Advances In Brief

Modification of the Growth Rates and Hypoxic Fractions of Xenografted A431 Tumors by Sialoadenectomy or Exogenously Supplied Epidermal Growth Factor

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Abstract

We studied A431 epidermoid carcinomas xenografted into male nude mice either in the unperturbed state or after either surgical removal of the salivary glands or i.p. injection of exogenous epidermal growth factor (0.2 mg/kg daily for 7 days). The percentage of hypoxic cells in unperturbed tumors was 10.5% (95% confidence limits, 6.6-16.8%). In mice that received epidermal growth factor injections, hypoxic percentages decreased to 3.7% (1.7-7.8%), and tumor growth rates increased. In sialoadenectomized mice, hypoxic percentages increased to 35.2% (27.1-45.6%), and tumor growth rates decreased. These data indicate that the biology of solid tumors can be significantly modified by the host status.

Introduction

In 1989, Inui et al. (1) showed that surgical removal of the submaxillary glands in mice bearing transplanted mammary cancers decreased tumor growth rate. This inhibitory effect could be reversed by supplying exogenous EGF.

Ozawa et al. (2) showed that growth rates of A431 human epidermoid xenografts could be markedly enhanced by EGF supplied by implanted osmotic pumps. These two sets of data indicate that the volumetric behavior of a solid neoplasm can be dramatically influenced by the changes in the physiological status of the host (e.g., circulating growth factor levels). Because of these results on growth dynamics, it appeared logical to us that such a modulation might also have an impact upon other end points of neoplastic physiology. Therefore, we chose to assay another important feature of solid tumors, i.e., the percentage of hypoxic cells. To our knowledge, there are no experimental data directly addressing the question of whether alterations in host growth factor status could affect the level of intraneoplastic hypoxia. Therefore, we investigated whether the growth rates or hypoxic percentages of xenografted A431 solid tumors could be modified by altering the EGF status of the host by either sialoadenectomy or i.p. injection of added exogenous EGF.

Materials and Methods

Cells. Because Ozawa et al. have shown that xenografted A431 solid tumors mice respond via increased growth rates to exogenously supplied EGF, we used A431 cells obtained from the American Type Culture Collection (Rockville, MD). These cells were routinely grown in RPMI 1640 containing 10% fetal bovine serum, 1% sodium bicarbonate, 1% anti-PPLO reagent, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 0.04% gentamicin (all reagents from the Grand Island Biological Co., Grand Island, NY). Cultures at approximately passage 25 were used.

Animals and Transplantation Procedures. Male nude mice (nu/nu genotype, 7-10 weeks of age) were obtained from the Charles River Breeding Laboratories, North Wilmington, MA. They were housed 5/large cage (with dustcovers) in a laminar flow hood (Thoren Industries, King of Prussia, PA) in a dedicated room in the Animal Care Facility of Brown University. Animals were quarantined for 1 week. For tumor cell implantation (1 tumor/animal), 1 x 10^7 cells from exponentially growing A431 cell cultures were injected into the right flank regions in a volume of 0.25 ml of HBSS.

Surgical Procedures and Growth Factor Administration. The surgical procedure has been described by Inui et al. (1). Briefly, mice were anesthetized using i.p. sodium pentobarbital (50 mg/kg), and a midline ventral neck incision was made. The submandibular glands were removed bilaterally, and the incision was closed using wound clips. Sham injections were done using HBSS.

For EGF administration, human recombinant EGF was obtained from Grand Island Biological Co. and was reconstituted from frozen stock in HBSS. EGF was administered i.p. at a rate of 0.2 mg/kg/day in a volume of 0.2 ml of HBSS daily for a period of 1 week. Sham injections were done using HBSS. Sham operations were performed in which the anesthesia, incision, and closure steps were carried out without removal of tissue.

Tumor bearing control animals received both sham injections and sham surgical procedures.

Volumetric Procedures. Tumors were measured in two orthogonal diameters using methods that we have previously published (3), and volumes (mm^3) were calculated. All measurements were made by a single individual. After injection, tumors were monitored until average tumor sizes were about 8 x 8 mm, at which time animals were randomly assigned to the sialoadenectomy, sham surgery, or control groups.

Irradiation Procedures. A Philips 250-kV X-ray machine (Philips, Ltd., Eindhoven, The Netherlands), operated at 250 kVp and 15 mA, was used to irradiate tumors. Exposure doses were measured using a Victoreen R-meter (Victoreen Co., Cleveland, OH), and absorbed doses were calculated using appropriate temperature, pressure, and rogent to Gy conversion factors. The absorbed dose rate was about 1 Gy/min.

Excision Assay Procedures. For determinations of hypoxic fractions, tumors were irradiated in either air-breathing, unanesthetized mice or mice that had asphyxiated by a 10-min exposure to nitrogen gas prior to exposure. For irradiations, the air-breathing mice were briefly anesthetized with Metofane (methoxyflurane; Pitman-Moore, Inc., Washington Crossing, NJ) and restrained on a Lucite irradiation platform. Animals were allowed to fully recover and were irradiated at room temperature.

For determination of survival of clonogenic cells by excision assay, we delivered graded doses of 0-25 Gy to oxic or hypoxic control tumors. In the sialoadenectomized or EGF treated mice, doses from 10-25 Gy were given. Immediately after irradiation, neoplasms were excised under sterile conditions, quartered, placed into ice-cold HBSS,
and weighed. Then the pieces were minced using opposed scalpel blades into approximately 1-mm³ fragments and placed into an enzyme cocktail containing 0.2% RNase free DNase (Sigma Chemical Co., St. Louis, MO), 0.25% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 0.25% neutral protease (Calbiochem Corp., San Diego, CA) in RPMI 1640 (minus FBS). Tumor fragments were digested for 40 min at 37°C in a stirred 250-ml trypsinizing flask. The digestion product was filtered through an 80 μm rectangular stainless steel mesh and pelleted (after addition of an equal volume of cold RPMI 1640 with FBS) at 1000 rpm for 10 min at 4°C. The pellet was resuspended in RPMI 1640 with FBS, tumor cells were counted using phase contrast microscopy, and appropriate numbers of cells were then seeded into 60- or 100-mm-diameter plastic dishes (B-D Labware, Trenton, NJ) at several dilutions for enumeration of survival by colony formation. Heavily irradiated (30 Gy, 137Cs γ-rays; J. L. Shepard Model 68A Irradiator, Glendale, CA) A431 feeder cells were added to all dishes to keep a minimum cell number of 10⁵ cells/dish. Colonies were fixed and stained with 0.5% crystal violet in absolute methanol. Colonies were inspected microscopically to ensure that no counting bias was incurred by the presence of giant cells. Excision assays were performed 8–13 days after commencement of respective treatments.

For the purposes of comparison, we also assayed the survival characteristics of A431 cells in vitro irradiated with graded single X-ray doses while in a fed plate state. A431 cells were allowed to reach confluency in T-25 plastic flasks with daily medium changes. Cells were then irradiated, trypsinized, dissociated into single cells, and plated in vitro for assessment of clonogenic survival as described above.

Results and Discussion

Volumetric growth responses are shown in Fig. 1. Tumors were measured after implantation until they reached an average size of 278.2 mm³ (95% confidence limits, 233.7–331.1 mm³) at which time they were randomly assigned to control, EGF supplemented, or surgery groups (day 0 in Fig. 1). Daily EGF injections produced increased growth as compared to that of unperturbed tumors. In the sialoadenectomized group, tumor volume continued to increase for about 1 day after surgery. Then, the volume decreased for about another day, after which growth increases, but at a slower rate than that of controls.

As an index of effect, the time taken for tumors in the 3 groups to reach twice their relative volume on day 0 was about 4.2 days in controls, 2.0 days in the EGF supplemented mice, and 7.8 days in the sialoadenectomized mice. Therefore, EGF increases growth by a factor of about 2.1, while sialoadenectomy decreases growth by a factor of about 0.5, i.e., roughly about a 100% change in either direction.

A summary of the excision assay data is presented in Table 1.

Confidence limits on HF were determined from the limits on log (S₀) — log (Sₜ) at a given dose (4). The clonogenic survival responses shown in Table 2. The dose-response curves for the cells from the various conditions (i.e., hypoxic, oxic, oxic plus EGF, oxic plus surgery) are parallel, fulfilling the requirement for determination of hypoxic fractions (4). Using the relative survivals at 20 Gy, the calculated hypoxic fractions were, respectively, 0.105 (0.066–0.168), 0.352 (0.271–0.457), and 0.037 (0.017–0.078) for unperturbed, sialoadenectomized, or EGF treated tumors (numbers in parentheses are 95% confidence limits), indicating that the hypoxic fraction was decreased by the EGF supplementation and increased by the surgical treatment.

These data indicate that manipulation of the host can affect both the volumetric growth and extent of hypoxia within solid tumors. The changes in volumetric growth recapitulate the previous results of Inui et al. (1) and Ozawa et al. (2). To our knowledge, however, this work represents the first demonstration of hypoxic fraction modulation by growth factor related mechanisms. The changes in growth rate and extent of hypoxia are roughly similar, being about 100% for both end points (growth rate, hypoxic percentage) for both types of manipulations (EGF, sialoadenectomy). It should be noted, however, that Robinson et al. (6) were unable to demonstrate changes in volumetric growth of either C3H mammary tumors or xenografted A431 epidermoid cancers after sialoadenectomy or EGF administration by osmotic pumps. The reason for the differences between the results of Robinson et al. (6) and the data presented by Inui et al. (1), Ozawa et al. (2), and herein is not known. However, a recent report by Yoneda et al. (7) has demonstrated that sialoadenectomy in nude mice bearing a
human squamous cell carcinoma (MH-85) which overexpresses the EGF receptor decreases tumor growth rate as described by Inui et al. (1), and this effect can be overcome by administration of i.p. EGF (5 μg/mouse every other day for a duration of 42 days) as described by Ozawa et al. (2).

Our results suggest that other manipulations of growth factor status might also affect intratumor hypoxia. For example, Gross et al. (8) have shown that daily injection treatments of nude mice bearing DLD-2 human colon carcinomas with FGF-β at a rate similar to that used in our studies (i.e., 0.25 mg/kg) results in increased tumor growth and tumor vascularization. Generalizing from our EGF results (Figs. 1 and 2), FGF-β treatment might then cause decreased hypoxic fractions in DLD-2 tumors. However, the unperturbed in vivo hypoxic status of the DLD-2 tumor has not been studied. Conversely, the data of Gross et al. (8) suggest that changes in hypoxic fractions of A431 tumors (Fig. 2) are mediated through effects on tumor vasculature.

The changes seen in hypoxic fraction as correlated with tumor growth kinetics in the A431 neoplasms are similar to changes previously shown for xenografted human or rodent tumors growing in X-irradiated sites where the vasculature has been damaged; i.e., hypoxic fractions of such tumors are typically increased, and growth rates are decreased as compared to unperturbed controls (9-11).

These data may have therapeutic implications. If decreasing the supply of mitogenic/angiogenic substances (e.g., EGF, FGF-β) to tumor parenchyma and/or to tumor vasculature increases in vivo hypoxic percentages, then agents such as suramin (12, 13) or monoclonal antibodies (7) against EGF receptors on tumor or vascular endothelial cells might be contraindicated. Based on the data presented herein, such treatment would slow tumor growth which might then be associated with an increased hypoxic fraction, leading to a worse clinical response to cytotoxic treatment (i.e., radiation therapy).

References

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