Andrographolide Inhibits the Adhesion of Gastric Cancer Cells to Endothelial Cells by Blocking E-selectin Expression

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Abstract. *Background: Andrographolide, an active component isolated from the Chinese official herbal Andrographis paniculata, has recently been reported to have anticancer activity. However the molecular mechanism responsible for its anticancer action has not been fully defined. In this study, we investigated the effect of andrographolide on the adhesion of gastric cancer cells to the activated endothelial cells and the expression of some cell adhesion molecules. Materials and Methods: Human endothelial cells were pre-incubated with andrographolide for 6 h and then incubated with the cytokine tumor necrosis factor for 4 h. Endothelial surface expression of E-selectin was evaluated by flow cytometry, immunostaining and ELISA. Further, we investigated E-selectin mRNA expression by RT-PCR. Surface expression of sialyl Lewis^X of three gastric cancer cell lines (SGC7901, MGC803, BGC823) and a normal gastric epithelial cell line GES-1 was evaluated by flow cytometry and immunostaining. Adherence of CFSE-labeled gastric cancer cells and GES-1 cells to endothelial cell monolayers was then determined. Results: Andrographolide significantly reduced E-selectin expression of activated endothelial cells, and inhibited the E-selectin expression on mRNA level. Three gastric cancer cell lines expressed high levels of sialyl Lewis^X, whereas GES-1 did not. Andrographolide also significantly decreased gastric cancer cells adherence to stimulated endothelial cells. The inhibition of E-selectin expression corresponded to the reduction of tumor cell adherence. The effects of andrographolide on tumor adhesion were almost nullified by pre-incubation with E-selectin and sialyl Lewis^X antibody. Conclusion: These findings demonstrate that andrographolide suppresses the adhesion of gastric cancer cells which express high level sialyl Lewis^X to human vascular endothelial cells by blocking E-selectin expression and, thus, may represent a candidate therapeutic agent for cancer.*

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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 metastases of malignant tumors (1). The process is initiated when tumor cells leave the primary site and invade the vessels to reach the blood stream. The tumor cells can then travel to distant sites *via* the blood stream, 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 on 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 direct interaction between tumor cells and endothelia, followed by tumor extravasation. Several laboratories have reported that sialyl Lewis^X (sle^x)/E-selectin molecules play an important role in cancer cell adhesion to endothelial cells *in vitro* (3) and *in vivo* (4).

Andrographolide (Andro) is a labdane diterpene isolated from the leaves of *Andrographis paniculata,* a traditional Chinese herbal medicine used for the treatment of viral infections and inflammatory disease (5). Andro is known to have anti-inflammatory (6, 7) and antiviral properties (8). Recently, it has been shown to have anticancer activity (9-12). However its molecular mechanism of anticancer action has not been fully defined. Activation of the NF-κB pathway is currently known to be essential for the *de novo* synthesis of high levels of E-selectin mRNA and protein, a member

of the selectin family of cell adhesion molecules (13). Upon challenges with cytokines TNF-α, IL-1β and bacterial LPS, the endothelial cells of the capillary and venular vessels *de novo* synthesize and express E-selectin, which reacts with leukocyte carbohydrate ligands bearing the tetrasaccharide sle^x and its derivatives as its minimal recognition motif. Recently, it was reported that Andro suppressed the activation of NF-κB in stimulating endothelial cells, which reduced the expression of the cell adhesion molecule E-selectin and prevented E-selectin-mediated leukocyte adhesion under flow (14). Thus, it is possible that Andro could inhibit the activation of endothelial cell adhesion molecules and thereby inhibit the adhesion of cancer cells to the endothelium, which can serve as a novel therapeutic target against cancer aggression. The present study was conducted to test the hypothesis that Andro inhibits the adhesion of gastric cancer cells to the activated endothelium by blocking E-selectin expression.

Materials and Methods

Materials. Andrographolide was purchased from Aldrich (Milwaukee, WI, USA). Trizol, DMSO, L-glutamine, endothelial cell growth supplement and trypsin were purchased from Sigma (St. Louis, MO, USA). RPMI1640, M199, heparin, penicillin-streptomycin and other cell culture supplies were from GIBCO-BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA). Monoclonal sle^x antibody (CD15s, mouse IgM) and monoclonal E-selectin antibody (CD62E, mouse IgG) were purchased from BD Pharmingen (San Diego, CA, USA). Fluorescein (FITC)-conjugated goat anti-mouse IgM and IgG antibodies were obtained through Southern Biotechnology Associates (Birmingham, AL, USA). Peroxidase-conjugated Affinipure Goat anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 5(6)-Carboxy fluorescein diacetate succinimidyl ester (CFSE) was from Molecular Probes (Eugene, OR, USA). Recombinant human TNF-α was purchased from PEProTech (CytoLab Ltd, Israel). The E-selectin and β-actin primers were from TakaRa Biotechnology, Japan.

Cell cultures and Andro treatment. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord by mild trypsinization. The endothelial cells obtained were grown on gelatin-coated tissue culture flasks in M199 medium supplemented with 20% fetal bovine serum, 50 μg/ml endothelial cell growth supplement, 5 U/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. For subculturing, the cells were dislodged using 0.125% trypsin/0.01M EDTA solution in Pucks saline and HEPES buffer. The cells were determined to be endothelial by their cobblestone morphology and the expression of the von Willebrand factor. 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 μg/ml of streptomycin. Tumor cells were grown as adherent monolayers but were used in a single cell suspension form for adhesion experiments. 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 and 50 μM Andro. Then human recombinant TNF-α at a concentration of 20 ng/ml was added, and cells were incubated for 4 h. For the control group, HUVEC monolayers were incubated at 37˚C for 10 h in serum-free media.

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

Immunostaining. Immunostaining was performed as reported previously (15, 16). Briefly, the Andro-treated HUVECs 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 the dark. Then slides were washed twice and evaluated using a fluorescence microscope (Olympus IX-500) 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 (17) *et al.* HUVECs were grown to confluence in 96 well, flat bottom, gelatin-coated plates. The cells were incubated with or without Andro 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, E-selectin antibody (3 μg/ml) was added to the cells and allowed to incubate overnight at 4˚C. Subsequently, unbound antibody was removed; the cells were washed three times with PBS, and then incubated with HRP-conjugated goat anti-mouse IgG (1:1000) for 1 h at room temperature. At the end of 1-h incubation, the unbound secondary antibody was removed and the cells were washed three times with PBS, and then exposed to the peroxidase substrate (*O*-phenylenediamine dihydrochloride, 40 mg/100 ml in citrate phosphate buffer, pH4.5). Color development reaction was stopped by the addition of 2 N sulfuric acid. Absorbance was determined at 492 nm by an automated microplate reader (Tecan Sunrise, Austria).

Reverse transcription and polymerase chain reaction (RT-PCR). The total RNA was isolated according to a modified guanidinium thiocyanate procedure (18) using Trizol. The expression of the transcripts for E-selectin was evaluated by RT-PCR. 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 s; 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 scan-analyzed. 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 (19). Briefly, HUVECs were plated at density of $1x10^4$ cells/well in collagen-coated 96-well plates. After incubation overnight at 37˚C, HUVECs were incubated with different concentrations of Andro, followed by 20 ng/ml of TNF-α for 4 h prior to 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 CO_2 incubator. In the case of sle^x, the antibody (CD15s, 50 μg/ml) was incubated with gastric cancer cells and GES-1 cells for 1 h at 37˚C. Then the gastric cancer cells and GES-1 cells were prelabeled with CFSE by the method of van Kessel *et al.* (20). The CFSE-labeled cells ($1x10^5$ cells/100 μl/well) were added onto a confluent monolayer culture of HUVECs 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 a fluorescent plate reader (Tecan GENios, Austria) at an excitation of 485 nm and emission at 530 nm. Cell adhesion was calculated as follows: % cell adhesion = mean fluorescence intensity of experimental wells/ mean fluorescence intensity of total cells plated x100%.

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

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

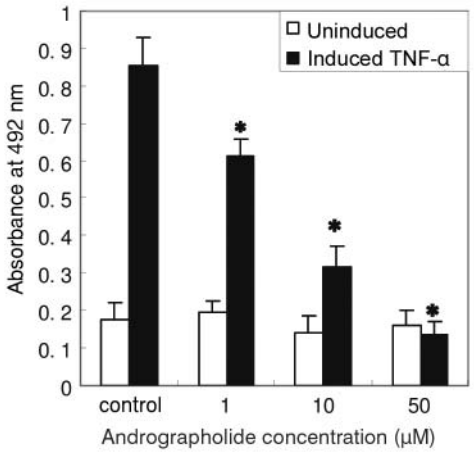

Figure 1. *Andro inhibited TNF-α-induced E-selectin expression: Endothelial cells grown to confluence in 96-well plates were incubated with or without the indicated concentrations of Andro for 6 h prior to induction without (open bars) or with TNF-α (closed bars) for 4 h. Following this, E-selectin level on the cells was measured by ELISA. Bars represent mean±SD of three independent experiments (four replicates per experiment), *p<0.05 as compared to control.*

Inhibition of E-selectin mRNA induction by Andro. To test whether the suppression of E-selectin antigen expression by Andro is due to the suppression of E-selectin gene expression, we investigated the effect of Andro on the steady-state levels of E-selectin transcripts by RT-PCR. Endothelial cells were incubated with different concentrations of Andro for 6 h prior to induction with TNF-α. As shown in Figure 3, there was a statistically significant increase in E-selectin mRNA following stimulating with TNF-α as compared to low levels in control cells. Andro, in a dose-dependent manner, suppressed TNF-α-induced E-selectin mRNA expression. A 50 μM concentration of Andro almost completely abrogated the TNF-α induction of E-selectin mRNA in endothelial cells whereas the levels of β-actin mRNA expressed under these conditions remained the same. These results indicate that Andro may inhibite E-selectin mRNA induction of endothelial cells.

Sle^x expression on gastric cancer cells. The carbohydrate antigen, sle^x, which is frequently present on human malignant cells, is known to be a ligand for the cell adhesion

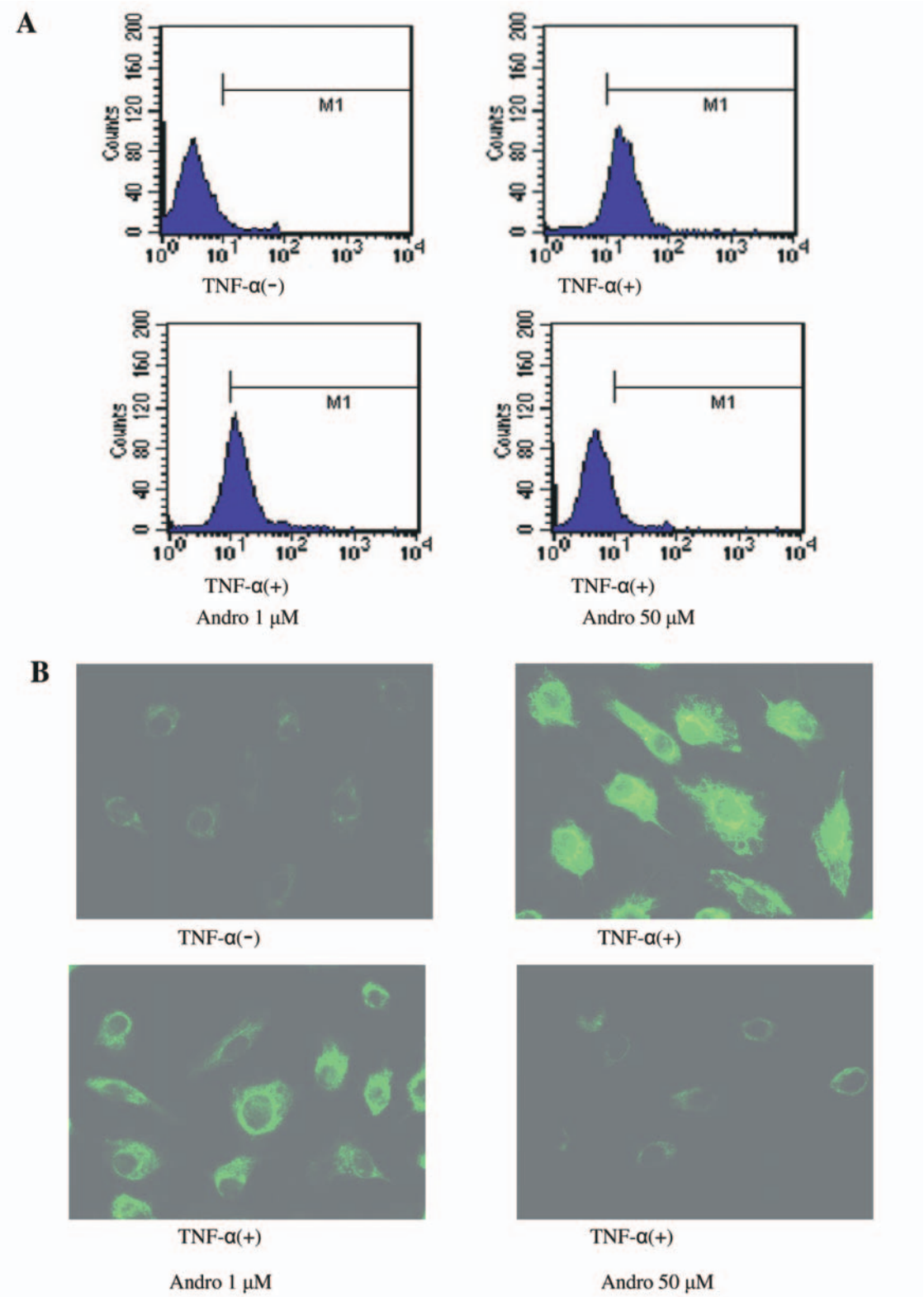

Figure 2. *Inhibition of E-selectin induction on endothelial cells by Andro. Before stimulation with TNF-α, endothelial cells were treated with 1, 10 or 50 μM Andro. A) The expression of E-selectin on the stimulated endothelial cells was determined by flow cytometry. Results were presented as histograms of the log fluorescence intensities from 10^4 cells from three independent experiments. B) E-selectin protein expression was determined on the surface of endothelial cells by immunostaining after treatment with Andro at the indicated concentrations.*

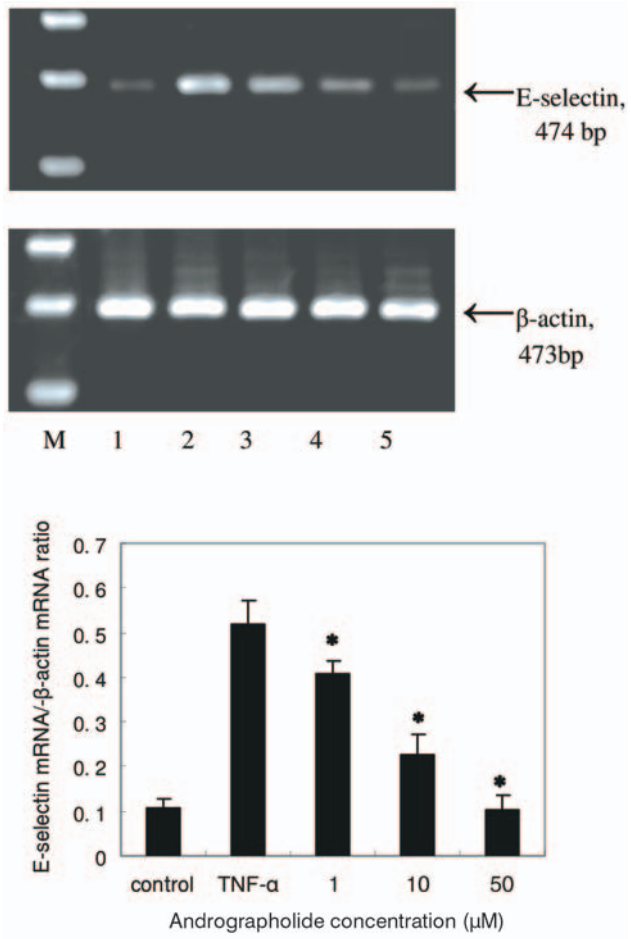

Figure 3. *Effects of Andro on E-selectin gene expression: The endothelial cells were incubated with various concentrations of Andro before stimulation with TNF-α. Following treatment exposure, RNA was isolated and the expression of E-selectin was analyzed by RT-PCR (upper panel), followed by densitomeric measurements. The experiments were repeated four times, and the ratios of E-selectin to β-actin mRNA levels were statically analyzed (lower panel). The data shown are the means±SD of four determinations. M: Marker 1: Control 2:TNF-α 3: Andro1 μM 4: Andro 10 μM 5: Andro 50 μM.*

molecule E-selectin. Therefore, we investigated the sle^x expression on three kinds of highly metastatic gastric cancer cell lines. As revealed by flow cytometry, SGC-7901, BGC-823 and MGC-803 had a high level of sle^x. In contrast, GES-1 only slightly expressed sle^x (Figure 4A). These results were further confirmed by immunostaining (Figure 4B).

Andro suppresses gastric cancer cell adhesion to activated endothelium through inhibiting the adhesion of sle^x/E-selectin molecules. To test the effect of Andro on gastric cancer cell adhesion to endothelial cells, confluent monolayers of HUVECs were pretreated with various concentrations of Andro 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 Figure 5, TNF-α simulation of endothelial cells significantly increased the adherence of those gastric cancer cells expressing high levels of sle^x. Furthermore, the Andro treatment suppressed the adhesion of gastric cancer cells to activated endothelial cells in a dose-dependent manner. A concentration of 50 μM Andro almost completely inhibited the adhesion. However, neither TNF-α stimulation of endothelial cells nor Andro or antibody significantly affected the adherence of GES-1, which had a low level of sle^x.

To confirm that the gastric cancer cell adhesion to endothelial cells was due to the cognate interaction between sle^x and E-selectin, specific antibodies were applied to block these molecules before 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 (Figure 5). When HUVECs were preincubated 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 preincubated with the antibody to sle^x, the gastric cancer cell adhesion to activated endothelial cells was also blocked. Additional experiments have shown that pretreatment of gastric cancer cells or GES-1 cells with Andro had no effect on their binding to activated endothelial cells (data not shown). Overall, these data suggest that Andro suppressed gastric cancer cell adhesion to endothelial cells through inhibiting adhesion of sle^x/ E-selectin molecules.

Discussion

Andrographis paniculata, which is a rich source for Andro (21, 22), has long been used as a folk remedy for alleviation of inflammatory disorders in Southeast Asian countries. Recently, Andro has been shown to have anticancer activity (9-12). However, Andro's role as an anticancer agent in humans and its molecular mechanism of action has not been fully elucidated. Andro is thought to exert its effect by modulating cell-cycle proteins and blocking the cell cycle at the G0-G1 phase in some kinds of cancer cells (9, 10). However, Kim (12) had demonstrated that Andro-induced cell death was achieved through the apoptotic pathway, *via* the activation of an extrinsic caspase cascade. Recently, it was reported that Andro suppressed the activation of NF-κB in stimulate endothelial cells, which reduced the expression of the cell adhesion molecule E-selectin and prevented E-selectin-mediated leukocyte adhesion under flow (14). E-selectin is considered to play an important role in hematogenous metastasis (3, 23-25). Previous studies have shown that among the cell adhesion molecules, including the selectins, immunoglobulin superfamily and integrins, E-selectin is the only molecule that has a significant role in

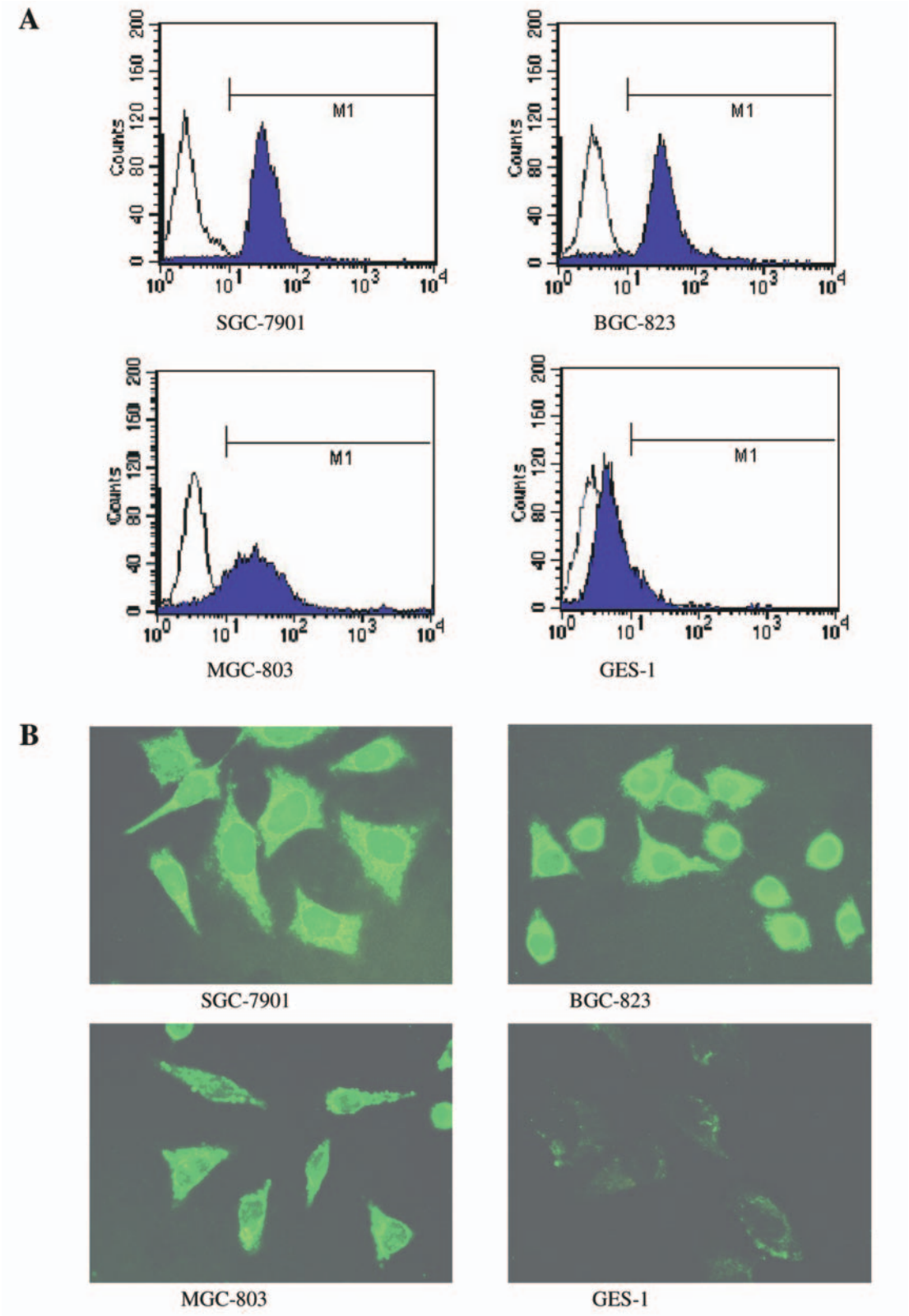

Figure 4. *Sle^X expression on the surface of three gastric cancer cell lines and a normal gastric epithelial cell line GES-1. A) Cells were stained with sle^X monoclonal antibody and treated with FITC-conjugated second antibody; fluorescence intensity associated with cells was analyzed by flow cytometry as described under Materials and Methods. B) Sle^X expression on the surface of the cells was determined by immunostaining.*

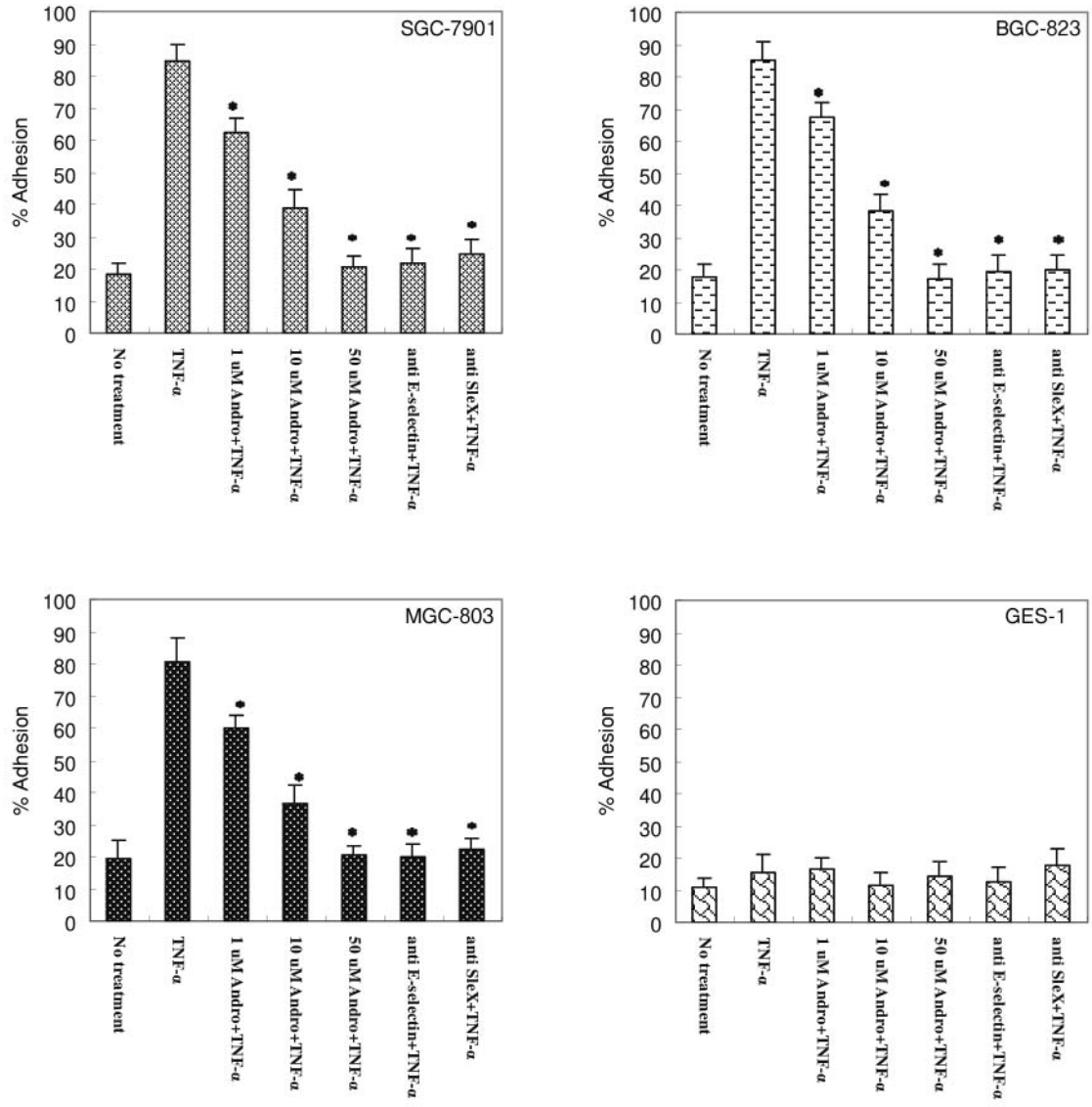

Figure 5. *Adhesion of gastric cancer cells to endothelial cells. Fluorescently labeled gastric cancer cells were overlaid on endothelial cells and incubated at 37˚C for 1h. After gentle washing to remove nonadherent cells, the fluorescence was measured with a fluorescent plate reader (EX=485 nm, EM=530 nm). Bars represent the mean±SD of three separate experiments (three replicates per experiment). *p<0.05 as compared to TNF-α group.*

the adhesion of colorectal cancer cells to vascular endothelium (26, 27). In addition, circulating levels of this adhesion molecule were identified as useful clinical markers of tumor progression and metastasis (28). Recent experimental evidence also indicated that the inhibition of E-selectin-mediated cancer cell adhesion may be an efficient strategy to inhibit cancer metastasis (29). These observations have led to the hypothesis that Andro could inhibit the adhesion of cancer cells to the endothelium by blocking the endothelial cell adhesion molecules, thus having the potential for being developed as a therapeutic agent against cancer. Hence in this study, we investigated the effect of Andro on the adherence of gastric cancer cells to the activated endothelium *in vitro*.

Our present study demonstrated that Andro can inhibit the TNF-α-induced overexpression of E-selectin and thus reduce the adhesion of gastric cancer cells to the activated endothelial cells. The data clearly document a marked reduction of down-regulation of E-selectin antigen expression on the endothelial cell surface when the endothelial cells were exposed to Andro before they were stimulated with TNF-α. Furthermore, Andro also was

shown to inhibit TNF-α-induced E-selectin mRNA expression. Moreover, the concentrations of Andro required to suppress gastric cancer cell adhesion to endothelial cells, as well as E-selectin expression were in a similar range. These findings suggest that Andro-mediated inhibition of gastric cancer cell adhesion stems from its inhibitory effect on E-selectin expression on the surface of endothelial cells. Consistent with this, anti- E-selectin antibody was shown to block the gastric cancer cell adhesion to endothelial cells.

Studies from other investigators showed that Andro suppressed the activation of NF-κB in stimulated endothelial cells (14) and HL-60-derived neutrophlic cells (31). The inducibility of the E-selectin gene by LPS and cytokines requires the activation of NF-κB, and the impairment of NF-κB activation leads to impairment in E-selectin gene expression (13). Thus, it is quite likely that Andro-mediated suppression of E-selectin mRNA expression is the result of inhibition of NF-κB activation by Andro.

Both sialyl Lewis^A (sle^a) and sle^x 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 an sle^a- and sle^x-dependent manner (3, 25). Many reports have noted that sle^x and sle^a/E-selectin or P-selectin cell adhesion molecules play an important role in the adhesion of epithelial cells, expressing more sle^x than do their poorly metastatic counterparts (32). Moreover, in human lung and colon carcinoma, highly metastatic tumor cells express more sle^x on the cell surface and bind more strongly to E-selectin than with their poorly metastatic counterparts (25, 33). Recently, B16-F1 cells which were stably transfected with α1,3-fucocyltransferaseIII (B16-TIII) to express sle^x structures produced larger numbers of lung tumor nodules than their parent cells (34). Furthermore, the peptide mimics E-selectin ligand, inhibits binding of B16-TIII to E-selectin and can inhibit lung colonization of B16-TIII cells (35).

We also found that three highly metastatic gastric cancer cell lines adhered to E-selectin-expressing endothelial cells, whereas GES-1 cells did not. Pre-incubation with sle^x antibody almost nullified the effect of Andro on adhesion of these gastric cancer cells. However, neither Andro nor these antibodies had effects on the adhesion of GES-1 to activated endothelial cells. These results indicated that these gastric cancer cells adhere to E-selectin-expressing endothelial cells through sle^x. In fact, flow cytometry analysis and cell immunostaining confirmed that these gastric cancer cells express high levels of sle^x. We also observed that GES-1 cells expressed low levels of sle^x. Selective suppression of adherence of gastric cancer cells and normal gastric epithelial cells by Andro was explained partly by the difference in expression of sle^x.

To our knowledge, this is the first study that demonstrates that Andro suppresses the adhesion of gastric cancer cells to human vascular endothelial cells by blocking E-selectin expression. These data suggest that Andro is an interesting pharmacophore with anticancer activities and has the potential to be developed as a cancer therapeutic agent. Additional analyses on the actions of Andro are necessary to identify a novel therapeutic target against cancer aggression.

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