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ORIGINAL ARTICLE

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An in vivo mouse reporter gene (human secreted alkaline phosphatase) model to monitor ovarian tumor growth and response to therapeutics

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Abstract *Purpose:* Developing new anticancer therapeutic regimens requires the measurement of tumor cell growth in response to treatment. This is often accomplished by injecting immunocompromised mice with cells from cancer tissue or cell lines. After treating the animals, tumor weight or volume is measured. Such methods are complicated by inaccuracies in measuring tumor mass and often animals must be killed to measure tumor burden. An in vivo tumor model system is presented in which the tumor cell line was stably transfected with a constitutively expressed marker gene: secreted human placental alkaline phosphatase protein (SEAP). The SEAP gene codes for a heat-stable protein that is produced at levels proportional to the amount of tumor cells in the animal. The SEAP protein is detectable in small blood samples so that animals can be repeatedly sampled over the trial period to monitor the course of tumor progression. *Methods:* OCC1 ovarian carcinoma cells were stably transfected with pCMV-SEAP. The OCC1-SEAP cells were maintained in vitro to monitor the relationship between cell number and SEAP production. Experiments were performed in vivo to determine whether SEAP levels in blood corresponded to tumor burden. OCC1-SEAP cells were injected s.c. or intraperitoneally into nude mice and tumor volume was measured as well as plasma SEAP levels as the tumors developed. *Results:* S.c. tumor volume correlated well with plasma SEAP levels ($R^2 = 0.95$). OCC1-SEAP cells were also injected intraperitoneally into nude mice and grown as abdominal tumors. After 3 weeks the animals were killed and the tumors were dissected and weighed. SEAP levels in plasma samples from the time of death correlated with intraperitoneal tumor weight ($R^2 = 0.87$). Experiments were performed to determine whether measuring SEAP levels could be used to monitor ovarian carcinoma cell response to platinum-containing chemotherapeutic drugs. OCC1-SEAP cells cultured in vitro were treated with the platinum-containing drug carboplatin. Carboplatin treatment decreased both cell proliferation and SEAP levels in culture medium. The constitutive rate of SEAP secretion per cell (nanograms SEAP per microgram DNA) was found not to be altered by carboplatin treatment. Therefore changes in SEAP level reflect changes in OCC1 tumor cell number, and not changes in regulation of SEAP secretion due to platinum containing chemotherapeutic drug treatment. OCC1 cells were injected intraperitoneally into nude mice and the mice were treated with the platinum-containing drugs cisplatin or carboplatin. Measurements of plasma SEAP over the treatment period showed OCC1-SEAP ovarian carcinoma growth to be inhibited by cisplatin and carboplatin treatment. *Conclusion:* The SEAP marker protein is constitutively expressed by tumor cells and blood levels are correlated with tumor cell number and burden. The results of these studies indicate that SEAP may be used as an in vivo reporter gene in a mouse model to monitor tumor growth and response to therapeutics. Future studies will utilize this model to investigate novel chemotherapeutic approaches to treating ovarian cancer.

Keywords Ovary cancer · Nude mouse · Chemotherapy · Tumor progression · Therapeutics

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Introduction

Developing new anticancer therapeutic regimens requires the measurement of tumor cell growth in response to treatment. This is often accomplished by injecting athymic nude mice or other susceptible animals with cells from cancer tissue or cell lines. After treating the animals with the chemotherapeutic agent the tumor weight or volume is measured at the end of the experi-

ment. This method is complicated by inaccuracies in measuring tumor weight and volume.

For tumors that primarily grow intraperitoneally (i.p.) in their host, such as ovarian carcinomas, the most appropriate experimental animal model is to inject and grow the tumor cells i.p. in the mouse. Measuring i.p. tumor growth and response to treatment in a living animal is difficult. Changes in body weight are difficult to measure because of the diluting effect of the weight of the animal itself and are complicated by weight loss due to tumor cachexia or anticancer drug therapy. Abdominal tumors may be dissected out of a host animal and weighed, but it is often difficult to find and isolate all the tumor mass from the host tissue. This procedure is also complicated by the fact that some tumors, including ovarian carcinomas, recruit host cells into the tumor itself [12]. In addition, the animal must be killed to dissect and measure an i.p. tumor. Therefore tumor size is only measured at the end of the trial, and may not be evaluated over the course of therapy.

The growth of subcutaneous (s.c.) tumors may be followed over time in a host animal by measuring the diameter of the s.c. mass. However, this method is sometimes inaccurate if the tumor is invasive and grows into the underlying tissue rather than spreading under the skin. Additional inaccuracy is introduced if the tumor forms a necrotic center. As mentioned above, tumors such as ovarian carcinomas are more appropriately grown i.p. than s.c. to examine tumor progression.

Ovarian cancer ranks fifth as a cause of cancer deaths among women, and results in more deaths than any other cancer of the female reproductive system. In the United States, it is estimated that there will be about 14,000 deaths this year from ovarian cancer. More than 95% of ovarian cancers originate from the surface epithelial cells of the ovary [1, 13]. The chemotherapeutic agents currently in use for treatment of ovarian cancer include the platinum-containing compounds cisplatin, carboplatin and oxaliplatin [8, 9, 17]. These agents are used alone, or more commonly in combination with cyclophosphamide or the Taxol analogue paclitaxel [6, 8, 9, 10, 17].

An in vivo tumor model system is presented in which the tumor cell line is transfected with a constitutively expressed marker gene. The marker gene codes for a heat-stable secreted human placental alkaline phosphatase protein (SEAP) [4]. SEAP is distributed through the body at levels proportional to the number of tumor cells in the animal. The SEAP protein is detectable in small blood samples collected into capillary tubes. Animals may be repeatedly sampled over the trial period to monitor the course of tumor progression. The current study confirms the work of Bao et al. [2], in which SEAP was transfected into the ovarian carcinoma line A2780 and monitored in severe combined immunodeficient (SCID) mice. The current study extends this model to use a different ovarian carcinoma cell line, OCC1 [19] and different chemotherapeutics. In addition, the athymic nude mouse was used rather than the SCID mouse. The current study also correlated in vitro response to drug therapy with response to treatment in the live animal. Observations demonstrate that treatment with platinum-containing drugs does not alter the rate of SEAP secretion per cell. Therefore, the nude mouse model using SEAP as a marker is an accurate indicator for monitoring ovarian tumor growth and progression.

Materials and methods

Transfection of SEAP gene into OCC1 cells

Dr. T. Hamilton of the Fox Chase Cancer Center kindly provided the expression vector pCMV-SEAP. This vector uses the SEAP gene and SV40 enhancer derived from p-SEAP2-enhancer plasmid (Clontech, Palo Alto, Calif.) cloned into pcDNA3. The pCMV-SEAP plasmid was transfected into the OCC1 [19] ovarian carcinoma cell line using transfection mediated by Fugene6 (Boehringer Mannheim). To each well of cells in a 24-well plate were added 200 ng plasmid DNA and 1 μl Fugene6 reagent. Cells with stably integrated SEAP were selected for neomycin resistance by treating with 600 μg/ml G418 (Cal-Biochem, La Jolla, Calif.). Clonal isolates were grown and culture medium tested for SEAP production. Of 18 clonal isolates of OCC1, one produced very high levels of SEAP (OCC1-SEAP-12). OCC1-SEAP-12 was used in subsequent in vivo and in vitro experiments.

Animal use and in vivo treatment protocols

Athymic nude mice (Nu/Nu; Charles River Laboratories, Wilmington, Mass.) weighing about 25 g were used for in vivo studies. Three mice were injected i.p. with 1×10^7 OCC1-SEAP-12 cells. After 1 week, experimental treatments were begun. Animals received i.p. injections of 3 mg/kg cisplatin divided over 2 weeks (one injection per week), 10 mg/kg cisplatin divided over 2 weeks or a vehicle control. Alternatively, four mice with well-developed tumors (2–4 weeks after injection) were treated with a high dose of carboplatin (60 mg/kg) three times over a period of 4 days. Blood samples were collected from saphenous vein lancings three times per week into heparinized capillary tubes. The capillary tubes were centrifuged and the plasma samples were frozen at –20°C until the time of SEAP assay. Blood samples were taken for SEAP assay from 13 mice within 24 h of death 2–3 weeks after i.p. injection of OCC1-SEAP cells. After death all visible i.p. tumors were dissected out and weighed.

In four nude mice 1×10^7 OCC1-SEAP-12 cells were injected s.c. into the dorsal flank region. Measurements of tumor length, width and height were taken three times per week. One-half the average diameter was used as the radius to calculate tumor volume using the equation: volume $= 4/3\pi r^3$. Blood samples were taken as above for the SEAP assay.

In vitro cell culture and biochemical assays

OCC1-SEAP-12 cells were grown in Ham's F-12 medium (Gibco) plus 10% calf serum then collected into Hank's balanced salt solution and counted for injection into nude mice. For in vitro assays OCC1-SEAP-12 cells were plated at sequential twofold dilutions and allowed to adhere to culture wells and incubated for 24 h. Alternatively, OCC1-SEAP-12 cells were grown to 80% confluence in Ham's F-12 medium plus 10% calf serum. Cells were then starved for 48 h in DMEM plus 0.1% BSA and 0.1% calf serum. Experimental treatments were then applied for 2 days in DMEM plus 0.1% BSA and 0.1% calf serum. At the end of the treatment period a sample of culture medium was taken for SEAP level determination. DNA assays were performed by discarding the remaining culture medium from over the cells, adding buffer solution, sonicating the cells in each well, and measuring the

fluorescence of cell solution into which SYBR Green I fluorescent dye (Molecular Probes, Eugene, Ore.) had been incorporated as previously described [11].

SEAP assay

Blood plasma and cell culture medium samples were assayed for SEAP activity using the Great EscAPe SEAP fluorescence detection kit (Clontech Laboratories, Palo Alto, Calif.). SEAP is secreted at a constant rate by transfected tumor cells and distributed throughout the body or into the culture medium. SEAP is heat stable, so any endogenous alkaline phosphatase activity in blood plasma is destroyed by heat treatment of the samples (65°C for 30 min) during the assay. The SEAP enzyme in each sample acts on the substrate 4-methylumbelliferyl phosphate during a 1-h incubation at room temperature to produce a fluorescent product with excitation and emission peaks at 360 and 449 nm, respectively. A fluorimeter is then used to measure SEAP activity. The intraassay and interassay coefficients of variation were 2.5% and 18.8%, respectively. Blood plasma samples were diluted 1:100 prior to the assay to bring values within the linear range of the standard curve.

Statistical methods

To determine the degree of correlations between tumor volume and SEAP levels a correlation coefficient was calculated from measurements of s.c. tumor volume and plasma SEAP levels of samples collected at the same time. A correlation coefficient was calculated for i.p. tumors between plasma SEAP levels of blood samples collected within 24 h of death and i.p. tumor weight.

Results

Plasma SEAP concentrations correlate with tumor size

OCC1-SEAP cells were injected s.c. into nude mice, and the average diameter of the growing tumor mass was measured three times per week to calculate tumor volume as described above. At the same time blood samples were taken and plasma SEAP levels were determined. While tumor volumes and tumor growth rates varied considerably between mice, SEAP levels were found to correspond closely to calculated tumor volume within individual mice. Results from a representative mouse out of four are shown in Fig. 1. The correlation coefficient was $R^2 = 0.95$.

It was demonstrated that tumor invasiveness could result in inaccurate s.c. tumor volume measurement (Fig. 2). Two mice were injected s.c. with OCC1-SEAP cells. The mouse with the smaller tumor volume measurement had higher plasma SEAP levels than the mouse with the apparently larger tumor volume measurement. However, at the termination of the experiment dissection showed that the "smaller volume" tumor had invaded into the underlying body wall, and actually weighed much more than the tumor in the other mouse. This indicates that plasma SEAP levels were a much more accurate indicator of tumor mass than were tumor diameter measurements.

The correlation between i.p. tumor mass and plasma SEAP levels was investigated by injecting OCC1-SEAP cells i.p. into 13 nude mice and letting the tumors develop for approximately 3 weeks. At the end of this time the animals were killed and all visible tumor was dissected from the abdominal cavity and weighed. Blood samples from the time of death were used to determine end-point plasma SEAP levels. The SEAP levels were correlated with tumor weight ($R^2 = 0.87$; Fig. 3).

OCC1-SEAP cells respond to platinum-containing drug treatment in vitro

In vitro studies were performed to verify that SEAP levels correspond to cell density after culturing OCC1-SEAP cells for 24 h. SEAP levels in the medium corresponded directly to the amount of DNA present in

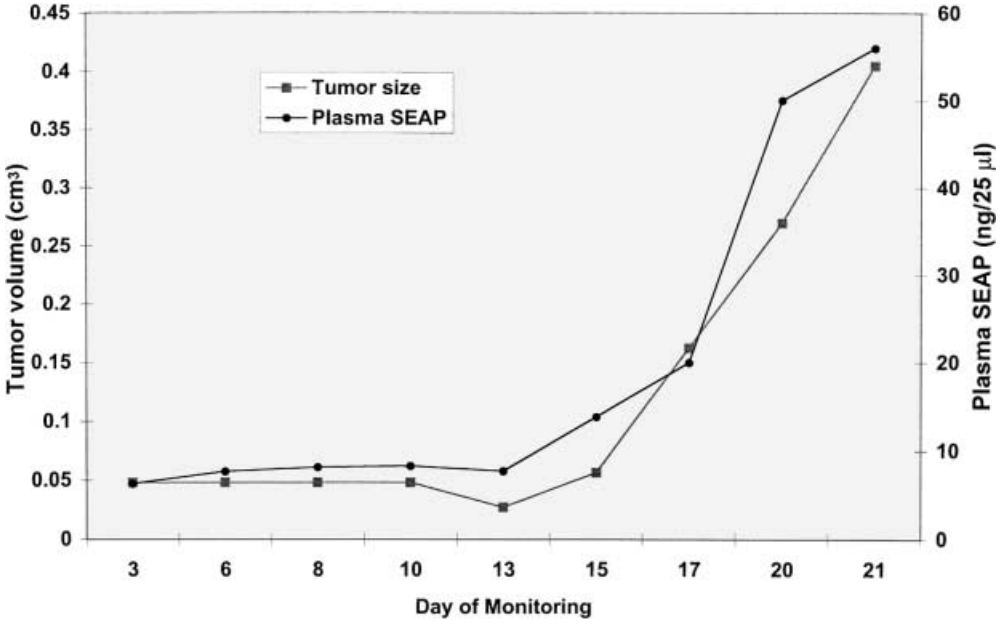

Fig. 1 Correlation between calculated s.c. tumor volume and plasma SEAP levels. Nude mice were injected s.c. in the dorsal flank region with 1×10^7 OCC1-SEAP cells. The volume of the tumors was calculated as described in Materials and methods. At the same time blood samples were taken for later plasma SEAP assay (nanograms SEAP per 25 µl). Representative results from four different mice and two experiments are presented

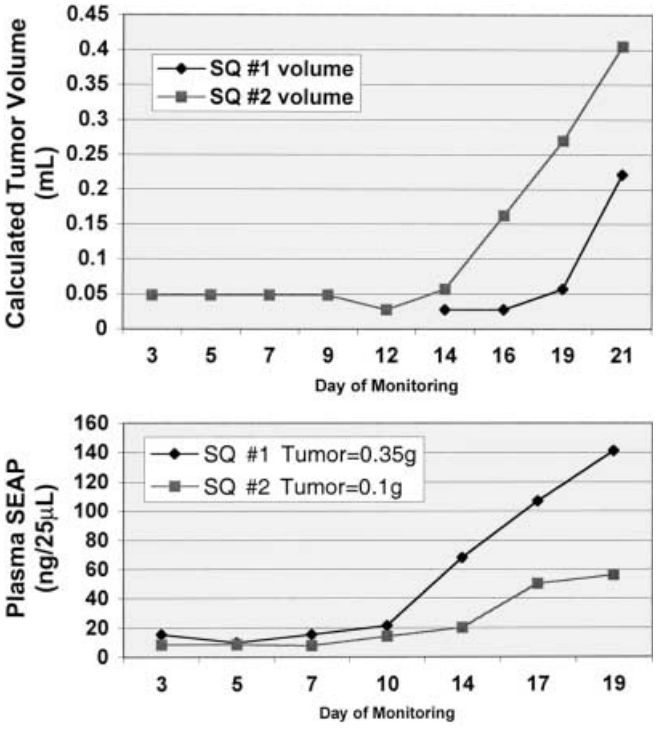

Fig. 2a, b Plasma SEAP levels versus tumor volume when the s.c. tumor becomes invasive. OCC1-SEAP cells (1×10^7) were injected s.c. into two mice. **a** Tumor diameter was measured through the skin and tumor volume calculated. **b** Blood samples were taken and plasma SEAP assayed. When the experiment was terminated the tumors were dissected and weighed. The tumor in SQ#1 had invaded the underlying body wall and was larger (0.35 g) than the tumor in SQ#2 (0.1 g)

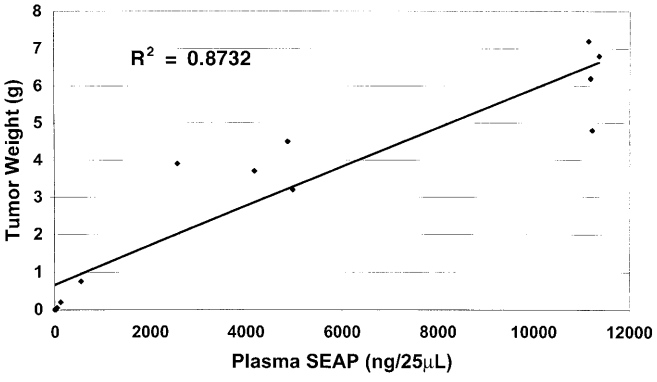

Fig. 3 Correlation between i.p. tumor mass and plasma SEAP levels. OCC1-SEAP cells (1×10^7) were injected i.p. into 13 nude mice. The tumors were allowed to develop for approximately 3 weeks. At the end of this time the animals were killed and all visible tumor was dissected from the abdominal cavity and weighed. Blood samples at the time of death were used to determine end-point plasma SEAP levels. Tumor SEAP levels were correlated with tumor weight ($R^2=0.87$)

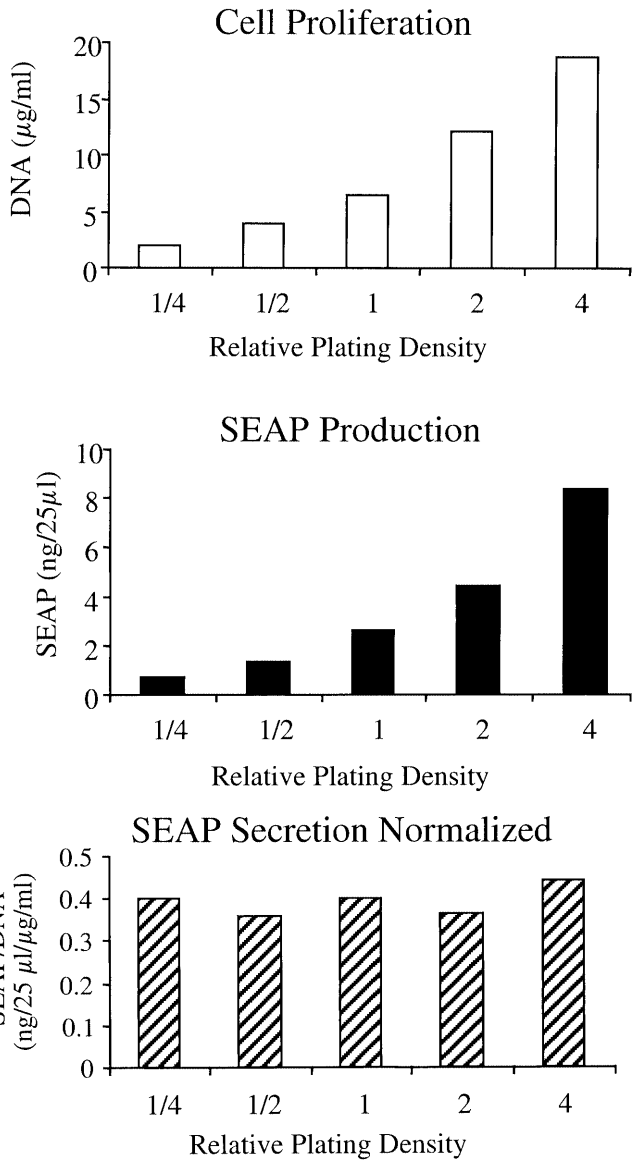

Fig. 4a–c Correlation between cell density and secreted SEAP levels in vitro. OCC1-SEAP cells were cultured at sequential twofold increases in cell density for 24 h. **a** DNA levels were determined to reflect cell number in each well. **b** SEAP levels were determined in the medium of cultured cells at 24 h. **c** SEAP secretion from OCC1-SEAP cells was normalized per microgram DNA in each well

a culture well (Fig. 4). The rate of SEAP secretion per cell was the same at all cell densities (Fig. 4). This indicates that SEAP levels were an accurate indicator of tumor cell number at different cell densities. OCC1-SEAP cells were cultured in the presence or absence of 60 μg/ml carboplatin (Sigma). Carboplatin treatment decreased tumor growth in vitro, as measured both by the amount of DNA per well and by the amount of SEAP in the culture medium (Fig. 5). In OCC1-SEAP cells the DNA levels decreased from 58 μg/ml for control cells to 35 μg/ml for carboplatin-treated cells. SEAP levels also decreased from 11.1 ng per 25 μl in control cells to 3.9 ng per 25 μl in carboplatin-treated cells. SEAP production was normalized by the amount of DNA per well (Fig. 5). This ratio reflects the amount of SEAP produced per cell. This normalized SEAP production did not change in the presence or absence of carboplatin treatment. The anticancer drug treatment

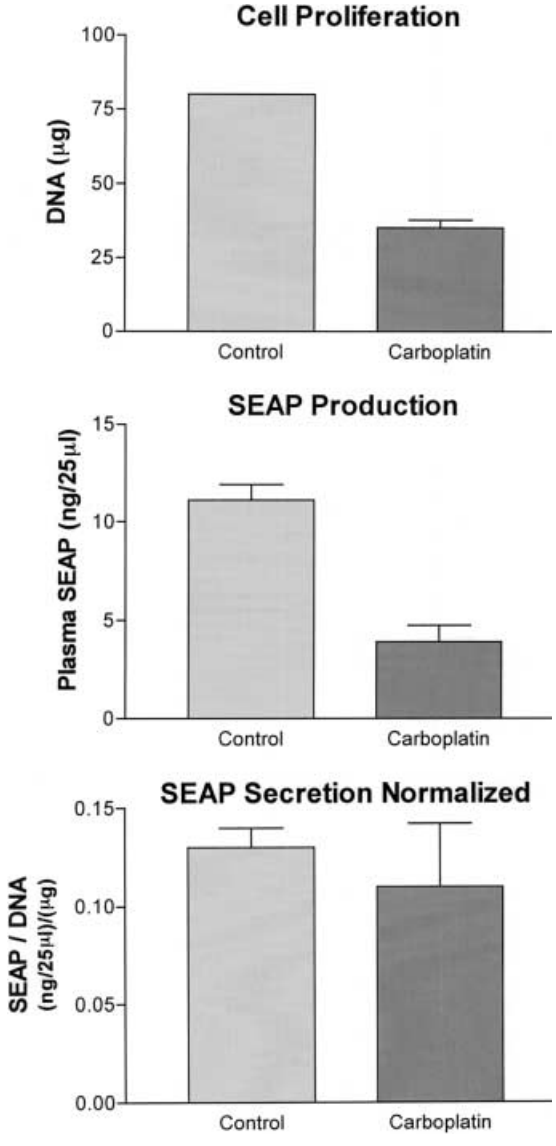

Fig. 5a–c Response of OCC1-SEAP cells to carboplatin chemotherapy in vitro. OCC1-SEAP cells were grown to 50% confluence in Ham's F-12 plus 10% calf serum and then starved for 2 days in DMEM plus 0.1% BSA and 0.1% calf serum. Cells were then treated with 60 μg/ml carboplatin for 48 h. **a** The amount of DNA per well was measured in control and carboplatin-treated cultures. **b** SEAP levels were assayed in the medium of cultured cells after the treatment period. **c** SEAP secretion from OCC1-SEAP cells was normalized per microgram DNA in carboplatin-treated and control wells. The bars indicate the means ± SEM from three different experiments

did not change constitutive SEAP production. Therefore, SEAP could provide a marker for tumor burden independent of carboplatin chemotherapeutic treatment.

OCC1-SEAP cells respond to platinum-containing drug treatment in vivo in the nude mouse model

Nude mice growing OCC1-SEAP tumor cells i.p. were treated with 3 or 10 mg/kg cisplatin every 48–72 h over a

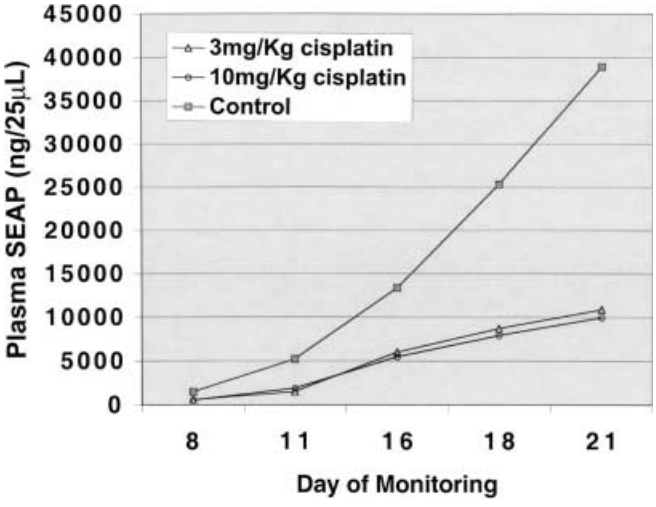

Fig. 6 OCC1-SEAP cell response to cisplatin chemotherapy in vivo. Three nude mice were injected i.p. with 1×10^7 OCC1-SEAP cells. After 1 week the mice were treated with 3 or 10 mg/kg cisplatin every 48–72 h over 2 weeks or treated with vehicle control. Blood samples were taken at intervals over the treatment period and assayed for SEAP. This results shown are representative of three different experiments

2-week period or treated with vehicle as controls. Blood samples over this time period were assayed for plasma SEAP. Plasma SEAP measurements indicated that cisplatin treatments decreased tumor growth compared to the untreated vehicle controls (Fig. 6). No difference was seen in tumor growth between mice treated with 3 and 10 mg/kg cisplatin. Additionally, nude mice having larger OCC1-SEAP i.p. tumors (2–4 weeks after injection) were treated with a high dose of carboplatin (60 mg/kg) three times over 4 days. Plasma SEAP levels indicated that carboplatin transiently decreased the tumor burden (Fig. 7). Tumor growth resumed after the treatment was terminated. Therefore, the SEAP tumor model is effective at monitoring tumor burden and response to platinum-containing chemotherapeutic drugs.

Discussion

In these studies an in vivo tumor model system was evaluated in which the tumor cell line was transfected with the constitutively expressed marker gene SEAP. The tumor cell line with the marker gene was then grown in nude mice. The SEAP protein is secreted by tumor cells and can be detected in blood samples. The mice were treated with anticancer chemotherapeutic drugs and the response of the tumors to treatment was evaluated by measuring SEAP levels in the blood over the duration of the experiment.

OCC1-SEAP cells were injected s.c. into nude mice to evaluate the accuracy of this model in determining tumor burden. The mice were monitored for tumor growth by measuring the s.c. mass through the skin and calculating tumor volume. At the same time plasma SEAP

levels were determined. Plasma SEAP was generally found to correlate closely with calculated tumor volume (Fig. 1) in s.c. tumors. Interestingly, in a mouse in which the s.c. tumor had invaded the underlying body wall, plasma SEAP levels were found to be a better indicator of tumor burden than tumor volume (Fig. 2). SEAP levels would be expected also to be a better indicator of tumor burden than volume for tumors that develop necrotic centers, because only viable tumor cells produce the SEAP protein.

Some tumors, including ovarian carcinomas, arise primarily in the abdominal cavity. For these types of cancers the s.c. environment may not be the appropriate system for investigation. OCC1-SEAP cells were injected i.p. into nude mice to evaluate the accuracy of this in vivo model system for determining i.p. tumor burden. At the end of 2–3 weeks of tumor growth the animals were killed and all visible tumor was dissected out and weighed. Blood samples from the time of death were assayed for SEAP levels. Tumor mass was found to correlate with SEAP levels ($R^2 = 0.8732$; Fig. 3). This correlation may have been improved if during dissection more of the disseminated tumor foci had been located and excised. Also the invasion of tumor cells into the body wall and abdominal organs made isolation of tumor tissue from host tissue problematic. Indeed, it has been shown that some carcinomas will incorporate host stromal tissue into the tumor mass itself [12]. Since SEAP levels can be measured over the course of an experiment, tumor burden can be monitored during treatment rather than just at the time of death. These considerations suggest blood SEAP levels are a more accurate and informative indicator of tumor burden than dissecting and measuring tumors at the end-point of the experiment.

One important purpose of a cancer model system is to test the response of tumor cells to anticancer therapeutic drugs. Platinum-containing drugs are used in anticancer therapies for ovarian carcinomas [8, 9, 17]. Experiments were performed in vitro and in vivo to test whether SEAP levels reflect the antiproliferative action of platinum-containing drug treatment. For in vitro experiments, OCC1-SEAP cells were cultured and treated for 2 days with carboplatin. Carboplatin treatment decreased cell proliferation compared to untreated control cells (Fig. 5). This was accompanied by a corresponding decrease in SEAP levels in the culture medium. As discussed above, accurate measurement of i.p. tumor mass is problematic. For this reason, and because repeated measurement of i.p. tumor mass over time in individual mice is extremely difficult, no attempt was made to correlate tumor mass with SEAP levels in these mice. Therefore, SEAP levels are a good indicator of cell number and response to carboplatin treatment in vitro.

Nude mice were injected i.p. with OCC1-SEAP cells and treated with the platinum-containing drug cisplatin to see whether the in vitro response also occurred in vivo. Cisplatin-treated mice had lower plasma SEAP levels indicating lower tumor burden than vehicle-treated control mice (Fig. 6). Similarly, when daily SEAP production by OCC1-SEAP cells in vitro was measured, SEAP levels corresponded to cell number on each day in both carboplatin-treated and untreated wells (data not shown). In another experiment, mice carrying established i.p. tumors were treated with a high dose of carboplatin. Carboplatin-treated mice had a transient decrease in plasma SEAP levels suggesting partial regression of the tumor (Fig. 7). Therefore, this in vivo mouse model system for monitoring ovarian tumor growth reflects responses to platinum-containing therapeutic drugs. In the current study OCC1 ovarian carcinoma cells were shown to respond to platinum-containing chemotherapeutic drugs in vitro and in vivo.

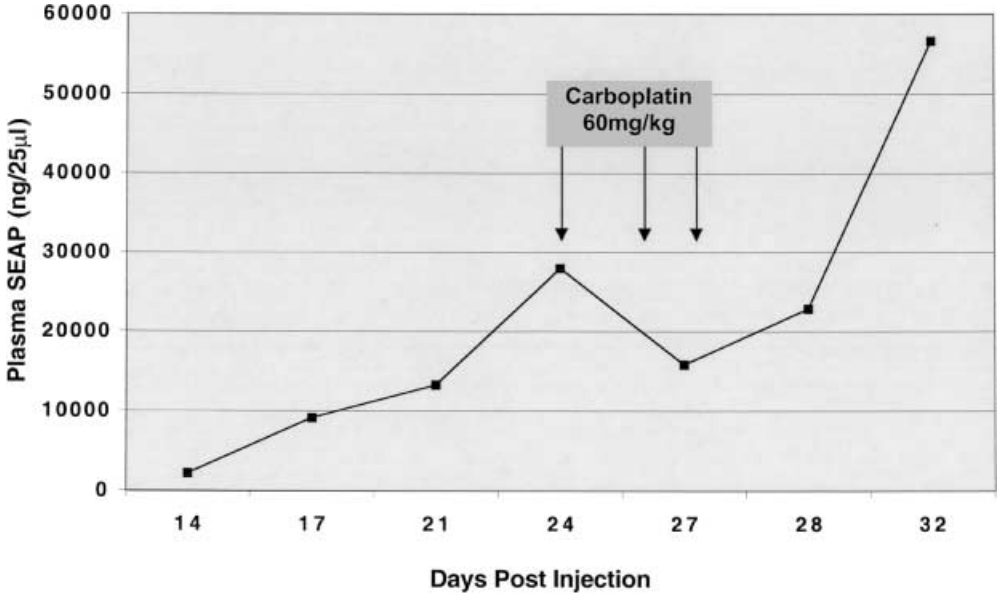

Fig. 7 Effects of carboplatin on i.p. tumor burden in vivo. Four nude mice with established OCC1-SEAP i.p. tumors (4 weeks after injection of 1×10^7 OCC1-SEAP cells) were treated with a high dose of carboplatin (60 mg/kg) three times over a period of 4 days. Blood samples were taken at intervals and assayed for SEAP. The results shown are the response of one mouse and are representative of two different experiments. The *arrows* indicate when mice were treated with carboplatin

It is important to note that SEAP secretion by OCC1-SEAP cells was not shown to be regulated independently of cell number. If treatment with a chemotherapeutic drug caused the constitutive levels of SEAP production and secretion to change, the use of SEAP levels as an indicator of tumor burden would be misleading. When SEAP levels were normalized per microgram DNA in in vitro cultures, it was shown that treatment with carboplatin did not change constitutive levels of SEAP secretion in these OCC1 cells (Fig. 5).

Other markers have been used to monitor tumor growth in vivo. The neomycin resistance gene (neo) has been transfected into tumor cells [16], but it is necessary to take surgical samples of the tumor and perform Southern blots to measure neo levels. This limits its usefulness for repeated monitoring. The luciferase gene has also been transfected into tumor cells, and chemiluminescence of the tumor in vivo has been measured as an indicator of tumor burden [20]. This procedure requires a luminometer capable of reading a whole mouse. The marker gene pVSneo-hCG has been transfected into HeLa cells [21]. When the transgenic cells are grown in nude mice, beta-human chorionic gonadotropin (βhCG) is excreted in the urine and assayed as a measure of tumor burden. This is noninvasive, but urine collection from mice can be problematic.

Endogenous markers exist for monitoring several tumors. Prostate adenocarcinomas secrete prostate-specific antigen (PSA) which is detected in serum and used clinically to screen for prostate cancer [15]. Serum alpha-fetoprotein is a biochemical marker for hepatocellular carcinoma [3, 7]. However, alpha-fetoprotein is secreted in other conditions such as testicular cancers and complications of pregnancy [14, 15, 18]. Carcinoembryonic antigens CA 125 and CA 15-3 have also been used as clinical markers of tumor progression [5]. However, it is rare for a cancer cell line to constitutively secrete a specific endogenous marker. Therefore, introduced reporter genes such as SEAP may be more universally applied and useful in research situations.

Bao et al. [2] have also examined the use of SEAP as a marker for tumor growth in vivo. The current work confirms their findings that SEAP levels correlate well with s.c. tumor volume. SEAP levels were also found to correlate with i.p. tumor weight at the time of death in the current study. Bao et al. transfected the CMV-SEAP into the ovarian carcinoma cell line A2780. In the current study OCC1 ovarian carcinoma cells were successfully transfected with pCMV-SEAP. SKOV3 cells have also been transfected in our laboratory (data not shown). This suggests that the SEAP gene may be used as a marker in a number of tumor cell lines. In the current study, the response of ovarian carcinoma cells to treatment with platinum-containing chemotherapeutic drugs was investigated. Similar to the response to paclitaxel seen by Bao et al. in SCID mice [2], the response to cisplatin treatment in nude mice was easily followed over the course of treatment by monitoring plasma SEAP levels.

In the current study, the in vitro response to platinum-containing drugs corresponded to the in vivo animal model. It was also demonstrated that platinum-containing drug treatment did not change the constitutive rate of SEAP secretion per OCC1 cell. The results of the current study indicate that SEAP can be used as an in vivo reporter gene mouse model to monitor tumor growth and response to therapeutic drugs. Future studies will utilize this model to investigate novel chemotherapeutic approaches to treating ovarian cancer.

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