Anti-tumor effect of cimetidine via inhibiting angiogenesis factors in *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced mouse and rat bladder carcinogenesis

YOSHITOMO CHIHARA[1], KIYOHIDE FUJIMOTO[1], MAKITO MIYAKE[1], YOSHIO HIASA[2] and YOSHIHIKO HIRAO[1]

[1]Department of Urology, Nara Medical University, Kashihara; [2]Pathology Division, Prefectural Nara Hospital, Nara, Japan

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Abstract. The aim of this study was to assess the anti-tumor effect and mechanisms of cimetidine in *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder carcinogenesis model. Sixty-three male BALB/c mice and 67 male Wister rats were treated with BBN and cimetidine to examine the anti-tumor effect of cimetidine. Immunohistochemistry (IHC) of vascular endothelial growth factor (VEGF), platelet-derived endothelial growth factor (PDECGF), and E-selectin were examined to compare their expression in the tumor tissues. In mice, the tumor growth was reduced by cimetidine (p=0.011). The expression of PDECGF was reduced in the cimetidine-treated group (p=0.016). In rats, treatment of cimetidine reduced tumor growth (p=0.0001). Moreover, the expression of VEGF and PDECGF was reduced (p=0.02 and <0.001, respectively). The expression of E-selectin did not correlate with the tumor growth in either mice or rats. In mice, long-term cimetidine treatment proved very effective for inhibiting the tumor growth, but in rats, BBN after treatment with cimetidine showed the least tumor growth-inhibitory effect. In conclusion, cimetidine may have an inhibitory effect on tumor growth in bladder carcinogenesis via reducing the expression of angiogenesis factors including VEGF and PDECGF.

Introduction

Cimetidine, a histamine-2 (H2) receptor antagonist, reportedly improves the survival of patients with colorectal cancer, melanoma and renal cell carcinoma (1-3). It is interesting that the other H2 receptor antagonists, such as ranitidine and famotidine, do not demonstrate such effects (4,5). Studies of the anti-tumor effects of cimetidine indicate multiple potential mechanisms of action, characterized by three overall features. One possible explanation is that cimetidine may show a direct inhibitory effect on tumor growth by blocking the cell growth-promoting activity of histamine-associated angiogenesis including the vascular endothelial growth factor (VEGF) (6,7). The second possibility is a cell-mediated immunomodulation by enhancing the host immune response to the tumor cells (8,9). The third is that cimetidine exerts a tumor-suppressive effect via E-selectin-mediated cell adhesion (10). However, the exact mechanism by which cimetidine exerts an anti-tumor effect is still poorly understood.

Angiogenesis is essential for the growth of bladder cancer, and several studies revealed overexpression of VEGF and platelet-derived endothelial growth factor (PDECGF) in the tumor tissue using animal models and bladder cancer patients, and that overexpression correlated with poor prognosis (11-14). Furthermore, cimetidine showed tumor-suppressor effect via inhibiting this angiogenesis and cell adhesion in several studies using animal cancer models as described above. Herein, we investigated the effect of cimetidine on tumor growth and the expression of several factors which were considered as targets using animal models of bladder cancer induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN).

Materials and methods

Animals and treatment. Sixty-three male BALB/c mice and 67 male Wister rats (SLC Japan, Shizuoka, Japan) at 5 weeks of age were used in this study. Cimetidine, courtesy of Dainippon Sumitomo Pharma Co., Ltd., Japan, was dissolved in autoclaved drinking water at a concentration of 1 mg/ml. This corresponded to a dose of 100 mg/kg/day, which reportedly produced an effective cimetidine level in murine plasma (15,16). Both BBN (Tokyo Kasai Kogyo, Tokyo, Japan) at a concentration of 0.05% and cimetidine were given in drinking tap water. After 1-week acclimation period; i.e., at the age of 6 weeks, the animals were divided into 4 groups as shown in Fig. 1; Group A: no treatment with BBN or cimetidine, Group B: BBN was administered for 9 weeks to rats and for 12 weeks to mice, Group C: after BBN treatment, cimetidine was given continuously until the animals were

Correspondence to: Dr Yoshitomo Chihara, Department of Urology, Nara Medical University, 840 Shijo-cho, Kashihara-City, 631-0846, Nara, Japan
E-mail: yychihara@gmail.com

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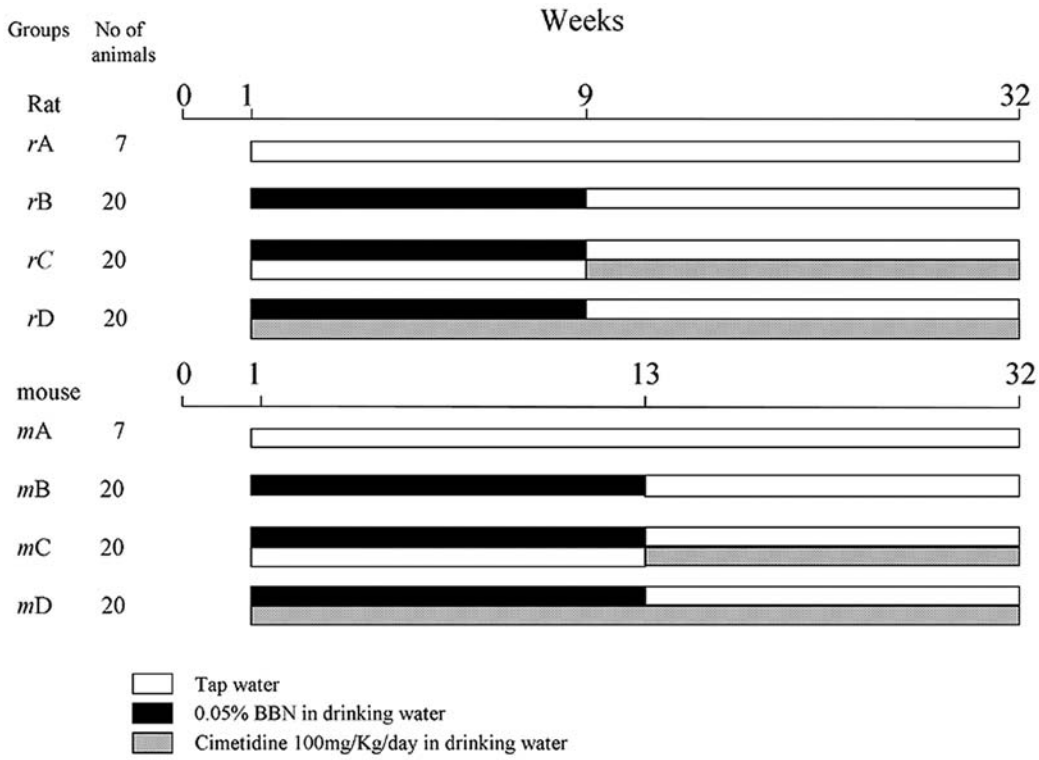

Figure 1. Schematic diagram showing the experimental design. The letters *r* and *m* correspond to rats and mice, respectively.

sacrificed, Group D: cimetidine was given from week 1. All surviving animals were sacrificed by ether anesthesia at the end of week 32. The bladders were then sectioned along the long axis of the maximum tumor, fixed in formalin, processed, and embedded in paraffin. The blocks were sectioned at 4-μm thickness and stained with hematoxylin and eosin (H&E) for microscopic examination. All animal experiments were carried our according to our university ethics guidelines.

Immunohistochemistry. Certain sections were processed for IHC of VEGF, E-selectin, and PDECGF as described previously (17). The primary antibodies were affinity-purified polyclonal rabbit anti-VEGF (clone Z-CVF3; Zymed Laboratories Inc., USA, diluted at 1:200), polyclonal rabbit anti-E-selectin (clone H-300; Santa Cruz Biotechnology Inc., USA, diluted at 1:200), and mouse monoclonal anti-PDECGF (clone PGF.44C; Lab Vision Corp., USA, diluted at 1:200). The intensity of staining was scored as follows: for VEGF; negative: <10% positive tumor cells, moderate: 10-50% positive cells, positive: >50% positive cells (13), for E-selectin; negative: <1% positive cells, moderate: <75% positive cells, positive: >75% positive cells (18), and for PDECGF; negative: no staining, moderate: weak staining, positive: strong staining. The tumors were considered positive for PDECGF when 25% of the neoplastic cells demonstrated moderate staining as described previously (19). All results were scored by one of the authors (H.Y.) without prior knowledge of the respective treatment of the animals (Fig. 2).

Statistical analysis. Statistical analysis was carried out by likelihood Chi-square analysis or Fisher's exact test. Probability values of $p<0.05$ were considered significant.

Results

Primary tumor growth and the effects of cimetidine. In mice, tumors developed in the all groups treated with BBN; i.e., Groups *m*B, *m*C, and *m*D (Table I, top). The incidence of tumors was 65% (13/20) in Group *m*B, 47.1% (8/17) in Group *m*C, and 25% (5/20) in Group *m*D. Furthermore, the incidence of tumor growth decreased according to the terms of cimetidine administration. The statistical difference between Groups *m*B and *m*D was significant (p=0.011). However, only 5 animals (25%) developed tumors in Group *m*D, but all tumors were of stage more advanced than pTa. Regarding the grade, no significant difference was found, and no dysplastic lesions were observed in either group.

In rats, all animals of Group *r*B developed tumors. Nine animals (45%) in Group *r*C and 11 cases (55%) in Group *r*D developed tumor growth (Table I, bottom). The decrease of tumor growth in Group *r*B was was significantly different from that in Group *r*C (p=0.00013) and Group *r*D (p=0.00085). Regarding the stage, the cimetidine-treated groups (Groups *r*C and *r*D) showed significant inhibition of tumor invasion as compared with the non-cimetidine-treated group (Group *r*B) (p=0.0019). Furthermore, concerning the grade, Group *r*C developed lower grade tumors as compared with Group *r*B (p=0.0035). However, there was no significant difference between Groups *r*B and *r*D.

The expression of VEGF, E-selectin, and PDECGF in tumors. The expression of VEGF, E-selectin, and PDECGF in the BBN-induced tumor tissue were examined by IHC. In mice, VEGF and E-selectin were expressed in the normal urothelium and background neighboring healthy tissue, but the expression of PDECGF was not detected in the healthy tissue (Table II,

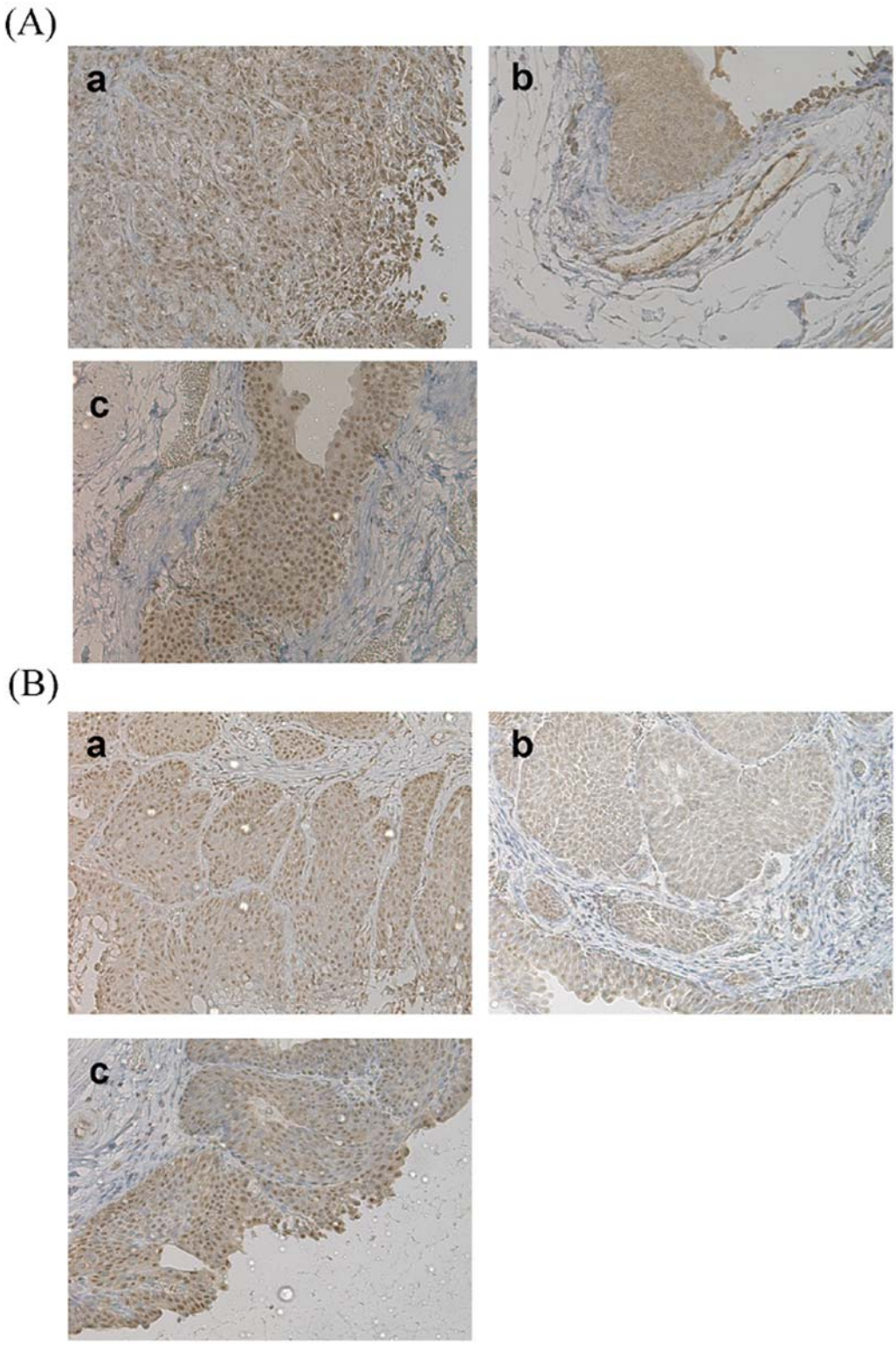

Figure 2. Immunohistochemical positive staining in tumors of VEGF (a), E-selectin (b), and PDECGF (c) in mice (A) and rats (B). The magnifications in all images are x100.

top). Cimetidine treatment did not affect the expression of VEGF and E-selectin. On the other hand, long-term (Group *m*D) cimetidine treatment significantly decreased the expression of PDECGF in the tumor tissue (p=0.016). In the rat BBN model, the healthy urothelium and adjacent healthy tissue did not express VEGF or PDECGF (Table II, bottom). The expression of VEGF decreased significantly by cimetidine treatment after BBN (i.e., Group *r*C) (p=0.021). The expression of PDECGF was detected in all tumor tissues of BBN alone-treated mice (Group *r*B). Moreover, the cimetidine-treated group showed significantly decreased expression of PDECGF in the tumors. These results were somewhat different from those in the mouse group, because the reduction of PDECGF expression was more remarkable in Group *r*C than

Table I. Incidence of tumors cancer, stage and grade in the bladder of mice (top) and rats (bottom).

	Treatment	No. mice	No. mice with carcinoma	Dysplasia	Stage			Grade		
					pTa	pT1	pT1<	G1	G2	G3
Group *m*A	None	3	0 (0%)	0	0	0	0	0	0	0
Group *m*B	BBN	20	13 (65%)	0	6	1	6	0	6	7
Group *m*C	BBN→CIM	17	8 (47%)	0	5	2	1	1	6	1
Group *m*D	BBN+CIM	20	5 (25%)	0	0	4	1	0	4	1
			*m*B vs. *m*C: NS		*m*B vs. *m*C: NS			*m*B vs. *m*C: NS		
			*m*B vs. *m*D: a		*m*B vs. *m*D: b			*m*B vs. *m*D: NS		
			*m*C vs. *m*D: NS		*m*C vs. *m*D: NS			*m*C vs. *m*D: NS		

	Treatment	No. rats	No. rats with carcinoma	Dysplasia	Stage			Grade		
					pTa	pT1	pT1<	G1	G2	G3
Group *r*A	None	6	0 (0%)	0	0	0	0	0	0	0
Group *r*B	BBN	19	19 (100%)	0	5	14	0	10	9	0
Group *r*C	BBN→CIM	20	9 (45%)	8	8	1	0	1	8	0
Group *r*D	BBN+CIM	20	11 (55%)	7	2	9	0	5	6	0
			*r*B vs. *r*C: c		*r*B vs. *r*C: b			*r*B vs. *r*C: a		
			*r*B vs. *r*D: c		*r*B vs. *r*D: NS			*r*B vs. *r*D: NS		
			*r*C vs. *r*D: NS		*r*C vs. *r*D: b			*r*C vs. *r*D: NS		

[a]p<0.05, [b]p<0.01, [c]p<0.001. NS, no significance; CIM, cimetidine.

in the long-term cimetidine-treated group (i.e., Group *r*D) (p=0.0047). Furthermore, the reduction of PDECGF expression was significantly different between Groups *r*C and *r*D (p=0.017).

Discussion

Cimetidine proved to be a potent growth inhibitor of tumor volume and weight in mice inoculated with colon adenocarcinoma cells, and these studies showed that cimetidine inhibited the angiogenic factors in the tumor tissue including VEGF (6,7). Another study showed that cimetidine might block cancer metastasis through blocking adhesion of tumor cells to the endothelium via interaction between E-selectin and sialyl-Lewis antigens (20). Several studies indicated that the expression levels of VEGF and PDECGF influenced the progression of transitional cell carcinoma (TCC) in the animal models (11-14), as well as the prognosis of the TCC patients (11). In this study, we examined the effect of cimetidine on tumor growth using BBN-induced carcinogenesis in the mouse and rat models.

In both mice and rats, the cimetidine treatment significantly reduced the development of tumors. These results were somewhat different in each group. In mice, the development of TCC was reduced in the group treated with long-term cimetidine (p=0.011). On the other hand, in rats, the group treated with cimetidine after BBN showed less frequency of tumor growth (p<0.00013).

PDECGF is the enzyme involved in the salvage pathway of pyrimidine nucleotide syntheses (21), and many studies revealed overexpression of PDECGF, among many angiogenic factors, in the bladder cancer, suggesting that stage and grade progression correlated with PDECGF expression (11,22,23). In our IHC, the expression of PDECGF was not detected in the healthy urothelium and adjacent healthy tissue. The expression of PDECGF in the tumor tissue was reduced in both mice and rats, and these results correlated with the frequency of tumor growth. VEGF is a well known potential mediator of tumor-associated neovascularization *in vivo* since it is up-regulated in various human cancers including bladder cancer. Furthermore, several studies demonstrated that VEGF and PDECGF were frequently co-expressed in the tumor tissue (24). In our study, the expression of VEGF was different between mice and rats. In mice, the healthy urothelium showed expression of VEGF, and this expression did not differ by cimetidine treatment. However, in rats, VEGF expression was not detected in the healthy tissue, and the cimetidine-treated groups showed significant reduction of the VEGF expression (p=0.021). In addition, we did not find any correlation between PDECGF and VEGF.

We referred to another mechanism of cimetidine concerned with cell adhesion via E-selectin. Previously, Kobayashi *et al* reported that cimetidine down-regulated the expression of E-selectin, a ligand for sialyl-Lewis antigens, in colon cancer cells, and that it suppressed cell adhesion to the endothelium resulting in suppression of metastasis (10). In our study, the

Table II. The expression of VEGF, E-selectin and PDECGF in tumors of mice (top) and rats (bottom).

	Treatment	No. mice with carcinoma	VEGF		E-selectin		PDECGF	
			Neg./Mod.	Pos.	Neg./Mod.	Pos.	Neg./Mod.	Pos.
Group *m*A	None	-	0	3	0	3	3	0
Group *m*B	BBN	13	3	10	3	10	1	12
Group *m*C	BBN→CIM	8	2	6	3	5	2	6
Group *m*D	BBN+CIM	5	3	2	1	4	3	2
			*m*B vs. *m*C: NS		*m*B vs. *m*C: NS		*m*B vs. *m*C: NS	
			*m*B vs. *m*D: NS		*m*B vs. *m*D: NS		*m*B vs. *m*D: a	
			*m*C vs. *m*D: NS		*m*C vs. *m*D: NS		*m*C vs. *m*D: NS	
	Treatment	No. rats with carcinoma	VEGF		E-selectin		PDECGF	
			Neg./Mod.	Pos.	Neg./Mod.	Pos.	Neg./Mod.	Pos.
Group *r*A	None	-	6	0	3	3	6	0
Group *r*B	BBN	19	11	8	4	15	0	19
Group *r*C	BBN→CIM	9	9	0	1	8	8	1
Group *r*D	BBN+CIM	11	10	1	8	3	4	7
			*r*B vs. *r*C: a		*r*B vs. *r*C: NS		*r*B vs. *r*C: b	
			*r*B vs. *r*D: NS		*r*B vs. *r*D: NS		*r*B vs. *r*D: a	
			*r*C vs. *r*D: NS		*r*C vs. *r*D: NS		*r*C vs. *r*D: a	

[a]$p<0.05$, [b]$p<0.001$. NS, no significance; CIM, cimetidine.

expression of E-selectin in the tissue was detected in 100% (3/3) of mice and 50% (3/6) of rats. Nonetheless, cimetidine treatment did not affect the expression of E-selectin in the tumor tissue in either mice or rats. Our results may be attributed to the difference of cancer cells and the expression of sialyl-Lewis antigens. However, our results probably indicate that the anti-metastatic effect of cimetidine did not correlate with carcinogenesis.

In conclusion, our findings in the BBN-induced bladder cancer models partially supported that the anti-tumor effect of cimetidine is mediated via suppression of angiogenic factors including VEGF and PDECGF. Cimetidine is widely used to manage gastro-esophageal reflex diseases and gastric and duodenal ulcers without serious adverse effects, and may be a useful adjuvant for bladder cancer. Further molecular studies are necessary to determine the mechanism of cimetidine-induced anti-tumor effect.

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