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# Production of thymine glycols in DNA by radiation and chemical carcinogens as detected by a monoclonal antibody

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**Summary** In order to understand the role in carcinogenesis of damage indirectly induced by chemical carcinogens, it is important to identify the primary DNA lesions. We have measured the formation and repair of one type of DNA modification, 5,6-dihydroxydihydrothymine (thymine glycol), following exposure of cultured human cells to the carcinogens N-hydroxy-2-naphthylamine or benzo(a)pyrene. The efficiency of production of thymine glycols in DNA by these carcinogens was compared to that by ionizing radiation and ultraviolet light. Thymine glycols were detected using a monoclonal antibody against this product in a sensitive immunoassay. We found that thymine glycols were produced in DNA in a dose dependent manner after exposure to the carcinogens and that their production was reduced if either catalase or superoxide dismutase or both were present at the time of treatment. The efficiency of thymine glycol production following exposure to the chemical carcinogens was greater than that following equi-toxic doses of radiation. Thymine glycols were efficiently removed from the DNA of human cells following treatment with either the chemical carcinogens, ionizing radiation or ultraviolet light.

There is increasing interest in the potential role of active oxygen species and free radicals as intermediates in carcinogenesis (Copeland, 1983; Ames, 1983). For chemical carcinogens such as aminoazo dyes and naphthylamines, there is good correlation between the formation of active oxygen, convertibility to free radicals, and carcinogenicity (Nakayama *et al.*, 1983). Active oxygen species and the ensuing lipid peroxidations could affect carcinogenic processes in at least two ways: by causing chromosomal damage and rearrangements, and by modulating cell growth and differentiation through epigenetic mechanisms (Cerutti, 1985). A common property of tumour promoters may be their ability to produce activated forms of oxygen, such as superoxide anions and peroxides. Phorbol ester-induced chromosomal damage (Emerit & Cerutti, 1982) and promotion of transformation (Borek & Troll, 1983) are suppressed by superoxide dismutase, as would be expected if the promoter was working through an oxidative mechanism. For ionizing radiation, it is known that active oxygen species are responsible for 60–70% of DNA strand breaks, chromosomal aberrations, mutations, and cell killing (Okada *et al.*, 1983). The mutagenic consequences of ionizing radiation and oxidative DNA damage in bacteria include base substitutions at both A:T and G:C base pairs (Glickman *et al.*, 1980; Levin *et al.*, 1982). In mammalian cells, similar events may lead to ionizing radiation-induced activation of protooncogenes (Guerro *et al.*, 1984).

Damage to DNA by active oxygen species result in single- and double-strand breaks, apurinic and apyrimidinic sites, and ring-saturated thymine derivatives, such as 5,6-dihydroxydihydrothymine (thymine glycol). In order to understand the role of indirect action produced by chemicals or radiation in carcinogenesis, it is important to identify the primary DNA lesions and ascertain how these lesions are processed at the molecular level. One type of DNA damage characteristic of indirect action that has been extensively studied are thymine glycols. The measurement of this damage, however, is made difficult by the lack of sufficiently sensitive assays to detect and distinguish chemically similar products at low doses. Although several chromatographic methods are available for the resolution of thymine glycols in hydrolysates of DNA (Hariharan & Cerutti, 1974; Frenkel *et al.*, 1981*a*, *b*), these techniques generally require large doses of radiation and the use of highly radiolabelled thymine producing a background of thymine damage from radiolysis products. Recently, a radioimmune assay for thymine glycols was developed using rabbit antiserum (West *et al.*, 1982). This assay is more sensitive than the chromatography methods and does not require radiolabelled thymine. However, the reaction of this antiserum with DNA appears to depend upon clustering of the glycols.

A monoclonal antibody that recognizes thymine glycols in DNA has also been developed and used in a sensitive immunoassay (Leadon & Hanawalt, 1983). The essential features of this assay are as follows: a constant amount of immobilized $OsO_4$-modified DNA and various amounts of a competitor in solution are reacted with a fixed amount of the antibody. The amount of antibody bound to the immobilized DNA is then determined and compared to the amount bound in the absence of the competitor. $OsO_4$-modified DNA is used in the assays because $OsO_4$ selectively oxidizes the 5,6-double bond of thymine to produce the glycol but reacts very little or not at all with other bases (Beer *et al.*, 1966). The enzyme-linked immunosorbent assay (ELISA) was calibrated using serial dilutions of radioactively labelled DNA modified by $OsO_4$ as the standard. The thymine glycol content of the standard DNA was determined chromatographically (Leadon & Hanawalt, 1983).

This antibody has been used in the ELISA to measure the production of thymine glycols in DNA by a variety of agents in normal fibroblasts (NF), in cells from a patient with xeroderma pigmentosum complementation group A (XPA), and in human mammary epithelial cells (HMEC). The repair of thymine glycols was measured by the disappearance of antibody binding sites in the DNA. We assume that this reflects removal of thymine glycols, although any modifications of the glycols that altered their antigenicity would also appear as removal. Since this assay was initially used to measure thymine glycol production in DNA following ionizing and ultraviolet irradiations, a discussion of the production of this base damage by chemical carcinogens requires a brief description of the results obtained with radiation as the source of the active oxygen species.

## Production of thymine glycols by radiation

### *Gamma-rays*

The production of thymine glycols in the DNA of NF was measured following various doses of gamma-rays. A linear dose-response for the production of thymine glycols was observed for doses up to 20 Krad (Figure 1a). The efficiency

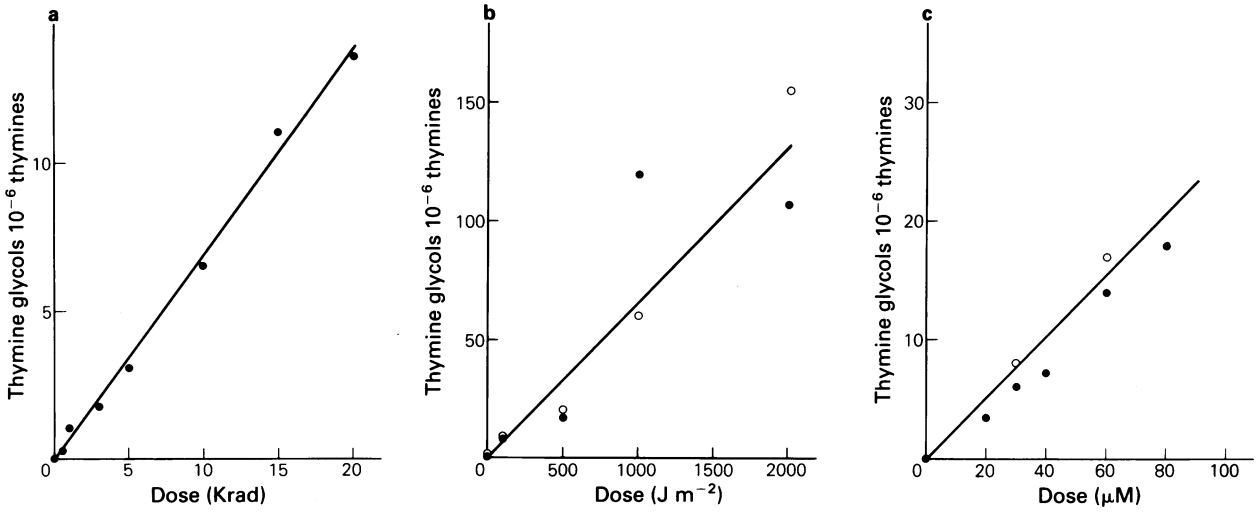


**Figure 1** Production of thymine glycols in DNA from normal (●) and XPA (○) fibroblasts exposed to gamma rays (a), ultraviolet light (b), and NOH-2-NA (c).

of product formation as detected by the ELISA was 0.66 thymine glycols per $10^6$ thymines per Krad. This is approximately a 3-fold lower level of thymine glycol formation than that observed by Mattern *et al.* (1975) using an alkali–acid degradation assay. The removal of thymine glycols was measured in NF and HMEC following a dose of 400 rad. While the initial frequency of thymine glycols varied, efficient removal of this base damage was observed for both cell types (Table I). A similarly efficient repair of thymine glycols has been reported for a number of other cell types but following much higher doses of radiation (Mattern *et al.*, 1975; Leadon & Hanawalt, 1983).

*Ultraviolet light*

Cyclobutane-type pyrimidine dimers are the major lesions produced in DNA by 254 nm ultraviolet (UV) light. Indirect effects of UV light, such as the formation of 5,6-dihydrothymine (Yamane *et al.*, 1967), thymine-methyl damage and thymine glycols (Hariharan & Cerutti, 1977) have also been detected. A comparison was made between the formation and removal of thymine glycols in NF and in XPA cells. A linear dose-response was observed for the formation of thymine glycols in both NF and XPA following doses up to 2000 J $m^{-2}$ (Figure 1b). There were 0.07 thymine glycols formed per $10^6$ thymines per J $m^{-2}$ in the DNA of irradiated cells. A direct comparison of thymine glycol formation as detected by the ELISA with previously published values is difficult to make since different wavelengths of UV were used for irradiation of the cells and the efficiency of thymine glycol formation appears to be dependent on the wavelength (Hariharan & Cerutti, 1977). The removal of thymine glycols was measured following a dose of 100 J $m^{-2}$ (Table I). Efficient repair of this lesion was found for both NF and XPA. This indicates that the pathway involved in the repair of thymine glycols is distinct from the one involved in the removal of cyclobutane-type pyrimidine dimers, which is defective in XPA cells.

**Production of thymine glycols by chemical carcinogens**

*N-hydroxy-2-naphthylamine*

One of the active metabolites of the bladder carcinogen 2-naphthylamine, N-hydroxy-2-naphthylamine (NOH-2-NA), can exhibit several types of interactions with cellular DNA: direct interaction leading to adducts of guanine and adenine (Kadlubar *et al.*, 1980), and indirect effects through the generation of active oxygen species that produce single-strand breaks (SSB) detected by alkaline elution (Kaneko *et al.*, 1984) and thymine glycols as detected by a monoclonal antibody (Kaneko & Leadon, 1986). The DNA lesions produced by the indirect effects were probably produced by hydroxyl radicals formed from hydrogen peroxide and superoxide anions by a metal catalyzed Haber–Weiss type of reaction. Hydrogen peroxide is produced from superoxide anions which were generated by the autoxidation of NOH-2-NA both extra- and intracellularly.

Thymine glycols were produced in DNA in a dose dependent manner following exposure to NOH-2-NA (Figure 1c) and were produced more efficiently *in vitro* than *in vivo*. While the initial levels of thymine glycols in DNA

**Table I** Production and removal of thymine glycols

| *Damaging agent* | *Thymine glycols $10^{-6}$ thymines* | | | | | | *Percent of initial thymine glycols after 45 min* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Initial* | | | *45 min* | | | | | |
| | *NF* | *XPA* | *HMEC* | *NF* | *XPA* | *HMEC* | *NF* | *XPA* | *HMEC* |
| 254 nm UV (100 J $m^{-2}$) | 8 | 8 | | 4.1 | 4.4 | | 51 | 55 | |
| NOH-2-NA (100 μM) | 7 | 14 | | 2.9 | 3.8 | | 41 | 27 | |
| B(a)P (1 μg $ml^{-1}$) | | | 16 | | | 7 | | | 44 |
| γ-rays (400 rad) | 0.23 | | 0.45 | 0.11 | | 0.26 | 48 | | 58 |

from cells varied between experiments, on average, the frequency of thymine glycols formed in the DNA was in the same order of magnitude as for SSB in the low dose range (i.e., $<20\,\mu M$). As with SSB, the formation of thymine glycols in the DNA was reduced by the presence of SOD or catalase both *in vitro* and *in vivo*. The presence of SOD and catalase together during treatment of cell cultures with NOH-2-NA reduced the formation of thymine glycols to undetectable levels. This indicates that these two enzymes together were reducing the extracellular levels of superoxide anions and hydrogen peroxide such that little or no hydrogen peroxide was being formed within the cell. These results support the hypothesis that superoxide anions and hydrogen peroxide are intermediates in the formation of thymine glycols after treatment with NOH-2-NA. No significant difference in the levels of thymine glycols or SSB was observed between the normal XPA fibroblasts treated with the carcinogen. Thymine glycols and SSB were efficiently repaired in both normal and XPA fibroblasts, with a 20 min half-life for thymine glycols and a 23 min half-life for SSB (Table I), indicating that the pathway involved in the repair of SSB and thymine glycols is distinct from the one involved in the removal of pyrimidine dimers. Treatment of $OsO_4$-modified DNA under the conditions used to detect SSB by alkaline elution, i.e., in 20 mM EDTA-tetrapropylammonium hydroxide (pH 11.9), did not reduce the amount of thymine glycols in this DNA as determined by either antibody binding or high-pressure liquid chromatography. Thus, thymine glycols were probably not precursors for the alkali-labile sites detected by alkaline elution.

*Benzo(a)pyrene*

Benzo(a) pyrene (B(a)P) can be converted to a very reactive electrophile, which forms covalent DNA adducts, as well as other metabolites (Huberman & Sachs, 1973). These metabolites can give rise to free radical intermediates and reactive reduced oxygen species as a result of autoxidative processes and oxygen-reduction cycles. These cycles are coupled with molecular oxygen to form reactive oxygen species such as superoxide anions and hydrogen peroxide. Ide *et al.* (1983) have shown that metabolism of B(a)P by cultured human cells results in damage at the methyl group of thymine in addition to covalent DNA adducts. The thymine damage was postulated to be due to the indirect action of B(a)P.

We tested the hypothesis that metabolism of B(a)P by human mammary epithelial cells (HMEC) might also be producing thymine glycols by indirect action. HMEC cultures, which readily metabolize B(a)P (Stampfer *et al.*, 1981), were exposed to $1\,\mu g\,ml^{-1}$ B(a)P for 4 h and the presence of thymine glycols and covalent DNA adducts were measured. As with the other agents studied, thymine glycols were produced in the DNA in a dose-dependent manner following exposure to B(a)P. At equi-toxic doses, the efficiency of thymine glycol formation was significantly greater in HMEC cultures exposed to B(a)P than gamma-rays (Table I). The frequency of thymine glycols formation was 4 per $10^6$ bases compared with 2.4 covalent B(a)P adducts per $10^6$ bases. Thus, the damage produced by the indirect action represents a large fraction of the total damage induced by B(a)P in actively metabolizing HMEC. Thymine glycols were removed from the DNA of HMEC with about the same efficiency as was found with the other agents that produce this damage (Table I).

## Conclusion

Thymine glycols, as detected by a monoclonal antibody, were produced in DNA following exposure of cultured human cells to ionizing radiation and 254 nm UV light. They were also produced when cells were exposed to an active metabolite of the carcinogen 2-naphthylamine and after the cells were allowed to metabolize B(a)P. The significance for the process of carcinogenesis of thymine glycol production after treatment with a chemical carcinogen is as yet unclear. Any chemical carcinogen which can generate free radicals can, in principle, induce the formation of thymine glycols. Thymine glycols in DNA could contribute to the formation of some of the SSB observed or accelerate abnormal DNA replication and recombination. It has been demonstrated that chromosomal abnormalities, such as translocations, deletions, or trisomy can play an important role in malignancy and could represent, at the molecular level, a mechanism to alter oncogene activity (Yunis, 1983). It has also been well documented that X-rays induce chromosomal mutations and can activate otherwise silent genomic elements in *Zea mays* (McClintock, 1984). In this context, it is interesting that most of the fragile sites for chromosomal abnormalities are located at the junction of Giemsa-negative and Giemsa-positive bands and that Giemsa-positive bands are enriched in middle-repetitive AT-rich DNA (Yunis & Soreng, 1984). Thus the formation of thymine glycols may be preferentially located near these fragile sites and could play an important role during the process of carcinogenesis.

The efficiency of repair of thymine glycols in various regions of the genome may also be important. Unrepaired damage in DNA of different functional states would be expected to have differing consequences for the cell. Mutations in active sequences or blockage of their transcription could have direct effects on cell survival and function. Different states of chromatin into which the DNA is packaged may modulate the actions of particular damaging agents and the various repair systems to different degrees. The deficient repair of bulky chemical adducts in the non-transcribed alpha sequence of monkey cells (Zolan *et al.*, 1982; Leadon *et al.*, 1983) and the preferential repair of pyrimidine dimers in the transcribed dihydrofolate reductase gene of hamster cells (Bohr *et al.*, 1985) indicate that damage in silent regions of the genome may have greater potential for engendering mutations and DNA rearrangements than damage in or near transcription units. Therefore, it will be important to determine the efficiency of repair of thymine glycols and other types of oxidative damage in specific regions of the genome.

This contribution summarizes and discusses research carried out in collaboration with M. Kaneko (N-hydroxy-2-naphthylamine) and J.C. Bartley and M.R. Stampfer (benzo(a)pyrene).

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## Discussion

*Butler:* Can you actually induce the repair systems?

*Leadon:* We are probably not looking at the induction of repair. For certain types of damage in mammalian cells, you can induce repair or an adaption to certain types of damage but this is probably not occurring in the system being studied.

*Butler:* We can actually show that radiation induces the $O^6$ methylguanine transferase.

*Leadon:* Right, that's the one type of repair activity you can induce.

*Elkind:* What is the molecular weight of the particular plasmid you use? What was the dose? What was the assay for the lesion that you are going to repair?

*Leadon:* The plasmid $pSV_2$-gpt is a 5.3 kb plasmid that is integrated as three copies tandemly repeated, so that you are measuring repair in a fifteen to sixteen kb fragment. The way the experiment is carried out is that you look at incorporation of bromodeoxyuridine into the DNA after irradiation so that you are looking at repair replication of various types of damage. You can do the same type of experiment under similar conditions looking at loss of thymine glycols by measuring the antibody binding, from fragments that contain transcribed sequence compared to fragments that don't contain the sequence, or that contain other specific sequences. The results you obtain are that the antibody binding sites for thymine glycols are removed more rapidly from the transcribed sequence than from the rest of the genome.

*Elkind:* The same dose, 25 Krad.

*Leadon:* Right.

*Cramp:* Do you need 25 Krad before you can see measurable effects?

*Leadon:* While I haven't done a full dose response, you need a high dose for measuring repair because you are looking at repair in three copies of a gene compared to the whole genome and you have a sufficiently high dose to increase the probability of getting damage in fifteen kb region containing the gene.

*Ward:* There may not be any discrepancy with Dr