Advances in Brief

Therapy of Human Pancreatic Carcinoma Implants by Irinotecan and the Oral Immunomodulator JBT 3002 Is Associated with Enhanced Expression of Inducible Nitric Oxide Synthase in Tumor-infiltrating Macrophages

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Abstract

We determined the therapeutic effect of irinotecan (CPT-11) combined with the immunomodulator JBT 3002, a synthetic bacterial lipopeptide (N-acylated derivative of ω-amino-C1-C3-alkane-sulfonic acid), against highly metastatic human pancreatic carcinoma cells injected into the pancreas of athymic nude mice. Mice received four courses consisting of three daily oral doses of JBT 3002, followed by once weekly i.p. injection of CPT-11. Control mice were treated with CPT-11 alone, JBT 3002 alone, or saline. Tumor growth and metastasis were assessed by gross pathology and confirmed by histological examination. Treatment with CPT-11 alone significantly decreased the median volume of pancreatic tumors and the incidence of metastasis, whereas treatment with only JBT 3002 did not. The combination therapy of CPT-11 plus JBT 3002 decreased tumor volume and incidence of metastasis significantly more than CPT-11 alone. The number of apoptotic cells (terminal deoxynucleotidyl transferase-mediated nick end labeling assay), the number of scavenger-receptor-positive macrophages, and expression level of inducible nitric oxide synthase (iNOS) within lesions directly correlated with therapeutic effects. Indeed, the \textit{in vitro} incubation of tumor cells with macrophages activated by JBT 3002 plus IFN-γ produced a significant lysis of tumor cells that could be blocked by a specific inhibitor of iNOS. Collectively, these data demonstrate that the oral administration of the immunomodulator JBT 3002 combined with i.p. injection of CPT-11 can decrease the growth of human pancreatic carcinoma and the incidence of metastasis in nude mice by both a direct antitumor effect and the activation of iNOS in infiltrating macrophages.

Introduction

Each year, cancer of the pancreas causes approximately 27,000 deaths in the United States and 50,000 deaths in Western Europe (1, 2). The late stage at which it is usually detected, its aggressiveness, and the lack of effective systemic therapies are responsible for the depressing survival rate: <1% of all patients with adenocarcinoma of the pancreas are alive 5 years after diagnosis (3). The most common early metastatic sites are regional lymph nodes (90%) and liver (70%; Refs. 4–6). Advances in surgery and chemoradiotherapy have not significantly improved prognosis and overall survival (1, 2). In fact, between 1991 and 1994, 28 Phase II clinical trials involving 25 different new agents reported a median objective response rate of 0% (range, 0–14.3%) and a median survival of 3 months (range, 2–8.3 months; Refs. 7, 8). Even the highly promising chemotherapeutic agent gemcitabine, a deoxyctydine analogue, produced a response rate of 27% with a median survival of 3.8 months in a recently completed Phase II trial (4, 5, 7).

CPT-11, a potent inhibitor of DNA topoisomerase I with a wide spectrum of antitumor activity (9–11), has been shown to produce a median survival of 5.2 months in a Phase II study of patients with pancreatic cancers; the main dose-limiting toxicity was severe diarrhea (12). A Phase I study in 46 patients with refractory solid malignancies (including pancreatic cancer) defined the dose-limiting toxicity as leukopenia and severe diarrhea (13).

In an effort to reduce the drug’s side effects, we treated mice given CPT-11 with oral doses of JBT 3002, a synthetic bacterial lipopeptide (N-acylated derivative of ω-amino-C1-C3-alkane-sulfonic acid). It prevented CPT-11 irinotecan-induced gastrointestinal toxicity by maintaining the integrity of the lamina propria, thereby allowing the administration of high-dose CPT-11 to mice with established syngeneic CT-26 colon cancer liver metastases (14). JBT 3002 has also been shown to activate macrophages to become tumoricidal (15, 16) and to induce the release of interleukin 1α, interleukin 6, tumor necrosis factor-α (15–17), and iNOS and, hence, the release of NO (15–17).

In the study reported here, we tested the combination of oral JBT 3002 and i.p. injection of CPT-11 against human pancreatic carcinomas implanted into the pancreas of nude mice. We hoped that the combination would allow us to administer higher, more effective doses of CPT-11. We show that this combination therapy could indeed reduce local (orthotopic) tumor growth and production of lymph node and liver metastasis. These significant therapeutic effects were directly correlated with the infiltration of macrophages into the lesions and the expression level of iNOS by the macrophages.

Materials and Methods

Cell Lines and Culture Conditions. The highly metastatic human pancreatic cancer cell line L3.6pl (18) was maintained in DMEM, supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, a 2-fold vitamin solution (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Monolayer cultures were maintained on plastic and incubated in a mixture of 5% CO2 and 95% oxygen at 37°C. The cultures were free of \textit{Mycoplasma} and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Thiel’s encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walk...
The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

**Reagents.** All antibodies were purchased from the suppliers as listed: mouse anti-PCNA clone PC10 (DAKO A/S, Copenhagen, Denmark), anti-mouse macrophage Scavenger-R and peroxidase-conjugated rat antimouse IgG2a (Serotec; Harlan Bioproducts for Science, Inc., Indianapolis, IN), antimacNOS clone 6 (Transduction Laboratories, Lexington, KY), and goat antirat IgG and goat antirat IgF(ab')2 fragment (Jackson Research Laboratories, West Grove, CA). NMA, a specific inhibitor of iNOS (19, 20), was purchased from Sigma Chemical Co. (St. Louis, MO), and CPT-11, irinotecan hydrochloride, was purchased from Pharmacia and UpJohn (Kalamazoo, MI). Recombinant mouse IFN-γ was purchased from Pharmingen (San Diego, CA), and JBT 3002 was obtained from Jenner Biotherapies (San Ramon, CA).

Animals. Male athymic nude mice (BALB/c background) and male BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8–12 weeks of age.

**Tumor Cell Injection Techniques and Necropsy Procedures.** Nude mice were anesthetized with methoxyflurane, a small left abdominal flank incision was made, and the spleen was exteriorized. A 40-μl tumor cell suspension (1 million cells) was injected subcapsularly into the pancreas, and the abdominal wound was closed in one layer with wound clips (Autoclip; Clay Adams, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C. Liver metastases were studied and enumerated using a dissecting microscope. Liver lesions were prepared for histological analysis (H&E). Microscopically enlarged regional (celiac and para-aortal) lymph nodes were harvested and processed for histological analysis and confirmation of metastasis.

**Treatment Schedules.** Groups of nude mice were injected in the pancreas with 1 × 10^6 viable L3.6pl cells (12). On days 5, 6, and 7 (after injection), the mice received oral feedings of JBT 3002 (1 μg/dose). On day 10, groups of mice were injected i.p. with 50 or 100 mg/kg CPT-11 (CPT-11_50 or CPT-11_100). This treatment schedule was repeated every week until the control (untreated) animals became moribund on day 41 of the study. The mice were necropsied, and tissues were harvested for analysis as described above.

**Immunohistochemistry.** Paraffin-embedded tissues were sectioned at 4–6-μm thickness and mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX). Sections were deparaffinized in xylene, followed by a graded series of alcohols (100, 95, and 80% ethanol) and rehydrated in PBS (pH 7.5). Then apoptotic cells (TUNEL) and PCNA protein were identified. The TUNEL assay (using fluorescein-conjugated dUTP) was performed using a commercial kit (Promega Corp., Madison, WI) according to manufacturer’s instructions. Fluorescent bleaching was minimized by treating slides with an enhancing reagent (Prolong; Molecular Probes, Eugene, OR), and the slides were examined under an Olympus inverted system IX70 microscope (Melville, NY). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval.” For the quantification of PCNA and TUNEL expression, positive cells were counted in 10 random 0.159-mm² fields at ×100. Frozen tissues were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific Co.), and air-dried 30 min. They were then fixed in cold acetone (5 min), acetone:chloroform (1:1; 5 min), and acetone (5 min) and used for identification of iNOS and Scav-R marker. For the quantification of Scav-R expression, the number of positive cells was counted in 15 random 0.039-mm² fields at ×200.

**Isolation and Activation of Macrophages.** BALB/c mice received i.p. injections of 1.5 ml of thioglycollate broth. Four days later, PEMS were collected by peritoneal lavage (21, 22). The cells were washed with HBSS, and 1 × 10^5 cells suspended in 0.1 ml serum-free MEM were plated into 38-mm² wells of 96-well microculture plates. One h later, the nonadherent cells were removed by washing with medium. At that time, >98% of adherent cell populations consisted of macrophages according to morphological, phagocytic, and immunostaining criteria (21, 22). PEMS were then incubated for 20 h with medium alone or with medium containing recombinant IFN-γ (10 units/ml) plus JBT 3002 (10 ng/ml) in the presence or absence of NMA (3 μM).

**Macrophage-mediated in Vitro Cytotoxicity Assay.** Macrophage-mediated tumor cytotoxicity was assessed by a radioactive release assay as described previously (21, 22). Briefly, L.3.6pl human pancreatic cancer cells in their exponential growth phase were incubated for 24 h in medium containing 0.2 μCi/ml [3H]thymidine (≈2500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA). The tumor cells were washed three times with HBSS to remove unbound radioligand, harvested by brief trypsinization, and resuspended in medium. The target cells were plated (1 × 10^4 cells/well) into wells containing control or test macrophages to obtain an initial macrophage:target cell ratio of 10:1. At this density, macrophages incubated in medium (control) were not cytotoxic to neoplastic cells. Radiolabeled target cells were also plated alone as a negative control. Three h after plating, 1 μg/ml CPT-11 was added to some of the cultures. At 72 h after plating, the cultures were washed twice with saline, and the adherent viable cells were lysed with 0.1 ml of 0.1 N KOH. The lysates were harvested with a Harvester 96 (Tomtec, Orange, CT) and counted in a liquid scintillation counter. The percentage cytotoxicity was calculated using the formula:

\[
\text{Cytotoxicity (\%) = } \frac{(A - B)}{A} \times 100
\]

where A is the cpm in cultures of target cells alone, and B is the cpm in test cultures.

**Statistical Analysis.** Pancreatic tumor weight, tumor volume, PCNA quantity, number of TUNEL-positive cells, and number of Scav-R-positive cells were compared by unpaired Student’s t test (two-tailed). P < 0.05 was regarded as significant.

**Results.**

**Therapeutic Effect of CPT-11 and JBT 3002.** An aliquot of 1 × 10^6 viable L3.6pl cells was injected into the pancreas of nude mice. Five days later, the mice were gavaged once daily for 3 consecutive days with JBT 3002 (1 μg/dose). Two days later (or 10 days after tumor cell injection), groups of mice were injected i.p. with saline, 50 mg/kg CPT-11, or 100 mg/kg CPT-11 (CPT-11_50 and CPT-11_100, respectively). At this time, the volume of pancreatic tumors was ~100 mm³. This treatment schedule was repeated weekly until day 41, when the control animals became moribund; at this point, all of the mice were killed and necropsied. Oral administration of JBT 3002 (1 μg/dose) was not therapeutic, but once-weekly i.p. injections of CPT-11_50 or CPT-11_100 significantly reduced the volume of primary pancreatic tumors (P < 0.0001; Table 1). A CPT-11 dose of 100 mg/kg was required to significantly reduce the incidence of liver and lymph node metastasis (P < 0.01). Combining oral JBT 3002 and i.p. CPT-11_100 reduced the volume of pancreatic tumors still more (P < 0.03 as compared with CPT-11_100 alone). The combination of oral JBT 3002 (1 μg/dose) plus CPT-11 at 50 mg/kg (CPT-11_100) significantly decreased the incidence of lymph node metastasis (P < 0.001 as compared with CPT-11_100 alone) but not the volume of the primary (pancreas) tumor and incidence of liver metastasis. The effectiveness of 25 mg/kg or 12.5 mg/kg doses of CPT-11 in the combined therapy (oral JBT 3002 at a constant 1 μg/dose) did not exceed that of CPT-11 alone.

**Tumor Cell Proliferation (PCNA) and Apoptosis (TUNEL).** To determine whether the regression in local pancreatic cancer and lymph node or liver metastases was attributable to a decrease in cell proliferation, an increase in apoptotic rate, or both, we analyzed tissue samples from mice treated with CPT-11 at 100 mg/kg alone or in combination with JBT 3002. Fig. 1 is a compilation of pathology and immunohistochemistry images from one mouse, and Fig. 2 summarizes the data. The number of PCNA-positive cells was significantly lower in the CPT-11 plus JBT 3002 group.
Table 1. Therapy of human pancreatic carcinoma implanted into the pancreas of nude mice by CPT-11 and JBT 3002

Groups of nude mice were given injections in the pancreas with $1 \times 10^6$ viable L3.6pl cells on day 0. Mice were given oral feedings of JBT 3002 (1 μg/dose) for 3 consecutive days beginning on day 5 after tumor cell injection. Five days later, groups of mice received CPT-11 by i.p. injection. The course was administered three more times, and all mice were killed on day 41 after tumor cell injection. Tumor volume was calculated by the formula $V = \frac{a \times b^2}{2}$, in which $a$ is the longest diameter and $b$ is the shortest diameter of the tumor.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Incidence</th>
<th>Volume (mm$^3$)</th>
<th>Liver</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>759 (355–1495)</td>
<td>5/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JBT 3002 (JBT)</td>
<td>10/10</td>
<td>870 (643–1244)</td>
<td>5/10</td>
<td>10/10</td>
</tr>
<tr>
<td>CPT-11 (50 mg/kg)</td>
<td>10/10</td>
<td>151 (37–33)$^a$</td>
<td>1/10$^a$</td>
<td>8/10</td>
</tr>
<tr>
<td>CPT-11 (50 mg/kg) + JBT</td>
<td>10/10</td>
<td>160 (45–301)$^b$</td>
<td>2/10</td>
<td>4/10</td>
</tr>
<tr>
<td>CPT-11 (100 mg/kg)</td>
<td>10/10</td>
<td>82 (56.3–243)$^c$</td>
<td>1/10$^d$</td>
<td>6/10</td>
</tr>
<tr>
<td>CPT-11 (100 mg/kg) + JBT</td>
<td>10/10</td>
<td>36 (11–70)$^d$</td>
<td>1/10$^d$</td>
<td>2–10$^d$</td>
</tr>
</tbody>
</table>

$^a$ Control versus CPT-11, $P < 0.0001$; JBT 3002 versus CPT-11, $P < 0.000001$.
$^b$ Control versus CPT-11, $P < 0.01$; JBT 3002 versus CPT-11, $P < 0.01$.
$^c$ Control versus CPT-11, $P < 0.0001$; JBT 3002 versus CPT-11, not significant.
$^d$ Control versus CPT-11, $P < 0.01$; JBT 3002 versus CPT-11, not significant.

The present results demonstrate that the oral administration of the immunomodulator JBT 3002, coupled with once weekly i.p. injections of CPT-11 to nude mice given pancreatic implants of human pancreatic cancer L3.6pl cells (18), significantly reduces primary tumor volume and lymph node and liver metastasis.
It is well established that systemic treatment of mice with liposomes that contain immunomodulators can activate macrophages to a tumoricidal state and lead to a decrease in regional lymph node, lung, and liver metastases (24). The efficacy of this immunotherapy, however, is limited to a minimal tumor burden, i.e., micrometastases, or residual tumors after conventional chemotherapy (25–27). Recent studies from our laboratory demonstrate the efficacy of combining CPT-11 as a chemotherapeutic agent with immunotherapy to prevent murine colon cancer liver metastasis (14). Weekly treatment with CPT-11 significantly reduced the incidence of liver metastasis, but oral JBT 3002 plus CPT-11 significantly enhanced the therapeutic results by activating macrophages (14).

The present results demonstrate the efficacy of combining CPT-11 with the immunomodulator JBT 3002 for therapy of human pancreatic cancer. Treatment with CPT-11 at 50 or 100 mg/kg once weekly significantly reduced primary tumor volume and lymph node and liver metastasis. However, the combination of JBT 3002 plus CPT-11 produced superior therapeutic results. JBT 3002 was effective in reducing primary tumor volume only when combined with 100 mg/kg CPT-11. Combination therapy with JBT 3002 and 100 mg/kg CPT-11, but not JBT 3002 or CPT-11 alone, led to the enhanced presence of macrophages within pancreatic tumors and metastases with increased expression of iNOS, suggesting that destruction of some tumor cells and inflammatory changes attributable to CPT-11 treatment are chemotactic to macrophages systemically activated by JBT 3002, which can lyse tumor cells by production of NO. Combination therapy using

Fig. 1. Histopathology and immunohistochemistry of human pancreatic carcinomas growing in the pancreas of nude mice. Nude mice were given injections in the pancreas of $1 \times 10^6$ viable L3.6pl cells. Therapy with saline, oral JBT 3002 alone, i.p. CPT-11 alone, or oral JBT 3002 and i.p. CPT-11 was carried out as described in “Materials and Methods.” Tissue sections harvested at necropsy from mice on day 41 of the experiment were stained for expression of PCNA (to show cell proliferation), TUNEL (FITC; to show cell death), Scav-R (to show infiltrating macrophages), and iNOS.
50 mg/kg CPT-11 and JBT 3002 significantly reduced regional lymph node metastases compared with 50 mg/kg CPT-11 alone, but a reduction in the dose of CPT-11 eliminated therapeutic benefits. The combination of the direct cytotoxic effect of CPT-11 and the indirect, macrophage-mediated cytotoxicity led to a significantly higher number of TUNEL-positive tumor cells and a decreased number of PCNA-positive cells in the tumor lesions.

Macrophages activated by lipopeptide immunomodulators are highly cytotoxic against tumor cells (19). A major cytotoxic mediator is NO. A previous study from our laboratory demonstrated the systemic administration of the lipopeptide CGP31362 (an analogue of JBT 3002) induces a high level of iNOS expression in macrophages and regression of liver metastases produced by a murine M5076 histiocytic sarcoma (19). The incubation of murine macrophages with JBT 3002 (plus 10 units of IFN-γ) activated iNOS expression and lysed L3.6pl target cells. The in vitro lysis was mediated, in part, by

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**Fig. 2.** Quantification of PCNA and TUNEL in human pancreatic carcinomas. The number of PCNA- or TUNEL-positive cells were derived from 10 random 0.159-mm² fields at ×100. A, control or JBT 3002 versus CPT-11 100 alone or CPT-11 100 plus JBT 3002, P < 0.00001. B, control or JBT 3002 versus CPT-11 100 alone or CPT-11 100 plus JBT 3002, P < 0.001. C, CPT-11 100 versus CPT-11 100 plus JBT 3002, P < 0.0001. Bars, SD.

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**Fig. 3.** Histopathology and immunohistochemistry of regional lymph node metastases produced by human L3.6pl pancreatic cancer cells. Nude mice were given injections in the pancreas of 1 × 10⁶ viable L3.6pl cells. Therapy with saline, oral JBT 3002 alone, i.p. CPT-11 alone, or oral JBT 3002 and i.p. CPT-11 was carried out as described in “Materials and Methods.” Tissue sections were stained for expression of Scav-R (to show infiltrating macrophages) and iNOS.

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**Fig. 4.** In vitro macrophage-mediated tumor cell lysis. L3.6pl cells in their exponential growth phase were incubated 24 h in medium containing 0.2 μCi/ml [³H]thymidine. The target cells were plated (1 × 10⁵ cells/well) into wells containing PEMs or PEMs activated by JBT 3002 (10 ng/ml) plus IFN-γ (10 units/ml) to obtain an initial macrophage:target cell ratio of 10:1. Three h later, 1 μg/ml CPT-11 and NMA (3 mM) were added to some groups. At 72 h after plating, the cultures were washed twice with physiological saline, and adherent viable cells were lysed with 0.1 ml of 0.1 N KOH. The lysates were harvested with a Harvester 96b (Tomec, Orange, CT) and counted in a liquid scintillation counter. The percentage of cytotoxicity was calculated by the formula: cytotoxicity (%) = (A - B)/A × 100, where A is cpm in cultures of target cells alone (no CPT-11), and B is the cpm in other test cultures. A, PEMs versus PEMs + JBT 3002, P < 0.00001. B, PEMs + CPT-11 versus PEMs + JBT 3002 + CPT-11, P < 0.0001. C, PEMs + JBT 3002 versus PEMs + JBT 3002 + CPT-11, P < 0.01. D, PEMs + JBT 3002 + NMA versus PEMs + JBT 3002, P < 0.001. E, PEMs + JBT 3002 + CPT-11 + NMA versus PEMs + JBT 3002 + CPT-11, P < 0.003. Bars, SD.
10. Matsuzaki, T., Yokokura, T., Mutai, M., and Tsuruo, T. Inhibition of spontaneous and pancreatic cancer. Is associated with infiltration of iNOS-positive macrophages into the mucosa can induce potent tumoricidal properties in tissue macrophages, probably initiated by CPT-11. Administration of high dose CPT-11, and it activates iNOS in macrophages that infiltrate the inflammatory zone in tumors, which was probably initiated by CPT-11.

In summary, we have demonstrated that p.o.-administered JBT 3002 can induce potent tumoricidal properties in tissue macrophages, thus enhancing the therapeutic effect of CPT-11 against primary tumor growth of human pancreatic cancer in the pancreas of nude mice and lymph node and liver metastasis. This combination therapy is associated with infiltration of iNOS-positive macrophages into the lesions. The ability of JBT 3002 to prevent CPT-11-mediated damage to the mucosa and activation of iNOS in tumor-infiltrating macrophages encourage clinical trials of this combination for therapy of pancreatic cancer.

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References

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