Focal adhesion kinase (FAK) is a nonreceptor type tyrosine kinase composed of ~1,030 amino acids that regulates integrin and growth factor signaling pathways, thereby playing an important role in cell proliferation, differentiation, and apoptosis (1–5). It is known that FAK is activated from integrin and growth factor receptors by autophosphorylation at Tyr397 (6), followed by subsequent activation of other functional phosphorylation sites to advance the signals to downstream pathways, such as AKT (7–11). Based on these facts, FAK is thought to play a critical role in malignant behavior including proliferation, survival, and invasion (12–16). Indeed, there are several studies reporting that FAK is overexpressed in human carcinomas including breast, thyroid, ovarian, head and neck, liver, pancreas, lung, and colon cancers and that the expression status of FAK in cancers is closely related with tumor progressiveness and clinical outcome (17–25). However, there are still many questions about detailed FAK functions with surrounding molecules and its downstream signaling.

Among gastrointestinal malignancies, there is only one report that FAK overexpression seems to be involved in tumor invasiveness and lymph node metastasis in squamous cell carcinoma of the esophagus (26), whereas no detailed information is available about FAK expression and function in Barrett's esophageal adenocarcinoma. Barrett's esophagus is an acquired condition in which the normal squamous cell epithelium of the esophagus is replaced by a metaplastic columnar epithelium due to chronic gastroesophageal reflux.
especially dissecting the apoptosis pathway. The exact mechanism of the effect needs to be elucidated. Our specific focus was on other types of malignancies, including Barrett’s esophageal adenocarcinoma (30, 31), although its anticancer effect on brain tumors (30, 31) is not well understood. The percentage of positive-stained tumor cells was calculated and categorized into three groups: -, 0% to 10% positive; +, 10% to 50%; and ++, >50%.

**Trypan blue exclusion assay.** SEG-1, FLO-1, and BIC-1 cells (1.0 × 10^4 per well) were plated directly in 24-well dishes with a culture medium. Subconfluent cells were treated with different concentrations of TAE226 (0.0.037, 0.1, 0.33, 1.0, and 3.0 μmol/L) for 48 h. After treatment, cells were harvested with trypsin, stained with trypan blue, and counted manually with a hemacytometer. Dose-effect plots were created to calculate the IC_{50} of TAE226 for each cell line using Calcsyn software (Biosoft).

**Scratch assay.** The ability of cell migration was determined by the scratch assay. SEG-1 and FLO-1 cells were grown to confluence in six-well tissue culture dishes and a single scratch was made in the confluent monolayer using a sterile 200-μL pipette tip. The monolayer was washed with PBS and then complete medium containing either 1.0 μmol/L TAE226 or DMSO alone was added. Serial photographs of the same scratched section were taken after 24 and 48 h. The number of cells that had migrated over the margins of the wounds was counted after 48 h.

**Immunofluorescence staining.** For indirect immunofluorescence staining, SEG-1 cells grown on Lab-Tek 8-well permanox chamber slides (Nunc) were fixed with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells were incubated with a blocking solution (1% bovine serum albumin in PBS) for 30 min and then with primary antibodies of Affinity-purified mouse anti-human phosphorylated-FAK (Y927; BD Biosciences) diluted in 1% bovine serum albumin in PBS for 90 min at room temperature. After three washings with PBS, the cells were incubated in the presence of secondary antibodies of rabbit anti-mouse IgG antibody labeled with FITC for 1 h. For actin staining, fixed cells were incubated with rhodamine-conjugated phalloidin (Chemicon) for 1 h at room temperature. After being washed twice with PBS, cells were counterstained with 1,000 4′,6-diamidino-2-phenylindole diluted to 1:100 with PBS for 5 min at room temperature. The slides were mounted with Vectashield (Vector Laboratories, Inc.) and examined under a confocal inverted fluorescence microscope (Zeiss Axiosvert 200M) and digitally processed with Zeiss LSM510 META software (Biosoft).
with an equivalent volume of the diluent DMSO (final concentration, 0.005%) as a control for 0 to 24 h (0, 1, 6, and 24 h). Apoptotic cells were assessed at the end of the experiment with a fluorescence microscope according to the percentage of TUNEL-positive cells.

**Cell cycle analysis by flow cytometry.** For cell cycle analysis, SEG-1 cells were plated in six-well tissue culture plates. Subconfluent cells were treated with 1.0 μmol/L TAE226; harvested at different time points of 0, 1, 6, and 24 h; and stained with 20 μg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACSscan, Becton Dickinson and Company) using CellQuest software (BD Biosciences).

**Western blotting.** Cells were collected by trypsinization and washed twice in cold saline. Cells were then dissolved in a protein lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% Triton X-100, and protease inhibitors [0.2 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L 4-(2-aminoethyl) benzenesulfonylfluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 μg/mL aprotinin] on ice for 30 min, and the collected cells were scraped into a microtube. After sonication and centrifugation at 15,000 rpm at 4°C for 15 min, the supernatant was collected for protein determination and Western blotting analysis. The concentration of protein lysates was measured with the bicinchoninic acid protein assay kit (Pierce). Equal amounts (20 μg) of protein were electrophoresed under reducing conditions in 5% to 20% (w/v) gradient polyacrylamide gels (ATTO). Proteins were then transferred onto GVHP filter membranes (Nihon Millipore) and incubated with primary antibodies at 4°C overnight, followed by incubation with peroxidase-linked secondary antibodies at room temperature for 1 h. For signal detection, a chemiluminescence Western system (Amersham ECM Plus Western Blotting Detection Reagents, GE Healthcare) was used for signal detection.

The antibodies used for Western blotting were the following; affinity-purified mouse anti-human phosphorylated-FAK (Tyr925; BD Biosciences), affinity-purified mouse anti-human total FAK (BD Biosciences), affinity-purified rabbit anti-human phosphorylated-IGF-IR (Cell Signaling Technology, Inc.), affinity-purified rabbit anti-human total IGF-IR (Santa Cruz Biotechnology), affinity-purified rabbit anti-human phosphorylated-AKT (Ser473; Cell Signaling Technology), affinity-purified rabbit anti-human total AKT (Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated extracellular signal–regulated kinase (ERK) 1/2 (Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated BAD (Ser112; Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated BAD-BAD (Ser536; Cell Signaling Technology), affinity-purified rabbit anti-human phosphorylated-BAD (Ser136; Cell Signaling Technology), affinity-purified rabbit anti-human total BAD (Cell Signaling Technology), affinity-purified rabbit anti-human caspase-3 (Upstate Cell Signaling Solutions), horseradish peroxidase–conjugated rabbit anti-mouse IgG (DakoCytomation), and goat anti-rabbit IgG (American Qualex Antibodies).

**Animal experiments.** BALB/cAJoCrl-nu/nu 8-week-old female mice derived from C.B-17/scid (Clea) were used in this study. All mice were maintained under pathogen-free conditions. SEG-1 cells (3.0 × 10⁶/100 μL) mixed with Matrigel (BD Biosciences; 100 μL) were s.c. inoculated (n = 6 each; day 0). From days 1 to 14, TAE226 (30 and 60 mg/kg) or methylcellulose as a vehicle was orally administered once a day and the width and length of each s.c. tumor were measured to calculate tumor volume. On day 15, all of the mice were sacrificed to take the tumors out and each tumor was weighed. Tumor volume (V) was calculated as (width) × (length)², as described elsewhere. Note that the final concentration of methylcellulose and TAE226 used in these experiments did not affect any organs or life span.

**Statistical analysis.** Student’s t test was used to compare data between two groups. Data represent the mean ± SD. P < 0.05 was considered statistically significant.

**Results**

The overexpression of FAK in Barrett’s esophageal adenocarcinoma. First, we determined the expression status of FAK in
Barrett's esophageal adenocarcinoma by immunohistochemical staining. We used 42 surgically resected tissues from patients with Barrett's esophageal adenocarcinoma and stained them with anti-FAK antibody. We observed the following parts in those tissues: 308 areas of Barrett's epithelia, 168 areas of adenocarcinoma, 93 areas of squamous epithelia, and 56 areas of gastric epithelia. We categorized them into the three groups defined in Materials and Methods. As shown in Fig. 1, FAK expression was increased in cancerous parts compared with noncancerous areas, and the strong expression (>50% positive-staining cells per area) was observed in 94.0% of Barrett's esophageal adenocarcinoma, compared with 17.9% of Barrett's epithelia. This tendency seemed to be obvious and we would say that, like other malignancies, the expression of FAK is also up-regulated in Barrett's esophageal adenocarcinoma as well. Interestingly, squamous epithelia revealed relatively higher percentage of the positive staining (43.0% for FAK+ and 47.3% for FAK++) than columnar epithelia such as gastric mucosa, whose staining status of FAK was quite similar with that of Barrett's epithelia (Fig. 1B), indicating that the expressions of FAK may differ among histologic configurations.

Inhibition of FAK by TAE226 suppresses cell growth and migration in esophageal adenocarcinoma cell lines. Because cultured adherent cells are quite dependent on adhesion to maintain their growth, it is easily hypothesized that the inhibition of FAK, which plays a critical role in integrin-mediated cell adhesion as well as in advancing signaling from integrin to its downstream effectors, should affect cell proliferation. To determine an inhibitory effect on cell growth by the chemical compound and to optimize its concentration for further experiments, the IC50 was measured to examine the effect of TAE226 in esophageal adenocarcinoma cell lines. All of three esophageal adenocarcinoma cell lines (SEG-1, FLO-1, and BIC-1) were treated with different concentrations of TAE226 for 48 hours and their susceptibility to TAE226 was assessed by the trypan blue exclusion method. The IC50 of SEG-1 cells was 0.47 μmol/L and those of FLO-1 and BIC-1 were 1.03 and 1.29 μmol/L, respectively (Fig. 2A-C). SEG-1 cells seemed to be the most sensitive to TAE226 among the three esophageal adenocarcinoma cell lines. TAE226 at 3 μmol/L seemed to be lethal to all of the cell lines because more than 90% of cells were dead within 48 hours (Fig. 2A-C).

Fig. 2. The effect of TAE226 on the inhibition of cell growth and migration. Representative cytotoxicity using the trypan blue exclusion method after treatment with FAK inhibitor for 48 h at the given concentrations in SEG-1 (A), FLO-1 (B), and BIC-1 (C) cells. Each column is given as a percentage of live cells compared with the control (treatment with DMSO alone), which was set as 100%. D, the ability of cell migration under TAE226 treatment was determined by the scratch assay as explained in Materials and Methods. Pictures were taken immediately after making a scratch (0 h) and at 48 h with or without 1 μmol/L TAE226. The number of cells that migrated into the scratched area was counted and displayed in a histogram (E). Columns, average number of cells from three independent experiments; bars, SD.
Second, we determined the inhibitory effect on cell migration by the FAK inhibitor. Monolayer cultures of SEG-1 cells were scratched with a micropipette tip at the time of full confluence (time 0) and then kept at 37°C for another 48 hours with TAE226 or its vehicle (DMSO). Figure 2D and E shows microscopic observations of the scratch assay. Certain areas within the scratched defect were occupied by migrated cells with DMSO treatment (332 ± 7 cells per area), whereas TAE226 treatment significantly decreased the number of cells that migrated to the defect (59 ± 1 cells per area), suggesting that FAK inhibition by the compound can negatively affect cell mobility. Cell migration of FLO-1 cells was also suppressed by TAE226 treatment at a concentration of 3 μmol/L (73 ± 26 versus 41 ± 11 cells per area; P < 0.05).

TAE226-treated SEG-1 cells displayed a structural change of actin fiber and a loss of cell adhesion with inhibited FAK activity. When observed under a microscope, TAE226-treated cells displayed an unhealthy morphology such as round, flattened, and burst shapes with a disordered nuclear structure and a cell density that was strongly decreased by the treatment (Fig. 3A). Some cells looked as if they were shrinking with easy detachment from the surface of the plastic plates, assuming that cell adhesion may be weakened by the inhibition of FAK.

To further explore the structural changes of the cell cultures from the inhibition of FAK, cells were stained with antibodies for actin, phosphorylated FAK, and 4ʹ6ʹ-diamidino-2-phenylindole, and immunofluorescent signals were observed by confocal microscopy. As shown in Fig. 3B and C, cells with FAK activity keep the actin fiber structure intact, accompanied by FAK phosphorylation and localization at the end of the actin fiber, assuming that activated FAK is located at focal adhesion points to maintain cytoplasmic structure. When cells were treated with TAE226, the actin fiber structure was dramatically destroyed and the signal of phosphorylated FAK was abolished.

FAK inhibition leads to apoptosis. To clarify whether the inhibition of FAK leads to cell death, the distribution of the cell cycle was analyzed by flow cytometry (fluorescence-activated cell sorting analysis). As shown in Fig. 4A and B, TAE226 treatment increased the population of the sub-G₀ phase. We did not see any obvious changes of cell distribution in the G₁ or G₂-M phase. Based on the results of the fluorescence-activated
cell sorting analysis, we speculate that the increase in cell numbers in the sub-G0 phase represents increasing cell death by the FAK inhibitor. The next question was whether cells undergo apoptosis with TAE226 treatment. To answer this question, TUNEL staining was carried out (Fig. 4C). TAE226 treatment apparently increased TUNEL-positive cells within 24 hours compared with DMSO treatment (Fig. 4D), suggesting that the inhibition of FAK activity by TAE226 leads the esophageal adenocarcinoma cells to apoptosis.

**TAE226 inhibits FAK and IGF-IR activity and suppresses their downstream signaling.** To explore which signaling pathway is involved in TAE226-induced apoptosis, we dissected out downstream molecules of FAK by Western blot. TAE226 inhibited phosphorylation of FAK in a dose- and timedependent manner (Fig. 5A and B). AKT activation was also suppressed, whereas the inhibition of ERK activity was modest. Phosphorylation of BAD, which is a key modulator for cell survival and is regulated by AKT and mitogen-activated protein kinase, was inhibited at Ser136 but not at Ser112, suggesting that suppression of FAK activity by TAE226 caused an inhibition in AKT phosphorylation, which prevents it from being able to phosphorylate BAD at Ser136, thereby promoting apoptosis. We looked further downstream along the apoptosis pathway. Caspase-3, a key molecule through caspase-dependent apoptosis, which is also regulated by BAD, was cleaved by TAE226 treatment. These results support our initial finding that the inhibition of FAK by TAE226 indeed leads to apoptosis through the AKT-BAD-caspase pathway.

**Fig. 4.** TAE226-induced apoptosis in esophageal adenocarcinoma cells. SEG-1 cells were treated with 1 μmol/L of TAE226 or DMSO and applied for cell cycle analysis by flow cytometry (A), and the cell distribution at each phase is drawn in histograms at the different time points indicated (B). C, TUNEL staining was done to assess TAE226-induced apoptosis in SEG-1 cells and the percentage of TUNEL-positive cells is displayed in histograms (D). White and black columns, percentage of cells with DMSO and TAE226, respectively.
It is reported that TAE226 also has a certain inhibitory effect on IGF-IR (≈25-fold less than on FAK; ref. 31). AKT is well known as a main downstream molecule of receptor tyrosine kinase such as IGF-IR. It is possible that the inhibition of IGF-IR may also contribute to TAE226-induced apoptosis through the AKT-BAD-caspase pathway. We saw a faint expression of IGF-IR phosphorylation in regular culture conditions without the inhibitor and its activity was shut down by the addition of TAE226, although the effect looked unclear. Therefore, the cells were treated with IGF to stimulate the receptor and to see specific inhibition of IGF-IR signaling by TAE226. As shown in Fig. 5C, 100 ng/mL IGF reasonably activated the receptor and its downstream effectors, whereas TAE226 suppressed IGF-induced activation of IGF-IR and AKT. In summary, TAE226 has a strong inhibitory effect on cell proliferation and migration in esophageal adenocarcinoma cells and leads the cells to apoptosis through the AKT-BAD-caspase pathway by inhibiting both FAK and IGF-IR signaling.

Oral administration of TAE226 suppresses s.c. tumor growth in vivo. We confirmed a potent inhibitory effect on cell growth and migration of esophageal adenocarcinoma cells in vitro. For the next step, we inoculated SEG-1 cells s.c. in nude mice (day 0). The animals were treated with an oral administration of either TAE226 (30 or 60 mg/kg) or methylcellulose as a vehicle. The daily administration started the day after inoculation and continued for 2 weeks (days 1-14).

**Fig. 5.** Western blotting analysis for TAE226-induced apoptosis. A, the activation of FAK, IGF-IR, and their downstream molecules was suppressed by TAE226 treatment in a dose-dependent manner. B, the inhibition of the cell survival pathway with 1 μmol/L TAE226 was prolonged for at least 24 h. C, TAE226 displayed a certain effect on the inhibition of IGF-IR signaling, which is specifically activated by IGF-I.
Tumor volume was measured twice a week during the treatment and the tumors were taken out of the mice and were weighed at day 15. Both tumor volume and weight were significantly decreased in mice that had TAE226 treatment compared with mice treated with vehicle only (Fig. 6; Supplementary table). No obvious side effects occurred during the treatment and there is not a statistical significance of tumor regression between the two different doses (30 and 60 mg/kg/daily).

**Discussion**

Here we first report that our immunohistochemical analysis using clinical samples revealed the up-regulation of FAK in Barrett’s esophageal adenocarcinoma (Fig. 1), suggesting that FAK might play a critical role for cancer progression in Barrett’s esophageal adenocarcinoma as it reportedly does in other types of malignancies (17–25). Because FAK is known as a key molecule for cell proliferation, migration, and invasion during cancer progression (1–5), targeting FAK can potentially be a good practice for cancer therapy. Indeed, a number of chemical compounds have recently been developed to target certain cancer-specific functions without apparent side effects (34, 35). TAE226 is one such small-molecule inhibitor specifically targeting FAK (30, 31). Thus, we determined that the inhibition of FAK by TAE226 can be a potential therapeutic strategy against adenocarcinoma of the esophagus, which is known as one of the most aggressive cancers (27–29).

TAE226 treatment displayed significant inhibitory effects on cancer proliferation and migration in vitro, and esophageal adenocarcinoma cells underwent apoptosis by the FAK inhibitor. TAE226 causes an inhibition in AKT phosphorylation, which prevents it from being able to phosphorylate BAD at Ser136, thereby promoting apoptosis (Fig. 5A and B). Because phosphorylation of BAD by Akt is known to keep it sequestered in 14-3-3 protein and to prevent the association between BAD and antiapoptotic Bcl-2 family proteins (36, 37), this is the first report to explore a major mechanism of anticancer effect by TAE226 that leads cancer cells to apoptosis through the AKT-BAD-caspase pathway.

TAE226 treatment also impaired the morphologic structure of the cytoplasm through the invalidation of actin fibers that stabilize focal adhesions via FAK (Fig. 3). Another importance of our findings is that TAE226 treatment of s.c. xenograft animals revealed a remarkable reduction of tumor volume by oral administration without any apparent side effects (Fig. 6; Supplementary table). It should be noted that a maximum of 100 mg/kg/daily administration of TAE226 for 14 days was done and no complication was found. We did not see a statistical significance of tumor regression by TAE226 in vivo, suggesting that a low dose of TAE226 could be sufficient in certain types of cancers. These data encourage us to advance FAK targeting as a novel cancer therapy with an eye toward clinical application.

TAE226 was originally synthesized as a specific inhibitor for FAK, and it is reported that the drug also has a certain inhibitory effect on IGF-IR activity, which is also well known as a major intracellular pathway for cell proliferation and survival, although this effect is 25-fold less than the effect on FAK (31). As we showed, TAE226 displayed a suppression of IGF-IR activation by IGF-I in SEG-1 cells (Fig. 5C), indicating that it should not be neglected that any anticancer effect on Barrett’s esophageal adenocarcinoma by TAE226 may be partially mediated through IGF-IR signaling. It is very meaningful that a single chemical compound can inhibit two major signaling pathways for cancer progression because a crucial issue that may often be faced in the clinical field is that targeting one particular molecule only is not sufficient enough to control cancer progression because most malignancies can be regulated by multiple signaling pathways (38) and that they can easily acquire a resistance to certain drugs through genetic alterations (39, 40). To overcome these problems, it is necessary to synthesize novel drugs that show multiple inhibitory mechanisms, such as TAE226, whose effect is mainly to inhibit...

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7 Personal observation.
activation of FAK and IGF-IR. Another strategy to gain the maximum effect for cancer therapy is a combination of two or more drugs (e.g., 5-fluorouracil and cisplatin). It is potentially intriguing to combine TAE226 with other compound(s) to test its synergistic effect on inhibiting cancer progression.

In this study, SEG-1 cells displayed the highest susceptibility to TAE226 compared with the other cells (Fig. 2A-C). This difference may have occurred due to the difference in their genetic background. It is reported that SEG-1 cells retain an intact function of wild-type p53 whereas FLO-1 and BIC-1 cells have acquired a p53 mutation (41). p53 status may partially affect the response to anticancer drugs as described previously (42–44).

In conclusion, we found that FAK is up-regulated in Barrett’s esophageal adenocarcinoma and that the inhibition of FAK by TAE226 led esophageal adenocarcinoma cells to apoptosis through the AKT-BAD-caspase pathway and displayed significant anticancer effects in vitro and in vivo. Targeting FAK can be a novel strategy for cancer therapy, and TAE226, which has a specific inhibition of FAK along with a certain inhibitory effect on IGF-IR activity, might be a potential candidate for clinical application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

Dual Tyrosine Kinase Inhibitor for Focal Adhesion Kinase and Insulin-like Growth Factor-I Receptor Exhibits Anticancer Effect in Esophageal Adenocarcinoma In vitro and In vivo

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