Etomidate Suppresses Invasion and Migration of Human A549 Lung Adenocarcinoma Cells

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Abstract. *Background/Aim:* Etomidate, an intravenous anesthetic, has been shown to have anticancer effects, including induction of cell-cycle arrest and apoptosis. However, to our knowledge, there are no reports about the anti-metastasis effects of etomidate on A549 human lung adenocarcinoma cells. *Materials and Methods:* The cell viability, cell adhesion, gelatin zymography assay, transwell migration and invasion assay, and western blotting analysis were used to investigate the effects of etomidate on A549 cells. *Results:* In our study, etomidate showed low cytotoxicity, inhibited cell adhesion, and suppressed the migration and invasion in A549 cells. The activity of matrix metalloproteinase 2 (MMP2) was reduced by 48 h treatment of etomidate. Results of western blotting analysis indicated that etomidate down-regulated the expression of protein kinase C, MMP7, MMP1,

MMP9, and p-p-38, but up-regulated that of RAS, phosphoinositide 3-kinase, and phosphor-extracellular-signal related kinase after 24 and 48 h treatment, in A549 cells. *Conclusion:* Etomidate suppressed the migration and invasion of lung adenocarcinoma A549 cells via inhibiting the expression of MMP1, MMP2, MMP7 and MMP9, and provides potential therapeutic targets for lung cancer treatment.

Cancer has been the leading cause of death in Taiwan for 31 years (1). According to the International Agency for Research on Cancer, it accounted for 1.8 million deaths (around 18.4% of all deaths) in 2018 worldwide (2). Since 90% of patients with cancer die due to metastases (3), inhibition of metastasis is a major concern in the care of such patients. Although surgery is a major approach for cancer removal, intraoperative manipulation of cancer tissues may release cancer cells into the vascular and lymphatic circulation or to neighboring tissues (4, 5). In those with a normal immune status, macrophage, dendritic cells, natural killer cells and T-cells have the ability to identify and to destroy cancer cells in the circulation. However, compromised immunity blocks these abilities and may lead to recurrence or progression of malignant diseases (6-9). Stressful perioperative events such as pain, surgery, transfusion, hypothermia, hypotension, electrolyte imbalance, and shock, inhalation anesthetics, and morphine depress immunological function can increase the incidence of cancer cell metastasis and cancer recurrence rate in patients treated for cancer with surgery (10, 11).

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Previous studies have revealed that some anesthetics inhibit cancer metastases, not only *via* blocking perioperative stress responses, but also through inducing anticancer effects (12-15). Etomidate is an intravenous anesthetic agent frequently used because it is devoid of cardiovascular side-effects (1, 16). Our previous study reported that etomidate had antitumor effects, including induction of cell-cycle arrest and apoptosis in a murine leukemia cell line (RAW264.7) (16). However, to our knowledge there is no report concerning the effect of etomidate on human lung adenocarcinoma cells. As etomidate is a common sedative and anesthetic, it is important to understand more mechanisms about the inhibition of cancer metastasis for its usage in surgery of invasive cancer.

In this study, we evaluated anti-metastasis effects, including cell viability, cell adhesion, cell invasion and migration, and the expression of metastasis-associated proteins on A549 human adenocarcinoma cells after treatment with etomidate.

Materials and Methods

Reagents and chemicals. Etomidate was purchased from Lipuro, B. Braun Co. Ltd. (Melsungen, Germany) and was dissolved in phosphate-buffered saline (PBS). Propidium iodide (PI) was from Sigma-Aldrich Corp. (St. Louis, MO, USA). F12K medium, fetal bovine serum (FBS), penicillin and streptomycin antibiotic mixture were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies to RAS, phospho-jun proto-oncogene (p-c-JUN), protein tyrosine kinase 2 (p-FAK), N-cadherin, AKT serine/threonine kinase 1 (p-AKT^{Thr308}), E-cadherin, ras homolog family member A (RHOA), matrix metalloproteinase 1 (MMP1), MMP2, MMP7, MMP9, MMP10, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), snail family transcriptional repressor 1 (SNAIL), SOS RAS/RAC guanine nucleotide exchange factor 1 (SOS1), p-p38, plasminogen activator, urokinase (uPA), phospho-extracellular signal-regulated protein kinases 1 and 2 (pERK1/2), growth factor receptor bound protein 2 (GRB2), β -catenin, tissue inhibitor of metalloproteinase 1 (TIMP1), phospho-c-Jun N-terminal kinases (p-JNK1/2) and β -actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell culture. Human lung carcinoma A549 cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.). Cells were cultured in 10-cm dishes and maintained in F12K medium supplemented with 10% FBS, 100 units/ml penicillin, 100 ng/ml streptomycin, and 2 mM L-glutamine at 37°C under 5% CO₂ and 95% air humidified at 1 Atm.

Determination of cell viability. Lung adenocarcinoma A549 cells (1×10⁵ cells/well) were cultured in 12-well plates overnight and then treated with different concentrations of etomidate (0, 0.2, 0.4, 0.8, 1 and 2 μ g/ml) for 48 h. After treatment, cells were observed and photographed under a phase contrast microscope at ×200 and then cells from each treatment were collected, stained by PI (1 μ g/ml) in PBS for 10 min, and analyzed by flow cytometry as previously described (17, 18). Cells that excluded (live cells) and

those that contained PI (dead cells) were analyzed using a flow cytometer (19, 20). The obtained values were compared with those for the control (PBS treatment).

Cell adhesion assay. A549 cells at a density of 1×10⁵ cells/well were placed in 12-well plates and then exposed to different concentrations of etomidate (0, 0.2, 0.4 and 0.8 μ g/ml) for 48 h. Then cells were harvested and seeded onto 24-well plates which were coated with 150 μ l type I collagen (10 μ g/ml) (Millipore, Temecula, CA, USA). After 2 h incubation, Non-adherent cells were removed and adherent cells were washed with PBS, fixed with paraformaldehyde, treated with methanol for 20 min, stained with 0.2% crystal violet for 10 min, and lysed in 0.2% Triton X-100 for 30 min. The lysed solution (150 μ l) was loaded into 96-well plates, and then the OD_{540 nm} was measured as previously described (21, 22).

Boyden chamber assay for migration assay. Collagen coated transwell assay was used for the measurements of migration of A549 cells (19, 23). Briefly, a 24-well transwell with 8 μ m pore size (Merck Millipore, Corp.) was coated with type I collagen (30 μ g; Merck Millipore, Corp.) overnight in an incubator. A549 cells (1×10⁴ cells/well) in serum-free F12K medium were placed in the upper chamber and then treated with etomidate (0, 0.2, 0.4 and 0.8 μ g/ml) for 24 and 48 h. Medium with 10% FBS was added to the lower chamber. After treatment, the transwell filters were individually fixed with 4% formaldehyde in PBS, treated with methanol, and stained with 2% crystal violet. Non-migrated cells were removed from the upper chamber by using a cotton swab and migrated cells adhering to the underside of the filter were observed and photographed using a light microscope at ×200 magnification. Quantification of migration inhibition utilized ImageJ software (version 1.49o software; National Institutes of Health, Bethesda, MD, USA). Etomidate-treated and control groups was assayed twice and three independent experiments were performed as previously described (20, 21). The treatment without etomidate (control group) was scored as 100%.

Boyden chamber assay for invasion assay. Matrigel-coated transwell assay was used to measure the invasion of cells as previously described (23). Briefly, transwell culture inserts were coated with diluted Matrigel overnight. A549 cells were trypsinized, collected and re-suspended in serum-free F12K medium. A549 cells (1×10⁴ cells/well) were added in the upper chamber of the transwell insert and then treated with different concentrations of etomidate (0, 0.2, 0.4 and 0.8 μ g/ml) for 24 and 48 h. F12K medium containing 10% FBS was added in the lower chamber. After incubation, the culture insert was individually wash with PBS, fixed with 4% formaldehyde in PBS, treated with methanol, and then stained with 2% crystal violet. The non-invasive cells in the upper chamber were removed by using a cotton swab. Invasive cells on the lower surface of the filter, indicating cells which had penetrated through the Matrigel, were observed, photographed using a light microscope at ×200 magnification, and counted as previously described (20, 21).

Gelatin zymography assay. A549 cells were placed in 12-well plates at a density of 2×10⁵ cells/well overnight and then incubated in serum-free F12K medium containing different concentrations of etomidate (0, 0.2, 0.4 and 0.8 μ g/ml) for 24 and 48 h. The conditioned medium was harvested and separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis containing

Figure 1. Etomidate reduced the viability of A549 cells. Cells were placed in F12K medium containing 10% fetal bovine serum and treated with 0, 0.2, 0.4, 0.8, 1 and 2 µg/ml of etomidate for 48 h. The cells from each treatment were collected and analyzed for cell viability by flow cytometry as described in the Materials and Methods. Each point is the mean±S.D. of three experiments. *Statistically significantly different at $p<0.05$ compared to untreated control.

Figure 2. Etomidate inhibited the adhesion of A549 cells. Cells were exposed to 0.2, 0.4, and 0.8 µg/ml of etomidate for 24 and 48 h, and phosphate-buffered saline (PBS) as control. The adhesion assay was performed on 24-well plates coated with collagen for 2-h incubation, as described in the Materials and Methods. The adhesion of the PBS-treated group was set as 100%. Each bar represents the mean±SD of three independent experiments. *Statistically significantly different at $p<0.05$ compared to untreated control.

0.18% gelatin (Sigma-Aldrich Corp.). After electrophoresis, the gels were immersed in 2.5% Triton X-100 twice for a total of 60 min at room temperature, and incubated with substrate buffer (50 mM Tris base, 0.2 M NaCl, 5 mM CaCl_2 and 0.02% Brij 35 in distilled water, pH 7.6) at 37°C for 24 h. The bands showing gelatinase activity of MMP2 were visualized by staining with 0.3% Coomassie blue (Sigma-Aldrich Corp.) in 50% methanol and 10% acetic acid as previously described (21).

Determination of metastasis-associated proteins by western blotting assay. A549 cells (1×10^6 cells/dish) were cultured in 10-cm dishes with F12K medium containing 10% FBS for 24 h. Cells were treated with different concentrations of etomidate (0, 0.2, 0.4, and 0.8 µg/ml) for 24 and 48 h. Cells were collected from each treatment and their total proteins were extracted by using PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea) as previously described (24, 25). The protein concentration of each sample was determined by Bradford assay then 30 µg of protein was mixed with loading buffer, boiled for 5 min at 100°C, and separated on an SDS polyacrylamide gel as described previously (26, 27). After electrophoresis, the gel was transferred to polyvinylidene fluoride membrane and the membrane was then stained overnight by primary antibodies to RAS, p-c-JUN, p-FAK, N-cadherin, p-AKT^{Thr308}, E-cadherin, RHOA, PKC, P13K, SNAI1, MMP1, MMP2, MMP7, MMP9, MMP10, SOS1, p-p38, uPA, p-ERK1/2, GRB2, β-catenin, TIMP1 and p-JNK1/2. After washing with phosphate-buffered saline with Tween-20, the blots were reacted with horseradish peroxidase-conjugated secondary antibody for enhanced chemiluminescence and anti-β-Actin was used as a loading control as described previously (26, 27). Bands were quantified using imageJ software.

Statistical analysis. All values are presented as the mean±standard deviation of three independent experiments. Differences between groups were analyzed by one-way analysis of variance and Dunnett test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

Etomidate affects the percentage of viable A549 cells. After A549 cells were exposed to different concentrations of etomidate for 48 h, cells assayed for cell viability. As shown in Figure 1, the percentage of viable A549 cells was not significantly reduced at low concentrations (0, 0.2, 0.4, and 0.8 µg/ml) of etomidate but was significantly reduced at higher concentrations (1.0 and 2.0 µg/ml) when compared to the control group.

Etomidate inhibits adhesion of A549 cells. Cells were treated with etomidate and then were harvested and assayed for cell adhesion. Significant inhibition of cell adhesion was observed in etomidate-treated A549 cells at 0.2 and 0.8 µg/ml after 24 h exposure and at 0.4 and 0.8 µg/ml after 48 h exposure when compared with the untreated (control) cells (Figure 2).

Etomidate inhibits migration and invasion of A549 cells. The anti-migration and invasion effects of etomidate on A549 cells were evaluated by using Boyden chamber (transwell) assay. After treatment with etomidate (0.2-0.8 µg/ml) for

Figure 3. Etomidate influenced migration and invasion of A549 cells. A: Cells were treated with or without 0, 0.2, 0.4 and 0.8 µg/ml of etomidate for 24 and 48 h. Cell migration was examined by the Boyden chamber assay and type I collagen-coated transwell. B: Cell invasion was examined by using Matrigel-coated transwell cell culture chambers. Ability for migration and invasion of A549 cells was quantified by counting the number of cells that migrated or invaded the underside of the porous polycarbonate membrane under a phase-contrast microscope. Data represent the average of three experiments. *Statistically significantly different at $p < 0.05$ compared to untreated control (C).

Figure 4. Etomidate altered enzymatic activity of matrix metalloproteinase-2 (MMP2) in A549 cells. Cells were incubated with 0, 0.2, 0.4 and 0.8 µg/ml of etomidate for 24 (A) and 48 (B) h. The supernatant was harvested after treatment and protein separated by gelatin zymography, as described in the Materials and Methods. MMP2 activity was quantified using the Image J software. *Statistically significantly different at $p < 0.05$ compared to untreated control (C).

24 h and 48 h, cells showed 28%-54% and 37%-77% inhibition of migration, respectively (Figure 3A). Penetration of the Matrigel-coated filter by A549 cells was significantly inhibited in the presence of etomidate. The invasion inhibition of etomidate treatment at 0.2-0.8 µg/ml was 42-84% and 58-82% after 24 and 48 h, respectively (Figure 3B).

Etomidate affects MMP2 enzyme activity in A549 cells. The effects of 24- and 48-h etomidate on MMP2 enzymatic activity of A549 cells were examined using gelatin zymography. After cells were treated with etomidate, the supernatant was harvested and gelatinase activity was analyzed by gelatin zymography. Figure 4 shows representative examples of zymography of activated MMP2 in A549 cells after 24 and 48 h of etomidate treatment. Etomidate at 0.2-0.8 µg/ml reduced MMP2 activity of A549 cells at 48 h treatment compared with control. Interestingly, after 48-h etomidate treatment at any concentration, the MMP2 activity of etomidate-treated cells was less than that of the control. Based on these observations, it indicated that etomidate significantly attenuated MMP2 activity in A549 cells.

Etomidate alters the levels of proteins associated with migration and invasion in A549 cells. To understand the molecular mechanism of etomidate in migration and invasion, A549 cells were treated with etomidate and

extracted proteins were examined by using western blotting. Results showed that etomidate diminished the expressions of p-FAK, p-AKT^{Thr308} (Figure 5A), E-cadherin, RHOA, MMP10, PKC, MMP7 (Figure 5B), SNAIL1, MMP1, MMP9, SOS1 (Figure 5C), p-p38, uPA (Figure 5D), β-catenin, TIMP1, and p-JNK1/2 (Figure 5E) after 24 h treatment in A549 cells. Etomidate reduced the expression of PKC, MMP7 (Figure 5B), MMP1, MMP9 (Figure 5C), and p-p-38 (Figure 5D) after 24 and 48 h treatments. The reduction of these protein levels may lead to the inhibition of migration and invasion in A549 cells. On the other hand, etomidate increased the protein level of RAS, N-cadherin (Figure 5A), PI3K (Figure 5C), MMP2 (Figure 5D), p-ERK1/2, and GRB2 (Figure 5E) after 24 h treatment, but elevated that of p-c-JUN, p-FAK, p-AKT^{Thr308}, E-cadherin, RHOA, MMP10 (Figure 5B), PI3K, SNAIL1, SOS1 (Figure 5C), uPA (Figure 5D), p-ERK1/2, and TIMP1 (Figure 5E) after 48 h treatment. The expressions of RAS (Figure 5A), PI3K (Figure 5C), and p-ERK1/2 (Figure 5E) were increased after 24 and 48 h etomidate treatment.

Discussion

In this study, we demonstrated that etomidate inhibited the migration and invasion of A549 human lung adenocarcinoma cells in a dose-dependent manner (Figure 3) by Boyden chamber assays. We also performed gelatin zymography assay to show the inhibition of MMP2 activity in A549 cells by etomidate (Figure 4). Furthermore, western blotting assay showed that etomidate down-regulated protein expression of PKC, MMP7 (Figure 5B), MMP1, MMP9 (Figure 5C), and p-p-38 (Figure 5D) and up-regulated that of RAS, PI3K, and p-ERK1/2 after 24 and 48 h treatment in A549 cells (Figure 5).

Etomidate is a favorable sedative and anesthetic drug for clinical use due to its reduced cardiovascular side-effects and better hemodynamic stability during the maintenance of anesthesia (28). Etomidate has less effect on immune function in patients with lung adenocarcinoma (29). In addition, etomidate induced apoptosis in N2a brain tumor cell line (30). In our previous study, we demonstrated that etomidate, citosol and propofol induced the expression of apoptosis-related genes, inhibited the expression of cell growth genes and altered the expression of apoptosis-related proteins in a mouse leukemia cell line (RAW264.7) (12-15). Etomidate had different effects on inhibiting migration in human cancer cell lines. Literature investigated the effects of intravenous anesthetics, such as propofol, etomidate and dexmedetomidine, on cell migration and showed etomidate promoted the migration of colorectal cancer cells both *in vitro* and *in vivo* by activating AKT signaling and inducing epithelial-mesenchymal transition (31). But etomidate had no influence on the number of migrating cells in breast

Figure 5. Etomidate affected the level of proteins associated with migration and invasion of A549 cells. Cells (1×10^6 cells/well) were treated with 0.2, 0.4 and 0.8 $\mu\text{g/ml}$ of etomidate for 24 and 48 h. Total proteins were collected and the levels of A: RAS, phospho-jun proto-oncogene (p-c-JUN), protein tyrosine kinase 2 (p-FAK), N-cadherin, AKT serine/threonine kinase 1 (p-AKT^{Thr308}); B: E-cadherin, ras homolog family member A (RHOA), matrix metalloproteinase 10 (MMP10), protein kinase C (PKC), MMP7; C: phosphoinositide 3-kinase (PI3K), snail family transcriptional repressor 1 (SNAI1), MMP1, MMP9, SOS RAS/RAC guanine nucleotide exchange factor 1 (SOS1); D: p-p38, MMP2, plasminogen activator, urokinase (uPA); and E: phospho-extracellular signal-regulated protein kinases 1 and 2 (p-ERK1/2), growth factor receptor bound protein 2 (GRB2), β -catenin, TIMP metalloproteinase inhibitor 1 (TIMP1) and phospho-c-Jun N-terminal kinases (p-JNK1/2) were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting as described in the Materials and Methods. Direct re-probing with β -actin antibody was used as an internal control.

cancer cells (MDA-MB-468) (32). No report has concerned the anti-metastasis effects of etomidate on human A549 lung adenocarcinoma cells. In this study, etomidate significantly inhibited migration and invasion in lung cancer A549 cells. Understanding the molecular mechanism of etomidate action may provide important information for the clinical application of anesthetics.

Here, we demonstrated that etomidate reduced the level of p-FAK, p-AKT^{Thr308}, E-cadherin, RHOA, MMP10, PKC, MMP7, SNAI1, MMP1, MMP9, SOS1, p-p38, uPA, β -catenin, TIMP1, and p-JNK1/2 after 24 h treatment and reduced the expression of PKC, MMP7, MMP1, MMP9, and p-p-38 after 24 and 48 h treatments. The results were consistent with our previous study that studied the effects of

Figure 6. The possible signaling pathways for etomidate inhibition of cell invasion and migration by A549 cells. *p-c-JUN*: Phospho-jun proto-oncogene; *p-FAK*: protein tyrosine kinase 2; *p-AKT^{Thr308}*: AKT serine/threonine kinase 1; *RHOA*: Ras homolog family member A; *MMP*: matrix metalloproteinase; *PKC*: protein kinase C; *PI3K*: phosphoinositide 3-kinase; *SNAI1*: snail family transcriptional repressor 1; *SOS1*: SOS RAS/RAC guanine nucleotide exchange factor 1; *uPA*: plasminogen activator; urokinase; *pERK1/2*: phospho-extracellular signal-regulated protein kinases 1 and 2; *GRB2*: growth factor receptor bound protein 2; *TIMP1*: tissue inhibitor of metalloproteinase 1; *p-JNK1/2*: phospho-c-Jun N-terminal kinases.

propofol on inhibition of invasion and migration by A549 human lung adenocarcinoma cells. Propofol regulated several mechanisms in order to suppress cell invasion and migration, including down-regulating the mRNA gene expression of *MMP2*, -7 and -9, reducing the activity of *MMP2*, inhibiting *GRB2*, *p-JNK1/2*, *p-p38*, *MMP2* and *MMP9*, and enhancing *TIMP1* and *TIMP2* (33).

Herein, our results demonstrated that etomidate significantly inhibited the gelatinase activity of *MMP2* at low concentrations (0.2-0.8 µg/ml) in A549 cells. Multiple regulatory mechanisms are involved in invasion and migration of cancer cells. MMPs play principle roles in angiogenesis, metastasis, and stimulating the release of growth factor from the extracellular matrix in cancer cells or tissues (34). Elevated expression of MMPs or *MMP* activity is observed in highly metastatic cancer cells (35). The inhibition of *MMP* expression also prevents cancer metastasis (34, 36, 37). *MMP2* and -9 are the main enzymes for degradation of type I and II collagens and the extracellular matrix (34, 36) and their expression and activity parallel the invasive and metastatic potential of cancer cells (38).

MMPs play important roles in cancer metastasis. *MMP2* is usually overexpressed by highly invasive tumors (39).

MMP9 can be induced by the activation of nuclear factor-κB (NF-κB), *PKC*, and activator protein-1 (AP1) (40, 41). In this study, etomidate inhibited the expression of *PKC*, *p-p38*, and *JNK*, indicating the downstream regulation of *MAPK* pathway (*p-p38* and *JNK*) may be involved in the effects of etomidate on suppression of *MMP2* expression and invasion by A549 cells. Moreover, etomidate down-regulated the levels of *MMP10*, *MMP7*, *MMP1*, *MMP9*, and *uPA* after 24 h treatment and reduced the expression of *MMP7*, *MMP1*, and *MMP9* after 24 and 48 h treatments. These results indicated that etomidate acted against migration and invasion. Overall, the possible signaling pathways of etomidate which suppressed the cell migration and invasion of A549 cells are presented in Figure 6.

Our results not only illustrate the effects of etomidate on metastasis of cancer cells, but also provide new insights into understanding the mechanisms of action of etomidate in cancer treatment. The anticancer effects of etomidate may assist to inhibit or ameliorate spontaneous- or manipulation-induced metastasis during cancer surgery. If so, our findings may have tremendous impact on the sedation and anesthetic care of patients with cancer. Further investigations for the potential therapy of etomidate in cancer are encouraged.

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