Non-specific Inhibition of DNA Repair Synthesis by Tumor Promoters in Human Diploid Fibroblasts Damaged with N-Acetoxy-2-acetylaminofluorene

Miriam C. Poirier, Benedict T. De Cicco, and Michael W. Lieberman

SUMMARY

The effects of selected tumor-promoting agents and their nonpromoting analogs on DNA repair synthesis were examined in human diploid fibroblasts (WI-38) damaged with N-acetoxy-2-acetylaminofluorene. Over a range of doses, three promoters (croton oil, 12-O-tetradecanoylphorbol-13-acetate, and anthralin) were found to inhibit DNA repair synthesis while their nonpromoting analogs (phorbol and 1,8-dihydroxyanthraquinone) had little effect. Another tumor promoter, phenol, inhibited DNA repair synthesis only at very high concentrations while an analog, 4-nitrophenol, produced inhibition of DNA repair synthesis at micromolar concentrations at which phenol had no effect.

To investigate the specificity of this phenomenon, the effects of these agents on DNA-replicative synthesis, RNA synthesis, protein synthesis, and cell morphology were evaluated. At equimolar concentrations, tumor promoters were found to inhibit DNA-replicative synthesis as effectively as repair synthesis. RNA and protein synthesis were similarly inhibited over the same range of concentrations. Extensive morphological changes, interpreted as evidence of toxicity, were seen at concentrations of promoters that inhibited the macromolecular syntheses studied. The nonpromoting analogs, with the exception of nitrophenol, had little effect on these processes and showed only slight morphological damage.

Thus tumor-promoting agents appeared to inhibit a number of macromolecular synthetic events, including DNA repair synthesis. It is suggested that the effect of tumor promoters on DNA repair synthesis is part of a general response to cellular injury rather than a selective response involving a single metabolic pathway. Furthermore, it is unlikely that the inhibition of repair synthesis represents the major mode of action of promoting agents in the carcinogenic process.

INTRODUCTION

The repeated administration of tumor promoters, after a single application of carcinogen, greatly enhances the rapidity of onset and the yield of tumors produced by the carcinogen (4, 6, 23). Relatively little is known about the molecular mechanism of tumor promotion. Recently, it has been shown that some promoters interfere with DNA repair synthesis (9, 10—12, 22, 31), and it has been suggested that reduced repair may account for the effect of these agents on the neoplastic process (9, 10). Because of the potential importance of this observation, we have investigated the effects of selected promoters and nonpromoting analogs on DNA repair synthesis in human diploid fibroblasts (WI-38) damaged with the direct-acting carcinogen NA-AAF1 (24).

Croton oil (3, 4), TPA (13), anthralin (the structure of anthralin is generally known to be 1,8,9-anthracenetriol, but a more probable structure, chemically, is 1,8-dihydroxy-9-anthrone; see Ref. 27.) (5), and phenol (7) are well-established skin tumor promoters that lead to the production of papillomas and carcinomas on initiated epidermis. In order to evaluate the specificity of any effects that these promoters might have on DNA repair synthesis, the following studies were performed. The effects of promoters on repair synthesis were compared to those of nonpromoting analogs [phorbol (13), 1,8-dihydroxyanthraquinone (H. Hennings and R. K. Boutwell, unpublished observations; this compound produced no tumors when tested on mouse skin after 7-12-dimethylbenz(a)anthracene initiation) and 4-nitrophenol (7)] at equimolar doses. The effects of promoters on DNA-replicative synthesis, RNA synthesis, protein synthesis, and cell morphology were also examined.

MATERIALS AND METHODS

Preparation of Cells. WI-38 human diploid fibroblasts were grown as previously described (18).

DNA Repair Synthesis. For DNA repair studies cells were grown to confluence in 150-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.). HU (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 1 ml Dulbecco's PBS (8) and added to a final concentration of 10 mM to suppress the remaining replicative synthesis in cells that had escaped contact inhibition. Twenty-five min later the cells were

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The abbreviations used are: NA-AAF, N-acetoxy-2-acetylaminofluorene; TPA, 12-O-tetradecanoylphorbol-13-acetate, phorbol myristate acetate; HU, hydroxyurea; PBS, phosphate-buffered saline (138 mM NaCl, 28 mM KCl, 81 mM Na₂HPO₄, and 14 mM KH₂PO₄, pH 7.3); DMSO, dimethyl sulfoxide.
treated with either a promoting agent or its nonpromoting chemical analog. The chemicals used were croton oil (Sigma Chemical Co.), TPA, phorbol (Consolidated Midland Corp., Brewster, N.Y.), 1,8,9-anthracenetriol (Pfaltz and Bauer, Flushing, N.Y.), 1,8-dihydroxyanthraquinone (Aldrich Chemical Co., Milwaukee, Wis.), phenol (Analar, British Drug House Chemicals Ltd., Poole, England), and 4-nitrophenol (Aldrich Chemical Co.). The latter was recrystallized before use from methanol:water (m.p. 115.5°). Promoters and analogs were added in 0.1 or 0.2 ml DMSO (packed under nitrogen; Pierce Chemical Co., Rockford, Ill.).

Twenty min later cells were damaged by administration of NA-AAF (kindly provided by Dr. E. K. Weisburger, National Cancer Institute, NIH, Bethesda, Md.) in 0.1 ml DMSO to give final concentrations of 10 or 100 μM. After an additional 15 min each Petri dish received 8 μCi of [3H]thymidine per ml (New England Nuclear, Boston, Mass.; 50 Ci/m mole) and incubation was usually continued for 4.25 hr before the cells were harvested.

**DNA Replication.** Cells to be used for replication studies were grown for 5 to 6 days after seeding to produce semiconfluent, asynchronous cultures. Equimolar concentrations of tumor promoters and analogs were added, followed 20 min later by 8 μCi of [3H]thymidine per ml. Cells were harvested at 4.25 hr.

**Preparation and CsCl Banding of Repaired and Replicated DNA's.** Petri dishes were scraped, and DNA was banded in CsCl buoyant density gradients as previously described (18). Specific activities (dpm/μg DNA) were calculated from absorbance at 260 nm and radioactivity analyses of gradient fractions. Aliquots of 0.1 ml were counted with 4 ml water and 10 ml Instagel (Packard Instrument Co., Downers Grove, Ill.) in a Beckman LS-250 liquid scintillation spectrometer at an efficiency of 27%.

**RNA and Protein Synthesis.** Experiments involving RNA and protein synthesis were essentially analogous to the DNA replication studies except that 2 μCi L-[4,5-3H(N)]-leucine per ml (5 Ci/m mole) or 4 μCi [5-3H]uridine per ml (New England Nuclear; 25 Ci/m mole) were added to each Petri dish already treated with a promoter or analog. Incorporation was allowed to continue for 4.25 hr at which time Petri dishes were washed twice and cells were scraped off in Dulbecco's PBS. Cells from two 150-mm Petri dishes were combined and pelleted by centrifugation (10 min, RC2-B Sorvall, 4°, 10,000 × g). The PBS was removed and the cell pellet was homogenized in Sarkosyl and centrifuged on a mixed CsCl-Cs2SO4 gradient (D. H. Janss, personal communication). After centrifugation, soluble and insoluble protein fractions were removed from the top of the gradient and combined. NaOH was added to a final concentration of 0.5 M, and samples were left for 18 hr at 22° to dissolve protein. Subsequent centrifugation (Beckman L-2-65B, 6660 × g) for 30 min at room temperature removed the insoluble residue. The supernatant was then dialyzed (5 Minicon Macrosolute concentrator; Amicon Corp., Lexington, Mass.) against water. A Lowry assay (21) with bovine serum albumin standard was used to determine μg protein. Liquid scintillation counting was carried out as described.

The RNA portion of the CsCl-Cs2SO4 gradient formed a pellet at the bottom of the tube. After removal of the proteins and DNA-containing portions of the gradient, the RNA pellet was dissolved with the addition of 2 ml 0.05 m Tris-HCl (pH 7.5) and dialyzed for 48 hr against 0.05 m Tris-HCl (pH 7.5), 4°. After dialysis the volumes were measured and specific activities (dpm/μg RNA) were calculated from absorbance measurements at 260 nm (1 A260 unit = 42 μg/ml) and radioactivity measurements.

**Morphological Studies.** WI-38 cells, grown as previously described (18), were plated on sterile coverslips (24 x 40 mm) in Lux multiplate tissue culture dishes (Lux Scientific Corp., Thousand Oaks, Calif.) with 24- x 67-mm wells. Croton oil, TPA, phorbol, anthralin, 1,8-dihydroxyanthraquinone, phenol, and 4-nitrophenol were each added in DMSO to separate wells containing semiconfluent cultures so that the final concentration in each case was the highest used in the replication and repair studies. The controls were given DMSO alone. At the end of 4.5 hr 1 set of coverslips was rinsed with Dulbecco's PBS, fixed with glacial acetic acid:ethanol (1:3), rinsed with water, air dried, and stained with Giemsa. At the end of 24 hr the same procedure was repeated on a 2nd set of coverslips. Coverslips were mounted on slides, covered with a 2nd set of coverslips, and examined by light microscopy.

**RESULTS**

**Structures.** The structures of the tumor-promoting agents and their nonpromoting chemical analogs used in these studies are presented in Chart 1.

**DNA Repair Synthesis.** DNA repair synthesis was measured in a system almost devoid of replicative synthesis, since WI-38 cells maintained at confluence have virtually no S-phase cells. Any residual replicative synthesis in these cells was further suppressed with HU.

CsCl buoyant density gradients were used to demonstrate repair synthesis as measured by [3H]thymidine incorporation into DNA from nonreplicating WI-38 cells (Chart 2, upper). In the absence of carcinogen little [3H]thymidine was incorporated into the DNA, while 10 μM NA-AAF induced approximately a 20-fold increase in incorporation. When croton oil at 0.01 or 0.05% (v/v) was introduced before NA-AAF damage, the amount of repair synthesis was reduced (Chart 2, lower). Similar gradients were used to measure the effects of the other promoters and analogs on NA-AAF-induced repair synthesis.

Croton oil administered after damage with NA-AAF did not inhibit repair synthesis extensively, possibly because the process of repair synthesis, once begun, is not easily arrested.

**Effects of Croton Oil and Derivatives on DNA Repair and Replicative Synthesis.** Croton oil at doses of 0.001, 0.01, and 0.05% inhibited DNA repair and replicative synthesis (Chart 3). When DNA repair synthesis was induced by 2 different levels of NA-AAF (10 and 100 μM), each showed an inhibition of 50% occurring at the same croton oil
tumor promoter (7), but it produced relatively little inhibition of DNA repair on DNA-replicative synthesis except at the highest concentrations (Chart 6). At concentrations at which TPA (50 μM) and anthralin (100 μM) were strongly inhibitory, phenol had no effect on either DNA repair or DNA replicative synthesis. Replication was more than 50% inhibited at 10^4 μM (1 mM) and DNA repair synthesis was 50% inhibited at 10^4 μM (10 mM). A nonpromoting analog, 4-nitrophenol, completely inhibited both repair and replicative synthesis at 10^3 μM.

**Effects of Croton Oil, TPA, and Anthralin on RNA and Protein Synthesis.** Since both DNA repair and DNA-replicative synthesis were affected by the promoters under study, we decided to examine the effects of these agents on other types of macromolecular synthesis. Experiments were designed to measure RNA and protein synthesis in cultures treated with inhibitory concentrations of croton oil, TPA, and anthralin. Interestingly enough, the results (Table 1) paralleled those obtained for DNA repair and replication. Inhibition of both RNA and protein synthesis occurred over the same range of concentrations found inhibitory for DNA repair and replicative synthesis.

**Effects of Anthralin and 1,8-Dihydroxyanthraquinone on DNA Repair and Replicative Synthesis.** The strong promoter, anthralin, also inhibited DNA repair and replicative synthesis at 10 and 100 μM (Chart 5). DNA replicative synthesis appeared to be more sensitive than repair synthesis since at 1 μM the latter function was only about 50% inhibited while replication was about 90% inhibited. A nonpromoting analog, 1,8-dihydroxyanthraquinone, was moderately inhibitory to both types of DNA synthesis at the highest concentrations.

**Effects of Phenol and 4-Nitrophenol on DNA Repair and Replicative Synthesis.** Phenol has been found to be a strong
Tumor Promoters and DNA Repair Synthesis

Morphology Studies. WI-39 cells growing on coverslips were treated for 4.5 or 24 hr with a tumor promoter or analog, fixed, stained with Giemsa, and observed under light microscopy. Concentrations of promoters were chosen to be those that produced substantial inhibitions of the macromolecular synthetic events (Charts 3 to 5; Table 1). Equimolar concentrations of the analogs were used.

Croton oil (0.05%), at 4.5 hr, produced mild chromatin clumping and nuclear vacuolization that progressed to severe chromatin clumping, nuclear pyknosis, cytoplasmic vacuolization, and cellular dissolution by 24 hr. At 50 μM TPA produced similar changes, but the onset was much more rapid and severe and cell lysis was well underway by 4.5 hr. Phorbol, at the same concentration, produced only occasional cells that showed chromatin clumping and nuclear swelling at 24 hr. Phenol at 10 μM produced effects

Thus, in general, it appears that, when 1 type of macromolecular synthesis is decreased by tumor promoters, other types are decreased at the same or similar concentrations.

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similar to TPA, with nuclear pyknosis and cytoplasmic retraction at 4.5 hr and widespread cell death at 24 hr. Nitrophenol at 10^4 μM produced nuclear swelling and loss of nuclear fine structure at 4.5 hr, with chromatin clumping, nuclear pyknosis, and sloughing of the cell sheet at 24 hr. Cells treated with anthralin (100 μM) showed a vital turquoise staining of the cytoplasm accompanied by a mild nuclear swelling at 4.5 hr and margination of the chromatin and mild cellular lysis at 24 hr. The analog, 1,8-dihydroxyanthraquinone (100 μM), produced a few changes at 4.5 hr and some chromatin clumping, nuclear pyknosis, and cell lysis by 24 hr.

DISCUSSION

Repair synthesis in WI-38 cells has been well characterized previously (18–20). This study utilizes incorporation of [³H]thymidine into nonreplicating DNA as a measure of repair synthesis. The data obtained with this system on the inhibition of DNA repair by tumor promoters are in general agreement with those obtained by other investigators. Inhibition of DNA repair synthesis by treatment with tumor promoters has been previously demonstrated in human lymphocytes, HeLa cells, and opossum lymphocytes (10, 22, 31). In 1 study croton oil, TPA, and anthralin reduced the uptake of radioactive thymidine by human lymphocytes damaged with UV radiation (10). In another study, TPA inhibited the release of thymine dimers from the DNA of HeLa cells radiated at certain dose levels (31). Experiments with opossum lymphocytes treated with TPA showed a decrease in UV radiation-induced DNA repair synthesis (22) and similar results were obtained when repair was measured by either autoradiography or bromodeoxyuridine treatment followed by CsCl buoyant density centrifugation. Concentrations of TPA that inhibited repair synthesis by 50% in human lymphocytes (25 μM) and in opossum lymphocytes (36 μM) are similar to the value obtained in our experiments using WI-38 cells (21 μM), in spite of the fact that different cell lines and different methods for measuring repair synthesis were used.

Since none of the promoters or structural analogs alone induced repair synthesis in the HU-suppressed cells under the conditions studied (Charts 3 to 6, controls), it is likely that these agents either do not damage DNA directly or do not produce DNA damage that is repairable by an excision repair mechanism.

Inhibition of DNA replication by TPA has been studied previously in opossum lymphocytes (22) and HeLa cells (30). The lymphocyte results showed a 50% inhibition of [³H]thymidine incorporation into replicating DNA at a TPA concentration of 50 μM. Replication in HeLa cells was 50% inhibited at 0.01 μM TPA; a 100-fold increase in TPA produced no additional inhibition (plateau phase) but concentrations of 100 μM produced total inhibition. This phenomenon was not observed in the opossum lymphocytes (22) or in the experiments reported here.

Most of the tumor promoters and analogs used in these experiments either inhibited both DNA repair and DNA replication synthesis, or had little effect on either type of synthesis. Anthralin and TPA completely inhibited both types of synthesis at concentrations between 10 and 100 μM, while phorbol and 1,8-dihydroxyanthraquinone were only slightly inhibitory. However, replicative and repair synthesis showed markedly different sensitivities to phenol; repair synthesis was only affected at a concentration 10-fold higher than that which inhibited replicative synthesis. Both replicative and repair synthesis were inhibited by the same concentration of the analog (4-nitrophenol), and both types of synthesis were more sensitive to the analog than to the promoter (phenol). This phenomenon may be related to the sensitivity of cellular processes (oxidative phosphorylation) to nitrophenol.

The tumor promoters tested inhibited RNA and protein synthesis (Table 1) over a range of dose levels, with an overall pattern similar to that seen for the inhibition of DNA repair and replicative synthesis. The similarities using anthralin and TPA are particularly striking, while croton oil appears to have a greater inhibitory effect on RNA synthesis than on other types of macromolecular synthesis. The fact that DNA repair and replicative synthesis, as well as RNA and protein synthesis, are all inhibited by the same or similar concentrations of tumor promoters, suggests that the results might be explained by a general toxic effect rather than a specific selective one.

The morphological data also support this contention. The cells treated with noninhibitory analogs, phorbol, and 1,8-dihydroxyanthraquinone showed few morphological changes compared to controls. Both 4-nitrophenol and croton oil produced a moderate degree of clumped chromatin, karyorrhexis, and granularity of the nucleus at the end of 4.5 hr at concentrations inhibitory to both DNA repair and DNA-.replicative synthesis. Phenol and TPA, at levels inhibitory to the macromolecular synthetic activities studied, produced extensive cell damage. At 4.5 hr the nuclei exhibited prominent clumping of chromatin, granularity, pyknosis, and karyorrhexis, while the cytoplasm was granular and retractive. In contrast, anthralin, although strongly inhibitory to the macromolecular synthetic activities, produced minimal changes compared to controls. The authors state that these results are in agreement with those obtained by other investigators. (Charts 3 to 6, controls).
inhibitory to all functions, produced few visible nuclear changes at the end of 4.5 hr other than some pronounced nuclear swelling.

These studies have been carried out in vitro using human fibroblasts; however, most of the work on tumor promotion has been done on mouse skin in vivo (3, 4, 6, 15). Whether or not findings from cell culture may be directly applicable to in vivo systems is uncertain, but similar effects on macromolecular synthesis and morphology have been observed with tumor promoters in in vivo systems (2, 14, 26).

In conclusion, tumor promoters produce a concerted inhibition of a number of macromolecular synthetic processes, including DNA repair synthesis. At inhibitory concentrations there is morphological evidence of cellular injury. These findings suggest that the effects of promoters on DNA repair synthesis are nonspecific. In view of the large number of biochemical and morphological changes induced by promoters in vivo (1, 2, 16, 25, 26, 28) and in vitro (17, 29, 30, 32), it is unlikely that alterations in DNA repair synthesis alone account for their mechanism of action in the process of tumor promotion.

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