Anti-angiogenic Treatment for Peritoneal Dissemination of Pancreas Adenocarcinoma: A Study Using TNP-470

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We established peritoneal dissemination-prone subcultures (PCI-43p3) using nude mice by a repetitive in vivo selection of intraperitoneally inoculated PCI-43, a pancreas adenocarcinoma cell line. The subcultures showed upregulated expression of matrix metalloproteinase (MMP)-9, but not MMP-2 in culture supernatants. They also produced increased amounts of vascular endothelial growth factor (VEGF), which was not associated with alterations in isoforms of VEGF mRNA. PCI-43p3 cells attached to cultured mesothelial cell monolayers more readily than did the parent PCI-43 cells. The angiogenesis inhibiting agent, TNP-470, at 30 mg/kg was administered to the model mice, resulting in a prominent suppression of the establishment of peritoneal nodules. The suppression was dependent on the duration of TNP-470 treatment. TNP-470 treatment significantly suppressed proliferation of tumor cells in disseminated nodules, assessed in terms of immunostaining for proliferating cell nuclear antigen (PCNA). TNP-470 did not affect the in vitro attachment between PCI-43p3 and mesothelial cells. The combined data show that anti-angiogenic treatment profoundly suppresses the in vivo process of peritoneal dissemination.

Key words: Pancreas cancer — Anti-angiogenesis — Peritoneal dissemination

Patients with ductal adenocarcinoma of the pancreas often develop peritoneal dissemination, the suppression of which would considerably improve these patients’ prognosis. Studies have addressed the importance of adhesion molecules,1, 2) matriolytic enzymes,3) aberrant oncogene/tumor suppressor gene expression4, 5) and cytokines/growth factors6) in the establishment of peritoneal implants. The importance of tumor angiogenesis is widely accepted in the case of blood-borne metastases. However, its importance in the establishment and growth of peritoneal dissemination remains largely unknown, as very few studies have been conducted.7) The peritoneal implants of pancreas adenocarcinoma patients are histologically composed of a proliferation of carcinoma cells accompanied by desmoplastic stromal reaction with neovascularization. Thus, inhibition of tumor angiogenesis might suppress peritoneal implants, as in the case of hematogenous metastatic nodules.8–11) We established and characterized dissemination-prone subcultures of a pancreas adenocarcinoma cell line to examine whether anti-angiogenic treatment, an effective modality for blood-borne metastasis, would also be effective against peritoneal dissemination.

MATERIALS AND METHODS

Animals Female nude mice (BALB/c nu/nu) 4 to 6 weeks old were used in this study (Clea, Tokyo). All mice were maintained under specific pathogen-free conditions at the Center for Animal Experimentation, Hokkaido University School of Medicine, in accordance with the Guide for the Care and Use of Laboratory Animals (Hokkaido University School of Medicine, 1988). The animals were provided with a γ-irradiated diet and sterilized water ad libitum.

Cells A pancreas carcinoma cell line, PCI-43, was established from the primary carcinoma tissue surgically resected at the Hokkaido University Hospital.12) One million PCI-43 cells in 0.5 ml of phosphate-buffered saline (PBS) were inoculated i.p. (n=3). Peritoneal implants were examined 6 weeks later, and several disseminated nodules were mixed together, then dispersed and suspended in PBS. These PCI-43 cells were temporarily cultivated in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). These cells were named PCI-43p1. One million PCI-43p1 cells in 0.5 ml of PBS were then inoculated i.p., and a similar procedure was done to obtain PCI-43p2 and PCI-43p3.

Mesothelial cells were prepared by the enzymatic digestion of the omental tissue. With the donors’ informed consent, surgically resected omentum was minced and treated...
with 0.25% trypsin for 15 min at 37°C. After centrifugation at 1500 rpm for 10 min, the floating lipid-rich layer was removed. This procedure was repeated until the lipid-rich layer was totally removed. The viable cells were cultivated in RPMI-1640 supplemented with 10% FBS. The polygonal monolayer was expanded to semiconfluency. The resultant cells, >90% keratin-positive, were used as mesothelial cells in the attachment assay.

**Zymographic detection of matrix metalloproteinases**

Gelatin zymography was done as described previously.13) Conditioned medium was prepared, using cultivated PCI-43 and PCI-43p3 cells, 1×10^6 in 5 ml of serum-free RPMI-1640 for 24 h. Conditioned medium from an ovarian carcinoma cell line, HTOA, was also prepared as a control. These conditioned media were electrophoresed at 4°C in 10% sodium dodecyl sulfate polyacrylamide cross-linked gels (SDS-PAGE), containing 0.1% gelatin. Gelatin was obtained from Difco (Detroit, MI). Following electrophoresis, the gel was washed with 2.5% Triton X-100 followed by incubation in Tris-HCl, 0.5 mM CaCl_2, 10⁻⁶ M ZnCl₂, pH 8.0, at 37°C for 16 h. Coomassie brilliant blue staining was subsequently done. The gel was washed in 5% acetic acid and 10% methanol in water. The molecular weights of the enzymes were determined by simultaneous SDS-PAGE of Rainbow markers (Amersham, Buckinghamshire, UK).

**Detection of vascular endothelial growth factor (VEGF)**

PCl-43 or PCI-43p3 cells were cultured in 24-well plates at 2×10⁵ cells/well with RPMI-1640 containing 10% FBS. When cells were subconfluently attached to the bottom of the plates, the culture medium was changed to RPMI-1640 containing no FBS, and the cultures were run for two additional days. Supernatants were collected on day 3 and stored at −20°C. VEGF in the supernatants was quantified by the use of an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, MA). The splicing variants of VEGF mRNA were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from cultured PCI-43 or its subcultures was reverse transcribed to yield cDNA. The primers used were the sense 5'-TCCAGGAGTACCTGTAGAG-3' and anti-sense 5'-TCACCCTCGCTTGTTCATAC-3'.14) The annealing temperature was 56°C. When the specific amplified products were 272, 404, 476, and 527 bp, the mRNAs were considered to represent VEGF₁₁⁵, VEGF₁₅₅, VEGF₁₈₅, and VEGF₂₅₅, respectively.

**Flow cytometric detection for surface molecules**

Surface intercellular adhesion molecule-1 (ICAM-1), very late antigen-3 (VLA-3) and E-cadherin were detected by indirect flow cytometry using a FACScan (Becton-Dickinson, Mountain View, CA). Monoclonal antibodies used in the cytometric analysis included HA58 (anti-ICAM-1) (kindly provided by Dr. K. Imai, Sapporo Medical College, Sapporo), anti-VLA-3 and anti-E-cadherin (Transduction Laboratories, Lexington, KY).

**Cell attachment assay**

The monolayer adhesion assay was performed in a microtiter plate, as described previously but with some modifications. In brief, mesothelial cells were cultured semi-confluently in a flat-bottomed, 96-well microtiter plate. Aliquots of 0.1 ml of 1×10⁷/ml PCI-43 or PCI-43p1–3 cells were incubated with 0.1 mCi of ⁵¹Cr for 1 h at 37°C, and after a wash in PBS, 1×10⁴/well labeled PCI cells were co-cultivated with confluent mesothelial cell monolayers for 60 min at 37°C. Following incubation, the plates were sealed with filters, inverted, and centrifuged at 800 rpm for 5 min to remove unattached PCI cells. The attached cells were solubilized by adding NaOH (0.2 N). The medium containing ⁵¹Cr was collected, and the radioactivity was measured with a γ-counter.

**Immunohistochemical detection of proliferative cell nuclear antigen (PCNA)**

Four-micrometer-thick sections were incubated with mouse monoclonal antibodies against PCNA (Dako Corp., Carpinteria, CA) as the primary antibody, followed by biotinylated anti-mouse immunoglobulin and avidin-biotin complex (Dako Corp). The PCNA indices were calculated as the percent ratio of positively stained PCI-43p3 cell nuclei to the total of PCI-43p3 nuclei in disseminated mesenteric nodules. A minimum of 500 cells was counted in triplicate.

**Anti-angiogenic agent**

TNP-470, O-(chloroacetyl-carbamoyl)flumagillol, was a kind gift from Takeda Chemical Industries (Osaka). In the in vivo experiments, TNP-470 was suspended in a vehicle of 3% ethanol and 5% gum arabic in saline. In in vitro experiments, TNP-470 was dissolved in dimethylsulfoxide and RPMI-1640 medium (Gibco, New York, NY). The final concentration of dimethylsulfoxide was 0.1%.

**Experimental design for anti-angiogenic treatment**

Experimental groups were designated as the TNP-long-treatment, TNP-short-treatment, and control groups. A single anti-angiogenic experiment was done according to the following design. In the TNP-long-treatment group, TNP-470 at 30 mg/kg was subcutaneously inoculated every other day from the beginning of 1×10⁷ PCI-43p3 inoculation (day 0) for 4 weeks. Test mice (n=4) were killed, and disseminated nodules on the mesentery were evaluated on the 28th day. In the TNP-short-treatment group, TNP-470 at 30 mg/kg was administered every other day beginning on day 14 and continuing through day 28, with the day of 1×10⁷ PCI inoculation being day 0. Test mice (n=5) were killed and disseminated nodules on the mesentery were evaluated on the 28th day. In the control group, 1×10² PCI-43p3 were intra-peritoneally inoculated and a vehicle without TNP-470 was subcutaneously administered every other day for 28 days. The test mice (n=7) were killed,
and disseminated nodules on the mesentery were evaluated. Body weights of each mouse in the three groups were measured both at the beginning of the experiment (day 0) and at the 28th day, to assess body weight gain for each mouse.

For the estimation of potential for peritoneal dissemination, tumor nodules in the mesentery were grossly identified and counted. The nodules were histologically examined for the presence of adenocarcinoma cells. We excluded peritoneal nodules if they were located at the parietal peritoneal surface. We also excluded bulky nodules, if present, found at the peritoneal injection sites. There were virtually no nodules on the surface of abdominal organs. Thus, we counted nodules only in the mesentery. In addition, we noted the diameter of the largest mesenteric nodule in each mouse.

Statistical analysis Unpaired Student’s t test was used. All in vitro experiments were carried out in triplicate.

RESULTS

Establishment of dissemination-prone PCI-43 subcultures Peritoneal dissemination was only rarely found in the original PCI-43 culture, while the PCI-43 subcultures were able to establish peritoneal implants (Fig. 1). The numbers of established nodules increased with in vivo passage in the peritoneum, with PCI-43p3 cells being superior to any other subculture (Table I). The dissemination nodules were composed of carcinoma cells and stromal fibroblasts and capillaries (Fig. 2). No ascites was observed in PCI- and its subculture-inoculated mice.

Characterization of dissemination-prone PCI-43p3 Zymographic examination revealed that the 92-kD matrix metalloproteinase (MMP)-9 was expressed in PCI-43p3, but not in conditioned supernatants from the original PCI-43 culture (Fig. 3). No obvious activated form of MMP-9 was seen in either PCI-43 or PCI-43p3 supernatant. In contrast, the 72-kD MMP-2 was expressed in supernatants from both cultures. No obvious activated form of MMP-2 was seen in either PCI-43 or PCI-43p3 supernatant. The culture supernatants from PCI-43 and PCI-43p3 produced VEGF at concentrations of 231±1.8 and 370±42 pg/ml, respectively (P<0.005). Two types of splicing variants, VEGF121 and VEGF165, were detected by RT-PCR in the original and all subculture PCI cells (Fig. 4). There was no apparent alteration in the relative density pattern of the two kinds of amplified product. Flow cytometry revealed that the PCI-43 subcultures, PCI-43p1, p2, and p3, all exhibited surface expressions of ICAM-1, VLA-3 and E-cadherin, and no difference was seen among these PCI cells.

The attachment assay revealed no significant adhesion between cultured mesothelial cells and PCI-43, PCI-43p1 or PCI-43p2 cells. In contrast, markedly augmented attachment was seen between mesothelial and PCI-43p3 cells (Table I), and this was not affected by the addition of TNP-470 to the medium at concentrations of 0.1, 1, 10, 100, and 1000 ng/ml (data not shown).

Suppression of in vivo growth of peritoneal implants by anti-angiogenic TNP-470 Numbers of mesenteric nodules were reduced in the TNP-short-treatment (9.4±2.1) and TNP-long-treatment (1.8±1.3) groups, compared with the control group (21.0±6.9) (P<0.01, control vs. TNP-short-treatment; P<0.0002, control vs. TNP-long-treatment group) (Fig. 5). The reduction of nodule numbers was more extensive in the long-treatment than in the short-treatment group (P<0.0002). The largest nodule diameters (mm) were smaller in the TNP-treatment groups (control, 5.4±1.0; TNP-short-treatment, 3.2±1.3; TNP-long-treatment, 1.0±0.7) (P<0.007, control vs. TNP-short/long-treatment groups), and the suppression was more extensive in the long-treatment than in the short-treatment group (P<0.02). The PCNA indices for the control, TNP-short-treatment, and TNP-long-treatment groups were 75.3±7.7, 49.7±9.0 and 52.7±10.4, respectively (P<0.003, control vs. TNP-short/long-treatment groups). Body weight gains (g) were 2.9±1.6, 2.9±1.0 and 0.2±1.0 for the control, TNP-short-treatment, and TNP-long-treatment groups, respectively (P<0.05, control vs. TNP-long-treatment group; P<0.02, TNP-short-treatment vs. TNP-long-treatment group).

DISCUSSION

The repeated in vivo selection of PCI-43 remarkably altered its phenotype, including augmented production of VEGF, induced expression of MMP-9, and augmented attachment to mesothelial cells. Although the phenotypic stability can be augmented by more than five in vivo passages, we utilized PCI-43p3 cell for analysis in this study, mainly because of the remarkable and statistically significant increase of dissemination of PCI-43p3 as compared with PCI-43p2 cells. The establishment of clinically harmful metastatic nodules requires neovascularization, and the suppression of angiogenesis in the microenvironment surrounding the tumors could prevent micrometastases from growing. An increased production of VEGF has been demonstrated in several clinical and experimental malignancies, and the upregulation of VEGF has been regarded as an important step for a tumor to produce distant metastasis. We extended this finding in the present study by noting that peritoneal dissemination is also associated with VEGF upregulation. Indeed, VEGF has been implicated in the peritoneal dissemination of carcinoma cells, and it has been shown to be a vascular permeability factor in peritoneal dissemination. The upregulation of VEGF was not accompanied by alteration in the mRNA isoform, although several metastasis-prone tumors express an altered mRNA splicing. As human VEGF can induce
Fig. 1. Peritoneal dissemination by PCI-43 (A) and its subculture, PCI-43p3 (B). Subcultures were established by a repetitive intra-peritoneal inoculation of PCI-43 cells followed by transient expansion in vitro. Arrows indicate disseminated tumor nodules.

Table I. Characterization of Parental and Subcultured PCI-43

| Characteristics                              | PCI-43 | PCI-43p1 | PCI-43p2 | PCI-43p3 |
|---------------------------------------------|--------|----------|----------|----------|
| Number of disseminated nodules per mouse    | 0      | 0.7±0.3  | 5.0±2.1  | 13.9±8.0 |
| In vitro adhesion to cultured mesothelial monolayers (×10⁴ cpm) | −0.8±0.5 | 1.9±1.5  | 0.3±1.3  | 10.4±8.2 |

a) One million PCI-43 cells in 0.5 ml of phosphate-buffered saline (PBS) were inoculated i.p. (n=3). Peritoneal implants were examined 6 weeks later, and several disseminated nodules were mixed together, then dispersed and suspended in PBS. These PCI-43 cells were temporarily cultivated in RPMI-1640 supplemented with 10% fetal bovine serum. These cells were named PCI-43p1. One million PCI-43p1 cells in 0.5 ml of PBS were then inoculated i.p., and a similar procedure was done to obtain PCI-43p2 and PCI-43p3.

Fig. 2. Histology of a disseminated nodule composed of PCI-43p3. The nodule was a sheet-like proliferation of PCI-43p3 cells (A), and inflammatory cells and neovascularatures are seen at the implantation sites (B). (A, B: hematoxylin and eosin staining; A, ×25, B, ×200). Arrows indicate examples of neovascularature.
Fig. 3. Zymographic demonstration of gelatinolytic activity in conditioned media from PCI-43 (lane 1) and PCI-43p3 (lane 2). The 92-kD lytic band (arrow) represents MMP-9; the 72-kD band (arrowhead) represents MMP-2. Conditioned medium from an ovarian carcinoma cell line, HTOA, was used as a control (lane 3).

Fig. 4. Expression of VEGF mRNA by PCI-43 and its subcultures. The upper band represents VEGF$_{165}$ and the lower, VEGF$_{121}$. M, size marker; lane 1, PCI-43; lane 2, PCI-43p1; lane 3, PCI-43p2; lane 4, PCI-43p3; lane 5, human umbilical vein endothelial cell culture.

Fig. 5. Dissemination of PCI-43p3 cells. A, control; B, TNP-short-treatment group; C, TNP-long-treatment group. Arrows indicate disseminated nodules.
angiogenesis in vivo in mice, the upregulated VEGF from PCI-43p3 most probably interacts with murine endothelial cells to induce tumor angiogenesis more effectively than does the original PCI-43. Angiogenic factors other than VEGF may also contribute to the phenotypic change in PCI-43p3. MMP-9, which has more potent collagenolytic activity than MMP-2, was weakly expressed in PCI-43p3, but not in PCI-43. Matrioletic enzymes other than MMP-2/9, including plasminogen activators, should be examined in future studies. As PCI-43p3 shows stronger adhesion activity than the remaining subcultures, differences in expression levels of adhesion molecules among subcultures are presumably important in determining the dissemination properties. As the expression of surface ICAM-1, VLA-3 or E-cadherin did not differ between PCI-43 and PCI-43p3, we were not able to identify the adhesion molecule(s) responsible for the augmented attachment.

The administration of TNP-470, an anti-angiogenic agent, suppressed peritoneal dissemination, when the agent was given from the beginning of the experiment for 28 days (TNP-long-treatment group), as well as from day 14 for 14 days (TNP-short-treatment group). The amount of suppression was dependent on the duration of TNP administration; the effect was more pronounced in the TNP-long-treatment than in the TNP-short-treatment group. The treatment period-dependent suppression was seen both in the establishment of nodules (number) and in the growth of nodules (largest diameter). In contrast, in vivo proliferation of PCI-43p3 cells, detected by PCNA staining, was suppressed in both groups to similar extents. The idea that TNP could suppress growth of established peritoneal implants is supported by the fact that the diameters of nodules (largest diameter). In contrast, in vivo proliferation of PCI-43p3 cells, detected by PCNA staining, was suppressed in both groups to similar extents. In addition, PCI-43p3 cells remained disseminated in the peritoneal cavity without TNP treatment for 14 days until the treatment began in the TNP-short-treatment group, where the numbers of nodules were also suppressed compared with those in the control group, TNP-470 did not alter the production of VEGF by PCI-43 in vitro in our previous work, and the anti-angiogenic drug did not affect the attachment to mesothelial cells in the present study. In addition, TNP-470 did not alter the proliferative activity of PCI-43 in vitro. These data imply that the suppressive in vivo effect was probably mediated mainly by the anti-angiogenic effect of the drug on host endothelial cells, rather than a direct effect on PCI cells. This is supported by our previous observation, using PCI-packed chambers in the dermis of host mice, that TNP-470 suppressed PCI cell-induced angiogenesis of host mice. However, it remains to be clarified whether TNP-470 intervenes in in vivo angiogenesis in dissemination nodules.

Recent data indicated that the anti-angiogenic therapy for several types of experimental malignancies can be used without inducing drug resistance, allowing repetitive administration of anti-angiogenics and raising the possibility of using anti-angiogenics in both the prevention and treatment of dissemination of pancreas adenocarcinoma and other human malignancies. The adverse effect of the treatment appears to be the suppression of body weight gain, but this could be managed at least in part by modifying the duration of drug administration, because this effect was not seen in the TNP-short-treatment group in this study. TNP treatment of short duration only partially suppressed the peritoneal dissemination, but a combination of short TNP treatment with other chemotherapeutic drugs should be considered, as shown by other work.

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