Cimetidine inhibits the adhesion of gastric cancer cells expressing high levels of sialyl Lewis x in human vascular endothelial cells by blocking E-selectin expression

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Received November 25, 2010; Accepted January 11, 2011

DOI: 10.3892/ijmm.2011.618

Abstract. Cimetidine has been shown to have anti-metastatic activity and improves the survival of patients with colorectal cancer. One hypothesis is its modulation of the expression of the cell adhesion molecule by target organ endothelial cells. Because of the inconclusive results in clinical trials of gastric cancer, we investigated the effects of cimetidine on the adhesion of gastric cancer cells to activated endothelial cells and on the expression of some cell adhesion molecules. Human endothelial cells were pre-incubated with cimetidine for 6 h, incubated with the cytokine tumor necrosis factor for 4 h, and the endothelial surface expression of E-selectin was evaluated by flow cytometry, immunostaining and ELISA. Further, we investigated E-selectin mRNA expression by RT-PCR. Three gastric cancer cell lines (SGC-7901, MGC-803, BGC-823) and a normal gastric epithelial cell line, GES-1, were studied for the surface expression of sialyl Lewis x by flow cytometry and immunostaining. Adherence of CFSE-labeled gastric cancer cells and GES-1 cells to endothelial cell monolayers was determined. Cimetidine significantly reduced E-selectin expression of activated endothelial cells, but did not influence E-selectin expression at the mRNA level. Three gastric cancer cell lines expressed high levels of sialyl Lewis x, whereas GES-1 did not. Cimetidine also significantly decreased gastric cancer cell adherence to stimulated endothelial cells. The inhibition of E-selectin expression corresponded to the reduction of tumor cell adherence. The effects of cimetidine on tumor adhesion were almost nullified by pre-incubation with E-selectin and sialyl Lewis x antibody. Furthermore, there was no significant change of GES-1 adherence to endothelial cells by TNF-α, cimetidine, E-selectin and sialyl Lewis x antibody. The inhibition of gastric cancer cell adherence to cytokine-stimulated endothelial cells treated with cimetidine appears to result from blocking endothelial E-selectin expression. These data support the hypothesis that cimetidine may exert its anti-metastatic effects in gastric cancer, in part, by inhibiting E-selectin/sialyl Lewis x-mediated adherence of gastric cancer cells to endothelial cells in the metastasis target organs.

Introduction

The formation of blood-borne metastasis is a complex process by which tumor cells spread out from the primary tumor. Evidence indicates that the endothelium is actively involved in the formation of blood-borne metastasis of malignant tumors (1). The process is initiated when tumor cells leave the primary site and invade the vessels to reach the bloodstream. The tumor cells can then travel to distant sites via the bloodstream, adhere to the vascular endothelium, penetrate the vessel wall, and establish metastasis (2). A growing body of evidence indicates that the direct adhesive interaction between tumor cells and endothelial cells is the critical step in the formation of blood-borne metastasis.

Both E- and P-selectins are expressed in activated endothelial cells. They recognize sialylated fucosylated lactosaminoglycans on the surface of various leukocytes and support leukocyte rolling in the first stage of cell adhesion, facilitating subsequent leukocyte arrest and extravasation. Tumor cells, particularly carcinoma and leukemic cell lines, can express large amounts of sialyl Lewis antigens on their surfaces. These oligosaccharides can mediate the direct interaction between tumor cells and the endothelium, followed by tumor extravasation. Several laboratories have reported that sialyl Lewis x (sLex)/E-selectin molecules play an important role in cancer cell adhesion to endothelial cells in vitro (3) and in vivo (4).

Cimetidine, a kind of histamine type 2 (H₂) receptor antagonist, has been shown to improve the survival of patients with colorectal cancer (5-9) in clinical trials. However, several clinical trials have assessed cimetidine in gastric cancer with
varied and inconclusive results. In 1988 it was reported that post-operative treatment with cimetidine improved survival of patients with any stage of gastric cancer (10). But, in a more recent and larger study organized by the British Stomach Cancer Group, cimetidine had no effect on survival when compared to the placebo group (11). Recently, Kobayashi et al (12) showed that cimetidine could block the adhesion of a colorectal tumor cell line to endothelial cells in vitro and could suppress the formation of hepatic metastasis in a nude mouse model via the down-regulation of the cell surface expression of the adhesion molecule E-selectin on endothelial cells. In order to evaluate the evidence for the use of cimetidine in clinical trials of gastric cancer, the present study tested the hypothesis that cimetidine inhibits gastric cancer cell adhesion to the activated endothelium mediated by the sLex/E-selectin molecules.

Materials and methods

Reagents. Cimetidine, TRIzol, DMSO, L-glutamine, endothelial cell growth supplement and trypsin were purchased from Sigma (St. Louis, MO). RPMI-1640, M199, heparin, penicillin-streptomycin, and other cell culture supplies were from Gibco-BRL (Grand Island, NY). Fetal bovine serum was from Hyclone (Logan, UT). Monoclonal sLex antibody (CD15s, mouse IgM) and monoclonal E-selectin antibody (CD62E, mouse IgG) were purchased from BD Pharmingen (San Diego, CA). Fluorescein (FITC)-conjugated goat anti-mouse IgM and IgG antibodies were obtained from Southern Biotechnology Associates (Birmingham, AL). Peroxidase-conjugated AffiniPure goat anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). 5(6)-carboxy fluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR). Recombinant human TNF-α was purchased from PeproTech (CytoLab Ltd., Israel). The E-selectin and β-actin primers were from Takara Biotechnology.

Cell cultures and cimetidine treatment. Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical cord by mild trypsinization. The endothelial cells were cultured in M199 supplemented with 20% fetal bovine serum, 50 µg/ml endothelial cell growth factor, 5 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. The cells between passage two and four were used in this study. Human gastric carcinoma metastatic lymph node cell line, SGC-7901, and normal gastric epithelial cell line,GES-1, were obtained from Cancer Research Institute of Beijing, China. Human gastric mucinous adenocarcinoma cell line, MGC-803, and gastric adenocarcinoma cell line, BGC-823 were obtained from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). They were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml of penicillin, 100 U/ml of streptomycin. Tumor cells were grown as adherent monolayers but were used for adhesion experiments as a single cell suspension. Rapid treatment with 0.05% trypsin and 0.5 mM EDTA in PBS released the cells from the monolayer.

For experiments involving cytokine-stimulated endothelial cells, HUVEC monolayers were incubated at 37°C for 6 h in serum-free media containing 0, 1, 10, 100 µM cimetidine. Then human recombinant TNF-α (20 ng/ml) was added and incubated for 4 h. For control group, HUVEC monolayers were incubated at 37°C for 10 h in serum-free medium.

Flow cytometry. The expression of E-selectin in endothelial cells and the sLex expression in gastric cancer cells or GE1-1 cells were measured by flow cytometry. Briefly, a single cell suspension of HUVEC after treatment and gastric cancer cells was created by treating monolayers with 0.05% trypsin and 0.5 mM EDTA. The suspension was washed with PBS twice and cells were resuspended in PBS containing 0.5% BSA and incubated with E-selectin (3 µg/ml) or sLex antibody (3 µg/ml) for 1 h at 4°C. To remove the unbound antibody the cells were washed twice with PBS, followed by incubation with 15 µg/ml FITC-conjugated goat anti-mouse antibody for 1 h at 4°C. Cells were again washed, centrifuged, then resuspended in PBS containing 1% paraformaldehyde and were analyzed by using a flow cytometer (FACScalibur; Becton-Dickinson). For each analysis, 10,000 events were collected and histograms were generated.

Immunostaining. Immunostaining was performed as previously reported (13,14). Briefly, the HUVEC cells after treatment and gastric cancer cells or GES-1 cells on Lab-Tek tissue culture chamber slides were fixed with acetone for 15 min at 4°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, they were incubated with the secondary antibody for 1 h at room temperature in a dark area. The slides were then washed twice and evaluated using a fluorescence microscope (Olympus IX-50) at a magnification of x400. Primary and secondary antibodies were diluted 1:100 in PBS containing 1% BSA.

Cellular ELISA assay for E-selectin. The cell surface expression of E-selectin on endothelial monolayers was quantified using cell-ELISA as described by Madan et al (15). HUVECs were grown to confluence in 96-well, flat bottom, gelatin-coated plates. The cells were incubated with or without cimetidine for 6 h, followed by induction with TNF-α (20 ng/ml) for 4 h. Cells were washed twice with PBS, and then fixed with 4% paraformaldehyde for 10 min at room temperature. After washing the cells three times with PBS, the E-selectin antibody (3 µg/ml) was added to the cells and allowed to incubate overnight at 4°C. Subsequently, the unbound antibody was removed; the cells were washed three times with PBS, then incubated with HRP-conjugated goat anti-mouse IgG (1:1000) for 1 h at room temperature. At the end of the 1 h incubation, the cells were washed three times with PBS, then exposed to the peroxidase substrate (O-phenylenediamine dihydrochloride, 40 mg/100 ml in citrate phosphate buffer, pH 4.5). The color development reaction was stopped by the addition of 2N sulfuric acid. Absorbance was determined at 492 nm by an automated microplate reader (TeCan Sunrise, Austria).

Reverse transcription and polymerase chain reaction (RT-PCR). E-selectin mRNA levels were assessed by the
RT-PCR method. The total RNA was isolated according to a modified guanidinium thiocyanate procedure (16) using TRIzol. cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase. PCR was performed with a set of primers: 5'-TTC GCC TGT CCT GAA GGA TG-3' (sense primer for E-selectin) and 5'-TCA GTT GAA GGC CGT CCT TG-3' (antisense primer for E-selectin); 5'-AAA TCG TGC GTG ACA TAA A-3' (sense primer for β-actin) and 5'-CTC GTC ATA CTC CTG CTT G-3' (antisense primer for β-actin). The PCR conditions were as follows: 94°C, 1 min; 57°C, 45 sec; 72°C, 1 min for 30 cycles. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, then photographed and scanned. Densitometric values of β-actin bands were used to standardize the results. The levels of mRNA were expressed as the ratio of the corresponding gene to β-actin expression.

**Cell adhesion assay.** Adhesion of gastric cancer cells to activated endothelial cells was examined as described by Hayashi et al (17). Briefly, HUVEC cells were plated at a density of 1x10^4 cells/well in collagen-coated 96-well plates. After incubation overnight at 37°C, HUVEC cells were incubated with or without different concentrations of cimetidine followed by 20 ng/ml of TNF-α for 4 h prior to the assay. For antibody-mediated blocking of cell adhesion, the TNF-α stimulated endothelial cells were incubated with antibody to E-selectin (50 µg/ml) for 1 h at 37°C in a humidified CO₂ incubator. In the case of sLex, gastric cancer cells and GES-1 cells were incubated with the antibody (CD15s, 50 µg/ml) for 1 h at 37°C. Then gastric cancer cells and GES-1 cells were pre-labeled with CFSE by the method of van Kessel et al (18). The CFSE-labeled cells (1x10^5 cells/100 µl/well) were suspended in a medium supplemented with 10% FBS and placed in 96-well plates containing endothelial cells, and incubated at 37°C for 1 h. Dishes were then washed three times to remove non-adherent cells. The adherence of CFSE-labeled gastric cancer cells was determined by measuring the fluorescence using the fluorescent plate reader (Tecan GENios, Austria) at an excitation wavelength of 485 nm and an emission of 530 nm. Cell adhesion was calculated as follows: % cell adhesion = mean fluorescence intensity of experimental wells/ mean fluorescence intensity of total cells plated x100%.

**Animal experiment.** This experiment was conducted in accordance with the guidelines issued by the State Food and Drug Administration (SFDA of China). The animals were housed and cared for in accordance with the guidelines established by the National Science Council of the Republic China.

Female BALB/c nude mice, 35–40 days old and weighing 20–22 g, were supplied by the Shanghai Slac Laboratory Animal Limited Co. The mice were kept under sterile conditions and fed a sterilized mouse diet and water. Animals were anesthetized via inhalation of isoflurane. To induce hepatic metastasis of tumor cells, 5x10^6 SGC-7901 cells were injected via the tail vein. The mice were randomly assigned to three groups (n=5 per group). To examine the effect of cimetidine, the mice were treated with 100 or 200 mg/kg/day cimetidine or saline (control) i.p. every other day for 4 weeks. Six weeks after the SGC-7901 cell injection, the animals were sacrificed, and the status of the liver metastasis was evaluated quantitatively.

**Statistical analysis.** All values in the text and figures are presented as mean ± SD. A one-way analysis of variance (ANOVA) was performed and a Bonferroni’s multiple comparison test was applied. Values of P<0.05 were taken to show a significant difference between means.

**Results**

**Cimetidine suppresses TNF-α induced E-selectin expression in endothelial cells.** To examine the effects of cimetidine on TNF-α induced E-selectin expression, endothelial cells were incubated with different concentrations of cimetidine for 6 h prior to induction with TNF-α (20 ng/ml) for 4 h. The time of incubation and concentration of cimetidine used in these experiments had no effect on the viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the morphology of the endothelial cells (data not shown). As detected by ELISA, E-selectin was expressed at very low levels in unstimulated endothelial cells. Upon induction with TNF-α, a significant increase in the expression of E-selectin was observed. Cimetidine had no effect on the basal level of E-selectin expression, whereas it led to a reduction in the TNF-α-induced E-selectin expression in a dose-dependent manner (Fig. 1). This was further confirmed by measuring the expression of E-selectin by flow cytometry (Fig. 2A) and immunostaining (Fig. 2B).

**Effects of cimetidine on E-selectin gene expression.** To test whether the suppression of E-selectin antigen expression by cimetidine is due to the suppression of E-selectin gene expression, we investigated the effect of cimetidine on the steady-state levels of E-selectin transcripts by RT-PCR. Endothelial cells were incubated with different concentrations of cimetidine for 6 h prior to induction with TNF-α. As shown in Fig. 3 there was a statistically significant increase in E-selectin mRNA following stimulation with TNF-α, as compared to low levels in control cells. However, treatment
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with different concentrations of cimetidine for 6 h prior to addition of TNF-α did not significantly change the transcription levels of E-selectin and the levels of β-actin mRNA expressed under these conditions remained the same. These results indicate that cimetidine may not affect the transcription of the E-selectin gene.

**SLex expression in gastric cancer cells.** The carbohydrate antigen, sLex which is frequently present in human malignant cells, is known to be a ligand for the cell adhesion molecule, E-selectin. Therefore, we investigated the sLex expression in three gastric cancer cell lines. As revealed by flow cytometry, SGC-7901, BGC-823 and MGC-803 had a high level of sLex. In contrast, GES-1 only slightly expressed sLex (Fig. 4A). These results were further confirmed by immunostaining (Fig. 4B).

**Cimetidine suppresses gastric cancer cell adhesion in the active endothelium through inhibition of the adhesion of sLex/E-selectin molecules.** To test the effect of cimetidine on gastric cancer cell adhesion to endothelial cells, confluent monolayers of HUVEC were pretreated with varying concentrations of cimetidine for 6 h and then stimulated with TNF-α (20 ng/ml) for 4 h, followed by incubation with gastric cancer cells or GES-1 cells for 1 h at 37˚C. As shown in Fig. 5, TNF-α stimulation of endothelial cells significantly increased the adherence of gastric cancer cells which expressed high levels of sLex. Furthermore, cimetidine treatment, in a dose-dependent manner, suppressed the adhesion of gastric cancer cells to activated endothelial cells. A concentration of 100 µM cimetidine almost completely inhibited the adhesion. However, TNF-α stimulation of endothelial cells did not significantly change the adherence of GES-1 which had a low level of sLex and treatment with cimetidine or antibody did not influence the adhesion.

To confirm that the adhesion of gastric cancer cells to endothelial cells was due to the cognate interaction between sLex and E-selectin, specific antibodies were applied to block these molecules prior to the cell adhesion assay. We incubated endothelial cells that had been stimulated with TNF-α with
the antibody to E-selectin and the monolayer cell adhesion assay was carried out (Fig. 5). When HUVECs were pre-incubated with the antibody to E-selectin, the TNF-α-induced gastric cancer cell adhesion to endothelial cells was abolished. Similarly, when gastric cancer cells were pre-incubated with the antibody to sLex, the gastric cancer cells adhesion to activated...
endothelial cells was also blocked. Additional experiments have shown that pre-treatment of gastric cancer cells or GES-1 cells with cimetidine had no effect on their binding to activated endothelial cells (data not shown). Overall, the data suggest that cimetidine suppressed the adhesion of gastric cancer cells to endothelial cells through inhibition of the adhesion of the sLex/E-selectin molecules.

Cimetidine inhibits the growth of transplantable tumors. The efficacy of cimetidine in blocking the E-selectin protein expression and the subsequent SGC-7901 cell adhesion to HUVECs prompted us to examine the effects of cimetidine on liver metastasis using a nude mouse model in vivo. Hepatic metastatic nodules for cimetidine-treated mice were visibly fewer than in saline-treated mice (Fig. 6A). As demonstrated in Fig. 6B, cimetidine prevented the incidence of liver metastasis in a dose-dependent manner. At the highest dose of cimetidine (200 mg/kg), liver metastasis was almost completely inhibited. None of the mice died during the treatment.

Discussion

Cimetidine, a kind of H2 receptor antagonist, has been used to inhibit the secretion of gastric acid in the clinic. It has also been demonstrated to prolong the survival of patients with colorectal cancer (5-9). However, the exact mechanism by which cimetidine may exert an anticancer effect remains uncertain. Cimetidine is thought to exert its effect by blocking high peritumoral concentrations of histamine and increasing systemic immunoreactivity via H2 receptor antagonism of circulating T suppressor cells or maintenance of natural killer cell activity (19-21). However, Rajendra et al (22) have demonstrated that cimetidine has a direct antiproliferative effect in the absence of histamine type 2 receptors and the induction of apoptosis. Recently, Kobayashi et al (12), found that cimetidine could block the expression of E-selectin on the surface of HUVECs, thus blocking the colorectal cancer cell adhesion to the endothelium and preventing the liver metastasis in the nude mouse model. Because of the inconclusive results in clinical gastric cancer treatment (10,11), we investigated the effect of cimetidine on the adherence of gastric cancer cells to the activated endothelium in vitro.

Our present study demonstrated that cimetidine mediated the suppression of gastric cancer cell adhesion to endothelial cells appears to be the result of the down-regulation of the expression of E-selectin in activated endothelial cells. The data clearly document a marked reduction of the down-regulation of the E-selectin antigen expression in the endothelial cell surface when the endothelial cells were exposed to cimetidine prior to stimulation with TNF-α. Moreover, the concentrations of cimetidine required to suppress the adhesion of gastric cancer cells to endothelial cells and the E-selectin expression were in a similar range, suggesting that the cimetidine-mediated inhibition of gastric cancer cell adhesion stems from its inhibitory effect on the E-selectin expression on the surface of endothelial cells. Consistent with this, the anti-E-selectin antibody was shown to block the gastric cancer cell adhesion to endothelial cells.

Furthermore, we assessed the E-selectin mRNA expression under cimetidine treatment. Even at the highest non-cytotoxic cimetidine concentration (100 µM), did not influence the TNF-α-induced E-selectin mRNA expression. This result agrees with a previous report (12). It has become obvious that the mechanism of cimetidine’s inhibitory function is present in a post-transcriptional step. p38MAPK (23), for instance, might be the regulatory molecule that activates the expression of a number of genes at the post-transcriptional level.

E-selectin is considered to play an important role in hematogenous metastasis (3,24-26). Previous studies have shown that among the cell adhesion molecules, including the selectins, the immunoglobulin superfamily and the integrins, E-selectin has been the only molecule that has a significant role in the adhesion of colorectal cancer cells to the vascular endothelium (27,28). Convincing experimental data have been generated on the involvement of E-selectin in breast and colon cancer metastasis (29). In addition, circulating levels of this adhesion molecule were identified as useful clinical markers of tumor progression and metastasis (30). Whereas E-selectin is not expressed in endothelial cells in vivo unless cells are stimulated by an inflammatory cytokine such as interleukin 1β or TNF-α, E-selectin is expressed in response to cytokines secreted by tumor cells in cancer patients (24,31). Recent experimental evidence also indicated that the inhibition of E-selectin mediated cancer cell adhesion may be an efficient strategy to inhibit cancer metastasis (32).

Both sialyl Lewis a (sLea) and sLex antigens are recognized by E-selectin expressed on the surface of endothelial cells (31). Indeed, many human adenocarcinoma cells adhere to E-selectin expression cells in vitro in a sLea- and sLex-dependent manner (3,26). Many reports have noted that sLex and sLea/E-selectin or P-selectin cell adhesion molecules play an important role in the adhesion of epithelial cells, which express higher levels of sLex than do their poorly metastatic compartments (33). Moreover, in human lung
and colon carcinomas, highly metastatic tumor cells express more sLex on the cell surface and bind more strongly to E-selectin than their poorly metastatic counterparts (26,34). Recently, B16-F1 cells, which were stably transfected with α-1,3-fucosyltransferase III (B16-TIII) to express sLex structures were reported to produce larger numbers of lung tumor nodules than their parent cells (35). Furthermore, the peptide mimic, E-selectin ligand, was shown to inhibit the binding of B16-TIII to E-selectin and to inhibit lung colonization of B16-TIII cells (36).

We also found that three gastric cancer cell lines adhered to E-selectin-expressing endothelial cells, whereasGES-1 cells did not. Pre-incubation with the sLex antibody almost nullified the effect of cimetidine on the adhesion of these gastric cancer cells. However, neither cimetidine nor these antibodies had any effects on the adhesion ofGES-1 to activated endothelial cells. These results indicate that the gastric cancer cells adhere to E-selectin-expressing endothelial cells through sLex. Flow cytometric analysis and cell immunostaining confirmed that these gastric cancer cells express high levels of sLex. In contrast, we observed thatGES-1 cells express low levels of sLex. Therefore, selective suppression of the adherence of gastric cancer cells and normal gastric epithelial cells by cimetidine is partly explained by the difference in the expression of sLex.

To examine the effect of cimetidine in vivo, we used the nude mouse model. We found that cimetidine could inhibit the hepatic metastasis of SGC-7901 cells in a dose-dependent manner. At the highest dose of cimetidine (200 mg/kg), liver metastasis was almost completely inhibited.

In summary, our present observations show that cimetidine suppresses gastric cancer cell adhesion to the activated endothelium mediated by the sLex/E-selectin molecules. The data provide evidence for the use of cimetidine in clinical trials of gastric cancer therapy. We propose that the inhibitory action of cimetidine on the metastasis is due to the blocking of the adhesion between endothelial cells and tumor cells, especially to those with a high expression of the sLex antigen. However, it is important to note that the anti-metastasis effect in vivo will depend not only on its potential anti-metastasis effect but also on many other factors, including its pharmacokinetics, absorption, distribution and metabolism. Therefore, additional analysis on the actions of cimetidine and more accurate clinical trials are warranted.

References