Induction of DNA-DNA Cross-Link Formation in Human Cells by Various Psoralen Derivatives

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ABSTRACT

Furocoumarin-induced DNA damage, monoadducts, and cross-links were measured in normal human, xeroderma pigmentosum, and Fanconi's anemia cells after exposure to near-UV (356 nm). At similar concentrations and near-UV doses, photoaddition by 8-methoxypsoralen was twice that by angelicin and the substitution of bromodeoxyuridine for thymidine in one strand of DNA did not alter the binding. The rate of cross-linking by 8-methoxypsoralen was twice that of 5-methoxypsoralen. Low frequencies of cross-links were detected from angelicin and 3-carbethoxypsoralen but none were detected from 5-geranylopsoralen at concentrations up to 25 µg/ml and near-UV doses up to 45,000 J/m².

INTRODUCTION

Furocoumarins are photosensitive compounds that are used therapeutically (for review, see Ref. 1) although they have recently been associated with high levels of cancer among treated individuals (2-5). The biological activity of these compounds is related to the formation of mono- or diadducts in DNA following cross-links, it is difficult to correlate a biological effect with a specific DNA lesion.

A comprehensive understanding of psoralen-induced carcinogenesis requires both qualitative and quantitative analysis of the DNA lesions produced by the various psoralen derivatives. Previous studies have sought to correlate both the mutagenic and carcinogenic effects with the degree of cross-linking (2, 7, 8). However, because the lesions formed by DNA cross-linking agents are a mixed population of monoadducts and interstrand cross-links, it is difficult to correlate a biological effect with a specific DNA lesion.

The psoralen derivative most commonly used as a monofunctional control has been the angular angelicin (9-12). Recent studies, however, indicate that angelicin also forms a low frequency of DNA interstrand cross-links in PM2 and λ-DNA (13, 14).

Previous studies have measured psoralen photoaddition in native DNA, bacterial cells, and in eukaryotic cells such as yeast, hamster cells, mouse cells, and human cells (1). However, the lack of information on the extent of cross-linking in human cells requires attention, especially in view of the use of psoralens in current dermatological practice. In this study we have compared the binding of 8-MOP and angelicin in normal cells and in cells from patients with two different hypersensitive diseases. We also measured the extent of interstrand DNA cross-linking from psoralen derivatives using a method that was modified from that of Ball and Roberts (15).

MATERIALS AND METHODS

Cells and Culture Conditions. SV40-transformed normal (GM637), excision-deficient xeroderma pigmentosum group A (XP12RO), and cross-link-sensitive Fanconi's anemia (FAH12) human fibroblasts (16) were used in this study. The FAH12 cells were the gift of Dr. K. Sperling (Institute für Human Genetik der Freien Universität, Berlin, Germany). Cells were maintained in Eagle's minimal essential medium supplemented with 15% fetal calf serum and antibiotics.

Psoralen Derivatives. Angelicin was chemically synthesized. 8-MOP was purchased from Sigma Chemical Co. and further purified by recrystallization. 5-GYP and 5-MOP were extracted from lime oil and oil of bergamot, respectively. 3-CP was the generous gift of Dr. D. Averbeck (Fondation Curie—Institut du Rayonnement, Section de Biologie, Paris, France). All compounds were characterized by nuclear magnetic resonance and mass spectroscopy.

The purity of psoralen derivatives was >99% as determined by high-performance liquid chromatography. Stock solutions of 8-MOP, angelicin, 5-MOP, 5-GYP, and 3-CP were stored at −20°C at concentrations of 0.25 to 1 mg/ml in 100% ethanol. Stock solutions were diluted to the appropriate concentrations with phosphate-buffered saline. Untreated and psoralen-treated cell cultures in 100-mm Petri dishes were exposed to near-UV filtered mercury arc lamp at a fluence of 25 J/m²/s (peak, 356 nm; Magna Flux Corp., Chicago, IL). Dosimetry was performed with a Yellow Springs Radiometer (Yellow Springs Instrument Co., Yellow Springs, OH).

Radiolabeled 8-Methoxypsoralen and Angelicin. 8-MOP and angelicin were labeled with 3H at the Amersharm Corporation (Chicago, IL) by a catalytic exchange process. The crude product was purified by silica gel column chromatography using CHCl₃ as solvent (17). Radiochemical purity was determined by analytical thin-layer chromatography (silica gel) and absorption spectrum analysis using unlabeled 8-MOP or angelicin as a standard. Samples containing purified material were dried and redissolved in 100% ethanol. Concentration was determined on a Cary 219 spectrophotometer and specific activity was determined on a Packard 3303 liquid scintillation spectrophotometer. The specific activity of [3H]MOP was 2.8 Ci/mmol and that of [3H]angelicin was 2.4 or 22 Ci/mmol.

The total amount of 8-MOP or angelicin bound to DNA was measured in confluent cell cultures prelabeled with [14C]dThd, 0.001 µCi/ml (57 mmCi/mmol). The cells were rinsed and exposed to near-UV in the presence of various concentrations of [8-3H]MOP or [3H]angelicin in phosphate-buffered saline on ice. The cells were harvested immediately after irradiation and the DNA was extracted and purified (18) to ensure removal of photoadducts in RNA and protein. Purified DNA was precipitated with ice-cold 100% ethanol and stored at −20°C for more than 1 h. The DNA was centrifuged at 10,000 rpm for 30 min at 4°C and then resuspended in SSC. To ensure removal of any psoralen that was not covalently bound, the precipitation and resuspension procedure was performed with phosphate-buffered saline; dThd, thymidine; SSC, standard saline citrate (0.15 M sodium chloride-0.015 M sodium citrate, pH 7.4).
repeated until the specific activity of the DNA remained constant (usually two replications). An aliquot of the DNA solution was precipitated on Whatman GF/A glass fiber filters and the radioactivity was determined by liquid scintillation counting. The concentration of the DNA solution was determined by measuring the absorption at 260 nm. Psoralen binding to BrdUrd-substituted DNA was determined in the same fashion. Some of the [3H]psoralen-labeled DNA was also centrifuged on alkaline CsCl gradients for further purification.

Isopycnic Gradient Analysis. Purified BrdUrd-substituted DNA was banded in neutral or alkaline CsCl-Cs2SO4 gradients (15, 19). The DNA in SSC and 2 mM EDTA was layered on top of either 5%–20% alkaline or neutral sucrose gradients. The gradients were centrifuged in a Beckman 50 Ti rotor at 42,000 rpm and 20°C for up to 48 h.

After centrifugation, the gradients were fractionated into 20 to 23 fractions/gradient while A600 was continuously monitored. The radioactivity profile was obtained by precipitating the DNA in a 50-μl aliquot from each fraction on Whatman GF/A glass fiber filters or on Whatman 3-mm paper filters and measuring the radioactivity by liquid scintillation counting.

Molecular Weight Measurements. A 100- or 200-μl aliquot of purified DNA in SSC and 2 mM EDTA was layered on top of either 5%–20% alkaline or neutral sucrose gradients. The gradients were centrifuged at 20°C (40,000 rpm) in a Beckman SW40 rotor for 2 h (alkaline gradients) and 3.5 h (neutral gradients). The gradients were calibrated with a H-labeled SV40 standard for determination of molecular weight (M0).

DNA Intercalation Cross-Link Analysis. Cells were prelabeled with [14C]dTdU, 0.01 μCi/ml, for 3–4 days to ensure uniform labeling of the DNA. Medium containing [14C]dTdU was replaced with fresh medium containing 10^{-8} M BrdUrd. After 1 h the medium was supplemented with 10^{-8} M fluorodeoxyuridine and either [14C]BrdUrd, 0.05 μCi/ml (57 mCi/mmol) or [3H]BrdUrd, 2 μCi/ml (50 Ci/mmol). [14C]dTdU, [3H]BrdUrd, and [14C]BrdUrd were obtained from New England Nuclear. The total incubation time of cells in the presence of BrdUrd was slightly less than one cell cycle. The 1-h incubation in nonradioactive BrdUrd ensures that the newly synthesized DNA containing label (either 14C or 3H) and BrdUrd is fully separated from the normal-density DNA minimizing trailing of radioactive activity at intermediate densities (15, 19). Cross linking was determined by establishing the amount of radioactivity remaining in the heavy (H) density single-stranded DNA peak of the isopycnic gradients relative to controls that were irradiated with near-UV only. Radioactivity is proportional to DNA content, so the radioactivity measured in the H peak, i.e., the area under the H peak, was representative of the single-stranded DNA in the entire gradient. The proportion of single-stranded DNA per gradient was determined by the calculations that follow where N0 is the radioactivity in the H peak of control DNA; N is the radioactivity in the H peak of treated DNA normalized to DNA content; N/N0 = F is the fraction of DNA remaining single stranded; and 1 - F is the fraction of cross-linked DNA.

The genomic DNA between cells is essentially the same, so we make the assumption that cross-linking is a random event occurring among a monodisperse population of double-stranded DNA target molecules of number average molecular weight M0. Random shearing of the DNA results in DNA molecules of number average molecular weight (Mn) in which cross-linking follows a Poisson distribution.

\[ P(n) = \frac{m^n \exp(-m)}{n!} \]  

where \( m \) is the mean number of cross-links per double-stranded DNA molecule.

The relative proportion of molecules that remain uncross-linked, i.e., \( n = 0 \), after treatment with a cross-linking agent is

\[ \frac{N}{N_0} = 1 + \exp(-m) \]  

\( N \) and \( N_0 \) are determined empirically from the alkaline isopycnic radioactivity profiles. Equation B can then be written as

\[ m = \ln \left( \frac{N_0}{N} \right) \]  

The cross-link frequency was then expressed as \( m/M_0 \), and \( M_0 \) is the number average molecular weight for double-stranded DNA which was determined on neutral or alkaline sucrose gradients. Measurement of \( M_0 \) on neutral sucrose gradients avoided artifacts caused by anomalous sedimentation of denatured DNA joined by cross-links (19).

\( N \) and \( N_0 \) were determined from the radioactivity profiles by measuring the radioactivity in equivalent fractions on the heavy side of the H peak. This empirical approach facilitated the measurement of DNA remaining single-stranded and minimized the contribution of radioactivity from cross-linked DNA in the H peak. Corrections for small shifts in the location of the peaks were made by measuring the distances between the peaks of banded DNA. Gradients showing unusual banding were not considered.

Three assumptions were required to determine the values of \( N \) and \( N_0 \): (a) radioactivity in the heavy side of the H peak was representative of the total single-stranded DNA in that peak; (b) the banding profile of H DNA for the control was the same as that for the treated DNA; (c) the banding of DNA in isopycnic gradients is Gaussian and because of the denaturing conditions of the gradient single-stranded and cross-linked DNA band independently. The sum of the radioactivity in several fractions in the control was then compared to that in equivalent fractions of treated samples.

RESULTS

Covalent Addition of Psoralens. Covalent addition of [8-3H]-MOP or [8-3H]angelicin to DNA increased as a function of near-UV dose and psoralen concentration (Chart 1, A and B). Addition of 8-MOP and angelicin saturated with respect to light dose. Saturation was not as evident at a fixed near-UV dose and increasing psoralen concentrations. The maximum observed value of 8-MOP addition for a concentration of 25 μg/ml and a near-UV dose of 7.5 kJ/m2 of near-UV and increasing concentrations of [8-3H]-MOP or [8-3H]angelicin. Data for all three cell types were combined. Points without bars, mean of two cell lines; bars, SE for all three cell types.
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Chart 2. Alkaline CsCl-CsSO₄ isopyknic gradient profiles of DNA from XP12RO cells labeled with [¹⁴C]dThd and [¹⁴C]BrdUrd. A, cells treated with near-UV alone (△) or exposed to 7500 J/m² plus 8-MOP, 25 μg/ml (△). Cross-linked DNA is reflected in the HL peak. B, ——, radioactivity profile of DNA from cells treated with 8-MOP, 10 μg/ml, and 7500 J/m² of near-UV; ——, normalized profile for the H peak of DNA from control cells and represents the distribution of single-stranded BrdUrd molecules in the treated DNA. Cells were incubated as described in "Materials and Methods." DNA content was normalized to the amount of DNA in cultures exposed to near-UV in the absence of 8-MOP.

A dose of 7500 J/m² was about 12 x 10⁻⁸ adducts/dalton (Chart 1B), whereas the comparable value for angelicin was 7 x 10⁻⁸. The binding of these psoralens to DNA was essentially the same in xeroderma pigmentosum and Fanconi's anemia cells as in normal cells. The ratio of angelicin and 8-MOP binding to DNA in which BrdUrd was substituted for dThd in one strand was 0.93 and 0.91, respectively. This is consistent with Ben-Hur and Riklis (20), although slightly greater binding to BrdUrd-substituted DNA was observed by Cassel and Latt (21).

The radioactivity profiles of [³H]MOP- or [³H]angelicin-treated DNA in alkaline isopyknic gradients indicated that all [³H] radioactivity was associated with the [¹⁴C]dThd-preflabeled DNA in which BrdUrd was substituted for dThd in one strand was 0.93 and 0.91, respectively. This is consistent with Ben-Hur and Riklis (20), although slightly greater binding to BrdUrd-substituted DNA was observed by Cassel and Latt (21).

The radioactivity profiles of [³H]MOP- or [³H]angelicin-treated DNA in alkaline isopyknic gradients indicated that all [³H] radioactivity was associated with the [¹⁴C]dThd-preflabeled DNA rather than dispersed throughout the gradient (22, 23). This is further evidence that noncovalently bound psoralen has been removed and that the psoralen associated radioactivity is covalently bound to the DNA.

DNA Cross-Linking from Psoralens. Verification of labeling time indicated that cells incubated in the presence of BrdUrd did not enter another round of replication up to 40 h incubation. Since SV40-transformed cells appear to have longer cell cycle times⁴ and BrdUrd inhibits progression through the cell cycle (24), lack of another round of DNA replication during a 40-h incubation is not unusual.

The radioactivity profiles in alkaline isopyknic gradients of control or 8-MOP-treated DNA, which was labeled with [¹⁴C]-dThd in one strand and [¹⁴C]BrdUrd in the opposite strand shows evidence of cross-linked molecules between H and L strand positions. At higher concentrations or higher UV doses, the H peak can be obscured by the banding of cross-linked DNA (Chart 2). However, our analysis (see "Materials and Methods") eliminates any ambiguity. The increase in cross-link frequency with dose was quantified from changes in the proportion of labeled DNA remaining single stranded and therefore lacking cross-links (see "Materials and Methods") (Chart 3).

Calculation of absolute cross-link frequency was dependent on molecular weights determined in neutral or alkaline sucrose gradients. Preferential breakage in DNA due to BrdUrd photolysis by near-UV or long exposure to alkaline conditions was a concern that was analyzed. The peak efficiency of BrdUrd photolysis is closer to 310 nm than the 356 nm used in our experiments (25). In addition, cells were irradiated through Petri dish covers and this filtered shorter wavelengths. Analysis on alkaline sucrose gradients of doubly labeled DNA which was unifilarly substituted with BrdUrd indicated no preferential breakage in the BrdUrd-substituted strand at near-UV fluences up to 60,000 J/m² at 356 nm nor did exposure of purified DNA to alkaline conditions for up to 48 h at 20°C.

The molecular weight of double-stranded DNA ranged from $M_w = 0.8$ to $8.4 \times 10^7$ daltons with a mean of $3.54 \times 10^7 \pm 1.68$ (SE) daltons. This range is not subject to significant centrifugation speed artifacts, which generally become important above $10^8$ daltons (26). The variation in molecular weight occurred as a

* L. N. Kapp, personal communication.
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result of the DNA isolation procedure. The higher molecular weights were obtained by isolating the DNA with minimal mechanical shear.

Cross-links/10⁸ daltons were determined for various psoralen derivatives as a function of drug concentration or near-UV dose (Chart 4). 8-MOP and 5-MOP showed a high degree of cross-linking, whereas angelicin and 3-CP showed slight but reproducible cross-linking and 5-GYP showed none. The graphs indicate that angelicin had 0.15 times the efficiency of cross-linking DNA as did 8-MOP.

DISCUSSION

This study has examined the binding of various psoralen derivatives to cellular DNA in intact cells. We observed a maximum level of 8-MOP and angelicin binding to DNA of about 7 x 10⁻⁸ and 3.5 x 10⁻⁸ adducts/dalton, respectively. Assuming approximately 10⁸ thymines/genome, there is only about 1 adduct/10⁷ thymines, which suggests that intact cells have a very low proportion of thymines which are potential binding sites. The level of angelicin binding is similar to previous measurements made in Ehrlich ascites tumor cells (27), although one study observed a 10-fold higher level (12). Saturation of covalent binding by psoralens may be due to limitations in binding sites imposed by chromatin structure (28, 29). Electron microscopic studies of photobinding in Drosophila melanogaster (30) and mouse chromatin (29) showed addition to every linker when isolated nuclei were treated with 4, 5, 8-trimethylpsoralen. Use of isolated nuclei and repeated applications of the psoralen may also have enhanced total binding above that possible in intact cells.

The association constants for noncovalent binding to DNA by 8-MOP and angelicin are 10 x 10³ and 13 x 10³, respectively (31), which suggests that intercalation is not responsible for the 2-fold difference in photoaddition we observed. Rather an analysis of the absorption spectra of psoralens indicates that greater photobinding of 8-MOP is due to differences in absorbance between angelicin and 8-MOP at the emission maximum of the near-UV source (λ_max = 356 nm) (1, 32).

The relative amount of cross-linking by 8-MOP, 5-MOP, and angelicin that we observed is consistent with the cytotoxicity (28, 33) and the photoaddition to purified DNA by each agent (34). 8-MOP has been shown to be more efficient in DNA interstrand cross-link formation than 5-MOP when studies were carried out using native DNA (35). The demonstration that DNA cross-linking in human cells by 5-MOP is one-half that of 8-MOP is consistent with this earlier study, although is has not been shown previously in human cells. 3-CP has a high photoaffinity for DNA (28) but forms very few cross-links. The low level of cytotoxicity detected in this previous study is consistent with less toxicity from monoadducts than from interstrand cross-links. Little is known about 5-GYP, although no cross-links were detected in human cells treated with this agent.

The studies showing that angelicin and 3-CP form only monoaadducts used different methods of analysis or were based on assumptions from work in nonmammalian systems (9, 12, 28). It is possible for 3-CP to form cross-links by photochemical cleavage of the carbethoxy group at wavelengths shorter than 340 nm (36). Because our near-UV source is not monochromatic, it is conceivable that there was enough near-UV of wavelength less than 340 nm to cause such a conversion. The resulting psoralen molecules would then be capable of cross-link formation.

DNA cross-linking by angelicin has been observed at high drug concentrations and high doses (14) or in highly condensed DNA (13); however, ours is the first indication that angelicin also cross-links human DNA in intact cells. The highly compact structure of mammalian chromatin may account for the cross-linking we
detected. The condensation and DNA supercoiling could cause different sections of the double helix to be adjacent making the formation of intermolecular cross-links possible. Intermolecular cross-links involving opposite strands would be indistinguishable from interstrand cross-links when measured by our density shift technique.

Although the possibility that outlying counts in the isopyknic distribution may be due to small diffusion-driven molecules cannot be ignored, we feel that the linearity demonstrated at high levels of cross-linking supports our empirical approach for interpretation of the data. In addition, we have made individual molecular weight determinations of the DNA in >75% of the isopyknic gradients used in this study. Variations in the value of $m$ were offset by differences in $M_*$ such that the values of $m/M_*$ were consistent for the same experimental conditions. This consistency is further demonstrated by the close agreement our measurements have with specific findings in previous investigations and by the linearity of the curve at high levels of cross-linking.

The use of 8-MOP therapeutically and of 5-MOP as a component of certain suntan preparations is cause for concern. Photoaddition and cross-linking will occur as long as the individual is exposed to a source of near-UV because of the presence of residual drug. It is therefore important that we define the repair of psoralen monoadducts and cross-links and the extent of the interaction between cross-links and other lesions in human cells (23, 37, 38). We have elucidated some of these phenomena in the accompanying study (39) to further our understanding of the role DNA cross-links play in mutagenesis and carcinogenesis.

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