Antitumor Effects of a Soluble Insulin-Like Growth Factor I Receptor in Human Ovarian Cancer Cells: Advantage of Recombinant Protein Administration in Vivo

Atsushi Hongo, Hiroyuki Kuramoto, Yojiro Nakamura, Kosei Hasegawa, Keiichiro Nakamura, Junichi Kodama, and Yuji Hiramatsu

Department of Obstetrics and Gynecology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

ABSTRACT

Antitumor effects of a soluble form dominant negative of the type I insulin-like growth factor receptor (IGF-IR) designated as 486/STOP were evaluated in CaOV-3 human ovarian cancer cells by establishing stable transformants overexpressing 486/STOP and by administration of 486/STOP recombinant protein. Expression of 486/STOP was detected from total cell lysates, as well as conditioned media collected from stable transformants. In stable transformants, growth in monolayer was slightly retarded, and anchorage-independent growth in vitro and tumorigenicity in vivo were markedly inhibited. Addition of conditioned media from 486/STOP cells inhibited anchorage-independent growth of parental cells. Although tumorigenicity of parental cells in vivo was abrogated when they were cocultured in monolayer with 486/STOP cells over 48 h before injection to nude mice, coinjection of parental cells and 486/STOP cells without preculture was not successful. In contrast, administration of 486/STOP partially purified recombinant protein inhibited tumorigenicity of parental cells in vivo. Because 486/STOP cells result in massive apoptosis in vivo within 48 h, usage of a recombinant protein has a great advantage to use its unique bystander effect in vivo for clinical application.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological malignancies. Over the last two decades, epoch-making drugs such as cis-platinum (and its derivatives) and paclitaxel have been introduced in clinical use. However, the mortality rate has not decreased dramatically. The main reason is the fact that ovarian cancer is generally asymptomatic, and at the time of diagnosis, the cancer has usually spread widely into pelvic and abdominal cavities. A novel strategy, possibly with a bystander effect, for the management of inoperable disseminated diseases is desirable for clinical use.

The IGF-IR and its ligands play a crucial role in establishment and maintenance of the transformed phenotypes in many malignant tumors (1, 2). Addition of IGF-I and/or overexpression and activation of the IGF-IR protect tumor cells as well as normal cells from apoptosis induced by a variety of causes, including c-myc overexpression (3), overexpression of interleukin 1β converting enzyme-related protease (4), p53 (5), ionizing radiation (6), tumor necrosis factor α (7), interleukin 3 withdrawal (5, 8–10), okadaic acid (11), UV-B (12), and anticancer drugs (13, 14). A series of previous articles have clarified that antiapoptotic signals through the IGF-IR are sent via ras-mitogen-activated protein kinase pathway, Insulin Receptor Substrate-(IRS)-1-phosphatidylinositol 3’-kinase-Akt pathway, and translocation of Raf-1 to mitochondria (12, 15–18). In tumor cells, impairment or modulation of the IGF-IR pathways by antisense strategies (19–25), triple-helix formation (26), a myristylated COOH-terminus of the IGF-IR (27, 28), and dominant negative mutants of the IGF-IR (29, 30) engender reversal of the transformed phenotypes and induces massive apoptosis, which is more prominent in vivo than in vitro. Our hypothesis is that the IGF-IR may also play an important role in survival of floating ovarian cancer cells within the ascites of patients’ abdominal cavity, where cancer cells are exposed to an anchorage-independent condition; it may allow them to form peritoneal dissemination. Among strategies for inhibiting the IGF-IR, the soluble IGF-IR designated as 486/STOP (31) was demonstrated to have a bystander effect and to induce apoptosis in vivo (31–33). This soluble IGF-IR is a truncated receptor at the 486th amino acid, which is located within the extramembranous α-subunit. According to this structural feature, 486/STOP is presumed to be partially secreted outside the cell membrane of expressing cells and to interfere with the IGF-IR. To evaluate the efficacy of this IGF-IR targeting against ovarian cancer, we established stable transformants of CaOV-3 human ovarian cancer cell line overexpressing 486/STOP and simulated their antitumor effects, including a bystander effect both in vitro and in vivo. We also evaluated administration of 486/STOP partially purified recombinant protein in vitro and in vivo and compared actual bystander effects between these two strategies.

MATERIALS AND METHODS

Plasmid. The expression vector for the soluble IGF-IR (486/STOP), pIGFIRsol, was constructed as described previously (31). Briefly, pCvn IGF-IR encoding the wild-type human IGF-IR cDNA under the control of SV40 promoter was cut by AgeI, overhangs were filled by Klenow, and then blunt ends were re-ligated to make a frame shift mutation. This procedure results in an early-stop codon formation after the 486th (excluding the first 30 amino acid signal peptide) amino acid within the α-subunit of the IGF-IR.

Cell Line and Transfection. CaOV-3 is a cell line derived from human epithelial ovarian carcinoma. CaOV-3 cells were originally purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FBS at 37°C. The expression vector pIGFIRsol or the pCvn empty vector was transfected into CaOV-3 cells by using Transfectam (Promega), and stable transformants were isolated and maintained in the presence of 800 μg/ml of G418 (Life Technologies, Inc.) until use.

Western Blot. Subconfluent cells were lysed in a lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100] containing 100 mM NaF, 0.2 mM sodium orthovanadate, 10 mM Na PPi, 1% phenylmethylsulfon fluoride, and 1 μg/ml aprotinin. Seventy-five μg of whole cell lysates from transfected clones were separated on a 5–15% gradient SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained with anti-IGF-IR α-subunit antibody (N20; Santa Cruz Biotechnology). For detecting the secreted 486/STOP protein from conditioned media of transfected cells, 486/STOP cloned or empty vector clones were incubated in SFM (DMEM with 0.1% BSA and 50 μg/ml transferrin) for 48 h, and conditioned media were recovered. Collected conditioned media were concentrated from 500–25 μl (20 times concentration) by Microcon 30 (Millipore) and applied into wells and electrophoresed, then blots were stained with...
anti-IGF-IR α-subunit antibody. Blots were developed with the enhanced chemiluminescence detection reagents (Amersham).

**Cell Growth in Monolayer.** Cells were plated at 5 × 10⁴ cells/well in 6-well plates in DMEM with 10% FBS and incubated for 24 h. Then cells were washed three times with Hank’s solution and shifted to SFM, SFM with 20 ng/ml human recombinant IGF-1 (Life Technologies, Inc.), or DMEM supplemented with 10% FBS. After an additional 48 h of incubation at 37°C, cells were counted by a hemocytometer. All of the points were results of triplicate experiments and evaluated as percentage increase after 48 h of incubation.

**Cell Growth in Soft Agar.** CaOV-3-derived cells were plated at 3 × 10⁴ cells/35-mm plate in 0.2% soft agar (with 0.5% agarose underlay) containing DMEM and 10% FBS. To evaluate a bystander effect of 486/STOP in vitro, fresh conditioned media collected from 486/STOP-expressing clones were used. Clones overexpressing 486/STOP and empty vector clones as a negative control were cultured in monolayer for 48 h in DMEM with 10% FBS, and conditioned media were collected. Then, wild-type CaOV-3 cells were seeded at 3 × 10⁴ cells/35-mm plate in 0.2% soft agar made of each conditioned medium from 486/STOP-expressing or empty vector clones. Colonies that grew >200 or 125 μm in diameter were counted after incubation at 37°C.

**Tumorigenesis in Nude Mice.** 486/STOP-transfected clones or empty vector clones were washed three times with Hank’s solution and incubated with SFM at 37°C for 24 h. Then cells were trypsinized and washed twice with PBS. As a xenograft, 5 × 10⁶ cells suspended in 100 μl of sterile PBS were injected s.c. above the hind leg of 5-week age male BALB/c nude mice (Charles River Japan, Yokohama, Japan). For evaluating a bystander effect in vivo, wild-type CaOV-3 cells were mixed with the same number of 486/STOP clones or empty vector clones and seeded in DMEM containing 10% FBS. After incubation at 37°C for 24 h in monolayer, mixed cells were washed three times with Hank’s solution and cocultured in SFM for 48 h. Mixed cells were trypsinized and washed twice with PBS, then 1 × 10⁷ mixed cells (∼5 × 10⁶ cells each) in 100 μl of PBS were injected into nude mice. As a positive control, wild-type CaOV-3 cells alone were treated in the same manner, and 5 × 10⁶ cells were injected in nude mice. To evaluate necessary time for coculture to get adequate bystander effect of 486/STOP, a mixture of wild-type CaOV-3 cells and 486/STOP no. 49 cells at 1:1 ratio were incubated 37°C for various time, and 1 × 10⁷ mixed cells were injected in nude mice. Established tumor volume was measured weekly as a spheroid from three-dimensional diameters. Each tumor volume represents mean and SD from five different mice inoculated with CaOV-3-derived cells.

**Detection of Apoptosis in Vivo.** Apoptosis of 486/STOP cells after injection into nude mice was detected by ApopTag Peroxidase In Situ Apoptosis Detection kit (Intergen) according to the manufacturer’s protocol. 486/STOP no. 49 cells (1 × 10⁷ cells) were injected s.c. in nude mice. After 48 h of incubation in vivo, mice were sacrificed, and established palpable tumors were fixed in 10% neutralized formalin, paraffin embedded, sectioned, and stained. DNA fragmentation was detected by the TUNEL assay followed by peroxidase staining, and specimens were counterstained in methyl green. As a control, established tumor of wild-type CaOV-3 cells after 48 h from injection was used. Morphological change of these tumors was also checked by H&E staining.

**Inhibition of Tumor Growth in Vivo by 486/STOP Protein.** To make preparations for 486/STOP partially purified recombinant protein, CaOV-3 486/STOP no. 49 cells cultured in 100-mm plates at 70% confluency were washed three times with Hank’s solution and incubated in SFM. Conditioned media were collected after 48 h of incubation at 37°C, then concentrated from 2 ml to 100 μl (20 times concentration) by centrifugation. Concentrated conditioned media were collected after 48 h of incubation at 37°C, then concentrated from 2 ml to 100 μl (20 times concentration) by centrifugation. Conditioned media were collected after 24 h, then washed three times with sterile PBS and concentrated again by centrifugation at 4°C. After incubation in SFM for 24 h, 5 × 10⁶ of wild-type CaOV-3 cells were resuspended in 100 μl of sterile PBS and injected into nude mice. After 1 week, when tumors could be palpable, 100 μl of concentrated 486/STOP suspension were injected nearby the established tumor in nude mice. Injection was continued weekly thereafter, and the growth of tumors in vivo was evaluated. Concentrated conditioned media prepared in the same manner from empty vector clone no. 21 was used as a control. The animal experiment protocol in the present study was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine and Dentistry (Okayama, Japan).

**RESULTS**

**Detection of 486/STOP from Total Cell Lysate.** According to its structural feature, 486/STOP is inferred to be partially secreted outside of the cell membrane from expressing cells, but a reasonable quantity of 486/STOP should remain in cytosol. Moreover, it is possible that some portions of the secreted 486/STOP may form heterodimer with endogenous IGF-IR and may remain on the cell surface. Therefore, we first evaluated expression levels of 486/STOP from total cell lysates. Seventy-five μg of total cell lysates collected from established stable transfectants with pIGFIRsol or pCvn empty vector were separated on SDS-PAGE and stained with anti-IGF-IR α-subunit (Fig. 1). Representative two 486/STOP-transfected clones nos. 48 and 49 express moderate and abundant 486/STOP protein at M, 72,000, respectively. It is noteworthy that these expression levels of 486/STOP are much higher than the endogenous α-subunit of the IGF-IR. In contrast, no product was confirmed from empty vector clones nos. 21 and 22.

**Detection of the Secreted 486/STOP from Conditioned Media.** Next, we tried to confirm the secretion of 486/STOP protein from conditioned media. Western blot of the concentrated conditioned media from each clone is shown in Fig. 2. The 486/STOP protein is secreted into the conditioned media according to expression levels in the total cell lysates (Fig. 1).

**Cell Growth in Monolayer Culture.** We have shown repeatedly that down-regulation of the IGF-IR pathway(s) has no effect or, if any, a minimal effect on cell growth in monolayer culture (19, 27). Fig. 3 shows that transfection of 486/STOP had no marked effect on monolayer cell growth of CaOV-3 cells either in SFM, SFM supplemented with IGF-I, or with 10% FBS.

**Cell Growth in Soft Agar.** Each clone was seeded in soft agar as described in “Materials and Methods” to check the inhibition of anchorage-independent cell growth by 486/STOP. Table 1 shows the inhibitory effect of 486/STOP on anchorage-independent growth of CaOV-3 cells. We used two empty vector clones as controls. The number of colonies formed by the two empty vector clones in soft agar was identical to that formed by the parental CaOV-3 cells comprising a mixed population (data not shown). In general, CaOV-3 cells express ∼8000 IGF-IRs/cell and are weakly transforming in soft agar. Moderate expression of 486/STOP in clone no. 48 suppressed and abundant expression in clone no. 49 dramatically suppressed their growth in soft agar. Subsequently, we tested the bystander effect of 486/STOP on anchorage-independent growth in vitro. Both 486/STOP clones or empty vector clones were incubated at 37°C in DMEM with 10% FBS for 72 h, then conditioned media were collected from each clone. Wild-type CaOV-3 cells were seeded in soft agar made of the conditioned media from each clone (Table 2). Conditioned media from 486/STOP clones showed an inhibitory
bystander effect is actually reproducible in vivo or not. Therefore, we next examined a bystander effect of 486/STOP on tumorigenicity in vivo. 486/STOP clones or empty vector clones were mixed at a 1:1 ratio with wild-type CaOV-3 cells, then they were incubated in monolayer in DMEM with 10% FBS. After 24 h, when cells were attached on plates, they were washed three times with Hank’s solution and cocultured in SFM for an additional 48 h. Then $1 \times 10^5$ of mixed cells were suspended in 100 µl of PBS and injected s.c. into nude mice. As shown in Fig. 4, we already confirmed that half of the cell number used in this experiment ($5 \times 10^5$ cells) was sufficient to form tumors in vivo. Table 3 shows a bystander effect of 486/STOP in vivo, it is related to the expression level of 486/STOP protein (Figs. 1 and 2). Subsequently, we checked a bystander effect of 486/STOP in detail by varying the time for coculture of wild-type cells and 486/STOP cells (Fig. 5A). We used CaOV-3 wild-type cells and 486/STOP clone no. 49 cells for this experiment. Tumorigenicity of CaOV-3 wild-type cells was abrogated when they were cocultured with 486/STOP cells for >48 h in monolayer before injection in nude mice. When they were preincubated only for 24 h, three of six mice formed palpable tumors. One mouse retained small tumor formation.

**Table 3. Bystander effect of 486/STOP in vivo**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt + empty vector no. 21</td>
<td>5 of 5</td>
</tr>
<tr>
<td>wt + empty vector no. 22</td>
<td>5 of 5</td>
</tr>
<tr>
<td>wt + 486/STOP no. 48</td>
<td>3 of 5</td>
</tr>
<tr>
<td>wt + 486/STOP no. 49</td>
<td>1 of 5</td>
</tr>
</tbody>
</table>

*Wild-type (wt) CaOV-3 cells were mixed with the same number of transfected clones and incubated in monolayer for 48 h. Then $1 \times 10^5$ of mixed cells were injected s.c. into nude mice. Palpable tumors were evaluated after 8 weeks.*
and two mice once formed tumors, but they regressed slightly within 6 weeks of observation. When they were coinjected in nude mice without preincubation in monolayer culture, tumor formation of these mixed cells \( (1 \times 10^7 \text{cells}) \) was identical to that of CaOV-3 wild-type cells alone \( (5 \times 10^6 \text{cells}) \). Because we used serum-free condition for preincubation of wild-type cells with 486/STOP cells in monolayer, we tested the effect of serum free condition on tumorigenesis of CaOV-3 wild-type cells. As shown in Fig. 3, the entire clone used in the present study could grow in SFM. Tumorigenicity of CaOV-3 wild-type cells was not affected, although they were incubated for 48 or 72 h in SFM (Fig. 5B).

**Behavior of 486/STOP Cells in Vivo.** Generally, when expression or function of the IGF-IR is impaired, tumor cells cause massive apoptosis \textit{in vivo} within 24 h after inoculation (23, 27). Our result from mixture experiments indicates that enough bystander effect may not be achieved if 486/STOP-expressing cells result in apoptosis within 48 h. So we try to figure out the fate of 486/STOP-expressing cells in nude mice after 48 h from injection. Wild-type CaOV-3 cells formed a tumor with viable cells (Fig. 6A). Only small percentage of cells were stained positively with TUNEL (Fig. 6B). In contrast, 486/STOP no. 49 cells were morphologically shrunk with condensed nuclei (Fig. 6C), and TUNEL staining suggested massive apoptosis (Fig. 6D).

**Tumor Treatment by 486/STOP Recombinant Protein.** Administration of 486/STOP partially purified recombinant protein, prepared as shown in “Materials and Methods,” significantly retarded tumor formation of CaOV-3 cells in nude mice (Fig. 7). In contrast, tumor formation was almost identical to that of CaOV-3 wild-type cells without any treatment when concentrated conditioned medium from an empty vector clone was used as a control. Although the 486/STOP protein we used in the present study is a partially purified protein, the difference between the 486/STOP clone and the empty vector clone suggests that the 486/STOP is the responsible protein for this inhibitory effect. This inhibitory effect of 486/STOP recombinant protein lasted during our observation of 6 weeks, thereafter, tumors began to grow again when we stopped recombinant protein administration.
DISCUSSION

The high mortality rate of the advanced ovarian cancer has two main causes. One is its tendency to spread into the abdominal cavity from its early stages. This intra-abdominal dissemination often makes complete resection of the diseases quite difficult. The other is its acquired tolerance to chemotherapeutic agents during cyclic chemotherapy. The present article is intended to elucidate antitumor effects of 486/STOP in human ovarian cancer because the possible bystander effect may be quite beneficial to the intra-abdominal disseminated diseases when it is administered intra-abdominally. In addition, although it is not discussed in the present study, its proapoptotic ability might act as chemosensitizer.

In recent years, the IGF-IR and its ligand IGF-I are considered not only as a growth factor but as a potent survival factor. In fact, only the IGF-I and overexpression of Bcl-2 were demonstrated to delay the onset of apoptosis in Rat-1 fibroblasts induced by c-myc, adenovirus E1A, and Bak expression (34). In previous studies, several truncated or mutated IGF-IRs were tested as dominant negatives (29–31). In the present study, we chose the 486/STOP because it cannot only reverse the transformed phenotype of tumors but can also cause apoptosis. More importantly, it is reported to have a bystander effect (33).

The proapoptotic mechanism of 486/STOP is not fully understood thus far. It may not arise from competitive ligand binding because 486/STOP is a truncated IGF-IR lacking amino acids 692–702, which is reported to be a critical portion for ligand binding within the α-subunit (35). It is rather speculated that the mechanism arises from heterodimer formation of 486/STOP with the endogenous full-length IGF-IR subunits (36). However, our vast expression of the 486/STOP over the endogenous IGF-IR did not detectably impaired either tyrosine autophosphorylation of the IGF-IR or serine phosphorylation of Akt (data not shown). These results may support the previously proposed hypothesis that the action of 486/STOP would not be the typical dominant effect, but it would be independent of the regular IGF-IR gene signal transduction pathways (36).

Our hypothetical advantages of the IGF-IR targeting for ovarian cancer using 486/STOP include: (a) down-regulation of the IGF-IR may reverse already established transformed phenotype of the existing ovarian carcinoma lesion in the abdominal cavity; (b) it may inhibit formation and progression of the peritoneal dissemination; (c) it may increase chemosensitivity to the chemotherapeutic agents because of the possible abrogation of antiapoptotic property of the disease. Furthermore, (d) a bystander effect is expected because we have chosen a soluble form dominant negative of the IGF-IR. This bystander effect is extremely beneficial for treatment of disseminated diseases of ovarian cancer.

In general, it is extremely difficult to get a stable transformant in which the IGF-IR expression or function is highly abrogated, e.g., antisense and dominant negatives, because the IGF-IR plays an important role in cell survival during transfection and clone selection. An inducible promoter has been used for the expression of these cytotoxic genes to overcome this difficulty (37). Fortunately, we were able to establish transformants that overexpressed 486/STOP. Therefore, we clearly demonstrated various antitumor effects of 486/STOP in CaOV-3 cell line in vitro and in vivo as well. It was also possible to prepare potent recombinant protein from the transformant because of its abundant expression of 486/STOP. Here, we demonstrated that the down-regulation of the IGF-IR in CaOV-3 ovarian cancer cells by 486/STOP could inhibit anchorage-independent growth in vitro and more vigorously inhibited tumorigenesis in vivo, and these inhibitory effects accompanied a bystander effect in vitro as well as in vivo.

Tumor formation in vivo indicated that expression of 486/STOP is sufficient to reverse the transformed phenotype of CaOV-3 cells. Therefore, we evaluated the bystander effect of 486/STOP in vivo. Our data from coculture of CaOV-3 wild-type cells with CaOV-3 486/STOP-expressing cells indicated that a bystander effect of 486/STOP was sufficient to abrogate tumorigenicity of wild-type CaOV-3 cells when they were cocultured >48 h in monolayer before inoculation into nude mice. When they were cocultured for only 24 h before injection, an antitumor effect of 486/STOP was not satisfactory. Mere coinjection into nude mice without prior coculture showed no inhibitory effect on tumorigenesis of wild-type CaOV-3 cells. This time lag of 48 h is crucial for us to obtain a bystander effect of 486/STOP in vivo.

There are two different possibilities for administrative strategies for the future application of the IGF-IR targeting by 486/STOP for ovarian cancer. One is viral vector-mediated gene therapy, and the other is molecular targeting therapy by administration of 486/STOP as a recombinant protein into the abdominal cavity. According to our experience of IGF-IR targeting, the latter seems even better for the clinical application. The main reason is that cancer cells in which the IGF-IRs are abrogated by antisense, dominant negative, or COOH terminus of the IGF-IR cause massive apoptosis in vivo within 24 h (23, 27). In the present study, it was also true that CaOV-3 cells expressing 486/STOP resulted in massive apoptosis within 48 h after injection in nude mice. This quick fate of 486/STOP-producing cells in vivo may not allow them to produce sufficient recombinant protein to produce a bystander effect. On this point, administration of 486/STOP into the abdominal cavity as a recombinant protein shows a good rationale for the clinical application. Simultaneously, it can avoid adverse effects arising from usage of viral vectors.

In conclusion, use of our overexpressing transformants clearly demonstrated that: (a) 486/STOP could reverse transformed phenotype of the CaOV-3 human ovarian cancer cell line in vitro; (b) it was able to inhibit tumorigenicity in vivo; (c) these effects were accompanied by a bystander effect; (d) administration of partially purified recombinant protein retarded already established tumor growth of CaOV-3 cells; and (e) administration of 486/STOP recombinant protein may offer advantages over 486/STOP gene transfer in terms of its bystander effect. These data present a good rationale for the clinical application of 486/STOP for treatment of advanced ovarian cancer with intra-abdominal dissemination. Additional investigation, including an animal treatment model, is needed.

ACKNOWLEDGMENTS

We thank Dr. Renato Baserga, Kimmel Cancer Center, (Philadelphia, PA) for generously providing pIGFIRsol vector.

REFERENCES


Antitumor Effects of a Soluble Insulin-Like Growth Factor I Receptor in Human Ovarian Cancer Cells: Advantage of Recombinant Protein Administration in Vivo

Atsushi Hongo, Hiroyuki Kuramoto, Yojiro Nakamura, et al.