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Cancer is one of the leading causes of death in America and breast cancer is particularly threatening for women. In America 10% of women will be diagnosed with breast cancer resulting in the death of more than 40,000 of these women each year. Inheriting a single defect in genetic material causes about 5% of the cases of breast cancer and a gene that is commonly mutated in these familial cases of breast cancer is called *BRCA2* (Breast Cancer susceptibility gene). *BRCA2* is considered a tumor suppressor gene because its function is essential for preventing cancer and deletion of this function predisposes women to familial breast cancer. BRCA2 is important for repairing damage to genetic material, DNA, by virtue of its association to a protein that repairs DNA called RAD51. It is believed that BRCA2 enhances the repair capacity of RAD51. Interestingly, many anti-cancer therapeutics have been developed that kill cancer cells by damaging DNA such as ionizing radiation and chemotherapy. We propose to develop anti-cancer therapeutics that hinders a cell s ability to repair DNA damage by disrupting the function of the RAD51-BRCA2 complex. We show that expression of a small region of the BRCA2 protein that associates with RAD51 kills cancer cells. Anti-cancer therapeutics that disrupt the RAD51-BRCA2 interaction should increase the effectiveness of treatment with either ionizing radiation or chemotherapy because these new therapeutics would decrease the cancer cell s ability to repair the damage caused by either ionizing radiation or chemotherapy. Administration of this new generation of anti-cancer therapeutic should allow the effective use of lower doses of ionizing radiation and chemotherapy, which would reduce the toxic side effects commonly associated with cancer therapy. In addition, these new therapeutics should increase the effectiveness of treatment against tumors that are resistant to ionizing radiation and chemotherapy.

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Introduction

BRCA2 is important for suppressing breast cancer. Women with one mutant copy of BRCA2 are predisposed to breast cancer with loss of heterozygosity being an important step in the oncogenic process. BRCA2 functions to repair DNA double-strand breaks by recombination. At the cellular level, disruption of recombination impedes proliferation and induces either senescence or apoptosis. In addition, cells are sensitized to agents that generate DNA double-strand breaks like ionizing radiation and DNA crosslinking agents. Thus, disruption of the association of BRCA2 with RAD51 should be efficacious in the treatment of cancer by acting as an adjuvant to radiation therapy and chemotherapy by decreasing the cell s ability to repair damage caused by these DNA damaging agents. Based on this reasoning we have developed a small peptide derived from a region encoded in exon 27 of BRCA2 that interacts with RAD51. Exposure to this peptide decreases proliferation and initiates apoptosis for cancer-derived cells grown in tissue culture. We propose to characterize this peptide by determining its cytotoxic effect on a variety of cells derived from tumors that are both radiosensitive and radioresistant. This peptide could prove to be an effective adjuvant to ionizing radiation and DNA crosslinking agents that would be especially useful for treating tumors that are resistant to these therapies and for lowering doses of these cytotoxic drugs, thereby reducing their deleterious side effects.

Body

RAD51 is important for repairing double-strand breaks in DNA by recombination (*1*); interestingly, this function is likely to be essential since mammalian cells deleted for RAD51 exhibit chromosomal instability, are unable to sustain proliferation and senesce or die (*2*). In order to function, RAD51 associates with BRCA2 (*3*), a protein important for suppression of breast cancer (*4*). RAD51 associates with BRCA2 in two domains: the most amino - terminal domain is mediated by the BRC motifs encoded in exon 11 (*5*) and in the most carboxy - terminal domain is mediated by a single region encoded in exon 27 (*3*). COOH terminal deletions that remove some but not all of these regions increase replicative senescence and sensitivity to ionizing radiation and cross-linking agents, suggesting that the RAD51 - BRCA2 association is biologically important (*6-8*). We describe in the grant a 26 amino acid peptide (PLPSPVSPICTFVSPAAQKAFQPPRS), derived from the RAD51 interacting region encoded in exon 27 of BRCA2 that biochemically interacts with a RAD51 filament formed on single stranded DNA. Biological activity of this 26mer was assessed by conjugating it to 16 amino acids from the third helix of the Antennapedia homeodomain (antp) that enables proteins to transverse biological membranes. Exposure to antp-26mer causes a reduction of cellular proliferation, disruption of RAD51 foci and programmed cell death when applied to tissue culture cells that were derived from cancer. We propose that antp-26mer forms a nonproductive association with RAD51 that inhibits its function and disables recombinational repair, leading to reduced cellular proliferation and induces either cellular senescence or apoptosis. In addition, compromised RAD51 function would increase sensitivity to ionizing radiation and to other clastogenic agents that are common cytotoxic drugs used to fight cancer. Our goal is to develop this RAD51-interacting peptide as an anti-cancer therapeutic. We previously proposed to characterize the peptide with tissue culture cells to limit the size and identify the residues in the smallest active peptide that are important for biological activity. We have accomplished this task and reduced the size to 5 amino acids and found at least one amino acid to be critical. These peptide derivatives could prove to be beneficial as anti-cancer agents by

hindering cellular proliferation and by sensitizing cells to chemotherapeutics and ionizing radiation. In order to determine the effectiveness of these peptides as anti-tumor agents, they will be tested for their ability to enhance the treatment of currently used chemotherapeutics and radiation. Towards this goal we are developing a genotoxic screen with mouse embryonic stem (ES) cells in order to test for possible synergy of the peptide with these agents. After this is completed we will test biological activity on cells derived from breast cancer and cells that posses either wild type or diminished BRCA2 function. We will also test cells with a variety of genetic backgrounds that may induce cancer. Antp-26mer and its derivatives could be highly efficacious against radio-resistant forms of cancer. These peptides will finally be tested for potential anti-cancer activity in a mouse model prone to mammary carcinoma. In addition, peptides will be tested as possible adjuvants to radiation therapy.

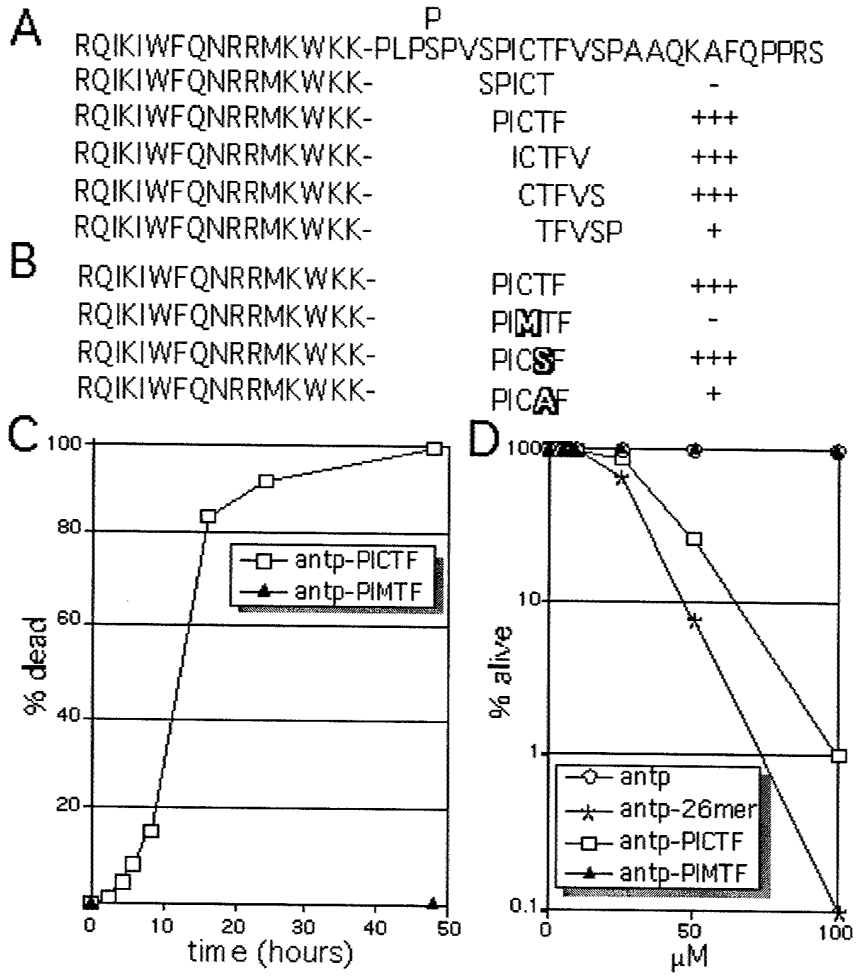

Figure 1. Cytotoxicity of Brca2 peptides. **A.** Analysis of antp (RQIKIWFQNRRMKW KKPL) conjugated to mouse Brca2 24mer (PSPVSPICTFV SPAAQKAFQPPRS). There is only one amino acid difference between mouse and human Brca2 in this peptide (S vs. P, respectively). Deletion of Brca2 shows that three sequential subunits of 5 amino acids exhibit substantial activity (+++) as judged by ability to inhibit colony formation. **B.** Substitution analysis of antp-PICTF. Substitute amino acids are shadowed. The C-M switch ablates activity (-), the T-A switch reduces activity (+) while the T-S switch does not alter activity (+++). **C.** Time course. HeLa cells exposed to 50 mM antp-PICT (square) or 50 mM antp-PIMTF (triangle). Cells that stain with trypan blue are counted as dead. **D.** Dose-response curve. The percent of alive cells are shown (cells that do not stain with trypan blue). Antp (circle), antp-24mer (•), antp-PICTF (square) and antp-PIMTF (triangle) are compared. The lines for Antp and antp-PIMTF are superimposed because both peptides fail to kill cells at all concentrations tested.

Since submission of the grant we have limited the size of the peptide from 26 amino acids to 5 amino acids (all peptides are conjugated to 16 amino acids of Antennapedia). A deletion analysis was performed to limit the region of peptide that reduces colony formation (Figure 1A). Antp was conjugated to sequential 5 amino acid segments of the 26mer and tested for effect on

colony formation. Three peptides substantially reduced colony formation and are contained within seven amino acids (PICTFVS). This region is completely identical between human and mouse. CTF is common to all three peptides. Therefore, this region is likely essential for reducing colony formation.

A substitution analysis was performed with PICTF conjugated to antp (antp-PICTF) to determine critical amino acids for reducing colony formation (Figure 1B). The focus was on cysteine and threonine. It is possible that phosphorylation status of threonine is important to reduce colony formation. To investigate this possibility, the threonine was changed to serine (antp-PICSF) and to alanine (antp-PICAF). Substitution of threonine with serine did not alter reduction of colony formation, as expected since this is a conservative change and both amino acids may be phosphorylated. However, substitution of threonine with alanine greatly reduced, but did not eliminate, the ability of the peptide to inhibit colony formation. Thus, this nonconservative change greatly inhibited activity of the peptide as measured by colony formation. Since antp-PICAF is marginally active, then phosphorylation may accentuate but is not essential for reduction of colony formation. To investigate the possibility that cysteine is important for activity of the peptide, cysteine was changed to methionine (antp-PIMTF). This change completely destroyed activity. Thus, methionine is absolutely detrimental for activity as measured by reduction of colony formation.

Onset of cell death was determine (Figure 1C). HeLa cells were stained with trypan blue at a variety of time points after exposure to 50 mM antp-PICTF or 50 mM antp-PIMTF. Less than 20% of cells stained with trypan blue 8 hours after exposure to antp-PICTF. However, greater than 80% of the cells stained blue after 16 hours of exposure to antp-PICTF. No cells stained blue after 48 hours exposure to antp-PIMTF. Therefore, antp-PICTF, but not anti-PIMTF, kills cells at a concentration of 50 mM and most cells are identified as dead between 8 and 16 hours after exposure.

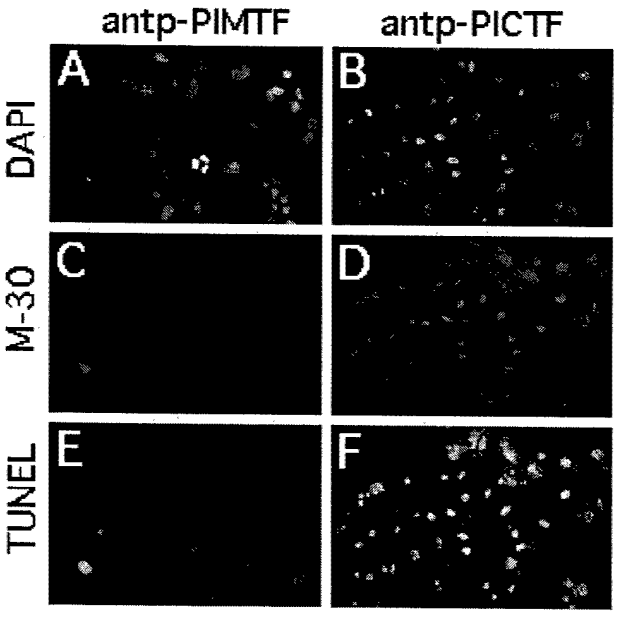

Figure 2. Programmed cell death. **(A, B)** Dapi stained HeLa cells. **(C, D)** antibody M30 CytoDEATH (M-30) stained HeLa cells. **(E, F)** TUNEL assay for nuclear fragmentation. **(A, C, E)** HeLa cells exposed to antp-PIMTF. **(B, D, F)** HeLa cells exposed to antp-PICTF.

A dose — response curve was established for HeLa cells exposed to antp, antp-24mer, antp-PICTF and antp-PIMTF (Figure 1D). HeLa cells were observed for death by staining with trypan blue 24 hours after exposure to peptide (a time when 92% of cells stain with trypan blue after exposure to 50 mM antp-PICTF, Figure 1C). Both antp-24mer and antp-PICTF killed cells starting at 25 mM and cell death progressively increased up to 100 mM concentration. Neither antp nor antp-PIMTF killed cells at the 100 mM concentration.

Programmed cell death was tested by analyzing asynchronous HeLa cells with either antibody M-30 CytoDEATH (M-30) or by TUNEL four hours after exposure to 50 mM antp-

PICTF or 50 mM antp-PIMTF, a time when trypan blue stains only 5% of cells exposed to antp-PICTF. M-30 recognizes a specific caspase cleavage site within cytokeratin 18 that is not recognized in its native state and TUNEL recognizes nuclear fragmentation. Cytokeratin 18 cleavage is an earlier step of apoptosis than nuclear fragmentation (*9*). Programmed cell death was detected after four hours exposure to antp-PICTF, but not antp-PIMTF, by both M-30 and TUNEL (Figure 2). Thus, apoptosis is induced in HeLa cells soon after exposure to antp-PICTF.

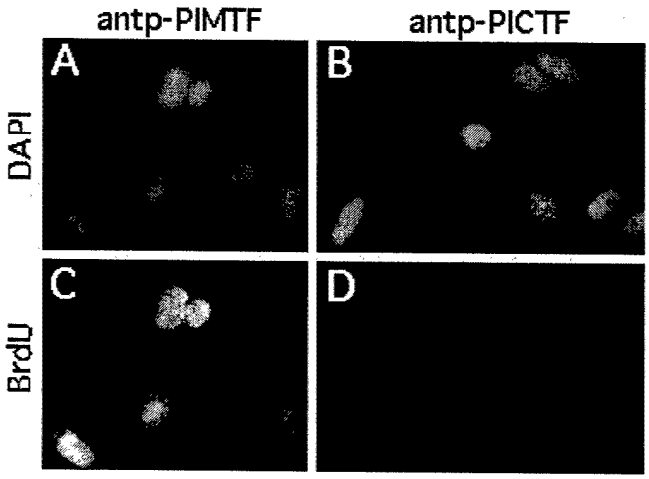

Figure 3. Unscheduled DNA synthesis. (**A, B**) Dapi stained HeLa cells. (**C, D**) anti-BrdU stained HeLa cells. (**A, C**) HeLa cells exposed to antp-PIMTF. (**B, D**) HeLa cells exposed to antp-PICTF.

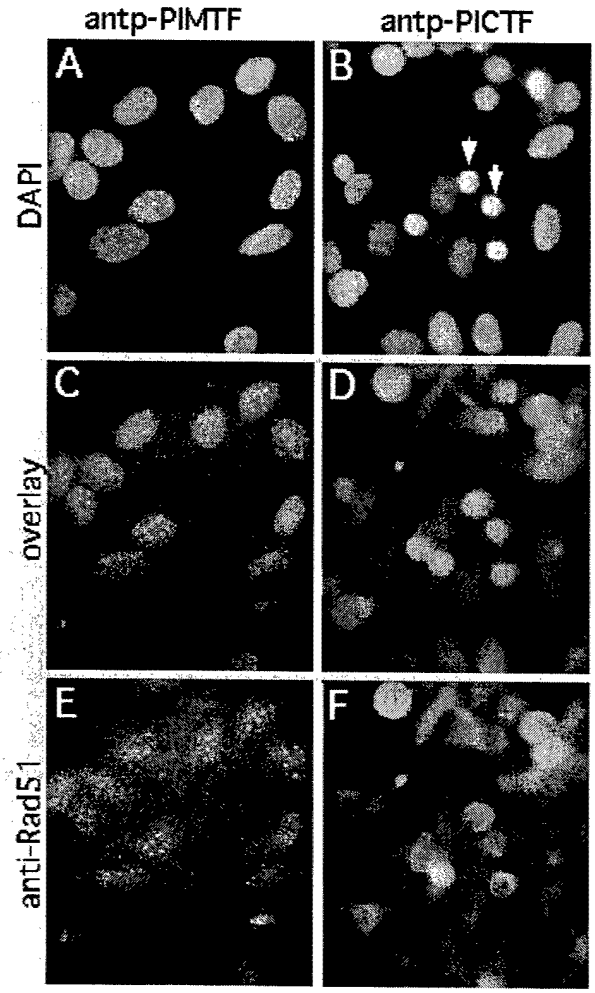

Figure 4. Rad51 foci. (**A, B**) Dapi stained HeLa cells. (**C, D**) Overlay of Dapi stain and anti-Rad51 stained cells. Demonstrates that Rad51 foci are in the nucleus for cells exposed to antp-PIMTF and Rad51 foci are no longer observed for is cells exposed to antp-PICTF. (**E, F**) Anti-Rad51 stained HeLa cells. (**A, C, E**) HeLa cells exposed to antp-PIMTF. (**B, D, F**) HeLa cells exposed to antp-PICTF.

We observed cells for unscheduled DNA replication and Rad51 foci in nonirradiated HeLa cells. Unscheduled replication, as judged by BrdU incorporation, was shown to occur mostly in cells that exhibit Rad51 nuclear foci, suggesting that cells containing concentrated Rad51 are also undergoing repair DNA synthesis (*10, 11*). Unscheduled replication (Figure 3) and Rad51 foci (Figure 4) were commonly observed four hours after exposure either to antp-PIMTF or to no peptide in HeLa cells. However, both unscheduled replication and Rad51 foci were no longer observed 4 hours after exposure to 50 mM antp-PICTF in HeLa cells. In addition, Rad51 seemed to be sequestered to the cytoplasm in some of the HeLa cells exposed to antp-PICTF.

Key Research Accomplishments

1) Narrowed the length of the peptide from 26 amino acids to 5 amino acids (have discovered three 5mers with activity).
2) Substitution of threonine with serine did not alter activity.
3) Substitution of threonine with alanine greatly reduced, but did not eliminate activity.
4) Substitution of cysteine with methionine ablates activity.
5) Antp-PICTF, but not antp-PIMTF, induces apoptosis within four hours.
6) Antp-PICTF, but not antp-PIMTF, disrupts Rad51 foci within four hours.
7) Antp-PICTF, but not antp-PIMTF, impairs unscheduled DNA replication within four hours.

Reportable Outcomes

Patent No.: 6,037,125
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51.

Patent No.: 6,057,104
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51 for Hindering Cell Proliferation

Conclusions

Based on these results we have narrowed the 26mer down to a 5mer with biological activity and the cysteine appears to be essential for activity. Antp-PICTF induces apoptosis, disrupts Rad51 foci, and interferes with unscheduled DNA replication. These results complete specific aim 2.

The activity of antp-PICTF will now be tested on a variety of cells to test its activity on a variety of genetic backgrounds (specific aim 1). In addition, we will test for synergy with chemotherapeutics and ionizing radiation (specific aim 1). Originally, I proposed to test antp-26mer for specific aim 1, but since submission of the grant decided specific aim 2 was more important to complete first. This way specific aim 1 is performed with the shortest possible peptide (antp-PICTF) and with a control peptide (antp-PIMTF). These experiments, to satisfy specific aim 1, should be completed within the year and those results presented for the next update.

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Appendices

none