Anthracyclines induce double-strand DNA breaks at active gene promoters

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ABSTRACT

Doxorubicin is a widely used chemotherapeutic drug that intercalates between DNA base-pairs and poisons Topoisomerase II, although the mechanistic basis for cell killing remains speculative. Doxorubicin and related anthracycline compounds have been shown to increase nucleosome turnover and/or eviction around promoters, which suggests that the resulting enhanced exposure of DNA might underlie cell killing. Previously, we showed that low doses of anthracyclines increase nucleosome turnover around active gene promoters, which suggests that loss of nucleosomes might contribute to cancer cell killing. Here we apply a genome-wide method to precisely map DNA double-strand breaks (DSBs) in cancer cells. We find that spontaneous DSBs occur preferentially around promoters of active genes, and that both anthracyclines and etoposide, a Topoisomerase II poison, increase DSBs around promoters, although CpG islands are conspicuously protected from DSBs. We propose that torsion-based enhancement of nucleosome turnover by anthracyclines exposes promoter DNA, ultimately causing DSBs around promoters.

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1. Introduction

Doxorubicin (also called Adriamycin) is one of the most effective anti-cancer compounds, although exactly how it kills dividing cells has been a matter of debate [1,2]. Doxorubicin and related anthracyclines consist of flat aromatic moieties that intercalate between DNA bases, each anchored tightly by one or more sugars in the minor groove [3]. Intercalation pushes apart the neighboring bases, which results in bidirectional transmission of positive torsion [3]. The resulting alterations in DNA structure can inhibit enzymes, including topoisomerases [4,5]. Doxorubicin can also trap Topoisomerase II (TopoII) in the double-strand cleavage form and prevent ligation, and so one model for cell killing is the direct introduction of a double-strand break (DSB) caused by TopoII poisoning [4]. However, whether the primary anti-cancer action of Doxorubicin is by trapping TopoII in its double-strand cleaved form, or by inhibiting TopoII with the consequent failure to relieve the positive torsion, or by some other mechanism, is uncertain.

We previously showed that sublethal doses of Doxorubicin (<0.5 μM) nevertheless enhance nucleosome turnover around promoters in mouse squamous cell carcinoma (SCC) cell lines [6], raising the possibility that cell killing at chemotherapeutic doses is a downstream consequence of the increased exposure of DNA when nucleosomes are disrupted. Indeed, Pang et al. [7] showed that histones were evicted around promoters using 9 μM of Doxorubicin or a related anthracycline, Daunarubicin. In both studies, Aclarubicin, an anti-cancer anthracycline compound that does not poison TopoII, likewise evicted nucleosomes around promoters at similar doses. Etoposide, a TopoII poison that does not intercalate into DNA, but rather covalently traps TopoII preferentially at induced DNA single-strand breaks [8], did not evict histones at therapeutic doses [7]. Taken together, these observations suggest that anthracycline intercalation enhances nucleosome depletion around promoters, perhaps by increasing torsion [2].

If anthracycline drugs kill cancer cells by their preferential action at mammalian promoters, then we might expect them to also cause DSBs at promoters. Here, we tested this hypothesis by applying a genome-wide method for sensitive detection of DSBs. Consistent with this prediction, we find that regions around active promoters are hotspots for DSBs caused by Doxorubicin, Aclarubicin and Etoposide.

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2. Materials and methods

2.1. Tissue culture, drug treatment and lysis

Mouse squamous cell carcinoma cell line MSCC-CK1 [6] was cultured in Dulbecco's Modified Eagle Medium (DMEM) media (Cat# 11965-092, Invitrogen) with 10% fetal bovine serum and 1X Antibiotic–Antimycotic at 37 °C. For drug treatment, MSCC-CK1 cells were treated with the indicated drugs for 24 h to maximize turnover with minimal toxicity based on our published finding that nucleosome turnover around promoters increases gradually up to 24 h when using low concentrations of anthracyclines [6]. We chose a clinically relevant concentration of 3 μM Doxorubicin based on two published sources: Namur et al. [9] who measured liver tissue dosages around drug-eluting beads in capillaries at 5 μM, and Goodman and Gilman's Pharmacological Basis of Therapeutics [10] which listed 1.7 μM as the peak plasma concentration during standard chemotherapy. Our high concentration of 3 μM was chosen to lie in between these two values. During chemotherapy, plasma concentration rapidly declines over 24 h to less than 0.05 μM. Therefore, our low concentration of 0.3 μM can be considered low relative to either high-dose standard but is equivalent to concentrations used in other in vitro experiments (e.g., [11]). As 0.3 μM anthracycline does not induce significant toxicity, these low-concentration experiments probably underestimate the degree of DSBs that occur during chemotherapy.

2.2. BLESS

BLESS (direct in situ breaks labeling, enrichment on streptavidin and next-generation sequencing) was performed as described by Crosetto et al. [12]. Drug-treated or untreated mouse SCC cells were cross-linked for 30 min at room temperature with 2% formaldehyde, followed by 5 min incubation with 125 mM glycine. The cells were then incubated with lysis buffer [10 mM Tris–HCl, 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2% NP-40, 1 mM DTT, and protease inhibitors (Roche cOmplete ULTRA)] for 90 min at 4 °C and nuclear break buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.3% SDS, and 1 mM DTT) for 45 min at 37 °C for nuclei isolation.

For yeast BLESS, yeast cells (W303) were grown in YPD and harvested at $OD_{600} = 0.6$–0.8. The cells were cross-linked and quenched as described above and yeast nuclei were isolation as previously described [13].

The mouse nuclei or yeast nuclei were quickly digested by proteinase K (100 μg/ml) at 37 °C for 4 min, and then washed twice with NEB Restriction Buffer 2 and once with quick blunting buffer followed by end polishing using the quick blunting kit (New England Biolabs). The nuclei were then washed and subject to ligation with biotin labeled proximal linker or non-biotin labeled proximal linker at 16 °C for 18 h. After ligation, genomic DNA was isolated and digested with HaeIII, followed by streptavidin bead capture, ligation of distal linker, digestion with I-SceI, PCR amplification, and XhoI digestion before Illumina sequencing. Illumina libraries were prepared as previously described [14]. For quantitative analysis (Fig. 4B), the same volume of mouse BLESS sample was mixed with 6 ng yeast BLESS DNA followed by library preparation as described [14]. The samples were sequenced using Illumina 50-bp paired-end sequencing.

2.3. Data analysis

After Illumina paired-end sequencing, each read in a pair was treated separately. Adaptors were removed using the program trim_galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Then, those reads containing the sequence 5'-TCGAGGTAGTA-3' derived from the BLESS first hairpin adapter sequence were selected and the sequence following it was retained. The selected reads were mapped as single-end reads against the MM9 release of the mouse genome using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). For the mapped reads, the cleavage site was determined as the first base pair for reads mapped to the forward strand and the last base pair for reads mapped to the reverse strand. Cleavage points were normalized by first aggregating them into 25-bp intervals and then multiplying the fraction of total counts in each interval by the size of the mouse genome. Ends analysis, k-mean clustering, and heat map construction were performed as previously described [6]. CpG island coordinates from Mus musculus MM9 were downloaded from the UCSC Genome table (mm9_cpgisland.bed.gz).

For the spike-in samples, selected reads were also mapped to yeast release sacCer3 and yeast-specific cleavage points from all samples were pooled before normalized counts were calculated using the yeast genome size. The greater yield of DNA for the more heavily cleaved drug-treated mouse samples will result in proportionally fewer yeast reads than in the untreated control. Multiplication by the spike-in untreated/treated ratio corrects for the fact that normalization equalizes the genome-wide average. Accordingly, to calibrate a mouse sample, each 25-bp interval value was multiplied by the total number of cleavage points over the yeast genome for the untreated mouse sample and divided by that for the drug-treated sample.

3. Results

3.1. Spontaneous DSBs occur preferentially around promoters

Control cells and cells treated for 24 h with anthracyclines or Etoposide were fixed with formaldehyde to stabilize chromatin and prevent artificial DSBs. The BLESS (direct in situ Breaks Labeling, Enrichment on Streptavidin and next-generation Sequencing) method was used for mapping DNA DSBs genome-wide. After brief Proteinase K digestion, ends were repaired and ligated to a biotinylated hairpin linker in situ, total DNA was extracted, cleaved with a 4-cutter restriction enzyme, and the new ends were ligated to a second hairpin linker. Cleavage of restriction
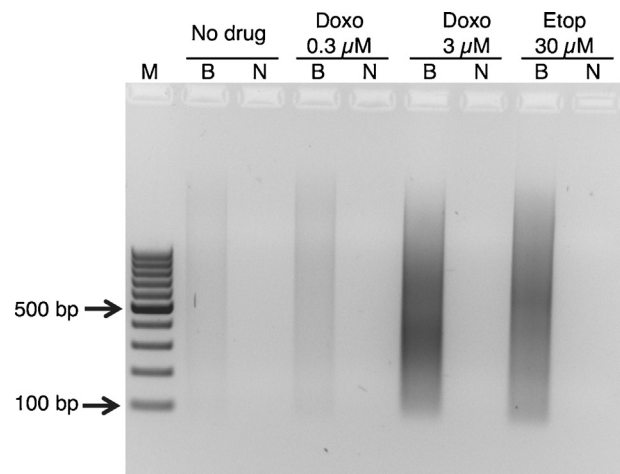

Fig. 1. Doxorubicin and Etoposide cause DNA double-stand breaks in mouse SCC cells. Agarose gel electrophoresis of BLESS fragments from a representative experiment is shown, where equal aliquots of DNA were loaded from samples prepared in parallel. BLESS DNA library samples derived from mouse SCC cells before and after Doxorubicin (Doxo) or Etoposide (Etop) treatment. M, 100 bp ladder DNA marker; B, biotin-labeled linker; N, non-biotin linker.