Marked Prevention of Tumor Growth and Metastasis by a Novel Immunosuppressive Agent, FTY720, in Mouse Breast Cancer Models

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ABSTRACT

FTY720 is a unique immunosuppressive agent that exerts its activity by inducing apoptosis in lymphocytes. We conducted the present study to investigate the effects of FTY720 on cancer growth and metastasis, as well as its mechanism of action. In vitro treatment with FTY720 induced dramatic cancer cell apoptosis in a mouse breast cancer cell line, Jyg-MC(A). Electron microscopy revealed distinct changes on the cell surface with decreased filopodias and microvilli in cancer cells treated with FTY720 at 2 μM and clear evidence of apoptosis at 10 μM. Interestingly, the effect of FTY720 was significantly less in the normal fibroblasts than in the cancer cells, indicating greater susceptibility of cancer cells to the agent. We then tested the in vivo effect of FTY720 in a mouse breast cancer model created by inoculating JygMC(A) cells (s.c.) in the flank region of BALB/c-nu/nu mice at three different dosages (2, 5, and 10 mg/kg/day; n = 30/group). Tumor growth was markedly suppressed at a dosage of 5 mg/kg or more without notable side effects. In addition, tumor metastasis, which was dramatically evident in control mice, was significantly prevented even at a low dose (2 mg/kg/day), resulting in a significant prolongation of animal survival. These data led us to additionally investigate the mechanism of action, especially the prevention of metastasis at a low dose. FTY720 treatment at 2 μM caused a remarkable cytoskeletal change with deformed and decreased filopodias in cancer cells. In addition, it significantly decreased the ability of cancer cells to adhere and migrate to extracellular matrix components, and markedly reduced the expression of integrins on the cancer cell surface. These results indicate that FTY720 is a potent anticancer agent that induces cancer cell apoptosis and is markedly effective for prevention of metastasis. The changes of cellular structure with reduction of integrin expression may be one of its underlying mechanisms of action.

INTRODUCTION

FTY720 is a synthetic compound that is produced by modification of a metabolite from Isaria sinclairii. The drug was originally developed as a new immunosuppressive agent and has subsequently been shown to prolong graft survival in several animal models of organ transplantation without causing severe adverse reactions. Induction of apoptosis in lymphocytes is considered to be one of its major mechanisms of action (1–6). We expected that this unique mechanism might have anticancer potential by inducing apoptosis in cancer cells. It has been demonstrated previously that FTY720 induces apoptosis in vitro in several cancer cell lines, including HL-60, Jurkat cells, WR19L, and DU145 (7–9). This background led us to investigate the in vivo anticancer potential of FTY720 and ascertain its exact mechanism in inducing apoptosis in cancer cells, which is still uncertain despite numerous studies.

The present study showed that FTY720 dramatically inhibited in vivo tumor growth with no severe side effects. In addition, it is noteworthy that the treatments with this agent significantly prevented metastasis, which is one of the major causes of mortality in cancer patients. The development of cancer metastasis is a complex cascade of events involving cell-cell interactions between tumor cells and host cells. The formation of tumor aggregates or emboli may be arrested nonspecifically in narrow capillaries; subsequently some of these tumor cells may penetrate the vascular wall, degrade the basement membrane and ECM, and then invade the secondary organ (10–13). The interaction of tumor cells with ECM components is thought to be critical in such metastatic cascade (14–16). In addition, the expression of integrins and adhesion molecules is reported to contribute to the events (14–18). Concerning cancer treatment, especially for prevention of metastasis, it may be possible that interference of cell-cell or cell-ECM adhesions by modulating the adhesive elements, such as integrins or ECM components, blocks, or suppresses cancer metastasis. Therefore, we conducted the present study to test the in vivo effect of FTY720 on prevention of cancer growth and metastasis. Furthermore, we investigated its mechanisms of action, especially for preventing metastasis directing on the process of cancer cell arrest.

MATERIALS AND METHODS

Cell Cultures and Reagents. We used a mouse breast cancer cell line, JygMC(A) and a mouse fibroblast cell line, NCTC-2525. JygMC(A) was originally isolated from mammary carcinomas arising in the M. m. musculus Sub-Jyg (Chinese wild mice). NCTC-2525 was purchased from American Type Culture Collection (Bethesda, MD). The cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% of heat inactivated FCS, 100 units/ml penicillin G, and 100 units/ml streptomycin. FTY720 supplied as a powder by Taito Company (Tokyo, Japan) in cooperation with Yoshitomi Pharmaceutical Industries (Osaka, Japan), was dissolved in distilled water and diluted in culture medium for each concentration study.

Colorimetric (MTT) Assay. Cell viability after FTY720 treatment was assessed using a Cell Survival and Proliferation kit (Chemicon, Temecula, CA). We plated 100 μl of a cell suspension (5000 cells) in each well of a 96-well flat-bottomed microtiter plate and incubated them overnight at 37°C in a humidified 5% CO2 atmosphere. We then added FTY720 in each well at the various concentrations (10, 30, 50, and 100 μM) by substituting new culture medium containing the drug at each concentration. After the incubation with FTY720 for 2 h, we added 10 μl of MTT prepared at a concentration of 5 mg/ml in PBS to each well and continued the cell culture for another 4 h at 37°C. We then added a color development solution to each well and measured absorbance using a microplate reader with a wavelength of 570 nm.

Received 8/22/01; accepted 1/4/02.

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2 The abbreviations used are: ECM, extracellular matrix; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Gp, group; TV, tumor volume; TUNEL, terminal deoxynucleotidyl transferase (Tdh)-mediated nick end labeling; VLA, very late antigen; F-actin, filamentous actin.
Cell viability was expressed as a percentage of absorbance in cells with FTY720 treatment to that in cells without FTY720 treatment.

**Flow Cytometry.** JygMC(A) cancer cells and NCTC-2525 fibroblasts were synchronized at the G<sub>0</sub>/G<sub>1</sub> phase by the double thymidine block method as follows. The cell number was adjusted to 5 × 10<sup>4</sup> for plating in fresh medium before the procedure. Cells were incubated with fresh medium containing 2.5 mM of thymidine without FCS for 12 h and then washed twice with PBS, followed by addition of fresh medium with 10% FCS and additionally incubated for another 12 h. After this, we again incubated cells with fresh medium containing 2.5 mM of thymidine without FCS for 12 h. To release the cells from the block, we washed the cells twice with PBS and replaced them in fresh medium with 10% FCS. After synchronization, we incubated the cells with or without 10 μM of FTY720 for 3, 6, 9, 12, 18, or 24 h. The cells were harvested after treatment with 0.2% EDTA, fixed with ice-cold 70% ethanol solution, hydrolyzed with 250 μg/ml of RNaseA (type 1-A; Sigma Chemical Co.) at 37°C for 30 min, and stained with propidium iodide (Sigma Chemical Co.) at 10 mg/ml for 20 min. We analyzed the DNA content of the cells by an EPICS ELITE flow cytometer (Coulter, Hialeah, FL) and calculated the cell cycle distribution using a MULTICYCLE program (Phoenix Flow Systems, San Diego, CA).

**Cell Growth Assay.** JygMC(A) cancer cells and NCTC-2525 fibroblasts were synchronized at the G<sub>0</sub>/G<sub>1</sub> phase by the double thymidine block method as described above. After synchronization, we incubated the cells with or without FTY720 at the various concentrations (2, 5, 10, 15, 30, and 50 μM) for 24 h. The number of cells was determined after the incubation with FTY720, and cell growth rate was expressed as the percentage of the cell number in the wells treated with FTY720 compared with that in the control wells.

**Electron Microscopic Analysis.** For electron microscopic observation, the cancer cells were incubated with FTY720 at the various concentrations (2, 5, and 10 μM) for 24 h. The cells cultured without FTY720 served as controls. We harvested floating cells together with adherent cells and centrifuged them at 2000 rpm for 5 min. The cell pellets were fixed overnight at 4°C in a 0.2 M sodium cacodylate buffer containing a 2% solution of glutaraldehyde. Samples were then postfixed in cacodylate-buffered 1% osmium tetroxide, dehydrated, and embedded in Epon 812 (Nacalai Tesque, Osaka, Japan) for ultrathin sectioning. We stained the ultrathin sections with uranyl acetate and lead citrate, and viewed them with an electron microscope (H7100; Hitachi, Tokyo, Japan).

**Fluorescent Staining of F-Actin with FITC-labeled Phalloidin.** Fluorescent staining of F-actin was carried out according to the method of Sasaki et al. (20) with minor modification. JygMC(A) cells were plated on 8-mm square Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) and incubated with FTY720 at the various concentrations (2, 5, 10, and 30 μM) for 2 h at 37°C in a CO<sub>2</sub> incubator. The cells were washed with PBS, fixed in 4% paraformaldehyde-PBS solution, permeabilized with 0.5% Triton X-100, and stained with FITC-labeled phalloidin. The slides were analyzed by a fluorescence microscope (UX-FX-DX; Nikon, Tokyo, Japan).

**Adhesion Assay.** We examined the influence of FTY720 on ability of JygMC(A) cancer cells to adhere to ECM components including laminin, collagen I, and fibronectin. Briefly, we coated a 96-well culture plate with laminin (50 μg/ml; Sigma Chemical Co.), collagen I (300 μg/ml; Chemicon), or fibronectin (20 μg/ml; Sigma Chemical Co.), and blocked the plate with 10 mg/ml BSA. JygMC(A) cells (2 × 10<sup>4</sup>) with or without 3-hour incubation of FTY720 at a concentration of 2 μM were added to each well. After another 3-hour incubation at 37°C, the cells were washed twice with PBS to remove nonadherent cells and fixed with 4% of paraformaldehyde solution for 10 min. Samples were stained with toluidine blue and rinsed in water. Cells were then solubilized by adding 1% SDS and quantified using a microtiter plate reader at 595 nm. The data were represented as absorbance; cells treated with FTY720 and control cells were then compared. The experiments described were repeated a minimum of three times.

**Migration Assay.** We performed cell migration assay by a modified Boyden’s chamber method using microchemotaxis chambers and polycarbonate filters (Neuro Probe, Gaithersburg, MD) with a pore size of 8.0 μm. The filters were coated with 20 μg/ml fibronectin, 50 μg/ml laminin, or 300 μg/ml collagen I, and placed between upper and lower chambers. We next prepared cell suspensions of JygMC(A) cells (5 × 10<sup>5</sup>) with or without treatment of FTY720 at 2 μM for 3 h in serum-free DMEM containing BSA. We then placed the cells (2.5 × 10<sup>4</sup> cells in 50 μl) into the upper compartment of chamber and placed 25 μl of DMEM containing 10% FCS in the lower chamber. Chambers were incubated at 37°C with 5% CO<sub>2</sub> for 3 h. Thereafter, we removed the filter and scraped off nonmigrating cancer cells remaining on the upper side of the filter. The cells that had migrated to the lower side of the filter were fixed in methanol, stained with toluidine blue, and counted in >10 fields of view under a microscope at ×200 magnification. Migratory activity was expressed as the mean number of cells that had migrated to the lower side of the filter, and results were represented as mean ± SD of cells per field of view. The experiments described were repeated a minimum of three times.
Flow Cytometric Analysis for Integrin Expression. JygMC(A) cells (2 × 10^5) cultured in DMEM containing 10% FCS were treated with or without FTY720 at a concentration of 2 μM for 6 h. The cells were harvested after treatment with 0.2% EDTA. After fixation with 4% paraformaldehyde solution, the cells were incubated with 10-fold dilution of goat antimouse polyclonal antibodies against the following integrins: VLA1 (α1β1), VLA2 (α2β1), VLA5 (α5β1), and VLA6 (α6β1); Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The cells were then incubated with an 80-fold dilution of FITC-conjugated donkey antigoat IgG (Santa Cruz Biotechnology) at 4°C for 30 min. After each incubation, the cells were washed three times with PBS containing 1% BSA and analyzed with an EPICS ELITE flow cytometer (Coulter).

In Vivo Effect of FTY720 on Tumor Growth in a Mouse Breast Cancer Model. We studied the in vivo effect of FTY720 in preventing tumor growth using a mouse breast cancer model (Group 1). Aliquots of JygMC(A) cancer cells (1 × 10^6) were s.c. inoculated into BALB/c nu/nu mice. The mice were housed in microisolator cages under positive pressure, and maintained at a constant temperature (20°C) and humidity. Surgical manipulations and drug treatments were performed in a laminar flow cabinet. When the tumors reached ~10 × 10 mm in size, the animals were assigned randomly to four subgroups, and the treatment with FTY720 was initiated at the three different dosages. Mice from three subgroups received an i.p. injection of FTY720 daily at a dosage of 2 mg/kg (Gp1–2; n = 30), 5 mg/kg (Gp1–5; n = 30), or 10 mg/kg (Gp1–10; n = 30); drug administration was continued until the end of the study. Mice without FTY720 treatment served as controls (Gp1-Cont; n = 90). Tumor size was measured every 3 days in two dimensions, the longest axis (a) and perpendicular shortest axis (b). TV was calculated using the formula TV = 0.4ab^2 and transformed into relative values (v) (v = Vx/V0, where V0 is the TV at initiation of treatment and Vx is the volume at any given day). Animals were sacrificed on days 2, 24, and 35, at which times tumors and tissues were harvested and prepared for morphological and molecular analysis.

The Influence of FTY720 on Tumor Metastasis In Vivo. Because the present animal model aggressively develops numerous metastatic lesions in the

Table 1  Number of filopodias on the cell surface (number of filopodias/cell, mean ± SD)

The number of filopodias on cancer cell surface was evaluated by staining of F-actin. The FTY720 treatment significantly decreased the number of filopodias on the cancer cell surface.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Number of Filopodias/cell (Mean ± SD)</th>
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<tbody>
<tr>
<td>2 μM</td>
<td>22.4 ± 4.70</td>
</tr>
<tr>
<td>5 μM</td>
<td>8.3 ± 2.05**</td>
</tr>
<tr>
<td>10 μM</td>
<td>6.94 ± 1.65*</td>
</tr>
<tr>
<td>30 μM</td>
<td>3.69 ± 1.49*</td>
</tr>
<tr>
<td>100 μM</td>
<td>2.25 ± 1.06*</td>
</tr>
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* αP < 0.01 versus control.
In Situ Assay for DNA Fragmentation. We observed in situ DNA fragmentation in the tumor sections with the TUNEL method. The sections were prepared as described previously (21). Staining for DNA fragmentation was performed using an In Situ Apoptosis Detection kit (Takara; Nihonbashi, Tokyo, Japan), according to the manufacturer’s instructions.

Transmission Electron Microscopy of Tumors from Mice. For electron microscopic observation, tumors were fixed, and ultrathin sections were prepared as described above and viewed with an electron microscope (H7100; Hitachi).

Ethics. All of the experimental protocols were conducted in accordance with the policies of the Animal Ethics Committee at our institution.

Statistical Analysis. The number of filopodias on the cell surface, the data from adhesion assay and migration assay, and the expression of integrins were subjected to the unpaired Student’s t test. Results from TV were subjected to one-way ANOVA without replication. When the ANOVA resulted in significance, individual comparisons were performed by the Student t test. The unpaired Student’s t test was applied to values obtained for quantitative evaluation of tumor metastasis including organ weight, number of metastatic colonies, and the ratio of metastatic area:total area. Animal survival was evaluated by Kaplan-Meier analysis. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

Significant Cancer Cell Death after in Vitro Treatment with FTYY720. MTT assay demonstrated that the FTYY720 treatment induced a dramatic loss in cell viability in JygMC(A) cancer cells in a dose-dependent fashion (Fig. 1A). This was confirmed by flow cytometric analysis where >80% of cancer cells died within 12 h after the initiation of FTYY720 treatment at 10 \( \mu \text{M} \), whereas the cells without FTYY720 treatment demonstrated normal cell viability with no remarkable cell death (Fig. 1B).

Drug Susceptibility Lower in Normal Fibroblasts, NCTC-2525, than in Cancer Cells. We analyzed the influence of FTYY720 treatment on cell viability in cancer cells as well as in normal fibroblasts lung, liver, and kidney, we investigated the influence of FTYY720 on tumor metastasis using this model. We set up another experimental group (Group 2) in which tumors were excised from mice when the tumors reached \(~10 \times 10 \text{ mm} \) in size to eliminate the influence of TV on the development of cancer metastasis. Animals were randomly assigned to four subgroups: mice not treated with FTYY720 (Gp2-Cont), and mice treated with FTYY720 i.p. at a daily dosage of 2 mg/kg (Gp2-2; \( n = 30 \)), 5 mg/kg (Gp2-5; \( n = 30 \)), or 10 mg/kg (Gp2-10; \( n = 30 \)). The drug administration was initiated immediately after tumor excision and continued until the end of the study. Animals were sacrificed on day 24 after initiation of drug administration, and harvested organs were weighed and fixed with 4% buffered formalin after perfusion. The paraffin sections were stained with H&E for light microscopic assessment. The number of metastatic colonies in each organ was counted in a histological section. For more accurate quantitative evaluation of cancer metastasis, the ratio of metastatic area:total area in a histological section was calculated using a public domain NIH image program (written by Wayne Rasband at NIH and available on the internet\(^1\)) and MCID program (Imaging Research Inc., Ontario, Canada). The results were represented as a percentage of metastatic area:total area ratio.

\(^1\) Internet address: http://info.nih.gov/nih-image/download.htm.
Disorganization of Actin Stress Fiber in Cancer Cells Treated with FTY720. We examined the influence of FTY720 treatment on cancer cell structures by staining with F-actin. FTY720 treatment caused remarkable cytoskeletal changes with marked disorganization of actin stress fiber, such as a decreased number and length of actin-containing microfilaments in cancer cells, in a dose-dependent manner (Table 1; Fig. 4). When cancer cells were treated with the drug at 2 μm, cells became relatively round with shortened stress fibers and deformed filopodias on the cell surface as compared with cells without FTY720 treatment. The stress fibers were markedly shortened, and cells became round with remarkably decreased and flattened filopodias at 10 μm. The filamentous actin structure was totally disrupted with marked deformity in cancer cells at 30 μm.

Significantly Decreased Cancer Cell Adhesion and Migration Because of FTY720 Treatment at 2 μm. We examined the influence of FTY720 treatment on the adhering ability of cancer cells to ECM components by a cell adhesion assay. Cancer cell adhesion to ECM components, especially laminin, was significantly reduced after 6 h of FTY720 treatment at 2 μm (Fig. 5A). In addition, we investigated the migration activity of cancer cells using a modified Boyden’s chamber method. The cancer cell migration was significantly suppressed after 6 h of FTY720 treatment at 2 μm, particularly when filters were coated with laminin (Fig. 5B).

Marked Decrease in the Expression of Integrins in Cancer Cells by FTY720 Treatment. The expression of integrins, including VLA1 (α1β1), VLA2 (α2β1), VLA5 (α5β1), and VLA6 (α6β1), that are known to be critical for cancer metastasis, was determined by flow cytometric analysis. As shown in Fig. 6, 6-h FTY720 treatment at 2 μm significantly decreased the expression of integrins, especially VLA1, VLA2, and VLA6, which bind to laminin and collagen type I.

Prevention of Tumor Growth in a Mouse Breast Cancer Model by FTY720 Treatment. In the control animals, the tumors became bulky masses that invaded the pleural cavity, and harvested tumors were soft and densely vascularized. In contrast, when mice were treated with FTY720 at a dosage of 5 mg/kg/day or more, tumor growth was significantly suppressed and remained s.c. with no evident pleural invasion; harvested tumors were elastically hard and poorly vascularized. Fig. 7 shows the time course of tumor growth in mice. Treatment with FTY720 at 2 mg/kg/day did not show any significant effect in preventing tumor growth.

Apoptotic Cell Death Appeared Frequently in Tumor Sections 2 Days after Initiation of FTY720 Treatment. We investigated cancer cell apoptosis in the tumors harvested on day 2 after initiation by cell growth assay and flow cytometry. Treatment with FTY720 markedly decreased cell growth rate in a dose-dependent fashion; no cells survived with the treatment of FTY720 at a concentration of 15 μm or more (Fig. 2A). Furthermore, the drug susceptibility to FTY720 was significantly lower in the normal mouse fibroblasts than in the cancer cells. Treatment with FTY720 at a concentration of 2 μm did not affect the cell viability in NCTC-2525, which exhibited normal cell proliferation (Fig. 2A). Although a decreased cell growth rate was noted in NCTC-2525 with the treatment of FTY720 at a concentration of 5 μm, the effect of FTY720 was significantly less in NCTC-2525 than in the cancer cells. These results were confirmed by flow cytometric analysis; only 16.7 ± 5.8% of NCTC-2525 cells underwent apoptosis at 24 h after FTY720 treatment at 5 μm, whereas 77.8 ± 4.68% of cancer cells underwent apoptosis in the same condition (Fig. 2B).

Morphological Changes in Cancer Cells Analyzed by Electron Microscopy. Cancer cells without FTY720 treatment exhibited innumerable microvilli and well-developed filopodias on the cell surface with intact nuclei (Fig. 3a). However, cancer cells treated with FTY720 at a low concentration of 2 μm demonstrated distinct changes on the cell surface with decreased filopodias and microvilli, despite no remarkable changes being noted in the nuclei (Fig. 3b). In contrast, FTY720 treatment at 10 μm induced features typical of apoptosis including fragmented nuclei with condensed chromatin accompanied by well-preserved cytoplasmic organelas, such as mitochondrias (Fig. 3c).
Fig. 8. Characteristic changes of apoptosis in the tumors harvested on day 2 after initiation of FTY720 treatment (10 mg/kg/day). H&E-stained tumors from the nontreated control mice (Gp1-Cont) show features typical of cancer growth representing aggressive cell proliferation accompanied by innumerable mitosis but no evidence of cell death (a). In contrast, structural deformity of the neoplastic glands and cell collapse were evident along with the characteristic changes of apoptosis, appearing as fragmented nuclei with highly condensed chromatin at 2 days after initiation of FTY720 treatment (Gp1-10; b). TUNEL-positive cells were rarely found in tumor sections from control mice (c), whereas many positive cells with marked nuclear fragmentation were present in the sections from mice treated with FTY720. Boxed area shows high-power magnification of TUNEL-positive cells (d). Electron microscopy showed that the tumor from untreated mice had undamaged cancer cell structures possessing fine nuclear chromatin pattern and intact mitochondrial architectures with no evidence of degenerative or apoptotic changes (e). In contrast, the tumor from mice treated with FTY720 exhibited the features typical of apoptosis, including fragmented nuclei with chromatin condensation (arrows) and cell shrinkage (f). Original magnification, ×400 in light microscopic appearance of a–d and ×600 in boxed area; ×3000 and ×3500 in electron microscopic appearance of e and f, respectively.

Prevention of Cancer Metastasis in Lung, Liver, and Kidney by Treatment with FTY720. In control groups (n = 90/group), >90% of the mice developed numerous metastatic lesions in the lung, and >50% of the mice developed metastasis in the liver and kidney by 3 weeks; these progressed aggressively and diffusely thereafter. Fig. 9a presented the in situ appearance of a mouse sacrificed at 5 weeks after inoculation of cancer cells. The mouse exhibited numbers of metastatic lesions in the lung, liver, and kidneys (Fig. 9b); only a few cancer cells were noted in the histological section of each organ even at a dosage of 2 mg/kg/day at which dose the treatment did not show any preventive effect on tumor growth (Fig. 9c).

We next tested the effect of FTY720 on cancer metastasis without the influence of primary tumor by excising tumors from mice when tumors reached ~10 × 10 mm in size. In the control group, most of the mice developed numbers of metastatic lesions in the lung, liver, and kidney by 3 weeks after the resection of tumors. Fig. 10 (a–c) shows numerous metastatic lesions as a hematoxylin-stained area in H&E sections of each organ. These lesions occupy more than half of the total area especially in the lung sections. Microscopic findings showed aggressive cancer growth with innumerable mitosis, which replaced normal organ construction and caused structural deformity in each organ (Fig. 10, a–c). In contrast, when mice were treated with FTY720, no remarkable metastatic lesions were observed macroscopically (Fig. 10, g–j), and only a few cancer cells were noted in the histological sections 24 days after resection of tumors (Fig. 10, d–f).

For quantitative evaluation of cancer metastasis, we examined organ weight, number of metastatic colonies, and the ratio of metastatic area:total area in a histological section; the results were presented in Table 2. The weight of organs harvested from FTY720-treated mice even at a low dose (2 mg/kg/day) significantly decreased and correlated with a reduced number of metastatic colonies as compared with those from control mice. The ratio of metastatic area:total area confirmed the data, demonstrating a significant de-
crease in the metastatic area in the organs from FTY720-treated mice (Table 2).

**Prolonged Animal Survival by Treatment with FTY720.** In the control group (Gp1-Cont), most mice showed substantial loss of body weight, and >50% died by 40 days. No animal survived >52 days after inoculation of the cancer cells (Fig. 11). In contrast, when mice were treated with FTY720, survival was significantly prolonged in all of the subgroups (Gp1–2, Gp1–5, and Gp1–10) as compared with control group. No animal died before 38 days, and >50% of the animals survived >52 days.

**Adverse Reaction.** All of the mice treated with FTY720 survived with a healthy appearance and no loss of body weight. Hematological data demonstrated neither myelosuppression nor biochemical abnormalities but did show a decrease in peripheral lymphocytes in mice treated with FTY720 (Table 3).

**DISCUSSION**

FTY720 is a new immunosuppressive agent that prolongs graft survival in various animal models of organ transplantation without causing severe adverse reactions. Induction of apoptosis in resting and activated lymphocytes is considered to be one of its major mechanisms of action (2–6). We have also demonstrated that FTY720 induces apoptosis *in vitro* in several cancer cell lines, including HL-60, Jurkat cells, and WR19L (7–9). Thus, we postulated that this mechanism might be effective for *in vivo* anticancer treatment by inducing apoptosis in cancer cells. Indeed, the present study showed that FTY720 powerfully inhibited *in vivo* tumor growth in a mouse model of breast cancer while causing no severe side effects. In these animals, histological sections of tumors stained by H&E and TUNEL methods demonstrated the characteristic features of apoptosis in many cancer cells. Apoptosis was confirmed by electron microscopy, affected cells exhibiting small cytoplasm and fragmented nuclei with condensed chromatin, and some exhibiting crescent formation. Despite such dramatic cancer cell apoptosis, no remarkable side effects were found in mice treated with FTY720, suggesting that cancer cells are more susceptible to FTY720 than normal cells.

*In vitro* treatment of cancer cells corroborated these results. MTT
and cell growth assays demonstrated a dramatic loss of viability of cancer cells with treatment of FTY720 in a dose-dependent fashion. The flow cytometric analysis showed that the initiation of drastic cancer cell death occurred by 9 h after incubation of FTY720, and most of cancer cell death was observed by 24 h. Morphological study by light and electron microscopy revealed typical changes of apoptosis after treatment with FTY720. Interestingly, the effect of FTY720 was significantly less in the normal fibroblasts than in cancer cells. In a previous study, we also found that FTY720 rapidly induced apoptosis in a human prostate cancer cell line, DU145, whereas normal human prostate stromal cells were resistant to FTY720 (22). These findings indicate that cancer cells are more susceptible to FTY720.

The mode of action of most currently used chemotherapeutic agents is based on the preferential toxicity to rapidly dividing cells, e.g., malignancies that have relatively rapid growth, such as germ cell tumors, are relatively chemosensitive, whereas slower growing neoplasms such as renal cell carcinoma are less sensitive. Similarly, toxicity from chemotherapeutic agents in normal, nonmalignant cells is also seen primarily in rapidly dividing cells, such as hematopoietic cells in the bone marrow, especially granulocyte, which has very high proliferation activity. Therefore, a reduction of granulocytes, which causes the most serious side effect, is frequently seen in patients treated with current chemotherapeutic agents. In contrast to these chemotherapy agents, FTY720 may exhibit its activity through a different mechanism. In our previous and present studies, the agent induced a drastic apoptosis in peripheral lymphocytes, whereas no remarkable change was noted in other hematopoietic cells, including granulocytes, megakaryocytes, and erythrocytes (5, 6). These findings may indicate that the drug-induced apoptosis is not based on the cell toxicity, but it is possible that FTY720 activates certain intracellular pathway of apoptosis, which may exist commonly both in cancer cells and lymphocytes. We have investigated the intracellular mechanisms of action of this agent in detail, providing the following evidence up to now. The intracellular ratio of Bcl-2:Bax proteins in the FTY720-exposed cultured human lymphocytes decreased immediately after treatment with FTY720 (4). This suggests that the drug induces Bcl-2-family associated apoptotic cell death. In addition, Fas-mutant mice (MRL-lpr/lpr) treated in vivo with FTY720 exhibited a widespread lymphocyte apoptosis in the thymus, spleen, and lymph nodes (23), indicating that the drug-induced cell death occurs via a Fas-independent pathway. Furthermore, recent studies have shown that the drug induces activation of caspase cascade (7) and that apoptosis is related to protein kinases, including c-Jun-NH2-terminal kinase, p38, and a novel Mr 36,000 myelin basic protein (8). Although additional investigation is necessary, FTY720 may be a new cancer-targeting agent, which induces cancer cell death by promoting the apoptotic signal pathways.

Table 2 Organ weight, number of metastatic colonies, and ratio of metastatic area: total area (mean ± SD)

Mice from group 2 were sacrificed 24 days after resection of tumors; we examined organ weight, number of metastatic colonies, and the ratio of metastatic area to total area for quantitative evaluation of cancer metastasis. The weight of organs harvested from FTY720-treated mice (2 mg/kg/day) significantly decreased and correlated with a reduced number of metastatic colonies as compared with those from control mice. The ratio of metastatic area to total area in a histological section confirmed the data, demonstrating a significant decrease in the metastatic area in the organs from FTY720-treated mice.

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<th></th>
<th>Lung (R+L)</th>
<th>Liver</th>
<th>Kidney (R+L)</th>
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<tbody>
<tr>
<td><strong>Gp2-Cont</strong></td>
<td></td>
<td></td>
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<tr>
<td>Organ weight (g)</td>
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<td>2.25 ± 0.24</td>
<td>0.90 ± 0.17</td>
</tr>
<tr>
<td>Number of metastasis</td>
<td>&gt;100</td>
<td>17.5 ± 5.56</td>
<td>9.09 ± 3.53</td>
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<tr>
<td>Ratio of metastasis (%)</td>
<td>45.7 ± 15.4</td>
<td>22.5 ± 8.82</td>
<td>20.2 ± 7.39</td>
</tr>
<tr>
<td><strong>Gp2-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ weight (g)</td>
<td>0.25 ± 0.04</td>
<td>1.67 ± 0.13</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Number of metastasis</td>
<td>15.1 ± 5.59</td>
<td>7.27 ± 2.76</td>
<td>4.91 ± 1.81</td>
</tr>
<tr>
<td>Ratio of metastasis (%)</td>
<td>15.9 ± 5.90</td>
<td>4.32 ± 1.99</td>
<td>9.11 ± 2.41</td>
</tr>
</tbody>
</table>

*p < 0.01 versus control.

Table 3 Hematological data in Gp1-Cont, Gp1-10, and age-matched naive mice (mean ± SD)

The data from hematological and biochemical examination at the time of harvesting in mice from Gp1-Cont, Gp1-10, and age-matched naive BALB/c-nu-nu mice (B/nu-naive) are presented in the table. Although a significant decrease in peripheral lymphatic cells (Lymph) was observed in mice treated with FTY720 compared with control mice (P < 0.01), no significant difference was observed in other hematological and biochemical data between FTY720-treated and control groups.

A. Complete blood count

<table>
<thead>
<tr>
<th></th>
<th>WBC (/µl)</th>
<th>Lymph (%)</th>
<th>RBC (x10^6/µl)</th>
<th>Hemoglobin (x10^3/g)</th>
<th>Platelet (x10^3/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gp1-Cont</td>
<td>1480 ± 228</td>
<td>27.7 ± 1.75</td>
<td>928 ± 75.4</td>
<td>14.0 ± 1.08</td>
<td>80.7 ± 5.10</td>
</tr>
<tr>
<td>Gp1-10</td>
<td>1560 ± 397</td>
<td>19.6 ± 3.79</td>
<td>929 ± 67.0</td>
<td>13.5 ± 0.75</td>
<td>81.6 ± 8.28</td>
</tr>
<tr>
<td>B/nu-naive</td>
<td>2060 ± 114</td>
<td>27.9 ± 2.22</td>
<td>1073 ± 105</td>
<td>14.7 ± 0.99</td>
<td>85.8 ± 5.34</td>
</tr>
</tbody>
</table>

B. Biochemical analysis

<table>
<thead>
<tr>
<th></th>
<th>Aspartate aminotransferase (IU/liter)</th>
<th>Alanine aminotransferase (IU/liter)</th>
<th>Blood urea nitrogen (x10^3 mg/dl)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Natrium (mEq/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gp1-Cont</td>
<td>60.6 ± 5.58</td>
<td>41.2 ± 8.58</td>
<td>33.9 ± 6.66</td>
<td>0.12 ± 0.04</td>
<td>158 ± 2.74</td>
</tr>
<tr>
<td>Gp1-10</td>
<td>66.4 ± 5.37</td>
<td>36.8 ± 7.33</td>
<td>31.8 ± 4.28</td>
<td>0.10 ± 0.00</td>
<td>160 ± 1.92</td>
</tr>
<tr>
<td>B/nu-naive</td>
<td>67.2 ± 3.49</td>
<td>60.8 ± 11.5</td>
<td>34.2 ± 3.13</td>
<td>0.10 ± 0.00</td>
<td>160 ± 1.58</td>
</tr>
</tbody>
</table>
Additionally, one of most important findings in this study is the marked prevention of tumor metastasis by FTY720 treatment even at a low dose. In control groups, most of the mice developed numerous metastatic lesions in the lung, liver, and kidney. Histological sections of these organs showed aggressive cancer growth accompanied by innumerable mitosis, which replaced normal organ tissue. In contrast, mice treated with FTY720, even at a low dose of 2 mg/kg/day, exhibited normal macroscopic appearance in the lung, liver, and kidney, although the agent did not influence tumor growth at this dosage. Only a few cancer cells were noted in the histological sections of the distant organs from these animals.

Although metastasis is one of the major causes of mortality in cancer patients, there are few therapeutic options for patients with advanced disease and metastases. The development of cancer metastasis is a complex cascade of events involving tumor dissemination from the primary site to distant organs. The cancer cells released from the primary tumor invade the surrounding tissues, enter into the vascular or lymphatic circulation (10, 11, 24), and then become arrested on the distant organ. The formation of tumor-tumor aggregates or emboli is an important event in this step (13). Tumor emboli may be arrested nonspecifically in narrow capillaries; subsequently, some of the tumor cells may penetrate the endothelium, degrade the basement membrane and ECM, and invade the secondary organ.

In the present study, the data from the staining of F-actin indicated that FTY720 caused remarkable cytoskeletal change with a marked disorganization of actin stress fiber, such as a decrease in number and length of actin-containing microfilaments in a dose-dependent manner. In addition, cells became round with decreased and deformed filopodias on the cell surface even at a low dose of FTY720 treatment. Electron microscopic findings confirmed these changes, revealing round cells with decreased filopodias and loss of microvilli on the cell surface. Such structural changes in cancer cells may contribute to the prevention of the metastatic cancer dissemination through the interruption of cancer cell arrest within the target organs.

After arrest, tumor cells must establish stable contacts with the endothelium, induce endothelial cell retraction, migrate, and attach to several connective tissue barriers that consist of ECMs such as laminin, fibronectin, and other glycoproteins and proteoglycans. Finally, they must proteolytically degrade these connective tissues and extravasate. During this process, the interaction of tumor cells with ECM components is thought to be mostly dependent on the presence of integrins (14, 17). The altered integrin-mediated adhesion of cancer cells to ECMs has been reported to be a critical factor in the metastatic cascade (15, 16, 18, 25); e.g., transfection of VLA-2 cDNA into rhabdomyosarcoma cells resulted in marked increase of adhesion to collagen and laminin in vitro and enhancement of lung metastasis in vivo (26). Cancer cell transformation is reported to be implicated in the altered expression of integrins (27–31). Human cells subjected to N-methyl-N-nitro-N-nitrosoguanidine transformed into highly tumorigenic cells with a significant increase in the expression of the integrins such as VLA1, VLA2, and VLA6 that promote the invasive ability through the basement membranes (29). The present study demonstrated that FTY720 treatment at a low concentration significantly decreased the ability of cancer cells to adhere to ECM components, especially laminin. In addition, the activity of cancer cell migration through ECMs was also significantly reduced by the treatment at a low concentration, especially when filters were coated with laminin. The results indicate that FTY720 treatment at a low concentration may decrease the ability of cancer cells to interact with ECM components. Furthermore, the expression of integrins, especially VLA1, VLA2, and VLA6, that are the ligands for laminin and collagen type I, was significantly decreased in cancer cells treated with FTY720. These findings suggest that the reduced expression of integrins may be implicated with a decreased ability of cancer cells to interact with ECM components, which is thought to be a critical process for the development of cancer metastasis.

In conclusion, the present study has demonstrated that FTY720 displays a potent anticancer activity by inducing apoptosis in vitro and in vivo. Additionally, it prevents dissemination of cancer metastases in animal models even at a lower dosage. Although additional investigation is necessary, the decreased ability of cancer cells to interact with ECM components, which is caused by FTY720 treatment, may be associated with the mechanism of the prevention of the cancer metastasis. Structural changes in cancer cells and decreased expression of integrins on the cell surface is possibly one of its underlying mechanisms of action. Thus, FTY720 may be a promising agent for the treatment of cancer patients even if they demonstrate evidence of metastatic spread of cancer.

REFERENCES


Marked Prevention of Tumor Growth and Metastasis by a Novel Immunosuppressive Agent, FTY720, in Mouse Breast Cancer Models

Haruhito Azuma, Shiro Takahara, Naotsugu Ichimaru, et al.


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