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Immunization with a Tissue Vaccine Enhances the Effect of Irradiation on Prostate Tumors

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Abstract. *Recent studies on immunotherapy as a means to treat prostate cancer presents an interesting opportunity for combination therapy with radiation. This work was undertaken to determine if immunization with a prostate cancer tissue vaccine could enhance the effect of radiation treatment. Groups of Lobund-Wistar (LW) rats were subcutaneously administered PAIII prostate cancer cells (Day 0). Tumor-bearing rats were then either left untreated, vaccinated on Days 11 and 14 with a vaccine composed of glutaraldehyde-fixed harvested tumor tissue (GFT), irradiated with 15 Gy on Days 21 and 23 (Irr), or vaccinated with the GFT vaccine on Days 11 and 14 and irradiated on Days 21 and 23 (GFT/Irr). The tumors were measured on Day 11 and at the time of euthanasia (Day 30) and tumor volume was calculated. On Day 30, the tumors were harvested, weighed and fixed for histopathological evaluation. The mean tumor weight was significantly less ($p \leq 0.05$) in the Irr rats (7.21 g) compared to the untreated (13.04 g) and GFT-treated (10.64 g) rats. In contrast, the mean tumor weight of the GFT/Irr rats (3.37 g) was significantly less than the untreated and the GFT-treated tumors ($p \leq 0.001$), as well as the Irr tumors ($p \leq 0.01$). The GFT/Irr rats were the only group in which the tumor volume decreased during the study (14% decrease) compared to the untreated (147% increase), the GFT-treated (115% increase), and the Irr (12% increase) rats. Immunization with a tissue vaccine prior to radiation treatment enhances the therapeutic effect of radiation.*

Cancer of the prostate gland is the most commonly diagnosed cancer among men and the second most common cause of cancer death, on an age-adjusted basis (1). Radiation therapy has been used extensively in the treatment of prostate cancer in three fundamentally different settings: as the primary mode of treatment with or without hormonal therapy (2-4); in the adjuvant setting for patients whose prostatectomy specimens demonstrate features indicating a high risk for recurrence (5-7); and following a rise in serum prostate specific antigen (PSA) in patients initially treated with a radical prostatectomy (8-10). While cure rates with radiation therapy alone are satisfactory for patients with low-risk features, no treatment is truly satisfactory for patients with high-risk disease (11-14).

One possible means of enhancing treatment for prostate cancer might be to combine radiotherapy with immunotherapy. The advantage of this approach is that focused localized therapy is provided and then adjuvanted with a method to target residual tumor tissue. External beam radiation has been demonstrated to alter the phenotype of murine MC38-CEA cells *in vitro* and make them more sensitive to cytotoxic lymphocyte-mediated lysis (15). Furthermore, external beam radiation has been shown to alter the phenotype of established MC38-CEA tumors *in vivo* as manifested by up-regulation of the death receptor Fas, and combined radiation therapy and vaccination with a recombinant poxvirus vaccine resulted in an infiltration of lymphocytes into the tumor not seen with either treatment alone (16, 17). In a study evaluating the use of vaccination with a recombinant poxviral vaccine expressing carcinoembryonic antigen (CEA), anti-CD25 monoclonal antibody treatment to diminish T lymphocyte-associated immunotolerance, and the co-stimulatory molecules lymphocyte function-related antigen-3 (LFA-3), B lymphocyte activation antigen B7-1, and intercellular adhesion molecule 1 (ICAM-1), it was demonstrated that established tumors could be eliminated only when vaccination was combined with radiation treatment (18). A randomized phase II clinical trial combining external beam radiation with a priming vaccine with recombinant vaccinia PSA plus recombinant vaccinia containing the T-cell co-stimulatory molecule B7.1 followed by monthly booster vaccines with recombinant fowlpox PSA resulted in at least

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Table I. *Grading scheme for cytoplasmic and nuclear radiation effect.*

Cytoplasmic changes	0	−No identifiable RT effect
	1	−Swelling and microvesicular change
	2	−Extensive vacuolation including macrovesicular changes, voluminous cytoplasm
	3	−Indistinct or ruptured cytoplasm, lipofuscin pigment accumulation Glands, when present, are dilated, often just single cell with no glandular formation
Nuclear changes	0	−No identifiable RT effect
	1	−Some swelling or smudging of nuclei but nucleoli still identifiable
	2	−Smudged and distorted chromatin −Nucleoli rare of absent −Large bizarre nuclei
	3	−Pyknotic, small, condensed nuclei

RT: radiotherapy.

3-fold increases in PSA-specific T-cells in 17 out of 19 patients *versus* non-detectable increase in patients receiving only radiotherapy (19).

Cancer vaccines composed of whole cancer cells offer a wide range of antigenic targets to the immune system. Michael *et al*. (20) investigated the utility of allogeneic whole cell prostate cancer vaccination. They reasoned that because tumor antigens are often conserved between tumors, allogeneic vaccines might stimulate cross-protective immunity (20). While the use of cultured monoclonal cells as cancer vaccine components have shown some utility, an even broader range of antigenic targets can be expected in vaccines prepared from harvested tumor tissue. Such vaccines would include targets not only limited to the neoplastic epithelial cells, but also those associated with the tumor stroma.

We have shown that a tissue vaccine composed of prostate tumor tissue harvested from Lobund-Wistar rats reduced the incidence of prostate cancer by 90% in syngeneic individuals (21). Furthermore, this same vaccine reduced the incidence of human PC-346C prostate tumors in xenogeneic mice by 70% when PC-346C cells were co-incubated with splenocytes from vaccinated immunocompetent mice and then administered orthotopically into the prostate glands of immunodeficient nu/nu mice (22). The study described here is an extension of that work and was undertaken to evaluate the utility of immunization with a tissue vaccine as an adjuvant to radiotherapy of prostate tumors.

Materials and Methods

Animals. The three- to four-months-old male Lobund-Wistar (LW) rats used in this study were obtained from the LW rat breeding colony at the University of Notre Dame. The rats were housed in polycarbonate cages provided with hardwood shavings. A natural ingredient diet, Teklad L-485 (Harlan Teklad, Inc., Madison, WI, USA) and fresh water were provided *ad libitum*. At the end of the study, the rats were euthanized by an overdose of inhaled carbon dioxide. All the animal studies were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. The studies were approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Generation of tumors. PAIII prostate adenocarcinoma cells from a passaged tumor were used to generate subcutaneous tumors. This cell line was originally isolated at the Lobund Institute of the University of Notre Dame from an autochthonous, metastatic prostate adenocarcinoma in a LW rat (23) and has been maintained as a passaged tumor line in LW rats. Cells were harvested from a subcutaneous tumor in a LW rat and mechanically separated by mincing with a scalpel blade followed by vigorous aspiration and rinsing with Modified Eagle's medium (MEM) using a 22-gauge hypodermic needle attached to a syringe. The LW rats were then administered 0.3 ml of cell suspension subcutaneously into the flank. This method reliably produces subcutaneous prostate tumors in 10-14 days.

Vaccination of rats. A glutaraldehyde-fixed tumor (GFT) tissue vaccine was prepared as previously described (21). Briefly, the vaccine was produced by harvesting from a LW rat, 3 g of a subcutaneous tumor produced by administration of $1x10^6$ PAIII cells 14 days prior to harvest. The tissue was finely minced, and the cells separated using an 80-mesh screen to create a cell suspension in MEM. The cell suspension was incubated in 2.5% glutaraldehyde (v/v) at 37˚C for 60 min and then washed thoroughly with medium to produce the GFT cell vaccine. One group of six tumor-bearing LW rats was vaccinated subcutaneously with a 50:50 mixture of the vaccine with complete Freund's adjuvant (CFA) for the first dose and incomplete Freund's adjuvant for the booster dose. Each dose consisted of $5x10^6$ GFT cells with adjuvant. The rats were vaccinated twice, 7 days apart, with the first dose being given when subcutaneous tumors were palpable, 11 days after the PAIII tumor cells had been administered to the rats. The vaccine dose was chosen based upon the author's experience with the GFT cell vaccine in rats (21).

Experimental design. Day 0 was defined as the day when the rats were administered the tumor cells. On Day 11, when all the rats had palpable subcutaneous tumors, they were divided into groups which underwent either no treatment, two doses of irradiation on Days 21 and 23, vaccination with a tissue vaccine on Days 11 and 14, or vaccination with a tissue vaccine on Days 11 and 14, followed by irradiation on Days 21 and 23. Each irradiation treatment consisted

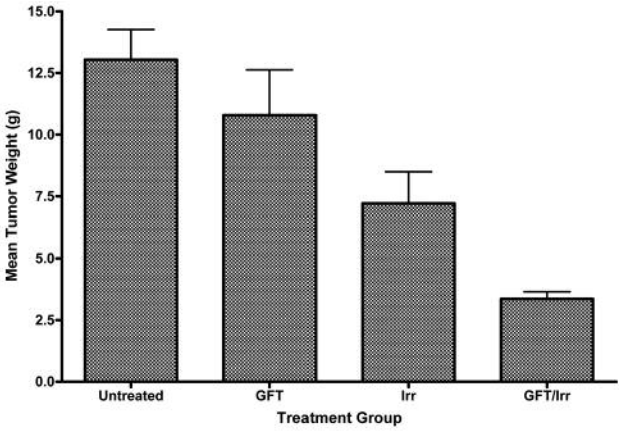

Figure 1. *Mean tumor weight in grams at Day 30. Rats were administered PAIII cells on Day 0 and were either: left untreated; vaccinated with the GFT cell vaccine (GFT) twice, 7 days apart on Days 11 and 14; irradiated twice, 3 days apart on Days 21 and 23 (Irr); or vaccinated and irradiated (GFT/Irr). Bars represent standard deviation. Tumors from Irr rats weighed significantly ($p \leq 0.05$) less than those from untreated and GFT rats and tumors from GFT/Irr rats weighed significantly less than tumors from untreated and GFT rats ($p \leq 0.001$) and Irr rats ($p \leq 0.01$).*

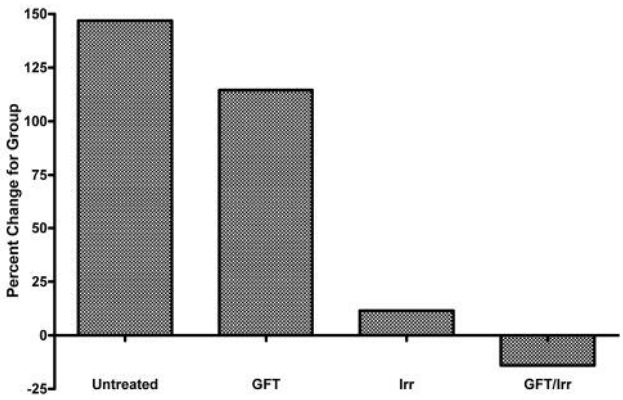

Figure 2. *Percentage change in tumor volume from immediately before any rats were vaccinated (Day 11) until the termination of the study (Day 30). Rats were either: left untreated; vaccinated with the GFT cell vaccine (GFT) twice, 7 days apart, on Days 11 and 14; irradiated twice, 3 days apart, on Days 21 and 23 (Irr); or vaccinated and irradiated (GFT/Irr). Tumor volume was calculated using the formula, tumor volume=$1/2ab^2$, where a is the larger and b is the shorter diameter (mm).*

of a dose of 15 Gy delivered with 9 MeV electrons produced by a clinical Varian 21 EX accelerator (Varian Medical Systems, Inc., Palo Alto, CA, USA) with 1.0 cm bolus and the dose specified at the 90% line using a Cerrobend block (Cerro Metal Products, Bellefonte, PA, USA) to treat a 3 cm area to encompass the tumor with a 1 cm margin. The tumors were measured, by an individual blind to treatment group, using a caliper in all the rats prior to vaccination and at the time of euthanasia, on Day 30. Tumor volume was estimated using the equation, tumor volume=$1/2ab^2$, where a is the larger and b is the shorter tumor diameter (mm) (24). At the time of necropsy, the tumors were excised, weighed, and fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin, sectioned at 4-5 μm and stained with hematoxylin and eosin. A board-certified pathologist (MY) graded the tissues for histological conformation, blind to treatment group, using a modification of an established scoring system for prostate tissue following external beam radiation (Table I) (25, 26). The differences in the mean tumor weights were evaluated for significance between groups using one-way analysis of variance with significance reached when $p \leq 0.05$.

Results

Effect on tumor size. As shown in Figure 1, the mean tumor weights were significantly ($p \leq 0.05$) less in animals which underwent radiation therapy only (7.21 g), compared to untreated (13.04 g) and GFT-vaccinated (10.64 g) rats. The mean tumor weight of the rats which underwent combined GFT-vaccination and radiation therapy (3.37 g) was significantly less ($p \leq 0.001$) than the untreated and GFT-vaccinated rats, as well as the rats which underwent radiation therapy only ($p \leq 0.01$). Furthermore, as shown in Figure 2, the only group in which the tumor volume decreased over the course of the study was the vaccinated-and-irradiated group (14% decrease), compared to the untreated group (147% increase), the vaccinated-only group (115% increase), and the irradiated-only group (12% increase).

Histological evaluation. In the untreated control animals, the tumors were high-grade carcinosarcoma with mixed carcinomatous and sarcomatous components (Figure 3). Poorly differentiated adenocarcinoma was present as irregular cords, clusters, and poorly-formed glands. The carcinoma cells had basophilic cytoplasm, pleomorphic nuclei with a high nuclear:cytoplasmic ratio, enlarged nuclei and prominent nucleoli. Mitotic figures were observed, with up to 34 mitoses/per 10 high power fields (34/10 HPF) and with scattered atypical mitotic figures. Tumor necrosis was present in the center, probably representing anoxic tumor necrosis. In the rats which underwent GFT vaccination only, there were minimal degenerative changes, but without obvious decreases in number and size of the carcinomatous glands. The mitoses count was 28 per 10 high power fields (28/10 HPF) which was decreased as compared with the control group. In the radiation therapy-only group, there were cytoplasmic and nuclear changes in the tumor which were more prominent in the carcinoma component (Figure 4). There was a decrease in the size and number of malignant glands and poorly-formed glands. The rats which underwent GFT-vaccination followed by radiation therapy had tumors characterized by marked treatment response, including decreased number and size of malignant glands, cytoplasmic and nuclear degeneration, and coagulative tumor necrosis (Figure 5), though focal areas of viable tumor were still present.

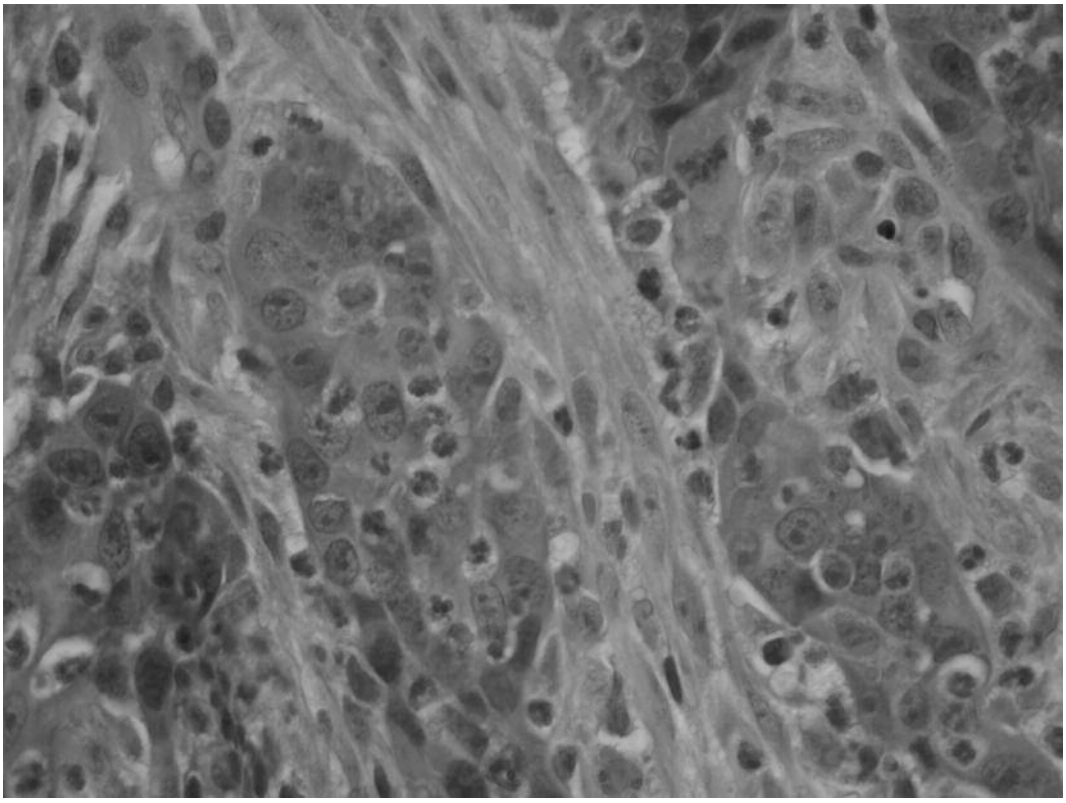

Figure 3. *Photomicrograph of a tumor from an untreated control rat. The tumor is characterized as a carcinosarcoma with mixed carcinomatous and sarcomatous components. Magnification x400.*

The tumors from the rats which underwent radiation treatment only had cytoplasmic changes which were scored 1-2 and which including swelling, microvesicular change, and focal extensive vacuolation and macrovesicular change. Nuclear changes in those rats were also scored 1-2 and included some swelling or smudging of nuclei and distorted chromatin, though identifiable nucleoli were still present. In the rats which underwent GFT-vaccination plus radiation treatment, the cytoplasmic effect was scored 2-3 and included macrovesicular changes, the presence of voluminous cytoplasm, indistinct cytoplasm, and reduced glandular formation. The nuclear change was scored 2-3 and demonstrated smudged chromatin, rare nucleoli, large bizarre nuclei, and pyknotic nuclei.

Discussion

Improvements in the care of locally advanced or recurrent prostate cancer might be achieved through combination therapy. For example, radiation therapy combined with vaccination with a recombinant poxvirus vector expressing PSA resulted in responses suggestive of immune-mediated killing of tumor cells as evidenced by the generation of PSA-specific T-cells in the patients (19). This result stands in contrast to studies showing that radiotherapy can decrease non-specific immune responses (27, 28).

There exist neither clinical nor pre-clinical reports examining the effects of combined radiation treatment and vaccination on the growth of prostate tumors. In the present study, combined immunization with a tissue vaccine and radiation treatment significantly reduced the growth of prostate tumors. Furthermore, the rats which underwent combined therapy were the only group in which a decrease in tumor volume occurred over the course of the study. The histological grading using an established system for irradiated tumors showed that the rats which underwent the combined treatment typically had greater degrees of tumor degeneration compared to the rats which underwent only radiation treatment. Taken together, these data demonstrate an additive benefit for treatment of prostate tumors with radiation therapy combined with immunization using a tissue vaccine.

Vaccines for prostate cancer have received substantial recent interest. A number of studies have examined the utility of vaccines composed of prostate specific antigen (PSA), or allogeneic whole prostate cancer cells (20, 29). A phase I clinical trial demonstrated T- and B-lymphocyte responses against autologous tumor antigens in patients vaccinated with the GVAX® prostate cancer vaccine (Cell Genesys, Inc.,

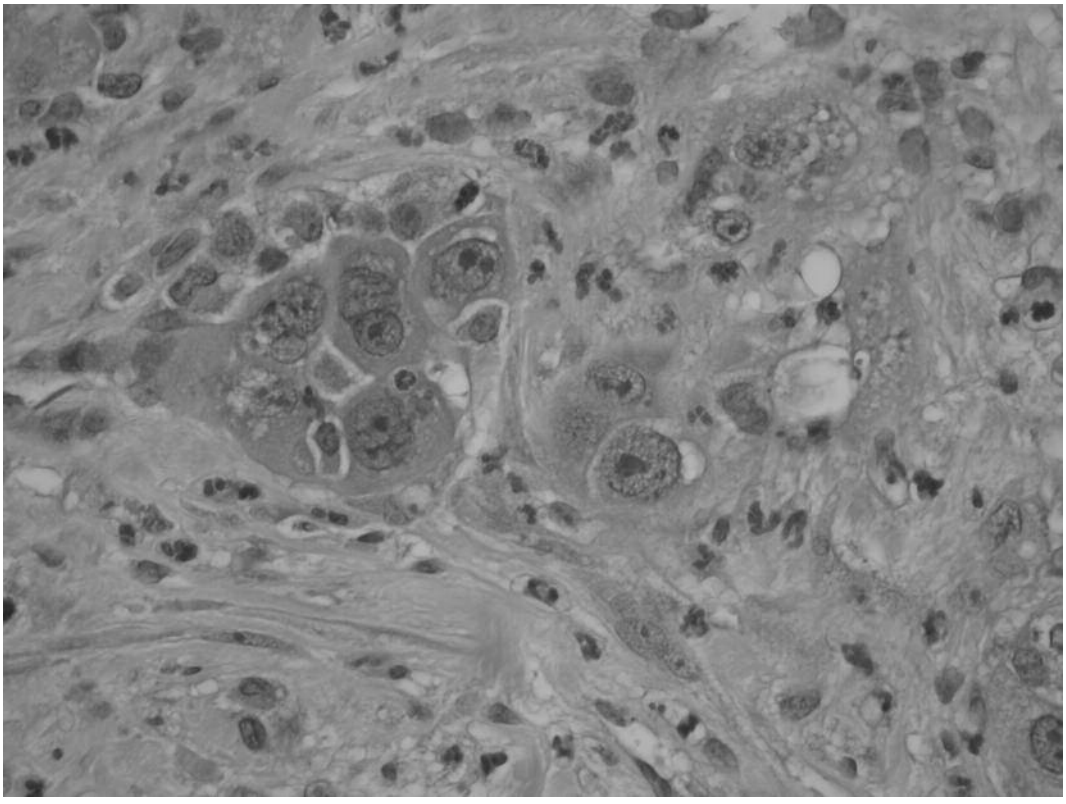

Figure 4. *Photomicrograph of a tumor from a rat treated with radiation on Days 21 and 23 (Irr). Compared to untreated controls, tumors from this group were characterized by swelling of cytoplasm and nucleoli, and had a decrease in the size and number of glands. Magnification x400.*

South San Francisco, CA, USA), a preparation composed of two irradiated, cultured allogeneic human prostate cancer cell lines which were engineered to secrete granulocyte macrophage-colony stimulating factor (GM-CSF) (30). In a follow-up phase I/II trial, the patients showed significantly reduced PSA velocity, dendritic cell and macrophage infiltrates at the vaccination sites, and antibody responses to at least five antigens present in the vaccine cells (31).

Tissue vaccines are derived directly from tumors and include neoplastic cells as well as connective tissue and stromal matrix, and, thus, present a very rich antigenic menu to the immune system. Furthermore tissue vaccines include antigens expressed following *in vivo* growth *versus* the more limited antigenic profile of cultured cells. For example, cultured renal carcinoma cells showed reduced expression of a variety of genes, including some known to be tumor-associated antigens (32). Similarly, gene expression profiling of human A549 lung adenocarcinoma cells grown in immunodeficient mice demonstrated selective induction and overexpression of genes important in tumor progression compared to cells grown *in vitro* (33). In a syngeneic system, we previously showed the GFT vaccine to reduce the incidence of prostate cancer by 90% (21) and to decrease by 70% the incidence of prostate cancer in a xenogeneic system (22).

Targeted radiation induces cell destruction by mechanisms including apoptosis (34). Furthermore, tumor cells are sensitized to antigen-specific cytotoxic lymphocyte attack by the up-regulation of Fas following radiation (15). Along with evidence that local external beam radiation combined with vaccination induced a specific T-cell response it is reasonable that refinement of this approach may yield an effective combined therapy for prostate cancer (19, 35). Because of their broad antigenic repertoire, tissue vaccines may represent a logical complement to radiation therapy as suggested by the data.

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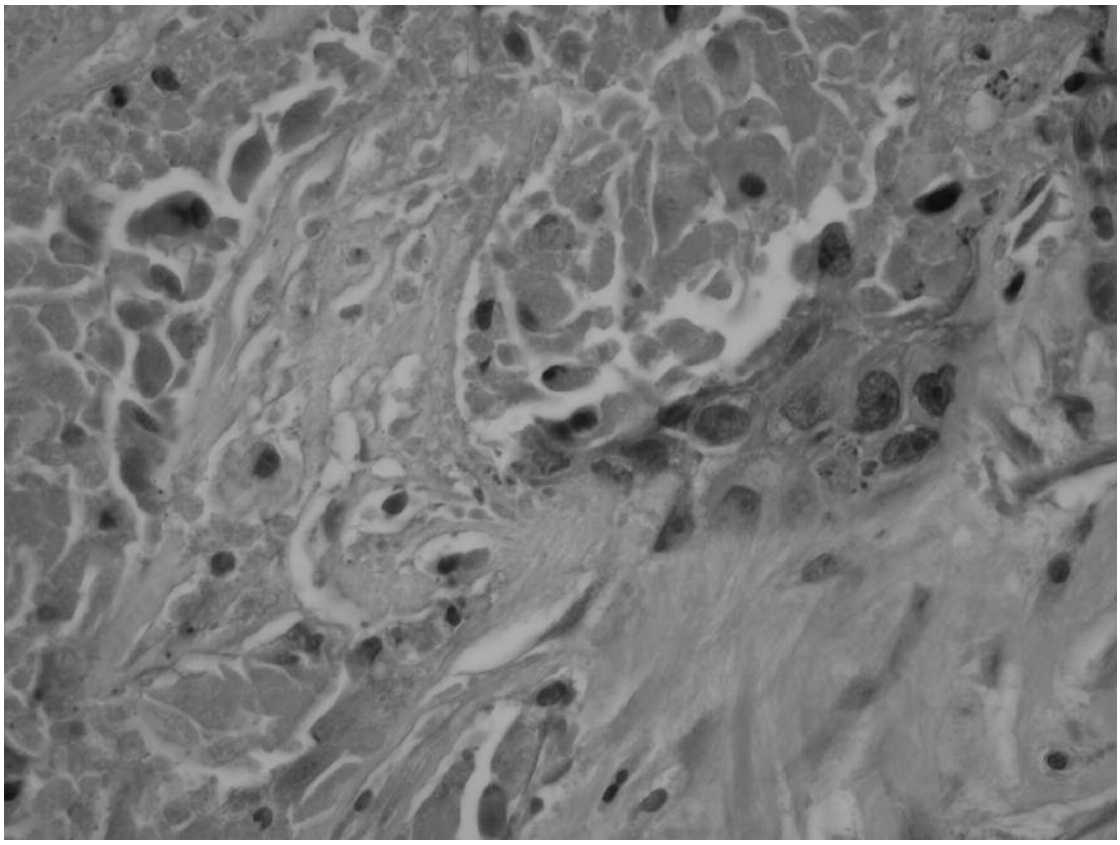

Figure 5. *Photomicrograph of a tumor from a rat treated with the GFT cell vaccine on Days 11 and 14 and radiation on Days 21 and 23 (GFT/Irr). Tumors from this group showed extensive degenerative treatment effect. Magnification x400.*

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