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DNA REPAIR SYNTHESIS IN MOUSE MAMMARY TISSUE TREATED WITH ALKYLATING AGENTS

by

William James Bodell

A DISSERTATION

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DNA Repair Synthesis in Mouse Mammary Tissue Treated with Alkylating Agents.

BY

William James Bodell

APPROVED	DATE
Mihir R. Banerjee	April 27, 1977
James Van Etten	April 27, 1977
Earl F. Ellington	April 27, 1977
Naba K. Gupta	April 27, 1977

SUPERVISORY COMMITTEE

GRADUATE COLLEGE **UNIVERSITY OF NEBRASKA**

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INTRODUCTION

In this section I examine DNA repair synthesis in mouse mammary epithelial cells treated with alkylating agents, and discuss the relationship between DNA repair, and mutagenesis, carcinogenesis, and cellular aging. The function of DNA repair in eukaryotic cells is to maintain the integrity of the cells genetic information. In addition to possible environmental exposure to chemical and physical agents, which can cause alterations of the cellular genetic information, spontaneous damage to the cellular genome can occur by depurination (1), depyrimidation (2), and deamination of cytosine (3). The spontaneous reactions occur at sufficient rates, that without DNA repair a considerable amount of DNA damage would accumulate in the cellular genome (4). DNA repair in eukaryotic cells occurs by three different mechanisms: excision repair, post replication repair, and photoreactivation. Each of these repair mechanisms are discussed separately in the following sections.

Excision Repair

Excision repair is the more thoroughly studied of the three DNA repair mechanisms. Excision repair occurs by the sequential action of at least 4 enzymes: a endonuclease, a exonuclease, a DNA polymerase, and a ligase. The first step in excision repair is the recognition of the altered base(s) by the endonuclease which hydrolyzes a phosphodiester bond near the altered base(s). It has been reported that base damage caused by physical and chemical agents results in alterations of

the secondary structure of double stranded DNA. Recent evidence suggests that the repair endonucleases may recognize alterations in the secondary structure of doubl stranded DNA, rather than the altered bases themselves. Thus different types of base damage, but which have the same influence on the secondary structure of double stranded DNA, appear to be recognized by the same repair endonuclease (5, 6). Cerruti (7) has proposed the following classification of types of DNA damage that may be recognized by repair endonucleases: Type I DNA damage causing negligible helix distortion, altered bases with essentially unaltered base pairing, and base stacking properties; Type II DNA damage causing minor helix distortion, altered bases with only slightly altered base pairing, and base stacking properties; Type III DNA damage causing major helix distortion.

An endonuclease has been purified from rat liver (8), calf thymus (9, 10), and Hela cells (11, 12), which is specific for apurinic and apyrimidinc acids. Since this endonuclease is inactive with alkylated DNA (10) it may work in conjunction with a glycosidase (13). Lindahl has recently isolated a glycosidase (13) which depurinates alkylated DNA (14) and depyrimidates uracil containing DNA (15). Since alkylated bases are removed from cellular DNA faster than can be explained on the basis of depurination (16-19), this glycosidase may work sequentially with the endonuclease specific for apurinic acids (13, 14). The glycosidase would recognize the alkylated base(s) catalyzing hydrolysis of the glycosidic bond, and converting the base to an apurinic acid which can be recognized by the apurinic acid endonuclease.

A second type of endonuclease has been isolated from mammalian tissues, which is specific for recognition of γ ray damage and minor

UV-photoproduct damage (12, 20). The exact photoproducts recognized by this endonuclease is not known at this time (12, 20), but Bacchetti and Benne (20) have ruled out that this endonuclease recognizes pyrimidine dimers. By the use of a photoreactivating enzyme, which is known to monomerize pyrimidine dimers, these investigators found similar enzymatic activity with their endonuclease both before and after photoreactivation of UV-irradiated DNA.

Van Lanker and Tomura (5, 6) have purified a third type of endonuclease which makes an endonucleolytic incision in DNA treated with UV light, N-acetoxy-2-acetylaminofluorene or 7-bromomethyl benz[a]anthracene. This enzyme apparently recognizes the distortion in the DNA double helix, which is known to occur with pyrimidine dimers (21, 22, 23), and with N-acetoxy-2-acetylaminofluorene bound to the DNA (24, 25). The same workers have shown that with this endonuclease in presence of alkaline phosphatase and DNA polymerase I results in excision of the pyrimidine dimers and the N-acetoxy-2-acetylaminofluorene-DNA adducts (5).

After endonuclease attack near the altered base(s), removal of the damaged base(s), plus the nucleotides adjacent to the damaged base(s) occurs, due to the action of an exonuclease. Lindahl has described the purification of an exonuclease from rabbit tissue, which can efficiently excise pyrimidine dimers from DNA (26). Similarly Mortelmans et al. (27), Cook et al. (28), and Duncan et al. (29) have reported exonuclease activity in crude extracts from a variety of human cell cultures. This exonuclease activity is capable of excising pyrimidine dimers from DNA (28, 29) or chromatin (27).

Another exonuclease, similar to the enzyme described above, has been purified from human placental tissue by Doniger and Grossman (30). This

exonuclease can excise pyrimidine dimers from DNA. The gap produced by this exonuclease is terminated by a 3' hydroxyl and 5' phosphoryl (30), and the 3' hydroxyl provides the proper site for the subsequent action of DNA polymerase.

Whether there is a specificity towards the excision of altered bases, similar to that seen by the repair endonucleases, is not known at this time. However, it is known that the number of excised bases does vary with the base damage (31). After treatment with UV light or N-acetoxy-2-acetylaminofluorene, excision of 100-140 bases per damaged base occurs (31), but after treatment with γ-rays or methyl methanesulfonate, 1-4 bases per damaged base are excised (31, 32). It is not known if this difference in the number of bases excised per base damaged by the exonuclease is due to different exonucleases.

After excision of the altered base(s), plus the adjacent nucleotides, the gap formed by the exonuclease is resynthesized by DNA polymerase using the complimentary strand as a template. There are two main DNA polymerases present in the cell (33, 34): DNA polymerase α, located in the cell cytoplasm; and DNA polymerase β, located in the cell nucleus. The properties of DNA polymerase α and β are similar (33). Both polymerases require a 3' hydroxyl group for chain elongation, and both require an initiator. However, there are differences in the properties of DNA polymerase α and β that may indicate which are involved in DNA repair. DNA replication is believed to be initiated with a RNA primer (33). DNA polymerase α can use an RNA initiator for DNA synthesis, but DNA polymerase β is not able to. <u>In vitro</u> synthesis of DNA on a chromatin template by DNA polymerase α is stimulated by the presence of an unwinding protein, while the presence of unwinding proteins does not affect

DNA synthesis by DNA polymerase β (35). The levels of DNA polymerase α varies with the stage of the cell cycle. It increases during DNA replication (33), while the concentration of DNA polymerase α is constant throughout the cell cycle (33). These results indirectly indicate that DNA polymerase α is involved in DNA replication, while DNA polymerase β may be involved in DNA repair.

Recently Lieberman and Poirier (36) have investigated the fidelity of excision repair in WI-38 cells, but the techniques they used in these studies detect only 1 misincorporation per 20 nucleotides (36). Since the fidelity of DNA polymerase α has been estimated at 1 misincorporation per 10^4 nucleotides inserted (37), it is apparent that more sensitive methods of detection have to be developed in order to study the fidelity of excision repair.

The final step of excision repair is the formation of a phosphodiester bond between the 3' hydroxyl group of the last nucleotide inserted by DNA polymerase, and the 5' phosphate group of the nucleotide on the 5' side of the excision gap. This process is performed by DNA ligase. There are two DNA ligases present in mammalian cells: DNA ligase I, found in the cell cytoplasm; and DNA ligase II, found in the nucleus (38, 39). Both enzymes are similar with respect to their pH optimum, divalent cation requirement, and need for ATP as a cofactor (38). The main difference between the two enzymes are their cell cycle dependency. The concentration of DNA ligase II is independent of the stage of the cell cycle, while the concentration of DNA ligase I increases during DNA replication (38). Thus it appears that DNA ligase II may be involved with DNA repair, while DNA ligase I may function during DNA replication.

An important question in DNA repair is the biological consequences of excision repair. This point has been extensively studied using cells derived from patients with the genetic disease xeroderma pigmentosum (XP). The pathology of this disease involves an extreme sensitivity to sunlight exhibited in the form of erythema, abnormal pigmentation, and a high incidence of sunlight induced skin cancer (40).

XP cells were first shown by Cleaver (41, 42) to undergo less UV induced DNA repair synthesis compared to normal human skin fibroblasts. The conclusion from these studies was that there was a defect in XP's excision repair pathway for UV induced DNA damage (41, 42). Further investigations of XP showed that these cells are not able to excise pyrimidine dimers from their own DNA as efficiently as normal human fibroblasts (43-45). Moreover, their capacity for host cell reactivation of UV irradiated adenovirus 2, or herpes simplex virus was reduced (45-48). However, studies with other chemical and physical agents showed that XP cells are capable of undergoing normal amounts of repair synthesis after treatment with X-rays (42, 49), and the monofunctional alkylating agents methyl methanesulfonate, N-methyl-N'-nitro-nitrosoguanidine (50, 51), N-methyl-N-nitrosourea (52), and nitrosated methylguanidine (53). In addition to UV light (41, 42, 52) a reduced repair activity is also evident after treatment with 4,5 epoxide benz[a]anthracene (54), 4-nitrosoquinoline-1-oxide (51, 58), N-hydroxy-2-acetylaminofluorene (56, 57), N-acetoxy-2-acetylaminofluorene (56, 57), 7-bromomethyl benz [a]anthracene (55), and methoxypsoralen (59). The findings of these studies strongly suggest that there are two pathways of excision repair. One repair pathway involves repair of DNA damage of monofunctional alkylating agents and X-rays, while the other is associated with DNA

repair invoked by arylalkylating agents and UV light. Thus it is apparent that XP cells are defective in the repair pathway for UV light and arylalkylating agents.

Although XP has been shown to undergo reduced DNA repair synthesis after UV-irradiation, compared to normal human fibroblasts, the amount of the reduction of repair synthesis varies for different XP cell strains (60,-63). Some XP cell lines showed: 1) no UV induced repair synthesis, 2) intermediate levels, but UV dose dependent repair synthesis, 3) normal or only slightly reduced repair synthesis (60-63). In order to understand the nature of the XP UV-repair synthesis defect, complementation studies were done. It was found that hybridizing XP cells to normal human fibroblasts resulted in the ability of the heterokaryons to undergo normal amounts of UV induced repair synthesis, as measured by autoradiography (64, 65). Similar studies showed that hybridizing XP cells from one cell line to another XP cell line, resulted in restoration of the UV-induced DNA repair abilities of the heterokaryon as measured by: 1) the amounts of unscheduled DNA synthesis (66-69), 2) DNA repair replication (70), 3) removal of UV-endonuclease sites (71), 4) host cell reactivation of UV-irradiated adenovirus 2 (72). Accordingly, the various XP cell lines have been classified into 5 complementation groups based on their capacity to restore UV-induced DNA repair in the heterokaryon (66-69). In addition to these 5 complementation groups, there is also the variant group which shows no reduction in UV-induced repair synthesis, but still has the clinical manifestations of XP (62, 63). These complementation groups thus indicate that there are multiple allelic defects in the XP repair pathway for UV induced DNA damage.

The nature of the biochemical defect responsible for XP cells reduced capacity to undergo UV-induced DNA repair has been investigated. Since XP cells were able to undergo DNA repair in response to X-rays and alkylating agents, but not to UV, it was considered that the defect was in the UV endonuclease. This was initially investigated by alkaline sucrose gradient centrifugation of DNA from UV irradiated normal human cells and XP cells. Setlow et al. (73) and Cleaver (74) found that UV-induced single strand breaks occurred in normal fibroblast DNA, but not in XP fibroblast DNA, suggesting that XP may be defective in a UV endonuclease. Recent experiments using purified E. coli DNA containing pyrimidine dimers, which had been incised by a T_4 UV-endonuclease, showed that the 5 XP complementation groups are able to excise the pyrimidine dimers as efficiently as WI-38 cells (28). Further work has shown that, while the 5 XP complementation groups are capable of excising pyrimidine dimers from purified DNA (not incised), these cells are inefficient in removing dimers from their own chromatin (27). The results indicate that the 5 XP complementation groups contain the necessary enzymes to remove pyrimidine dimers from purified DNA, but one or more of the enzymes appear ineffective when acting on a chromatin template containing pyrimidine dimers. Thus, further investigations are required to determine the biochemical nature of the XP defect for UV-induced DNA repair.

Since XP cells have been shown to have reduced excision repair after treatment with UV-light (41-45) and arylalkylating agents (54-58), but normal repair after treatment with monofunctional alkylating (50-53) agents, it was important to analyze the biological responses of XP cells to treatment with these chemical and physical agents. It has been shown

that the biological response, as measured by colony formation of XP cells after treatment with chemical or physical agents, is positively correlated with their capacity to repair the DNA damage caused by these agents. Thus XP cells which show a reduced repair capacity for DNA damage induced by UV irradiation and arylalkylating agents, also exhibit a reduced capacity for colony formation after treatment with these agents (45, 51, 54, 57). The level of repair synthesis after treatment of XP cells with methyl methanesulfonate, N-methyl-N-nitrosourea, and N-methyl-N'-nitro-nitrosoguanidine correlates with a normal colony forming ability after treatment with N-methyl-N'-nitro-nitrosoguanidine (51). A supramolecular manifestation of DNA damage is often associated with the formation of chromosome aberrations, and a reduced capacity to repair the DNA-damage induced by chemical and physical agents may result in an increased frequency of structurally aberrant chromosomes. It has been shown that the chemical agent 4-nitrosoquinoline-1-oxide, which causes DNA damage that XP cells are not able to repair (51, 58), results in a higher frequency of chromosome aberrations than in normal cells (77, 78). While the alkylating agents methyl methanesulfonate, N-methyl-N'-nitro-nitro-soguanidine, and nitrosated methylguanidine, which case DNA damage that XP cells repair to the same extent as normal human cells (50-53), they do not cause an increase in the frequency of chromosome aberrations (53, 77, 78). The above results strongly indicates that fibroblasts derived from patients with xeroderma pigmentosum have a decreased repair capacity for UV induced DNA damage. This reduced excision repair capacity is correlated with a decreased cell survival, and an increased frequency of chromosome aberrations. The association of reduced DNA repair capacity for UV-induced pyrimidine dimers with xeroderma pigmentosum seems to

raise some significant questions concerning a link between UV-induced DNA repair abilities, and UV-induced malignant transformation of these cells.

Photoreactivation Repair

Irradiation of cells with UV light (220-300 nm) results in the formation of pyrimidine dimers, protein-nucleic acid adducts, and other minor UV photoproducts (79, 80). In addition to excision repair, photoreactivation repair is another mechanism to remove these UV-induced lesions from the cellular DNA (81, 82).

The mechanism of photoreactivation repair is very different than that of excision repair. It is a light dependent, enzymatically mediated reaction, which monomerizes pyrimidine dimers. The following is a general description of photoreactivation repair (83-85). The photoreactivating enzyme binds to the locally denatured region of DNA containing the pyrimidine dimers. Absorption of light (300-600 nm) by the chromophore of the photoreactivating enzyme results in monomerization of the pyrimidine dimer to the corresponding pyrimidine monomers. The photoreactivation enzyme is specific for pyrimidine dimers, and does not remove the UV-induced protein-nucleic acid adducts or other minor UV photoproducts (86, 87).

The biological effectiveness of photoreactivation repair can be determined by the dose reduction factor (88). The dose reduction factor is simply the ratio of UV doses X/Y, where X is a UV dose producing a given biological effect in the dark (no photoreactivation repair), and Y is the UV dose producing the same biological effect in the light (with photoreactivation repair). The photoreactivation has appeared to reduce the UV dose by X/Y.

Photoreactivation repair has been shown to result in restoration of the biological integrity of UV irradiated cellular DNA in many different ways: 1) decreased cell death after UV irradiation (89), 2) decreased cellular transformation after UV irradiation (90), 3) increased host cell reactivation of UV irradiated herpes simplex virus (91, 92), and 4) decreased formation of sister chromatid exchanges after UV irradiation (93, 94).

The photoreactivating enzyme is widely distributed throughout both plant and animal kingdom, but until recently had not been found in mammalian tissues except for marsupials (88). Recently Sutherland (95-98) has detected photoractivating enzyme activity in human leucocytes, human skin fibroblasts, and established mouse cell lines. This photoreactivating enzyme activity was active at low ionic strength, and inactive at the ionic strength normally used for the photoreactivating enzyme assay (95). This difference in ionic strength requirement, may explain the previous failure to find the photoreactivating enzyme in mammalian tissues.

Post-replication Repair

DNA replication is bidirectional (99, 100), and DNA replication initiates at a site termed replicon (101, 102). It has been shown that UV irradiation (103, 104) and alkylating agents (105, 106) can cause a depression in the rate of DNA synthesis. Further investigations have shown that after treatment with UV irradiation (103, 107-109) or alkylating agents (105, 106, 110) the size of the newly replicated DNA was shorter than that of control DNA. The size of the newly replicated DNA corresponded to the distance between pyrimidine dimers formed after UV irradiation (103, 108), or the distance between base alkylation

products (106). On further incubation this newly synthesized DNA obtained the size of control DNA (103-110). The restoration of the size of the newly synthesized DNA was sensitive to the presence of caffeine or theophyline in rodent cells, and excision deficient human cells (103, 110), but not normal human cells (111).

Lehmann has postulated the following mechanism to account for the restoration of the size of the newly synthesized DNA, and has termed this process post-replication repair (108, 112). DNA replication may continue until the replication complex encounters a lesion in the template such as a pyrimidine dimer, and this may cause a premature termination of the DNA replication process. Reinitiation of DNA replication occurs at a point approximately 1000 nucleotides from the point of termination (108, 113), creating a gap in the newly synthesized DNA. At a later time this gap is filled in using the complementary strand as a template, and this gap filling process is believed to be caffeine or theophylline sensitive (108, 109, 112).

Although the mechanism of post-replication repair proposed by Lehmann appears to explain the sedimentation profiles of newly synthesized DNA at early periods after UV irradiation, or treatment with alkylating agents; the proposed mechanism of post-replication repair does not account for some additional observations. Rauth (114, 115) has shown that soon after UV irradiation the size of the newly synthesized DNA strands in mouse L cells was greater than the distance between pyrimidine dimers. Indicating, that in this case the pyrimidine dimer did not act as a block for chain elongation. Lehmann (117) and Buhl et al. (116) have also shown that if the cells were pulsed with ^{3}H-Tdr several hours after UV-irradiation, the size of the newly synthesized DNA was the same

size as control. Excision of pyrimidine dimers during the period after UV-irradiation could not have accounted for the results, since similar results were found in cells incapable of excising pyrimidine dimers (116, 117). It is also difficult to explain, that immediately after UV-irradiation the pyrimidine dimers act as blocks to chain elongation, but not at a later time.

The basis of Lehmann's proposal for post-replication repair is that the size of the newly synthesized DNA is approximately the distance between pyrimidine dimers (103, 108). This conclusion assumes a random distribution of pyrimidine dimers in the UV-irradiated cellular DNA. However, recent findings indicate that the formation of pyrimidine dimers may occur in clusters (118-121). Similarly, work with the alkylating agents, dimethylnitrosoamine (122), N-hydroxy-2-acetylaminofluorene (123, 124) and 7-bromomethyl benz[a]anthracene (125) have shown that certain chromatin fractions are more susceptible to base alkylation than other.

Another possible difficulty with the proposed mechanism of post-replication repair is that of reinitiation of DNA synthesis by DNA polymerase on the template strand, after it has come to a dimer or an alkylated base. Isolated DNA polymerases require a double stranded initiator sequence with a 3' hydroyl group available (33, 34). It is currently believed that an RNA-DNA hybrid serves as an initiator for DNA polymerase (33). Lehmann's model of post-replication repair also does not take into consideration for this requirement of DNA polymerase for initiation of DNA synthesis. While the above discussion is not intended to rule out the proposed mechanism of post-replication repair, the evidence does indicate the need for further investigations.