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13. ABSTRACT (Maximum 200 Words)

Ataxia-telangiectasia mutated (ATM) is a protein kinase that acts as a master switch controlling the cell cycle in response to ionizing radiation-induced DNA double-strand breaks (DSBs). Carriers of *ATM* mutations are at increased risk for breast cancer. Since many anti-tumor chemotherapeutics used in breast cancer treatment also have the capacity to induce DNA DSBs, I have investigated the requirement for ATM in the cellular response to these agents. Using human ATM-positive and ATM-negative cell lines, I examined the cellular response to five common chemotherapeutics. Although I observed robust p53-dependent responses with 80% of the agents, only doxorubicin demonstrated a dependence upon ATM. Further characterization of the ATM-dependent response to doxorubicin revealed ATM-dependent p53 nuclear accumulation and phosphorylation of p53 on seven serine residues. I have also investigated doxorubicin-treated cell extracts for ATM autophosphorylation, ATM-dependent changes in p53-DNA binding affinity and alterations in the phosphorylation of downstream signaling molecules. Co-incubation of cells with antioxidants attenuated the doxorubicin-induced effect, suggesting that oxygen radicals may play a role in doxorubicin-induced activation of ATM-dependent pathways. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications for the treatment of breast cancer patients harboring mutations in *ATM*.

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Annual Summary

Award Number: DAMD17-02-1-0318
Project Title: Activation of ATM by DNA Damaging Agents
Principal Investigator: Ebba U. Kurz, Ph.D.
Reporting Period: 1 Sept 02 – 31 Aug 03

Introduction

ATM is a nuclear protein kinase required for the arrest of the cell cycle at G_1/S, S and G_2/M in response to ionizing radiation (IR)-induced DNA damage (reviewed in 1). Inherited defects in ATM lead to the development of ataxia telangiectasia (A-T), a progressive neurodegenerative disorder characterized by profound sensitivity to IR, cancer predisposition, immunodeficiency, and a progressive loss of motor control due to cerebellar ataxia (reviewed in 2). Although A-T is relatively rare, studies suggest that 1% of the normal population is heterozygous for *ATM* mutations and that *ATM* heterozygosity could play a more significant role than BRCA1 and BRCA2 in breast cancer.

Exposure to IR causes DNA double-strand breaks leading to the activation of ATM in the cell (1). Interestingly, many of the anticancer drugs used in the treatment of breast cancer also have the capacity to induce DNA double-strand breaks (4,5), however little has been known about the role of ATM in response to damage induced by these drugs. The first aim of my three-year training grant from the US Army Breast Cancer Research Program is to examine the effects of DNA damaging chemotherapeutics on the activation of ATM *in vivo*. The experimental efforts of the previous year towards this aim are summarized in this report.

Body (Detailed Research Accomplishments)

As outlined in the approved Statement of Work, the initial experimental task undertaken in examining the effects of DNA damaging chemotherapeutics on the activation of ATM *in vivo* was to determine, by cell proliferation assay, the IC_{50} of each test compound in the two pairs of ATM-proficient and ATM-deficient lymphoblastoid cell lines to be used in my studies. Using the WST-1 cell proliferation reagent (Roche Molecular Biochemicals), cytotoxicity assays were performed on cells four days following a two hour exposure to drug. Five chemotherapeutic agents were tested: doxorubicin, cyclophosphamide, melphalan, methotrexate and 5-fluorouracil.

Following the determination of IC_{50} for each chemotherapeutic to be surveyed, ATM-proficient and ATM-deficient cells were treated with each of the five agents (using concentrations at or below the determined IC_{50}) and nuclear extracts were prepared. As a marker for the activation of ATM, extracts were assayed by immunoblot for phosphorylation of p53 at serine 15 and the nuclear accumulation of p53. Four of the agents tested (doxorubicin, melphalan, methotrexate and 5-fluorouracil) induced a robust phosphorylation and accumulation of p53 within two hours. However, among these, only doxorubicin demonstrated a dependence upon ATM for this response. Cyclophosphamide failed to induce either p53 phosphorylation or nuclear accumulation. This is likely attributable to the fact that cyclophosphamide is an alkylating agent that requires metabolic activation to a cytotoxic species and the majority of cultured cell lines lack the cytochrome P450 isozyme essential for this activation.

As the aim of this research study is to evaluate the effects of DNA-damaging chemotherapeutics on the activation of ATM, all subsequent studies are focused on the cellular

effects following doxorubicin treatment. All experiments are carried out on at least two normal cell lines and two A-T cell lines. Cells exposed to IR serve as a positive control.

In the research project proposal submitted to the USAMRMC, I proposed to evaluate p53 phosphorylation at serines 15 and 20. At the time of submission, due to limited access to reagents, these were the only phosphoserine residues in p53 that could be evaluated by immunoblot. I am pleased to report that reagents are currently available for the analysis of seven phosphoserine residues in p53 all of which have been demonstrated to play a role in the DNA damage response. Consequently, I have expanded my proposed study. Using these reagents, ATM-proficient and ATM-deficient cells were treated with doxorubicin (1 μM) and cell lysates prepared at 0, 0.5, 1, 2, and 4 hours after initiation of drug treatment. p53 was immunoprecipitated from the extracts and immunoblotted with the available phosphospecific antisera. Reminiscent of the well-characterized response to IR, ATM-deficient cells demonstrated delayed phosphorylation of p53 on serines 6, 9, 15, 20, 37, 46 and 392 in response to treatment with doxorubicin (Figure 1).

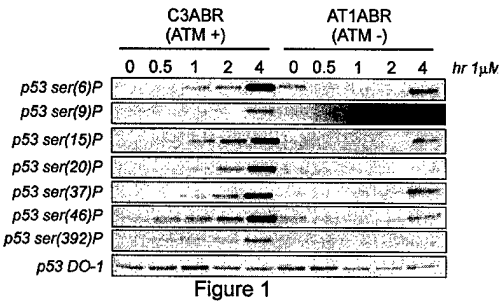

Figure 1

To further confirm the role of ATM in the phosphorylation and accumulation of p53 in response to doxorubicin, cells were pretreated with wortmannin, a fungal metabolite that binds irreversibly to the ATP binding site of phosphatidylinositol 3-kinase-like serine/threonine protein kinases (PIKKs) (6). Pretreatment of ATM-proficient cells with wortmannin prior to the addition of doxorubicin inhibited the phosphorylation and accumulation of p53, further supporting the involvement of a PIKK, such as ATM, in the cellular response to doxorubicin.

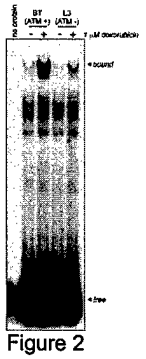
Figure 2

Having observed doxorubicin-induced nuclear accumulation of p53 and phosphorylation of p53 on seven serine residues, I next evaluated changes in p53-DNA binding affinity by electrophoretic mobility shift assay. As shown in Figure 2, treatment of ATM-positive cells with doxorubicin (1 μM) was found to increase dramatically the ability of p53 to bind its cognate DNA-binding site, indicating that the effect of doxorubicin in activating p53 to a transcriptionally competent species is very similar to that seen with IR exposure.

A very early stage in the response of mammalian cells to DNA double-strand breaks, such as those induced by IR and etoposide, is the phosphorylation of histone H2AX at serine 139 (7,8). Moreover, in the case of IR-induced DNA damage, H2AX phosphorylation is largely ATM-dependent (7). Phosphorylation of histone H2AX on serine 139 can, therefore, serve as an indicator of DNA damage and, more specifically, DNA double-strand break induction. Consequently, as a downstream target of ATM and marker of DNA damage, I evaluated the effect of treating ATM-proficient and ATM-deficient cells with doxorubicin (1 μM) on ATM-dependent histone H2AX phosphorylation by immunoblotting with a phosphospecific antibody. In a manner similar to IR, doxorubicin induced ATM-dependent phosphorylation of histone H2AX on serine 139, suggesting the presence of DNA double-strand breaks (Figure 3). Currently, I am expanding these studies to evaluate other signaling molecules and effectors in the ATM-mediated cellular response to doxorubicin.

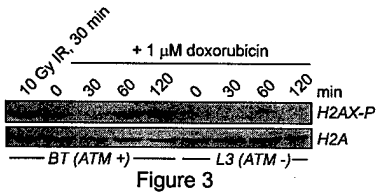

Figure 3

Exposure to IR results in a rapid increase in the protein kinase activity of ATM (9,10). Activation of ATM kinase activity following irradiation has recently been demonstrated to occur after autophosphorylation of ATM on serine 1981 (11). To determine if doxorubicin also induces phosphorylation of ATM at serine 1981, extracts were prepared from doxorubicin-treated cells and probed with a phosphospecific antiserum to phosphorylated serine 1981 of ATM. As has been reported previously for IR, exposure of ATM-proficient cells to doxorubicin (1 μM) induced phosphorylation of ATM on serine 1981 (Figure 4). Complementary studies are ongoing to assay the effect of doxorubicin on the protein kinase activity of ATM using ATM immunoprecipitates from treated or mock-treated cells and a known physiological substrate of ATM.

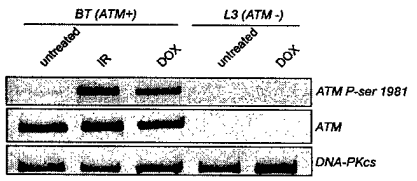

Figure 4

Although generally classified as a topoisomerase II-stabilizing drug that induces DNA double-strand breaks, doxorubicin also intercalates DNA and generates reactive oxygen species. Previously, etoposide, another topoisomerase II-stabilizing drug, has been shown to induce p53 accumulation and phosphorylation in an ATM-*independent* manner (12). Given the striking contrast between these two agents, I speculated that the ability of doxorubicin to induce reactive oxygen species might contribute to its cellular effects. Pretreatment of cells with the superoxide scavenger, ascorbic acid, had no effect on the doxorubicin-induced phosphorylation and accumulation of p53 (Figure 5). In contrast, pre-incubation of cells with the hydroxyl radical scavenger, N-acetyl cysteine, significantly attenuated the doxorubicin-mediated phosphorylation and accumulation of p53, suggesting that hydroxyl radicals may play an important role in doxorubicin-induced activation of ATM-dependent pathways. While these observations are provocative and may provide insight in the mechanisms influencing ATM activation, they lie beyond the scope of the proposed research and will be limited in nature.

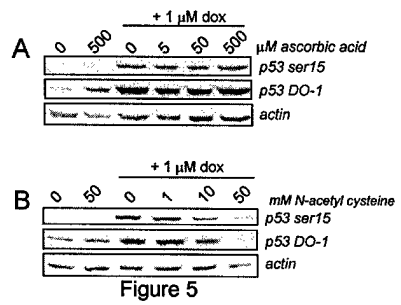

Figure 5

Given my early success, an abstract describing these studies was submitted and accepted for presentation at the Annual Meeting of the American Association for Cancer Research scheduled for April 5-9, 2003 in Toronto, Canada. Unfortunately, due to concerns over the outbreak of Severe Acute Respiratory Syndrome (SARS) in Toronto, the meeting was cancelled. Although the data were not presented, an abstract describing the work remains a citable publication (13).

In addition to conducting the research described herein, my training has been enriched through my interactions with other members of the Cancer Biology Research Group at the University of Calgary. As a member of this research group, I attend and participate in a weekly journal club and a weekly Work In Progress seminar series. My interaction with fellow members of the Cancer Biology Research Group has been greatly enhanced by the recent relocation of Dr. Lees-Miller's laboratory from the Biosciences building on the main campus of the University of Calgary to the Health Sciences Centre, home of the Faculty of Medicine, the Southern Alberta Cancer Research Centre and fifteen interdisciplinary research groups. As a member of the Department of Biochemistry and Molecular Biology, I also participated in their annual research retreat. As a senior member of Dr. Lees-Miller's laboratory, I also mentored an undergraduate student conducting an honors research project and a summer student working in our laboratory through the Heritage Youth Research Studentship program.

Key Research Accomplishments

- I identified doxorubicin as an anti-tumor chemotherapeutic that activates ATM in manifesting its cellular effects.
- I determined that ATM is required for the phosphorylation of p53 on seven serine residues in response to doxorubicin treatment.
- I observed that ATM is required for the doxorubicin-induced activation of p53 to a transcriptionally competent species.
- I established that doxorubicin induces DNA double-strand breaks and stimulates the ATM-dependent phosphorylation of histone H2AX on serine 139.
- I determined that doxorubicin induces ATM activation and autophosphorylation on serine 1981.
- I observed that co-treatment of cells with N-acetyl cysteine and doxorubicin significantly attenuates the phosphorylation and accumulation of p53, suggesting that hydroxyl radicals may play an important role in doxorubicin-induced activation of ATM-dependent pathways.

Reportable Outcomes

1. An abstract describing this work was published in the Proceedings of the American Association for Cancer Research (Volume 44 (1st ed), 6166, 2003).
2. My studies contributed to the work described in the provisionally accepted manuscript entitled "Genistein induces multisite phosphorylation of human p53 via an ATM-dependent DNA damage response pathway" by Ye R, Goodarzi AA, Kurz EU, Saito S, Higashimoto Y, Lavin MF, Appella E, Anderson CW and Lees-Miller SP.
3. A manuscript detailing the work described herein is currently in preparation.

Conclusions

It has been reported that carrying mutations in ATM could account for up to 5% of breast cancers, thus playing a more significant role in breast cancer than BRCA1 and BRCA2. Characterization of a role for ATM in the cellular response to anti-tumor chemotherapeutics could have significant implications leading to modified treatment protocols with fewer side effects for breast cancer patients who carry mutations in ATM.

The research conducted in the first year of my three-year training grant from the U.S. Army Breast Cancer Research Program identified doxorubicin as a chemotherapeutic agent that induces DNA double-strand breaks and requires ATM for the phosphorylation and activation of numerous cell cycle effectors and signaling molecules. These fundamental findings pave the way for the study of doxorubicin on the activation of ATM in breast cancer cells and ultimately to the identification of proteins in that interact with ATM in breast cancer cells following IR or doxorubicin treatment. These studies may provide clues as to the preferential predisposition of ATM heterozygotes to breast cancer.

References

1. Shiloh Y (2001) ATM and ATR: networking the cellular responses to DNA damage. *Current Opinion in Genetics and Development* 22, 71-77.
2. Lavin MF and Shiloh Y (1997) The genetic defect in ataxia-telangiectasia. *Annual Review of Immunology* 15, 177-202.
3. Khanna KK (2000) Cancer risk and the ATM gene: a continuing debate. *Journal of the National Cancer Institute* 92, 795-802.
4. Fritsche M, Haessler C and Brandner G (1993) Induction of nuclear accumulation of the tumor suppressor protein p53 by DNA-damaging agents. *Oncogene* 3, 307-318.
5. Vock EH, Lutz WK, Hormes P, Hoffmann HD and Vamvakas S (1998) Discrimination between genotoxicity and cytotoxicity in the induction of DNA double-strand breaks in cells treated with etoposide, melphalan, cisplatin, potassium cyanide, Triton X-100 and γ-irradiation. *Mutation Research* 413, 83-94.
6. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT (1998) Inhibition of phosphatide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Research* 58, 4375-4382.
7. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *Journal of Biological Chemistry* 276, 42462-42467.
8. Rogakou EP, Pilch DR, Orr AH, Ivanova VS and Bonner WM (1999) DNA double-stranded breaks induce histone H2AX phosphorylation at serine 139. *Journal of Biological Chemistry* 273, 5858-5868.
9. Banin S, Moyal L, Shieh S-Y, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y and Ziv Y (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 281, 1674-1677.
10. Canman CE, Lim D-S, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB and Siliciano JD (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* 281, 1677-1679.
11. Bakkenist CJ and Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499-506.
12. Ye R, Bodero A, Zhou B-B, Khanna KK, Lavin MF and Lees-Miller SP (2000) The plant isoflavanoid genistein activates p53 and Chk2 in an ATM-dependent manner. *Journal of Biological Chemistry* 276, 4828-4833.
13. Kurz EU and Lees-Miller SP (2003) Doxorubicin induces ATM-dependent multi-site phosphorylation of p53 through the generation of reactive oxygen species. *Proceedings of the American Association for Cancer Research* 44 (1st ed), 6166.

Appendices

Appendix 1: Published abstract from the annual meeting of the American Association for Cancer Research.

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Abstract Number: 6166

Doxorubicin induces ATM-dependent multi-site phosphorylation of human p53 through the generation of reactive oxygen species.

Ebba U. Kurz, Susan P. Lees-Miller. University of Calgary, Calgary, AB, Canada.

Ataxia-telangiectasia mutated (ATM) is a cellular protein kinase that acts as a master switch controlling if and when cell cycle progression arrests in response to ionizing radiation (IR). Exposure to IR results in an increase in the protein kinase activity of ATM, ATM-dependent up-regulation of p53 protein, and the direct and indirect phosphorylation of p53. In addition, ATM contributes to p53-independent mechanisms of cell cycle checkpoint activation. IR-induced DNA double-strand breaks (DSBs) are thought to be an important trigger leading to ATM activation. Since many antitumour chemotherapeutics also have the capacity to induce DNA DSBs, we have investigated the requirement for ATM in the cellular response to doxorubicin, a topoisomerase II-stabilizing drug. Using several human ATM-positive and ATM-negative cell lines, we have observed ATM-dependent nuclear accumulation of p53 and ATM-dependent phosphorylation of p53 on seven serine residues. We have also investigated doxorubicin-treated cell extracts for changes in ATM protein kinase activity, ATM-dependent changes in p53 DNA binding affinity and alterations in the levels of downstream cell cycle effectors. Co-incubation of cells with antioxidants attenuated the doxorubicin-induced effect, suggesting oxygen free radicals play an important role in doxorubicin-induced activation of ATM-dependent pathways.

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