Extrachromosomal Chromatin: Novel Target for Bleomycin Cleavage in Cells and Solid Tumors†

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ABSTRACT: The preference of bleomycin, a DNA strand scission antitumor agent, to damage extrachromosomal (episomal) DNA was investigated. These episomes contain transcriptional promoters, replication origins, and oncogenes from MMTV, BPV, and *v-Ha-ras* and confer a neoplastic phenotype to a mouse fibroblast cell line. We found that bleomycin induces dose-dependent single- and double-stranded cleavage of intracellular episomes as measured by topological forms conversion. Bleomycin scission of episomes occurs within 1 min, and upon drug removal, damaged episomes are as rapidly repaired. By expressing the episomal and genomic damage as breaks per nucleotide, bleomycin has a 30–50-fold cleavage preference for episomal chromatin compared to genomic DNA. The episomes have preferred regions of the bleomycin-induced damage, particularly within the MMTV LTR and BPV origin of replication. Also, it is possible to assess bleomycin action on episomes in solid tumors in mice. Single intravenous injections of BLM into tumor-bearing mice result in single- and double-stranded cleavage of episomes that are dose related and occur within 1 min. Specific double-stranded breaks occur in the same regulatory regions of episomes in solid tumors and in cultured cells. Finally, we observe that damage to the episomal drug target occurs at therapeutic doses in mice.

Bleomycin (BLM)[1] is an antitumor agent whose cytotoxic behavior is related to its intrinsic DNA cleavage activity (Suzuki et al., 1969; Fujiwara & Kondo, 1973). There is evidence, primarily acquired in cell-free studies with naked DNA or in subcellular assays, that DNA structure and possibly function are important determinants in the cleavage reaction. For example, BLM treatment of nuclei isolated from erythrocytes results in preferential cleavage of an active β-globin gene in comparison to damage induced in an inactive oviduct gene (Kuo, 1981). With a mouse mammary tumor virus long terminal repeat (MMTV LTR) sequence as a gene target, BLM treatment of nuclei results in preferential damage to the glucocorticoid receptor binding enhancer region (Beckmann et al., 1987). Although DNA targets associated with unique chromatin structures appear to be unusually vulnerable to the cleaving action of BLM, this phenomena has not been well studied in the more complex environment of whole cells or in animals. In fact, a recent study demonstrated that BLM treatment of SV40-infected cells leads to preferential cleavage at multiple sites that are not limited to the SV40 promoter but rather dispersed throughout the SV40 genome (Grimwade et al., 1986). While the SV40 system is amenable to studies of DNA damage in a cellular environment, the assay is conducted under conditions where drug effects are evaluated on nonviable cells and where SV40 DNA is replicating outside of normal growth controls (Tooze, 1980). Moreover, SV40-infected cells cannot be used in an in vivo environment, thus precluding evaluation of BLM damage to DNA in animals.

To overcome these deficits with the SV40 system and still maintain the advantages of being able to track damage on defined DNA targets in cells, an extrachromosomal element (episome) was evaluated as a potential BLM target. The episomes in the 935.1 cell line were derived by transforming normal mouse fibroblast cells (C127) to a neoplastic phenotype with a bovine papillomavirus (BPV) vector linked to a dexamethasone-inducible MMTV LTR–*v-Ha-ras* fusion gene (Ostrowski et al., 1983). Thus, the episome consists of two transcribed gene sequences: the MMTV LTR, which controls expression of the adjacent *v-Ha-ras* gene, and the 69% transforming sequence of BPV, which includes the promoter region and replication origin (Waldeck et al., 1984). The 935.1 cell line has been used previously in our laboratory to evaluate DNA damage induced by topoisomerase II targeted drugs in cultured cells (Cullinan & Beerman, 1989). Recently, 935.1 cells were developed for in vivo assays of solid tumors in mice and were found to be capable of detecting m-AMSA-induced topoisomerase II mediated episomal DNA breaks (Cullinan et al., 1990).

The susceptibility of this extrachromosomal DNA to BLM-induced damage was assessed as a means of deriving new insight into the molecular mode of BLM action in cells and tumor-bearing mice. BLM-induced cleavage was readily detected on episomes in whole cells by changes in topology. Sites of specific DNA cleavage were identified to determine whether active chromatin regions such as the origin of replication and the MMTV LTR were unusually sensitive to drug cleavage. By quantitative analysis of genomic damage, comparisons were made between the frequency of BLM cleavage of episomal and genomic DNA. Treatment of mice bearing subcutaneous 935.1 tumors with single intravenous (iv) doses of BLM resulted in strand breakage of episomes at therapeutically relevant doses of the drug.

Materials and Methods

Materials

Blenoxane (BLM, NDC 0015-3010-20) was generously supplied by Dr. Bradner at Bristol-Myers Co. (Syracuse, NY).

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[1] Abbreviations: BLM, bleomycin; VM26, teniposide; m-AMSA, amsacrine; BPV, bovine papillomavirus; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; SV40, simian virus 40; DSB, double-stranded break; SSB, single-stranded break; bp, base pair(s); nt, nucleotide; SDS, sodium dodecyl sulfate.

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A 10 mg/mL stock solution was made in water and stored at −20 °C. For animal use, BLM was diluted in Travenol. Restriction endonucleases, proteinase K, and RNase A were purchased from Boehringer-Mannheim. Nitrocellulose filters were purchased from Schleicher and Schuell. Polycarbonate filters, 0.2-μm pores and 25-mm diameter, were purchased from Millipore. All other chemicals were reagent grade or better and were purchased from Sigma Chemical Co. or Fisher Scientific. The Amersham Multiprime DNA-labeling system was used to label the *Eco*RI–*Sma*I BPV fragment.

The 935.1 cell line is described elsewhere (Ostrowski et al., 1983) and was generously provided by Dr. Gordon Hager. Cells were grown as monolayer cultures in Dulbecco's-modified Eagle's medium (GIBCO) and supplemented with 10% fetal bovine serum (GIBCO). The bacterial plasmid pM19 (Ostrowski et al., 1983), containing the entire episomal sequence, was also obtained from Dr. Hager. Taconic NIH Swiss female mice were kept in a biocontainment facility supplied with sterilize food, water, bedding, and microisolator system cages (Lab Products, Inc.).

Methods

Topological Forms Conversion. Damage to the episome was measured by using a topological forms conversion assay that detected single- and double-stranded breaks in superhelical DNA. The assay consisted of drug treatment of cells followed by DNA extraction. The purified DNA was separated in 0.7% agarose gels, transferred to nitrocellulose filters by Southern blotting, and hybridized to a ^{32}P-pM19 plasmid probe. Autoradiographs of the transferred bands were quantitated with use of a densitometer. The method has been described in detail elsewhere (Cullinan & Beerman, 1989). The episomal single-stranded break frequencies were calculated by dividing the decrease in percent supercoiled DNA by the number of episomal nucleotides (17 588 nt). The episomal double-stranded break frequencies were calculated by dividing the increase in the percent linear DNA by the number of episomal base pairs (8794 bp).

High-Sensitivity Filter Elution. Analysis of drug- or X-ray-induced single-stranded breaks in genomic DNA was made according to the high-sensitivity alkaline elution technique (Kohn et al., 1976). Intracellular DNA was labeled by the addition of [^{14}C]thymidine (56 mCi/mmol; Moravek Biochemical, Brea, CA). A total of 2.5×10^5 935.1 cells were treated with drugs or X-rays and then impinged on polycarbonate filters and lysed in situ. Fractions were collected at 90-min intervals with a flow rate of 0.033 mL/min. From the initial slopes of the elution curves, the X-ray calibration curve and the BLM rad equivalent damage curve were calculated. Rad equivalent damage was converted to genomic single-stranded break frequency by using the 0.9×10^{-9} SSB rad^{-1} nt^{-1} conversion factor (Kohn et al., 1976).

The neutral filter elution technique was used to assay for double-stranded breaks and has been described elsewhere (Cullinan & Beerman, 1989; Bradley & Kohn, 1979). Briefly, 935.1 cells were treated as described above except that a neutral elution buffer was used. From the initial slopes of the neutral elution curves, the X-ray calibration curve and BLM rad equivalent damage curve were calculated. Rad equivalent damage was converted to genomic double-stranded break frequency by using the conversion factor of 4.5×10^{-11} DSB rad^{-1} bp^{-1} (0.9×10^{-9} SSB rad^{-1} nt^{-1} divided by 20) (1 DSB for every 20 SSB induced by X-ray) (Kohn et al., 1976; Bradley & Kohn, 1979).

Analysis of DNA Breaks in Vivo. The method of DNA isolation has been described previously (Cullinan et al., 1990). In short, female NIH Swiss Taconic nude mice were implanted subcutaneously (sc) with 50 mg of 935.1 tumor pieces with use of 13-gauge trocar syringes. After 10 days of growth, tumors were palpable and mice were then treated through the tail vein with a bolus (0.2-cm^3) iv injection of the indicated doses of BLM or saline alone. At the times indicated after drug injection, mice were sacrificed by cervical dislocation and tumors immediately excised and immersed in liquid nitrogen. The frozen tumors were pulverized with a pulverizing apparatus cooled by liquid nitrogen. The pulverized tumor was transferred to ice-cold homogenizing solution (10% sucrose, 10 mM Tris-HCl, pH 7.6, and 20 mM EDTA), and nuclei were prepared by 10 strokes in a Potter-Elvehjem homogenizer and then pelleted at 2000 rpm for 10 min at 4 °C. Pellets were resuspended in 5 mL of lysis buffer [1% (w/v) SDS, 10 mM Tris-HCl, pH 7.8, and 20 mM EDTA], and then total DNA was purified as previously described (Cullinan & Beerman, 1989). Total DNA (1 μg) was separated in 0.7% agarose gels (50 mM Tris-HCl, pH 8.3, 20 mM sodium acetate, and 2 mM EDTA buffer) at 0.1 V/cm for 20 h. The DNA was transferred to nitrocellulose and hybridized to radiolabeled pM19 probe (Maniatis et al., 1980).

Site-Specific Damage. Site-specific damage to the BPV origin of replication and the MMTV LTR was mapped according to the indirect end-labeling technique (Wu, 1980), where a purified *Eco*RI–*Sma*I fragment of BPV from pM19 was labeled to high specific activity and hybridized to *Eco*RI-digested 935.1 cellular or tumor DNA. An additional step involving a modification of the Hirt supernatant technique, which enriched for episomal DNA, was required for the DNA purification from solid tumors and is described below. After nuclei were lysed, 200 μL of crude lysate was further diluted in 400 μL of lysis buffer. NaCl was added to the diluted lysate to a final concentration of 1 M. Lysates were placed at 4 °C for 24 h and then centrifuged at 10000*g* for 30 min. The supernatant was collected and incubated at 50 °C for 4 h with 400 μg/mL proteinase K and then with 40 μg/mL RNase. Purified DNA (10 μg) was digested with *Eco*RI, which has a single site on the episome (see Figure 6) and generates either full-length linear DNA (8.9 kb) from the supercoiled and nicked circular population or two or more smaller fragments from the linear drug-damaged population. DNA was separated by electrophoresis in 1% agarose gels (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, and 2 mM EDTA running buffer at 0.1 V/cm for 20 h). The DNA was transferred to nitrocellulose filters, and blots were hybridized as described above except that the probe was a short *Eco*RI–*Sma*I fragment of BPV (specific activity 1×10^9 cpm/μg). Purified episomal DNA used as markers for determining sites of cleavage in the regulatory regions was isolated by a salt extraction method (Ostrowski et al., 1983) and digested with restriction enzymes as indicated.

Therapeutic Efficacy Trials. Fragments of 935.1 tumors (50 mg) were implanted (bilateral, sc) into nude mice 1 week prior to BLM treatment. Mice were randomized three per treatment group, and all mice had palpable tumors after 1 week. On day 1, the mice (average tumor burden of 50 mg) were treated with single iv doses (0.2 cm^3) of BLM or saline as indicated. Tumor weight was calculated from the length (L) and width (W) dimension (mm) of the tumor where tumor weight (milligrams) = $(L \times W^2)/2$ (Bissery et al., 1988). Tumors were measured daily.

Results

Topological Forms Conversion. Single- and double-stranded breaks were analyzed by topological forms conversion

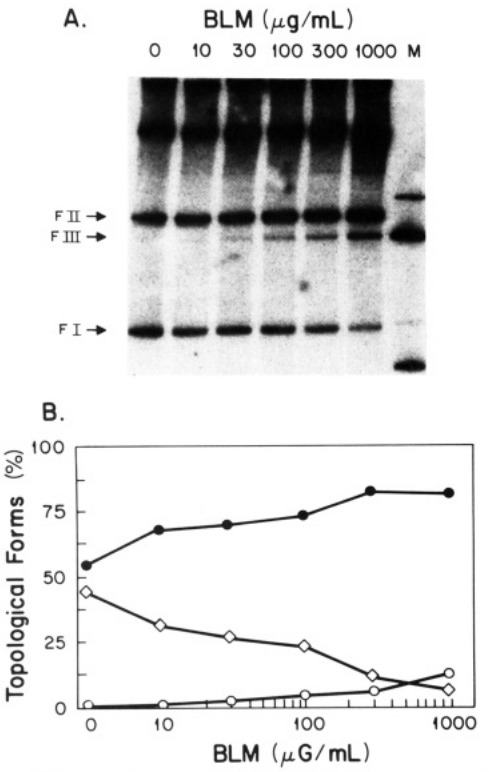

FIGURE 1: Episomal cleavage in cultured 935.1 cells related to BLM concentration. (A) The Southern blot of the topological forms of the episome separated in a 0.7% agarose gel and hybridized to the pM19 probe. Cleavage is depicted as a decrease in form I (FI) and an increase in form II (FII) and form III (FIII). 935.1 cells were treated for 30 min with the indicated concentrations of BLM. (B) The distribution of monomeric topological forms in panel A was expressed as percent supercoiled form I (◇), nicked circular form II (●), and linear form III (○). Densitometric scans of the episomal bands were performed in the linear range of detection. The data are representative of at least three independent experiments.

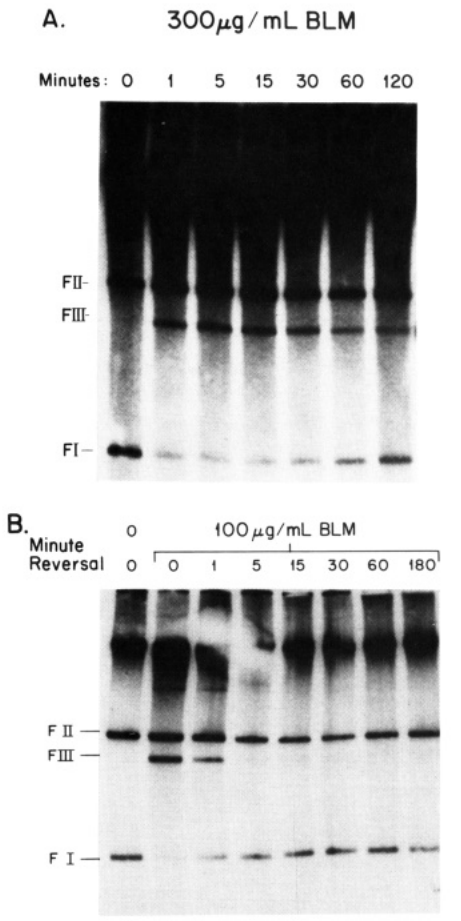

FIGURE 2: Time dependence and reversal of BLM cleavage on intracellular episomes. Southern blots of episomal DNA after (A) treatment of cells with 300 μg/mL BLM for various times as indicated and (B) treatment of cells with either no drug (first lane) or with 100 μg/mL BLM for 30 min (all remaining lanes) followed by washing in drug-free media and incubating in drug-free media for the times indicated before lysis. Blots were hybridized to radiolabeled pM19. Experiments were performed twice with similar results.

of monomeric, circular episomes following BLM treatment of cultured 935.1 cells (Figure 1A). Single-stranded breaks were detectable as a reduction of supercoiled DNA (FI), and double-stranded breaks were seen as an increase in linear DNA (FIII). Single-stranded breaks were also apparent by the increase in nicked circular DNA (FII), although a nicked molecule may have more than one single-stranded break. The intense uppermost bands seen in Figure 1A are replication intermediates of the episome, which although cleaved by BLM do not produce free monomeric episomes. Episomal damage is first evident at 10 μg/mL BLM and increases with increasing BLM concentration (Figure 1A). The quantitation of topological forms is shown in Figure 1B. It is interesting to note that BLM-induced episomal cleavage is readily detectable at 20 μg/mL BLM, a dose at which only 10% of the 935.1 cells survive. At 1000 μg/mL BLM, a substantial fraction of episomal DNA (18%) contains double-stranded breaks while a smaller proportion of episomes (12%) has yet to receive single-stranded breaks. An analogue of BLM, tallysomycin S10b, showed a similar ability to cleave episomal DNA (Beerman and Gawron, unpublished observation). It was also noted that the incidence of BLM-induced cleavage was unaffected by dexamethasone, a glucocorticoid that induces expression of the *ras* gene from the MMTV LTR (see Figure 6 for a map of the episome) (data not shown).

The rate of BLM cleavage and repair was determined. The cleavage reaction is extremely rapid, with the strongest net cleavage occurring within the first 5 min (Figure 2A). As the reaction proceeds, the amount of linear episomal DNA is reduced and the amount of supercoiled episomal DNA increases, although not to untreated control levels. These data imply that the BLM-induced episomal cleavage reaction reaches a stasis with drug inactivation and damage repair that is similar to published observations of BLM damage to genomic DNA (Miyaki et al., 1973; Saito & Andoh, 1973; Iqbal et al., 1976; Hurt et al., 1981). Because the extent of DNA damage approaches maximal cleavage at the earliest time points, we also demonstrated that the cleavage reaction was actually halted at the time of cell lysis. Lysis buffer containing exogenous SV40 DNA was added to 935.1 cells that were either untreated with BLM, treated with 100 μg/mL BLM and then immediately lysed, or treated with 100 μg/mL BLM for 30 min before lysis. Cleavage of SV40 DNA in the cell lysates would indicate that BLM damage could occur after cell lysis. However, while episomal DNA was cleaved by the short 30-min treatments, SV40 DNA remained completely intact, suggesting strongly that in these assays BLM-induced cleavage occurs in whole cells and not in subsequent DNA-purification steps (data not shown). To measure DNA repair, cells were treated with BLM, incubated for various times in drug-free media, lysed, and then analyzed for episomal DNA damage. As shown in Figure 2B, the initial 30-min BLM treatment yields mostly nicked and linear DNA molecules. Within the first 1 min of drug-free incubation, half of the linearized episomes are repaired, and repair is essentially complete by 60 min. Therefore, the cellular response to repair

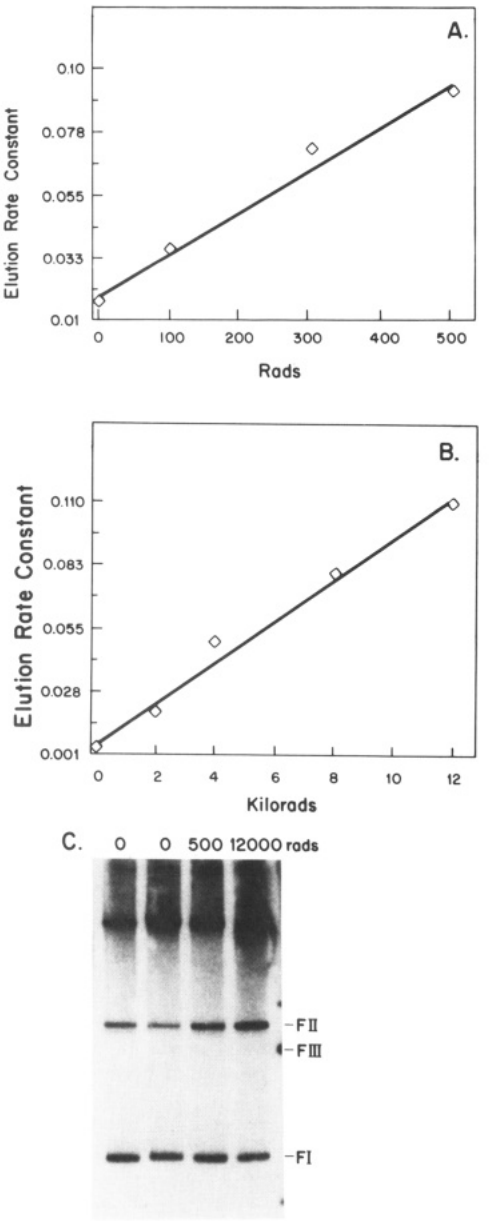

FIGURE 3: Analysis of X-irradiation-induced single- and double-stranded breaks on genomic and episomal DNA. Single- (A) and double-stranded (B) elution rate constants (the slope of the first 3 h of elution) were plotted as a function of the dose of ionizing radiation received by 935.1 cells. (C) 935.1 cells were treated with 0, 500, or 12000 rad of ionizing radiation, and then damage of the episome was assessed by Southern blotting.

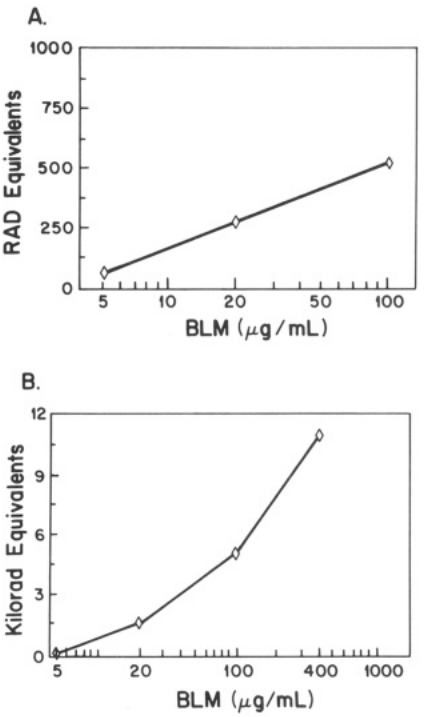

FIGURE 4: Single- and double-stranded rad equivalent damage induced by BLM. (A) Single-strand rad equivalent damage is plotted as a function of increasing BLM dose. Initial elution rates of DNA from BLM-treated 935.1 cells were compared to equivalent elution rate constants in Figure 3A and then converted to rad equivalents. (B) Double-stranded break kilorad equivalent damage is plotted as a function of BLM dose. The initial elution rates of DNA from BLM-treated 935.1 cells were compared to equivalent elution rate constants in Figure 3B and then converted to kilorad equivalent damage. The data are representative of at least two independent experiments.

of episomal DNA is similar to the rapid repair phenomenon previously reported for genomic DNA (Miyaki et al., 1973; Saito & Andoh, 1973; Iqbal et al., 1976; Hurt et al., 1981; Moore et al., 1985).

Comparison between Episomal and Bulk Genomic DNA Damage. An important question was whether the episome was a preferred drug target over genomic DNA. To make this comparison, genomic DNA damage in 935.1 cells was assessed by filter elution techniques. Since irradiation is presumed to induce damage randomly throughout chromosomal DNA, it served as a standard to which BLM damage was compared. X-ray elution data clearly display single-stranded breaks between 0 and 500 rad expressed as a dose-related increase in the elution rate constant (Figure 3A). These data are similar to elution data obtained from L1210 cells from which a conversion factor for equating rad equivalent damage to breaks per nt is derived (Kohn et al., 1976). Similarly, the double-stranded break analysis shows a relationship between elution rate constant and irradiation doses up to 12000 rad (Figure 3B). In addition, irradiation of 935.1 cells gives a single- to double-stranded break ratio for chromosomal DNA of 23:1, very close to the ratio of 20:1 previously reported (Bradley & Kohn, 1979). To quantitatively compare DNA breaks of the genome with those of the episome, Southern blot analysis was used to follow changes in the topological forms of the episome when cells were irradiated at 500 and 12000 rad (Figure 3C). Doses of 500 and 12000 rad caused limited single-stranded breakage, observed as a 13% and 27% reduction in supercoiled DNA, respectively. After 12000 rad, double-stranded breaks were detectable as 0.5% linear episomal DNA. The potential for preferred single- or double-stranded cleavage of the episome by X-ray was then expressed as a ratio of actual episomal damage to predicted episomal damage (based on genomic break frequencies). As shown previously, 12000 rad produces double-stranded breaks of genomic and episomal DNA with equal frequency with a ratio of episomal to genomic damage of 1 (Cullinan & Beerman, 1989). A ratio of 1 would be predicted for random cleavage. Similarly, a comparison of the incidence of single-stranded breaks induced by 12000 rad also results in a ratio of 1. Interestingly, at a low dose of 500 rad single-stranded breaks are measured more frequently (15 times) on episomal versus genomic DNA. Others have reported that low doses of radiation can preferentially cleave transcribing chromatin over the bulk of the genome (Chiu et al., 1982; Chiu & Oleinick, 1982).

BLM-induced genomic DNA damage was determined by filter elution and expressed in rad equivalents (Figure 4). Single-stranded breaks of genomic DNA are first detectable

Table I: BLM Single-Stranded Break Comparison[a]

	10 μg/mL	100 μg/mL	1000 μg/mL
episomal SSB/nt	6.8×10^{-6}	1.1×10^{-5}	2.1×10^{-5}
genomic SSB/nt	1.5×10^{-7}	4.6×10^{-7}	7.9×10^{-7}
episomal to genomic ratio	45	24	27

[a] A comparison of episomal to genomic damage was made at 10, 100, and 1000 μg/mL BLM. The genomic single-stranded break frequency was calculated by multiplying the rad equivalent damage (167, 510, and 875 rad at 10, 100, and 1000 μg/mL BLM, respectively) by 0.9×10^{-9} SSB rad^{-1} nt^{-1} (Kohn et al., 1976). From the data in Figure 1, the episomal single-stranded break frequency was calculated by dividing the reduction in form I (12, 19, and 36%, respectively) by the number of nucleotides in the episome (17588 nt). The potential for preferred single-stranded cleavage of the episome by BLM was expressed as a ratio of episomal to genomic damage.

Table II: BLM Double-Stranded Break Comparison[a]

	10 μg/mL	100 μg/mL	300 μg/mL
episomal DSB/bp	1.1×10^{-6}	6.8×10^{-6}	1.1×10^{-5}
genomic DSB/bp	3.3×10^{-8}	2.3×10^{-7}	4.5×10^{-7}
episomal to genomic ratio	33	30	24

[a] A comparison of episomal and genomic double-stranded damage was made at 10, 100, and 300 μg/mL BLM. The genomic double-stranded break frequency was calculated by multiplying the rad equivalent damage (750, 5150, and 10000 rad at 10, 100, and 300 μg/mL BLM, respectively) by 4.5×10^{-11} (Kohn et al., 1976; Bradley & Kohn, 1979). From the episomal damage shown in Figure 1, the double-stranded break frequency was calculated by dividing the percent of form III (1, 6, and 10%, respectively) by the number of base pairs in the episome (8794 bp). The potential for preferred double-stranded cleavage was expressed as a ratio of episomal to genomic damage.

at 5 μg/mL BLM and increase up to 100 μg/mL (Figure 4A). Double-stranded breaks are detectable at 20 μg/mL and increase up to 400 μg/mL (Figure 4b). For chromosomal 935.1 DNA, the ratio of double- to single-stranded breaks of genomic DNA is about 1:5, which is similar to the ratio previously reported in L1210 cells (Bradley & Kohn, 1979) and suggests that the chromosomal DNA of 935.1 cells does not show unusual sensitivity to bleomycin. On the assumption that, like X-ray, BLM cleaves chromosomal DNA randomly, rad equivalent data were converted to genomic break frequencies by using the conversion factor of 0.9×10^{-9} SSB rad^{-1} nt^{-1} or 4.5×10^{-11} DSB rad^{-1} bp^{-1} for single- and double-stranded DNA breaks, respectively. Episomal damage was converted to break frequencies from data obtained by Southern blots of actual episomal cleavage. The calculations of SSB/nt or DSB/bp at three different concentrations of BLM are presented in Tables I and II. The ratios of episomal to genomic strand breaks between 10 and 1000 μg/mL BLM indicate that the drug shows a significant degree of preference (~20–40-fold) in damaging episomal DNA (Tables I and II). The data for BLM-induced double-stranded breaks are also consistent with data obtained with m-AMSA where a 30–40-fold sensitivity for episomal DNA was also found (Cullinan & Beerman, 1989).

In Vivo Investigations of BLM Action on Episomal DNA. The finding that the episome was a sensitive target for BLM under in vitro conditions encouraged an evaluation of BLM-induced damage in vivo. Effects of single iv doses of BLM on the episome were observed in 935.1 tumors[2] in vivo (Figure 5). On the basis of earlier therapeutic efficacy studies

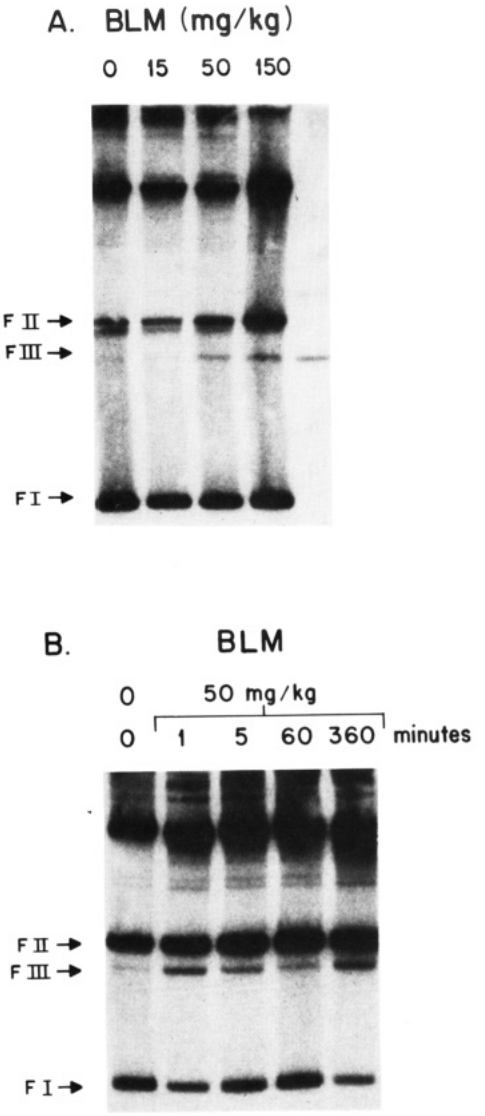

FIGURE 5: BLM cleavage of episomes in 935.1 solid tumors; Southern blot analysis of tumor DNA isolated from nude mice treated with BLM. Blots were hybridized to labeled pM19 probe. (A) nude mice were given iv injections of the indicated BLM doses. After 60 min, animals were sacrificed by cervical dislocation and tumor DNA was isolated as described under Materials and Methods. The data are representive of at least two independent experiments. (B) Tumor-bearing mice were injected with 50 mg/kg BLM and sacrificed at the times indicated, and tumor DNA was isolated immediately.

(Schurig et al., 1984), the doses of drug administered to nude mice were between 15 and 150 mg/kg (well below the lethal dose that kills 50% of the mice). BLM cleavage activity in 935.1 tumors is revealed as an increase in nicked circular and linear molecules and a decrease in supercoiled episomes (Figure 5A). At 15 mg/kg BLM, there is little damage beyond control levels. However, the episome is clearly sensitive to BLM cleavage at 50 and 150 mg/kg. There are 4%, 8%, and 15% reductions in supercoiled episomes at 15, 50, and 150 mg/kg BLM, respectively, with concomitant increases in nicked circular episomes. In addition, linear episomal molecules accumulate to 1, 3, and 4% at 15, 50, and 150 mg/kg BLM, respectively. Damage to the episome was also detected within this dose range by intraperitoneal delivery of BLM (data not shown). Tallysomycin S10b was also able to cleave episomal DNA when given iv to tumor-bearing mice (Beerman and Gawron, unpublished observation).

The time dependence of bleomycin-induced cleavage in tumors was measured (Figure 5B). Like the onset of damage

[2] Initial attempts to grow 935.1 cells as subcutaneous tumors in a syngenic RIII mouse line failed (Cullinan, Rustum, and Beerman, unpublished observation). Successful growth of the 935.1 cells as a fibrosarcoma was accomplished with use of NIH Swiss Taconic nude mice.

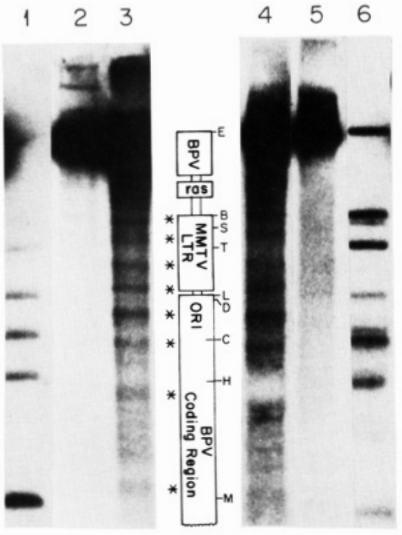

FIGURE 6: Preferential cleavage sites on episomes in 935.1 tumors and cultured cells detected by indirect end-labeling. Lanes 2 and 3 contain DNA digested with *Eco*RI after isolation from tumors of mice treated with vehicle alone (lane 2) or treated with 50 mg/kg BLM (lane 3). Lanes 4 and 5 contain DNA digested with *Eco*RI after isolation from cultured 935.1 cells either treated with 100 μg/mL BLM (lane 4) or untreated (lane 5). Lanes 1 and 6 contain purified episomal DNA digested with *Eco*RI and other restriction enzymes to yield fragments of known size that define the regulatory regions of the episome. The Southern blots were hybridized to radiolabeled *Eco*RI–*Sma*I BPV fragments. The center map denotes the area of the episome studied. Restriction enzyme sites that define regulatory regions are defined by E (*Eco*RI), B (*Bam*HI), S (*Sac*I), T (*Stu*I), L (*Sal*I), D (*Hin*dIII), C (*Cla*I), H (*Hpa*I), and M (*Sma*I). The stars indicate regions that are cleaved by BLM in both cells and tumors.

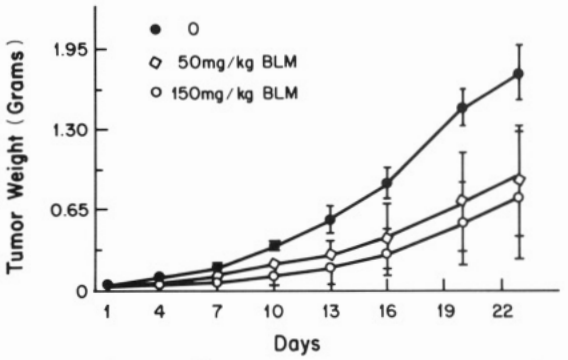

FIGURE 7: Antitumor efficacy of BLM on the 935.1 tumor in nude mice. The antitumor activity of single iv doses of 50 and 150 mg/kg BLM is plotted as a function of tumor growth. Tumor-bearing mice were treated with the indicated doses of BLM or saline (day 1). Tumors were measured daily. Each data point represents the mean ± SE of six tumors.

in cultured 935.1 cells, episomal DNA damage in tumors occurs rapidly. At 50 mg/kg BLM, cleavage occurs as early as 1 min and is maintained for at least 360 min postinjection (Figure 5B).

The active chromatin regions of episomal DNA were examined by the indirect end-labeling method (Wu, 1980), which enabled sites of specific BLM-induced DNA damage in solid tumors to be examined for the first time (Figure 6). Strongest cleavage (denoted by stars) is observed within the origin of replication. In addition, four double-stranded breaks also occur within the MMTV LTR. Most notably, the cleavage sites induced by BLM on episomal DNA from 935.1 cells and tumors are identical, suggesting strongly that BLM alone induces the breaks. Furthermore, in BLM-treated cells repair of specific double-stranded episomal breaks occurs rapidly and uniformly upon removal of the drug (data not shown).

Antitumor Efficacy. Experiments were conducted to determine whether cleavage activity in vivo reflected antitumor efficacy. These trials were designed to evaluate both antitumor and episomal cleavage activities at the same BLM doses. A single iv dose schedule of BLM at 50, 100 (not shown), and 150 mg/kg results in statistically significantly therapeutic responses of the tumors in treated mice (Figure 7). For example, on day 20, the tumor growth inhibition (weight of treated tumor/weight of control tumor) is 48% for 50 mg/kg BLM and 37% for 150 mg/kg. Therefore, significant tumor growth inhibition occurs at doses that induce detectable single- and double-stranded breaks to the episome.

Discussion

The episomal element contained in the 935.1 cell line has allowed for the first time an evaluation of the mode of action of a DNA strand scission agent on a common experimental target in both cultured cells and solid tumors. In a previous study using cultured 935.1 cells, we found that episomes sustained readily detectable damage when cells were treated with either of the topoisomerase II inhibitors VM26 or m-AMSA (Cullinan & Beerman, 1989). What was not apparent was whether bleomycin, which directly damages DNA, would produce detectable damage of a DNA target that makes up less than 0.01% of the genome. Our results demonstrate that the episome is very sensitive to the action of bleomycin and that both single- and double-stranded breaks are detected at cytotoxic drug concentrations of 20 μg/mL (Figure 1). At drug concentrations where the single-stranded breaks would average one per episome (10 μg/mL), the ratio of double- to single-stranded breaks is 1:6. The fact that this ratio is significantly greater than if the breaks occur randomly (1:23) is consistent with the finding that damage of this extrachromosomal target occurs with preference for certain regions of the episome. It is also known that bleomycin cleavage of naked DNA occurs nonrandomly, yielding a ratio of double-stranded to single-stranded breaks of 1:10 (Povirk et al., 1977). That our calculated ratio is somewhat higher is not surprising since it is known that when the experimental target possesses a nucleosomal structure there is an enhancement of the ratio of double- to single-stranded breaks (Grimwade & Beerman, 1986).

Direct evidence that bleomycin damage is occurring nonrandomly is demonstrated in Figure 6, which shows that there are multiple preferential cleavage sites, particularly at the origin of replication and the MMTV LTR regions. Two topoisomerase II directed agents also showed a strong tendency to damage these regions of the episome although the cleavage patterns were different (Cullinan & Beerman, 1989). Another study from our laboratory showed that bleomycin preferentially cut an integrated MMTV LTR sequence (Beckmann et al., 1987). These studies, as well as those by Kuo (1981), suggest that regions of chromatin that may be actively transcribing and thus sensitive to DNase I digestion are often also especially vulnerable to the action of bleomycin.

It is likely that the drug's targeting of active or potentially active chromatin contributes to the observed 30–40-fold enhancement of both single- and double-stranded breaks of the episomal compared to the total genomic DNA (Tables I and II). That additional factors are involved is evidenced by observations that while VM26 and m-AMSA preferentially damage episomes within promoter regions, only m-AMSA displays an enhanced preference to cleave the episomal compared to the genomic DNA (Cullinan & Beerman, 1989). The preference for bleomycin-induced single-stranded breaks of

episomal DNA determined at low rad equivalent doses (500 rad) is paralleled by the observation that irradiation itself shows enhanced damage (15-fold) of episomal DNA. In addition, the amounts of episomal cleavage are unchanged regardless of whether or not the MMTV promoter has been activated by glucocorticoids. However, the hormone-independent BPV promoter may have an influence on the MMTV LTR and may mask potential differences in drug-induced damage observed in the presence and absence of dexamethasone (Ostrowski et al., 1983). There also appears to be little difference between the rate of drug-induced damage and the rapid repair of genomic and episomal DNA that could account for the greater damage to the episomes. We are currently evaluating a broader spectrum of agents to determine whether other drugs have a strong preference for active chromatin domains.

By adopting a DNA-isolation procedure that minimizes spontaneous cleavage and enzymatic repair, damage of the episome was detected in vivo at doses as low as 15 mg/kg BLM. BLM-induced cleavage of the episome occurs very rapidly in solid tumors and in cells and thus resembles the characteristics of genomic DNA damage in vitro (Miyaki et al., 1973; Saito & Andoh, 1973; Iqbal et al., 1976; Hurt et al., 1981), as well as in a recent study of BLM-induced DNA damage of lung fibroblasts in vivo (Harrison et al., 1989). Although double-stranded breaks are rapidly repaired when BLM is withdrawn from the 935.1 culture medium, it is unlikely that the episomal lesions apparent in 935.1 tumors are repaired during the tumor procurement or DNA purification because they are done at extremely cold temperatures. However, failure to suppress rapid repair could explain why BLM-induced cleavage had previously remained undetectable in vivo (Cox et al., 1974). Additional evidence for the conclusion that BLM cleaves episomal DNA in vivo is the finding that the limited number of sites that are strongly preferred for BLM cleavage are identical on episomal DNA of both cells and solid tumors.

Episomal damage in intact cells is readily detected at cytotoxic drug levels. In mice, episomal damage is seen at doses that produce a therapeutic effect. While it is certain that the episome is responsible for the neoplastic phenotype, further studies are needed to determine whether episomal damage reflects or contributes to therapeutic efficacy. Our work indicates that DNA-reactive agents can be targeted to episomes and that a simplified model system may contribute to a rational therapeutic approach based on selective cleavage of extrachromosomal DNA. Currently, we are investigating whether antineoplastic agents induce preferential damage to those naturally occurring episomes frequently found in tumor cells (Von Hoff et al., 1988; Ruiz et al., 1989).

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