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PRINCIPAL INVESTIGATOR: Kathryn D. Held, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

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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 these manipulations to enhance cell killing and, thus, improve radiation therapy of breast cancer. To date, six human breast cancer cell lines and nine human non-breast cancer lines have been treated *in vitro* with ionizing radiation or tamoxifen, and the ability of the cells to undergo apoptosis has been evaluated using gel electrophoresis. None of the breast lines and only three of the other lines show radiation-induced apoptosis with this assay. Expression of p53 and bcl-2 has been investigated in most of the lines. Studies with four breast cancer lines have shown that high dose tamoxifen or estradiol slows growth or causes cell death, and in the two lines studied to date this treatment increases radiation sensitivity. These data suggest that breast cancer cells may contain strong anti-apoptotic mechanisms or have lost the ability to undergo apoptosis, perhaps as part of their neoplastic development. In the next year we will confirm and extend these observations and continue to investigate possible mechanisms for this apparent resistance to apoptosis, with emphasis on *p53*, *bcl-2* and related genes.

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Kevin D. Hall Sept. 25 1996

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TABLE OF CONTENTS

INTRODUCTION

It is estimated that 1 in 9 women in the US will develop breast cancer during her lifetime. Hence, there is great interest in developing better treatments for 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 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 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 undergo apoptosis. (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

Radiation-induced apoptosis in human tumor cell lines

As discussed in last year's report, the appearance of the DNA "ladder" pattern on agarose electrophoresis gels is frequently considered to be a hallmark of apoptosis (15,16). We mentioned in that report that none of the five breast cancer cell lines we had tested showed evidence of radiation-induced ladders, although some showed larger molecular weight DNA fragments what might be consistent with apoptosis. Because aim 1 of our project was to test whether there are differences between breast cancer cells and cells from other types of human cancers with regards to ability to undergo radiation-induced apoptosis, in the past year we have stressed testing other human cancer cell lines, those listed in Table I. In all these studies, the HL-60 cell line was used as a control, because it forms very clear DNA ladders after radiation doses of about 20 Gy (17). In these studies, a range of radiation doses up to 30 Gy was used, and the assay for apoptosis was conducted out to 7 days after irradiation to ensure that late occurring apoptosis was not missed. As shown in Table I, only the STS26T soft tissue sarcoma and the HP555 glioblastoma showed significant evidence of radiation-induced apoptosis in this gel assay. Hence, initially, it appears that the majority of cell lines from human solid tumors (5/5 breast cancer lines, reported last year, and 6/8 lines from these new data) do not form the DNA ladders characteristic of apoptosis after irradiation. As mentioned in last year's report, there is not always a correlation between the appearance of DNA ladders and the appearance of other characteristics of apoptosis (18,19); hence, we are beginning to test all six breast lines and the lines listed below for apoptosis using other assays, including morphological endpoints and the TUNEL method (20,21).

Table I
Non-Breast Cancer Cell Lines Tested for Radiation-Induced Apoptosis

Cell line	Cancer type	Radiation-induced apoptosis?	p53 status
HL-60	leukemia	yes	null (22)
SaOS	osteosarcoma	no	null (23)
U2OS	osteosarcoma	no	wild-type (23)
FaDu	squamous cell carcinoma	no	mutant (24)
STS26T	soft tissue sarcoma	yes	null (this report)
U87	glioblastoma	no	wild-type (25)
HP555	glioblastoma	yes	mutant (this report)
HGL-21	glioblastoma	no	?
MMC1	glioblastoma	no	?

Growth curves of breast cancer cells during hormonal manipulation

We mentioned in last year's report that we had begun to obtain growth curves for untreated, irradiated and tamoxifen-treated breast cancer cell lines. These studies are important for two reasons: from a practical point of view, such studies provide information that is necessary for optimal design of the cell survival and apoptosis experiments testing combined radiation and

hormonal manipulation and, second, these studies will give preliminary indications of whether apoptosis is occurring after some treatments if the cell growth is slowed or there is a decrease in cell numbers with time of treatment.

Also as mentioned last year, it is known that phenol red and bovine estrogens (from the fetal bovine serum (FBS) used in culture medium) can have effects on growth of human breast cancer cells (26). Hence, based on initial experiments, we have now established uniform control conditions for these experiments in which all cell lines are grown for one week prior to the start of an experiment in phenol red-free medium containing serum that has been pretreated with dextran-coated charcoal to remove hormones (prf medium). Table II shows the effect of continuous treatment of breast cancer cell lines with varying concentrations of estradiol, progesterone, and tamoxifen in prf medium. Of the cell lines tested, MCF-7 and T47D are estrogen receptor (ER) positive and HS578t and HTB26 are ER negative. The growth rate of all four cell lines is somewhat slower in prf medium compared to that in complete medium. Addition of 0.1 - 1.0 μM estrogen to the medium enhances the growth rate of MCF-7 cells, as expected for an ER^+ line, but these doses of estrogen have no effect on the other cell lines. High doses of estrogen (10 μM) decrease the growth of all four cell lines, with a particularly large effect, greater than a 4-fold decrease, in the T47D line. In most cases, the addition of progesterone has little effect on the growth rate of the four lines, but growth of the T47D cells is slowed appreciably by 10 μM progesterone. Both ER^+ cell lines are greatly affected by the addition of tamoxifen: 1 or 3 μM tamoxifen increases the cell doubling times about 2-fold, while 10 μM tamoxifen causes cell killing as indicated by a decrease in the number of cells in the cultures. On the other hand, in the ER^- lines, low concentrations of tamoxifen have no effect on cell growth, although 10 μM slows growth dramatically in HS578t cells and slows growth then begins to cause cell death after long exposures in HTB26 cells. The effect of tamoxifen on the ER^+ cell lines presumably reflects, largely, the antiestrogenic properties of tamoxifen. Growth inhibitory effects of tamoxifen and high concentrations of estradiol have been demonstrated before [e.g., (27)]. The effect of high dose tamoxifen on the ER^- cells may be a reflection of its activity as an inhibitor of protein kinase C (28-30), as an antagonist of calmodulin (31), or its reduction of bcl-2 expression (see below). These possibilities may be investigated in later studies.

Table II
Effects of Estrogen, Progesterone and Tamoxifen on Growth Rate of Breast Cancer Cell Lines

	Doubling Time (h)			
Treatment	MCF-7	T47D	HS578t	HTB26
Complete medium	35.3	47.0	30.4	32.2
Phenol red-free (prf) medium	39.5	59.9	37.1	35.4
prf + 0.1 μM estradiol	26.0	60.9	38.0	35.7
prf + 1.0 μM estradiol	26.7	65.4	35.4	35.6
prf + 10 μM estradiol	116	>200	57.4	73.3
prf + 0.1 μM progesterone	35.8	60.0	39.6	34.8
prf + 1.0 μM progesterone	27.9	63.8	33.1	35.3
prf + 10 μM progesterone	34.9	179	49.1	41.9
prf + 1.0 μM tamoxifen	61.8	106	42.7	33.9
prf + 3.0 μM tamoxifen	66.9	97.0	44.2	35.8
prf + 10 μM tamoxifen	cell number decreased	cell number decreased	175	47.8*

Data are averages from 1-3 separate experiments.
* At times longer than 150 h, cell number decreased.

Plating efficiency determinations also have been made on cells exposed to hormonal manipulation for long times (2-7 days in some experiments). In these experiments, following drug treatment, the cells were replated into complete medium (containing phenol red and untreated serum). It is interesting to note from the results that, in all cell lines, even under conditions where the growth rate was slowed dramatically or the cell number even decreased (e.g., 10 μM tamoxifen), the plating efficiency in treated cells was about the same as that in control untreated cells. In other words, all the cells present after any given time of drug exposure were equally viable as control cells if the drug was removed.

Radiation cell survival curves of breast cancer cell lines without and with hormonal manipulation

We have begun to obtain clonogenic survival curves for the breast cancer cells exposed to ionizing radiation following treatment with the concentrations of estrogen, progesterone and tamoxifen listed in Table II. In all our experiments cells were grown for one week in estrogen-free, phenol-red-free medium prior to initiation of drug treatment. Our data indicate that pre-culture in estrogen-free, phenol-red-free medium does not alter the radiation sensitivity of either MCF-7 or T47D cells compared to controls grown in complete medium. This finding contrasts slightly with a recent publication that showed that culture of MCF-7 BUS cells for 72 h in estrogen-free medium resulted in an increased radioresistance, although there was no change in the radiosensitivity of T47D B8 cells (32). It is unclear at this time why our results differ from theirs with regards to the MCF-7 cells, but the differing *p53* status of the strains may be a factor (see below). Our initial studies also indicate that treatment of either MCF-7 or T47D cells with low concentrations (0.1 and 1.0 μM) of estradiol, progesterone or tamoxifen does not alter radiation sensitivity of the cells, consistent with the lack of effect of these treatment on cell growth rate (Table II). However, treatment of the cells for 48-96 h with 10 μM of any of the drugs increases radiation sensitivity. Repeat determinations of these survival curves and studies with HS578t and HTB26 cells are in progress.

Molecular analysis of cancer cell lines

In last year's report we discussed the potential importance of the tumor suppressor gene *p53* and the oncogene *bcl-2* and its related family to radiation-induced apoptosis. We also presented information that was available in the literature regarding the status of *p53* and *bcl-2* in the breast cell lines we have been using. Since some relevant information was not available in several lines and we felt it was necessary to confirm the molecular analyses ourselves on some cell lines, we have begun to perform Western blots, using standard protocols [e.g., (33,34)] to ascertain the *p53* and *bcl-2* status of cell lines we are using. The data we have obtained thus far are summarized in Table III and compared to available literature data. In terms of *p53* status, our findings agree with published literature for T47D, HS578t and BT20 cells. However, it is generally reported in the literature that MCF-7 cells are wild-type *p53*, although we could find no evidence of any p53 expression in control or irradiated MCF-7 cells. MCF-7 cells are known to sporadically, spontaneously lose the *p53* gene when grown in culture (personal communication from Patrick O'Connor to Simon Powell in our Department). Hence, it appears that we have a strain of MCF-7 cells that have lost *p53*. We will obtain a *p53* wild-type strain and repeat the experiments shown above with the wild-type cells. Having this pair of MCF-7 cells will provide a unique opportunity to compare the effects of p53 expression on cell survival and apoptosis following radiation and or hormonal manipulation in two cell lines that are similar genetically except for *p53*.

Table I includes a listing of the *p53* gene status, from the literature where available, of the non-breast cancer cell lines we have tested for radiation-induced apoptosis. For those cell lines

on which we have been unable to find any information in the literature, we have begun to determine the *p53* status. Our initial studies indicate that the soft tissue sarcoma STS26T line is p53 null and the HP555 glioblastoma line appears to contain mutant p53. As noted above, both of these lines undergo radiation-induced apoptosis.

It has been reported in the literature that bcl-2 is expressed in MCF-7 cells, and our MCF-7 strain is consistent with that (Table III). It also has been reported that HS578t cells do not express bcl-2, but we have consistently found low, but significant, levels of bcl-2 protein in the HS578t cells we use. In our work, T47D cells show expression of bcl-2, and that expression appears to be increased by exposing the cells to estradiol for 24 h and decreased by a similar exposure to tamoxifen. This apparent modulation of bcl-2 expression by hormonal manipulation has been reported for MCF-7 cells (35).

Table III
Status of p53 and bcl-2 Determined by Western Blotting in our Laboratory Compared with Literature Reports

	p53		bcl-2	
Cell line	This Study	Published	This Study	Published
MCF-7	null	wild-type (36)	expressed	expressed (35)
T47D	mutant	mutant (36)	expressed	?
HS578t	mutant	mutant (37)	expressed (low)	not expressed (37)
BT20	mutant	mutant (36)	?	not expressed (37)

Discussion

Based on the new data we have presented herein and those data presented in last year's report, it appears that most cell lines derived from human solid tumors, breast or non-breast, do not undergo radiation-induced apoptosis as measured using DNA ladder formation in electrophoresis gels. Few studies have been published to date on radiation-induced apoptosis in cells from human solid tumors. Consistent with our data is a publication showing that only 2 of 6 soft tissue sarcoma cell lines and none of 14 glioma cell lines had any demonstrable radiation-induced apoptosis (38). There are several possible explanations for these findings. First, as mentioned in last year's report, it is possible that cells from solid tumors undergo apoptosis but don't fragment their DNA to ladder size pieces. Studies using other assays for apoptosis are needed to check this possibility. Second, also as discussed last year, these cancer cells may contain strong anti-apoptosis mechanisms related to the status of genes such as *p53*, *bcl-2*, *bax*, 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 (39,40). Since most of the cell lines we have tested to date contain mutant p53 or are p53 null (Tables I and III), that may be the cause of their resistance to radiation-induced apoptosis. Alternatively, it has been shown quite recently that overexpression of bax sensitizes MCF-7 cells to radiation-induced apoptosis (41). We have begun to determine the bax levels in breast cancer cell lines, also, and to determine whether radiation or tamoxifen alters these levels. Elucidation of these possible molecular mechanisms continues to be a main emphasis of this research project. The importance of such studies has been emphasized by a recent review article that suggested decreased apoptosis may play an important role in the biological aggressiveness of breast cancer (42).

It is interesting to speculate that in some way the decreased bcl-2 expression we have seen in tamoxifen treated T47D cells and that has been reported in the literature for MCF-7 cells (35) may be involved in the loss of cells seen in growth curves and in the increased radiation

sensitivity of these cells at high tamoxifen doses. Apoptosis studies are now needed to parallel these growth curve and molecular analyses.

Our ongoing and immediately planned experiments include: (1) additional assays for apoptosis applied to all the breast and non-breast cancer cell lines we have been using, with cells exposed to radiation and/or hormonal manipulation; (2) flow cytometry studies on the breast cancer lines, to be conducted in parallel with the apoptosis studies, to determine if the hormonal manipulations are altering cell cycle distribution and whether that, in turn, alters radiation sensitivity or apoptosis induction; (3) continuation of the molecular characterization of the cell lines, as mentioned above. All of these studies are continuations of our work addressing the technical objectives in the original grant application and summarized here in the Introduction.

CONCLUSIONS

The data we have acquired to date in this project continue to indicate that most human breast cancer cell lines tested and a number of cell lines from other human solid tumors do not readily undergo radiation-induced apoptosis as measured using the appearance of DNA ladders on electrophoresis gels. Possible reasons for this finding are discussed above, and the possibility that breast cancer cells contain strong anti-apoptotic mechanisms that may contribute to both the development of the cancer initially and the resistance to therapy is being investigated.

Tamoxifen does affect the growth of the breast cancer cell lines in a manner that may be consistent with the induction of apoptosis, and tamoxifen increases the radiation sensitivity of the breast cancer cell lines. Of particular relevance for this project is the possibility that bcl-2 expression, or the expression or bcl-2-related proteins, may be involved in these effects. This is being studied.

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