Cimetidine Inhibits Cancer Cell Adhesion to Endothelial Cells and Prevents Metastasis by Blocking E-selectin Expression

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ABSTRACT

Although the beneficial effect of cimetidine on survival in cancer has been clinically demonstrated in colorectal cancer patients, the mode of action of cimetidine has not been elucidated. In this report, we have demonstrated for the first time that cimetidine can block the adhesion of a colorectal tumor cell line to the endothelial cell monolayer in cell culture and that it can suppress the metastasis of the tumor cell in a nude mouse model. We also demonstrated that these ant metastasis effects of cimetidine might occur through down-regulation of the cell surface expression of E-selectin on endothelial cells, a ligand for sialyl Lewis antigens on tumor cells. We found that the cimetidine-mediated down-regulation of E-selectin did not involve down-regulation of E-selectin mRNA or blocking of the nuclear translocation of nuclear factor \( \kappa \)B, a transcriptional activator of E-selectin gene expression. Because two other histamine type 2 receptor antagonists, famotidine and ranitidine, did not show any similar effect, these actions of cimetidine probably do not occur via blocking of the histamine receptor. These observations support the idea that cancer metastasis can be blocked by cimetidine administration through blocking the adhesion of tumor cells to the endothelium when an interaction between E-selectin and sialyl-Lewis antigens plays a role.

INTRODUCTION

Cimetidine has been shown to improve the survival of patients with colorectal cancer, melanoma, and renal cell cancer (1–9). Although it is not clear whether this effect of cimetidine on cancer is direct or indirect, it has been proposed that cimetidine may act by enhancing the host immune response against tumor cells (10, 11) or by blocking the cell growth-promoting activity of histamine in colon cancer and melanoma cell lines (9, 12–14). Other \( \mathrm{H}_{2} \)R antagonists including ranitidine and famotidine did not have such an effect on the survival of cancer patients (13–15), indicating that the anticancer actions of cimetidine might not be mediated via histamine antagonism. Therefore, the mechanism of action by which cimetidine prolongs the survival of patients with various forms of cancer remains to be clarified.

In this study, we have attempted to examine the action of cimetidine by investigating its effect on the cancer cell adhesion to endothelial cells, one of the critical steps of cancer invasion and metastasis. We have also applied an in vivo metastasis model to confirm the effect of cimetidine. We demonstrated previously (16) that the induction of E-selectin in HUVECs by IL-\( \beta \)B induced the adhesion of cancer cells expressing the E-selectin ligand sialyl Lewis antigens (17, 18) to HUVECs and that inhibitors of NF-\( \kappa \)B activation including pentoxifylline, aspirin, and N-acetyl-L-cysteine could block the cancer cell adhesion by inhibiting the IL-\( \beta \)-mediated induction of E-selectin. Here we attempted to examine the effect of cimetidine by applying a similar approach, and we found that cimetidine blocked cancer cell adhesion to HUVECs. We also demonstrated that cimetidine can prevent liver metastasis in a nude mouse model in which tumor cell line HT-29 was injected intrasplenically.

MATERIALS AND METHODS

Cells. HUVECs were isolated from fresh human umbilical cords by treatment with 1 mg/ml collagenase and dispase and cultured in RPMI 1640 supplemented with 50 \( \mu \)g/ml endothelial cell growth supplement (UBI, Lake Success, NY), 1 \( \mu \)g/ml epidermal growth factor (UBI), 292 \( \mu \)g/ml L-glutamine, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, 5 \( \mu \)g/ml heparin, and 15\% fetal bovine serum. HUVECs were characterized by expression of von Willebrand factor using immunostaining with a specific antiserum (Zymed, San Francisco, CA). HUVECs between passage 4 and 8 were used in this study. HUVECs obtained from three unrelated donors were assessed in this study, and we ensured that the observed effects were not batch specific. HUVECs from a single donor were used within each experiment. Human tumor cell line HT-29 expressing sialyl Lewis antigens (X and A) and derived from well-differentiated adenocarcinoma of the colon (19) was a gift from Dr. M. Iigoh (National Cancer Center, Tokyo, Japan). This cell line was maintained in McCoy’s medium supplemented with 10\% fetal bovine serum.

Cytotoxicity of Compounds. To evaluate the cytotoxicity of each compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed by incubating HUVECs or HT-29 cells with various concentrations of each compound according to the method described previously (20).

Reagents. Recombinant IL-\( \beta \)B was a gift from Otsuka Pharmaceutical Co. (Tokushima, Japan). Immunostaining and flow cytometry (FACScan) of E-selectin were carried out using a peptide-specific mouse monoclonal antibody to human E-selectin as a primary antibody (R&D Systems, Minneapolis, MN) as reported previously (16, 21). For ELISA, mouse monoclonal antibodies to human E-selectin (CD62E; PharMingen, San Diego, CA) and human ICAM-1 (CD54; Becton Dickinson, San Jose, CA) were used. The secondary antibodies used for immunostaining and ELISA were FITC-conjugated rabbit antirabbit IgG or rhodamine-conjugated goat antirabbit IgG (Cappel, Durham, NC) and horseradish peroxidase-conjugated sheep antirabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), respectively. For the immunostaining of NF-\( \kappa \)B, the peptide-specific rabbit antibody to human p65 was used as a primary antibody (22). Commercial antibodies to p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, CA) were also used for this study. For blocking of the HT-29 cell adhesion to HUVECs, antibodies to E-selectin (CD62E) and to sialyl Lewis (23) (CD15s; PharMingen) were used. Pentoxifylline and aspirin were purchased from Sigma Chemical Co. (St. Louis, MO). Cimetidine, famotidine, and ranitidine were kindly provided by Smith Kline Beecham Japan (Tokyo, Japan), Yamanouchi (Tokyo, Japan), and Sankyo (Tokyo, Japan), respectively. Aspirin was dissolved in 99\% ethanol. H2R blockers and pentoxifylline were dissolved in PBS.

Monolayer Cell Adhesion Assay. HUVECs were stimulated with 10 ng/ml IL-\( \beta \)B for 0–4 h in a 10-cm\(^2\) plate. HT-29 cells (1 \( \times 10^7 \) cells/200 \( \mu \)l/well) were added onto a semiconfluent monolayer culture of HUVECs, incubated for 20 min at 37\°C with rotation at 120 rpm, and washed extensively to exclude nonspecific cell attachment. The number of attached cells was counted directly under a microscope as reported previously (16). For antibody-mediated blocking of cell adhesion, the IL-\( \beta \)B-stimulated HUVECs were
incubated with antibody to either E-selectin (CD62E) or ICAM-1 (CD54; final dilution of 1:400 for both antibodies) for 3 h at 37°C in humidified CO₂ incubator, and then HT-29 cells were added. In the case of sialyl Lewis X, the antibody (CD15s) was incubated with HT-29 for 1 h and added to the IL-1β-stimulated HUVECs.

**Northern Blot Analysis.** Poly(A) RNA was purified from HUVECs treated with various concentrations of cimetidine in the absence or presence of IL-1β using a commercial mRNA isolation kit (Boehringer Mannheim, Mannheim, Germany). Two μg of poly(A) RNA were loaded onto each lane. The electrophoresis and blotting to Hybond-N membrane filter (Amersham Pharmacia Biotech) were performed as recommended by the supplier. Hybridization was carried out with a specific cDNA probe for E-selectin that did not hybridize with other selectin genes. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Radiolabeling was carried out using a commercial kit (Random Primer Labeling Kit; Stratagene, La Jolla, CA).

Fig. 1. Inhibition of HT-29 tumor cell adhesion to HUVECs by cimetidine. HT-29 cells (1 × 10⁵ cells/200 μl/well) were added onto a semiconfluent monolayer culture of HUVECs, incubated for 20 min at 37°C with rotation at 120 rpm, and washed extensively to exclude nonspecific cell attachment. A, various concentrations of H2R antagonists were added, and HT-29-HUVEC interaction was examined in the presence of IL-1β (10 ng/ml). Phase-contrast microscopic pictures of representative experiments are shown. B, quantitation of HT-29-HUVEC interaction. The number of HT-29 cells adhering to the HUVEC monolayer was counted. Values were obtained from the results of three independent experiments; bars, SD.
Immunostaining, Flow Cytometry, Immunoblotting, and Confocal Laser Microscopy.

Immunostaining was performed as reported previously (16, 21). Briefly, semiconfluent HUVECs on Lab-Tek tissue culture chamber slides (Nunc, Inc., Naperville, IL) were fixed with acetone for 10 min at 22°C and washed three times with PBS. They were subsequently incubated with the primary antibody for 1 h at 37°C. After washing three times with PBS containing 0.05% Triton X-100, they were incubated with the secondary antibody for 20 min at 37°C. Slides were mounted with buffered glycerol for fluorescence (BX50; Olympus Co., Tokyo, Japan) or confocal laser microscopic examination (MRC-600UV; Bio-Rad, Hercules, CA). Primary and secondary antibodies were diluted 1:100 in PBS. For analysis of cell surface E-selectin expression, unfixed cells were stained with the same anti-E-selectin antibody and FITC-conjugated rabbit antimouse IgG, and cytofluorometric examination was carried out as described previously (Ref. 21; FACScan; Becton Dickinson). For immunoblotting, total cell extracts prepared from HUVEC cultures were analyzed with the mouse monoclonal anti-E-selectin antibody according to the method reported previously (21).

ELISA. An ELISA method was developed, according to the work of Wellicome et al. (23), to quantitatively measure the amount of cell adhesion molecules, including E-selectin and ICAM-1, expressed on the cell surface of HUVEC cultures. Basically, the ELISA was performed at room temperature with three washes of 0.1% BSA in PBS between each step. HUVECs were incubated for 1 h with the primary antibody, antihuman E-selectin monoclonal antibody (CD62E2) or antihuman ICAM-1 monoclonal antibody (CD54). After washing, the secondary antibody, horseradish peroxidase-conjugated sheep antimouse IgG antibody, was added and incubated with HUVECs for 30 min. The enzyme substrate O-phenylenediamine (1 mg/ml) and 0.03% hydrogen peroxide in citrate-phosphate buffer (pH 5.0) were then added. Color development was stopped with 2 N sulfuric acid, and the absorbance of each well was read at 450 nm in a Titertek ELISA plate reader (SLT Lab Instruments, Salzburg, Austria). Test and control samples were examined in triplicates for each experiment. The degree of specific antibody binding was calculated by subtracting the mean negative control value (without the primary antibody), and the results were expressed as the fold activation of surface E-selectin expression (mean ± SD). In each experiment, HUVECs at the fourth or the fifth passage were used to minimize the interassay variation. Although HUVEC cultures from three unrelated donors were tested, there was no

Fig. 2. Inhibition of HT-29 tumor cell adhesion to HUVECs by E-selectin, sialyl Lewis x, and ICAM-1 antibody. A, E-selectin, sialyl Lewis x, and ICAM-1 antibody were added (at a final dilution of 1:400), and HT-29-HUVEC interaction was examined in the presence of IL-1β (10 ng/ml). Phase-contrast microscopic pictures of representative experiments are shown. B, quantitation of HT-29-HUVEC interaction. The number of HT-29 cells adhering to the HUVEC monolayer was counted. Values were obtained from the results of three independent experiments; bars, SD.

Fig. 3. Effects of cimetidine on E-selectin gene expression. A, Northern blot analysis of the E-selectin mRNA level in HUVEC's pretreated with various concentrations of cimetidine before stimulation with IL-1β (10 ng/ml). Positions of E-selectin (closed arrowhead) and glyceraldehyde-3-phosphate dehydrogenase (open arrowhead) as an internal control are indicated. B, effects of cimetidine on the nuclear translocation of NF-κB in response to IL-1β. Treatment of HUVECs with H2R antagonists was as described in the legends to Figs. 1 and 2. Cells were fixed after 30 min of IL-1β stimulation, and immunostaining was performed using rabbit polyclonal antibody to the p65 subunit of NF-κB as a primary antibody.
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Quantification of Hepatic Metastasis in Nude Mice. Ten weeks after HT-29 cell injection, animals were sacrificed, and the status of liver metastasis was evaluated quantitatively. The livers were excised and cut into 2-3-mm-thick slices that were then fixed in ice-cold acetone for subsequent H&E staining. The area of tumor nodules was measured with the aid of a video image processor (VIP-21C; Olympus Co.,) and it was expressed as a percentage of occupancy in the liver according to the method of Hirose et al. (24).

Statistical Analysis. Differences between the results of experimental treatments were evaluated by means of the two-tailed Student’s t test. Fisher’s exact probability test was used to assess the difference in the incidence of metastatic liver lesions. StatView-J 4.0.2 computer software was applied for these analyses.

RESULTS

Suppression of Tumor Cell Adhesion to Endothelial Cells by Cimetidine. To examine the effects of cimetidine on HT-29 tumor cell adhesion to HUVECs, a monolayer cell adhesion assay was carried out. As shown in Fig. 1A, the adhesion of HT-29 cells to HUVECs was strongly induced on stimulation with IL-1β (10 ng/ml). The maximum induction of cell adhesion by IL-1β was obtained after 4 h of stimulation. Using this model, we investigated the effects of various H2R antagonists at a range of noncytotoxic concentrations [from 10^{-8} to 10^{-6} M (9, 13); also confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in this study]. Cultured HUVECs were pretreated with cimetidine or other H2R antagonists 2 h before the addition of IL-1β. After 4 h of IL-1β stimulation, HT-29 cells were added, and the cell adhesion assay was performed. As shown in Fig. 1, pretreatment of HUVECs with pentoxifylline blocked the adhesion with HT-29 cells, as reported previously (16). Similarly, this adhesion of HT-29 cells to HUVECs was inhibited by cimetidine in a dose-dependent manner. However, other H2R antagonists, famotidine and ranitidine, had no inhibitory effect.

To confirm that the HT-29 adhesion to HUVECs was due to the cognate interaction between sialyl Lewis^X (on HT-29) and E-selectin (on HUVECs), specific antibodies were applied to block these molecules before the cell adhesion assay. In Fig. 2, we incubated HUVECs that had been stimulated with IL-1β with the antibody to E-selectin or ICAM-1, and the monolayer cell adhesion assay was carried out. When HUVECs were preincubated with the antibody to E-selectin, the IL-1β-induced HT-29 adhesion to HUVECs was abolished (Fig. 2, A and B). Similarly, when HT-29 cells were preincubated with the antibody to sialyl Lewis^X, the HT-29 adhesion to HUVECs was also blocked. In contrast, preincubation of HT-29 cells with anti-ICAM-1 antibody did not significantly reduce cell adhesion. There was no significant effect of antibodies to E-selectin or sialyl Lewis^X on the basal level of HT-29 cell adhesion to HUVECs (without IL-1β stimulation; data not shown). These findings were consistent with previous observations by others (18, 25, 26) and indicate that both E-selectin and sialyl Lewis^X are primarily involved in our experimental cell adhesion assay system using HT-29 cells and HUVECs.

Effects of Cimetidine on E-selectin Gene Expression and IL-1β-mediated NF-κB Activation. To investigate the effect of cimetidine on the E-selectin mRNA level, Northern blot analysis was carried out (Fig. 3A). Poly(A) RNA purified from HUVECs treated with various concentrations of cimetidine in the absence or presence of IL-1β was hybridized with the E-selectin cDNA probe. As shown in Fig. 3A, IL-1β could induce E-selectin gene expression. However, even at the highest concentration, cimetidine could not significantly reduce the E-selectin mRNA level. We also examined the effect of cimetidine on the IL-1β-induced nuclear translocation of NF-κB (Fig. 3B). In contrast to aspirin, which effectively blocked the nuclear translocation of NF-κB as reported previously (16, 21), cimetidine or...
other H2R antagonists did not block the nuclear translocation of NF-κB elicited by IL-1β (Fig. 3A).

Cimetidine Blocked Cell Surface Expression of E-selectin Induced by IL-1β. We then examined the effect of H2R antagonists on the E-selectin protein level in HUVECs stimulated by IL-1β using immunohistochemistry and cytofluorometry (Fig. 4). Maximum induction of E-selectin by IL-1β (10 ng/ml) was obtained after 4 h of treatment (Fig. 4A), which coincided with the HT-29 adhesion to HUVECs. Interestingly, a significant suppression of E-selectin induction was observed on pretreatment of HUVECs with cimetidine in a dose-dependent manner at noncytotoxic concentrations (from 10^(-8) to 10^{-4} M), which again correlated with the effect of cimetidine on the adhesion of HT-29 cells to HUVECs (Fig. 1). It was noted by confocal microscopic examination that there was no significant change in the intracellular localization of E-selectin (Fig. 4A). Neither famotidine nor ranitidine showed any inhibitory effect on E-selectin levels. There was no change in the expression level of sialy Lewis antigens on the HT-29 cell surface by cimetidine (data not shown). There was no significant effect of cimetidine on the ICAM-1 level in HUVECs in either the presence or absence of IL-1β (data not shown). In spite of our repeated attempts to identify the change of E-selectin protein levels by Western blotting using a number of commercially available antibodies, we were not able to detect the monospecific band corresponding to E-selectin, a failure we believe to be due to the low affinity of the antibodies in solution or the presence of interacting proteins that interfered in the antibody reaction.

Quantitation of the Cell Surface E-selectin Expression by ELISA. We thus developed a cell ELISA system, as described by Wellicome et al. (23), to quantitate the E-selectin level on the HUVEC cell surface. Using this system, the effects of H2R antagonists on E-selectin and ICAM-1 expression on HUVECs with or without IL-1β stimulation were examined. As shown in Fig. 5A, the E-selectin level on HUVECs was augmented by 6.0 ± 0.36-fold on stimulation with IL-1β. When HUVECs were pretreated with cimetidine 2 h before IL-1β stimulation, the extent of E-selectin induction decreased to 4.9 ± 0.35-, 1.9 ± 0.06-, and 1.1 ± 0.12-fold at cimetidine concentrations of 10^{-8}, 10^{-6}, and 10^{-4} M, respectively (P < 0.01). The cell surface ICAM-1 expression was also augmented by 6.37 ± 0.43-fold on IL-1β treatment (Fig. 5B). However, ICAM-1 expression was not significantly inhibited by cimetidine pretreatment. Neither famotidine nor ranitidine showed any inhibitory effect (Fig. 5, C and D).

Suppression of Liver Metastasis in Nude Mice by Cimetidine. The efficacy of cimetidine in blocking E-selectin protein expression and the subsequent HT-29 cell adhesion to HUVECs prompted us to examine the effects of cimetidine on liver metastasis using a nude mouse model in vivo. Mice were inoculated with HT-29 (1 x 10^6 and 1 x 10^7 cells in experiments 1 and 2, respectively) intraplacently, and the effects of cimetidine on the incidence and the extent of liver metastasis were evaluated. Ten weeks after the injection of HT-29 cells, mice were sacrificed, and the status of liver metastasis was examined. The areas of metastatic nodules in the liver were measured from the excised liver slices using a video image processor according
to the method developed by Hirose et al. (24). As demonstrated in Table 1, cimetidine prevented the incidence of liver metastasis in a dose-dependent manner. At the highest dose of cimetidine (daily doses of 200 mg/kg), liver metastasis was completely inhibited when 1 × 10⁶ HT-29 cells were injected intrasplenically (none of the nine mice had detectable liver metastasis; Table 1, experiment 1), whereas there was no notable difference in the size of the primary tumor in the spleen (data not shown). Famotidine or ranitidine at doses equivalent to that of cimetidine had no inhibitory effect on liver metastasis (data not shown). A repeated experiment using a greater number of HT-29 cells (1 × 10⁷) for inoculation demonstrated similar effects of cimetidine on the prevention of liver metastasis (Table 1, experiment 2). Furthermore, the extent of liver metastasis (the percentage of occupancy of the metastatic nodules in the liver slices) was significantly diminished in the cimetidine-treated animals (Table 2).

**DISCUSSION**

The clinical benefit of cimetidine for the prolonged survival of cancer patients has been established previously (1–9). In this study, we investigated the effects of cimetidine using an in vitro cell culture system and an in vivo nude mouse cancer metastasis model. These experiments clearly demonstrated that cimetidine effectively blocked HT-29 adhesion to HUVECs by preventing E-selectin induction and the trans-portal liver metastasis of HT-29 cells in nude mice.

E-selectin is considered to play a primary role in initiating the adhesion of cancer cells to vascular endothelial cells through its interaction with its specific ligand sialyl Lewis antigens (16–18, 25–28). In a previous study (16), we reported that adhesion of tumor cell line QG90 to HUVECs was dependent on E-selectin expression on the cell surface of HUVECs and was induced by IL-1β. Similar results were reported with HT-29 cells by Dejana et al. (26). In these studies, IL-1β was used to activate E-selectin on HUVECs to mimic the local inflammatory response in the metastasized region (29, 30), and some tumor cells were reported to produce IL-1β (31, 32). In fact, expression of E-selectin in endothelium adjacent to the metastatic tumor lesion was reported by others (33, 34). It is also known that E-selectin is induced on ischemia-reperfusion injury (35), which may be associated with tumor embolism during metastasis. Thus, it is likely that E-selectin is induced by such mechanisms in our nude mouse model.

We found that the effect of cimetidine did not appear to be at the level of E-selectin gene expression because the E-selectin protein level was reduced without significantly reducing its mRNA level. The failure of cimetidine to block NF-κB nuclear translocation also supported this possibility. Previous studies have indicated a variety of unexpected actions of cimetidine. For example, cimetidine was reported to have direct growth-inhibitory effects on certain cancer cell lines (1, 14) and direct stimulatory effects on lymphocyte function (10–12). It was also reported that histamine was released into the blood stream during the operation, presumably from tumor cells (36) or the adjacent mast cells (37), and that histamine interfered with the host immune system (10, 11, 38). It was then suggested that histamine might promote the growth of tumor cells (12, 14, 39). However, we did not observe any cytotoxic or cytostatic effects of cimetidine on HT-29. In addition, we found that other H2R antagonists did not have the same effect as cimetidine in our experimental systems, indicating that this action of cimetidine is probably not mediated via the H2R. In addition, although some reports have indicated that cimetidine may have antioxidant activity (40–42), and antioxidants have been shown to block E-selectin gene expression by blocking the activation cascade of NF-κB (16), cimetidine did not block IL-1β-induced NF-κB activation (Fig. 3B).

The action of cimetidine on E-selectin expression does not appear to involve the transcription step. Therefore, the experimental observations obtained in this study suggest that cimetidine may block E-selectin expression at a step after transcription. In this regard, it may be worth noting that other regulatory molecules such as p38 MAPK, in addition to NF-κB, were also involved in IL-1β signaling (43, 44). Because it has been implicated that E-selectin expression requires p38 MAPK (43), and p38 MAPK activates the expression of a number of genes at the level of posttranscription (44–46), the issue of whether cimetidine but not other H2R antagonists might interfere with such signaling mediators should be explored.

Our observations support the previous clinical findings that cimetidine increased the survival of cancer patients. It is likely that cimetidine may block cancer cell adhesion to endothelial cells and thus prevent metastasis. In fact, we found in our recent randomized clinical study that cimetidine treatment significantly reduced cancer metastasis and recurrence, thus resulting in the prolonged survival of patients with colorectal cancer whose tumors had high levels of sialyl Lewis antigens.4 There were no effects when the tumor had no sialyl Lewis antigens or low levels of sialyl Lewis antigens. Additional analyses on the actions of cimetidine are needed to identify a novel therapeutic target against cancer aggression.

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