to be lower in group B than in group A, though the differences were not statistically significant (Fig. 4F).

Cytotoxicity tests. Cytotoxicity tests were performed using spleen cells with a ^{51}Cr releasing assay at week 28. The percent lysis in group A was the lowest, while that in group B was the

second highest among the 4 groups. In rats not treated with DEN, the percent lysis in group D was higher as compared to group C, though the difference was not significant (Fig. 5).

Flow cytometric study. The proportion of NK cells among total splenic lymphocytes was analyzed by flow cytometry, with representative results from a group A rat shown in Fig. 6A. The percentage of CD161-positive cells among CD45-positive cells was not significantly different among the 4 groups ($23.32 \pm 3.79\%$ in group A, $23.32 \pm 2.94\%$ in group B, $21.14 \pm 2.25\%$ in group C, $21.12 \pm 2.40\%$ in group D; Fig. 6B).

Discussion

Cimetidine is widely used to treat peptic ulcers and has also been shown to have clinical benefits in cancer patients. Previous studies have identified anti-tumor effects and a number of mechanisms of activity, such as direct inhibition of tumor growth, stimulation of host immune response and inhibition of cancer cell adhesion to endothelial cells.

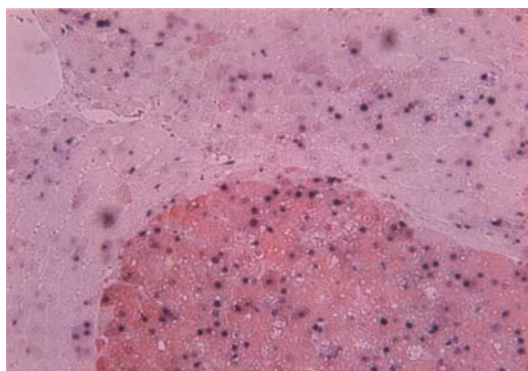
Cimetidine may have a direct inhibiting effect on tumor cell growth by blocking the cell growth activity of histamine, which is known to be a growth factor, with high concentrations in some types of cancer (24,25). The presence of a large number of mast cells, which produce histamine, in colorectal



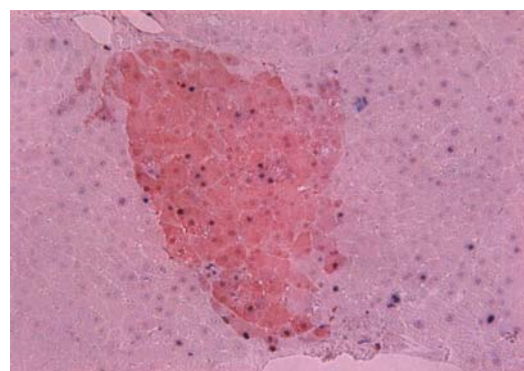
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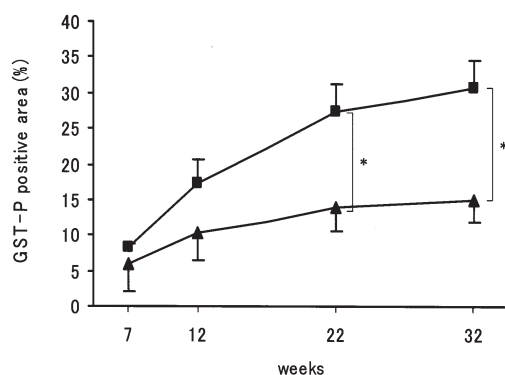
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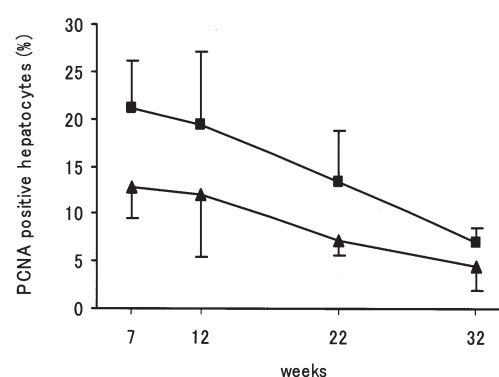
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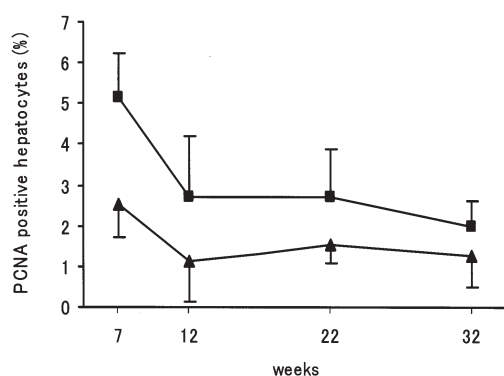


Figure 4. Immunohistochemical findings. GST-P staining at week 22 in groups A (A) and B (B) (magnification x40). Double staining with PCNA and GST-P at week 12 in groups A (C) and B (D) (magnification x100). (E) Proportions of GST-P-positive areas in microscopic fields of liver tissues. The areas of GST-P-positive hepatocytes gradually expanded over time in groups A and B, though those in group B grew at a slower rate. ■, group A; ▲, group B; * $p < 0.05$. (F) Rates of PCNA-positive hepatocytes in GST-P-positive areas. (G) Rates of PCNA-positive hepatocytes in GST-P-negative areas. PCNA-positive hepatocytes in both GST-P-positive and negative areas tended to be lower in group B than in group A. ■, group A; ▲, group B.

cancer tissue was shown to be associated with a worsened prognosis in patients (26). Adams *et al* reported that histamine stimulated the proliferation of human colorectal tumor cell lines, which was inhibited by cimetidine treatment, though cimetidine did not affect the basal proliferating ability of

these cell lines in the absence of histamine (27). Previously, we used a ^3H -methyl-thymidine incorporation assay with human HCC cell lines (HepG2 and KYN-2). Our results showed that a high concentration of histamine (10^{-6} to 10^{-4} M) stimulated the proliferation of HCC cell lines, while

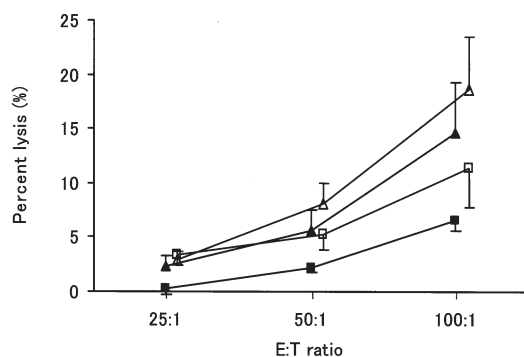


Figure 5. Cytotoxicity assay of splenic lymphocytes performed at different E:T ratios using a ^{51}Cr releasing assay. YAC-1 cells were used as the target cells and 4 rats from each group were evaluated. The percentage of lysed cells tended to be enhanced by cimetidine administration in both groups of DEN-treated rats (A,B), with that in group B greater than in group A, as well as in rats not treated with DEN (C,D), with that in group D greater than in group (C). The assays were performed in triplicate. ■, group A; ▲, group B; □, group C; △, group D.

cimetidine tended to inhibit cell growth stimulated by histamine, but did not have an effect on the basal proliferating ability of the cell lines (data not shown). In a study of L-histidine decarboxylase (HDC)-deficient mice, which lack the capability to synthesise endogenous histamine, Takahashi *et al* reported that cimetidine did not suppress the growth of colon cancer xenografts, whereas suppression was observed in wild-type mice (28).

Together, these findings suggest that the anti-tumor effects of cimetidine require a stimulating effect of histamine toward tumor cell growth, which is mediated via histamine H_2 receptors on tumor cells. However, other H_2 receptor antagonists such as ranitidine and famotidine did not exert anti-tumor effects in previous *in vitro* and *in vivo* studies, despite their stronger acid-inhibiting activity as compared to cimetidine (29-31). In a cell proliferating assay previously performed, famotidine did not have an effect on either histamine-stimulated or basal proliferation of HCC cell lines (data not shown), which shows that the mechanisms of the

inhibitory effects of cimetidine on tumor cell proliferation are not limited to blocking the H_2 receptor. Recently, Fujikawa *et al* reported that cimetidine inhibited cell proliferation by blocking the epidermal growth factor receptor (EGFR) pathway in HCC cell lines (18).

Cimetidine has demonstrated immunomodulatory effects in a number of studies (32-36), while histamine has been shown to have immunosuppressive effects (37,38). These results suggest that the effect of cimetidine on immunity is mediated by blocking the immunosuppressive effect of histamine via the H_2 receptor. However, other H_2 receptor antagonists possess no immunomodulatory effects, and no inhibitory effect on tumor cell proliferation as noted above. Hahm *et al* (39) reported comparative data regarding the immunomodulatory effects of various H_2 receptor antagonists, including cimetidine, ranitidine, and famotidine, on peripheral blood mononuclear cells in patients with gastric cancer. Their findings showed that the strongest immuno-modulatory substance among these H_2 receptor antagonists was cimetidine, while famotidine had the lowest effect. They speculated that the results were due to structural differences, i.e., ranitidine and famotidine lack imidazole nuclei, which are common to cimetidine. Their findings also suggested that H_2 receptors, which are present on immune cells, are structurally different from those present on gastric parietal cells (40).

NK cell activity has been measured and proposed as a possible mechanism of the immunomodulatory effects of cimetidine (41,42). Further, it was reported that low NK cell activity in patients with liver cirrhosis increased the risk of hepatocarcinogenesis (43), and caused early development of and invasion by HCCs (44), while preoperative NK cell activity was found to have an influence on recurrence and prognosis after hepatectomy procedures in patients with HCC (45). Lee *et al* studied changes of NK cell activity in DEN-induced hepatocarcinogenesis model rats and demonstrated that the activity was significantly decreased in comparison with untreated control rats at 30 weeks after the first administration, while there was no difference before that time point (46). Since DEN-induced immunosuppression is considered to occur at a later stage, we evaluated the NK cell

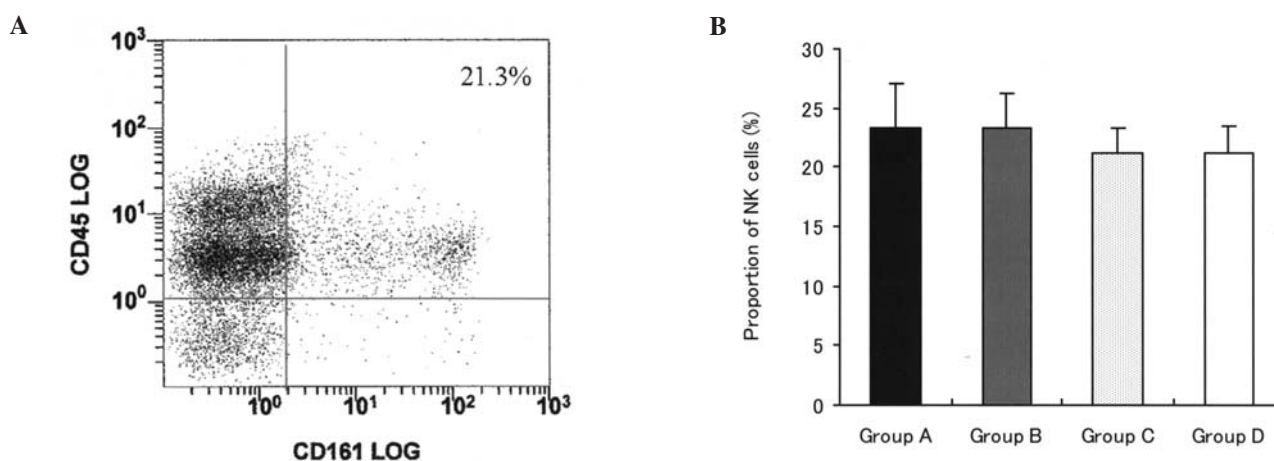


Figure 6. Proportion of NK cells in splenic lymphocytes (n=4 rats from each group). (A) Flow cytometric analysis of double staining for CD161 (x-axis) and CD45 (y-axis) cells in group A. Double-positive cells are represented in the upper-right quadrangle as the population of NK cells. (B) There were no significant differences among the 4 groups.



of rats at week 28 and the results suggested that cimetidine increases NK cell activity that was previously reduced by DEN treatment. In addition, an increase in NK cell activity was also observed in rats not treated with DEN, though the difference was not statistically significant. We determined NK cell activity with a cytotoxicity test using total splenic lymphocytes, which consist of many kinds of lymphocytes and NK cells. Consequently, we also evaluated the proportion of NK cells among total lymphocytes using flow cytometry. CD161-positive cells (NK cells) accounted for ~20% of the CD45-positive splenic lymphocytes, which remained similar regardless of treatment with cimetidine and DEN. Therefore, cimetidine may not increase the proportion of NK cells, but rather tends to enhance their activity.

Kobayashi *et al* demonstrated that cimetidine blocks the adhesion of colorectal tumor cells to endothelial cells through the down-regulation of E-selectin cell surface expression (47). In a clinical study, their group also showed cimetidine treatment dramatically improved survival in patients with colorectal cancer expressing high levels of sialyl Lewis antigen, a ligand for E-selectin (13). Although this mechanism may play a crucial role in tumor cell metastasis, it may not be important in the present hepatocarcinogenesis model, as no apparent distant metastasis was found throughout the experimental period.

Glutathione S-transferase (GST) is an enzyme family with multi-molecular forms and multiple functions for the detoxification of drugs (48). Rat GST-P, which belongs to class *pi* in the species-independent classification of GST, has been shown to be a marker enzyme for rat preneoplastic lesions (48). Rat chemical hepatocarcinogenesis comprises 3 stages; initiation, promotion and progression (49), and GST-P is strongly expressed in rat preneoplastic lesions such as enzyme-altered foci, which emerge in the promotion stage. Preneoplastic enzyme-altered foci have been proposed to play a key role in the process of hepatocarcinogenesis (48). In the present study, cimetidine reduced the percentage of GST-P-positive areas in DEN-treated rat livers. Simultaneously, PCNA-positive cells in both GST-P-positive and -negative areas tended to be decreased by cimetidine treatment, though statistical significance was not reached. A reduction in the number of GST-P-positive preneoplastic hepatocytes by cimetidine treatment may be closely related to a decrease in the number of PCNA-positive hepatocytes. Indeed, cimetidine was reported to inhibit PCNA-positive cells in carcinogen-induced colon cancer model rats (21).

We found that administration of cimetidine reduced the number and size of liver nodules, with a significant difference in the number of liver nodules between groups A and B observed at week 22, but not week 32. It was considered that the lack of significant difference between the 2 groups after 32 weeks was caused by fusion of the growing liver nodules. At week 32, the maximum diameter of the largest nodule was significantly larger in group A than in group B, while liver weight, which is thought to reflect tumor volume, was also significantly heavier in group A.

In summary, our results showed that cimetidine has an inhibiting effect on hepatocarcinogenesis and we are the first to show the anti-tumor effects of cimetidine on HCCs *in vivo*. Thus, this classic and safe drug may be suitable for the

prevention and treatment of HCC, though additional studies are needed.

Acknowledgements

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