Abstract. To examine the drug efficacy of a novel farnesyltransferase inhibitor (FTI), CH4512600, in vivo, we developed a reliable liver metastasis model of human colon cancer using NOD/Shi-scid IL2Rγnull (NOG) mice. Eleven human colon cancer cell lines were examined for their ability to form diverse metastatic foci in the livers of NOG mice. When inoculated with 10⁴ COLO320DM, HCT 116, HT-29, WiDr, LoVo and LS174T cells, liver metastasis was evident in 100% (6/6), 100% (6/6), 88.9% (8/9), 87.5% (7/8), 83.3% (5/6) and 50.0% (3/6) of the NOG mice, respectively. CaCo2, COLO201, LS123, SW48 and SW1417 showed no metastasis when seeded at 10⁴ cells even in NOG mice. The mRNA expression levels and genetic mutations of N, H and K-RAS genes, which directly affect the levels of cellular RAS protein that would be molecular target for FTI, were also examined in these six metastatic human colon cancer cell lines for molecular biological and genotypic characteristics. Only three cell lines had a point mutation in the RAS oncogene. LS174T cell line had a point mutation of the K-RAS gene at codon 12 (gly12→asp; G12D), and HCT 116 and LoVo cell lines had a point mutation of the K-RAS gene at codon 13 (gly13→asp; G13D). Relative gene expression levels of N, H and K-RAS genes in the HCT 116 cell line were 2.6-5.0-fold lower than that of LS174T and LoVo cell lines. We selected HCT 116 cell line from our liver metastasis model for evaluation of FTI CH4512600 efficacy in vivo.

Using the NOG mouse liver metastasis model, we demonstrated the effectiveness of FTI CH4512600 to suppress tumor growth in vivo and to prolong mouse survival significantly from 36.9±2.9 to 50.3±9.4 days.

Introduction

Colon cancer is one of the major cancers (1,2). Despite resection of colorectal cancer, about half of the patients suffer recurrence from metastasis. The liver is the major site of metastasis of colon cancer (3-6); but only limited numbers of patients who suffer metastasis are candidates for surgery. The alternative method to treat liver metastasis is chemotherapy; therefore, establishment of a liver metastasis model effective for evaluating compounds is important.

RAS is a guanine nucleotide binding GTPase that transduces developmental and proliferative information from extracellular signals to the nucleus (7,8). In normal cells, activation of RAS is mediated by nucleotide exchange factors that catalyze the exchange of GDP (inactive form) with GTP (active form), whereas its inactivation is mediated by GTPase-activating proteins (7). A large number of human cancers (30%) harbor mutations in the RAS protein that result in GTP-locked RAS, which is constitutively activated and is believed to contribute to uncontrolled malignant growth (9). Therefore, blocking the reckless RAS signal is an attractive target for chemotherapy. RAS requires a lipid post-translational modification (farnesylation) for its cancer-causing activity (10,11). The modification is catalyzed by FTase, which attaches farnesyl to the cysteine of the RAS carboxyl-terminal tetrapeptide CAAX (12-16). Because farnesylation of RAS is required and sufficient for its transforming activity (17,18), an intense search for FTase inhibitors (FTIs) with potential anticancer activity is under way (10,11). Many FTIs are reported to have potent tumor growth inhibitory activity, although their anti-tumor effect is caused not only by blocking of the RAS signaling pathway (19-24).

New strategies for treating metastatic colon cancer will also require the development of appropriate animal models for
studying their effectiveness. NOD/Shi-scid IL2R<sup>null</sup> (NOG) mice have been used for many in vivo models with engrafted human cells and tissues, such as such human hematopoietic stem cells (25,26), myeloma cells (27,28) and endometrial tissue (29). Moreover, when compared with NOD/Shi-scid mice, NOG mice were a superior xenotransplantation system for the engraftment of human cancer cells (30). Recently, we developed a reliable new model system for assaying hematogenous liver metastasis of pancreatic cancer using NOG mice (31). The liver metastasis incidence and grade of each of the pancreatic cancer cell lines were quantitatively evaluated, and were dose-dependent over a wide range of inoculation doses.

In this study, we first examined the in vivo biological behavior of eleven human colon cancer cell lines using the NOG mouse liver metastasis model. Then, we examined human colon cancer cell lines that possessed liver metastatic potentials in terms of the molecular biological behavior of the target molecules. In this case, RAS oncogene mutation and gene expression are molecular targets for the FTI. We selected the HCT 116 cell line from our liver metastasis panel for evaluation of FTI CH4512600 efficacy in vivo and demonstrated its effectiveness through significantly prolonged mouse survival. This liver metastasis model using NOG mice should respond flexibly in efficacy evaluations of all types of anti-cancer drugs.

Materials and methods

Cells. CaCo2, COLO201, COLO320DM, HCT 116, HT-29, LoVo, LS123, LS174T, SW48, SW1417, and WiDr were obtained from the American Type Culture Collection (Manassas, VA). COLO201 and COLO320DM were maintained in RPMI1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Hyclone, UT). HCT 116 and HT-29 were maintained in McCoy's 5A (Sigma), LoVo was maintained in Ham's F12K (Sigma), SW48 and SW1417 were maintained in Leibovitz's L-15 (Sigma), and LS123, LS174T and WiDr were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma). All culture medium were supplemented with antibiotics and 10% FBS, except for the CaCo2 cell line. DMEM containing 20% was used for CaCo2. Cells were incubated in a humidified (37˚C, 5% CO2) incubator and passaged on reaching 80% confluence. SW48 and SW1417 were incubated in a 100% air (37˚C) incubator and passaged on reaching 80% confluence.

Liver metastasis assay. This study was performed in accordance with the institutional guidelines and was approved by the Animal Experimentation Committee of the Central Institute for Experimental Animals. We bred NOD/Shi-scid IL2R<sup>null</sup> (NOG) mice, and used them at the age of 7-9 weeks. Sub-confluent culture of colon cancer cells was harvested with trypsin-EDTA solution. Collected cells were washed and suspended in serum-free medium at a concentration of 2x10<sup>5</sup> cells per ml. Experimental liver metastases were generated by intrasplenic injection of 10<sup>5</sup> of cancer cells (50 μl of cell suspension) and splenectomy (9). The mice were sacrificed 6 weeks later, and liver metastases were enumerated immediately, without fixation.

RAS oncogene mutation and gene expression analyses. Total cellular RNA was obtained from 90% confluent cultures of colon cancer cell lines using the RNeasy Mini kit (Qiagen K.K., Tokyo, Japan). The reverse transcription polymerase chain reaction (RT-PCR) was performed using the GeneAmp RNA PCR Kit (Applera Corp., Applied Biosystems, CA). N, H and K-RAS oncogene mutations were analyzed by the PCR-direct sequencing method with the following primer sets, H-Nras-F: TGTTGTCCTTAAACCTGTGCAAAAAGC and H-Nras-R: GTCAGTGACGTGGAAATGTCG, H-Hras-F: GTGAACGGTTGGGCAAGGAC and H-Hras-R2: CTG CACCTGCACCTCATGT, and H-Kras-F: CGGGAGAG AGGGCTCGTGAAG and H-Kras-R: AAATGCCCAGCTGG ACTAGTATGCGT, respectively. For quantitative analysis, an aliquot of cDNA was added to the Master Mix of SYBR Premix Ex Taq™ (Perfect Real Time, Takara Bio Inc., Shiga, Japan), and quantitative gene expression data were acquired using an ABI PRISM 7700 Sequence Detection System (Applera Corp., Applied Biosystems). PCR primers for detecting each RAS oncogene were obtained from Takara Bio Inc. RT-PCR of GAPDH RNA was used to standardize results.

Gene expression analysis for angiogenesis-related VEGF isoforms. Novel FTI compound, CH4512600 (see structure in Fig. 1) was added to sub-confluent HCT 116 cultures at concentrations of 1 and 10 μM, and further incubated for 24 h. Total cellular RNA isolation was as described above. Quantitative analyses for VEGF-A and VEGF-A isoform (VEGF121, VEGF165, and VEGF189) expression were performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers used in this study were as follows: VEGF-A-F: CTTGCTTGCTGCTCCTACCT, VEGF-A-R: GATTTGGCCTCTCCCTTCTC, VEGF-A-probe: CATGCGAACGTGTCG, VEGF189-F: GCGGAA ATCCCGGTATAAGT, VEGF189-R: GCCTCCTCCTGCGCT TGAACAA, VEGF189-probe: CCTGAGAGCTTCTCCCTG, VEGF165-F: ACAACAAATGTAATGACCCACGCA, VEGF165-R: CTGTTCTCGCTCTCGACCA, VEGF165-probe: CCACAGGATTTTCT, VEGF121-F: ACAACAAATGTAATGACCCACGCA, VEGF121-R: CTGAGGAG...
GCTCCTTCCT, VEGF121-probe: CAAGAAAAATGTGA CAAGCCG, and for the internal control, ß-actin-probe-primer mix (PE Applied Biosystems). Quantitative gene expression data were acquired using an ABI PRISM 7000 Sequence Detection System (Applera Corp., Applied Biosystems).

Immunohistochemical staining. Sections (4-μm-thick) were cut from formalin-fixed and paraffin-embedded tissue blocks. Immunohistochemical staining was performed using the streptavidin-biotin method. An affinity-purified rabbit polyclonal anti-VEGF antibody (A-20, Santa Cruz) at 1:100 dilution was used. After 30 min of incubation at room temperature, sections were incubated with peroxidase-labeled anti-rabbit Ig antibody, Histofine Simple Stain MAX PO (Nichirei Bioscience, Japan), for another 30 min at room temperature. Peroxidase activity was detected with diamino benzidine (Dojin, Japan). Sections were counter-stained with hematoxylin and dehydrated.

Drug efficacy studies of CH4512600 in a NOG mouse liver metastasis model. Thirty-two NOG mice were intrasplenically injected with 10^4 HCT 116 cells. After three days, the mice were randomized and divided into 4 groups in order to start administration. Control animals received a saline vehicle. FTI CH4512600 was administered daily for three weeks via peroral administration (50 and 250 mg/kg). Type I DNA topoisomerase inhibitor, CPT-11, was administered weekly for three weeks via intravenous injection as positive control.

Statistical analysis. The Kaplan-Meier log-rank method was used with SAS preclinical package software ver. 5.0.

Results

Selection of a suitable cell line for evaluating the FTI drug efficacy from the panel of liver metastasis models. To examine novel FTI CH4512600 (Fig. 1) efficacy in liver metastasis, we established a liver metastasis model of human colon cancer using NOD/Shi-scid IL2Rγnull (NOG) mice. Liver metastasis capability was first examined at autopsy within 6 weeks after 10^4 cells were transplanted intrasplenically. Eleven human colon cancer cell lines (COLO320DM, HCT 116, HT-29, LoVo, LS123, LS174T, SW48, SW1417 and WiDr), were intrasplenically implanted into NOG mice. The mice were sacrificed 6 weeks later, and liver metastases were enumerated immediately, without prior fixation.

Figure 2. Representative gross findings of liver metastases of human colon cancer cell lines. Eleven human colon cancer cell lines: CaCo2, COLO201, COLO320DM, HCT 116, HT-29, LoVo, LS123, LS174T, SW48, SW1417 and WiDr, were intrasplenically implanted into NOG mice. The mice were sacrificed 6 weeks later, and liver metastases were enumerated immediately, without prior fixation.
116 cell growth in the liver metastasis model. Furthermore, CH4512600 discernibly suppressed the transplanted HCT FTI CH4512600 slightly suppressed, and 250 mg/kg FTI after the start of administration), administration of 50 mg/kg after the injection of HCT 116 cells. At day 28 (25 days were administered daily for 3 weeks starting from 3 days periodically. Doses of 50 and 250 mg/kg of FTI CH4512600 liver; the degree of liver metastasis was monitored angiogenesis was thought to be affected by RAS oncogene status, which is the molecular target for FTIs. We examined VEGF expression in our FTI efficacy-testing model. Immunohistochemical staining with anti-VEGF antibody was performed in HCT 116 colon cancer cell metastasized NOG liver. In a non-FTI CH4512600-treated liver metastatic focus at day 35, VEGF-expressing cells were observed to have a strong reaction product for immunoperoxidase staining (Fig. 4b). On the other hand, at day 35 when the most effective drug efficacy at 250 mg/kg FTI CH4512600 appeared, no VEGF-expressing cells, which showed a strong positive reaction in the non-treated group, were observed. Only a few, weakly VEGF-expressing cells were observed. To confirm the effect of FTI CH4512600 on VEGF expression, mRNA levels of VEGF isoform were compared between non-treated and of FTI CH4512600 on VEGF expression, mRNA levels of VEGF isoform were compared between non-treated and FTI CH4512600-treated HCT 116 cells also resulted in suppression of VEGF expression (Fig. 5). Addition of FTI CH4512600 to culture medium of HAMADA et al.: DRUG EFFICACY TESTING WITH NOG MICE LIVER METASTASIS MODEL

Table I. Comparison of RAS oncogene mutations and relative gene expression among six colon cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>N-RAS</th>
<th>H-RAS</th>
<th>K-RAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Point mutation</td>
<td>Gene expression</td>
<td>Point mutation</td>
</tr>
<tr>
<td>COLO320DM</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>HCT116</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>HT29</td>
<td>-</td>
<td>2.3</td>
<td>-</td>
</tr>
<tr>
<td>WiDr</td>
<td>-</td>
<td>2.3</td>
<td>Gly 15, silent</td>
</tr>
<tr>
<td>LoVo</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td>LS174T</td>
<td>-</td>
<td>4.3</td>
<td>-</td>
</tr>
</tbody>
</table>

*All RAS gene (N, H and K) expressions are normalized to the each RAS gene expression level in HCT 116, which were given a value of 1.0 in each experiment.*

Drug efficacy studies of FTI CH4512600 in the NOG mice liver metastasis model. We selected the HCT 116 cell line from our panel of liver metastasis models using NOG mice for evaluating FTI CH4512600 efficacy in vivo. HCT 116 cells (10⁴) were injected intrasplenically into the NOG mouse liver; the degree of liver metastasis was monitored periodically. Doses of 50 and 250 mg/kg of FTI CH4512600 were administered daily for 3 weeks starting from 3 days after the injection of HCT 116 cells. At day 28 (25 days after the start of administration), administration of 50 mg/kg FTI CH4512600 slightly suppressed, and 250 mg/kg FTI CH4512600 discernibly suppressed the transplanted HCT 116 cell growth in the liver metastasis model. Furthermore, administration of 250 mg/kg FTI CH4512600 dramatically suppressed HCT 116 tumor growth in this model at day 35 (32 days after the start of administration) (Fig. 2). However, low-dose (50 mg/kg) administration was not sufficient to inhibit tumor growth completely.

Vascular endothelial growth factor (VEGF)-dependent angiogenesis was thought to be affected by RAS oncogene status, which is the molecular target for FTIs. We examined VEGF expression in our FTI efficacy-testing model. Immunohistochemical staining with anti-VEGF antibody was performed in HCT 116 colon cancer cell metastasized NOG liver. In a non-FTI CH4512600-treated liver metastatic focus at day 35, VEGF-expressing cells were observed to have a strong reaction product for immunoperoxidase staining (Fig. 4b). On the other hand, at day 35 when the most effective drug efficacy at 250 mg/kg FTI CH4512600 appeared, no VEGF-expressing cells, which showed a strong positive reaction in the non-treated group, were observed. Only a few, weakly VEGF-expressing cells were observed. To confirm the effect of FTI CH4512600 on VEGF expression, mRNA levels of VEGF isoform were compared between non-treated and FTI CH4512600-treated HCT 116 cells in vitro. After 24 h, relative mRNA levels of VEGF121, VEGF165, and VEGF189 were reduced dose-dependently by addition of FTI CH4512600 (Fig. 5). Addition of FTI CH4512600 to culture medium of HCT 116 cells also resulted in suppression of VEGF expression in vitro.

We described the effectiveness of FTI CH4512600 in complete suppression of HCT 116 cell growth in the NOG mouse liver metastasis model. Finally, we confirmed the systemic anti-cancer effects of FTI CH4512600; a survival study was carried out with or without administration of drugs in the NOG mouse liver metastasis model. Thirty-two mice were intrasplenically injected with 10⁴ HCT 116 cells and 3 days later, they were divided into 4 groups (n=8) for a survival study. Type I DNA topoisomerase inhibitor CPT-11 was used as a positive control. As shown in Fig. 6a, neither FTI CH4512600 nor CPT-11 treatment had any effect on body weight change throughout the experiment. Unfortunately, 2 of 8 CPT-11-treated mice died from the toxicity of the compound immediately after administration; these two mice...
were excluded from the statistical analysis. Untreated mice died within 44 days after HCT 116 injection (range 34-41 days after treatment initiation, mean ± SD: 36.9±2.9 days), which was statistically no different than the mean survival for 50 mg/kg FTI CH4512600-treated mice (39.3±3.3 days). These data corresponded to the degree of metastasis in mice treated with 50 mg/kg FTI CH4512600 on day 35 as shown in Fig. 2. On the other hand, treatment with 250 mg/kg of FTI CH4512600 (50.3±9.4, P<0.0001) and 75 mg/kg CPT-11 (46.3±5.1, P=0.0003) significantly prolonged mouse survival compared with that of vehicle control (Fig. 6b).

Discussion

The human xenograft model with cancer cells implanted in subcutaneous spaces of athymic nude mice or severe combined immunodeficiency (scid) mice has been the mainstream for evaluating drug efficacy in vivo. When a novel FTI compound was developed, efficacy studies were first performed using the xenograft model. Kohl et al (32) first reported the effectiveness of FTI L-739,749 on Ras-dependent tumor growth in nude mice. Thereafter, various FTIs such as FTI-276, LB42722 and SCH66336 were developed and their efficacies were evaluated in the xenograft model (33-35). We are interested not so much in suppression of primary tumor growth as in suppression of metastatic tumor growth in situ, particularly liver metastasis of colorectal tumors. However, the previous
models only evaluated in vivo cell growth. Recently, we developed a reliable and quantitative liver metastasis model using NOG mice (31). With this model, we observed differential in vivo growth characteristics between the subcutaneous space and liver. Human pancreatic cancer cell lines, BxPC-3 and Capan-2, grew well in the subcutaneous space but when injected intrasplenically, these cells were scarcely able to grow in the liver. Therefore, we had to first select a suitable colon cancer cell line, which can metastasize and grow well after intrasplenic injection, to be the FTI target. In this study, we developed a reliable liver metastasis model for colon cancer using NOG mice as an in vivo FTI efficacy evaluation model. This liver metastasis model using NOG mice more closely mimics the in vivo conditions in patients with colon cancer. Another in vivo FTI efficacy evaluation model was reported by Lantry et al (36). They used a lung adenoma chemically induced in A/J mice by 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) administration as the FTI target, and demonstrated FTI efficacy in a primary tumor model. In addition, the reduction in total tumor volume with treatment is the first demonstration of inhibition of tumor development by FTI in a murine primary lung tumor model.

Antitumor efficacy of FTI-276 was evaluated in a nude mouse xenograft model using two human lung carcinoma cell lines: one (Calu-1) with a K-RAS oncogenic mutation and the other (NCI-H810) with no RAS mutations (33). The Calu-1 tumors from control animals (treated with saline once and the other (NCI-H810) with no cell lines: one (Calu-1) with a model.

The process of tumor metastasis can be divided to several steps: initially tumor cells separate from the original tumor mass and invade a blood vessel; then they are carried in the bloodstream, and finally adhere to the site of metastasis and proliferate (40). Our liver metastasis model mimics only the latter part of metastasis. Angiogenic factor VEGF is thought to be one of the important factors for developing liver metastasis (40). Oncogenic RAS is known to upregulate expression of VEGF, acting via either the Raf/MAPK or the PI3K pathway (41,42). In contrast, FTI led to a down-regulation of VEGF mRNA in RAS-transformed cells (34). Furthermore, L-744,832 potently inhibits the secretion of VEGF by these cells and, hence, may also demonstrate an antiangiogenic effect in vivo (43). Our in vitro experiment also demonstrated that HCT 116 human colon cancer cells down-regulated VEGF gene expression by addition of FTI CH4512600. Feldkamp et al reported that viable regions of vehicle-treated human glioblastoma multiforme (GBM) showed robust VEGF expression; in contrast, few components of drug-treated FTI SCH66336-treated GBM were viable, with a disorganized histological architecture, and weak VEGF expression (35). Immunohistochemical staining with anti-VEGF antibody in NOG mouse liver, after intrasplenic injection with HCT 116 cells revealed that administration of 250 mg/kg FTI CH4512600 induced not only a decrease in the positive cell number but also weakened the positive signals. These results suggested that angiogenic factor VEGF is partially involved in developing liver metastasis.

In conclusion, we demonstrated that treatment with novel FTI CH4512600 strongly suppressed transplanted HCT 116 cancer cell growth in the liver of NOG mice used as a liver metastasis model and significantly prolonged survival without any toxicity. Our liver metastasis model developed using NOG mice is reliable and more closely mimics in vivo conditions in patients with colon cancer compared with the traditional xenograft model created by cancer cell transplantation into a subcutaneous space. Our panel of eleven colon cancer cell lines for liver metastasis is applicable to evaluation of the safety, efficacy, and medicinal benefits of new anticancer drugs.

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