X-Linked Dominant Growth Suppression of Transplanted Tumors in C57BL/6J-scid Mice

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Abstract
Tumor susceptibility, angiogenesis, and immune response differ between mouse strains. We, therefore, examined the growth rates of tumor xenografts in three genetically isolated strains of severe combined immunodeficient mice (C.B-17-scid, C57BL/6j, and C3H). Tumors grew at significantly reduced rates in the C57BL/6j-scid strain. Engrafting bone marrow from the C57BL/6j-scid strain onto C.B-17-scid mice did not transfer the slow-growing tumor phenotype to the recipient mice; this counters the supposition that the slow-growing tumor phenotype is caused by a greater immune response to the xenograft in the C57BL/6j-scid strain. To establish the inheritance pattern of the slow-growing tumor phenotype, we reciprocally crossed C.B-17-scid mice and C57BL/6j-scid mice. Tumor growth was suppressed in all of the F1 progeny except the male mice derived from the cross between C.B-17-scid female and C57BL/6j-scid male mice. The F1 male mice that received the X chromosome from the C.B-17 strain displayed a fast-growing tumor phenotype. These results confirm that there are significant strain differences in capacity to support the growth of tumor xenografts. In addition, these results reveal the existence of a dominant allele involved in host suppression of tumor growth on the X chromosome of C57BL/6j mice. (Cancer Res 2005; 65(13): 5690-5)

Introduction
Genetically divergent strains of mice exhibit differences in their inherent ability to resist cancer. C57BL/6j mice, for example, have a low incidence of spontaneous tumors and are less susceptible to diethylaminoethyl-induced hepatocarcinogenesis when compared with C3H mice (1). The BALB/c strain is particularly susceptible to methylcholanthrene-induced skin carcinogenesis (2). The capacity of a strain to resist tumor growth is likely to depend on a variety of phenotypic characteristics, including the ability to resist DNA damage (3–5), the ability to metabolize carcinogens (6, 7), and the immunologic response to tumor cells (8). Additionally, unique host traits that are likely to be associated with the ability to resist cancer have been genetically mapped in mice: the capacity to grow new blood vessels in response to angiogenesis factors (9) as well as the ability to resist immune suppression (10, 11).

In the present study, we compared the growth of xenografts in several strains of immunodeficient mice, including C.B-17-scid, C57BL/6j-scid, and C3H-scid. Transplanted human tumors were found to grow significantly more slowly in the C57BL/6j-scid strain when compared with the other two strains. In addition, C57BL/6j-scid and C.B-17-scid mice were reciprocally crossed and the F1 progeny were characterized with respect to their capacity to support tumor growth. The rates of tumor growth in the F1 females were equivalent to the parental C57BL/6j-scid strain, whereas tumor growth in the F1 males depended on the parental strain that contributed the X chromosome. These results show genetic differences in host capacity to support the expansion of tumors and that a dominant allele involved in suppressing tumor growth resides on the X chromosome of C57BL/6j mice.

Materials and Methods

Mouse strains. All of the studies utilized severe combined immunodeficient (SCID) mice. C.B-17-scid mice (C.B-17/ice-SCID/Scl-Pdkcsclid) were purchased from Massachusetts General Hospital (Boston, MA). The C57BL/6j-scid (B6.CB17-Pdkcsclid5J), C3H/HeSnJ-scid (C3SnJsn.CB17-Pdkcsclid/J), and BALB/cBySmn (CBBySmn.CB17-Pdkcsclid/J) strains were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were fed autoclaved water and chow ad libitum and housed in microisolator cages. Male F1 progeny were generated by reciprocal crosses between C.B-17-scid and C57BL/6j-scid mice. Mice between 5 and 10 weeks of age were used for the tumor studies. The C57BL/6j-GFP-scid mice were generated by crossing the green fluorescent protein (GFP) transgenic line UBI-GFP/B6, which was created in the C57BL/6j strain (12) with C57BL/6j-scid mice (The Jackson Laboratory). The heterozygous progeny were intercrossed to generate mice that were homozygous for both the GFP transgene and the SCID mutation. The C.B-17-GFP-scid mice were GFP-positive heterozygote offspring that were back-crossed into C.B-17-scid mice for seven generations.

All animal work was carried out in the animal facility of the Children’s Hospital in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee.

Tumor studies. The cell lines used in this study are routinely cultured at Children’s Hospital Boston (13) and were chosen for convenience. The MG63Ras tumor line is an osteogenic sarcoma line (MG-63) that has been stably transfected with the activated c-Ha-ras oncogene (HRAS), and the ST2V165 tumor line was derived by transfecting the gastric tumor line ST-2 with human VEGF165 (13). The ST2RFP tumor line was derived by transfecting ST-2 with both the activated c-Ha-ras oncogene and the red fluorescence protein vector pDSRed2N1Hygro, which was created by subcloning DSRed2 flanked by from BambH-NotI sites from pDSRed2-N1 (BD Biosciences, San Jose, CA) into pcDNAHygroPlus (Invitrogen Life Technologies, Carlsbad, CA). The A375SM melanoma line was obtained from Dr. Isaiah Fidler (M. D. Anderson Cancer Center, Houston, TX) and maintained in MEM containing 10% fetal bovine serum. The red fluorescence protein vector pDSRed2N1Hygro, which was created by subcloning DSRed2 flanked by from BambH-NotI sites from pDSRed2-N1 (BD Biosciences, San Jose, CA) into pcDNAHygroPlus (Invitrogen Life Technologies, Carlsbad, CA). The A375SM melanoma line was obtained from Dr. Isaiah Fidler (M. D. Anderson Cancer Center, Houston, TX) and maintained in MEM containing 10% fetal bovine serum. The A2058E1AGFP melanoma line is a derivative of the melanoma A2058E1 (14) that stably expresses GFP from the transfected plasmid pEGFP-C1 (BD Biosciences).

Tumor lines were maintained in a humidified 37°C incubator (10% CO2) and cultured in DMEM (Invitrogen) containing 5% fetal bovine serum and 200 U/ml penicillin/streptomycin (Invitrogen). Xenografts were created with adherent cells that had been rinsed once with 1 × PBS containing 2.5 mM EDTA and then dispersed into a single cell suspension using a 0.05% trypsin solution (Invitrogen). Trypsin was

Note: M. Wood and T. Udagawa contributed equally to this work.

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neutralized by the addition of 10 volumes of serum-containing medium, and the cells were harvested by centrifugation at 4,000 g for 5 minutes. The tumor cells were suspended in saline at a density of 2 × 10^7 cells/mL and a volume of 50 μL was injected in the s.c. space midline between the shoulder blades in the backs of shaved mice. Tumor volumes were calculated by measuring the width and length of tumors and applying the following formula: volume = width^2 × length^0.5.

Tumor proliferation and apoptosis assay. MG63Ras tumor tissue in C.B-17-scid and C57BL/6J-scid mice was minced using scalpels into 3 mm pieces. Digestion buffer containing 2.4 units/mL dispase grade II (Roche, Indianapolis, IN), 0.3 units/mL of liberase blendzyme 3 (Roche), 20 Kunitz units/mL of DNase (Sigma, St. Louis, MO), and 20 units/mL hyaluronidase (ICN, Costa Mesa, CA) was added at a rate of 1 mL of digestion buffer to 50 mg of tissue. The minced tissue was triturated 10 times with a 5 mL pipette and then incubated with shaking for 10 minutes at 37°C. The digest was diluted with an equal volume of medium containing 10% serum and filtered through a 40 μm nylon mesh to remove particulates.

Apoptotic cells were labeled using an Annexin V-PE apoptosis kit (BD Biosciences) according to the manufacturer's direction and analyzed by flow cytometry. Proliferation was determined by analyzing proliferating cell nuclear antigen (PCNA) expression by flow cytometry. Intracellular PCNA expression of the enzymatically digested tumor tissue was measured by first fixing and permeabilizing the cells with BD Cytofix/Cytoperm solution before staining with the PC-3 monoclonal antibody directly conjugated to phycoerythrin (BD Biosciences).

Bone marrow transplantation. GFP transgenic SCID donor animals were anesthetized with avertin and sacrificed by cervical dislocation. Bone marrow cells were collected by irrigating the femur and tibia with saline using a syringe and a 26 G needle. The marrow cells were passed 10 times through a 26 G needle and then filtered through a 40 μm nylon mesh. The cells were washed once and suspended in saline at a density of 5 × 10^7 cells/mL. C57BL/6J-scid recipients were irradiated at a dose of 2.5 Gy (15) and injected i.v. with 5 × 10^6 donor bone marrow cells in a 100 μL volume. Drinking water was supplemented with Sulfatrim Pediatric (Alphapharma, Baltimore, MD) to prevent infections following bone marrow transplantation. To monitor bone marrow engraftment, peripheral blood was obtained from the retroorbital sinus of lightly anesthetized (isofluorane) mice using heparinized microhematocrit capillary tubes and
Table 1. Proliferation and apoptotic indices of MG63Ras tumors in C.B-17-scid and C57BL/6J-scid mice

<table>
<thead>
<tr>
<th></th>
<th>PCNA</th>
<th>Annexin V</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B-17-scid</td>
<td>91.9 ± 1.2</td>
<td>26.8 ± 4.4</td>
</tr>
<tr>
<td>C57BL/6J-scid</td>
<td>73.0 ± 8.1</td>
<td>28.3 ± 10.8</td>
</tr>
</tbody>
</table>

NOTE: The proliferation and apoptotic indices were analyzed, as described in Materials and Methods, for tumors that were approximately the same size. The average volume ± SD of the tumors analyzed from C.B-17-scid and C57BL/6J-scid mice was 324 ± 115 mm$^3$ (days 24-28 postimplantation) and 475 ± 176 mm$^3$ (15-19 days postimplantation). Statistical significance was determined using an unpaired t test.

Results

The growth rate of MG63Ras tumors was initially measured in three different strains (BALB/c, C57BL/6J, and C.B-17) of male SCID mice (Fig. 1A). Tumor growth was evident in all of the mice ~4 days after tumor implantation. After 9 days, tumors were ~1.4 times larger in C.B-17-scid mice than in C57BL/6J-scid mice, but the difference was not significant (P > 0.1). By days 19 and 23, the differences in tumor volumes were significant (P < 0.05). There was no difference in tumor volume when C.B-17-scid mice were compared with BALB/c-scid mice for all time points (P > 0.05). Identical tumor growth rates were expected because C.B-17 and BALB/c are congenic strains that differ only at the immunoglobulin heavy chain locus (C.B-17 mice are Igh-1b, whereas BALB/c mice are Igh-1a on chromosome 12).

To exclude the existence of a sexual dimorphism in the capacity to support tumor growth, MG63Ras cells were implanted into male and female C.B-17-scid and C57BL/6J-scid mice. Significant differences in tumor growth (Fig. 1B) were observed between the two strains for all comparisons (P < 0.01), but there was no significant difference in the size of tumors implanted in male and female mice within the same strain (P > 0.05).

To determine if the differential tumor growth rates were limited to the s.c. space, peritoneal tumor growth was tested. The GFP-expressing tumor line MG63RasGFP was injected (10^6 cells/mouse) into the peritonea of C.B-17-scid and C57BL/6J-scid mice. After 14 days, the peritoneal cavities were examined and tumors were counted in an 8× field by fluorescent microscopy. Tumor growth was evident in all of the mice; however, the C.B-17-scid strain had significantly more tumors (P < 0.0001) in the peritoneum than the C57BL/6J-scid strain (Fig. 1C and D).

Tumor growth rate is determined by the fraction of cells that are undergoing proliferation as well as apoptosis. Therefore, we investigated the proliferative and apoptotic indices of MG63Ras tumor tissue taken from C.B-17-scid and C57BL/6J-scid mice. The fraction of cells in tumor tissue undergoing proliferation and apoptosis was determined by PCNA staining and cell surface Annexin V binding by fluorescence-activated cell sorting (FACS) analysis. The proliferation index of MG63Ras significantly decreased when tumor volume became >1,000 mm$^3$ (not shown). Therefore, we compared tumors that were comparable in size and <800 mm$^3$ from the two strains of mice. As shown in Table 1, there was no significant difference in the apoptotic index, as determined by cell surface Annexin V binding (P = 0.791). The proliferation rate, however, as determined by the fraction of cells that expressed PCNA, was significantly higher in C.B-17-scid mice than in C57BL/6J-scid mice (91.9 ± 1.2% versus 73.0 ± 8.1%, respectively; P = 0.002). Thus, the difference in tumor growth rate between the two strains of mice is largely due to a difference in the proliferation index.

The growth of additional tumor lines implanted in the s.c. space was measured in the C.B-17-scid and C57BL/6J-scid strains. Although there was a considerable difference in the rate of growth between different tumor lines (Table 2), in general, tumors grew more slowly in C57BL/6J-scid mice than in C.B-17-scid mice. For example, after 28 days, the red fluorescent protein expressing gastric tumor line ST2RRFP was ~80 times larger in the C.B-17-scid strain than in C57BL/6J-scid strain. On day 28, some of

Table 2. Differential growth of human tumor lines implanted in C.B-17-scid and C57BL/6J-scid mice

<table>
<thead>
<tr>
<th>Tumor lines</th>
<th>C.B-17, mm$^3$ ± SD (n)</th>
<th>C57BL/6J, mm$^3$ ± SD (n)</th>
<th>P*</th>
<th>Relative volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG63Ras (day 21) osteosarcoma</td>
<td>621 ± 285 (9)</td>
<td>187 ± 113 (15)</td>
<td>0.00002</td>
<td>3.3</td>
</tr>
<tr>
<td>ST2RRFP (day 28) gastric</td>
<td>185 ± 139 (10)</td>
<td>23 ± 6.7 (8)</td>
<td>0.002</td>
<td>80.4</td>
</tr>
<tr>
<td>ST2GFPV/165 (day 28) gastric</td>
<td>231 ± 280 (15)</td>
<td>5 ± 0.9 (4)</td>
<td>0.036</td>
<td>38.5</td>
</tr>
<tr>
<td>A375SM (day 9) melanoma</td>
<td>232 ± 81 (5)</td>
<td>64 ± 20 (4)</td>
<td>0.005</td>
<td>3.6</td>
</tr>
<tr>
<td>A2058E1AGFP* (day 58) melanoma</td>
<td>219 ± 234 (37)</td>
<td>&lt;1 (8)</td>
<td>—</td>
<td>—</td>
</tr>
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</table>

NOTE: The volume of each tumor line implanted in the s.c. space was calculated as described in Materials and Methods.

*Statistical significance was determined using an unpaired t test.

Relative volume = (tumor volume in C.B-17-scid mice) / (tumor volume in C57BL/6J-scid mice).

There were no externally visible tumors in C57BL/6J scid mice on day 58.
the tumors implanted in C57BL/6j-scid mice were not palpable but were clearly visible using an epifluorescent microscope (Fig. 1D). The gastric tumor line ST2VEGF165 and two melanoma lines, A375SM and A2058E1AGFP, grew significantly more slowly in the C57BL/6j-scid strain. Slower tumor growth rates were not limited to the MG63Ras cell line, but instead seemed to be a general phenomenon associated with the C57BL/6j-scid strain. The growth of MG63Ras was tested in additional strains of immunodeficient mice, including C3H-scid, CBx7-GFP-scid, and C57BL/6j-GFP-scid mice (Fig. 2). CBx7-GFP-scid and C57BL/6j-GFP-scid mice were GFP transgenic mice generated by breeding the transgene from UBI-GFP/BL6 mice into C.B-17-scid and C57BL/6j-scid mice, respectively. Tumor growth was equivalent in C.B-17-scid, BALB/c-scid, C3H-scid, and CBx7-GFP-scid mice. Tumor growth was equally reduced in both C57BL/6j-scid and C57BL/6j-GFP-scid mice.

To determine if a nonspecific immune response was responsible for tumor growth suppression, we transplanted donor marrow from a C57BL/6j-GFP-scid strain into C.B-17-scid mice. C57BL/6j-GFP-scid, which has the identical tumor growth phenotype as non-GFP C57BL/6j-scid (Fig. 2), permitted the monitoring of hematopoietic marrow reconstitution in the recipient mice. As shown in Fig. 3A, ~93% and 88% of the peripheral blood mononuclear cells in the recipient C.B-17 and control C57BL/6j mice, respectively, were derived from the C57BL/6j-GFP donor mice after 8 weeks, as determined by coexpression of CD45 and GFP by FACS analysis. Despite almost full reconstitution of C.B-17-scid mice with hematopoietic cells from the C57BL/6j-GFP-scid strain, tumors implanted in these mice grew ~3-fold faster than in the control mice when measured on day 28 (Fig. 3B). These observations suggest that tumor growth suppression in the C57BL/6j-scid strain was not due to a greater specific or nonspecific immune response against the transplanted tumor cells.

Reciprocal crosses of C57BL/6j-scid and C.B-17-scid mice were done to identify the inheritance pattern of the slow-growing tumor phenotype; tumor growth in the F1 progeny was compared with parental mice (Fig. 4). In reciprocal crosses, the genotypes of the F1 females are the same, whereas in the males only the sex chromosomes differ (Fig. 4A). As shown in Fig. 4B, tumors implanted in female F1 progeny were approximately the same size as tumors implanted in the parental C57BL/6j-scid strain. The F1
males that inherited an X chromosome from C57BL/6j-scid exhibited a phenotype, with respect to tumor growth, that was similar to the F1 females and to the C57BL/6j-scid strain. In contrast, the F1 males that inherited an X chromosome from the C.B-17-scid strain were similar in phenotype to C.B-17-scid. There was no difference in tumor growth rate in male and female mice of the same strain (Fig. 1B); taken together, these observations indicate that a dominant allele involved in tumor growth suppression is on the X chromosome of C57BL/6j-scid mice.

Discussion

Cancer susceptibility genes are frequently determinants that influence the conversion of normal cells to malignant cells. Previous studies have shown that C57BL/6j mice are relatively resistant to skin tumor promotion in a two-stage mouse skin carcinogenesis model (16). A dominant C57BL/6 allele that results in decreased sensitivity to 12-O-tetradecanoylphorbol-13-acetate skin tumor promotion has been mapped near D19Mit38 on chromosome 19 (16).

We have shown that human tumors grafted onto C57BL/6j-scid mice grow dramatically slower when compared with tumors grafted onto C.B-17-scid mice. No differences in the capacity to support tumor growth were observed between males and females within the same strain. The offspring derived from reciprocal crosses between C57BL/6j-scid and C.B-17-scid inherited a tumor growth phenotype that distributed in a bimodal fashion; the male F1 mice displayed a tumor growth phenotype that depended on the parental strain that contributed the X chromosome, whereas all of the female F1 mice displayed a tumor growth rate that was similar to the C57BL/6j strain. This inheritance pattern indicated that a dominant allele implicated in suppressing the growth of transplanted tumors resides on the X chromosome of C57BL/6j mice.

A similar pattern of susceptibility to UV light–induced carcinogenesis was reported for progeny produced by reciprocal crosses between immune competent C57BL/6j and BALB/c mice (8). In these studies, F1 males that carried a BALB/c X chromosome (CB6F1) were susceptible to UV carcinogenesis, whereas F1 males that inherited a C57BL/6j X chromosome (B6CF1) were resistant. Susceptibility to UV light–induced carcinogenesis correlated with a delayed-type hypersensitivity response, so the strain differences in tumor susceptibility were explained in terms of an immune surveillance mechanism (8). An alternative to an immune surveillance mechanism for tumor resistance in the C57BL/6j strain is supported by several observations. First, transplanted tumors grew more slowly in immunodeficient SCID mice that carried the X chromosome from the C57BL/6j strain. Second, tumor growth suppression was not transferable in bone marrow engraftment experiments. Third, the proliferation rate of tumors in C57BL/6j-scid mice was lower than in C.B-17-scid mice, but there was no difference in the apoptotic rate; a higher apoptotic fraction in C57BL/6j-scid mice would be expected if tumor suppression was due to immune-mediated tumor cytotoxicity.

Human leukemia and lymphomas have been reported to grow significantly faster in C.B-17-scid mice compared with C57BL/6j-Rag1 mice (17), but the authors attributed the difference in tumor growth to the differential effects of SCID and Rag1 mutations on the immune system (17). Based on our results, the previously reported difference in tumor growth attributed to SCID and Rag1 mutations could have been due to the background strain of the mutations, rather than to the defect per se (17). We tested our MG63ras xenograft model in the C57BL/6j-Rag1 background and compared it to the BALB/c-Rag1 background (data not shown). As expected, tumor growth was significantly reduced in the C57BL/6j-Rag1 (50%) when compared with the BALB/c-Rag1 background.

Although differential immunity cannot be entirely ruled out as a factor for strain differences in the capacity to support tumor growth, this hypothesis is not satisfactory for several reasons. First, the “leakiness” phenomena, in which some detectable levels of serum immunoglobulins as well as functional B and T cells are found, is elevated in the C57BL/6j-scid and BALB/c-scid strains (18, 19). An elevated level of leakiness would correlate with a decrease in the tumor growth rate. In the present study, the strain with the greatest degree of leakiness (BALB/c-scid and C.B-17-scid) exhibited the fastest rate of tumor growth.

Other host-tumor cell interactions, such as tumors angiogenesis (20), are critical for tumor growth. The magnitude of a response to purified angiogenesis factors in a corneal micropocket assay was shown to be strain-dependent, and C57BL/6j mice exhibited a less robust response compared with BALB/c mice (9). The angiogenic response of C57BL/6j-scid mice was also lower than that of C.B-17-scid mice using the same assay; the relative angiogenic response of the F1 progeny, however, did not correlate with tumor growth rates (data not shown). Therefore, angiogenesis alone does not seem to account for the observed influence of the X-linked loci on tumor growth. Genetic mapping experiments also have not
yet revealed X-linked genes that affect angiogenesis (21, 22). Nevertheless, a number of X-linked genes such as \textit{Timp-1}, \textit{apoptosis-inducing factor}, and \textit{bone morphogenic protein}, have been shown to play a role in cancer (23, 24). For some of the X chromosome, such as \textit{Timp-1}, there are isoforms that may in turn influence tumor growth (25–27). In humans, regions of the X chromosome have been implicated in prostate (Xq27–28; ref. 28), testis (Xq27; ref. 29), and bladder cancer (Xq11-12; refs. 24, 30). The X chromosome is unique in that synteny is conserved as single group in mice and in humans (31). As a consequence, human X-linked diseases, for example Menkes disease (32) and Alport syndrome (33), often have a murine equivalent. Further mapping of the \textit{X} chromosome linked to tumor growth in mice may lead to the identification of host genes that mediate critical host-tumor cell interactions in human cancers.

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