Brief Definitive Report

Inhibition of Tumor Growth by Elimination of Granulocytes

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Summary

As observed for many types of cancers, heritable variants of ultraviolet light–induced tumors often grow more aggressively than the parental tumors. The aggressive growth of some variants is due to the loss of a T cell–recognized tumor-specific antigen; however, other variants retain such antigens. We have analyzed an antigen retention variant and found that the variant tumor cells grow at the same rate as the parental tumor cells in vitro, but grew more rapidly than the parental cells in the T cell–deficient host. The growth of the variant cells was stimulated in vitro by factors released from tumor-induced leukocytes and by several defined growth factors. In addition, the variant cancer cells actually attracted more leukocytes in vitro than the parental cells. Furthermore, elimination of granulocytes in vivo in nude mice by a specific antigranulocyte antibody inhibited the growth of the variant cancer, indicating that this tumor requires granulocytes for rapid growth.

The appearance of heritable variants with increased malignant potential is one of the hallmarks of cancer. Such variants fall into one or more of three broad categories: those that have acquired resistance to inhibition by host defenses or chemotherapy, those that more effectively invade or metastasize, and those that acquire a faster growth rate. Determining the mechanisms that account for escape of variants from host control may help in developing new therapeutic approaches tailored to interfere with particular mechanisms of tumor progression.

UV light–induced cancers, when transplanted into normal syngeneic hosts, are usually rejected after a period of initial growth (1). Rejection depends on $CD8^+$ CTL specific for unique antigens expressed by the cancer cells (2, 3). Occasionally these "regressor" (RE) tumors give rise to variants that grow progressively and kill normal hosts. Some "progressor" (PRO) variants have lost T cell–recognized unique tumor-specific antigens. More commonly, however, such antigens are retained (3). We have compared the growth of such an antigen retention PRO with that of the parental RE in vitro and in vivo. We found that the parental and the variant tumors, designated 4102-RE and 4102-PRO, differ significantly in the rate of growth in mice devoid of functional T cells. We show that 4102-PRO requires host granulocytes for rapid growth and that elimination of the host cells by an antigranulocyte antibody reduces tumor growth.

Materials and Methods

Mice and Tumors. Athymic *nu/nu* mice (from the National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD) were used for all experiments except for those using anti-NK1.1, where B6CByF1/J *nu/nu* mice (which express the NK1.1 marker on NK cells) from The Jackson Laboratory (Bar Harbor, ME) were used. The 4102 RE tumor was induced by UV light at the LaRabida-University of Chicago Institute, adapted to culture in MEM and 10% FCS (CMEM), and maintained as described (2, 3). The tumor is called a regressor because it is rejected by normal euthymic mice even without prior immunization (2), but the tumor grows regularly in nude mice and kills such mice. Unlike the parental 4102-RE tumor, the 4102-PRO variant when transplanted into euthymic syngeneic mice grows progressively to kill. All tumor cell lines were tested regularly for mycoplasma contamination by staining with HOECHST 33258 and examination of a fluorescence microscope.

Antibodies. RB6-8C5 (anti-Gr-1) was a gift of Dr. Robert L. Coffman (DNAX Research Institute, Palo Alto, CA). GK1.5, which recognizes the CD4 marker on a subset of T cells (4), was used as an IgG2b isotype control in nude mice, which lack such T cells. Anti-NK1.1 was a gift of Dr. Paul Leibson (Mayo Clinic, Rochester, MN). The efficiency of the anti-Gr-1 antibody for depleting neutrophils was determined by examining peripheral blood smears and smears of peritoneal exudate cells prepared by a cytospin centrifuge (Shandon Inc., Pittsburgh, PA).

Tumor Growth In Vivo. 5×10^6 tumor cells were injected subcutaneously into the flanks of three to four athymic nude mice. Tumor growth was measured every 3–4 d with a caliper. Size in cm^3 was calculated as $(abc)/2$ where a, b, and c are three orthogonal diameters. This formula is derived from the formula for the volume of an ellipsoid $= \frac{\Pi\, abc}{6}$.

Recovery of Cells from the Peritoneal Cavity. Peritoneal exudate cells (PEC) were harvested from nude mice that were either naive (N-PEC) or that received an i.p. injection 3 d earlier of 2 ml of aged thioglycolate medium (I-PEC) (Difco Laboratories, Inc.,

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Detroit, MI) or 250 μl containing 5 × 10^6 cultured tumor cells (T-PEC). PEC (containing tumor cells or not) were removed after 3 d by injecting 5 ml of sterile PBS witha 5-ml syringe and 20-gauge needle, massaging the abdomen, and then aspirating the fluid. The recovered cells were sedimented at 225 *g* for 5 min, resuspended in 12 ml CMEM, and put into culture dishes as described below.

Assay for Quantifying Viable Cells. To assess cell growth, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each culture containing 100 μl of medium (after removal of 100 μl of the original medium) for 4–6 h, followed by 100 μl of 10% SDS plus 0.01 N HCl. OD were read on an ELISA reader at 570 nm, subtracting the background at 650 nm.

Chemoattraction Assay. Medium conditioned for 24 h by 1.5 × 10^6 tumor cells in 10 ml of CMEM was added to two bottom wells of modified Boyden chambers (NeuroProbe, Cabin John, MD). 2 × 10^5 thioglycolate-elicited I-PEC were added to the top chamber in a volume of 200 μl. The I-PEC were suspended in either fresh CMEM (to test for directional movement) or in the same tumor cell–conditioned media as in the lower chamber (to distinguish from increased random movement). The top chambers with the I-PEC were separated from the lower chambers containing tumor cell–conditioned media by a polycarbonate filter with a pore size of 5 μm (Neuro Probe). Chambers were incubated for 90 min. Unmigrated cells were washed off the tops of filters by aspirating the 200 μl of medium that contained the I-PEC, wiping the filter with a Q-tip, adding distilled water with a squirt bottle, and repeating three times. The filters were stained by dipping them into a staining solution (Diff-Quick; Baxter, McGaw Park, IL). Cells that had migrated through the filter pores were counted using a magnification of 400, and the average of six fields from the duplicate filters ± SEM was plotted.

Results

We first compared the growth of the parental 4102-RE tumor and the 4102-PRO variant in nude mice. Fig. 1 (*left*) shows that 4102-RE grows much more slowly during the first 25 d, though the regressor does continue to grow and eventually kills nude mice. However, the growth rates in vitro (Fig. 1, *right*) were identical in repeated trials, indicating that the 4102-RE and 4102-PRO interact differently with a host component(s). The growth of PRO but not RE cells was stimulated in culture by T-PEC from nude mice. The stimulation was most likely due to a soluble factor since similar effects were observed when T-PEC and tumor cells were separated by a filter in transwell plates (Fig. 2, *left*). Growth factors that have been shown to stimulate some malignant cells (5) were added to tumor cells in vitro. These included TGF-β, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF). Surprisingly, the PRO variant was stimulated by each of the factors (Fig. 2, *right*), while the parental tumor was either inhibited or slightly stimulated. It is possible that a common regulatory mechanism has been mutated in the PRO, leading to upregulation of the mitogenic response to several growth factors.

To determine whether stimulatory cells were attracted to the tumor cells, supernatants of cultured tumor cells were tested for chemotactic activity. In repeated experiments, conditioned media from 4102-PRO had considerably higher levels of chemotactic activity for thioglycolate-elicited I-PEC compared with conditioned media from 4102-RE (Fig. 3). This chemotactic activity was directional, as we could find no evidence of increased random movement (Fig. 3). We also found that small clusters of 4102-PRO tumor cells, present in the peritoneal lavage of nude mice 3 d after tumor cell injection, attracted in vitro large numbers of T-PEC. This gradient of T-PEC was not found surrounding clusters of 4102-RE tumor

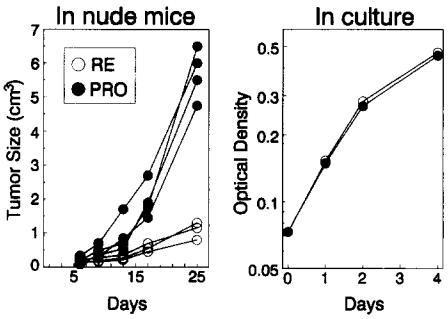

Figure 1. Growth of 4102-PRO (●) and 4102-RE (○) in nude mice (*left*) and in culture (*right*). 5 × 10^6 tumor cells were injected subcutaneously into three to four athymic nude mice. 10^3 tumor cells were cultured per well (eight replicates) in 96-well flat-bottom plates. Growth was measured on indicated days in eight replicate cultures by the MTT assay (See Materials and Methods).

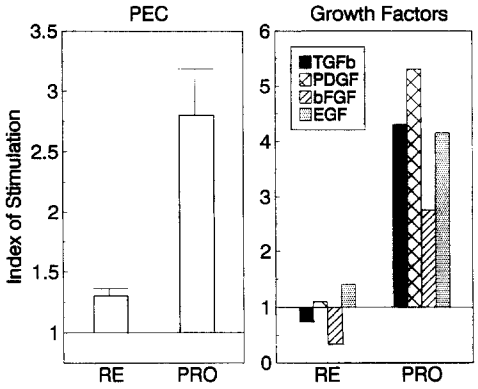

Figure 2. Stimulation of tumor cells by tumor-induced PEC and defined growth factors. In the left panel, 2.5 × 10^5 lavaged T-PEC were added to the bottom of a transwell plate. 10^3 tumor cells were plated in the top of the transwells (on a filter of 0.4-μm pore size) and cultured for 4 d in triplicate. The top wells were then transferred to a new plate for an MTT assay. Index of stimulation is calculated as the average OD of tumor cells in the presence of T-PEC divided by the average OD of tumor cells with medium alone. Error bars show SEM for triplicate wells. In the right panel, exponentially growing cells were incubated for 12 h with 0.5% FCS. 7 × 10^3 cells were plated per 96 well in the presence or absence of growth factors for 30 h. TGF-β_1 was at 10 ng/ml, PDGF at 100 ng/ml, bFGF at 25 ng/ml, and EGF at 20 ng/ml. All growth factors were purchased from R&D Systems, Inc. (Minneapolis, MN). Wells were pulsed with 1 μCi [^{3}H]thymidine; incorporation was measured 12 h later. Each result is the mean for three replicate wells. Index of stimulation is equal to the average incorporation in the presence of growth factors/incorporation in 0.5% FCS alone. Similar results were found in eight repeated experiments.

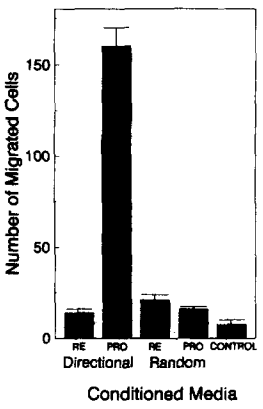

Figure 3. Chemoattraction of inflammatory cells by the 4102-PRO. Tumor cell-conditioned medium (1.5×10^6 cells/10 ml/24 h) was placed in the bottom of modified Boyden chambers. 2×10^5 PECs were added to the top of the chambers in either fresh CMEM (to test for directional movement) or the same conditioned media as in the bottom chamber (to control for random movement). Cells that had migrated through the 5-μm pores of the filter into the lower chamber were counted. (See Materials and Methods). The average (± SEM) of six fields from duplicate Boyden chambers is shown.

cells. Experiments are in progress to identify the chemoattractant(s) responsible fo this difference.

If non-T cell inflammatory cells were providing stimulatory factors, then removal of such cells might reduce tumor growth in nude mice. The Gr-1 antigen, defined by the RB6-8C5 monoclonal antibody, is highly expressed on mature granulocytes, transiently expressed on cells of the monocyte lineage, and absent from mature monocytes or macrophages (6–8). Pretreatment of nude mice with the antigranulocyte antibody (anti-Gr-1) resulted in a 94 ± 6% decrease in granulocytes 2 d after treatment as assessed by differential counts of peripheral blood smears. The subcutaneous growth of the PRO variant was significantly decreased by anti-Gr-1 treatment given every 3 d, while the growth of the parental RE tumor remained virtually unchanged (Fig. 4, *left*). Treatment of B6CByF1/J nude mice with anti-NK1.1 every 3 d did not affect tumor growth (Fig. 4, *right*), although spleen cells from these mice had a 94% decrease in NK lytic activity against YAC cells compared with spleens from nontreated mice.

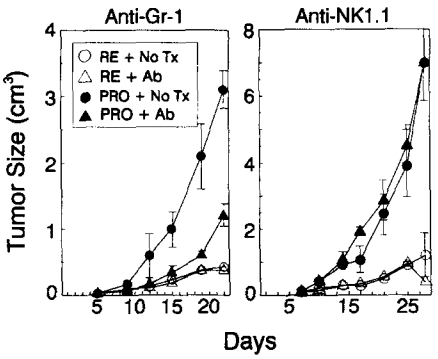

Figure 4. Anti-Gr-1 treatment reduces subcutaneous tumor growth of the 4102-PRO variant. 5×10^6 tumor cells were injected subcutaneously and tumor growth measured as described in Materials and Methods. (*Left*) Effect of anti-Gr-1 treatment. Mice were treated intraperitoneally with 200 μl anti-Gr-1 of ascites, every 3 d beginning on the day of s.c. tumor cell challenge. Multiple linear regression was used to relate tumor volumes to treatment variables. Three experiments were analyzed separately. In each case, treatment significantly decreased tumor growth (p = 0.005, 0.0012, and 0.0005, respectively.) (*Right*) Treatment of B6CByF1/J nude mice with anti-NK1.1 had no significant effect on tumor growth. Mice were treated intraperitoneally with 0.2 mg purified NK1.1. For both panels, each line represents the average tumor size for three to four mice per experiment ± SEM.

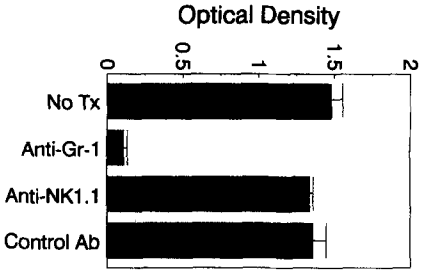

Figure 5. Anti-Gr-1 antibody treatment results in reduced outgrowth of 4102-PRO tumor cells recovered from the peritoneal cavity. 200 μl of anti-Gr-1 ascites (~0.4 mg IgG) or an isotype control ascites was injected intraperitoneally into athymic nude mice 2 d before tumor challenge. 0.2 mg NK1.1 was injected into B6CByF1/J nude mice. 5×10^6 tumor cells were injected intraperitoneally into naive or antibody-treated mice. After 3 d T-PEC were lavaged in 5 ml PBS and resuspended in 12 ml complete MEM. Recovery of liquid was always greater than 97%. 0.5 ml of cell suspension was plated per well of a 24-well plate. An MTT assay was done after 10 to 12 d in culture. Methods were as described in Materials and Methods. Data are pooled from two to four experiments ± SEM.

To measure the effect of anti-Gr-1 treatment on early tumor cell growth independent of vascularization, 4102-PRO tumor cells were recovered after growth in the peritoneal cavity for only 3 d. Fig. 5 shows that the recovery and subsequent outgrowth of 4102-PRO tumor cells from the peritoneal cavities of mice were significantly decreased when mice were pretreated with anti-Gr-1. Pretreatment with either an isotype control antibody or the anti-NK1.1 antibody had no effect. We do not know the absolute numbers of tumor cells recovered from the peritoneal cavities since the cells tend to form aggregates, which are difficult to break up. Using the MTT assay allows for reliable quantification of viable tumor cells whether in clusters or dispersed. The MTT metabolism by nonmalignant PEC in the cultures must be negligible since only tumor cells remain viable after the 10 to 12-d culture period. It was essential to determine whether anti-Gr-1 actually inhibited granulocyte induction in the peritoneal cavity after tumor cell challenge. PEC washed out of naive mice had no detectable neutrophils or eosinophils, 27% lymphocytes, 71% macrophages/monocytes, and 2% basophils. 4 h after injection of tumor cells, ~36% of the PEC were neutrophils. However, PEC of tumor-injected mice pretreated with the anti-GR-1 antibody 2 d earlier had <1% neutrophils (little change was detected in the small number of basophils or eosinophils).

Discussion

Findings in this study strongly suggest that Gr-1$^+$ cells are required for the rapid growth of the 4102-PRO variant in vivo. However, it is not clear which cell type(s) is directly responsible for the stimulation. Both granulocytes and macrophages are known to produce growth factors, and both are present in the peritoneal lavage surrounding PRO tumor cell clusters. Pretreatment of mice with the granulocyte-specific anti-Gr-1, and not an isotype control antibody, led to a large decrease in the number of peritoneal macrophages that could be induced with thioglycolate (Pekarek, L., unpublished data).

This is supported by previous work in the mouse or rat showing that neutrophil depletion leads to an inhibition of macrophage recruitment (9) or other mononuclear cells in delayed-type hypersensitivity (10). Therefore, granulocytes may be stimulatory themselves, or may be involved in the recruitment of other stimulatory cells. Granulocytes have been found to produce factors that are chemotactic for macrophages (11). In any event, it appears that the PRO variant attracts host leukocytes that in turn provide factors to stimulate tumor growth. We do not know if this type of stimulatory loop occurs frequently in tumor progression, but we have made similar findings for a second randomly selected progressive tumor variant that also retains CTL-recognized antigens (data not shown), suggesting that this stimulatory loop may not be rare.

An optimal growth factor environment may play a key role in tumor progression. Some tumors may provide factors through an autocrine mechanism, while other tumors may depend on host factors. It is known that certain tumors preferentially spread to specific tissues. For example, human prostatic cancer most often metastasizes to bone, and two prostatic carcinoma cell lines were stimulated by bone marrow–conditioned media (12), an effect attributed to transferrin (13). Similarly, human melanomas may be stimulated by factors released by normal dermal fibroblasts (14), and certain murine B cell tumors depend on stimulation by specific $CD4^+$ T cells for growth in vivo (15, 16).

Tumor cells that do not migrate to a more stimulatory environment may undergo heritable changes that enable them to attract host cells that provide growth factors. We found stimulation of the 4102-PRO variant by several growth factors, including TGF-β, which is growth inhibitory to some tumors, including the parental 4102-RE, but stimulatory for other tumor cells (5). TGF-β is secreted by I-PEC (Pekarek, L., unpublished results) and has been shown to be produced by both monocytes and granulocytes (17–19). We do not know if the other factors that stimulated 4102-PRO growth in vitro are secreted by the PEC, although PDGF and bFGF have been found to be secreted by peritoneal macrophages (20, 21). Granulocytes can produce platelet-activating factor (PAF) (22, 23), which stimulates platelets to produce TGF-β and PDGF (24). In addition, PDGF has been shown to induce the expression of MCP-1, a potent monocyte chemoattractant (25–27). Furthermore, 4102-PRO produces latent TGF-β (28) which, if activated in vivo by macrophages, could stimulate the growth of 4102-PRO. Finally, the previously described less-effective immunological rejection of 4102-PRO tumor cells embedded in syngeneic stroma (29) may be the result of the local growth-promoting environment of the stroma. Resolving the local mechanism involved in paracrine stimulation of tumor growth will be complicated by the fact that not only multiple cell types and cytokines but also the extracellular matrix may contribute significantly (for review, see reference 30).

Several previous experiments have suggested that granulocytes can have cytocidal activity in vitro and antitumor activity in vivo against murine cancers (7, 31–35) even though human cancer cells are remarkably resistant to oxidative cytolysis (36). However, the granulocytes studied had been induced by bacteria, chemicals, or recombinant proteins, or by cytokines released from gene-transfected tumor cells (7, 31–35). By contrast, naive granulocytes and macrophages may be tumor stimulatory. This is in agreement with our experiments showing that naive PEC or tumor-induced PEC stimulate 4102-PRO and not the 4102-RE tumor cells, while the same PEC suspension is tumoricidal to both the RE and PRO tumor cells when first activated by cytokines in vitro (Pekarek, L., and H. Schreiber, unpublished results). Thus, for controlling certain leukocyte-dependent cancers, the tumor-infiltrating leukocytes must either be activated to become tumoricidal, or the naive stimulatory leukocytes must be eliminated. In agreement with the latter notion is our recent finding that anti-Gr-1 treatment alone can lead to complete tumor rejection in normal mice (rather than the mere growth inhibition observed here in nude mice). Granulocytes may, therefore, not be required for an effective T cell response in this tumor model (Pekarek, L., and H. Schreiber, manuscript in preparation).

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