Induction of Granulocytic Hyperplasia, Thymic Atrophy, and Hypercalcemia by a Selected Subpopulation of a Murine Mammary Adenocarcinoma

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ABSTRACT

Three tumor subpopulation lines derived from a single, spontaneously occurring BALB/cfC3H mammary tumor were evaluated for their ability to cause a leukemoid effect in mice. One of the BALB/cfC3H tumor cell lines (410.4) produced leukocytosis with neutrophilia, hypercalcemia, and thymic atrophy. A second line (66) produced none of these effects. The third line (168) was intermediate in its ability to affect neutrophil counts and splenomegaly but did not produce thymic atrophy or hypercalcemia. These studies demonstrated that the hemopoietic effect of tumor cells derived from a single tumor was variable and that a tumor cell line which caused neutrophilia also induced hypercalcemia and thymic atrophy, the same association that was reported previously in the case of murine CE mammary carcinoma. These observations will be useful in the further investigation of pathophysiology of tumor-induced leukemoid reactions, hypercalcemia, and other paraneoplastic syndromes.

INTRODUCTION

The leukemoid response has been observed for decades in humans and animals in association with various illnesses, including cancers not involving the bone marrow. Chen and Walz (1) studied the bone marrow of patients with leukemia reactions and observed a "definite pattern" of increased granulocyte precursors and decreased erythroid precursors similar to but distinct from that of true leukemia; they postulated substances were produced by the tumor that stimulate the hematopoietic system. In more recent years, various tumors of laboratory animals have been found to produce leukemoid reactions (2, 3). We have observed that a severe neutrophilia induced in mice by transplantable CE 1460 mammary carcinoma (4) was also associated with depletion of lymphocytes in the primary lymphoid organs accompanied by marked thymic atrophy and severe hypercalcemia (5, 6). The mechanism of this tumor effect is of interest because it influences hemopoiesis, an imperfectly understood process in normal organisms, and because its distortion of blood cell populations, and particularly its reduction of lymphocytes, may be significant in the course of malignant disease. In the present study we tested three different subpopulation lines derived from a single spontaneous mammary adenocarcinoma for similar effects on hemopoiesis.

MATERIALS AND METHODS

Mice. BALB/c mice were from Jackson Laboratories (Bar Harbor, ME). Mice were 12 to 17 wk old at tumor implantation and were of both sexes. Tumor-bearing and control mice were matched for age and sex. Mice were housed in the vivarium at the University of Washington. They were fed a standard diet, Mouse Breeder Blox and Rodent Blox, both from Wayne Pet Food Division, Continental Grain Co., Chicago, IL.

Tumor Cells. Tumor cell lines 410.4, 66, and 168 were kindly provided by Dr. G. H. Heppner (Michigan Cancer Foundation, Detroit, MI). As described previously in detail (7–9), these cell lines were derived from a single murine mammary carcinoma. Tumor cells were received growing as s.c. implants in carrier BALB/c mice. All experiments were performed during the first two in vivo passages of the tumor cells in our laboratory. When experiments were repeated, frozen cells from the first passage were inoculated into mice, and experiments were performed on the second passage of frozen tumors in vivo. Tumors were excised from mice, pressed through a stainless steel sieve in cold sterile HBSS (Grand Island Biological Co., Grand Island, NY), and washed once with HBSS as described before (4). Mice received 10⁵ viable cells s.c. in sterile HBSS; control mice were given injections of an equal volume of HBSS alone.

Experimental Design. For each tumor line, a group of 16 mice were given injections s.c. of tumor cells, and a group of 8 mice served as controls. Tumor growth was recorded, and blood cell counts were determined at 1-wk intervals for 4 wk on all mice alive at each time point. Four tumor and two control mice were killed each week, their spleens and thymuses were weighed, and bone marrow smears and sections were prepared from the femora of each mouse. At the same time, blood was collected and plasma was separated after centrifugation of the heparinized capillary tubes. Plasma specimens were stored at 4°C until the end of the experimental period and were assayed for plasma calcium and phosphorus.

Blood Cell Counts. WBC counts were determined on orbital sinus blood using a Coulter Counter, and differential counts were done on 200 cells on a Wright-Giemsa-stained smear for each sample, as described before (4).

Bone Marrow Cells. Bone marrow cell suspensions were prepared by flushing one femur from each mouse with 10 ml of HBSS using a needle and syringe. After centrifugation at 200 x g for 5 min, cells were smeared on glass slides with a drop of fetal calf serum and stained with Wright-Giemsa. Five hundred cells were differentiated on each specimen (4).

Bone Marrow Sections. Histological sections were prepared from the other femur of each mouse. These bones were fixed in 10% neutral buffered formalin for 9 h at 4°C, decalcified with 5% tetrasodium EDTA in 0.2 M phosphate buffer (pH 7.4) for 14 days at 4°C, and dehydrated with ethanol. They were embedded in plastic (JB4; Polyscience, War- rington, PA), and 2-μm-thick sections were cut and stained for histochemical demonstration of acid phosphatase in osteoclasts as described before (10). The number of osteoclasts was counted along the endosteal surface in the diaphyseal region in longitudinal sections of the femur as described previously (6).

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2 Recipient of support from a stipend from the Medical Student Research Training Program of the University of Washington.
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Calcium Determinations. Plasma calcium and phosphorous levels were measured by standard autoanalyzer methods.

RESULTS

Tumor Growth. All cell lines of transplanted tumors grew in host mice at approximately equal rates and reached the size of 1.5 to 2.0 cm in diameter by the fourth week after transplantation. At the time of sacrifice, there were no metastases of the tumor in bone marrow or spleens.

Blood Neutrophil Counts. As shown in Chart 1, mice with tumor 410.4 acquired high peripheral neutrophil counts, significantly higher (P < 0.005) than the control value after the second week post-tumor transplantation. Those with Tumor 168 had an intermediate degree of neutrophilia which appeared only at the fourth week when all mice were in their terminal condition. At this stage, there were considerable degrees of variation in neutrophil counts. Mice carrying Tumor 66 did not show a rise in neutrophil counts at all during the observation period. Neutrophils of mice with neutrophilia demonstrated hypersegmentation as seen in other types of tumor-induced neutrophilia (2).

Bone Marrow. The bone marrow of mice bearing Tumor 410.4 for 3 wk revealed a significant increase in the number of neutrophilic granulocytes as shown in Table 1. Severe neutrophilic granulocyte hyperplasia was also evident on histological bone marrow sections from these mice, and erythroblasts and lymphocytes were found infrequently. In contrast, the bone marrow of mice bearing Tumors 168 and 66 did not show remarkable changes in marrow differential counts throughout the study period.

Thymus. A dramatic thymic involution was observed in the 410.4 tumor-bearing mice as shown in Chart 2, similar to that previously described for the CE tumor (5), whereas mice bearing lines 168 and 66 demonstrated no change in thymus weight when compared with controls.

Spleen. Changes in the spleen weight from the second experiment are shown in Chart 3. Spleens of mice bearing the 410.4 tumor were enlarged to nearly 3 times normal at 2 wk and about 2 times normal at 3 wk after tumor transplantation. The spleens of Tumor 168 mice also increased to 3 times normal at the end of the study period, while Tumor 66 caused no change. Spleen changes showed considerable variation between the first and second experiments.

Table 1

<table>
<thead>
<tr>
<th>Tumor lines</th>
<th>No. of wk</th>
<th>No. of mice</th>
<th>% of neutrophilic granulocytes</th>
</tr>
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<tbody>
<tr>
<td>410.4</td>
<td>3</td>
<td>4</td>
<td>83.2 ± 2.1*</td>
</tr>
<tr>
<td>168</td>
<td>4</td>
<td>4</td>
<td>59.5 ± 23.8</td>
</tr>
<tr>
<td>66</td>
<td>4</td>
<td>4</td>
<td>62.2 ± 2.6</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td></td>
<td>54.9 ± 10.8</td>
</tr>
</tbody>
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*Mean ± SD

Data were pooled from all controls (2 for each tumor line) at the same week as shown for the tumor mice.
Serum Calcium. As shown in Chart 4, plasma calcium in the 410.4 tumor-bearing mice rose significantly (P < 0.005) by the second week after tumor transplantation and remained elevated until the end of the study. Plasma phosphorus was within normal limits until the fourth week when it dropped. In contrast, plasma calcium of mice bearing the 168 and 66 tumor lines did not show significant changes. As in the case of CE mammary carcinoma (6), the bone marrow sections of 410.4 tumor-bearing mice revealed an increased number of endosteal osteoclasts which eroded the endosteal bone margin (Fig. 1), but the section of 168 and 66 tumor-bearing mice did not show such changes.

DISCUSSION

In this study, we found that there is a heterogeneity in the ability to cause leukemoid reactions among subpopulations of a single tumor and that a cell line which causes leukemoid reactions also causes significant hypercalcemia, thymic atrophy, and an increase in endosteal osteoclasts. So far, the association of these phenomena has only been reported for the CE mammary carcinoma in mice (5, 6). As described in detail elsewhere by Lee et al. (4-6), the CE 1460 mammary carcinoma causes the following in CE or BALB/c × CE F, mice: blood neutrophil counts rise to 10 to 20 times normal at 3 to 4 wk after tumor implantation; the hematocrit drops moderately; in the marrow, granulocytic precursors decrease; the spleen weight rises and falls variably with extramedullary hemopoiesis; the thymus consistency shows marked involution; the marrow spaces are enlarged by osteoclastic bone resorption; and the plasma calcium rises significantly.

The tumor cell lines we used in this study have been extensively investigated in the laboratory of Heppner and coworkers (Michigan Cancer Foundation, Detroit, MI) regarding their cellular heterogeneity for a variety of phenotypes including metastatic potential, morphology, immunogenicity, and association with mammary tumor virus (9, 11, 12). It appears that metastatic potential or prostaglandin production by the tumor does not seem related to the tumor's ability to cause leukemoid reactions, but there is a chance that the tumor's immunogenicity may be related to the observed phenomenon. It is also noted that line 410.4 is polygonal, but lines 66 and 168 are fusiform in morphology, and the virus production was negative only for line 66 (Refs. 9, 11, and 12; Footnote 5). Our report adds functional cellular heterogeneity in the ability of the tumor to cause leukemoid reactions in hosts. Because of this heterogeneity, it is possible that the clinical occurrence of leukemoid reactions can be influenced by the competing effects of different clones in a given tumor. Those tumors that do not produce leukemoid reactions as a whole tumor may still contain cells that are capable of doing so as an individual clone. Although the tumor lines tested vary in these above-mentioned phenotypes, more data are necessary to know whether the ability of line 410.4 to cause leukemoid reactions correlates with any of them.

Tumor-induced leukemoid reactions are not limited to mammary carcinoma in mice nor a particular type of tumor. The reactions have been reported in mice bearing fibrosarcomas (3, 13), salivary gland tumors (14), lung tumors (15), and in patients with various kinds of tumors (16). The mechanism of leukemoid reactions associated with tumor-bearing hosts in humans and animals is not clearly understood. Kodama et al. (3) investigated a number of tumors in several different strains of mice and found a wide range of severity of leukemoid reactions, depending on both the tumor line and the host strain. These effects are apparently constant for a given stable tumor line and host. Their results suggest that the leukemoid changes are not a general response to the stress of carrying a tumor or to inflammation caused by tumor necrosis, but they are due to specific actions of only some tumors at the hemopoietic level.

Tumors shown to produce CSF which stimulates growth of colonies of granulocytes and macrophages in vitro have been intensely investigated in an effort to understand the mechanism of neutrophilia (17-19). However, the role of such CSF production by the tumor in leukemoid reaction is still uncertain. As shown by Burlington et al. (17), a subpopulation of a granulocytosis-inducing tumor failed to cause neutrophilia in vivo, but it retained the capacity to produce CSF in vitro. Although the physiological significance of CSF in leukemoid reactions is still controversial, it is of interest to note that Burlington et al. also found heterogeneity in ability to produce leukemoid reactions among subpopulations of a tumor. Further investigations of specific clones which cause hemopoietic changes may be warranted.

The association of hypercalcemia in tumor-induced leukemoid reactions has only recently been appreciated and described, mainly in nude mouse systems with human tumors (6, 20-23). We have shown previously that the hypercalcemia associated with granulocytosis in CE mammary tumor-bearing mice was
likely due to an increased bone resorption by abundant osteoclasts and was not due to other metabolic causes of hypercalcemia (6). Although we have not conducted detailed metabolic studies in 410.4 tumor-bearing animals, it is conceivable that the hypercalcemia of 410.4 tumor-bearing mice is also related to the increase in osteoclasts as we observed in the marrow sections. Therefore, it would seem that the hypercalcemia, the granulocytic hyperplasia, and the increase in osteoclasts are associated or related aspects of some tumor-induced leukemoid reactions.

The mechanism by which the tumor affects hemopoiesis and the effect of these changes on the immunological response to the tumor remain to be characterized. In a case of CE mammary tumor system, an increased number of marrow granulocyte progenitors in tumor-bearing animals has been demonstrated (24), and a possible tumor-derived humoral factor which stimulates bone resorption by local prostaglandin production has been shown (25). Similar studies using 410.4, 168, and 6 cell lines will be helpful to elucidate the mechanism.

Thymic atrophy has been noted in association with other tumors in experimental animals (5, 26, 27). Ahernem et al. suggested that this is due to physiological stress, since dexamethasone caused similar effects on the thymus. However, Efskind et al. (27) found decreased plasma corticosterone levels in mice asonest caused similar effects on the thymus. However, Efskind et al. sug

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REFERENCES

Fig. 1. Photomicrographs of sections from femoral diaphyses of normal and tumor-bearing mice, showing marrow cells and endosteal margin. A, from control mouse. Note smooth endosteum. B, from mouse bearing tumor line 410.4 at Wk 3. Note increased neutrophil precursors in marrow, increased number of osteoclasts (arrows), and eroded appearance of endosteal bone margin. Acid phosphatase and toluidine blue, $\times$ 620.
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