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This project was designed to study the role of apoptosis in breast cancer cells exposed to ionizing radiation and hormonal manipulation in vitro and to investigate the potential for using these manipulations to enhance tumor cell killing, thus improving radiotherapy of breast cancer. Several new, and potentially important, observations are reported herein. First, breast cancer cell lines *in vitro* generally do not appear to be particularly sensitive to induction of apoptosis, and even when they do undergo apoptosis in response to some treatments, do not fragment their DNA to oligonucleosomal sized pieces. Second, breast cancer cell lines *in vitro* appear to be resistant to radiation-induced apoptosis. At this time, the reasons for this observation and its potential clinical significance are not clear, but the finding clearly deserves further investigation because of its potential clinical importance. Third, in some breast cancer cell lines tamoxifen treatment prior to irradiation does not alter the radiation response, but in others tamoxifen pre-treatment causes radiation sensitization. On the other hand, some published data have shown radiation protection by tamoxifen. Since this is clearly a clinically important issue, attempts should be made to clarify the discrepancies.

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K D. Held 9/25/98

PI - Signature Date

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# INTRODUCTION

It is estimated that 1 in 9 women in the US will develop breast cancer during her lifetime. 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 (6-10)]. 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 (11-15). Therefore, the overall goals of this research project have been 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 apoptotic cell killing as a means of improving the efficacy of radiation therapy in the treatment of breast cancer.

The specific technical objectives of this research project have been: (i) To test the hypothesis that, because breast tissue normally undergoes apoptosis in certain physiological situations, breast cancer cells are more sensitive to apoptosis induced by ionizing radiation than are cancer cells from tissues that do not normally undergo apoptosis. (ii) 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. (iii) 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. (iv) 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. (v) 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 has been determined using quantitative assays, and the relationship between apoptosis induction and cell killing (colony formation and/or growth curves) has been 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

## Experimental Methods, Assumptions and Procedures

*Cell lines and cell culture*

The breast cancer cell lines used in the studies reported herein were MCF-7, T47D, HS578t and HTB26. Most cell lines were obtained from the American Type Culture Collection (ATCC). All cells are maintained in exponential growth by twice weekly transfer in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (MCF-7 and T47D) or 20% (HS578t and HTB26) fetal bovine serum, antibiotics and Hepes buffer (all reagents from Sigma Chemical Co., St. Louis, MO). During the course of these studies, we found that the MCF-7 cells (obtained from the ATCC) we were using were p53 null, rather than p53 wild-type, as they have been widely reported in the literature to be. MCF-7 cells are genetically unstable and can spontaneously lose the p53 gene in culture (P. O'Connor, personal communication to S. Powell of our Department). We subsequently obtained a strain of MCF-7 cells (from Dr. H. Nagasawa, Harvard School of Public Health) that have been shown to be p53 wild type (16) and have also performed studies with them. Relevant characteristics of the cell lines are listed in Table I.

Table I
Relevant Characteristics of Breast Cancer Cells Used in these Studies

| Cell line | ER status | p53 status[a] | bcl-2 status[a] |
|---|---|---|---|
| MCF-7 (ATCC) | positive | null | expressed |
| MCF-7 (HN) | positive | wild-type | ND[b] |
| T47D | positive | mutant | expressed |
| HS578t | negative | mutant | expressed (low) |
| HTB26 | negative | ND | ND |

[a] p53 status and bcl-2 status determined in our laboratory using Western blots, except for the MCF-7 (HN) variant which has been shown by Dr. Nagasawa to be functionally p53 wild-type by Westerns for increased expression of p53 and p21 after irradiation and by cell cycle analysis showing $G_1$ arrest (16).

[b] ND = not determined yet

For each experiment, cells are grown in phenol red-free DMEM containing dextran-coated charcoal treated serum (prf/dcc medium) for one week prior to the initiation of drug treatment. Cells are then replated into DMEM or prf/dcc DMEM containing an appropriate concentration of estrogen, progesterone or tamoxifen for varying lengths of time prior to irradiation or assay for cell growth, plating efficiency, etc.

*Irradiation of cells*

For each radiation experiment, cells are trypsinized, counted, diluted to 2 x $10^5$ cells/ml, then placed in specially designed stirring, irradiation vessels. Details of our standard irradiation methods have been published (17,18). Cells are irradiated with a range of doses using a Siemens Stabilipan 2 X-ray generator operated at 250 kVp, 12 mA, dose rate about 1.4 Gy/min. Following irradiation, cells are diluted and plated in triplicate into petri dishes at cell numbers that should yield between 30 and 200 colonies per plate. The plates are incubated at 37°C for 2-6 weeks, depending on the growth rate of the cells, and colonies containing more than 50 cells are counted. Survival curves are generated according to standard protocols, and data are fit to the target model equation

$$SF = 1 - (1 - e^{-D/D_0})^n \quad (1)$$

where SF is surviving fraction at each dose D, $D_0$ is the reciprocal of the slope of the cell survival curve, a measure of radiation sensitivity, particularly at high radiation doses, and n is the intercept. Alternatively, survival data were also fitted to the linear/quadratic equation

$$SF = e^{-(\alpha D + \beta D^2)} \quad (2)$$

where $\alpha$ and $\beta$ are the coefficients of the linear (dependent on dose) and quadratic (dependent on dose$^2$) components of the response, respectively. SF2, the surviving fraction at 2 Gy, is calculated from this later equation and gives an indication of cellular radiation sensitivity at low, clinically relevant, radiation doses.

*Flow cytometry analysis for apoptosis*

For each treatment point, media is aspirated off cells and saved, then attached cells are trypsinized, counted, and added back to the saved treatment medium which contains detached cells. Cells are pelleted by centrifugation for 5 min at 2000 rpm, washed once in 10 ml PBS and fixed in 70% ethanol at 4°C for at least 60 min. Cells are held at 4°C until further processing. Cells are washed again in PBS, resuspended in PBS + 0.5 mg/ml RNAse (Sigma) and incubated at 37°C for 30 min. Propidium iodide and NP-40 are added to the cells to a final concentration of 0.1 mg/ml and 0.1%, respectively. Cells are kept in the dark at 4°C until analyzed. DNA content is measured using a Coulter EPICS XL-MCL flow cytometer, and data are analyzed using Coulter System 2 or winMDI 2.6 software.

## Results

*Radiation-induced loss of clonogenicity and apoptosis in breast cancer cell lines*

An overall goal of this research project was to determine the relationship between apoptosis induction and total cell killing (measured using a clonogenic assay) in breast cancer cells treated with ionizing radiation without and with chemical modifiers. In last year's report we presented our preliminary data on clonogenic survival of four breast cancer cell lines following irradiation. Table II now presents all the data obtained to date on the radiation survival response for the five breast cancer cell lines irradiated in the absence of any drug treatments. These much more complete data are consistent with the preliminary data presented in last year's report. The following conclusions can be made from the data in Table II. Based on the $D_0$ values (which largely reflect the cellular response to higher doses of radiation), the sensitivity of the five cell lines differs significantly, with the most resistant cells, the HS578t line, being almost two-fold less sensitive to ionizing radiation than the sensitive MCF-7 lines. However, if one assesses radiation sensitivity using SF2 values, which provide more information on the clinically relevant, low dose region of the cell survival curve, the HS578t cell line is not more radiation resistant than the MCF-7 lines. Lastly, it is noteworthy that, for all five cell lines, the radiation response of the cells is not altered significantly by growth in hormone stripped, phenol red-free (prf/dcc) medium for periods ranging from 2 days to 2 weeks prior to irradiation, compared to the response of cells irradiated in complete medium. This lack of an effect of removal of hormones occurs despite the fact that the growth rate of the ER$^+$ cells is slowed in the charcoal-stripped serum (see below).

Objective 1 of this project was to test the hypothesis that breast cancer cells are more sensitive to induction of apoptosis by ionizing radiation than are cancer cells derived from tissues that do not normally undergo apoptosis in response to physiological stimuli. We reported

Table II
Radiation Response of Breast Cancer Cells

| | DMEM | | prf/dcc DMEM[a] | |
|---|---|---|---|---|
| Cell line | $D_0$[b] | SF2 | $D_0$ | SF2 |
| MCF-7 (p53 wt) | 1.05 ± 0.18 (9) | 0.72 ± 0.09 | 0.98 ± 0.16 | 0.68 ± 0.09 |
| MCF-7 (p53 null) | 1.03 ± 0.18 (11) | 0.62 ± 0.10 | 1.07 ± 0.09 | 0.65 ± 0.09 |
| T47D | 1.23 ± 0.23 (10) | 0.54 ± 0.10 | 1.25 ± 0.23 | 0.58 ± 0.06 |
| HS578t | 2.10 ± 0.37 (7) | 0.65 ± 0.10 | 1.99 ± 0.36 | 0.61 ± 0.10 |
| HTB26 | 1.34 ± 0.15 (6) | 0.53 ± 0.09 | 1.33 ± 0.14 | 0.50 ± 0.08 |

[a] Cells were grown in phenol red-free DMEM containing 10% charcoal-stripped serum for 2 days to 2 weeks prior to irradiation. Since the radiation response did not vary with the duration of growth in prf/dcc DMEM, survival data for all time points have been averaged together.

[b] $D_0$ is the reciprocal of the slope of the cell survival curve determined using equation 1. SF2 is the surviving fraction at 2 Gy determined using equation 2. All data are means ± standard deviation of the number of experiments in parentheses in the $D_0$ column.

previously that none of the five breast cancer cell lines exhibited DNA fragmentation to oligonucleosomal sized pieces ("ladders") on agarose gels following irradiation. Since there are mixed reports in the literature concerning whether breast cancer cells undergo "typical" apoptosis and degrade their DNA to oligonucleosomal pieces, this year we have used additional assays for apoptosis on irradiated breast cancer cells and extended our studies to longer times after irradiation. We chose to use a flow cytometry assay for apoptosis which assesses the amount of cells having DNA content less than that of $G_1$ cells, the so-called sub-$G_1$ apoptotic cells (19), because we have had good results using this assay for apoptosis in other types of cells and in breast cancer cells exposed to hormonal manipulation (see below). The studies have been unable to demonstrate the occurrence of radiation-induced apoptosis in any of the five cell lines at times from 6 h to 7 days after irradiation. This lack of radiation-induced apoptosis observed in our studies is consistent with the results of two papers published by others during the course of our research (20,21). Because there are numerous assays for apoptosis and misleading results can be obtained by using only one or two assays, especially when the result appears to be negative, we are currently confirming these negative results by using two additional assays for apoptosis that do not depend on DNA fragmentation: Annexin V binding, which indicates changes in membrane composition that occur during apoptosis (22), and activation of caspases, enzymes that cleave a subset of cellular proteins, resulting in cell disassembly (23). Both these assays are in use in our laboratory for other studies, so the experiments on irradiated breast cancer cells should be completed shortly.

*Growth, viability and apoptosis in breast cancer cells during and after hormonal manipulation*

As part of our ongoing studies, we have continued to accumulate additional data on the effects of hormonal manipulation on the growth rates and plating efficiencies of all five breast cancer cell lines exposed to varying concentrations of estrogen, progesterone and tamoxifen. In short, the accumulating data continue to be consistent with those shown in last year's report, so they will be not shown in detail here. The important findings from the growth rate studies are as follows. First, culture of cells in hormone depleted medium significantly slows the growth of the $ER^+$ cells, but has little effect on the $ER^-$ cells. This effect is largely reversed by the addition of low doses of estrogen to the culture medium. High doses (10 μM) of estrogen, progesterone or tamoxifen have different results on different cell lines. For example, tamoxifen dramatically slows the growth rate or even causes cell loss in all three $ER^+$ lines and in the HS578t cells, but has little effect on growth of HTB26 cells. And several of the cell lines, especially the p53 null

MCF-7, T47D, and HS578t, are quite sensitive to slowed growth and initial cell loss when exposed to high dose progesterone.

Last year we reported the interesting and important finding that cells exposed to all three hormonal manipulations for up to 9 days showed no changes in plating efficiencies compared to cells in complete medium without any drugs. These studies have now been extended to 14 day drug exposures (in conjunction with the radiation/drug combination experiments described below), and the results continue to show no decreases in plating efficiencies in treated cells. In other words, even though the growth of the cells exposed to some of the hormonal manipulations (e.g., high dose tamoxifen) is slowed dramatically and in some cases cells are even lost from the populations, those cells present at the time they are transferred to complete, drug-free medium have the same viability as untreated cells.

In our earlier reports, we noted that there was no evidence of DNA ladders on electrophoresis gels of breast cancer cells treated by hormonal manipulations, although pulsed field gel electrophoresis results suggested the occurrence of DNA fragments of approximately 50 and 300 kbp, which may be indicative of a poptosis (24,25). Because apoptosis can not be quantified from pulsed field gels, this year we have used the sub-$G_1$ flow cytometry assay described above to assess hormone-related apoptosis in the breast cancer cells. Results of experiments for the three ER+ cell lines are shown in Figure 1. The results of all our studies indicate that exposure to varying concentrations (0.1 - 10 μM) of estrogen, progesterone and tamoxifen does not elicit production of sub-$G_1$ apoptotic-like cells in T47D, HS578t or HTB26 cells, but in the MCF-7 p53 wild-type cells apoptosis is caused by high doses of estrogen or progesterone and in MCF-7 p53 null cells significant apoptosis is caused by high dose tamoxifen and some apoptosis is caused by lower concentrations of tamoxifen and high dose estrogen.

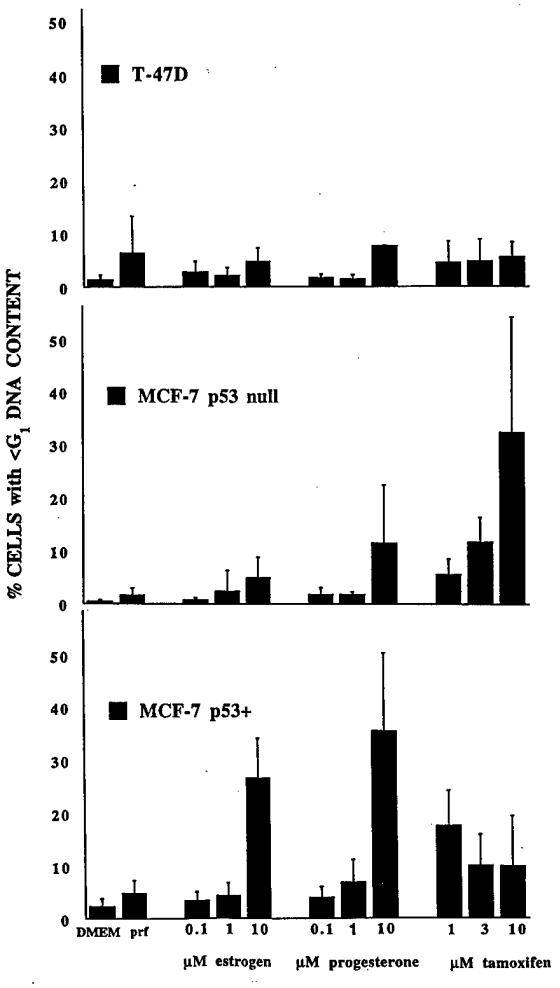


Figure 1. Percentage of breast cancer cells with sub-$G_1$ content after treatment for 7 days with the indicated concentrations of estrogen, progesterone or tamoxifen in prf/dcc DMEM. Sub-$G_1$ DNA content was determined using flow cytometry of propidium iodide-stained cells. Data are averages ± SD of 2-6 experiments.

*Survival curves and apoptosis induction in breast cancer cells exposed to hormonal manipulation and ionizing radiation.*

We mentioned in last year's report that in the course of conducting these experiments combining hormonal manipulation and radiation exposure in the breast cancer cell lines, it had become evident that long (1 week) pre-treatment of cells in prf/dcc medium followed by long (1-2 weeks) treatment with drugs were needed prior to irradiation to maximize effectiveness of the combination. Hence, these experiments have been very time-consuming and are not yet completed. Figure 2 shows typical survival curves that have been obtained and Table III summarizes the data for breast cancer cells exposed to prf/dcc DMEM for one week, followed by one week exposure to estrogen, progesterone or tamoxifen, then irradiation. The control data for untreated cells and for cells cultured for two weeks in prf/dcc DMEM were shown in Table I. The most important observations from these data are that the MCF-7 p53 null cells are sensitized significantly (enhancement ratio about 1.7, based on the ratio of the $D_0$ values) by exposure to tamoxifen prior to irradiation and the T47D cells are sensitized slightly by the same treatment. The radiation response is not altered in any of the other cells lines, including the other ER+ line, MCF-7 p53 wild-type cells. Neither estrogen nor progesterone alter the radiation response of any of the five cell lines. In experiments still in progress, the effects of 14 day drug treatment, rather than the 7 day treatment shown here, are being investigated. To date, it appears that the longer drug treatment gives the same response as that shown here for 7 day treatments.

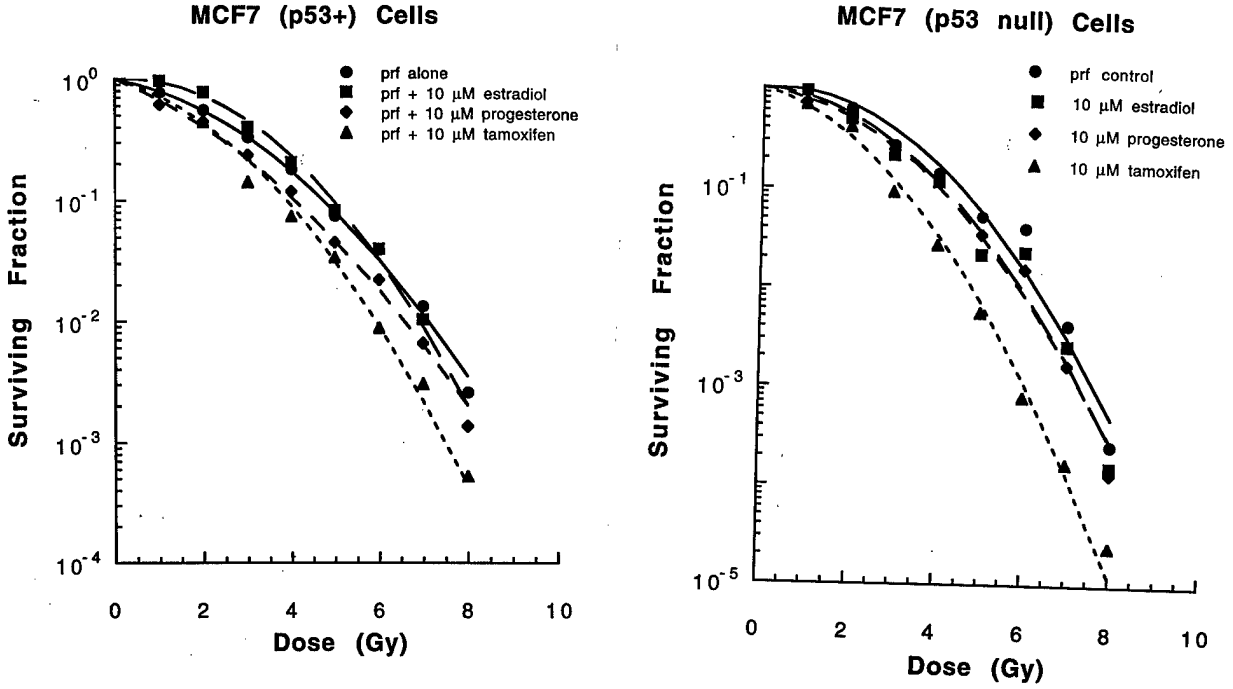


Figure 2. Radiation survival curves for MCF-7 p53 wild type (left panel) and p53 null (right panel) breast cancer cells. Cells were grown in prf/dcc DMEM for one week prior to the start of hormone manipulation, then cultured for an additional week in prf/dcc DMEM containing the indicated concentration of drug. Data are from a single, typical experiment.

We have also assayed for apoptosis, using the flow cytometry sub-$G_1$ assay, in the breast cancer cell lines treated with hormone manipulation and radiation, in parallel with the clonogenic studies shown in Table III. In all cases tested to date, the radiation exposure does not cause any additional apoptosis above the levels caused by the hormone manipulation itself. Although these experiments are still being completed, it appears that the increased radiation sensitivity caused by tamoxifen pre-treatment of MCF-7 cells, for example, can not be due to apoptosis.

Table III
Effect of Hormonal Manipulation on Radiation Sensitivity of Breast Cancer Cell Lines[a]

| | 10 μM estrogen | | 10 μM progesterone | | 10 μM tamoxifen | |
|---|---|---|---|---|---|---|
| | $D_0$ | SF2 | $D_0$ | SF2 | $D_0$ | SF2 |
| MCF-7 (p53 wt) | 1.12 ± 0.14 (6) | 0.54 ± 0.11 | 1.01 ± 0.12 | 0.61 ± 0.14 | 1.05 ± 0.14 | 0.59 ±0.11 |
| MCF-7 (p53 null) | 0.98 ± 0.07 (3) | 0.64 ± 0.07 | 0.91 ± 0.09 | 0.57 ±0.06 | 0.68 ±0.03 | 0.35 ± 0.01 |
| T47D | 1.45 ± 0.25 (2) | 0.59 ± 0.01 | 1.23 ± 0.24 | 0.60 ± 0.03 | 1.21 ± 0.09 | 0.49 ± 0.03 |
| HS578t | 1.82 ± 0.07 (2) | 0.68 ±0.11 | 1.82 ± 0.27 | 0.64 ± 0.02 | 1.81 ± 0.10 | 0.57 ±0.01 |
| HTB26 | 1.26 ± 0.08 (2) | 0.41 ± 0.01 | 1.20 ± 0.07 | 0.54 ± 0.05 | 1.19 ±0.04 | 0.47 ± 0.03 |

[a] Cells were cultured for 7 days in prf/dcc DMEM, then treated with the indicated drug concentrations for 7 days prior to irradiation.

## Discussion

*Induction of apoptosis in breast cancer cell lines*

We have mentioned in this report and previously that we have been unable to demonstrate induction of apoptosis using the assay of DNA "ladders" on electrophoresis gels for any breast cancer cell lines treated with radiation and/or hormone manipulation. This contrasts with a report that tamoxifen induces oligonucleosomal DNA fragmentation in MCF-7 cells (26). However, in a review by McCloskey *et al.*(27) it is noted that quite variable results have been reported by different investigators on whether tamoxifen causes apoptosis in breast cancer cells *in vitro* and *in vivo*. They note that these variable results may reflect the existence of several phenotypic variants of MCF-7 cells. It should be noted that in the new data presented here (Figure 1), apoptosis is seen in the MCF-7 cells in response to certain hormonal manipulations when the flow cytometry sub-$G_1$ assay is used. This demonstrates clearly the importance of choosing apoptosis assays carefully and the need to use more than one assay for apoptosis prior to reaching conclusions.

Although radiation-induced apoptosis has been demonstrated in an ever-increasing number of other cancer cell lines [reviewed in (10)], we have been unable to demonstrate radiation-induced apoptosis in breast cancer cell lines using the flow cytometry assay with which we are able to demonstrate drug-induced apoptosis. This lack of radiation-induced apoptosis has also been shown in two papers (20,21) published while these studies were in progress. This observation is important, since it is consistent with the suggestions that decreased apoptosis may play a role in the biological aggressiveness of breast cancer (28) and/or in its resistance to some therapies (20). Although p53 status appears to be a determinant of radiation-induced apoptosis in cells of hematopoietic origin [reviewed in (10)], p53 status is apparently not a factor in this lack of radiation-induced apoptosis in breast cancer cells because there is no radiation-induced apoptosis in the p53 wild-type MCF-7 cells. Clearly the possible roles of other oncogenes or tumor suppressor genes need to be investigated in an attempt to understand why breast cancer cells generally appear to be resistant to radiation-induced apoptosis.

*Effect of hormonal manipulation on breast cancer cells*

We have shown in this report and previously that treatment of breast cancer cells with high concentrations of estrogen, progesterone and tamoxifen, especially tamoxifen, slows cell growth and can cause cell loss in all five breast cancer cell lines. This result with tamoxifen has been reported previously (29). These effects of tamoxifen on the $ER^+$ cell lines presumably reflect, largely, the anti-estrogenic properties of tamoxifen. The effect of high dose tamoxifen on the $ER^-$ cells may be a reflection of its activity as an inhibitor of protein kinase C (30-32), as an antagonist of calmodulin (33), or its reduction of bcl-2 expression (34). Growth inhibitory effects of high concentrations of estradiol also have been demonstrated before (35).

A particularly relevant point that has come from our data, however, is the observation that although the drug treatments may cause some cell loss, when the drugs are removed the cells which are present at that time are fully viable and capable of beginning regrowth of the culture. By extrapolation to the clinical situation, this is consistent with the idea that tamoxifen is largely cytostatic, and after a patient is taken off the drug, the tumor may begin to regrow if all tumor cells were not killed by the hormone therapy or other therapies given with it.

*Effects of combined hormonal manipulation and radiation exposure*

A large number of women with breast cancer receive treatment with both ionizing radiation and tamoxifen, however, clinicians typically avoid giving the two agents concurrently because there is a concern that the tamoxifen may increase the radiation resistance of the breast cancer cells. This concern is based on the idea that tamoxifen treatment decreases cell proliferation, causing accumulation of cells in $G_1/G_0$, where they are more resistant to radiation. However, the data in the literature addressing this issue are somewhat controversial. Some investigators have shown that $ER^+$ MCF-7 cells show slight radiation protection when treated with tamoxifen prior to irradiation (36-38), but others have found no change in the radiation response of MCF-7 cells after tamoxifen treatment (39) or radiation sensitization *in vivo* (40). The data presented herein are most consistent with those last two reports; some cell lines show no change in radiation sensitivity with tamoxifen treatment, whereas others, especially the one MCF-7 line, show significant radiation sensitization. The reasons for these differences are not clear. On possibility is that different treatment regimens, including drug concentration, drug exposure time, length of pre-treatment in hormone-depleted medium, etc., may result in different results. Another possible explanation is that since many variants of MCF-7 cells exist (27) and these cells are known to undergo genetic changes in culture, the different variants may have quite different responses to tamoxifen and radiation.

There is no clear correlation between enhancement of apoptosis by the combination of hormone manipulation and radiation with radiation sensitivity. For example, in the MCF-7 p53 wild type cells, apoptosis is induced by treatment with estradiol or progesterone (Figure 1), but that apoptosis is not increased by addition of radiation, nor is the radiation sensitivity altered significantly by the combinations (Figure 2). On the other hand, in the MCF-7 p53 null cells, tamoxifen alone causes apoptosis, the addition of radiation does not increase the level of apoptosis, but the clonogenic survival is significantly decreased by the combination.

Although in cells of hematopoietic origin p53 status appears to be important for both radiation sensitivity and radiation-induced apoptosis, as mentioned above, in the breast cancer cell lines p53 status appears to be irrelevant for radiation sensitivity or apoptosis induction. The p53 null cells are actually slightly more sensitive (although not significantly so) to radiation exposure without drugs in the clonogenic assay (Table II) and are sensitized to radiation by tamoxifen to a much greater degree than the p53 wt cells (Figure 2).

## Recommendations

Experiments should be designed to address the discrepancies in our data and published data on the effects of tamoxifen treatment on radiation response. This is a critical clinical question. Clearly, if tamoxifen does decrease radiation response, clinical use of the two agents concurrently must be avoided. On the other hand, if, at least in some circumstances, the combination can result in greater cell killing, as the data presented here suggest, ways should be developed to identify those circumstances and take advantage clinically of the increased cell killing to increase tumor cure probabilities.

Another critically important aspect of these studies and other related ones cited above, is the observation that breast cancer cells may be resistant to radiation-induced apoptosis. Several aspects of this need further investigation. First, is this a general finding that occurs in the clinic in primary breast cancer as well as in these established cell lines? Second, if this is a general finding in primary breast cancers, is this resistance to radiation-induced apoptosis a factor in the ability to cure breast cancer with radiation, or is radiation response in breast cancer independent of apoptosis? Third, does the genetic make-up of the breast cancer cells compared to normal breast epithelial cells provide clues about the lack of radiation-induced apoptosis? In relation to this last question, there is clearly a strong need for more full characterization in these cell lines of other genetic factors that might be important for apoptosis induction and/or radiation sensitivity. This characterization might include, for example other pro-apoptosis genes such as *bax* or *c-myc*, and anti-apoptosis genes such as *bcl-2*. Other researchers have begun such work. For example, Watson *et al.* (21) have been investigating the relationship between *c-myc* and apoptosis, and Sakakura *et al.* (20) have presented data showing that *bax* overexpression increases radiation sensitivity.

## CONCLUSIONS

In summary, the data obtained in this research project have yielded several interesting and potentially important findings. First, breast cancer cell lines *in vitro*, generally, do not appear to be particularly sensitive to induction of apoptosis and even when they do undergo apoptosis in response to some treatments, do not fragment their DNA to oligonucleosomal sized pieces as seen on electrophoresis gels. This highlights the importance of selection of appropriate assays when conducting apoptosis studies and the need for use of more than one assay.

Second, breast cancer cell lines *in vitro* appear to be resistant to radiation-induced apoptosis. At this time, the reasons for this observation and its potential clinical significance are not clear, but the finding clearly deserves further investigation because of its potential clinical significance.

Third, in the data presented here, it is shown that in some cell lines tamoxifen treatment prior to irradiation does not alter the radiation response, but in others tamoxifen pre-treatment causes radiation sensitization. On the other hand, some published data have shown radiation protection by tamoxifen. Since this is clearly a clinically important issue, attempts should be made to clarify the discrepancies.

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