ABSTRACT

Cryopreserved cell suspensions of freshly excised melanoma metastases from nine patients were injected s.c. into C.B-17 severe combined immunodeficiency (SCID) mice. All 9 tumors grew as s.c. masses and six of nine were successfully transplanted into other SCID mice. Transplant inocula as low as 5 x 10⁶ cells resulted in 100% tumor incidence. Moreover, seven of nine tumors metastasized, five from the original s.c. implants and two from transplanted s.c. tumors. Metastases were detected mainly in the lungs but also were found in abdominal viscera (liver, spleen, and pancreas) and thoracic lymph nodes. Flow cytometric analysis showed that expression of a panel of melanoma antigens, melanoma-associated proteoglycan, ganglioside GD₃, and ganglioside GD₂, was maintained with SCID passage. The original tumor inocula contained a variable percentage of tumor-associated lymphocytes (1-76%). Flow cytometry analysis indicated that these were mainly CD₃⁺ T-cells. However, there was no correlation between the percentage of tumor-associated lymphocytes and the time required for development of a palpable tumor after s.c. injection or the ability to metastasize. These results demonstrate the growth and spontaneous metastasis of fresh human melanoma in SCID mice and suggest that this model could be important for therapeutic and basic biological studies.

INTRODUCTION

The growth of human tumor cells in immunodeficient animals, particularly athymic “nude” mice, has provided an in vivo model for the study of tumor progression and metastasis and for the testing of therapeutic modalities (1, 2). However, in nude mice the success with growth of tumor from fresh, uncultured human melanoma tissue has been low and spontaneous metastasis from successful tumor implants has been rare (3-5). While the incidence of s.c. growth of established cell lines is higher, manipulation is usually required to induce metastases (6, 7). These limitations have discouraged investigators from using the nude mouse model as a screen for therapeutic modalities and have limited the clinical relevance of basic biological studies performed in that system.

The growth of human tumors in nude mice might be limited by their residual immunity (1). Young or immunosuppressed nude mice are much better hosts for human tumor growth than adult mice, which suggests that some immune resistance may develop with age (8). Thus a mouse with broader immune deficiencies might serve as a better host for human tumor grafts. The C.B-17 SCID1 mouse is more severely immunocompromised than the nude mouse. It lacks both functional B- and T-cells because of nonfunctional rearrangements of immunoglobulin and T-cell receptor genes (9, 10). Natural killer cells, macrophages, and other hematopoietic cell lineages do not appear to be affected by the autosomal recessive SCID mutation (11-13). Histological abnormalities in the SCID mouse are limited to those associated with severe lymphopenia (14).

Recent studies have shown that human tumors grow readily in SCID mice. Established cell lines (15, 16) and fresh tumor tissue (5, 17) formed palpable tumors after s.c. injection. However, fresh tumor tissue did not metastasize, and metastases from cell lines were generally observed only when tumor cells were injected i.v. (15, 16, 18).

A human xenograft model in which tumors spontaneously metastasize from the s.c. injection site would more closely resemble cancer-bearing patients and might be useful for preclinical investigation of new therapies. To develop such a model, we examined the ability of SCID mice to support the growth of human tumor cells dissociated from metastatic melanoma. Here we show that 9 of 9 fresh human melanomas grew after s.c. injection into SCID mice. Moreover, 7 of 9 spontaneously metastasized, mainly to the lungs but also to other organs.

MATERIALS AND METHODS

Mice. C.B-17 scid/scid mice of either sex were bred and maintained in a protected environment in microisolator cages. Breeding pairs were a generous gift of George Carlson, McLaughlin Research Institute, Great Falls, MT. Animals were given autoclaved food and acidified water (pH 2.5) ad libitum, and all manipulations were performed in a laminar flow hood. Mice of either sex were used at 6-16 weeks of age.

Tumor Cells. Tumors were obtained from nine patients who were undergoing therapeutic surgery for metastatic melanoma. Seven specimens represented lymph node metastases and two were from bowel and s.c. tissue, respectively.

A single cell suspension of melanoma cells was prepared as described previously (19). Briefly, freshly excised tumors were minced in cold HBSS supplemented with 1% human albumin and 0.1% EDTA. Minced tumor pieces were placed in HBSS plus collagenase (140 µg/ml) and digested in a 37°C water bath with constant stirring. The tumor cells were cryopreserved in RPMI 1640 plus 1% human albumin plus 10% dimethyl sulfoxide and stored in liquid nitrogen. Prior to inoculation, tumor cells were quickly thawed, washed 3 times, and resuspended in HBSS.

Inoculations. For primary inoculations, melanoma cells were suspended in 0.2 ml HBSS and the suspension (2.5-6.0 x 10⁶ cells) was injected s.c. in the lower left flank. In some experiments, injections were given i.m. or i.p. Tumors that grew as primary inocula were transplanted to other SCID mice. This was accomplished by excising the tumors and preparing single cell suspensions by mechanical dissociation; then 0.5-1.0 x 10⁶ cells, either freshly harvested or cryopreserved, were injected s.c. Mice were examined twice weekly for tumor growth at the site of injection. Tumors were allowed to grow until they reached 2 cm. Euthanasia was accomplished with carbon dioxide.

Histology. Tissues were placed in 10% buffered formalin, paraffin embedded, cut, and stained with hematoxylin and eosin. Multiple sections of each organ were examined by three of the authors (C. J., G. M., P. M.).

Flow Cytometry Analysis. Melanoma cell surface antigens were studied with previously described monoclonal antibodies according to a
standard procedure (19). The following monoclonal antibodies were used: (a) 9.2.27, anti-melanoma-associated proteoglycan (obtained from the Biological Response Modifier Program, National Cancer Institute) (20); (b) R24, anti-ganglioside GD3 (gift of A. Houghton, Memorial Sloan-Kettering Cancer Center, New York, NY) (21); and (c) 14G2a, anti-ganglioside GD2 (gift of R. Reisfeld, Scripps Institute, La Jolla, CA) (22). After staining, the cells were analyzed with an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL). Gates were set to exclude debris, dead cells, and leukocytes. For antibody 9.2.27, only mechanically dissociated tumor cells were tested because of our previous observation that the proteoglycan is sensitive to collagenase (19).

Tumor-associated lymphocytes were identified by a monoclonal antibody to the common leukocyte antigen, CD45 (MB 12; American Type Culture Collection). They were enumerated and characterized phenotypically as described previously (23).

RESULTS

SC Growth of Human Melanoma in SCID Mice. Tumor cells were obtained by enzymatic dissociation of metastatic masses excised from nine patients; seven of these were lymph node metastases, and two were s.c. and small bowel metastases, respectively (tumors DE5 and GO6). In all cases, the cells were cryopreserved and thawed when needed for injection.

After the s.c. injection of 2.5–6.0 × 10⁷ tumor cells, all nine melanomas grew as s.c. masses in SCID mice (Table 1). Of 45 mice inoculated, 43 developed tumors. The time to development of a palpable tumor varied considerably, from 4 to 37 weeks (median, 8 weeks). Six of the nine tumors were successfully transplanted into other SCID mice using cell doses that were about 80% lower (0.5–1.0 × 10⁷ cells) than had been used for primary inoculation. Transplanted tumors appeared more rapidly than the original tumors (median time, 4 weeks) and grew large enough to necessitate sacrifice of the mice by 6–8 weeks.

One tumor (BA1) was transplanted into groups of mice at graded cell dosages: 1 × 10⁸, 5 × 10⁸, 1 × 10⁹, 5 × 10⁹, or 1 × 10¹⁰ cells. All inocula except the lowest (1 × 10⁸) resulted in 100% s.c. tumor development. The time to development of a 2-cm³ tumor was 10 weeks after injection of 5 × 10⁸ cells and 5 weeks after injection of the higher doses.

In initial experiments, three of the melanomas (BA1, JA4, CR3) grew i.p. after what was considered to be s.c. injection into the flank. Thus, in addition to developing a s.c. mass at the injection site, the mice exhibited abdominal distension, which at autopsy was shown to be due to multiple tumor masses in the peritoneal cavity, some as large as 1 cm in diameter. This was presumed to be due to inadvertent puncturing of the peritoneum during inoculation and was avoided in subsequent experiments by slightly modifying the injection technique.

Other Routes of Injection. Injection i.p. of 1.5 × 10⁷ BA1 cells, which had been transplanted once, resulted in the development of massive abdominal distension after 3 weeks. Autopsy showed multiple large tumor nodules in the mesentery and attached to the peritoneal surfaces of the liver, spleen, and kidneys and a small amount of ascitic fluid containing viable tumor cells.

BA1 cells also grew with 100% incidence after i.m. injection. Tumors were detectable at 21 days and grew progressively to a size of about 2 cm³ by 35–40 days.

Metastasis of Human Melanomas in SCID Mice. As indicated in Table 1, 7 of the 9 melanomas spontaneously metastasized, 5 from primary s.c. implants and 2 from transplanted s.c. tumors. Metastases were detected mainly in the lungs and lung-associated lymph nodes. Three tumors also metastasized to abdominal viscera (liver, spleen, or pancreas). Metastatic de-

Table 1 Growth and metastasis of human melanomas in SCID mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Source</th>
<th>Injected No. of mice</th>
<th>s.c. tumor</th>
<th>Metastases</th>
<th>Sites of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA1</td>
<td>Original</td>
<td>4</td>
<td>4*</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>2</td>
<td>2*</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>Passage 2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td></td>
<td>Passage 3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td>JA4</td>
<td>Original</td>
<td>2</td>
<td>2*</td>
<td>0</td>
<td>LALN</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>2</td>
<td>2*</td>
<td>0</td>
<td>LALN</td>
</tr>
<tr>
<td></td>
<td>Passage 2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Passage 3</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CR3</td>
<td>Original</td>
<td>2</td>
<td>2*</td>
<td>1</td>
<td>Lung, liver, spleen, LALN</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Passage 2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td>DE5</td>
<td>Original</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Lung, LALN</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>Lung</td>
</tr>
<tr>
<td>HU7</td>
<td>Original</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
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<td>1</td>
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<td>0</td>
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<td>Original</td>
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<td>R12</td>
<td>Original</td>
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<td>1</td>
<td>1</td>
<td>Lung, liver, pancreas</td>
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<tr>
<td>Total</td>
<td></td>
<td>45</td>
<td>43</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

* i.p. as well as s.c. tumor growth observed.

LALN, lung-associated lymph node.
HUMAN MELANOMAS IN SCID MICE

posits were detected only microscopically and varied in size from clusters containing 10 to 500 cells. The histology of a representative lung metastasis is illustrated in Fig. 1, and a liver metastasis is shown in Fig. 2.

Expression of Melanoma-associated Antigens. Flow cytometric analysis was performed for a panel of cell surface antigens. The profile of two tumors (CR3 and JA4) excised and cryopreserved after s.c. growth in SCID mice was compared with that of the original metastatic lesions removed from the patients which had been similarly processed. As shown in Table 2, the expression of melanoma-associated proteoglycan (antibody 9.2.27), ganglioside GD3 (antibody R24), and ganglioside GD2 (antibody 14G2a) of melanomas passaged in SCID mice was similar to that of the original inoculum.

Representative histograms showing ganglioside GD3 expression on human-derived versus SCID-passaged tumors are shown in Fig. 3. The fluorescence intensity after staining with antibody R24 plus fluorescein isothiocyanate-conjugated second step antibody is similar in the two preparations.

Role of Tumor-associated Lymphocytes. Since seven of the original tumors were lymph node metastases, it was not surprising that the tumor cell suspensions contained lymphocytes. However, the degree of lymphocyte contamination varied widely (range, 1.3–75.6%; median, 13.9%). The phenotypes of tumor-associated lymphocytes are shown in Table 3. The majority were CD3+ T-cells with a wide range of ratios of CD4+ to CD8+ cells. Natural killer cells (CD56+) were rare, as we have reported previously (23). There was no correlation between the percentage of tumor-associated lymphocytes and the time required for development of a palpable tumor after s.c. injection into SCID mice or the ability to metastasize. For example, of the two tumors with the highest lymphocyte content, one, HU7, grew in 8 weeks whereas the other, RI2, required 37 weeks. Moreover, RI2 spontaneously metastasized.

Table 2 Melanoma antigen expression

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Source</th>
<th>Proteoglycan (9.2.27)</th>
<th>GD3 (R24)</th>
<th>GD2 (14G2a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JA4</td>
<td>Original</td>
<td>48.5*</td>
<td>43.1</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>49.3</td>
<td>49.0</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>Passage 2</td>
<td>97.5</td>
<td>55.4</td>
<td>40.6</td>
</tr>
<tr>
<td>CR3</td>
<td>Original</td>
<td>95.9</td>
<td>69.4</td>
<td>57.6</td>
</tr>
<tr>
<td></td>
<td>Passage 1</td>
<td>97.7</td>
<td>75.8</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>Passage 2</td>
<td>98.3</td>
<td>94.1</td>
<td>87.2</td>
</tr>
</tbody>
</table>

* Percentage of melanoma cells positive by flow cytometry in tumors freshly excised from patients ("original") or in tumors serially transplanted into other SCID mice ("passaged").
ND, not determined.

Fig. 1. Photomicrographs of a lung metastasis that developed after s.c. injection of human melanoma. In A, at low power (× 100), a tumor nodule is seen surrounded by normal lung. In B, higher magnification (× 400) shows very large malignant cells with abundant, foamy cytoplasm and pleomorphic nuclei with clearly visible nucleoli.

Fig. 2. Photomicrographs of metastatic melanoma in liver. In A, at low power (× 100), a metastatic tumor nodule is present within the hepatic parenchyma taking up the left two-thirds of the section. Right, normal liver tissue. The tumor cells are growing as an expansile mass compressing the adjacent liver sinusoids. In B, higher magnification (× 400) shows sheets of neoplastic cells with abundant amphophilic cytoplasm and markedly atypical nuclei on the left, with normal hepatocytes to the right.

Fig. 3. Representative histograms showing ganglioside GD3 expression on human-derived versus SCID-passaged tumors.


**DISCUSSION**

These experiments show that 9 of 9 human melanomas grew progressively after s.c. injection into SCID mice and that 6 of 9 were successfully transplanted 1 to 3 times. Even more noteworthy was the demonstration that 7 of 9 melanomas spontaneously metastasized, mostly to the lungs, but in some mice, to liver, spleen, and pancreas as well.

Our demonstration of spontaneous metastases from s.c. implanted fresh human tumor tissue in SCID mice appears to be unique, since previously published studies have described growth of tumor at the s.c. site, but no spread of tumor beyond that site. Phillips et al. (5) reported that 10 of 23 tumors, not including melanoma, grew after implantation of 1-mm³ pieces of tissue with no evidence of dissemination. Bankert et al. (17) found that only a small proportion of fresh human lung tumors grew s.c. in SCID mice and none metastasized. Finally, Charley et al. (24) engrafted skin from a single patient with a cutaneous T-cell lymphoma; they observed infiltration of the graft site with human T-cells but no human cells outside the graft.

Spontaneous metastasis of human cancer in SCID mice is unusual even after injection of cell lines. Reddy et al. (15) reported that a lung tumor cell line grew after s.c. injection but did not metastasize; lung metastases were observed only after i.v. inoculation. Similar results were obtained by Ghetie et al. (16) with the Daudi B-cell lymphoma. Kamel-Reid et al. (18) reported that a human acute lymphoblastic leukemia cell line disseminated widely after i.v. but not i.p. injection. A noteworthy exception is the work of Mueller et al. (25); these authors isolated a variant of a melanoma cell line that metastasized to lymph nodes and lungs.

The high rate of s.c. tumor growth and spontaneous metastases that we obtained may be an indication that melanoma propagates and disseminates in SCID mice more readily than other human tumors. It is also possible that our success was due to technical factors. We injected enzymatically dissociated suspensions of viable tumor cells rather than solid implants used by other investigators. Dissociated cells might become established more readily because they are more easily vascularized.

When compared with the data published by other investigators, our results suggest that the SCID mouse may be a better host for human melanomas than other congenitally immunodeficient strains. The growth of freshly excised tumor tissue in athymic nude mice is unpredictable and metastasis of these tumors is rare (3–5). For example, Fogh et al. (3) reported that of 381 human tumor specimens, only 28.3% grew when injected s.c. and 1.0% metastasized. Recently, Mule et al. (26) reported that 10 of 12 human melanomas, either small tissue fragments or single cell suspensions from enzymatic dissociation, grew as s.c. tumors in beige/nude/xid mice. However, none of the tumors metastasized to distant sites.

Bankert et al. (17) proposed that the growth of human tumors in SCID mice could be limited by the tumor-associated lymphocytes that were admixed with the tumor cells. To support that hypothesis, they showed that mice given injections of tumors that did not grow had high levels of circulating human immunoglobulin, which suggested that those mice had been engrafted with human B-lymphocytes. In contrast, we found no correlation between the percentage of lymphocytes in tumor cell suspensions and the growth rate of the s.c. tumor or the incidence of spontaneous metastases. Whether tumor growth is inhibited by concomitant injection of autologous lymphocytes is an important question that can be answered definitively only by adoptive transfer studies in which the number and phenotype of the lymphocytes are carefully controlled.

We propose that the frequency of metastasis of human tumors in SCID mice might be increased by optimizing the system. For example, excision of the large s.c. masses would allow observation of the mice for an additional 4–8 weeks, which would provide more time for metastases to become established. Metastasis might also be potentiated by excluding “leaky” SCID, which have small numbers of functioning lymphocytes (27). Finally, preliminary data from our laboratory indicate that the development of metastases after i.v. injection of melanoma cells is augmented by depletion of natural killer cells.

Our results suggest that the SCID mouse could prove to be an important tool for studying human melanoma. On the most practical level, it provides a reliable way of quickly growing large numbers of tumor cells from an individual patient without the risks of biological and chemical contamination that have been associated with tissue culture. The system also could be used as a preclinical screen for new therapeutic agents and even for determining the most appropriate chemotherapy for a particular patient. It could be of value in investigating the biology of the metastatic process. Finally, the recent reports that SCID mice can be reconstituted with human T-cells (28, 29) raise the intriguing possibility of being able to study and manipulate the anti-melanoma immune response in an “autologous” system.

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Growth and Metastasis of Fresh Human Melanoma Tissue in Mice with Severe Combined Immunodeficiency

Laurie L. Hill, Robert Korngold, Christine Jaworsky, et al.


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