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13. ABSTRACT *(Maximum 200 words)*

This project is designed to investigate the possible role of apoptosis as a mode of cell death in irradiated and tamoxifen-treated breast cancer cells and to study the potential for using therapeutic manipulations to enhance this cell killing as a means of improving radiation therapy for treatment of breast cancer. To date, six breast cancer cell lines have been treated **in vitro** with ionizing radiation or tamoxifen, and the ability of the cells to undergo apoptosis following treatment has been evaluated using gel electrophoresis techniques. Although radiation inhibits cell growth in all the cell lines and tamoxifen inhibits cell growth in the two estrogen receptor positive lines, neither tamoxifen nor radiation appear to cause apoptosis in any of the cell lines under the experimental conditions used. These data suggest that breast cancer cells may contain strong anti-apoptotic mechanisms or have lost the ability to under apoptosis, perhaps as part of their neoplastic development. In the next year we will confirm and extend these observations to additional cell lines and begin to investigate possible mechanisms for this apparent resistance to apoptosis, with emphasis on the tumor suppressor gene **p53** and the anti-apoptosis oncogene **bcl-2** and related genes.

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TABLE OF CONTENTS

INTRODUCTION

It is now estimated that 1 in 9 women in the US will develop breast cancer during her lifetime. Hence, there is great interest in development of improved ways to treat breast cancer. Although local treatment of breast cancer, especially early breast cancer, by surgery and/or radiation therapy is quite effective, recurrence and metastases remain substantial problems limiting the cure rate of this disease. Radiation therapy plays a prominent role in the treatment of breast cancer, both as a primary and an adjuvant therapy, so increased knowledge of the mechanisms involved in ionizing radiation-induced inactivation of breast cancer cells might be expected to translate into gains in the efficacy of treating breast cancer with radiation. It has been demonstrated in other cell types that radiation can induce apoptosis, a type of cell death which is biochemically and morphologically distinct from necrosis [for general reviews on apoptosis see (1-5); for examples of studies on radiation-induced apoptosis see (6-9)]. It has also been shown that apoptosis can occur in breast tissue and breast cancer cells under normal physiological conditions and in response to hormonal manipulations (10-14). Therefore, the overall goals of this research project are to investigate the possible role of apoptosis as a mode of cell death in irradiated breast cancer cells and to study the potential for using therapeutic manipulations to enhance this cell killing as a means of improving the use of radiation therapy in the treatment of breast cancer.

The specific approaches to be used to achieve the overall technical objectives of this research are: (1) To test the hypothesis that, because breast tissue undergoes apoptosis in some normal situations, breast cancer cells are more sensitive to apoptosis induced by ionizing radiation than are cancer cells from tissues that do not normally apoptose. (2) To test the hypothesis that radiation-induced apoptosis in breast cancer cells is dependent on the proliferative status of the cells and the cell cycle phase at the time of irradiation. (3) To ascertain whether hormonal status of breast cancer cells affects the radiation sensitivity of apoptosis induction and whether hormone-induced changes in cell proliferative status alter radiation-induced apoptosis. (4) To test the hypothesis that the level of apoptosis induced by radiation in breast cancer cells can be modified by agents that modify cell survival after irradiation. (5) To ascertain whether the cellular proto-oncogene *bcl-2* plays a role in radiation-induced apoptosis and loss of clonogenicity in breast cancer cells. In all these studies, apoptosis will be determined in a quantitative assay, and the relationship between apoptosis induction and cell killing (colony formation and/or growth curves) will be determined in order to test whether apoptosis contributes significantly to long-term cell killing, i.e., whether apoptosis would be expected to contribute significantly to tumor cure.

BODY OF THE REPORT

Breast cancer cell lines

To date, experiments have been conducted using six human breast cancer cell lines cultured *in vitro*. The lines are listed in Table I, together with information on their estrogen receptor (ER), *p53* and *bcl-2* status, where available. Also included in Table I is information on the human leukemia cell line, HL-60, which was used as a positive control in these experiments because it undergoes apoptosis within a few hours of exposure to ionizing radiation, although only after relatively high radiation doses (15). All the cell lines were obtained from the American Type Culture Collection (ATCC) and cultured according to their recommendations.

Table I
ER, *p53* and *bcl-2* Status in the Breast Cancer Cell Lines Used to Date in this Study

Cell line	ER status	*p53* status	*bcl-2* status	References
HL-60	yes	null	normal	(16)
BT20	no	point mutation	unknown	(17,18)
BT549	no	point mutation	unknown	(18)
Hs578T	no	mutant	unknown	ATCC (19,20)
HTB26	no	point mutation	unknown	(18,21,22)
MCF-7	yes	normal	normal	(20,23-25)
T-47D	yes	unknown	unknown	ATCC

Testing for apoptosis induction by radiation or tamoxifen in cultured breast cancer cells

An important biochemical event frequently seen in apoptosis is double-stranded cleavage of DNA at the linker regions between nucleosomes (26,27). This cleavage produces a characteristic "ladder" pattern of DNA fragments on agarose gel electrophoresis, where the fragments represent the 180-200 base pair nucleosome and multiples thereof. This specific DNA fragmentation in apoptosis is quite different from the random degradation of DNA which occurs in the late stages of necrosis and appears on electrophoresis gels as smears of DNA. Hence, the appearance of DNA "ladders" is generally considered to be a hallmark of apoptosis.

We have tested all the cell lines listed in Table I except T-47D for the appearance of radiation-induced apoptosis. Exponentially growing cells were exposed to a single, relatively high dose of radiation (25 Gy), then assayed for apoptosis, appearance of oligonucleosomal DNA ladders, by conventional gel electrophoresis using the methods of Sellins and Cohen (6). The apoptosis assay was conducted on cell samples taken at daily intervals out to 7 days after irradiation. None of the five breast cancer cell lines tested showed any DNA fragmentation to ladders after exposure to ionizing radiation, although the control HL-60 cells routinely showed ladders within 5 h of radiation exposure. However, at early times (e.g., days 2-3 after irradiation) all the irradiated breast cancer cell lines did show large molecular weight DNA pieces that moved out of the wells into the electrophoresis gel but remained above the largest molecular weight marker (12 kb). As time increased (e.g., beyond day 3), smears of low molecular weight DNA become noticeable in most cell lines. This smearing had the appearance of necrosis, rather than apoptosis.

Using conventional gel electrophoresis, all six cell lines have also been tested for the ability to undergo apoptosis after exposure to tamoxifen. Cells were treated with 1, 3, or 10 μM tamoxifen continuously and assayed for apoptosis at daily intervals for up to 8 days. The results

were similar to those obtained for exposure to ionizing radiation. No evidence of apoptotic DNA ladders was seen in any of the cell lines at any time points, although fragmented DNA of high molecular weight was seen at early times, and some DNA smears were seen at later days.

Some circumstances have been identified where there is not a correlation between DNA fragmentation to oligonucleosomal sized pieces and the morphological appearance of apoptosis (28,29), and recently it has been shown that the oligonucleosomal cleavage of DNA is preceded by DNA cleavage into large fragments of about 50 kb, and that cleavage may be preceded by fragments of about 300 kb (30-32). Although the DNA ladders are not always observed, it appears that DNA cleavage to the 50 kb fragments is widely, perhaps universally, observed (33). Therefore, we wondered whether the high molecular weight fragmented DNA seen in the conventional electrophoresis gels was DNA of 50 or 300 kb, also indicative of apoptosis. We have used pulsed field gel electrophoresis to separate high molecular weight DNA, according to protocols similar to those used by others (34). With HL-60 cells, 6 h after irradiation, fragmented DNA of 50 and 300 kb was readily seen using pulsed field gel electrophoresis. To date we have only obtained pulsed field gel electrophoresis analyses of two breast cancer lines, HTB26 and MCF-7, exposed to 25 Gy of X-rays. The HTB26 cell line shows a band of DNA at 50 kb for both unirradiated and irradiated cells at all days tested. The MCF-7 cells showed both 300 kb bands after irradiation and 50 kb bands in unirradiated cells. These pulsed field gel electrophoresis experiments are currently being repeated and extended to additional breast cancer cell lines.

Growth curves of breast cancer cells after irradiation and tamoxifen treatment

Growth curves were obtained for untreated, irradiated and tamoxifen-treated breast cancer cell lines by performing daily hemocytometer counts of cells following treatment. Cells from all untreated lines had doubling times between 1.5 and 2.5 days. Following exposure to 25 Gy, as expected for this high radiation dose, none of the cell lines showed any growth, and in all cases the cultures contained decreasing numbers of intact cells, particularly from day 2 on, and increasing numbers of detached cells floating in the medium. With some cell lines, detached cells have been shown to be apoptotic (35); we have not yet tested only the detached cells for apoptosis. In addition, the T47-D cells appear to be sensitive to intracellular radioactivity; cells which had their DNA radiolabeled by growth in a relatively low level of ^{3}H-labeled thymidine (0.1 μCi/ml) grew only slightly for 2 days, then declined in numbers. These radiolabeled cells have not been tested yet for apoptosis.

The effect of tamoxifen on cell growth is dependent on the cell line and drug concentration. The cells were exposed continuously to 1, 3 or 10 μM tamoxifen. Ten micromolar tamoxifen had no effect on the growth rates of BT20, BT549, HS578t or HTB26 cells, a not unexpected result since these lines are all ER^- (Table I). Exposure of MCF-7 cells to 1 and 3 μM tamoxifen resulted in little effect on the cell growth rate compared to control, but 10 μM tamoxifen slowed the growth rate of MCF-7 cells significantly. This growth inhibition was enhanced by removal of phenol red from the medium; phenol red has been shown to have a weak estrogenic effect on cells (36). The T47-D cells showed growth inhibition by all three tamoxifen concentrations, with the inhibition increasing with increasing drug concentration.

In parallel with the growth curves on the MCF-7 and T47-D cell lines, cells exposed to varying times and concentrations of tamoxifen were plated for determination of their colony forming ability. Those plates are currently being counted, so results are not yet available.

Discussion

These experiments have provided us with some very interesting data related to specific aims 1 and 3 of this project. Probably most interesting, and somewhat unexpected in light of our hypothesis that breast cancers would be prone to apoptosis, is our observation of a lack of apoptosis in these breast cancer cell lines exposed to radiation or tamoxifen. This leads to a potential alternative hypothesis, namely, that breast cancer cells contain a strong anti-apoptotic mechanism(s) or have lost the ability to express apoptosis. In fact, it may be this anti-apoptosis process that has allowed or at least contributed to the breast cancer cells becoming neoplastic, as others have suggested for breast and other cell types [e.g., (14,5,37)]. The nature of the anti-apoptotic pathway(s) in breast cancer cells is, at this time, unknown, although one could postulate involvement of changes in *53*, *bcl-2* and its related family of genes, *c-myc*, etc. In particular, a role for the tumor suppressor gene *p53* may be indicated, in light of observations that, at least in hematopoietic cells, wild-type *p53* is involved in radiation-induced apoptosis (38,39). Since most of the cell lines we have tested to date contain mutant *p53* (Table I), that may be the cause of their resistance to radiation-induced apoptosis. We are not aware of any literature on whether wild-type *p53* plays a role in tamoxifen-induced apoptosis. The role of the anti-apoptotic oncogene *bcl-2* in apoptosis may be a particularly interesting one for this study because of the demonstrations that *bcl-2* expression inhibits radiation-induced tamoxifen in thymocytes (40,41), but a recent study indicates, paradoxically, that breast cancer patients with elevated BCL-2 immunostaining appeared to derive the greatest benefit from endocrine therapy (42). Elucidation of possible mechanisms for the apparent loss of the ability to undergo apoptosis in breast cancer cells will be a subject for continuing study and emphasis in this project (specific aim 5).

Other ongoing and immediately indicated experiments for our laboratory are: (1) To extend these apoptosis and cell growth studies to additional ER^+ and ER^- breast cancer cell lines treated with tamoxifen and/or ionizing radiation. This is necessary in order to test the generalization of our observation of little induced apoptosis in breast cancer line *in vitro*. Since most of the breast cancer cell lines we have studied to date have mutant *p53*, there is a particular need to include in our studies breast cancer cell lines containing wild-type *p53*, such as MCF-7. Since *p53* mutations are especially common in breast cancer (43), it may be difficult to find lines that are wild-type *p53*; in that case we will transfect wild-type *p53* into one or more of the cell lines we have investigated to-date and study the effect of that added *p53* on radiation and tamoxifen sensitivity. (2) To test for apoptosis using additional assays for that endpoint, e.g., morphological assessment of the treated cells or the DNA end labeling, or TUNEL, method (44,45). The need for additional assays is indicated by observations of others that there is not always a correlation between the appearance of fragmented DNA on conventional electrophoresis gels and the morphological appearance of apoptosis (28,29). (3) To test whether the presence of phenol red and bovine estrogens (from the fetal bovine serum) in the media in which these breast cancer cells are grown can alter the cell growth and apoptosis potential in the absence and presence of treatment with tamoxifen and radiation.

CONCLUSIONS

The data presented here show a lack of induction of apoptosis by ionizing radiation or tamoxifen in six breast cancer cell lines tested *in vitro*. This may be consistent with the presence of strong anti-apoptotic mechanisms or the loss of the ability to express apoptosis in breast cancer cells compared to normal breast epithelium. Elucidation of the relevant processes might lead to development of ways to regain apoptosis in breast cancer cells, hence making the cells more sensitive to therapeutic interventions. In the immediate short term (next few months), our emphasis will be on repeating and extending these observations, as discussed above. If the observations continue to hold-up, in the longer term (next one to two years) we expect to place increasing emphasis on elucidation of the possible relevant anti-apoptosis or lost apoptotic pathways in breast cancer cells.

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