Effect of Host Age on the Transplantation, Growth, and Radiation Response of EMT6 Tumors

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ABSTRACT

The characteristics of EMT6 tumors in young adult (3- to 4-month-old) and aged (20- to 28-month-old) BALB/c KaRw mice were compared. The number of tumor cells implanted s.c. necessary to cause tumors in 50% of the injection sites was lower in aging than in young adult mice (69 and 138 cells for young adult mice versus 8.8 and 16 cells for aging mice in two experiments). The latent period of intradermally implanted tumors was shorter in aging mice than in young animals; however, the growth curves of established tumors were similar. The number and appearance of lung colonies after injection of cells i.v. and the pattern of spontaneous metastases were similar in young and aged animals. The cloning efficiencies (viabilities) of cells suspended from tumors in young adult and aging animals were the same (approximately 30% in both groups). Radiation dose-response curves for the cells of tumors in young and aging mice were different and suggested that the proportion of hypoxic cells was higher in tumors on aging animals and that such factors may influence the natural history of the tumor and the response of the tumor to treatment.

INTRODUCTION

The spectrum of cancers seen in young adult and aging patients is quite different (1, 11). Moreover, the prognosis for tumors of similar histological types presenting in young and aging individuals often differs, apparently reflecting both differences in the abilities of the patients to tolerate aggressive treatment and differences in the sensitivities of the tumors to treatment. It is probable that some of the observed differences in tumor sensitivity reflect intrinsic differences in the malignant cells of the diseases arising at different ages. However, the development and therapeutic responsiveness of malignant neoplasms are also influenced by tumor-host interactions and therefore may reflect age-related changes in host factors such as the activities of the immune system, the function of the vasculature and hematopoietic systems, and the composition and organization of the connective tissues. Such factors may be examined in the laboratory by implanting malignant cells into animals of different ages and observing the characteristics and therapeutic responses of the resulting tumors. As the implanted cells are identical in origin, any observed differences in the tumors must reflect differences in tumor-host interactions arising as a result of the differing physiological characteristics of the young and aged animals. Using this approach, it has been shown that the transplantation and growth of several antigenic tumor lines are different in young and aging animals and that these differences are correlated with the diminution of immunocompetence in senescent mice (3–5, 9–11, 22, 23). Immunotherapy of line 1 carcinoma with Corynebacterium parvum has been shown to be correspondingly less effective in aging than in young mice (23). The effects of radiotherapy and chemotherapy on transplanted tumors in young and old mice have not been compared thoroughly. This paper presents our experiments defining the transplantation, growth, and metastatic pattern of EMT6 mouse mammary tumors implanted into hosts of different ages and our preliminary experiments examining the response of the tumor cells to irradiation. This tumor system has several advantages as a model for such experiments: (a) the characteristics of the cells and tumors in young animals have been studied extensively (6, 8, 12–18, 20); (b) because it is an in vivo-in vitro cell line, cultures of malignant cells which are well characterized and free of host cell contamination may be used for implanting tumors; and (c) the viability of cells suspended from the tumors may be assayed in cell culture, allowing the effects of therapy to be examined in terms of tumor cell survival as well as in situ tumor response.

MATERIALS AND METHODS

Animals. BALB/c KaRw mice were used throughout these experiments. These animals were bred and maintained in a barrier in our institution and were removed from the breeding barrier to an isolated experimental room 1 to 2 weeks before the experiments. Animals in the barrier were routinely monitored to determine their microbiological status. They were known to have low antibody titers to Sendai virus and to be associated with several nonpathogenic bacteria but were without bacteriological or serological evidence of infection with other pathogenic viruses, bacteria, or other microorganisms. Animals from this colony showed no evidence of infection with mouse mammary tumor virus when tested by competitive radioimmunoassay (19). Aging animals were mice retired from our breeding colony because of age, falling productivity, or the death of one of the pair and were between 20 and 28 months of age at the beginning of the experiments. Weanling animals were 4 weeks old. Young adults were 3 to 4 months old. Aging animals were housed individually (males) or in groups of 2 to 3 (females). Young adult and weanling mice were housed in cages containing 2 to 5 animals of the same sex. The number of animals per cage over this density range has not been found to alter the characteristics of tumors in young adult animals during past experiments.

Tumor Cells. EMT6 cells (subline EMT6 Rw) were maintained by alternate passage in mice and in cell culture using the techniques described previously (12, 16) and were routinely tested and found to be free of mycoplasma contamina-
tion. Experimental tumors were implanted by injecting 1 to 2 x 10^5 cells, harvested from exponentially growing cell cultures, into the skin of the flank as described previously (16). Inoculated cell suspensions were therefore free of the stromal elements and immune cells which might be found in cell suspensions prepared from solid or ascites tumors.

**Tumor Growth Curves.** Tumor growth was followed by serial external caliper measurements of the 3 diameters of the neoplasms. Tumors were measured 3 to 4 times/week. Tumor volumes were calculated from the 3 diameters, assuming that the tumors were hemiellipsoidal (16).

**TD0** Analyses. TD0’s were determined as described in detail previously (6, 7, 16). Briefly, a single-cell suspension of EMT6 tumor cells from exponentially growing cell cultures was prepared and counted. Six serial 5-fold dilutions of cells were made, and 0.1-ml aliquots from these suspensions were injected s.c. into the 2 axillae and 2 groins of each animal. Four mice of each age were given injections of each dilution. Cell numbers in the injected suspensions ranged from 0.643 to 2000 cells/inoculum. Heavily irradiated cells were not admixed with the injected cell suspensions. Old and young mice were given injections of cells from the same cell suspensions during a single experiment. Animals were palpated to detect developing tumors beginning 1 week after injection and were observed twice weekly during the first 6 weeks and then once weekly for the remainder of the 100-day observation period. Whenever possible, animals dying before the end of the observation period were autopsied to determine whether tumors were present at negative or equivocal sites and to analyze the pattern of metastases. TD0’s were calculated by probit analysis using standard techniques (2, 6, 7).

**Lung Colonies.** The ability of the EMT6 cells to form tumor nodules in the lungs was assayed by injecting 0.1-ml aliquots of cell suspensions into the tail veins of mice (20, 22, 23). The lungs were not preirradiated, and heavily irradiated cells or microspheres were not admixed with the viable cell suspensions. Animals were sacrificed 14 days after injection, and the lungs were removed, cleared of extraneous tissues, fixed in Bouin’s solution overnight, washed with 100% ethanol, and stored in ethanol until they were counted. Tumor nodules on the surface of the lungs were counted with a dissecting microscope.

**Cellular Radiosensitivity.** The radiation dose-response curve for the tumor cells was determined using i.d. flank tumors that were implanted 2 weeks earlier. Animals received whole-body irradiation with 250-kV X-rays at a dose rate of approximately 200 rads/min with the mice in individual radially arranged chambers of a Lucite box as described previously (13, 17). The animals were irradiated without anesthesia and with ample ventilation. Immediately after irradiation, the mice were sacrificed, and the tumors were removed, minced, and trypsinized to form a single-cell suspension. The viability of the suspended tumor cells was determined from their ability to form colonies in cell culture (12, 15, 16). Survival curves for tumor cells from aging and young adult mice were obtained simultaneously on the same day during the same experiments. Plating efficiencies were determined for unirradiated tumors in mice of each age during these experiments.

Survival curves were analyzed by using a computer analysis technique which fit the data, assuming that the surviving fractions were obtained from linear portions of multitarget single-hit survival curves. Hypoxic fractions were calculated by comparing survival curves for tumor cells from air-breathing animals with those for tumor cells from nitrogen-asphyxiated young adult animals, using a variation of the method described by van Putten and Kaliman (21). The mathematical and biological techniques used in this analysis are described in detail in a paper which is in preparation and in previous papers examining hypoxic fractions in EMT6 tumors (6, 15).

**RESULTS**

Chart 1 shows typical growth curves for EMT6 tumors implanted into the flanks of weanling, young adult, and aging mice. In this and replicate experiments, the pattern of tumor growth was similar in male and female mice at each age (data not shown), which is in agreement with our previous observations on young adult animals (12, 16). The latent period, i.e., the time between injection of tumor cells and development of palpable tumor, was longer in young adult mice than in weanling or aging animals. The growth of established tumors was similar in animals of different ages.

Chart 2 shows the pattern of tumor development in mice of different ages as a function of the number of tumor cells injected into each site. The TD50’s derived from these data are shown in Table 1. The TD50’s were lower in aging mice than in young adult mice.

Table 2 shows the number of lung tumors produced per 2000 i.v. injected cells in young adult and aging mice. There was no difference in the number, size, or appearance of the tumor nodules in animals of different ages.

Young adult and aging animals from the tumor growth and TD50 experiments were autopsied to examine the pattern of metastatic spread. No difference in the metastatic pattern was observed. Both young and old animals showed a low rate of metastasis, and only a few metastases (to lungs, peritoneal surfaces, and lymph nodes) were observed.

Plating efficiencies were determined for tumor cells from young adult and aging animals in 2 experiments: the plating efficiencies of cells from tumors in aging animals in these experiments were 34.6 and 26.8%; those for cells from tumors in young animals were 29.2 and 28.0%. These values are quite similar and are similar to the plating efficiencies generally found for tumors in young adult animals, which average 33 ± 6% (S.E.) (12). This shows that the clonogenicsities (viabilities) of cells from tumors in young and aging animals were the same.

Chart 3 shows radiation dose-response curves for cells from tumors in young adult and aging mice. The points are individual determinations of surviving fractions, each of which was performed with a pool of 4 to 5 tumors. Data from 3 experiments are shown. Survivals shown for tumors from young and old mice were determined during the same experiments. The thin line is the radiation dose-response curve for cells from tumors in aged mice, which was determined by analysis of the data shown on the chart. The thick line is a composite dose-re-

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2 The abbreviations used are: TD0, number of tumor cells necessary to cause tumors in 50% of the injection sites; i.d., intradermal.
in young adult animals obtained in these experiments were not significantly different from the composite cell survival curve or from the cell survival curves reported previously (6, 15, 17). The slopes of the cell survival curves for tumors on young and aging mice were not significantly different from each other or from the slope of the survival curve for cells of tumors in young adults rendered artificially hypoxic by asphyxiating the host mice with nitrogen before irradiation (data from Refs. 6 and 17). The dose-response curve for the cells of tumors in aging mice lies significantly above that for tumors in young adult mice (p < 0.01). If it is assumed that this represents a change in the hypoxic fraction and if the hypoxic fraction is estimated by comparing the survival curve for aging air-breathing animals with that for nitrogen-asphyxiated young adult mice, these data imply that the hypoxic fraction increases from the value of 35% (95% confidence limits, 21 to 55%) found in young adult animals to a value in excess of 60% (95% confidence limits, 57 to 100%).

**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Young adults</th>
<th>Aged mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>138 (87-220)*</td>
<td>8.6 (4.4-17.3)</td>
</tr>
<tr>
<td>2</td>
<td>69 (35-135)</td>
<td>15.6 (7.2-33.7)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, 95% confidence limits.

**Table 2**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of animals</th>
<th>Colonies/animal</th>
<th>No. of animals</th>
<th>Colonies/animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>22.2 ± 5.2a</td>
<td>8</td>
<td>26.8 ± 5.7</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>17.7 ± 2.2</td>
<td>10</td>
<td>16.9 ± 2.9</td>
</tr>
</tbody>
</table>

*a Mean ± S.E.

**Chart 1.** Growth of EMT6 tumors in aging, weanling, and young adult mice. Time, days after injection of 2 x 10^5 tumor cells; volume, mean tumor volume for group of 8 to 10 mice. O, young adults; ●, aged mice; △, weanlings.

**Chart 2.** Percentage of injected sites developing tumors as a function of the number of cells inoculated. Circles and diamonds, 2 independent experiments. ● and ○, aged mice; O and △, young adult mice. Lines, probit curves resulting from analysis of pooled data for aging mice (left curve) and young adult mice (right curve).

**Chart 3.** Radiation dose-response curves for cells from tumors irradiated in vivo. O, tumors in young adults; ●, tumors in aged mice. ---, dose-response curve for tumors in aged mice; ——, composite dose-response curve for young adult mice; ----, dose-response for tumor cells in hypoxic young adult mice (from Ref. 17).
DISCUSSION

The characteristics of tumors growing in aging mice may be different than those of tumors growing in young adult animals even when tumors are produced by injecting cultured cells from the same in vivo-in vitro tumor cell line. This probably reflects the different tumor-host interactions in young and aging animals.

The immune system of the aging mouse has been studied using several mouse strains, including the BALB/c. Aging BALB/c mice have been shown to have a markedly decreased immunocompetence, which involves defects in both cellular and humoral immunity (10, 22, 23). Spleen cells from aging BALB/c mice have a decreased ability to respond to stimulation with phytohemagglutinin, mount graft versus host reactions in neonatal F, mice, and produce antibodies to horse and sheep RBC (10). Decreased numbers of peritoneal exudate cells are found in unstimulated aging mice and in aging mice stimulated by i.p. injections of C. parvum (23). Aged BALB/c mice also show a decreased resistance to oncogenic viruses (23), decreased lymphoproliferative responses in lymph nodes draining areas implanted with P815 mastoma cells (10), and increased tumor development after injections of P815 mastoma cells (10), or EMT6 tumor cells (23). Although the immunocompetence of the aging mouse of the BALB/c KaRw substrain has not been studied, there is no reason to expect that this substrain should differ from those studied by other investigators.

EMT6 tumor cells are known to elicit an antitumor-immune response in either the BALB/c Ka or BALB/c KaRw substrains, which influences the TDso, the latent period, and curability of the neoplasms (12, 14, 16). The nature and therapeutic implications of this immune response have been examined in several laboratories (8, 14, 18). We have shown previously that preimmunization of young adult animals with radiation-sterilized tumor cells increases the TDso of the tumor cells and lengthens the latent period between injection of the tumor cells and the appearance of palpable tumors but does not alter the growth curve of established tumors (14, 16). The results reported here show that the TDso of the tumor cells is lower and that the latent period is shorter in aging mice than in young adult mice. However, the growth curve of tumors is similar once the tumors reach palpable sizes. The similarity of the growth curves in aged and weanling animals (which have not yet become fully immunocompetent) and the analogies between the results shown here and those resulting from deliberate immunostimulation of the hosts (14) suggest that the decreased TDso and latent period observed in aging animals reflect the decreased immunocompetence of the aging hosts, which would be expected to be most evident when the hosts are faced with only small burdens of tumor cells. Our data for EMT6 tumors are in agreement with the findings of others (3–5, 10, 22, 23) who have demonstrated similar changes in the growth of other transplanted tumor lines in aged animals and with the hypothesis proposed by these other investigators that many of these differences are related to the changing immunological status of the aging animals. As discussed above, previous studies by others have showed that the changes in the immune system with aging are complex (1, 3, 9, 11) and that many aspects of the interaction between tumors and the immune system are altered in aging animals (1, 3–5, 10, 11, 22, 23). Additional work will be required to define the immunological interactions between EMT6 tumors and young and aging hosts.

The incidence of lung nodules was similar in young adult and aging mice. This is in contrast to the results obtained by Yuhas et al. (22) and Yuhas and Ulrich (23) who observed increased numbers of lung nodules after i.v. injection of line 1 carcinoma cells and increased numbers of spontaneous pulmonary metastases from s.c. implanted line 1 carcinomas in aged mice. The reason for this difference is not known but may reflect biological differences in the tumors. The line 1 is a lung adenocarcinoma which metastasizes to the lung within 4 to 6 days after implantation, whereas EMT6 is a mammary tumor which rarely metastasizes. Our data on lung colonies are consistent with our necropsy observations, which suggested that the incidence of spontaneous metastases from s.c. or i.d. EMT6 tumors was not increased in aging animals.

The similarity of lung colony formation in young and aged mice after i.v. injection of EMT6 tumor cells contrasts with the reduction of the TDso of s.c. injected cells in aged animals. The reason for this difference is not known; it might be related to a differential effect of aging on the components of the immune response influencing tumor development in the lung and s.c. tissues or to differences in the effects of age on the connective tissue and vasculature in the s.c. and pulmonary tissues.

The radiation dose-response curves for tumors in young adult and aging animals were different and suggested a higher hypoxic fraction in aging animals than in young adult animals. This might reflect the inability of the aging host to provide an adequate vascular bed for this rapidly growing tumor. A definitive, quantitative evaluation of the magnitude and mechanism of the change in cellular radiosensitivity with host age will require additional data, including radiation dose-response curves for hypoxic tumors in aging mice and observations of the radiation response of tumors remaining in situ after treatment. This will be examined in future studies.

It has often been observed clinically that tumors in aging patients are more difficult to treat successfully than are tumors of similar histology in young adults. This is probably caused in part by the less vigorous medical status of aging patients, who are often unable to tolerate radical surgery, intensive chemotherapy, or aggressive radiotherapy and must therefore be treated with less aggressive and less effective therapy. However, the data reported here show that aging animals may sometimes host tumors which are more resistant to therapy than are the corresponding tumors in young adult animals, probably as a result of differences in the interactions between the malignant cells and the vasculature. This raises the possibility that human tumors may likewise be more resistant to therapy when presenting in aging patients. Furthermore, if human tumors are immunogenic, like EMT6 tumors and the other experimental tumors used in the studies described above, the declining immunocompetence of the aging patient would also be of clinical importance. In this case, a smaller number of viable tumor cells would be necessary to cause a recurrence, and more intensive therapy would be necessary for the aging patient than for the young patient. The possible clinical importance of tumor-directed immune responses and of the decreasing immunocompetence during aging has been discussed in detail by others (1, 3, 5, 11).

The data reported here suggest that both immunological and nonimmunological tumor-host interactions in the aged animal may be different from those in the young animal generally used as a model in cancer research and that such factors may

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influence the natural history of tumors and the response of tumors to therapy. Further studies of tumor-host interactions in aging animals and of the response of tumors in aging animals to treatment with radiation, chemotherapy, and other agents are needed.

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