

## Vaccination Effect of Interleukin-6-producing Pancreatic Cancer Cells in Nude Mice: A Model of Tumor Prevention and Treatment in Immune-compromised Patients

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**In an effort to explore properties important in hematogenous metastasis of pancreatic adenocarcinoma, we previously demonstrated that tumor-derived interleukin (IL)-6 is a crucial factor that conveys resistance to liver metastasis. Here we extend the study to examine a possible vaccination effect of tumor-derived IL-6 in T-cell-deficient nude mice, as a model for predicting the effect in immune-compromised patients. We used a pair of IL-6-nonproducing and highly producing pancreatic adenocarcinoma cell lines, PCI-43 and PCI-43h, respectively. The reaction intensity of anti-PCI IgG antibodies in host nude mice was maximal 28 days after inoculation of PCI-43h cells, and remained high thereafter. A fraction of the pancreatic carcinoma cell lines, namely, PCI-6, -10, and -43, expressed surface antigenic determinant(s) reactive with the IgG; but the others, PCI-19, -24, -55, -64, -66, -68, -72, and -79, did not. Inoculation of PCI-43h but not PCI-43 suppressed growth of simultaneously inoculated PCI-43, but not PCI-24 xenografts. In addition, administration of PCI-43h, but not PCI-43 suppressed the growth of PCI-43 that was xenografted 4 weeks later, thus revealing a vaccination effect of IL-6-producing PCI-43h, but not IL-6-nonproducing PCI-43. These data, obtained from T-cell-deficient nude mice, suggest an *in vivo* role for IL-6 in inducing IgG-mediated, pancreatic carcinoma-specific vaccination against a thymus-independent antigen.**

Key words: Pancreatic carcinoma — IL-6 — Vaccination — Anti-tumor immunity

Tumor-derived cytokines, especially interleukin-6 (IL-6), can mediate various functions such as proliferation of tumor cells in an autocrine fashion,<sup>1)</sup> local permeability increase,<sup>2)</sup> and stimulation of host immunity.<sup>3)</sup> Importantly, an IL-6-mediated antimetastatic effect in mice has been shown in colon cancer<sup>4)</sup> and in pancreatic cancer,<sup>3)</sup> providing hope for an IL-6-mediated tumor vaccination. However, other studies failed to show the effectiveness of the procedure.<sup>5)</sup> The ambiguity may have resulted from the heterogeneity of host immune status and/or from the heterogeneity of antigenicity of tumor cells. Addressing these issues appears to be important in the light of the possibility of applying cytokine-based tumor vaccination to patients.

Any anti-tumor or vaccination effect that is achievable in immune-compromised hosts is important, because of possible immune suppression resulting from chemotherapy for cancer patients. Moreover, malignant tumors arising in allograft recipients or in patients with acquired immunodeficiency syndrome (AIDS) have caused medical problems, and vaccination may be useful to prevent or treat such

problems. Since a T-cell deficiency with a relative preservation of B-cell function is a usual outcome of immunosuppressive treatment for patients,<sup>6)</sup> a cytokine-based vaccination against thymus-independent tumor antigens should be considered.

Our previous studies have focused on IL-6 because the production of this cytokine inversely correlates with blood-borne metastasis of pancreatic adenocarcinoma cells in T-cell-deficient nude mice.<sup>3)</sup> Transfection of IL-6 cDNA into metastasis-prone, IL-6-negative PCI-43 cells dramatically altered their phenotype to a non-metastatic one without changing either *in vitro* proliferation or ability for invasion.<sup>3)</sup> Furthermore, spontaneous regression occurred in tumor xenografts composed of IL-6-producing transfectant cells.<sup>3)</sup> Interestingly, no regression has been observed in T/B-cell-deficient severe combined immunodeficiency (SCID) mice, implying that the immune status of the host greatly affects the action of tumor-derived IL-6 and that the regression was probably mediated by humoral immune response. Circulating IgG antibodies, which had an *in vitro* activity of antibody-dependent cellular cytotoxicity (ADCC),<sup>3)</sup> have been detected in nude mice that apparently rejected human pancreatic adenocarcinoma xenografts. The IgG antibodies were not seen in sera of mice injected with IL-6-nonproducing PCI-43.<sup>3)</sup> The present study, in this context, attempts further analysis, focusing especially on the specificity and kinetics of the

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IgG and on a possible vaccination effect in the T-cell function-deficient animal model.

## MATERIALS AND METHODS

**Animals** Female nude mice (BALB/c *nu/nu*) 4 to 6 weeks old were used (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).  $\gamma$ -Irradiated diet and sterilized water were provided to the animals *ad libitum*.

**Cells** Pancreatic carcinoma cell lines PCI-6, -10, -19, -24, -43, -55, -64, and -66 were established from surgically resected, primary carcinoma tissues.<sup>7-10</sup> PCI-68, -72, and -79 were established from a 69-year-old man, a 50-year-old woman, and a 56-year-old man, respectively. Histologic types of the primary pancreatic cancer of the PCI-68, -72, and -79 patients were all moderately differentiated tubular adenocarcinoma of ductal origin. In brief, 1-mm<sup>3</sup> cubes of primary tumor tissue were cut with scissors and gently stirred into RPMI-1640 medium supplemented with 1 mg/ml of collagenase type I (Wako, Osaka) and 1 TRU/ml of hyaluronidase (Amano, Nagoya) for 2 h at room temperature (RT). The dispersed cells were washed 3 times in RPMI-1640 and cultured in RPMI-1640 supplemented with 20% fetal bovine serum (FBS). Continuously grown cell lines exhibited a pavement-like arrangement. These cells were passaged by trypsinization and gentle scraping. All the PCI lines were xenotransplantable into BALB/c nude mice, when  $1 \times 10^7$  cells/0.5 ml in phosphate-buffered saline (PBS) were injected subcutaneously. The generation of PCI-43h, an IL-6 cDNA transfectant that produces and releases IL-6, has been reported.<sup>3</sup> The transfectant clone was maintained in media containing 1.0 mg/ml of G418. PCI-43h used in the present study produced IL-6 in culture media at a concentration of 1300 pg/ml.

**Xenograft model** In order to assess whether or not spontaneous regression also occurs in xenografts remote from the injection sites, tumorigenesis of PCI cells in locations away from the inoculation site was examined. PCI-43h or PCI-43 cells at  $1 \times 10^6/100 \mu\text{l}$  of PBS were inoculated subcutaneously on the left side of the dorsum of nude mice. At the same time, PCI-43 or PCI-24 cells at  $1 \times 10^7/100 \mu\text{l}$  of PBS were subcutaneously injected on the right side of the dorsum. The largest (*a*) and smallest (*b*) diameters of each nodule on the right side of the dorsum were measured at 3-day intervals, and tumor volume (mm<sup>3</sup>) was estimated by using the formula  $0.4ab^2$ .<sup>11</sup> Each group consisted of 5 nude mice.

The vaccination effect of IL-6-producing PCI-43h was investigated by the following procedure. PCI-43h or PCI-43 cells at  $1 \times 10^6/100 \mu\text{l}$  of PBS were inoculated subcuta-

neously on the left side of the dorsum of nude mice. Four weeks later, PCI-43 cells at  $1 \times 10^7/100 \mu\text{l}$  of PBS were subcutaneously injected on the right side of the dorsum. The largest (*a*) and smallest (*b*) diameters of each nodule on the right side of the dorsum were measured at 3-day intervals, and the tumor volume (mm<sup>3</sup>) was estimated by using the formula described above. Each group consisted of 5 nude mice.

**Specificity and kinetics of serum IgG reactive for PCI-43** The presence of antigenic determinant(s) on the surface of human cell lines was investigated by flow cytometry. The cell lines or culture used were PCI-6, -10, -19, -24, -43, -55, -64, -66, -68, -72, and -79, OSRC-2 (a renal cell carcinoma line), MKN-28 (a gastric carcinoma line), HSC-42 (a gastric carcinoma line), HTOA (an ovarian carcinoma cell line), Takigawa (an  $\alpha$ -fetoprotein-producing gastric cancer line), SQ-5 (a lung cancer line), and human umbilical vein endothelial cell (HUVEC). Circulating IgG purified from mouse sera after PCI-43h inoculation was prepared by the use of a protein A column and employed as the primary antibody, followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG. Live, unfixed cells from these cell lines and culture cells were used, so the procedure exclusively detected surface, but not cytoplasmic antigens.

Flow cytometric detection of serum IgG reactive for PCI-43 was done 1, 2, 3, 4, and 6 weeks after subcutaneous inoculation of PCI-43h at  $1 \times 10^6/\text{ml}$ .

**Statistical analysis** Student's *t* test was done to determine statistical significance.

## RESULTS

**Specificity of serum IgG** Table I summarizes the specificity of IgG affinity-purified from sera of IL-6-producing PCI-43h-bearing nude mice, collected 4 weeks after the inoculation of  $1 \times 10^6$  cells of PCI-43h. A fraction of the pancreatic adenocarcinoma cell lines, PCI-6, -10, and -43 expressed surface antigen(s) that reacted with the IgG, but the other cell lines, PCI-19, -24, -55, -64, -66, -68, -72, and -79, did not. OSRC-2, a renal cell carcinoma line; MKN-28, a stomach carcinoma cell line; and Takigawa, an  $\alpha$ -fetoprotein-producing gastric cancer cell line, also expressed the antigen(s). On the other hand, HSC-42, a gastric cancer line; HTOA, an ovarian carcinoma cell line; and human umbilical endothelial cell culture were negative for the antigen(s). These findings, together with our previous observation that non-specific, pre-existent tumor-reactive IgM was seen in naive nude mice as a natural antibody, indicate that the IgG was not a natural antibody, but resulted from a specific immune response. The PCI-43-reactive IgG in sera of PCI-43h-bearing mice first appeared one week after inoculation, and the reactivity increased during the second and third weeks. The peak

Table I. Reaction of Serum IgG in Nude Mice Inoculated with IL-6-producing PCI-43h

Cell line	Origin	Reaction with serum IgG <sup>a)</sup>
PCI-6	Pancreas carcinoma	±
PCI-10	Pancreas carcinoma	+
PCI-19	Pancreas carcinoma	-
PCI-24	Pancreas carcinoma	-
PCI-43	Pancreas carcinoma	+
PCI-55	Pancreas carcinoma	-
PCI-64	Pancreas carcinoma	-
PCI-66	Pancreas carcinoma	-
PCI-68	Pancreas carcinoma	-
PCI-72	Pancreas carcinoma	-
PCI-79	Pancreas carcinoma	-
OSRC-2	Renal cell carcinoma	+
MKN-28	Gastric carcinoma	±
HSC-42	Gastric carcinoma	-
Tagigawa	Gastric carcinoma	+
HTOA	Ovarian carcinoma	-
SQ-5	Lung carcinoma	±
HUVEC	Umbilical vein endothelial cell	-

a) +, positive; ±, weakly positive; -, negative.

reactivity was observed at the fourth week (Fig. 1), and it remained high at the sixth week.

**Suppression of remote pancreatic adenocarcinoma xenografts by inoculation of IL-6-producing pancreatic adenocarcinoma** Our previous study demonstrated a spontaneous regression of PCI-43h xenografts, accompanied by the appearance of PCI-43-reactive IgG in host sera. We attempted here to see if the regression could be observed in remotely located xenografts. In addition, we examined whether or not the remote effect can be seen in PCI-24 xenografts. PCI-24 cells were used as negative controls, because they were not reactive with IgG purified from PCI-43h-bearing mouse sera.

The inoculation of either PCI-43h or PCI-43 did not result in regression of control PCI-24 xenografts at the remote site. In contrast, inoculation of PCI-43h, but not PCI-43, suppressed growth of PCI-43 xenografts located at the remote site ( $P < 0.003$ ) (Fig. 2). Therefore, regression of remote xenografts requires production of IL-6, and is specific to PCI-43.

**Vaccination effect by pre-injection of IL-6-producing tumor** We next addressed whether or not the effect of PCI-43h inoculation on the regression of PCI-43 xenografts is maintained for a certain period. For this purpose, we investigated whether or not inoculation of PCI-43h has a suppressive effect on the growth of PCI-43 xenografts grafted 4 weeks later. The growth of PCI-43 was greatly suppressed when PCI-43h but not PCI-43 cells were inoculated 4 weeks earlier ( $P < 0.008$ ) (Fig. 3), thus

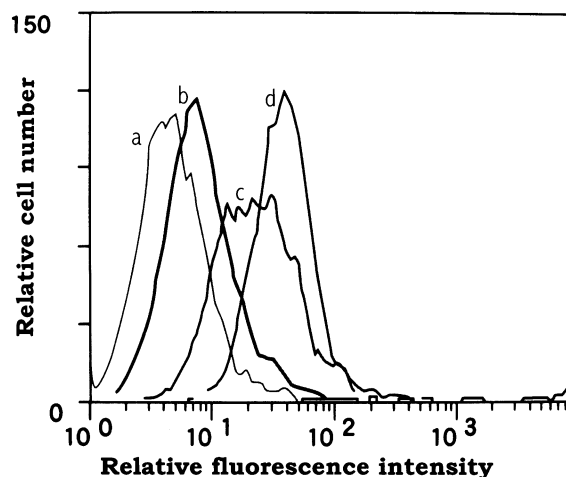


Fig. 1. An increased reaction of IgG in sera from PCI-43h-bearing mice. Reaction of IgG with live PCI-43 cells was examined by flow cytometry. IgG was purified from host sera 1 (a), 2 (b), 3 (c), and 4 (d) weeks after inoculation of PCI-43h cells. Reaction at 6 weeks was almost identical with that at 4 weeks, and is omitted from the figure.

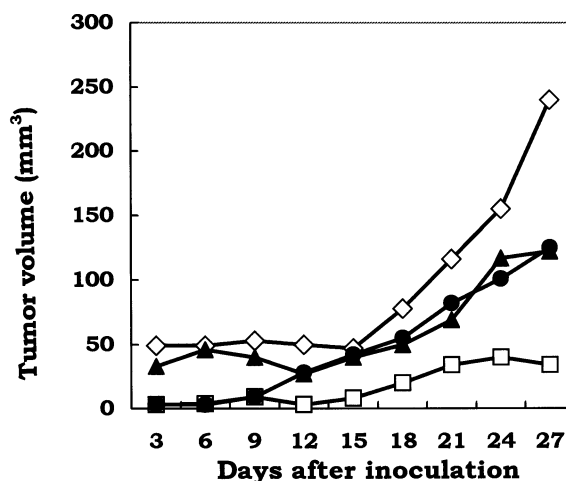


Fig. 2. Inhibition of growth of PCI-43 xenografts in the right dorsum by simultaneous inoculation of PCI-43 or PCI-43h in the left dorsum of nude mice. The growth of control PCI-24 xenografts in the right dorsum was also examined. ◇, Lt: PCI-43, Rt: PCI-43; □, Lt: PCI-43h, Rt: PCI-43; ▲, Lt: PCI-43, Rt: PCI-24; ●, Lt: PCI-43h, Rt: PCI-24.

revealing a vaccination effect mediated by tumor-derived IL-6.

## DISCUSSION

Reaction of serum IgG in host mice inoculated with PCI-43h but not PCI-43 increased for up to 4 weeks and

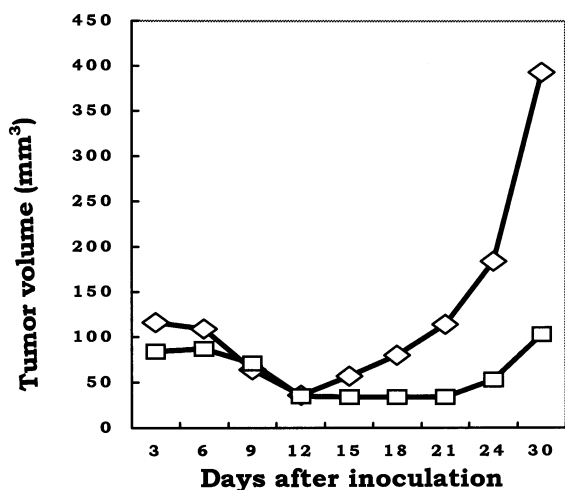


Fig. 3. Vaccination effect of PCI-43h inoculation against the growth of PCI-43 xenografts grafted 4 weeks after the PCI-43h inoculation. As a control, PCI-43, instead of PCI-43h, was inoculated 4 weeks before PCI-43 was xenografted. ◇, Lt: PCI-43, Rt: PCI-43; □, Lt: PCI-43h, Rt: PCI-43.

remained high thereafter. This corresponds to the previous observation that the spontaneous regression of PCI-43h xenografts began around 4 weeks after inoculation.<sup>3)</sup> The production of IL-6 *in vivo* by PCI-43h has been demonstrated by the specific detection of human but not mouse IL-6 in host mouse serum.<sup>3)</sup> The IgG had an *in vitro* ADCC activity,<sup>3)</sup> and the hypothesis of humoral immune response-mediated tumor regression in the present model is further supported by the current observation that regression can be seen in xenografts composed of IgG-reactive (PCI-43) but not IgG-nonreactive (PCI-24) pancreatic adenocarcinoma cells. In addition, the anti-tumor effect of PCI-43h injection was observed in PCI-43 xenografts located at a remote site. The anti-tumor effect was maintained more than 4 weeks after PCI-43h inoculation, revealing it to be a vaccination effect. We used the parent PCI-43 cells rather than PCI-43n, an IL-6-transfected subculture with no IL-6 production, because PCI-43n did not differ from PCI-43 in terms of kinesis, invasiveness, and metastasis. Although changes in phenotype might have occurred in PCI-43h cells during the selection, the effect was most likely mediated by B cell immunity invoked by IL-6 production. We did not examine the effect of IL-6 alone in the vaccination procedure. We believe IL-6 alone would generate no vaccination effect, because of the obvious requirement of PCI-specific IgG response, which in turn necessitates the simultaneous presence of PCI cells as an immunogen.

The presence of the surface antigenic determinant(s) for the IgG is restricted to a fraction of pancreatic adenocarcinoma cell lines, thus excluding the possibility that it is a

natural anti-human antibody. In addition, such anti-pancreas carcinoma IgG antibodies were not seen in mice inoculated with IL-6-negative PCI-43. Anti-human natural antibodies were detected in naive nude mouse, but they were of IgM nature and not IgG.<sup>3)</sup> The surface determinants are also seen in human cell lines other than pancreatic adenocarcinoma. These observations indicate that a thymus-independent antigen on the surface of pancreatic cancer cells invoked an IgG response in nude mice through the action of tumor-derived IL-6 in the microenvironment.

IL-6-producing cell lines such as PCI-6, -10, -19, -64, and -66 generated very few liver metastases in the nude mouse model, while IL-6-nonproducing PCI-24, -43, and -55 generated them frequently (Fig. 1 in Ref. 3). PCI-6 and PCI-10 cells have the surface antigenic determinant(s) that reacts with the IgG in PCI-43h-bearing nude mice sera. Therefore, metastasis of PCI-6 and PCI-10 cells might be suppressed through the action of tumor-derived IL-6 and humoral immunity against the antigen(s) on the surface of PCI-6 and PCI-10. In contrast, IL-6-producing PCI-19, -64, and -66 generate few metastases, even in the absence of the IgG-reactive determinant on the cell surface. This could be explained either by the lack of properties important in hematogenous metastasis in these cell lines or by the presence of a dominant surface antigenic determinant(s), which can induce thymus-independent humoral response, other than the surface antigen(s) commonly present on PCI-43, -6, and -10.

The present study, together with our previous one,<sup>3)</sup> partially explains the ambiguity of IL-6-mediated tumor suppression. Even if tumor cells generate a large amount of IL-6, the absence of a predominant surface antigenic determinant may result in no effect. The immune status of the host also greatly affects the outcome; a deficiency in T/B-cell immunity as seen in SCID animals, for instance, resulted in no effect.<sup>3)</sup> The preservation of T-cell immunity may profoundly modify the *in vivo* effect of IL-6; IL-6 secreted by renal cancer cells appears to be tumor-protective by inhibiting the function of cytotoxic, tumor-infiltrating leukocytes (TIL).<sup>12)</sup> The status of the complement system and/or natural killer cell activity may also affect the efficacy, because of the possible involvement of Fc-dependent cytotoxicity. Thus, the *in vivo* effects of tumor-derived IL-6 have been reported to be as follows: 1) humoral immunity-mediated anti-tumor effect as shown in this study, 2) tumor promotion by suppressing TIL cytotoxicity,<sup>12)</sup> and 3) tumor promotion through interaction with the non-immune host environment.<sup>13)</sup>

The athymic nude mouse is a model of deficient T-cell function, whose response to thymus-independent antigens is preserved.<sup>14)</sup> The humoral immune response to the surface antigen seen in the present study appears to be exerted in a thymus-independent manner. Although the

immune response to a thymus-independent antigen is usually mediated by IgM, several such antigens have been shown to induce IgG response,<sup>15)</sup> as was seen in the present study. Tumor vaccination in T-cell function-impaired patients is worth considering in the light of the frequent development of malignant tumors in transplant

recipients and AIDS patients. It may also be effective in the prevention of further dissemination of malignant tumors in patients under chemotherapy.

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