

BIOLOGY CONTRIBUTION

BASE EXCISION REPAIR OF BOTH URACIL AND OXIDATIVELY DAMAGED BASES CONTRIBUTE TO THYMIDINE DEPRIVATION-INDUCED RADIOSENSITIZATION

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Purpose: Increased cellular sensitivity to ionizing radiation due to thymidine depletion is the basis of radiosensitization with fluoropyrimidine and methotrexate. The mechanism responsible for cytotoxicity has not been fully elucidated but appears to involve both the introduction of uracil into, and its removal from, DNA. The role of base excision repair of uracil and oxidatively damaged bases in creating the increased radiosensitization during thymidine depletion is examined.

Methods and Materials: Isogenic strains of *S. cerevisiae* differing only at loci involved in DNA repair functions were exposed to aminopterin and sulfanilamide to induce thymidine deprivation. Cultures were irradiated and survival determined by clonogenic survival assay.

Results: Strains lacking uracil base excision repair (BER) activities demonstrated less radiosensitization than the parental strain. Mutant strains continued to show partial radiosensitization with aminopterin treatment. Mutants deficient in BER of both uracil and oxidatively damaged bases did not demonstrate radiosensitization. A recombination deficient *rad52* mutant strain was markedly sensitive to radiation; addition of aminopterin increased radiosensitivity only slightly. Radiosensitization observed in *rad52* mutants was also abolished by deletion of the *APN1*, *NTG1*, and *NTG2* genes.

Conclusion: These data suggest radiosensitization during thymidine depletion is the result of BER activities directed at both uracil and oxidatively damaged bases. © 2006 Elsevier Inc.

Thymidine deprivation, Radiosensitization, Base excision repair, Yeast.

INTRODUCTION

Thymidine is the only base unique to DNA. Inability to import or synthesize the nucleotide thymidine triphosphate (TTP) is lethal to proliferating cells. This phenomenon, termed *thymidineless death*, was originally described in 1954 by Barner and Cohen (1). The exact mechanism behind the toxicity of TTP deprivation is not known. Thymidylate (dTMP) is produced *de novo* by thymidylate synthase, an enzyme that transfers a one carbon group from reduced folate to dUMP (2). Thymidylate production can therefore be blocked either by inhibiting thymidylate synthase or by the production of reduced folate. Conversion of fluorodeoxyuridine (FdUR) to FdUMP by thymidine kinase produces a structural analog of dTMP able to inhibit thymidylate synthase. Inhibiting thymidylate synthase by agents such as

fluorodeoxyuridine or depleting reduced folate by agents such as methotrexate, or its analog aminopterin, can lead to thymidylate depletion (2). Inhibiting thymidylate synthesis by either means leads to accumulation of the precursor dUMP. Likewise, accumulation of dUMP leads to a build-up of its precursor, dUTP. During thymidylate depletion, the ratio of dUTP to TTP rises dramatically (3). dUTP may serve as an analog of TTP for most DNA polymerases (4) and dUTP incorporation into DNA during thymidylate deprivation has been considered a key event in the toxicity of thymidylate deprivation (5). Despite intensive study, the exact mechanism of thymidineless death remains incompletely understood.

The budding yeast *S. cerevisiae* has been used as an informative model system to explore the toxicity and processes occurring during thymidylate deprivation. S.

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This work was supported in part by a grant from the American Cancer Society through the Holden Comprehensive Cancer Center,

University of Iowa Hospitals and Clinics.

Acknowledgments—We are grateful for the masterful expertise of Ms. Amanda Kalen in irradiating the yeast cultures. We also appreciate the sage guidance of Douglas Spitz, Ph.D., and Eric Radany, M.D., Ph.D.

Received Oct 4, 2005, and in revised form Jan 23, 2006. Accepted for publication Mar 14, 2006.

cerevisiae lacks thymidine kinase activity and therefore cannot metabolize 5-fluorouracil or fluorodeoxyuridine to fluorodeoxyuridine monophosphate (FdUMP), the active inhibitor of thymidylate synthase. Therefore, most studies examining thymidylate depletion in *S. cerevisiae* have used aminopterin or methotrexate to block reduction of folate together with sulfanilamide to block *de novo* synthesis of reduced folate. Alternative strategies using strains able to import FdUMP directly have also been employed (6). Using these techniques, several events occurring during thymidylate deprivation have been described in yeast. Among these events are uracil (dUTP) incorporation into DNA and subsequent removal by uracil base excision repair (5). Interruption of uracil base excision repair, particularly at the abasic endonuclease cleavage step mediated by Apn1, produces profound sensitivity to thymidylate deprivation (7). Cell cycle arrest (8), single- and double-strand DNA breaks, recombination induction (6, 9) and mitochondrial damage (10) have all been described as consequences of thymidylate depletion.

Thymidine triphosphate-depleted cells are also more sensitive to the toxic effects of ionizing radiation (11, 12). The synergistic toxicity seen with TTP depletion and ionizing radiation is the fundamental basis of clinical radiosensitization for many cancers. The overall magnitude of this synergistic toxicity seen with radiation and TTP deprivation is modest, with dose enhancement ratios typically ranging from 1.2 to 1.8 (12). However, significant improvements in local control and overall survival result from repeating this enhancement with each radiation dose throughout a fractionated course of treatment. Genetic studies in *S. cerevisiae* have identified processes of central importance for response to radiation and thymidylate deprivation as single treatments. Seminal work in *S. cerevisiae* identified recombination as a key process in mediating the toxicity of ionizing radiation, because recombination-deficient cells (*rad52* mutants) are extraordinarily sensitive to radiation (13). The mechanism of radiosensitization during thymidylate deprivation, like the toxicity of thymidylate deprivation itself, remains unknown. Although genetic approaches in *S. cerevisiae* have been productive in exploring the individual toxicity of radiation and thymidine depletion, this approach has not yet been used to characterize the unique synergistic toxicity of combined thymidylate depletion and ionizing radiation.

This article uses mutants of yeast deficient in key aspects of response to either ionizing radiation or thymidylate depletion to examine the synergistic toxicity of radiosensitization during TTP depletion. Mutants lacking various steps in recombination or uracil-base excision repair (BER) show altered responses to radiation during thymidylate depletion. Mutations in activities involved in BER of oxidized bases also influence radiosensitization by thymidylate depletion.

METHODS AND MATERIALS

Strains

The parental strain used in this study is the haploid SSL204 *MATa ade2 trp1 leu2 ura3 his3* (14). The *rad52* mutant has been described previously and contains a disruption of the majority of the *RAD52* open reading frame and insertion of *LEU2* (15). The remaining strains have been described elsewhere (7). Mutants used for this study were generated by deletion of the entire open reading frame of the target and insertion of a selectable marker by transformation of a polymerase chain reaction (PCR) fragment containing a selectable marker flanked by ends homologous to the gene to be disrupted. The PCR fragment was introduced by lithium transformation, as previously described (16).

Drug exposure conditions

Aminopterin treatment. Cultures were grown overnight in minimal medium supplemented with 0.2% norite-treated casamino acids; 20 μ g/mL of tryptophan, adenine, and uracil, pH adjusted to 6.0 with succinic acid; and NaOH (17). Cultures were diluted 1:100 into fresh medium and outgrown 4 hours to ensure cultures were in exponential growth phase. Cell density was determined and 10^5 cells/mL were placed into fresh medium, described above, that also contained 6 mg/mL sulfanilamide and 0.2 mg/mL aminopterin (Sigma Aldrich, St. Louis, MO). Sulfanilamide was added to medium before autoclaving (18). Cultures without drug were also established. After three hours incubation, aliquots from the culture were removed and exposed to varying doses of radiation, as described below.

Methoxylamine treatment. A filter-sterilized 1 M methoxylamine (Sigma Aldrich) stock solution was freshly prepared in water and diluted to 10 mM in the appropriate culture. Methoxylamine was added when the culture was initially established at 10^5 cells/mL (at the same time that indicated cultures received aminopterin). Cultures were then returned to a shaking incubator at 30°C for 3 hours, followed by irradiation at the indicated doses.

Radiation exposure conditions

Logarithmically growing cultures were diluted to 10^5 cells/mL into the same medium described above with sulfanilamide (6 mg/mL) and aminopterin (0.2 mg/mL). Control cultures without drug were established by diluting these logarithmically growing cultures into the same medium without sulfanilamide or aminopterin at 5×10^4 cells/mL. Cell concentrations of the freshly diluted cultures were determined by plating appropriate amounts to yeast extract, peptone, dextrose (YPD) solid medium. The cultures were grown at 30°C with shaking for 3 hours. Portions of the cultures were removed and irradiated using a J.L. Shepherd 81-16A Cesium 137 irradiator (J.L. Shepherd & Associates, San Fernando, CA) at a dose rate of 30.3 Gy per minute. Immediately after irradiation, appropriate dilutions were made into water and plated to YPD agar medium (17). Plates were grown for 3 days and the numbers of colonies were counted. Unless stated otherwise, survival is reported relative to the portion of the culture that did not receive radiation. Toxicity of aminopterin/sulfanilamide was determined by comparing the colony-forming units in culture before and after the 3-hour incubation from the nonirradiated culture.

Flow cytometry analysis

Logarithmically growing cultures were grown in supplemented minimal liquid medium described above and diluted to 10^6

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