Severe Combined Immunodeficiency (SCID) Mouse Modeling of P-Glycoprotein Chemosensitization in Multidrug-resistant Human Myeloma Xenografts

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ABSTRACT
We have established a reproducible in vivo model of human multiple myeloma in the severe combined immunodeficiency (SCID) mouse using both the drug-sensitive 8226/S human myeloma cell line and the P-glycoprotein-expressing multidrug-resistant 8226/C1N subline. As demonstrated previously, the SCID mouse is well suited as a model for myeloma because: (a) human SCID xenografts are readily attained; (b) human myeloma xenografts are readily detected by their immunoglobulin secretion; and (c) differential therapy effects in drug-sensitive versus drug-resistant cell lines are readily demonstrable by monitoring mouse urinary human immunoglobulin output. In the current study, we have utilized this model to evaluate the in vivo efficacy of chemomodulators of P-glycoprotein-related multidrug resistance. In our initial experiments, doxorubicin alone was effective in treating the 8226/S human myeloma xenografts but had no effect on the drug-resistant 8226/C1N xenografts, in the absence of the chemosensitizing agent verapamil. In subsequent experiments, the combination of verapamil and doxorubicin resulted in both a decrease in human light chain urinary excretion and an increase in survival of those animals bearing the 8226/C1N tumor. The median survival time of animals injected with 8226/C1N cells and subsequently treated with doxorubicin was 48.6 ± 7 days, which compared to a survival of 89.6 ± 18 days in animals receiving the 8226/S cell line and treated with doxorubicin alone (P < 0.001). When verapamil was added to the treatment regimen of those animals bearing the 8226/C1N xenografts, there was a 179% increase in their life span (P < 0.001), which corresponded with the observed decreased light chain in the urine. In animals receiving multiple courses of chemotherapy, an attenuated response to verapamil and doxorubicin was observed, in a manner analogous to the clinical setting of human drug-resistant myeloma escape from chemosensitivity. The SCID human myeloma xenograft model thus offers a means of evaluating the in vivo efficacy and potential toxicities of new therapeutic approaches directed against P-glycoprotein in multidrug-resistant human myeloma.

INTRODUCTION
Multiple myeloma is a plasma cell malignancy which is generally incurable in spite of a high initial response to chemotherapy. Relapsing disease commonly heralds increasing drug resistance. Previous studies indicate that terminal drug-resistant myeloma commonly expresses P-glycoprotein (1–5). The P-glycoprotein is a cellular efflux pump encoded by the MDR-1 gene, and is frequently overexpressed in chemotherapy-refractory cancers in the clinic (6). One approach to modulate drug resistance due to P-glycoprotein overexpression has involved the use of agents known as chemosensitizers/chemomodulators which inhibit its function. Using a murine leukemia model, Tsuruo et al. (7) were the first to observe that a number of compounds, including the calcium channel blocker verapamil, were able to reverse P-glycoprotein-mediated MDR both in vitro and in vivo. Clinical trials have shown that the use of chemomodulators such as verapamil and cyclosporin A may be beneficial, particularly in hematological malignancies (8–13). Agents such as verapamil, however, have been shown to be toxic at the concentrations required to be fully effective (14), and, therefore, new chemosensitizers are sought that will be more potent and less toxic than the initial compounds. Confounding the development of more potent chemosensitizers is the observation that P-glycoprotein is expressed in normal tissues including the kidney, adrenal gland, liver, and endothelial cells lining the capillaries of the brain (15–17). The effects of new chemosensitizers on these tissues is of great importance because of the possibility of untoward toxicities as a consequence of enhanced tissue uptake or altered tissue distributions of either the chemomodulator or the chemotherapeutic agents (18–21). Such interactions are best assessed through in vivo models.

We have established a reproducible in vivo model of human multiple myeloma in the SCID mouse using both the drug-sensitive 8226/S human myeloma cell line and the P-glycoprotein-expressing multidrug-resistant 8226/C1N subline (1). Although murine models of myeloma exist, these systems do not specifically model human myeloma associated with drug

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The abbreviations used are: MDR, multidrug resistance; SCID, severe combined immunodeficiency.
resistance. The SCID mouse, as we have previously demonstrated, is well suited as a model for myeloma because: (a) human SCID xenografts are readily attained; (b) tumor cells are readily detected by their immunoglobulin secretion; and (c) differential therapy effects in drug-sensitive versus drug-resistant cell lines are readily demonstrable (1). The objective of this study was to evaluate our model for its ability to assess the in vivo efficacy and toxicities of chemosensitizers directed against P-glycoprotein-mediated MDR using verapamil as the prototypical chemosensitizer.

MATERIALS AND METHODS

Generation of Human Multiple Myeloma in SCID Mice. The generation of human SCID myeloma xenografts has been described in detail in a previous publication (1). BALB/c/C.17 mice homozygous for the SCID defect (scid/scid) were bred and maintained in a dedicated facility at the Arizona Health Science Center, which is accredited by the American Association for Accreditation of Laboratory Animal Care. The animals were housed in microisolator cages under specific pathogen-free conditions and were handled in a laminar flow hood. Principles of animal care as set forth in NIH Publication No. 85–23, revised 1985, were followed throughout. Mice were screened at regular intervals for the presence of bacteria, Sendai virus, mouse hepatitis virus, and Mycoplasma. All mice were evaluated for the presence of mouse IgG by an ELISA, and those animals with $\geq 1$ mg/liter of mouse immunoglobulin were excluded from the studies. In brief, 5–8-week-old male and female animals received either the P-glycoprotein-negative human 8226/S cells or the P-glycoprotein-expressing multidrug-resistant subtype 8226/C1N given i.p. Prior to injection, the tumor cells were washed twice and resuspended in sterile PBS (pH 7.4). In all instances, low passage number cells in logarithmic growth were used. Cell viability was $>95\%$ as assessed by trypan blue dye exclusion. Both 8226 cell lines produce and secrete $\lambda$ immunoglobulin light chain, and the development of human tumors in the recipient mice was monitored at 4–5-day intervals using a radial immunodiffusion assay (The Binding Site Ltd., Birmingham, England) to measure the presence of human $\lambda$ light chain in the urine. The sensitivity of this assay is $\leq 0.7$ mg/liter.

Immunophenotyping. Tissue section phenotyping was performed on formalin-fixed or snap-frozen tissues using a standard three-stage immunohistochemistry method on an automated Ventana 320 immunostainer (Ventana Medical Systems, Tucson, AZ; Refs. 22 and 23). Snap-frozen tissues were permeabilized in 4°C acetone and assayed using relevant primary monoclonal antibodies (anti-$\lambda$, anti-$\kappa$, mouse antihuman; Becton Dickinson, Mountain View, CA) followed by incubation with secondary biotin-avidin conjugates and horseradish peroxidase labeled with diaminobenzidine tetrahydrochloride as the detection agent. P-glycoprotein was detected using monoclonal antibodies JSB-1 (Accurate Chemicals, Westbury, NY) and C-494 (Centocor, Malvern, PA) using a biotin-avidin detection method. An irrelevant isotype-matched primary antibody was substituted as a negative control.

In Situ Hybridization. Paraffin sections were cut 4-μm thick and heated at 60°C for 2 h. The sections were then deparaffinized in two changes of xylene for 12 min each, two changes of 100% ethanol for 5 min each, and were taken through a graded series of alcohols (95%, 80%, 70%) followed by two changes of diethylpyrocarbamate H₂O. Proteinase K (100 μg/ml; Boehringer Mannheim, Indianapolis, IN) was then placed on the sections for 60 min at 37°C and removed by two washes in PBS. The sections were then placed through a second series of graded alcohols (70%, 80%, 95%, 100%) and allowed to air dry prior to hybridization. The slides were then heated to 95°C and hybridization to a biotinylated human DNA probe enriched for the repetitive Alu and Kpn sequences (Cot-1; Gibco-BRL, Grand Island, NY) was carried out in 25% formamide, 10% dextran sulfate, $5 \times$ Denhardt’s, $4 \times$ SSC, 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, and 100 μg/ml salmon sperm DNA overnight at 37°C. Two stringency washes of 2.5% BSA/0.1× SSC were carried out at room temperature for 10 min each. The slides were then washed in PBS and blocked with 1% BSA in PBS for 30 min at room temperature. For detection of the biotinylated probe, the ExtrAvidin Alkaline Phosphatase system was used according to the manufacturer’s protocol (Sigma, St. Louis, MO).

Tumor Treatment with Doxorubicin and Verapamil. Prior to the initiation of therapy, tumor growth was confirmed through urinary $\lambda$ measurement. Approximately 21 days after inoculation with either 8226/S or 8226/C1N cells, treatment with doxorubicin in the presence or absence of verapamil was initiated. Both doxorubicin and verapamil were obtained from Sigma Chemical Corp. and were dissolved in 0.9% saline. Drug concentrations were adjusted so that a maximum volume of 100 μl was injected per animal.

Three doses of doxorubicin (1.5 mg/kg) were administered i.p. every 4 days. This dosing regimen was established previously as the maximum tolerated dose for doxorubicin in this model (1). In the initial experiments, verapamil was administered via continuous infusion using an Alza 2001 osmotic pump (ALZA Corp., Buena Vista, CA) implanted s.c. through a small incision made on the dorsal surface. Pumps were implanted 24 h prior to administration of the initial dose of doxorubicin. The infusion rate was 1.01 μl/h and resulted in a dose of 150 mg/kg/day verapamil.

In subsequent experiments, verapamil was administered as a bolus i.m. injection immediately prior to each dose of doxorubicin. The maximally tolerated dose of verapamil by bolus injection in our model system was determined to be 50 mg/kg (data not shown). To minimize drug-induced toxicities, animals were given injections of a dose of 40 mg/kg verapamil.

Following drug administration, the animals received food and water ad libitum and were monitored for urinary $\lambda$ light chain excretion as well as weight loss and other signs of toxicities. A response to therapy was defined as a stabilization or a decrease in the urinary light chain values following drug administration. All mice were necropsied at the time of tumor relapse to document the presence of tumor.

RESULTS

Human SCID xenografts using the 8226 myeloma cell lines were established rapidly and reproducibly under the conditions of i.p. growth. Xenografts of both drug-sensitive and -resistant
Fig. 1  Human plasmacytoma xenograft in a SCID mouse. A and B, xenograft of 8226/C1N drug-resistant myeloma cells infiltrating the pancreas of a SCID mouse. The large blastic or anaplastic plasma cells infiltrate the pancreas, producing a mass which displaces normal pancreas. C and D, an assay for cytoplasmic light chains (κ and λ, respectively) reveals a monoclonal cytoplasmic presence of λ light chain with absent κ light chains.

<table>
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<tr>
<th>Table 1</th>
<th>Immunophenotype of 8226 cell lines</th>
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<td></td>
<td>8226/S</td>
</tr>
<tr>
<td>Light chain (κ)</td>
<td>pos.*</td>
</tr>
<tr>
<td>CD-38 (Leu17)</td>
<td>neg.</td>
</tr>
<tr>
<td>CD-56 (N-CAM)</td>
<td>pos.</td>
</tr>
<tr>
<td>P-glycoprotein JSB-1</td>
<td>neg.</td>
</tr>
<tr>
<td>C-494</td>
<td>neg.</td>
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* pos., positive; neg., negative.

8226 cell lines appeared histologically as plasmacytomas (Fig. 1). Immunophenotyping revealed that all tumors were human λ light chain positive and human κ light chain negative (Fig. 1 and Table 1). The human origin of the tumors was confirmed by in situ hybridization as shown in Fig. 2. The human SCID 8226/C1N xenografts showed strong surface P-glycoprotein expression as evidenced by reactivity with both JSB-1 and C-494, whereas the human SCID 8226/S xenografts were negative (Table 1).

Previous work with this model demonstrated that the latency period from injection of tumor cells to the appearance of human light chain in the urine was identical for mice given injections of either the 8226/S or 8226/C1N cells (1). In addition, the survival times of untreated animals bearing either tumor type were not statistically different (1).

When tumor-bearing SCID mice were treated with 1.5 mg/kg doxorubicin administered i.p. every 4 days for three doses, beginning 21 days after injection of cells, there was a marked decrease in the level of human λ light chain in the urine of those animals bearing the P-glycoprotein-negative 8226/S cells (Fig. 3A). In contrast, there was an increase in the λ light chain excretion of those animals inoculated with the multidrug-resistant 8226/C1N cells under the same treatment schedule (Fig. 3B). Thus, doxorubicin was effective in treating the 8226/S xenografts but had no effect on 8226/C1N xenografts in the absence of a chemosensitizer. In a second set of experiments, shown in Fig. 4, we demonstrated that when verapamil was added to the treatment regimen, either as a continuous infusion or as a bolus injection, there was a marked decrease in the level of human λ light chain in the urine of those animals bearing the drug-resistant 8226/C1N cells in contrast to those without verapamil, thus demonstrating a measurable chemosensitizing effect. As shown in Fig. 4A, when doxorubicin is administered in the absence of verapamil, there is a continuous increase in the λ
light chain values, thus indicating a failure of therapy. When doxorubicin is administered in conjunction with verapamil to 8226/C1N-bearing mice, as shown in Fig. 4B, there is a rapid decline in the λ values. The tumor is not completely eradicated however as demonstrated by the gradual increase in λ values following cessation of therapy. Our initial studies were based on the observation that, to maintain adequate tissue and plasma levels in the range demonstrated to be effective for in vitro chemosensitization, verapamil should be administered continuously in the presence of chemotherapy (24). We therefore utilized a previously published protocol for continuous infusion of verapamil using surgically implanted osmotic pumps (25). Subsequent studies, using bolus injections in nontumor-bearing SCID mice, revealed that we could achieve a mean plasma verapamil level of 598 ± 75 (range, 540–710; n = 9) ng/ml following i.m. injection of 50 mg/kg. Although bolus dosing does not achieve the continuous serum levels which are felt to be optimal, we were able to achieve a treatment effect similar to that observed with the osmotic pumps. Regardless of whether verapamil was administered as a continuous infusion or as a series of bolus injections, we did not observe any increase in toxicities, as assessed by increased weight loss or mortality, in those animals receiving the combination of doxorubicin and verapamil over those animals receiving doxorubicin alone. When verapamil was administered alone, under conditions of continuous infusion, we observed no treatment effect against either tumor type (data not shown). There were no treatment differences observed based on the sex of the animal.

With the addition of verapamil to the doxorubicin regimen, the reduction in the light chain values of those mice given injections of the 8226/C1N tumors translated into a significantly prolonged animal survival (Fig. 5 and Table 2). The median survival time of animals given injections of 8226/C1N cells and subsequently treated with doxorubicin was 48.6 ± 7 days, which compared to a survival of 89.6 ± 18 days in animals receiving the 8226/S cell line and treated with doxorubicin alone (P < 0.001). The addition of verapamil to the treatment regimen did not alter the survival of animals bearing the 8226/S xenografts, which was consistent with our observations that verapamil did not alter the excretion of λ light chain in the urine. When verapamil was added to the treatment regimen of those animals bearing the 8226/C1N xenografts, there was a 179% increase in their life span (P < 0.001), which corresponded with the observed decreased light chain in the urine. This increase in the survival time allowed us, in a third set of experiments, to administer multiple courses of chemotherapy to mimic the situation of escape from chemosensitization observed in human MDR clinical trials. In Fig. 6, a group of animals bearing the 8226/C1N tumors were treated with both doxorubicin and verapamil administered as bolus injections under our standard treat-
Fig. 3. A and B, human λ light chain excretion in the urine of 8226/S and 8226/C1N tumor-bearing SCID mice before and after therapy with 1.5 mg/kg doxorubicin administered every 4 days. Tumor cells were injected on day 0, and doxorubicin therapy was initiated (arrows). A, 8226/S cells; B, 8226/C1N cells (n = 5 mice in each group). Symbols, individual mice.

Fig. 4. A and B, human λ light chain excretion in the urine of 8226/C1N tumor-bearing SCID mice before and after therapy with 1.5 mg/kg doxorubicin administered every 4 days in the presence or absence of the chemosensitizer verapamil (150 mg/kg/day s.c.). Tumor cells were injected on day 0, and doxorubicin therapy was administered (arrows). A, λ excretion in the absence of verapamil; B, λ expression in the presence of verapamil. Symbols, individual mice.

ment protocol. We observed a sharp decrease in tumor burden following the first course of treatment, which was consistent with our previous studies. The animals were then treated with a second course of doxorubicin and verapamil once the light chain values had increased to approximately pretreatment levels. Following the second course of chemotherapy, an attenuated therapeutic response was observed.

DISCUSSION

The resistance of tumor cells to the cytotoxic effects of chemotherapy continues to be a major obstacle to the successful treatment of human cancers. Chemotherapy is the primary treatment modality of multiple myeloma but despite improvements in survival and quality of life, this remains an incurable disease in part due to the development of drug resistance. The evaluation of novel experimental therapeutic approaches for multiple myeloma has been hampered by the lack of an appropriate animal model. Although murine models of myeloma are known, i.e., pristane-induced BALB/c peritoneal plasmacytomas and spontaneous C57BL marrow myelomas (26-29), these systems do not specifically model human myeloma associated with drug resistance. We have recently developed a reproducible in vivo human xenograft model in SCID mice which utilizes both the P-glycoprotein-negative RPMI 8226 human myeloma cell line and the P-glycoprotein-expressing multidrug-resistant 8226/C1N subline, a derivative of the 8226/DOX40 MDR cell line (1).

The SCID xenograft model was developed to assess the efficacy and toxicities of new chemotherapeutic agents and chemosensitizers directed against the P-glycoprotein-mediated MDR phenotype and should serve to complement current in vitro assays which are better suited to initially identify compounds effective in overcoming P-glycoprotein-mediated drug resistance. The advantages of the SCID human myeloma xenograft model have been previously stated and allow for quantification of tumor burden via urinary monoclonal light chain measurement and a reproducible, defined time frame of tumor growth and survival allowing for assessment of outcome in a
Chemosensitization in SCID Human Myeloma Xenografts

Fig. 5 In vivo activity of verapamil in combination with doxorubicin in SCID mice bearing 8226/S or 8226/C1N xenografts. Mice were given i.p. injections of tumor cells and treated with doxorubicin with or without verapamil as described in “Materials and Methods.” Data presented are the results of three separate experiments (n = 21–30 animals/group). Points, surviving animals at the time on the abscissa divided by the initial number of animals treated.

Table 2 Mean survival times of myeloma-bearing SCID mice (n = 21–30/group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean survival time (days) ± SE</th>
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<tr>
<td>8226-Sens + Dox/saline</td>
<td>89.6 ± 3.6</td>
</tr>
<tr>
<td>8226-Sens + Dox/verapamil</td>
<td>87.8 ± 6.1</td>
</tr>
<tr>
<td>8226-C1N + Dox/saline</td>
<td>48.6 ± 1.2</td>
</tr>
<tr>
<td>8226-C1N + Dox/verapamil</td>
<td>86.0 ± 5.63</td>
</tr>
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*Sens, definition; Dox, doxorubicin.

Both in vitro and clinical trials have shown that chemomodulators such as verapamil and cyclosporin A may be beneficial in multiple myeloma (8, 9, 11, 31). While these compounds have demonstrated a proof of concept, the clinical results obtained to date in myeloma have been limited. Myeloma patients, after an initial beneficial chemosensitizing effect, may escape chemosensitization and experience remission. Thus, new chemosensitizers are sought that will be more potent and less toxic than the initial compounds in an effort to improve the duration and number of responses. Development and testing of candidate compounds has been hampered by the lack of a suitable in vivo model. The objective of this study was to evaluate our SCID mouse xenograft model for its ability to assess the in vivo efficacy and toxicities of chemomodulators directed against P-glycoprotein, using verapamil as the prototypical chemosensitizer. The maximum tolerated dose of doxorubicin in this model was established as 1.5 mg/kg every 4 days × 3 (1) and reflects the increased sensitivity of SCID mice to agents which induce DNA damage (32). When doxorubicin was administered i.p. directly to the site of the tumor, we were unable to observe a therapeutic response in those animals bearing the 8226/C1N tumor in the absence of verapamil. When verapamil was added to the doxorubicin regimen using a second route of administration, either s.c. or i.m., we were then able to observe an effect on the tumor. The combination of verapamil and doxorubicin resulted in both a decrease in light chain excretion and an increase in survival of those animals bearing the 8226/C1N tumor.

Thus, although the i.p. route of drug delivery was not ideal from a clinical perspective, we were nevertheless able to demonstrate a chemomodulating effect for verapamil. To avoid the potential confounding variables associated with administering the drug to the same site as the tumor, we have initiated studies using the ARH-D60 human myeloma cell line, designed to evaluate the i.v route of drug delivery. This cell line, when injected into SCID mice, results in osteolytic lesions and may
thus represent a more orthotopic xenograft model of myeloma (33).

In addition to evaluating drug efficacy, the SCID xenograft model allows investigators to examine pharmacokinetic and pharmacodynamic parameters, which are not easily studied in the in vitro setting, and to identify the limiting organ-specific toxicities resulting from the use of new chemomodulators. Indeed, we have used this model to identify increased toxicities in the SCID mouse as a consequence of the administration of doxorubicin and cyclosporin A (34).

Using this model, we may not be able to differentiate the effects of inhibition of P-glycoprotein from that of altered pharmacokinetics solely by examining changes in the urinary light chain excretion nor by prolonged survival times. What we will be able to do, however, is to provide information relating to the efficacy or toxicities of a given compound under the actual conditions of its proposed use. Several studies have demonstrated that the use of chemomodulators directed against the P-glycoprotein may alter the pharmacokinetics of antineoplastic agents, resulting in decreased clearance and/or increased area under the curve (18–21). Such reports are of importance due to the potential of increased toxicities when chemomodulators are utilized. To assess whether the observed effects of a compound being examined in our model are due to alterations in pharmacokinetics, we will utilize measurements of tissue and plasma levels of the drugs as we have done previously (34).

Future uses of our model may also include studies of new agents and therapeutic strategies directed against non-P-glycoprotein-mediated MDR in myeloma. The SCID model may also serve as a more general model of multiple myeloma, providing a means to evaluate the effectiveness of various therapeutic strategies aimed at this disease, and to elucidate additional mechanisms of resistance by which myeloma cells may escape from the effects of chemosensitization. Escape from chemosensitization has been the clinical experience and was suggested by the attenuated therapeutic effect of verapamil observed in animals receiving multiple treatment courses. The current model would seem to be ideal for studying the specifics of escape from chemosensitization. We are currently examining this avenue of drug resistance using the SCID xenograft model.

These observations confirm that the SCID mouse is an excellent recipient for the human multiple myeloma cells and may be very suitable for evaluating the efficacy and toxicities of new chemomodulators of the P-glycoprotein. This in vivo model should be useful not only for studying drug-resistant human multiple myeloma but also as a more general model of multiple myeloma, providing a means to evaluate the effectiveness of various therapeutic strategies aimed at this disease, and to elucidate the mechanisms by which myeloma cells may escape from chemosensitization.

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