Transplantation of human or rodent tumors into cyclosporine-treated mice: A feasible model for studies of tumor biology and chemotherapy

(human tumor xenografts/nude mice/cisplatin)

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ABSTRACT

Total growth of transplanted human or rodent tumors in the subrenal capsule of mice was much improved by treatment with cyclosporine (CSA, cyclosporin A). Tumor size increased rapidly between days 6 and 12 after implantation. CSA injected on days 1–5 or 2–8 prevented tumor regression. In contrast, immunologic regression occurred after 6 days in absence of the drug. Tumor growth was comparable in CSA-treated mice, athymic nude mice with human tumors, or normal mice with syngeneic rodent tumors. Studies with rodent tumors in syngeneic mice showed that the CSA treatments had no antitumor effect. Inflammatory infiltration was seen on days 6–12 after tumor implantation into control mice. Immunoperoxidase staining showed murine T cells to be prominent in the infiltrate. In contrast, tumors in CSA-treated mice contained minimal inflammatory infiltrate even 12 days after implantation. Allogeneic tumors in CSA-treated mice caused neovascularization, metastases, and local invasion into the kidney. cis-Diamminedichloroplatinum showed highly significant activity against human tumors in CSA-treated mice during the period 6–10 days after tumor implantation but showed no statistically significant antitumor activity 6–12 days after implantation in mice not treated with CSA. We suggest that in CSA-treated mice the subrenal capsule assay for tumor growth provides a rapid, economical model for investigations in vivo of human tumor biology, for drug screening with a standard tumor, or for determination of optimal treatment of particular human tumors.

The ability to grow human tumors outside of the natural host has been a continuous challenge for investigations of human tumor biology, for development of antineoplastic drugs, and for selection of optimal therapy based on specific drug sensitivities of clinical tumor samples. Although many curative treatments for rodent tumors have been demonstrated in laboratory models, it is often difficult to transfer such promising results from animal models into clinical therapy with significant benefit for patients with cancer. In contrast to rodent tumors, which grow readily in numerous test systems, human tumors are difficult to propagate in laboratory models for testing cancer therapeutic agents (1). Selection of optimal cancer treatments for clinical trials would be greatly facilitated by a simple, reliable, and economical model for determination of efficacies of various treatments against human cancers. Since most current cancer treatments have clinical activity limited to specific tumor types (e.g., breast cancer vs. melanoma), an ideal model would allow testing in several human tumors so that antitumor activity of promising new treatments could be determined in many types of human cancer.

Several assays for growth in vitro of clonogenic human tumor cells have been developed, and these may be useful for certain tumor types. However, results with these methods have not been easily reproducible, and many human tumors give cloning efficiencies too low to evaluate (2). Moreover, clonogenic assays do not provide a system to study (i) toxicity of drugs to normal tissues, (ii) host responses to tumor growth, or (iii) localization or activation of drugs and other agents in tumor vs. normal tissues. Monoclonal antibodies and angiogenesis inhibitors are two promising therapeutic approaches that cannot be evaluated by clonogenic assays.

Subcutaneous implantation of human or rodent tumors into athymic nude mice is an alternative method for investigations of tumor–host interactions and new treatment strategies. This technique, although generally usable, is time-consuming, and nude mice and the facilities necessary to maintain them are expensive enough to limit its practicality (3). The subrenal capsule (SRC) assay developed by Bogden and coworkers (3, 4) permits precise in situ measurement of transplanted human tumors by use of a stereomicroscope with an ocular micrometer. Beneath the transparent renal capsule of mice is an advantageous site for delivery of nutrients, drugs, or other substances to the implanted tumor. In contrast to the subcutaneous site, which often requires weeks or months for appreciable tumor growth, in nude mice the SRC assay can quantify small changes in tumor size after 6–12 days.

With normal mice, a 6-day protocol is used for the SRC assay; this is an attempt to avoid the period of greatest immunologic regression, which occurs 6–12 days after tumor implantation (4). A comparison (5) of the SRC assay with a human tumor cloning method (bilayer soft agar) developed by Hamburger and Salmon (6) showed that the SRC method gave about twice as many evaluable assays. This observation suggests that the subcapsular site is a more favorable medium for growth of solid human tumor explants than is soft agar. Initial studies with fresh surgical explants of human cancers in the 6-day SRC assay also indicate a high success rate for several common human tumors, exceeding 80% for breast, ovarian, lung, and colon cancers (7).

Because of the rapid end point and economy of the SRC assay in normal mice, these initial studies suggested that it could be useful for other investigations of human cancer biology or therapeutic strategies. However, two major problems prevent such applications: (i) Minimal growth of primary human tumors limits the capacity of the 6-day assay for discrimination of drug treatments. (ii) Inflammatory responses of immunocompetent mice are evident by day 6 af-

Abbreviations: CSA, cyclosporine (cyclosporin A); SRC, subrenal capsule; mmu, ocular micrometer units; cis-Pt, cis-diamminedichloroplatinum.
ter tumor implantation, and these infiltrates adversely affect subsequent measurements of tumor size. In a detailed histologic analysis of primary human tumor xenografts in the 6-day SRC assay in normal mice, Levi et al. (8) found that 18 of 23 tumors (78%) contained moderate to severe lymphocytic infiltration on day 6. These authors questioned the reliability of measuring gross tumor size on day 6 to quantify chemotherapeutic responses and proposed a complex multiparameter histological score to quantify antitumor activity of chemotherapy on day 4 after tumor implantation.

We report here experiments designed to improve the SRC assay by extending tumor growth in normal mice by treatment with cyclosporine (CSA). CSA is a cyclic polypeptide that prevents organ-transplant rejection without inducing bone-marrow suppression, probably through selective inhibition of helper T cells (9). We also studied the inflammatory infiltration of grafted tumors in control animals and in animals treated with CSA, using immunoperoxidase staining with monoclonal antibodies to murine T-cell subsets. Cisplatin [cis-diaminedichloroplatinum (II), cis-Pt] treatment of mice with human tumor xenografts, after 6 days in control mice or after 10 days in CSA-treated mice, tested the capacity of the SRC model to discriminate antitumor drug activities under these two conditions.

Our experiments provide evidence that (i) T-cell activity is a prominent early component of the immunologic rejection of grafted tumors in the SRC assay with normal mice, (ii) CSA dramatically reduces T-cell infiltration and allows extended growth of human or rodent tumors, (iii) accurate measurement of tumor vs. inflammatory cell mass is greatly facilitated by CSA treatment, (iv) CSA has no antitumor effects at the doses used in these studies, and (v) the capacity to test cancer treatments is much improved by extending tumor growth beyond the 6-day period. Thus, we introduce a valid and economical method to investigate tumor biology, host-tumor interactions, or new anticancer treatments by using human tumor xenografts in CSA-treated mice.

**MATERIALS AND METHODS**

**SRC Assay.** The SRC assay followed the technique of Bogden and co-workers (3, 4). Tumor obtained from subcutaneous passage in donor mice was trimmed, and 1 mm³ fragments were excised. Female mice (6–9 weeks old) were anesthetized with chloral hydrate, placed on a sterile surface, and swabbed with ethanol/Zephiran chloride solution. An incision about 1 cm long was made through skin and body wall in the region of the left kidney. The kidney was partially exteriorized and a small slit was made in the renal capsule. A tumor fragment was then loaded onto the tip of a 16-gauge trocar, the trocar was inserted through the slit, and the tumor tissue was deposited under the capsule by moving the plunger forward. Immediately after implantation, the longest and shortest diameters of the implanted tumor were measured in situ with a dissecting microscope equipped with an ocular micrometer calibrated so that 10 ocular micrometer units (omu) equaled 1 mm. The kidney was then replaced into the body cavity, and the incision was closed with sterile wound clips. Mice were randomly assigned to treatment groups (5–7 mice per group) after tumor implantation. At the termination of the assay, each animal was weighed and then killed by cervical dislocation. The abdominal cavity was exposed, and the left kidney was removed and placed under the dissecting microscope for measurement of final tumor size. Tumor sizes were expressed as the average of longest and shortest diameters, as described by Bogden et al. (3). The tumor implantation procedure required <2 hr for a typical experiment involving 30–40 mice.

**Tumors.** The MX-1 human breast tumor was maintained subcutaneously in female athymic nude (BALB/c nu/nu) mice bred in our laboratory. The MX-1 tumor is currently used by the Division of Cancer Treatment, National Cancer Institute, for preclinical screening of new anticancer drugs. This tumor originated from a female patient with primary breast cancer and was obtained from the Breast Cancer Human Cell Culture Bank, E. G. & G. Mason Research Institute (Worcester, MA). The human karyotype was confirmed in our laboratory after passage in nude mice.

The MBT-2 tumor originated from a bladder tumor in C3H/He mice exposed to the carcinogen N-(4-(3-nitro-2-furyl)-2-thiazoyl)formamide by M. Soloway (University of Tennessee Center for Health Sciences). C3H/He and CD-1 (outbred Swiss) mice were purchased from Charles River Laboratories and were maintained in a standard mouse facility in the Surgical Research Laboratory, Harvard Medical School.

**Drugs.** CSA was purchased from Sandoz Pharmaceuticals as a liquid suspension in oil (100 mg/ml) and diluted in olive oil prior to injection. Control groups received olive oil alone. CSA injections (80 mg/kg of body weight, in 0.1 ml of oil) were done subcutaneously on a daily basis starting 1–2 days after tumor implantation. cis-Pt was purchased from Bristol Laboratories (Syracuse, NY), dissolved in 0.9% saline, and stored frozen at −70°C prior to use. Control mice received 0.9% saline alone.

**Histologic and Immunoperoxidase Staining.** At the termination of assay, tumor-bearing kidneys were removed and randomly assigned to fixation in Hanks' balanced salts solution/formalin or freezing in OCT (optimal cutting temperature) compound (Ames, Elkhart, IN). Formalin-fixed kidneys were subsequently sectioned at the limits of the xenograft and embedded in paraffin. Representative serial sections were stained with hematoxylin/eosin. Frozen kidneys were prepared for immunoperoxidase staining according to methods reported in detail by Bhan and DesMarais (10). Monoclonal antibodies directed against murine T-cell subsets were used, including anti-Lyt1 and anti-Lyt2 obtained from Becton Dickinson (Sunnyvale, CA). Identification of helper T-cells was done using an anti-L3T4 monoclonal antibody (11), generously provided by H. Cantor, Dana-Farber Cancer Institute.

**RESULTS**

**Growth of Human Tumor Xenografts.** CSA treatment allowed MX-1 xenografts to grow comparably in normal (CD-
tumor implantation allowed tumor growth for 10–12 days after implantation. Without CSA, average tumor diameters after 6 days were ~17 omu, an increase of ~30% compared to the original tumor sizes (12.5 omu). In contrast, the tumors on day 10 in CSA-treated mice were ~30 omu, an increase of ~130%. The growth rate of tumors during days 6–10 was greater than during days 0–6, especially because tumors grew more rapidly in depth after day 6. Gross inspection of kidneys from CSA-treated mice after 10 days revealed healthy, discrete tumor grafts. In contrast, control tumors were difficult to measure because of hemorrhage and necrosis (Fig. 2).

**Growth of Murine Tumor Allografts.** MBT-2 tumors grew in allogeneic CD-1 mice during the first 6 days after implantation (Fig. 3), and no statistical difference in tumor size was observed on day 6 in allogeneic vs. syngeneic (C3H) mice. CSA treatment of CD-1 mice allowed tumor growth for 12 days that was not statistically different from tumor growth in syngeneic C3H mice. These results are similar to results with MX-1 human tumor xenografts in CSA-treated normal mice vs. nude mice. In these experiments MBT-2 tumor growth also was more rapid during days 6–12 than during days 0–6. CSA treatments given to C3H mice implanted with syngeneic MBT-2 tumors had no detectable antitumor effect; in fact, a slight increase in tumor size was noted on day 12 in syngeneic mice (P < 0.05 for treated vs. controls). Similar results were obtained in two other experimental groups.

**Histologic and Immunohistologic Studies.** Although tumors increased in total size on day 6 in control mice, histological analysis revealed areas of inflammatory infiltrates and hemorrhagic necrosis (Fig. 4A). In contrast, fragments from the same tumors in CSA-treated mice showed much less inflammation and necrosis even on days 6–12 (Fig. 4B). The differences were similar for both mouse (MBT-2) and human (MX-1) tumors.

Tumors in CSA-treated mice also showed prominent neovascularization and invasion of the kidney on days 6–12 after implantation, and some mice with allogeneic tumors had liver metastases. Although minor neovascular changes were observed on day 6 in control mice with allogeneic tumor grafts, only CSA-treated mice exhibited metastases or prominent tumor invasion into the kidney.

Immunoperoxidase staining with monoclonal antibodies revealed that a prominent portion of the cellular infiltrate consisted of host (murine) T cells (Fig. 4C). Both helper and
suppressor T-cell subsets were observed on day 6 after tumor implantation into control mice, using anti-L3T3, anti-Lyt1 and anti-Lyt2 antibodies. Both T-cell subsets were dramatically reduced in CSA-treated mice.

**Antitumor Activity of cis-Pt.** The capacity of the SRC assay to delineate antitumor activity of cis-Pt against growing MX-1 tumors was significantly improved when the drug was tested in CSA-treated mice (Fig. 5). A single injection of cis-Pt (8 mg/kg) on day 1 in untreated mice produced a slight difference in tumor size observed on day 6 compared to controls (Fig. 5A), but the difference was not statistically significant (P > 0.2). However, the same cis-Pt treatment given on day 6 to CSA-treated mice produced a much greater difference in tumor size by day 10 (P < 0.001 for treated vs. controls). Although an increase in control tumor size was observed on day 6 in CSA-treated mice (Fig. 5B), this difference was not statistically significant compared to mice without CSA (Fig. 5A). Similar results with cis-Pt were obtained in two additional experimental groups when CSA (80 mg/kg per day) was injected on days 1–5 after tumor implantation or when cis-Pt was given on day 1 before CSA (days 2–8).

**DISCUSSION**

We report that CSA allowed extended growth of human and rodent tumors in the SRC assay. The most rapid growth of

**FIG. 4.** Histology of MBT-2 murine tumors in the SRC assay. A and B show staining with hematoxylin/eosin. C shows immunoperoxidase stain of frozen section. (A) Control tumor (T) on day 6 with necrosis (N) and prominent inflammation (I) at border with kidney (K). (B) The same tumor on day 12 in CSA-treated mice shows homogeneous malignant cells, less necrosis, and very little inflammation at border with kidney. (C) Using anti-L3T4 monoclonal antibody on frozen sections from control mice, immunoperoxidase staining (darker cells) shows murine (helper) T cells at border between tumor and kidney (compare with A). CSA-treated mice showed dramatic reduction of both total inflammation and T-cell response 6–12 days after tumor implantation (×36).

**FIG. 5.** Antitumor activity of cis-Pt against MX-1 human tumor xenografts in the SRC assay. Shown are mean values of treatment groups (5–7 mice per group) ± SEM. CSA (80 mg/kg per day) was given subcutaneously on days 2–8 after implantation. cis-Pt (8 mg/kg) was given subcutaneously on days indicated by arrows. (A) Mice not treated with CSA. Difference between cis-Pt-treated and control tumors on day 6 was not significant (P > 0.2). (B) CSA-treated mice. Difference between cis-Pt-treated and control tumors on day 10 was significant (P < 0.001).
implanted tumors in the SRC assay occurred after day 6, both in mice with syngeneic rodent tumors and in CSA-treated mice with human tumors. These results suggested that statistical discrimination of experimental treatments would be much improved 6–12 days after tumor implantation, since the difference between treated vs. control groups (or between various treatment groups) should be much greater during this later period. Our results with cis-Pt are consistent with this hypothesis, since significant differences between control and cis-Pt-treated tumors were observed on day 10, compared to insignificant differences on day 6.

Other factors producing this greater activity of cis-Pt at later times after implantation may include (i) increased drug delivery due to improved neovascularization in the older growing tumors, and (ii) increased cytotoxicity due to the higher percentage of replicating cells in tumors at the later time of treatment. CSA-treated mice should therefore be advantageous hosts for investigations of cancer therapies that depend on cell proliferation or developed blood supply in the tumor mass. Extended growth of tumors after CSA treatment suggests that antineoplastic drugs could be tested after (or before) CSA administration, thereby obviating direct drug–drug interactions.

Slowly growing primary human tumor specimens from clinical biopsies may require longer growth periods (>10 days) for optimal discrimination of antitumor-drug activity, and the SRC assay in CSA-treated mice may make such studies feasible.

In contrast to other immunosuppressants, which have antineoplastic as well as antilymphocytic properties, CSA is highly selective against T cells, at doses that have no effect on tumor growth or bone marrow (9, 12, 13). The rapid end point of the SRC assay allows brief CSA treatments, which have minimal toxicity to other vital host tissues. Using immunoperoxidase stains of tumors in the SRC assay, we observed that murine T cells were a prominent early component of the inflammatory response on days 6–10 after implantation. CSA effectively prevented T-cell infiltration, thereby improving reliability of tumor size (average diameter) for rapid quantification of total tumor cell growth. Our observations are consistent with previous studies of CSA in organ-transplant models, which showed selective immunosuppressive activity against T-cell function (9) and graft survival for extended periods after cessation of CSA treatment (12, 13).

Using a transplanted rodent tumor in syngeneic mice, we found that CSA can be utilized in simple, daily regimens that have no antitumor effect per se, as previously shown with other syngeneic rodent tumors grown subcutaneously in CSA-treated animals (13). Slightly increased growth of MBT-2 tumors in CSA-treated syngeneic mice also suggests that CSA could be used to improve growth of human tumors even in nude mice. CSA has been reported to inhibit natural-killer-cell activity (14), which may be important for growth or rejection of some xenografted tumors in athymic nude mice.

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