TECHNICAL PROGRESS REPORT

DIRECT ASSAY OF RADIATION-INDUCED DNA BASE LESIONS IN MAMMALIAN CELLS

DOE Grant DE-FG05-86ER60464 DOE/ER/60464--6

November 1, 1989 - September 1, 1992 DE93 001832

This progress report covers the period from November 1, 1989 to September 1, 1992. The first year of this grant period had an operating budget of ≈ $99,000 supporting 2.2 FTE that worked on 6 specific aims. Three of these specific aims involved continued development of the GC/MSD technique to improve its sensitivity for the detection of DNA base lesions, while studying how the DNA sequence, conformation, hydration state, and oxygenation status influenced the spectrum and frequency of DNA lesions induced by ionizing radiation. The remaining three specific aims involved determining the spectrum and frequency of DNA base lesions produced in irradiated C3H10$T_{1/2}$ cells under oxic and anoxic conditions, with and without potential modulators of DNA damage induction or DNA damage repair. A main component of the latter projects involved modulating the internal antioxidant environment by transfecting human antioxidant genes into C3H10$T_{1/2}$ cells, and then measuring their ability to modify DNA lesion induction, cell kill, and cell transformation.

In the first year, the grant was being run as if it were a program project. Dr. Daret St. Clair, Assistant Professor, was responsible for the antioxidant molecular biology portion of the program. Dr. William St. Clair, Assistant Professor, was responsible for the cell kill and cell transformation portion of the program. Dr. Steven Swarts, a postdoctoral fellow, and I were responsible for the instrumentation development, the methodology development, and radiation chemistry portion of the program.

In October 1990, I was informed that the second year operating budget would be reduced to ≈ $58,000. Consequently, a significant restructuring of the program was required. In June of 1991, this grant was reviewed in Chicago by a DOE panel of external reviewers. The reviewers severely criticized the management of the grant as a program project. The concern was that the PI did not have a good grasp of the molecular biology aspects of the program where his expertise was minimal. Given the budget cuts and the criticism raised in the review, the program was restructured to eliminate all the cellular and molecular biology work and concentrate on the technology development and radiation chemistry portion of the grant which received a very favorable review by the external panel. In November 1990, Dr. Swarts was appointed to the faculty at Bowman Gray as a Research Assistant Professor and assumed a greater role in the daily supervision of the technician and in the design of the experiments to improve and validate the GC/MSD technique for quantitatively measuring DNA base lesions. At the present time, his role is more like a Co-PI than a Co-I. The following is a summary of the accomplishments on DOE grant DE-FG05-86ER60464, including publications and presentations at national and international meetings on each individual topic. In some cases, many of the experiments were performed in the previous grant period, but were not completed and published until the present grant period.

EFFECT OF SEQUENCE, CONFORMATION AND OXYGENATION STATUS ON RADIATION-INDUCED DAMAGE IN DNA MODELS

Adenine (Ade), 2'-deoxyadenosine (dAdo), 5'-deoxyadenosine monophosphate (dAMP), single stranded poly adenylic acid [poly (dA)], double stranded deoxyadenylic-thymidylic acid [ds poly (dA-T)]

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and salmon testis DNA were irradiated with 500 Gy under oxic and anoxic conditions. The major damage products were analyzed by HPLC with optical detection and quantitated in terms of the percentage of the adenosine in each model compound found as a specific damage product. As previously reported by many investigators, the major damage products were different under oxic and anoxic conditions. Outside of the Ade free base, 8-OH-dAdo was the major oxic damage product from each model compound. However neither the monomeric nor the polymeric compounds gave results identical to that found for salmon testis DNA.

The type and quantity of the major damage products depended on the sequence and conformation of the model compounds under anoxic conditions. When dAdo and dAMP were irradiated under anoxic conditions, the major damage product was either the R or S isomer of 8,5'-cdAdo and little Ade or α-dAdo was observed. However, when poly(dA), poly(dA-dT), and salmon testis DNA were γ-irradiated under nitrogen, the major deoxyadenosine damage product (excluding the liberated Ade base) was identified as the α-anomer of deoxyadenosine. The yields of α-deoxyadenosine from poly(dA), poly(dA-dT), and salmon testis DNA irradiated with a dose of 500 Gy under anoxic conditions were 1.5, 1.3, and 1.3%, respectively. No α-deoxyadenosine was detected after irradiation under oxic conditions. The presence of nucleotides with the α-configuration at the anomeric carbon atom in the DNA chain may have a significant effect on its tertiary structure and possibly modify its biological activity.

These data indicate that DNA sequence and conformation plays an important role in determining the spectrum and quantity of lesions formed during anoxic irradiation. Moreover, the failure of the results from the monomeric compounds to predict the results obtained with the polymers suggests that model studies should be cautiously extrapolated to cellular DNA. Cadet and his group have recently confirmed these observations for anoxic irradiations and have extended the concept to include the induction of damage products under oxic conditions. In addition, our studies indicated that base damage detection and quantitation by HPLC methods did not have the sensitivity to study cellular DNA damage at biologically relevant doses, even when fluorescent derivatives were generated and analyzed. It is anticipated that the use of a laser as the excitation source and the improved resolution of the capillary electrophoresis technology (Specific Aims 1-4), will overcome many of the difficulties encountered in these HPLC studies.

Publications:

1. Lesiak, K.B., Wheeler, K.T., Formation of α-deoxyadenosine in polydeoxynucleotides exposed to ionizing radiation under anoxic conditions. *Radiat. Res.* 121: 328-337, 1990.

Presentations:

1. Swarts, S.G. "Is there relevance of *in vitro* DNA damage to *in vivo* DNA damage"? Invited talk presented at the 9th International Congress of Radiation Research, Toronto, Ontario, Canada, July 7-12, 1991.

RADIATION-INDUCED DNA DAMAGE AS A FUNCTION OF HYDRATION: BASE RELEASE

The release of unaltered bases from irradiated DNA, hydrated between 2.5 and 32.7 molecules of water per mole of nucleotide (Γ), was investigated using HPLC. The objective of this study was to elucidate the yield of the four DNA bases as a function of dose, extent of hydration, and the presence

or absence of oxygen. The increase in the yield of radiation-induced free bases was linear with dose up to 90 kGy, except for the DNA with $\Gamma = 2.5$, for which the increase was linear only to 10 kGy. The yield of free bases as a function of Γ was not constant in either the absence or the presence of oxygen over the range of hydration examined. For DNA with Γ between 2.5 and 15, the yield of free bases was nearly constant under nitrogen, but decreased under oxygen. However, for DNA with $\Gamma > 15$, the yield increased rapidly under both nitrogen and oxygen. The yield of free bases was described by a model that depended on two factors: 1) a change in the DNA conformation from a mixture of the A and C conformers in vacuum-dried DNA to predominantly the B conformer in the fully hydrated DNA, and 2) the proximity of the water molecules to the DNA. Irradiation of the inner water molecules ($\Gamma < 15$) was less efficient than irradiation of the outer water molecules ($\Gamma > 15$), by a factor of ≈ 3.3, in forming DNA lesions that resulted in the release of an unaltered base. This factor is similar to the previously published relative efficiency of 2.8 with which hydroxyl radicals and base cations induce DNA strand breaks. Our irradiation results are consistent with the hypothesis that the G value for the first 12-15 water molecules of the DNA hydration layer is the same as the G value for the form of DNA to which it is bound (i.e., the pseudo-C or the B form). Thus, we suggest that the release of bases originating from irradiation of the hydration water is obtained predominantly: (1) by charge transfer from the direct ionization of the first 12-15 water molecules of the primary hydration layer and (2) by the attack of hydroxyl radicals generated in the outer, more loosely bound water molecules.

RADIATION-INDUCED DNA DAMAGE AS A FUNCTION OF HYDRATION: BASE DAMAGE

In order to verify the mechanisms proposed in the base release work, an analysis of base damage produced in irradiated DNA that was hydrated from $\Gamma = 2.5$ to 32.7 was performed. The base damage products formed in these hydrated DNA samples were assayed using gas chromatography/mass spectrometry techniques. Several base damage products have been analyzed including: 1) 5,6-dihydrothymine (DHT), 2) 5-hydroxymethyluracil (HMU), 3) 5,6-dihydroxy-5,6-dihydrothymine (TG), 4) 4,6-diamino-5-formamidopyrimidine (FAPYA), 5) 8-hydroxyadenine (HOA), 2,6-diamino-4-oxo-5-formamidopyrimidine (FAPYG) and 7) 8-hydroxyguanine (HOG). DHT and HOG are the predominant DNA base damage products that were formed for DNA with $\Gamma = 2.5$-13 and irradiated under nitrogen. The levels of HOG were approximately 2 times those found for DHT in DNA with $\Gamma \leq 13$. Theoretically, the DHT/HOG yield ratio is expected to be ≈ 1 after direct ionization of the DNA, assuming the electron and the hole localize exclusively on thymine and guanine, respectively. This difference between the theoretical and experimental yields is likely due to the fact that the electron does not exclusively localize on thymine. Thus, the formation of DHT and HOG at $\Gamma \leq 13$ is consistent with the expectation that the damage induced by the direct ionization of DNA and the innermost water molecules of the hydration layer is derived mainly from the transfer of the electron and hole.

The yields of DHT and HOG maximizes at $\Gamma = 10$. The yield of DHT decreases at $\Gamma = 13$ and remains constant to $\Gamma = 33$. Conversely, the yield of HOG decreases at $\Gamma = 13$ and then increases as Γ is increased to 33. The lower yield of DHT at $\Gamma = 13$ is likely due to an increase in the incidence of recombination reactions involving the electron and hole. A progressive increase in the yield of HOG at $\Gamma > 13$ is likely due to the increasing incidence of hydroxy radical attack on the guanine moiety from the radiation damage in the outer water molecules. However, the increased HOG yield was not paralleled by increased yields of DHT over this same hydration range. It was expected that the electrons formed from irradiation of the outer water molecules would also react with thymine and, thus, should have resulted in increased yields of DHT for $\Gamma > 13$.

In the presence of oxygen, HOG is the predominant DNA base damage product formed over the range of DNA hydrations examined. In comparison to the nitrogen results, only a small amount

of DHT was observed for $\Gamma < 13$. This is consistent with the hypothesis that electrons are scavenged by oxygen and, thus, prevent the formation of DHT. In summary, the results of the DNA base damage study provide support for the mechanisms proposed in the base release study.

Publications:

1. Swarts, S.G., Sevilla, M.D., Becker, D., Tokar, C.J., Wheeler, K.T. Radiation-induced DNA damage as a function of hydration. I. Release of unaltered bases. *Radiat. Res.* 129: 333-344, 1992.

Presentations:

1. Swarts, S.G., Sevilla, M.D., Wheeler, K.T. "Radiation-induced DNA damage as a function of the degree of hydration." Presented at the UCLA Symposium on ionizing radiation damage to DNA: molecular approaches, Lake Tahoe, CA, January 16-21, 1990.

2. Swarts, S.G., Sevilla, M.D., Wheeler, K.T. "Radiation-induced damage in single- and double-stranded DNA as a function of the degree of hydration." Presented at the annual meeting of the Radiation Research Society, New Orleans, LA, April 6-12, 1990.

3. Swarts, S.G., Smith, G.S., Sevilla, M.D., Becker, D., Wheeler, K.T. "Specific DNA base damage produced by irradiation of hydrated DNA. Presented at the 9th International Congress of Radiation Research, Toronto, Ontario, Canada, July 7-12, 1991.

4. Swarts, S.G. "Radiation-induced DNA damage as a function of hydration". Invited talk presented at the International Conference on Pathways to Radiation Damage in DNA, Meadow Brook Hall, Oakland University, Rochester, MI, June 14-18, 1992.

STABILITY OF DNA DAMAGE PRODUCTS DURING ACID HYDROLYSIS

Characterization and quantitation of base damage products that are formed in cellular DNA as a result of reactions with activated oxygen species or exposure to ionizing radiation is important in understanding how these lesions are involved in mutagenesis or carcinogenesis. One technique that has been used in detecting and quantitating DNA base damage lesions is gas chromatography/mass spectrometry (GC/MS) because of its selectivity and sensitivity. This technique often employs the use of an acid hydrolysis step to degrade the DNA to the individual free bases before the subsequent derivation and GC/MS analysis. However, there is evidence that acid hydrolysis will cause modification or degradation of some of the DNA damage products. Consequently, we have studied a number of DNA base damage products to determine if they could be modified or degraded by acid hydrolysis both in the presence and absence of DNA, using the GC/MS assay. Several acid hydrolysis procedures were examined to ascertain their impact on the quantitation of the DNA base damage products, and where effects were noted, alternative procedures were developed to improve the quantitation of the products.

We have identified a number of factors that can modify quantitation of the DNA base damage lesions. These include the acid stabilities of the bases, base damage lesions, and the internal standards, hydrolysis temperature, source and grade of the formic acid, and the sample mass during acid hydrolysis. Although steps can be taken to minimize the influence of these factors on the quantitation of some base damage products, no single procedure will solve the quantitation problem for all the base damage products that we have studied. Under the best of circumstances, GC/MS analysis of acid hydrolyzed DNA can quantitatively measure 1 damaged base in 10^4 to 10^5 bases with a variance of 5

to 10%. In the worst cases, the variance can exceed 25% of the mean value. As described in the grant, enzymatic hydrolysis of the DNA at moderate to high doses produces undigestible fragments that can not be assayed by GC/MS. Consequently, both methods of preparing samples for GC/MS analysis have deficiencies when quantitative, rather than qualitative measurements are to be made. This emphasizes the need to explore the proposed CE-LIFD technology to measure DNA base lesions, because small undigested fragments can be handled with this technology.

Publications:

1. Swarts, S.G., Smith, G.S., Wheeler, K.T. Product stability during formic acid hydrolysis in quantitative gas chromatography/mass spectrometry analysis of DNA base damage. *Anal. Biochem.* (submitted)

Presentations:

1. Swarts, S.G., Smith, G.S., Wheeler, K.T. "Radiation-induced DNA base damage product stability to acid hydrolysis". Presented at the 40th Annual Meeting of the Radiation Research Society, Salt Lake City, Utah, March 14-18, 1992.

2. Swarts, S.G., Smith, G.S., Wheeler, K.T. "Stability of DNA damage products during acid hydrolysis". Presented at the 204th National Meeting of the American Chemical Society, Washington, DC, August 23-28, 1992.

CELLULAR DNA REPAIR STUDIES

The radiosensitivities and the kinetics for removal of radiation-induced DNA damage were compared for proliferative (P) and quiescent (Q) cells of the lines 66 and 67 derived from a mouse mammary adenocarcinoma. As determined from cell survival assays, the 66 and 67 Q cells were more radiosensitive than their 66 and 67 P counterparts. The rank order of their radiosensitivity was: 67 Q > 66 Q ≥ 67 P > 66 P. Induction of radiation damage in the DNA of these cells, as measured by the alkaline elution technique, was identical for 66 and 67 P and Q cells. The repair of this DNA damage was biphasic for 66 and 67 P and Q cells. The half-times for the fast and slow repair phases in 66 Q cells were identical to those previously measured in 67 Q cells. The half-times of the fast and slow repair phases in 66 P cells were also identical to those previously measured in 67 P cells. However, the half-times for the fast and slow repair phases in 66 and 67 Q cells were longer than those measured in their 66 and 67 P counterparts. The 66 cell data are consistent with our previously published hypothesis that Q cells are more radiosensitive than their corresponding P cells because they repair their radiation-induced DNA damage slower. However, our results are not consistent with hypotheses that attempt to explain the radiosensitivity differences between lines 66 and 67 solely on the basis of measurable induction and repair of DNA damage. These studies provide the kinetic data base for the studies proposed in Specific Aim 6 of this grant.

Publications:

1. Swarts, S.G., Nelson, G.B., Wallen, C.A., Wheeler, K.T., Radiation-induced cytotoxicity, DNA damage and DNA repair: Implications for cell survival theory. *Radiat. Environ. Biophys.* 29: 93-102, 1990.

Presentations:

1. Swarts, S.G., Wallen, C.A., Wheeler, K.T., "Repair of radiation-induced DNA damage in proliferative (P) and quiescent (Q) cells from a heterogenous mouse mammary tumor". Presented at the annual meeting of the Radiation Research Society, Seattle, WA, March 18-23, 1989.

INTRACELLULAR MODULATION OF THE ANTIOXIDANT, MANGANESE SUPEROXIDE DISMUTASE

Manganese superoxide dismutase (MnSOD) is a nuclear encoded mitochondrial matrix enzyme that functions to scavenge superoxide radicals. The human MnSOD cDNA under the transcriptional control of a human β-actin promoter was introduced into mouse C3H10$T_{1/2}$ cells by cotransfection with a recombinant plasmid containing the Neo^R selectable marker. C3H10$T_{1/2}$ transformants (C3H-SOD) were obtained that expressed high levels of authentic enzymatically active human MnSOD. Overexpression of the MnSOD gene did not affect the protein levels for CuZnSOD, catalase (CAT), or glutathione peroxidase (GPX) in the transformants. Treatment of cells with paraquat was less toxic to the C3H-SOD cells than to the control cells. Similarly, treatment with low doses of ionizing radiation was less toxic to these C3H-SOD cells. These results are consistent with the possibility that superoxide radicals are mediators of paraquat and radiation cytotoxicity.

Publications:

1. St. Clair, D.K., Oberley, T.D., and Ho, Y-S. Overproduction of human MnSOD modulates paraquat-mediated toxicity in mammalian cells. *FEBS Letters* 293: 199-203, 1991.

Presentations:

1. St. Clair, D.K., and Oberley, T.D. and Ho, Y-S. "Overproduction of human manganese superoxide dismutase modulates oxidative stress-mediated toxicity in C3H10$T_{1/2}$ cells." Presented at the annual meeting of the American Association for Cancer Research in Houston, TX, May 15-18, 1991.

2. St. Clair, D.K., and Daniel, L.W. "The role and regulation of MnSOD expression in oxidative stress and drug resistance". Presented at the 9th International Congress of Radiation Research, Toronto, Canada, July 7-12, 1991.

Summary:

As can be seen from the above progress report, our philosophy for the past several years has been to, 1) construct a library of authentic DNA base lesions (see the Experiment Design and Methods Section), 2) evaluate and optimize the operating parameters of each specific technique using these authentic compounds to maximize its selectivity and sensitivity, 3) use this technique to study an important radiation chemistry question in model DNA systems to determine the quantitation limits under controllable, but practical conditions, and finally 4) apply the approach to an important radiobiological problem in mammalian cells, if the technology has sufficient selectivity and sensitivity

to warrent it. The present grant proposal follows this same philosophy. It is important to remember that the task of developing a technique for measuring DNA base lesions at biologically relevant doses is a difficult one that requires a time consumming, careful, systematic approach. Although we have not yet achieved our overall goal during the past 3 years, we believe our progress has been steady, our productivity has been good (4 publications, 1 submission and 10 presentations), and our contributions to the understanding of how radiation produces DNA base damage in model systems and mammalian cells has been significant. Given funding, we anticipate similar progress over the next three years.

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