Bikunin is a Kunitz-type protease inhibitor, acting at the level of tumor invasion and metastasis. The goal of this study was to investigate the effect of bikunin-dependent signal transduction involved in the expression of a plasminogen activator (PA) system and invasion. We report here the following. 1) The human ovarian cancer cell line HRA produced secreted and cell-associated urokinase-type PA (uPA) and PA inhibitor type 1 (PAI-1). The plasma membrane of the cells showed enzymatically active uPA even in the presence of high level of PAI-1, as measured by zymography, Western blot, chromogenic assay, enzyme-linked immunosorbent assay, and Northern blot. 2) HRA cells leading to invasion are induced through up-regulation of uPA expression. 3) HRA cells specifically released transforming growth factor-β type 1 (TGF-β1) participating in an autocrine/paracrine regulation of cell invasion. 4) Elimination of endogenous TGF-β1 could induce change in uPA/PAI-1 expression, which could in turn modify the invasive behavior of the cells. 5) The constitutive expression of TGF-β1 as well as up-regulation of the PA system observed in HRA cells was inhibited by preincubation of the cells with bikunin or calcium channel blocker SK&F 96365 but not with nifedipine or verapamil, with an IC50 of ~100 nM for bikunin or ~30 μM for SK&F 96365, respectively, as measured by enzyme-linked immunosorbent assay. Bikunin showed no additive effect on SK&F 96365-mediated suppression of TGF-β1 expression. 6) The ability of TGF-β1 to elevate free intracellular Ca2+ followed by activation of Src and ERK, was reduced by preincubation of the cells with bikunin. In conclusion, bikunin could inhibit the constitutive expression of TGF-β1 and TGF-β1-mediated, Src- and ERK-dependent, PA system signaling cascade, at least in part, through inhibition of a non-voltage-sensitive calcium channel.

Tumor cell invasion is dependent on finely regulated extracellular proteolytic activity, which allows tumor cells to invade the extracellular matrix (1). Among the proteolytic enzymes involved in this process are PAs, whose expression in cultured cells is regulated by several types of growth factors and cytokines (2). Invasive tumor cells not only express cell-associated proteases but also secrete anti-proteases, preventing the over-digestion of the extracellular matrix, which leads to a loss of cell attachment. The balance between proteolytic activity and inhibition is crucial in the invasive and metastatic event (3). Indeed, the increment of a specific PA inhibitor, PAI-1, could have an important regulatory role on the extracellular proteolysis and might explain the decrease of net PA and gelatinolytic activities measured in the medium (3, 4). Even in the high levels of PAI-1 in the medium, however, several growth factors, including TGF-β1, induced the increase of net PA and gelatinolytic activities on the plasma membrane, which results in strong proteolytic activity in some specific sites, such as areas of cell-to-cell or cell-to-extracellular matrix contacts. For this reason, drugs that manipulate or suppress signal transduction on the PA system in malignant cells offer a potentially new approach to anti-cancer therapy.

One prototype signal transduction therapy agent is bikunin, which is a Kunitz-type protease inhibitor with tumor-suppressive potential in several malignant cell types, acting at the level of tumor invasion and metastasis (5–7). It was subsequently demonstrated that bikunin inhibits tumor invasion, at least in part, by a direct inhibition of plasmin activity as well as by inhibiting uPA (5, 6) and uPAR (7) expression at the gene and protein levels. Interestingly, in cell-free solutions, bikunin does not inhibit uPA activity effectively. Mechanistic studies in several cell types demonstrated that bikunin interferes with an upstream target(s) of selected MAP kinase signaling processes such as phosphorylation of MEK and ERK, leading to overexpression of uPA (6). A recent study in our laboratory demonstrated that bikunin could interfere with selected calcium-sensitive signaling processes such as agonist-induced cytokine expression in several types of cells, including human umbilical vein endothelial cells, uterine myometrial cells, vascular endothelial cells, neutrophils, HL60 leukemia cells, and tumor cells (8, 9). It has been established that calcium mobilization is a common signaling response in malignant and nonmalignant cells treated with a variety of growth factors, including TGF-β and TNF-α, which are associated with tumor progression (10). These findings underscore the importance of calcium in the biology of malignant cells.
We have been reported that in preclinical studies with several types of rodent and human malignant cells, bikunin has proven effective as an anti-invasive and anti-metastatic agent (5–7). In recently completed clinical studies, bikunin has shown promise at stabilizing disease progression in patients with advanced ovarian cancer (11). Despite the recent biochemical evidence that bikunin specifically inhibits expression of uPA and uPAR mRNA and proteins, the molecular mechanism underlying the tumor-suppressive effect of bikunin remains elusive.

A growing body of evidence has recently implicated that members of TGF-β family are potent regulators of multiple cellular functions, including cell proliferation, differentiation, migration, organization, and death (4, 12, 13). TGF-β1 is produced by host cells and/or tumor cells in a latent form and is activated by proteases in a cell-dependent manner. TGF-β1 produced by breast cancer (12) and prostate cancer (4) and breast cancer (12–14) is a candidate responsible for the induction of PA system expression. The TGF-β1 may promote invasion of tumor cells by increasing uPA activity and PAI-1 levels. In contrast, the TGF-β1 signaling pathway is considered to be one of the most important mechanisms for tumor suppression (16). Therefore, the role of TGF-β1 on tumor cell invasion is controversial.

In our experimental system, we chose the human ovarian cancer cell line HRA because these cells mediate plasminogen activation primarily by uPA but produce low amounts of matrix metalloproteases (MMPs), and the cell invasion is a uPA-dependent process. We examined whether bikunin can reduce cell invasion by modulating cell surface expression of PA system through suppression of tumor-associated production of cytokines, including TGF-β1. We also investigated whether bikunin can inhibit tumor-associated production of TGF-β1 and the TGF-β1-stimulated PA system through suppression of the calcium-dependent signaling.

**MATERIALS AND METHODS**

**Cell Culture**—The human ovarian cancer cell line HRA was grown and cultured as described previously (6). The HRA cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum in a cell culture incubator (constantly set at 37 °C with 5% CO2).

**Chemicals and Reagents**—Bikunin was purified from human urine as described previously (5–7). Reagents of molecular biology grade for protein gel electrophoresis, protein staining, and protein concentration analyses were purchased from Bio-Rad. Proteolytic enzymes, protease substrates, and specific protease inhibitors were obtained from American Diagnostica (Greenwich, CT) and include recombinant single-chain tPA, high molecular weight recombinant uPA, synthetic chromogenic substrate of uPA (Spectrozyme TPA), Glu-type plasminogen, purified plasmin, chromogenic plasmin substrate (Spectrozyme PL), purified tPA-neutralizing monoclonal antibody (373B), purified uPA-neutralizing monoclonal antibody (394), and anti-PAI-1 antibody (379). Monoclonal antibodies against MMP-2 and MMP-9 were from Oncogene Science (Cambridge, MA) and R & D Systems (Minneapolis, MN). Rabbit polyclonal antibodies against the total and phosphorylated ERK were from Zymed Laboratories Inc. (San Francisco, CA) and Calbiochem, respectively. Polyclonal antibodies that recognize the total and phosphorylated (activated) forms of JNK and p38 were from Santa Cruz Biotechnology. Ultrarapid natural human TGF-β1 was from Genzyme (Cambridge, MA) and R & D Systems. Neutralizing antibody against TGF-β1 was purchased from R & D Systems and Sigma. Neutralizing mouse monoclonal IgG antibodies to TNF-α or IL-1 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). MEK inhibitor PD98059 was from New England BioLabs (Beverly, MA). Matrigel was purchased from Collaborative Research (Bedford, MA). PP2 (Calbiochem), genistein, nifedipine, sodium vanadate, and verapamil were from Sigma. SK&F 96365 was obtained from BioMol (Plymouth Meeting, PA) and dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration was held constant throughout each experiment at either 0.5% or 1%. Control experiments indicated that neither of these dimethyl sulfoxide concentrations affected gene expression or cell viability. Unless otherwise specified, all other chemicals and reagents were of the highest purity and obtained from Sigma.

**Preparation of Conditioned Medium and Cell Lysate**—Cells were seeded in 35-mm dishes and basically 24 h later washed twice with phosphate-buffered saline and then treated with several reagents, including bikunin or TGF-β1 in serum-free medium. The conditioned medium was produced by incubating the cells (~90% confluent) for 24 h in a serum-free medium. All conditioned medium thus obtained was concentrated ~10-fold in Centri-10 units (Amicon). The cell monolayers were extracted with 0.2 M Tris-HCl, pH 7.5, 25% Triton X-100 (v/v) in the presence of inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin) at 4 °C for 15 min and scraped with a rubber policeman. Cell extracts were then centrifuged at 3,000 × g to remove cell debris. All samples were stored at −20 °C until use. In parallel, cells treated in the same condition in different dishes were harvested and counted using a hemocytometer.

**Quantitation of uPA, PAI-1, and TGF-β1 Levels**—The amount of uPA was measured in the cell-conditioned medium and in the cell lysate using an Imubind uPA ELISA kit from American Diagnostica. PAI-1 levels in the cell-conditioned medium were determined using an Imubind PAI-1 ELISA kit from the same vendor. Lower detection levels were set at 10 pg/ml for the uPA kit and below 1 ng/ml for the PAI-1 kit. Total and total (active plus latent) TGF-β1 were measured using a human TGF-β1 ELISA kit (BIOSOURCE International Company, Tokyo) according to the manufacturer’s instructions. To measure total TGF-β1, samples were acidified with acetic acid and then neutralized as described previously (17).

**Detection of Cell Surface-associated PAs by Colorimetric Assay**—HRA cells were seeded at 10,000 cells/well in 96-well plates in the maintenance medium and allowed to reach ~90% confluence. The cells were washed once with phosphate-buffered saline and analyzed in a reaction buffer (100 μl/well) containing 0.8 mM MgCl2 and 0.2 μg/ml leupeptin. The reactions were initiated by the addition of the chromogenic amido-lytic substrate specific for either uPA (Spectrozyme UK) or tPA (Spectrozyme TPA) to a final concentration of 0.2 mM in the absence or presence of 1 mM amiloride or neutralizing antibodies to 10 μg/ml uPA or 10 μg/ml tPA. No amiloride substrate was added in the blank reactions. The photometric absorbance of the reaction mixtures at 405 nm was monitored at 37 °C over the next 30 min. The trypan blue cell staining procedure was performed as described by Broman et al. (18). Plasmin activities in cell-conditioned medium and cell lysate were determined spectrophotometrically using a Spectrozyme PL as described (12).

**3HThymidine Incorporation**—Cells were seeded in 24-well plates (Corning Costar Corp.). After 2 days, cells were washed with phosphate-buffered saline and treated with the indicated concentrations of TGF-β1 for 4 h in serum-free medium. Four h before the end of culture, cells were incubated with 2 μCi/ml [3H]thymidine (ICN, specific activity 2 Ci/mmol). Cells were then washed with 10% trichloroacetic acid at 4 °C followed by four washes with water and solubilized with 0.5 M sodium hydroxide; the radioactivity was measured with a liquid scintillation counter (LKB).

**Cell Growth Assay**—HRA cells were grown on a flat bottomed 96-well plate (9,000 cells/well) in the serum-free medium including 10% fetal bovine serum. 24 h later, cells were grown in RPMI 1640 medium with or without several reagents. After 1, 3, and 5 days of culture, cell proliferation was assayed by the addition of 10 μl/well dimethyl sulfoxide and its absorbance was directly proportional to the number of cells.

**Substrate Zymography for Protease Activities**—Confluent monolayers of HRA cells in 35-mm tissue culture dishes were washed twice with serum-free medium and incubated for 12 h in the presence of serum-free medium, the indicated concentrations of TGF-β1, neutralizing antibody to TGF-β1, TNF-α, IL-1β, or prionsome IgG. At the end of the incubation, cell extracts and supernatants were analyzed by zymography as described (19). Conditioned medium (10 μl of 10 × concentrated) and 20 μg of cell lysate were electrophoresed in nonreducing SDS-PAGE conditions in gel copolymerized with 0.1% gelatin for gelatinase activities and with 0.1% casein plus 15 μg/ml Glu-
plasminogen for PA activities. Plasminogen-dependent gelatinolytic zymogram was performed as described previously (20).

Effects of Inhibitors on uPA Expression—HRA cells were cultured and serum starved. Treatment was achieved by adding 0.5–50 μM SK&F 96365 to the medium for 20 h. In addition, cells were incubated for 12 h with one of the following agents: 50 μM MEK inhibitor PD98059, 50 μM t-type calcium channel blocker nifedipine or verapamil, 25 μM general tyrosine kinase inhibitor genistein, or 10 μM selective Src tyrosine kinase inhibitor PP2. Control cells received just vehicle (0.1% dimethyl sulfoxide for PD98059, SK&F 96365, genistein, and PP2; 0.1% ethanol for nifedipine and verapamil).

Isolation of Cytoplasmic RNA and Northern Blot Analysis—The HRA cells were plated at a density of 10^6 cells/100-mm dish. When cells grown in monolayer reached an early phase of confluence, total cellular RNA samples were isolated from cells using a Qiagen RNeasy kit (Qiagen Ltd.) according to the manufacturer’s protocol. For Northern blot analysis, 20 μg of cytoplasmic RNA was electrophoresed onto a formaldehyde and 1.0% agarose gel and blotted onto a nylon filter. After transfer, RNA was cross-linked onto the membranes by UV irradiation. RNA samples were isolated from cells using a Qiagen RNeasy kit according to the manufacturer’s instructions. The experiments were repeated three times.

Invasion Assay—The ability of cells to migrate across a Matrigel barrier (invasion) was determined by the modified Boyden chamber method (25). Briefly, HRA cells (10^5/chamber) were added to polycarbonate filters coated with 50 μg/ml Matrigel and incubated with complete medium containing 0.1% bovine serum albumin. Filters were removed from the chambers and stained with hematoxylin. Cells were counted at a 100× magnification, and the mean numbers of cells/field in five random fields were recorded. Duplicate filters were used, and the experiments were repeated three times.

FIG. 1. Plasminogen activator system in the cell lysate and the conditioned medium of HRA cells. A and B, Zymographic analysis. Gelatinolytic zymographic profiles of plasminogen activators (A) and matrix metalloproteases (B) in the cell lysate (CL) and conditioned medium (CM) of HRA cells are shown. A total of 20 μg of the lysate protein or 10 μl of the 10× concentrated conditioned medium was loaded on the zymogram gels. Purified high molecular weight uPA (0.1 NIH unit) and tPA (0.2 NIH unit) were used as standards. The detected signals were identified on the basis of their relative molecular masses (arrows). C and D, Western blot analysis. The cell lysate and conditioned medium protein of HRA cells were analyzed by Western blotting using anti-uPA antibody (C) or anti-PAI-1 antibody (D), respectively. E, colorimetric analysis. The monolayer of HRA cells was analyzed by colorimetric assay in the presence of the Spectrozyme UK or Spectrozyme TPA in the presence or absence of amiloride, neutralizing antibodies to uPA, or tPA. Open circles, Spectrozyme UK alone; open squares, Spectrozyme TPA alone; open triangles, Spectrozyme UK plus 1 mM amiloride; filled squares, Spectrozyme UK plus 10 μg/ml neutralizing anti-uPA antibody; filled circles, Spectrozyme TPA plus 10 μg/ml neutralizing anti-tPA antibody; and filled triangles, Spectrozyme UK plus 10 μg/ml neutralizing anti-tPA antibody. The data represent an average result of triplicate repeats, and the error bars indicate the S.D. values, which are less than 15%.
Bikunin and TGF-β1 Signaling

7793

Fig. 2. HRA cell invasion through cell surface-mediated uPA. The cell invasion assay was performed in the presence of no additional factor, 2.5, 5, and 10 µg/ml uPA-neutralizing antibody; 10 µg/ml tPA-neutralizing antibody; 10 µg/ml MMP-2-neutralizing antibody; 10 µg/ml MMP-9-neutralizing antibody; 10 µg/ml preimmune IgG; 1 mM amiloride; 1 mM E64; 1 µM bikunin; and 1 µM bikunin plus 10 µg/ml uPA-neutralizing antibody. Results are expressed as the average number of cells present in five fields/membrane and are the mean ± S.D. of three different determinations; unlike letters (a–e) stand for statistical differences (p < 0.05).

HRA Cells Produce Primarily uPA, but Not tPA or MMPs—

The human ovarian cancer cell line HRA was chosen for our study because we have shown previously that HRA cells exhibits a uPA-dependent invasion and metastasis (6). In this study, HRA cells were evaluated for their capability to secrete and activate PAs and MMPs in great detail. Fig. 1, A and B, shows the zymographic profiles of the plasminogen-dependent (for PA (A)) and plasminogen-independent (for MMP (B)) gelatinolytic activities of both the cell lysate and the conditioned medium (10× concentrated) of HRA cells. Compared with the purified tPA, uPA, MMP-2, and MMP-9 standards, both the lysate and the conditioned medium of HRA cells contained primarily uPA, although a trace amount of tPA was also detected (Fig. 1A). Gelatin zymography (Fig. 1B) and Western blotting (not shown) indicate that HRA cells basally secreted detectable but low amounts of 92- and 65-kDa gelatinases whose positions identify the produced molecules as pro-MMP-9 and pro-MMP-2 (Fig. 1B). As shown in Fig. 1, C and D, analysis of the uPA and PAI-1 levels using Western blotting showed a band of 33 kDa (for low molecular mass uPA), 50 kDa (for high molecular mass uPA), 60 kDa (for PAI-1), 110 kDa (for uPA-PAI-1 complex), and 130 kDa (for tPA-PAI-1 complex) in the conditioned medium and the cell lysate. Moreover, HRA cells produced extremely low amounts of PAI-2 as revealed by ELISA (data not shown). ELISA data showed that the basal cell-associated uPA levels measured in HRA cells were 14.8 ± 2.1 ng/mg cell lysate protein (mean ± S.D.), whereas the basal level of uPA released in the medium by the cells during a 24-h period was 12.0 ± 3.8 ng/ml/10⁶ cells/24 h. Furthermore, the cells also produced cell-associated PAI-1 and secreted PAI-1 (5.2 ± 1.7 ng/mg protein for cell-associated PAI-1; 30.2 ± 8.7 ng/ml/10⁶ cells/24 h for secreted PAI-1).

The conditioned medium of HRA cells was not very active in converting Glu-plasminogen to plasmin, which in turn did not lead to the cleavage of Spectrozyme PL. In contrast, when Spectrozyme UK or Spectrozyme TPA was added to the monolayer of HRA cells, the Spectrozyme UK was specifically cleaved (Fig. 1E). 1 mM amiloride (a catalytic inhibitor of uPA) and 10 µg/ml uPA-neutralizing antibody, but not 10 µg/ml tPA-neutralizing antibody, blocked the cleavage of Spectrozyme UK on the plasma membrane of the HRA cells. It is worth noting that throughout this biochemical assay, cells remained intact, showing little or no plasma membrane permeability by trypan blue and a full recovery of proliferation in the subsequent cell growth assay (data not shown).

HRA Cells Invade through a uPA-dependent Mechanism—

Effects of bikunin at concentrations from 1 to 1,000 nM on the cell proliferation were tested in dose-response and time course experiments over a 14-day period. Bikunin concentrations varying from 1 to 1,000 nM did not show any significant effect on growth (data not shown). Thus, the invasion assay could be performed at a time (36 h) when there was no growth inhibition. To test whether the HRA cells had an ability to invade by a PA system-dependent manner, cells were preincubated with E64 and amiloride as well as specific neutralizing antibodies to uPA, tPA, MMP-2, or MMP-9 and analyzed using a chemoinvasion assay. As shown in Fig. 2, the uPA-neutralizing antibody specifically inhibited HRA cell invasion in a dose-dependent manner. Antibody against uPA A-chain 3471 (containing the domain interacting with uPA receptor) also strongly blocked the invasion. We have shown previously that bikunin inhibits the in vitro invasion of several types of cancer cells, including HRA cells. Cells preincubated with the uPA-neutralizing antibody were not inhibited further by bikunin in the Matrigel invasion assay. Neutralizing antibodies to tPA, MMP-2, and MMP-9 neither inhibited cell invasion nor altered the invasion-inhibitory effect of bikunin (data not shown). In addition, enhanced invasion of HRA cells was inhibited markedly by the uPA-specific inhibitor, amiloride. E64, which totally inhibits soluble cathepsins and uPA, had a weak inhibitory effect.

We next examined whether the uPA level is modulated by
bikunin in the medium collected from the upper chamber during the invasion experiment. The presence of bikunin caused a dramatic reduction of the uPA level (107 ± 21.5 [±bikunin] versus 283 ± 43.6 pg/ml/10^5 cells/36 h [−bikunin]). At a concentration of 1 μM, the uPA level is inhibited by 62%. These data support that the HRA cells leading to invasion are induced through up-regulation of uPA expression and a specific inhibitory activity of bikunin toward the endogenous expression of uPA.

Neutralization of Endogenous TGF-β Specifically Suppresses uPA and PAI-1 mRNA Expression—To investigate whether HRA cells released several types of cytokines participating in an autocrine/paracrine regulation of HRA cell invasion, the conditioned medium of HRA cells was tested by ELISA for TGF-β, TNF-α, IL-1β, or nonimmune IgG. A, total RNAs were extracted and hybridized with uPA, PAI-1, tPA, MMP-2, MMP-9, and GAPDH cDNA. Each filter was loaded with 20 μg of total RNAs. Filters were exposed for 5 h (PAI-1 and β-actin), 24 h (uPA, MMP-2, and MMP-9), or 3 days (tPA). The uPA (B) and PAI-1 (C) levels in the conditioned medium were analyzed by ELISA. Results are from at least three separate experiments. *p < 0.05 compared with the control D, a cell suspension, 200 μl (500,000 cells/ml) in RPMI 1640 containing 1% fetal bovine serum, was placed on the Matrigel-coated surface of the upper chamber. The indicated concentrations of neutralizing monoclonal antibodies to TGF-β1, TNF-α, IL-1β, or nonimmune IgG were added to the upper compartment. After a 36-h incubation, cells that invaded through the Matrigel-coated membrane were stained and counted under the microscope. All experiments were performed three times, and typical data are shown. Results are the mean ± S.D. of three different determinations; unlike letters (a–d) stand for statistical differences (p < 0.05).

ELISA data revealed that in the conditioned medium, the TGF-β1-neutralizing antibody inhibited up-regulation of uPA and PAI-1 mRNA levels. In contrast, antibodies to other cytokines (TNF-α and IL-1β) or nonimmune IgG had no significant effect on expression of uPA and PAI-1 mRNA. No morphologically identifiable signs of cytotoxicity were observed in cells incubated with these antibodies.

Inhibition of Invasion of HRA Cells through TGF-β1-Mediated Modulation of the Plasminogen Activator System—Treatment of the HRA cells with the neutralizing anti-TGF-β1 antibody reduced cell invasion (Fig. 3D). The responses were found to be concentration-dependent. Antibodies against TNF-α and IL-1β reduced HRA cell invasion to a lesser degree, demonstrating that both TNF-α and IL-1β were less potent than TGF-β1. These data allow us to hypothesize that neutralization of endogenous TGF-β1 by the anti-TGF-β1 antibody
could induce change in uPA/PAI-1 expression, which could in turn modify the invasive behavior of HRA cells.

**Effect of Exogenously Added TGF-β1 on Expression of uPA and PAI-1 by HRA Cells and Tumor-promoted Plasmin Activity**—We next investigated whether exogenously applied TGF-β1 could enhance expression of cell surface and secreted uPA and PAI-1 by colorimetric, zymographic, ELISA, and Western blot analyses (Fig. 4). In the cell lysate and cell-conditioned medium, we found that TGF-β1 induced a significant increase in the amount of cell-associated and secreted uPA and PAI-1 compared with untreated cultures. TGF-β1 stimulated uPA production in a dose-dependent manner. The incubation with 10 ng/ml TGF-β1 resulted in about a 5-fold increased cell-associated uPA expression (51.8 ± 1.5 ng/mg protein) (Fig. 4A) and in about 2.5-fold increased expression of uPA in the conditioned medium (30.5 ± 3.3 ng/ml/10^6 cells/24 h versus 12.1 ± 1.1 ng/ml/10^6 cells/24 h) (Fig. 4B). Furthermore, TGF-β1 increased cell-associated PAI-1 about 2-fold and cell-secreted PAI-1 4-fold in HRA cells.

**Zymography** (Fig. 4C) and Western blot (Fig. 4, D and E) analyses revealed that exogenously added TGF-β1 could enhance expression of cell surface (data not shown) and secreted uPA and PAI-1 levels in a dose-dependent manner. Anti-TGF-β1 antibody abrogated the TGF-β1-mediated expression of uPA (Fig. 4F). Concomitantly, plasmin activity decreased to 30% of untreated cells in the cell-conditioned medium and increased 3-fold on the plasma membrane for the TGF-β1-treated cultures by colorimetric assay (data not shown). In contrast, TGF-β1-treated HRA cells secreted low amounts of tPA (data not shown).

**Effect of TGF-β1 on Cell Proliferation**—To examine the sensitivity of ovarian cancer cells to TGF-β1, we measured the anchorage-dependent growth of HRA cells for a 24 h-period of treatment with the drug. Different concentrations of TGF-β1 (0.1–10 ng/ml) were tested for their effect on cell proliferation. At any of the doses tested, TGF-β1 had no effect on cell proliferation during the incubation period because no change in cell growth could be detected for either untreated or TGF-β1-treated samples as assessed with the [3H]thymidine incorporation test. These data indicate that TGF-β1 has no immediate effects on cell proliferation.

**Bikunin Specifically Suppresses Invasion through Down-regulation of TGF-β1**—To assess the specificity of the effect of bikunin, the conditioned medium of HRA cells was tested by ELISA for uPA, PAI-1, and TGF-β1. ELISA data revealed that bikunin specifically suppresses uPA and PAI-1 secretion into the conditioned medium in a dose-dependent manner (Fig. 5A). We showed that treatment of HRA cells with bikunin induced a suppression of endogenously secreted TGF-β1 with doses as low as 30 nM, with an IC50 of ~300 nM (Fig. 5A), which is associated with suppression of uPA/PAI-1 secretion. Confluent
monolayers of HRA cells treated with bikunin showed no evi-
dence of cytotoxicity as measured by the trypan blue exclusion
test or by the MTT assay. We also found that treatment of cells
with bikunin induced a suppression of the TGF-β1-stimulated
secretion of uPA and PAI-1 in a dose-dependent manner (Fig.
5B). In addition, we found that bikunin could reduce the in-
vansion of the HRA cells treated with or without TGF-β1 (Fig.
5C). Thus, bikunin specifically inhibited tumor cell invasion
through suppression of endogenous and exogenous TGF-β1-
mediated secretion of uPA/PAI-1.

Analysis of the Signals Regulating Bikunin-induced Sup-
pression of TGF-β1 and uPA Expression—To analyze the sig-
als regulating bikunin-mediated suppression of TGF-β1, cells
were treated with calcium channel blockers (the L-type volt-
age-sensitive calcium channel blockers nifedipine (50 μM) and
verapamil (50 μM) and the non-voltage-sensitive calcium chan-
nel blocker SK&F 96365 (0.5–50 μM)), protein tyrosine kinase
inhibitor (genistein (25 μM), PP2 (10 μM)), MAP kinase inhibi-
tor (PD98059 (50 μM)), and protein phosphatase (sodium van-
adate (0.1 mM)), or bikunin (1–1,000 nM), aspirate cell-condi-
tioned medium, and assayed for TGF-β1 (Fig. 6A) and uPA
(Fig. 6B) levels by ELISA. The constitutive expression of
TGF-β1 observed in HRA cells was inhibited by preinoculation
of the cells with bikunin or SK&F 96365 but not with nifedip-
ine, verapamil, genistein, PP2, PD98059, or sodium vanadate.
The addition of increasing doses of bikunin and SK&F 96365
strongly decreased TGF-β1 secretion in a dose-dependent man-
ner, with an IC50 of ~30 μM for SK&F 96365. Further, we tried
to establish whether bikunin causes any modulation of SK&F
96365-dependent down-regulation of TGF-β1. However,
bikunin showed no additive effect on SK&F 96365-mediated
suppression of expression of TGF-β1.

In addition, the constitutive expression of uPA observed in
HRA cells was inhibited by preinoculation of the cells with
bikunin, SK&F 96365, genistein, PP2, or PD98059, but not
with nifedipine, verapamil, and sodium vanadate. Again,
bikunin showed no additive effect on SK&F 96365-, genistein-,
or PP2-mediated suppression of uPA expression.

Suppression by Bikunin of TGF-β1-induced Calcium Influx
Leading to Inhibition of ERK Activation—To investigate TGF-
β1-induced signaling in the HRA cells, we examined the Src
and MAP kinase family using HRA cells. Among MAP kinase
family members, expression of ERK, JNK, and p38 proteins
was determined by Western blotting (Fig. 7A). When HRA cells
were exposed to TGF-β1 for various periods, the phosphoryla-
tion of ERK was enhanced from 5 min after addition of 10 ng/ml
TGF-β1 and peaked at 15 min. In contrast, JNK and p38 did
not appear to be activated by TGF-β1. Thus, activation of ERK
was most relevant among three subsets of MAP kinase family
members in response to TGF-β1. Previous reports have dem-
ostrated that an increase of intracellular calcium is important
for activation of ERK (26). Therefore, we examined the con-
tribution of extracellular calcium to the TGF-β1-stimulated ERK
activation using EGTA and SK&F 96365. When HRA cells were
incubated in the presence of EGTA (data not shown) or SK&F
96365, TGF-β1-stimulated phosphorylation of ERK was abro-
gated (Fig. 7B). In addition, the TGF-β1-stimulated ERK activation was also abrogated when HRA cells were treated with bikunin, PP2, or PD98059. Again, bikunin showed no additive effect on SK&F 96365-, PP2-, or PD98059-mediated suppression of the TGF-β1-stimulated activation of ERK. These results suggested that an increase of intracellular calcium via non-voltage-gated calcium channels is required for the TGF-β1-stimulated ERK activation in HRA cells.

We hypothesized that bikunin-mediated suppression of calcium influx could inhibit the constitutive expression of TGF-β1 by HRA cells. We examined the influence of bikunin on calcium mobilization in response to TGF-β1 and TNF-α, two agonists previously shown to elevate cellular calcium in ovarian cancer cells rapidly (10) and stimulate expression of uPA. Using suspension of HRA cells loaded with the calcium fluoroprobe Fura-2, we observed that the ability of TGF-β1 to elevate free intracellular Ca\(^{2+}\) was reduced 50% by a preincubation of the cells with 1 μM bikunin (Fig. 8). Similar attenuations of calcium signaling were observed in bikunin-treated HOC-I cells (human ovarian cancer cell line) (data not shown). SK&F 96365 also inhibited TGF-β1-stimulated elevation of intracellular Ca\(^{2+}\), with 10 μM SK&F 96365 causing significant inhibition. Higher concentration of SK&F 96365 produced further inhibition, with 50 μM agent causing around 85% inhibition. In contrast, nifedipine did not suppress TGF-β1-stimulated intracellular Ca\(^{2+}\) elevation. These results suggest that the bikunin inhibitory effect on TGF-β1 signaling may act, at least in part, through a non-voltage-sensitive calcium-dependent pathway.

**DISCUSSION**

The results of this study extend earlier observations about the inhibitory effect of bikunin on tumor cell invasion (5–7). Our previous data provide evidence that bikunin not only has the capacity of functioning as a protease inhibitor to suppress directly cell surface-associated plasmin activity but it can also reduce cell invasion by modulating uPA mRNA and protein expression through inhibition of an upstream target(s) of protein kinase C- and/or MAP kinase-dependent signaling cascade (6). More recent data (7) demonstrate that uPAR expression is also down-regulated by bikunin in human chondrosarcoma HCS-2/8 cells and two human ovarian cancer cell lines, HRA and HOC-I.

The novel findings of the present study are as follows. First, HRA cells have an ability to invade through a PA system-dependent manner. This is an extension of our previous findings that HRA cells exhibited a uPA-mediated fibrinolytic activity (6). Even in the high levels of PAI-1 in the medium, HRA cells exhibited an increase of net PA activity on the plasma membrane, which results in strong proteolytic activity in some specific sites, such as areas of cell-to-cell or cell-to-extracellular matrix contacts (26, 27). Therefore, up-regulation of net PA activity on the plasma membrane is important for the invasive process of HRA cells. Secreted PAI-1 inhibited activation of plasminogen to plasmin in the growth medium, thereby preventing plasmin-induced detachment of cells (28).

Second, we found that expression of the PA system in HRA cells is dependent on endogenously secreted TGF-β1, that exogenously added TGF-β1 can specifically induce uPA and PAI-1 secretion and promotes binding of uPA at the cell surface, which results in strong proteolytic activity in some specific sites, such as areas of cell-to-cell or cell-to-extracellular matrix contacts (26, 27). Therefore, up-regulation of net PA activity on the plasma membrane is important for the invasive process of HRA cells. Secreted PAI-1 inhibited activation of plasminogen to plasmin in the growth medium, thereby preventing plasmin-induced detachment of cells (28).
Bikunin and TGF-β1 Signaling

Fig. 7. Effects of bikunin on the TGF-β1-stimulated activation of the MAP kinase family members. A, time-dependent MAP kinase activation by TGF-β1 in HRA cells. HRA cells were exposed to 10 ng/ml TGF-β1 for the periods indicated. Cell lysates were prepared; and phosphorylation of ERK, JNK, and p38 was measured by Western blotting analysis using specific antibodies against the phosphorylated proteins. The same filters were immunoblotted with each of the specific antibodies to demonstrate total amounts of ERK, JNK, and p38. B, HRA cells were exposed to 10 ng/ml TGF-β1 in the absence or presence of bikunin, SK&F, PP2, PD98059, SK&F plus bikunin, PP2 plus bikunin, or P98059 plus bikunin for 15 min. Total and phosphorylated ERK were measured by Western blotting. Results are from at least two separate experiments with similar results.

Fig. 8. Effects of bikunin and SK&F 96365 on TGF-β1-induced elevation of [Ca²⁺] in HRA cells. HRA cells were loaded with Fura-2 and preincubated with the 0 mM bikunin (trace 1), 100 nM bikunin (trace 2), 500 nM bikunin (trace 3), or 1,000 nM bikunin (trace 4) or 50 μM SK&F (trace 5) for 30 min before the addition of 10 ng/ml TGF-β1, indicated by the dip in the traces between 0 and 1 min. The results shown are representative of three experiments. Variability between replicates generally was less than 20% in a given experiment.

Fig. 9. Putative mechanism of bikunin-mediated suppression of tumor invasion capacity through the TGF-β1-dependent signal transduction cascade. Bikunin can directly inhibit production of TGF-β1 and TGF-β1-mediated MAP kinase activation through suppression of calcium influx. Reduction of TGF-β1-dependent signals by bikunin might cause inhibition of the PA system, leading to suppression of cell invasion.

Our recent data that the ERK cascade, often an event downstream of Src kinase cascade. A part of this is in agreement with previous data that the ERK cascade involved in phorbol ester-mediated uPA expression to a variety of agents, including phorbol ester (29, 30) and that bikunin could inhibit an upstream component of the ERK cascade involved in phorbol ester-mediated uPA expression. In addition, we found that TGF-β1-stimulated phosphorylation of ERK and uPA/PAI-1 expression was significantly inhibited by bikunin, suggesting that bikunin may function at the upstream target of Src tyrosine kinase and MAP kinase cascade. Therefore, we hypothesize that TGF-β1 and TGF-β1-stimulated phosphorylation of ERK and uPA/PAI-1 expression, suggesting that bikunin may function at the upstream target of Src tyrosine kinase and MAP kinase cascade and the uPAuPAR system (Fig. 9).

Third, SK&F 96365 is an agent known to inhibit non-voltage-gated calcium influx (34). The suppression by bikunin of TGF-β1 and uPA expression could be mimicked by the addition of exogenous SK&F 96365. Thus, bikunin could inhibit the constitutive expression of TGF-β1 and TGF-β1-mediated PA system signaling cascade possibly through suppression of the intracellular calcium ion influx. Removal of extracellular calcium and the non-voltage-sensitive calcium channel blocker SK&F 96365 but not the L-type voltage-sensitive calcium channel blockers nifedipine and verapamil, inhibited both the constitutive expression of TGF-β1 and TGF-β1-stimulated phosphorylation of ERK and uPA/PAI-1 expression, suggesting that the non-voltage-sensitive calcium channel is specifically involved in these processes.

Finally, TGF-β1-stimulated phosphorylation of ERK and uPA/PAI-1 expression was significantly inhibited by bikunin, SK&F 96365, PP2, and by P98059. Bikunin showed no additive effect on SK&F 96365-, PP2-, or P98059-mediated suppression of uPA expression, suggesting that bikunin may function at the upstream target of Src tyrosine kinase and MAP kinase cascade. A part of this is in agreement with previous data that the ERK cascade, often an event downstream of Src tyrosine kinase activation, has been shown to mediate uPA expression to a variety of agents, including phorbol ester (29, 30) and that bikunin could inhibit an upstream component of the ERK cascade involved in phorbol ester-mediated uPA expression in human chondrosarcoma cell line HCS-2/8 (6).

SK&F 96365 and another non-voltage-sensitive calcium channel blocker carboxamidotriazol were shown previously to have anti-metastatic properties at low micromolar concentrations. Above 10 μM, carboxamidotriazol inhibited all second messenger pathways (36). Therefore, the influx of calcium seems to be an event upstream of the activation of Src and ERK, followed by uPA/PAI-1 expression. In addition, we found that TGF-β1 activity needs active uPA because inhibitors of uPaplasm activity (p-aminobenzamidine and anti-catalytic antibodies) block the activation of latent TGF-β1 present in the conditioned medium (data not shown). This suggests that...
TGF-β1 is secreted by HRA cells as a latent form and is activated successively by tumor cell-secreted uPA/plasmin. Taken together, we conclude that expression of uPA/PAI-1 in HRA cells is dependent upon continuous activation of the ERK signal transduction cascade, which is downstream of an influx of extracellular calcium, and activation of Src tyrosine kinases. Thus, bikunin may function as a voltage-independent calcium influx antagonist.

The differences in bikunin sensitivity could be the result of variability in drug metabolism, a higher dependence on calcium-sensitive functions for cell invasiveness in certain types of cells, or a variability in the amounts of bikunin receptors on tumor cell membranes. There appeared to be a specific interaction between bikunin and the tumor cell surface. HRA cells do not produce endogenous bikunin, but they do exhibit two types of bikunin receptors. Bikunin at low concentrations (<200 nm) bound to HRA cells in a dose-dependent manner. As the concentration of bikunin exceeded 200 nm, the amount of cell surface-bound bikunin was not further increased, suggesting that the binding of bikunin had reached a level of saturation (data not shown).

Because calcium is a pleiotropic second messenger in cell regulation and function, it is likely that bikunin will affect several cellular events simultaneously. A recent study of our laboratory demonstrated that bikunin has a specific inhibitory effect on calcium influx (8, 9). The inhibition of the uPA expression seen in bikunin could be caused by efficient inhibition of calcium influx, rather than direct inhibition of protein tyrosine kinase or phosphorylation of MAP kinase cascade, which was confirmed by the in-gel kinase assay.2 It is strongly supported that bikunin interferes with selected calcium-sensitive transmembrane signal transduction events.

Proteins of the Kunitz family may act as a calcium channel blocker. Bovine pancreatic trypsin inhibitor is a representative member of a widely distributed class of serine protease inhibitors known as Kunitz inhibitors. Bovine pancreatic trypsin inhibitor is also homologous to dendrotoxins from mamba snake venom, which has been characterized as an inhibitor of various types of voltage-dependent K channels (39). Bovine pancreatic trypsin inhibitor interferes directly with conductance to the channel and is found to inhibit a large conductance Ca2+-activated K+ channel. The trypsin inhibitory loop of bovine pancreatic trypsin inhibitor recognizes a specific site on the channel protein. Calcitulin isolated from the green sponge (37, 38). There appeared to be a specific interaction between the uPA-binding site on the channel protein and the in-gel kinase assay. It is likely that bikunin will affect regulation and function, it is likely that bikunin will affect.

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