Cobalt Enhances DNA Cleavage Mediated by Human Topoisomerase IIα *in Vitro* and in Cultured Cells†

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ABSTRACT: Although cobalt is an essential trace element for humans, the metal is genotoxic and mutagenic at higher concentrations. Treatment of cells with cobalt generates DNA strand breaks and covalent protein−DNA complexes. However, the basis for these effects is not well understood. Since the toxic events induced by cobalt resemble those of topoisomerase II poisons, the effect of the metal on human topoisomerase IIα was examined. The level of enzyme-mediated DNA scission increased 6−13-fold when cobalt(II) replaced magnesium(II) in cleavage reactions. Cobalt(II) stimulated cleavage at all DNA sites observed in the presence of magnesium(II), and the enzyme cut DNA at several "cobalt-specific" sites. The increased level of DNA cleavage in the presence of cobalt(II) was partially due to a decrease in the rate of enzyme-mediated religation. Topoisomerase IIα retained many of its catalytic properties in reactions that included cobalt(II), including sensitivity to the anticancer drug etoposide and the ability to relax and decatenate DNA. Finally, cobalt(II) stimulated topoisomerase IIα-mediated DNA cleavage in the presence of magnesium(II) in purified systems and in human MCF-7 cells. These findings demonstrate that cobalt(II) is a topoisomerase II poison *in vitro* and in cultured cells and suggest that at least some of the genotoxic effects of the metal are mediated through topoisomerase IIα.

Although cobalt is an essential trace element necessary for the formation of vitamin B_{12} in humans, the metal also has been classified as a probable carcinogen based on experimental animal studies (*1*). Furthermore, at higher concentrations, it is genotoxic and mutagenic to mammalian cells (*2*−*4*).

Treatment of cultured cells with cobalt generates DNA strand breaks (*5*), induces the formation of covalent protein−DNA complexes (*6*), and triggers recombination pathways (*7*). The basis for cobalt genotoxicity is not fully understood; however, two general mechanisms have been proposed (*2*−*4*). First, since cobalt can enhance the production of reactive oxygen species in the presence of H_2O_2, it has been suggested that the toxic effects of the metal are caused by the accumulation of oxidative lesions in the genome (*8*, *9*). Second, since replacement of active site divalent cations with cobalt can lead to inactivation of metal-containing enzymes and zinc finger proteins, it has been postulated that cobalt toxicity results from inactivation of DNA repair or signal transduction pathways (*10*). In support of this latter suggestion, treatment of cells with the metal inhibits the repair of DNA lesions generated by ultraviolet radiation (*11*, *12*).

Clearly, multiple modalities may be contributing to the genotoxic effects of cobalt. Therefore, additional mechanisms for the actions of the metal may need to be considered. To this point, it is notable that the generation of DNA strand breaks and covalent protein−DNA complexes are hallmarks of topoisomerase II poisons (*13*−*16*).

Topoisomerase II is a ubiquitous enzyme that alleviates DNA over- and underwinding and resolves knots and tangles in the genetic material (*17*, *18*). It is required for DNA replication and recombination, as well as chromosome structure and segregation (*13*, *17*, *19*−*21*). Lower eukaryotes, such as yeast and *Drosophila*, contain only a single form of topoisomerase II (*22*, *23*). Alternatively, vertebrates contain two isoforms of the enzyme, topoisomerase IIα and topoisomerase *β* (*24*, *25*). Topoisomerase IIα levels increase dramatically during periods of cell growth, and this isoform appears to be primarily responsible for the required roles of the enzyme during mitosis (*26*−*28*).

Topoisomerase II carries out its essential functions by passing an intact double helix through a transient double-stranded DNA break that it generates in a separate segment of DNA (*13*, *16*, *19*, *29*, *30*). To maintain the integrity of the genome during the DNA strand passage event, topoisomerase II forms covalent bonds between its active site tyrosyl residues and the newly created 5′-termini of the cleaved double helix (*31*−*33*). This covalent enzyme−DNA complex is termed the topoisomerase II *cleavage complex*. Cleavage complexes normally are present at very low steady-state concentrations and are tolerated by the cell. However, conditions that significantly increase the concentration of these covalent enzyme−DNA complexes generate permanent

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double-stranded breaks in the genetic material, trigger recombination events, and lead to the formation of chromosome aberrations (*13*, *14*, *16*, *30*, *34*, *35*). When topoisomerase II-generated DNA breaks overwhelm the cell, they induce death pathways (*14*, *35*).

Topoisomerase II poisons increase the concentration of cleavage complexes and convert topoisomerase II from an essential enzyme to a potent cellular toxin that generates strand breaks in the genetic material (*13*−*16*, *30*). Some of the most well studied topoisomerase II poisons, including etoposide, mitoxantrone, and doxorubicin, are among the most active anticancer drugs in clinical use (*36*). Despite their therapeutic value, these agents (as well as naturally occurring topoisomerase II-active compounds, such as bioflavonoids) have been linked to the generation of specific leukemias (*37*−*41*). Finally, several different DNA lesions are position-specific topoisomerase II poisons (*42*). For example, abasic sites enhance enzyme-mediated DNA scission with a potency that is ~1000 times greater than that of etoposide (*43*−*45*).

Topoisomerase II requires a divalent cation to carry out its DNA cleavage reaction (*31*, *32*, *46*). Although magnesium(II) is believed to fulfill this role in the cell, other divalent cations can substitute *in vitro*. For example, previous studies with *Drosophila* topoisomerase II indicate that the level of DNA cleavage is higher in the presence of calcium(II) (*47*). Since cobalt generates DNA strand breaks and covalent protein−DNA complexes in treated mammalian cells (*5*, *6*), the effect of cobalt(II) on human topoisomerase IIα was examined. Results indicate that levels of enzyme-mediated DNA cleavage increased 6−13-fold when magnesium(II) was replaced by cobalt(II). Furthermore, the cellular concentration of topoisomerase IIα−DNA cleavage complexes rose when human cells were treated with the metal ion. These findings indicate that cobalt(II) acts as a topoisomerase II poison *in vitro* and in cultured human cells. They further suggest that at least some of the genotoxic effects of cobalt are mediated through topoisomerase IIα.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Wild-type human topoisomerase IIα and topoisomerase II*β* and *Saccharomyces cerevisiae* topoisomerase II were expressed in *S. cerevisiae* (*48*) and purified as described previously (*49*, *50*). Negatively supercoiled pBR322 DNA was prepared using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. Cobalt(II) (as $CoCl_2$) was obtained from Fisher Scientific. All divalent cations were ACS certified grade, and were prepared as 1 M chloride stocks and stored at room temperature. Etoposide (Sigma) was prepared as a 20 mM stock solution in 100% DMSO and stored at room temperature.

DNA Cleavage. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff (*51*). For assays that utilized human type II topoisomerases, each reaction mixture contained 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of human topoisomerase II reaction buffer [10 mM Tris-HCl (pH 7.9), 100 mM KCl, and 2.5% glycerol]. For assays that utilized yeast topoisomerase II, each reaction mixture contained 10 nM negatively supercoiled pBR322 DNA in a total of 20 μL of 10 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10% DMSO, and 2.5% glycerol. Reaction mixtures contained 1 or 5 mM divalent cation ($MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $CdCl_2$, $BaCl_2$, $SrCl_2$, $CuCl_2$, or $ZnCl_2$) as indicated in the figures. Reactions that included etoposide utilized 10 nM pBR322 as the DNA substrate. DNA cleavage was initiated by the addition of enzyme (final concentration of 220 nM), and mixtures were incubated for 6 min at 37 °C (human) or 28 °C (yeast). Enzyme−DNA cleavage intermediates were trapped by adding 2 μL of 5% SDS and 1 μL of 375 mM EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45 °C to digest the type II enzyme. Samples were mixed with 2 μL of agarose gel loading buffer [60% sucrose in 10 mM Tris-HCl (pH 7.9)], heated for 15 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. Cleavage was monitored by the conversion of negatively supercoiled plasmid DNA to linear molecules. DNA bands were visualized by ultraviolet light and quantitated using an Alpha Innotech digital imaging system.

In reactions that determined whether DNA cleavage by human topoisomerase IIα was reversible, NaCl (an additional final concentration of 250 mM) or EDTA (final concentration of 18 mM, in lieu of post-SDS addition) was added prior to treatment with SDS. To determine whether cleavage was protein-linked, proteinase K treatment was omitted.

Site-Specific DNA Cleavage. DNA cleavage sites were mapped using a modification of the procedure of O'Reilly and Kreuzer (*52*). The pBR322 DNA substrate was linearized by treatment with *Hin*dIII. Terminal 5′-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The DNA was treated with *Eco*RI, and the 4332 bp singly end labeled fragment was purified from the small *Eco*RI−*Hin*dIII fragment by passage through a CHROMA SPIN+TE-100 column (Clontech).

Site-specific DNA cleavage assays were based on the procedure of Burden *et al*. (*53*) and included 0.7 nM labeled pBR322 DNA substrate and 90 nM human topoisomerase IIα in 50 μL of human topoisomerase II reaction buffer that contained 1 or 5 mM $MgCl_2$ or $CoCl_2$. Reactions were started by the addition of topoisomerase IIα, and mixtures were incubated for 6 min at 37 °C. Cleavage intermediates were trapped by adding 5 μL of 5% SDS followed by 3.75 μL of 250 mM EDTA (pH 8.0). Topoisomerase IIα was digested with proteinase K (5 μL of a 0.8 mg/mL solution) for 30 min at 45 °C. DNA products were precipitated twice in 100% ethanol, washed in 70% ethanol, dried, and resuspended in 6 μL of 40% formamide, 10 mM NaOH, 0.02% xylene cyanole FF, and 0.02% bromophenol blue. Samples were subjected to electrophoresis in a denaturing 6% polyacrylamide sequencing gel in TBE buffer [100 mM Tris-borate (pH 8.3) and 2 mM EDTA]. The gel was fixed in a 10% methanol/10% acetic acid mixture for 2 min and dried. DNA cleavage products were analyzed on a Bio-Rad Molecular Imager FX.

DNA Religation. DNA religation mediated by human topoisomerase IIα was monitored as described previously (*54*). DNA cleavage−religation equilibria were established for 6 min at 37 °C as described above in the presence of 5

mM $MgCl_2$ or 1 mM $CoCl_2$. Religation was initiated by shifting samples from 37 to 0 °C. Reactions were stopped at various time points up to 60 s by the addition of 2 μL of 5% SDS followed by 1 μL of 375 mM EDTA (pH 8.0). Samples were treated with proteinase K and analyzed as described for DNA cleavage reactions. Religation was monitored by the loss of linear DNA over time.

DNA Relaxation. DNA relaxation assays were based on the procedure of Fortune and Osheroff (*51*). Reaction mixtures contained 2.5 nM human topoisomerase IIα and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of human topoisomerase II reaction buffer containing 1 mM ATP and either 1 mM $CoCl_2$ or 5 mM $MgCl_2$. Reaction mixtures were incubated at 37 °C, and DNA relaxation was stopped at various time points up to 15 min by the addition of 3 μL of 0.77% SDS, 77 mM NaEDTA (pH 8.0). Samples were mixed with agarose gel loading buffer and subjected to electrophoresis in a 1% agarose gel in TBE buffer. Gels were stained with 1 μg/mL ethidium bromide, and DNA bands were visualized with ultraviolet light and analyzed as described above.

Decatenation of Kinetoplast DNA. Decatenation assays were performed as described previously (*55*). Reaction mixtures contained 20 nM human topoisomerase IIα and 0.3 μg of kinetoplast DNA in a total of 20 μL of 10 mM Tris-HCl (pH 7.9), 175 mM KCl, 2.5% glycerol, 1 mM ATP, and either 1 mM $CoCl_2$ or 5 mM $MgCl_2$. Decatenation was carried out at 37 °C for 15 min. Reactions were stopped and products analyzed as described above for DNA relaxation.

Topoisomerase II-Mediated DNA Cleavage in MCF-7 Cells. MCF-7 human breast adenocarcinoma cells were cultured under 5% CO_2 at 37 °C in RPMI 1640 medium (Cellgro by Mediatech, Inc.), containing 10% heat-inactivated fetal calf serum (Hyclone) and 2 mM glutamine (Cellgro by Mediatech, Inc.). The *in vivo* complex of enzyme bioassay (*54*, *56*) (as modified on the TopoGEN, Inc., Web site) was employed to determine the ability of cobalt(II) to induce topoisomerase II-mediated DNA breaks in treated MCF-7 cells. Briefly, exponentially growing cultures were treated with 200 μM $CoCl_2$ for 24 h. Alternatively, cells were treated with 100 μM etoposide for 1 h for comparison. Cells ($\sim 4 \times 10^6$) were harvested by centrifugation and lysed by the immediate addition of 3 mL of 1% sarkosyl. Following gentle douncing, cell lysates were layered onto a 2 mL cushion of CsCl (1.5 g/mL) and centrifuged at 42 000 rpm for 20 h at 20 °C. DNA pellets were isolated, resuspended in 5 mM Tris-HCl (pH 8.0) and 0.5 mM EDTA, and blotted onto nitrocellulose membranes using a Schleicher and Schuell slot blot apparatus. Covalent complexes formed between DNA and topoisomerase IIα were detected using a polyclonal antibody directed against human topoisomerase IIα (Kiamaya Biochemical Co.) at a 1:2000 dilution.

RESULTS

Cobalt(II) Increases the Level of DNA Cleavage Mediated by Human Topoisomerase IIα. The genotoxic effects of cobalt, including the generation of DNA strand breaks and covalent protein−DNA complexes (*5*, *6*), are characteristic of topoisomerase II poisons (*13*). Therefore, the effect of cobalt(II) on DNA cleavage mediated by human topoisomerase IIα was examined and compared to that of several other divalent cations.

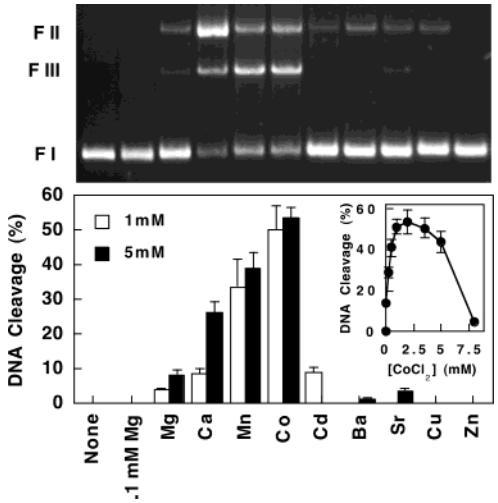

FIGURE 1: Effect of divalent cations on DNA cleavage mediated by human topoisomerase IIα. The ability of different divalent cations to support DNA cleavage mediated by human topoisomerase IIα was assessed. An agarose gel of DNA cleavage reactions carried out at 5 mM divalent cation is shown at the top. The mobilities of negatively supercoiled DNA (form I, FI), nicked circular plasmid (form II, FII), and linear molecules (form III, FIII) are indicated. Levels of DNA cleavage were quantified and expressed as the percentage of negatively supercoiled substrate that was converted to linear molecules (bottom). Assays were performed at 1 (□) or 5 mM (■) divalent cation. Error bars represent the standard error of the mean of four independent experiments. The inset shows DNA cleavage over a range of $CoCl_2$ concentrations. Error bars represent the standard deviation of four independent experiments.

As seen in Figure 1, levels of DNA cleavage observed in the presence of $CoCl_2$ were higher than those seen in reactions that included equivalent concentrations of $MgCl_2$, $CaCl_2$, $MnCl_2$, $CdCl_2$, $BaCl_2$, $SrCl_2$, $CuCl_2$, or $ZnCl_2$. Cobalt(II) displayed maximal enhancement between 1 and 5 mM (inset). Over this range, levels of DNA cleavage were 6−13-fold higher than that observed in the presence of magnesium(II), the physiological divalent cation used by topoisomerase IIα.

Under conditions that support the production of reactive oxygen species, cobalt generates DNA damage (including strand breaks) in an enzyme-free manner (*8*, *57*−*59*). Therefore, a number of controls were carried out to ensure that the DNA cleavage seen in Figure 1 was mediated by topoisomerase IIα (Figure 2). First, no linear DNA was generated in the absence of enzyme. Second, all of the cleaved plasmid molecules were covalently attached to topoisomerase IIα, as determined by omitting proteinase K treatment. In the absence of protease digestion, the electrophoretic mobility of cleaved (i.e., linear, FIII) DNA decreased and the band broadened significantly (see the band near the origin). Third, cleavage was reversed when EDTA or salt was added to the reaction mixture prior to topoisomerase II denaturation with SDS. This reversibility is inconsistent with an enzyme-free reaction and demonstrates that cobalt(II) can support both DNA cleavage and religation mediated by human topoisomerase IIα.

DNA cleavage observed in reactions that included $CoCl_2$ cannot be explained by the presence of contaminating magnesium(II). The $CoCl_2$ preparation that was used contained less than 0.005% magnesium(II) (as reported by the

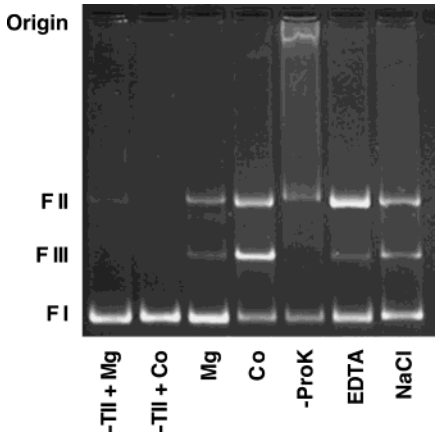

FIGURE 2: DNA cleavage in the presence of cobalt(II) is mediated by topoisomerase IIα. The ability of human topoisomerase IIα to cleave pBR322 in the presence of $CoCl_2$ was assessed. An agarose gel of the DNA cleavage reactions is shown. DNA controls in the presence of 5 mM $MgCl_2$ (−TII + Mg) or 5 mM $CoCl_2$ (−TII + Co) in the absence of the enzyme are shown. DNA cleavage mediated by human topoisomerase IIα in the presence of 5 mM $MgCl_2$ or 5 mM $CoCl_2$ was examined. To determine whether the DNA cleavage observed in the presence of cobalt(II) was protein-linked, proteinase K treatment was omitted (−ProK). The reversibility of reactions that included $CoCl_2$ was analyzed by adding EDTA or NaCl prior to SDS treatment. The positions of negatively supercoiled, nicked, and linear plasmid DNA are as in Figure 1. The position of the gel origin also is shown.

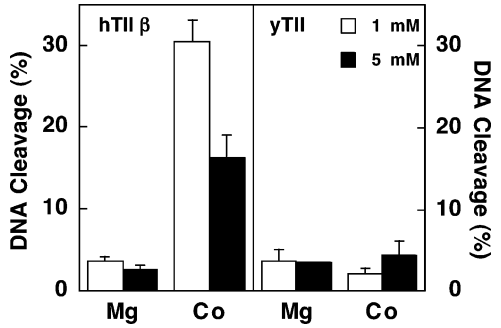

FIGURE 3: Ability of cobalt(II) to support DNA cleavage mediated by human topoisomerase IIβ or yeast topoisomerase II. The ability of human topoisomerase IIβ (hTIIβ) or yeast topoisomerase II (yTII) to cleave DNA in the presence of $CoCl_2$ was assessed. Levels of DNA cleavage were expressed as the percentage of negatively supercoiled substrate that was converted to linear molecules. Assays were performed at 1 (□) or 5 mM (■) divalent cation. Error bars represent the standard error of the mean of two independent experiments.

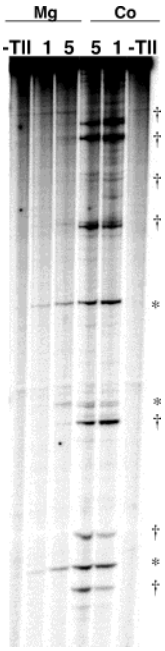

FIGURE 4: Cobalt(II) alters the DNA cleavage site utilization of human topoisomerase IIα. The DNA cleavage site specificity of human topoisomerase IIα was determined in the presence of $CoCl_2$. A polyacrylamide gel is shown. Cleavage reactions included 1 or 5 mM divalent cation. Control reaction mixtures contained the indicated divalent cation in the absence of human topoisomerase IIα (−TII). Sites that were significantly stimulated in the presence of cobalt(II) are denoted with asterisks. Cobalt-specific sites are labeled with daggers.

manufacturer), which would lead to the presence of <250 nM contaminant in reaction mixtures. As shown in Figure 1, 0.1 mM $MgCl_2$ does not sustain DNA cleavage mediated by the human enzyme. On the basis of these findings, it appears that cobalt(II) can substitute for magnesium(II) as the divalent cation used by the catalytic center of human topoisomerase IIα to mediate DNA cleavage and religation. Furthermore, cobalt(II) should be classified as a topoisomerase II poison, at least *in vitro*.

In addition to topoisomerase IIα, human cells contain a second isoform of the enzyme, topoisomerase IIβ. Topoisomerase IIβ appears to be present at low levels in most human cells, irrespective of their proliferative status (*28*, *60*−*62*). Similar to results with topoisomerase IIα, the level of DNA breaks generated by the β isoform increased 6−9-fold when $CoCl_2$ was substituted for $MgCl_2$ in cleavage assays (Figure 3). Conversely, the DNA cleavage activity of yeast topoisomerase II was not stimulated by the presence of $CoCl_2$.

Cobalt(II) Alters the DNA Cleavage Site Utilization of Human Topoisomerase IIα. While some topoisomerase II poisons act primarily by increasing the level of DNA cleavage at sites normally utilized by the enzyme, others work by creating novel sites of DNA scission (*13*, *30*, *63*). As shown in the DNA cleavage map in Figure 4, cobalt(II) appears to utilize both modes of action. While the divalent cation significantly stimulated DNA cleavage at all of the sites observed in the presence of magnesium(II) (marked with asterisks), several additional strong sites appeared in reactions that included $CoCl_2$ (marked with daggers). These "cobalt-specific" cleavage sites were not observed in the absence of topoisomerase IIα. Furthermore, they only appeared as very weak bands (if at all) in reactions that included $MgCl_2$ when DNA cleavage was carried out in the presence of a strong topoisomerase II poison such as etoposide or when autoradiograms were overexposed (not shown).

Effect of Cobalt(II) on DNA Religation Mediated by Human Topoisomerase IIα. Topoisomerase II poisons increase levels of enzyme-mediated DNA breaks by two nonmutually exclusive mechanisms (*13*, *16*, *30*). Some poisons, such as quinolones, genistein, and DNA lesions, appear to act primarily by enhancing the forward rate of DNA scission. Conversely, others such as etoposide and amsacrine appear to act primarily by inhibiting the ability of topoisomerase II to religate DNA breaks.

To determine the mechanistic basis for the actions of cobalt(II) against human topoisomerase IIα, the effect of the

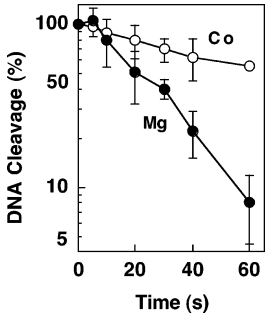

FIGURE 5: Rates of DNA religation mediated by human topoisomerase IIα are slower in the presence of cobalt(II). DNA religation mediated by human topoisomerase IIα was examined in the presence of 5 mM $MgCl_2$ (●) or 1 mM $CoCl_2$ (○). DNA cleavage−religation equilibria were established at 37 °C, and religation was initiated by shifting samples to 0 °C. The amount of DNA cleavage observed at equilibrium for each divalent cation was set to 100% at time zero. Error bars represent the standard deviation of four independent experiments.

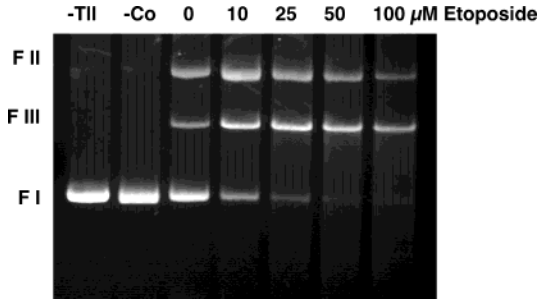

FIGURE 6: Etoposide increases the level of DNA cleavage mediated by human topoisomerase IIα in reaction mixtures that contain cobalt(II). An agarose gel of DNA cleavage reactions is shown. Reaction products from assays that omitted human topoisomerase IIα (−TII) or divalent cation (−Co) are shown as controls. Reactions were carried out in the presence of 0.5 mM $CoCl_2$ over an etoposide concentration range of 0−100 μM. The positions of negatively supercoiled, nicked, and linear plasmid DNA are as in Figure 1.

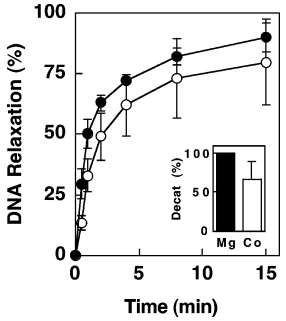

FIGURE 7: Cobalt(II) supports DNA relaxation and decatenation catalyzed by human topoisomerase IIα. DNA relaxation and decatenation (inset) reactions catalyzed by human topoisomerase IIα were carried out over 15 min in the presence of 5 mM $MgCl_2$ (● or ■) or 1 mM $CoCl_2$ (○ or □). DNA relaxation was quantified by the disappearance of the negatively supercoiled DNA substrate, and DNA decatenation was quantified by the appearance of monomeric circular molecules. Error bars represent the standard deviation of four independent experiments.

divalent cation on enzyme-mediated religation was examined (Figure 5). The rate of religation in the presence of $CoCl_2$ was ~3-fold slower than that observed in the presence of $MgCl_2$. Since this decrease cannot fully account for the 6−13-fold increase in the level of topoisomerase II−DNA cleavage complexes observed in the presence of $CoCl_2$ (see Figure 1), it suggests that cobalt(II) is both stimulating scission and inhibiting religation.

Human Topoisomerase IIα Retains Characteristic Catalytic Properties in the Presence of Cobalt(II). Although levels of topoisomerase II-mediated DNA cleavage are higher in the presence of $CoCl_2$ and the site utilization of the enzyme is altered, human topoisomerase IIα appears to retain many of its fundamental catalytic properties when cobalt(II) replaces magnesium(II) as the active site divalent cation. For example, cleaved DNA is enzyme-linked and cleavage complexes are reversible (see Figure 2). Therefore, two additional experiments were performed to determine whether topoisomerase IIα retains other characteristic catalytic properties in the presence of cobalt(II).

First, the human enzyme is highly sensitive to topoisomerase II poisons such as etoposide (*54*), an anticancer drug that is widely used for the treatment of human malignancies (*36*). This agent functions in the active site of the enzyme and strongly inhibits the ability of topoisomerase II to religate cleaved DNA molecules formed when magnesium(II) is used as the divalent cation (*54*). To determine whether topoisomerase IIα maintains its sensitivity toward etoposide when cobalt(II) is used as the divalent cation, the effect of the anticancer drug on DNA cleavage was assessed. As seen in Figure 6, the level of DNA breaks (monitored by the conversion of the negatively supercoiled plasmid to unit-length linear molecules) doubled when etoposide (≤25 μM) was added to reaction mixtures that contained $CoCl_2$. At higher drug concentrations (>25 μM), the negatively supercoiled plasmid was converted primarily to a DNA smear that contained shorter than unit-length linear molecules. This smear is indicative of multiple scission events per plasmid. In addition, levels of cleavage at the DNA bands marked in Figure 4 approximately doubled when 100 μM etoposide was added to cobalt-containing reaction mixtures (data not shown). These results demonstrate that human topoisomerase IIα maintains its sensitivity toward etoposide in the presence of cobalt(II).

Second, to determine whether cobalt(II) can support the overall catalytic activity of the human enzyme, DNA relaxation and decatenation assays were performed. Topoisomerase II requires a divalent cation at two separate catalytic centers, the DNA cleavage−religation center and the ATP binding−hydrolysis center. Overall catalytic activity requires that the divalent cation functions at both catalytic centers and supports coordination between the two. Under optimal conditions (5 mM $MgCl_2$ and 1 mM $CoCl_2$), cobalt(II) supported the DNA relaxation reaction of topoisomerase IIα. Rates of DNA relaxation in the presence of $CoCl_2$ were within a factor of 2 of those observed in reactions that included $MgCl_2$ (Figure 7). Similar results were seen in decatenation reactions (inset). These results indicate that cobalt(II) can support the overall catalytic activity of the human enzyme.

Cobalt(II) Increases the Level of DNA Cleavage Mediated by Human Topoisomerase IIα in the Presence of Magnesium(II). Previous studies indicate that cobalt can have multiple effects on cellular processes (*2*−*4*). To establish the potential for a direct effect of the metal ion on topoisomerase II in physiological systems, it is critical to determine whether cobalt(II) can alter the DNA cleavage−religation activity of

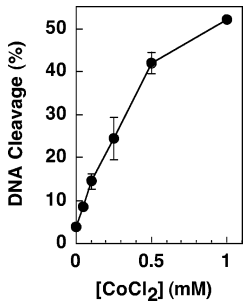

FIGURE 8: Cobalt(II) can compete with magnesium(II) as the divalent cation utilized by human topoisomerase IIα. To determine whether cobalt(II) can compete with magnesium(II) for the DNA cleavage−religation center of human topoisomerase IIα, DNA cleavage reactions were carried out in the presence of 1 mM $MgCl_2$ along with increasing concentrations of $CoCl_2$. Levels of DNA scission were expressed as the percentage of negatively supercoiled substrate that was converted to linear molecules. Error bars represent the standard error of the mean for two independent experiments.

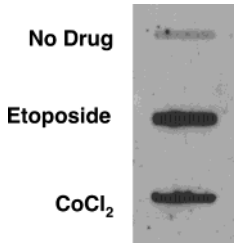

FIGURE 9: Cobalt(II) enhances DNA cleavage mediated by human topoisomerase IIα in treated human MCF-7 cells. The *in vivo* complex of enzyme bioassay was used to monitor the level of cleavage complexes in cells treated with $CoCl_2$. DNA (10 μg) from cultures treated with no drug, 100 μM etoposide, or 200 μM $CoCl_2$ was blotted onto nitrocellulose. Blots were probed with a polyclonal antibody directed against human topoisomerase IIα. Results are typical of two independent experiments.

the enzyme in the presence of magnesium(II). Therefore, as a prelude to cellular studies, a competition experiment using human topoisomerase IIα was performed (Figure 8). Consistent with the data shown in Figure 1, ~4% DNA cleavage was observed in the presence of 1 mM $MgCl_2$, and the level of double-stranded breaks increased dramatically as increasing concentrations of $CoCl_2$ were added to reaction mixtures. Levels of DNA scission observed with a mixture of 1 mM $CoCl_2$ and 1 mM $MgCl_2$ (52%, Figure 8) were similar to those seen in the presence of 1 mM $CoCl_2$ alone (51%, Figure 1). This finding provides strong evidence that cobalt(II) stimulates DNA cleavage mediated by human topoisomerase IIα in the presence of magnesium(II).

Cobalt(II) Is a Topoisomerase II Poison in Cultured Human Cells. The data presented above demonstrate that cobalt(II) poisons human topoisomerase IIα *in vitro*. Consequently, experiments were performed to determine whether the metal acts as a topoisomerase II poison in cultured cells.

The physiological effect of cobalt(II) on DNA cleavage mediated by topoisomerase IIα was monitored in cultured human MCF-7 breast cancer cells using the *in vivo* complex of enzyme bioassay (*54*, *56*) (Figure 9). In this assay, cells are lysed with an ionic detergent, and proteins that are covalently attached to genomic DNA are separated from free proteins by sedimentation through a CsCl cushion. The pelleted DNA from cultures treated with no drug, 100 μM etoposide (for comparison), or 200 μM $CoCl_2$ was blotted and probed with a polyclonal antibody specific for human topoisomerase IIα. As determined by the increased levels of covalent enzyme−DNA complexes, the stimulation of DNA cleavage mediated by topoisomerase IIα in cobalt(II)-treated cells approached that observed in the etoposide-treated culture. Therefore, we conclude that cobalt(II) acts as a topoisomerase II poison in cultured human cells.

DISCUSSION

Cobalt is genotoxic to mammalian cells and is a suspected human carcinogen (*1*−*4*). It induces DNA breaks and covalent protein−DNA complexes in treated cells (*5*, *6*). Several potential mechanisms for explaining the genotoxicity of cobalt have been proposed, including the generation of reactive oxygen species and the inhibition of DNA repair enzymes (*2*−*4*). While evidence that supports both hypotheses exists, there is no consensus about the precise mechanism by which cobalt damages the genetic material.

Although cobalt genotoxicity can be mediated by many different pathways, the DNA strand breaks and covalent protein−DNA complexes formed after exposure to the metal ion are suggestive of the actions of topoisomerase II poisons. Therefore, the effect of cobalt(II) on the DNA cleavage activity of human topoisomerase IIα was examined. Results indicate that the metal ion is a strong enhancer of DNA cleavage mediated by the enzyme. DNA breaks increase by an order of magnitude when cobalt(II) is substituted for magnesium(II), the physiological divalent cation used by topoisomerase II. In addition, cobalt(II) stimulates enzyme-mediated DNA scission in the presence of magnesium(II) and induces the formation of covalent topoisomerase IIα−DNA cleavage complexes in cultured cells. On the basis of these findings, we conclude that cobalt(II) is a topoisomerase II poison, both *in vitro* and in cultured human cells.

Two broad classes of topoisomerase II poisons have been described. The first is comprised of anticancer drugs and other organic compounds (*13*, *14*, *16*, *30*). These agents are believed to increase the level of enzyme-mediated DNA cleavage either by disrupting the orientation of the DNA termini within the cleavage complex (thus impairing religation) or by altering the structure of the DNA (thus creating a preferential substrate for cleavage) (*16*, *64*, *65*). The second class is comprised of DNA lesions (*13*, *42*). When situated within a topoisomerase II cleavage site, these lesions are believed to act by altering DNA structure (once again, creating a preferential cleavage substrate). We propose that cobalt (and potentially other divalent cations) constitute a third class of topoisomerase II poisons. In contrast to the other two classes, divalent cations play a direct role in the DNA cleavage−religation reaction mediated by type II topoisomerases.

The mechanistic basis for the cobalt-induced increase in the level of DNA cleavage is not yet understood. The divalent cation appears to promote both faster rates of cleavage and slower rates of religation, and thus may act by favoring a transition state of the DNA during the scission event. Since cobalt is known to alter DNA structure (*66*−*70*), it is unclear whether the effects of the metal ion on topoisomerase II-mediated DNA cleavage ultimately result from an effect on the active site of the enzyme, the structure of the nucleic acid substrate, or the complex of the two.

Results of this study support the hypothesis that topoisomerase IIα plays a role in mediating the genotoxic effects

of cobalt in human cells. The contributions of topoisomerase IIα relative to the other proposed mechanisms of cobalt genotoxicity have yet to be determined. Since cobalt(II) increases the level of topoisomerase II-mediated DNA cleavage *in vitro*, even in the presence of magnesium(II), it is tempting to speculate that cobalt generates topoisomerase II-linked DNA breaks in treated cells by directly altering the cleavage−religation equilibrium of the enzyme. Alternatively, cobalt may increase the level of topoisomerase II-mediated DNA breaks by an indirect mechanism. Cobalt generates oxidative DNA lesions (*8*, *57*−*59*) that have been shown to act as modest poisons of human topoisomerase IIα (*45*). Furthermore, these lesions are processed by base excision repair pathways that create abasic sites as requisite intermediates (*71*−*73*). Since abasic sites formed during DNA repair are strong topoisomerase II poisons (*42*, *43*, *45*, *50*), it is possible that cobalt alters enzyme-mediated DNA cleavage in treated cells through the generation of DNA lesions.

In summary, cobalt(II) is a strong topoisomerase II poison both *in vitro* and in cultured human cells. These results strongly suggest that at least some of the genotoxic effects of the metal ion are mediated by topoisomerase IIα in mammalian cells.

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REFERENCES

1. International Agency for Research on Cancer (1991) *IARC Monogr. Eval. Carcinog. Risk Chem. Man 52*, 363−472.
2. Beyersmann, D., and Hartwig, A. (1992) *Toxicol. Appl. Pharmacol. 115*, 137−145.
3. Hartwig, A. (1995) *Biometals 8*, 3−11.
4. Lison, D., De Boeck, M., Verougstraete, V., and Kirsch-Volders, M. (2001) *Occup. Environ. Med. 58*, 619−625.
5. Hartwig, A., Kasten, U., Boakye-Dankwa, K., Schlepegrell, R., and Beyersmann, D. (1990) *Toxicol. Environ. Chem. 28*, 205−215.
6. Wedrychowski, A., Schmidt, W. N., and Hnilica, L. S. (1986) *Arch. Biochem. Biophys. 251*, 397−402.
7. Helleday, T., Nilsson, R., and Jenssen, D. (2000) *Environ. Mol. Mutagen. 35*, 114−122.
8. Kawanishi, S., Inoue, S., and Yamamoto, K. (1994) *Environ. Health Perspect. 102* (Suppl. 3), 17−20.
9. Kawanishi, S., Hiraku, Y., Murata, M., and Oikawa, S. (2002) *Free Radical Biol. Med. 32*, 822−832.
10. Hartwig, A., and Schwerdtle, T. (2002) *Toxicol. Lett. 127*, 47−54.
11. Hartwig, A., Snyder, R. D., Schlepegrell, R., and Beyersmann, D. (1991) *Mutat. Res. 248*, 177−185.
12. Kasten, U., Mullenders, L. H., and Hartwig, A. (1997) *Mutat. Res. 383*, 81−89.
13. Fortune, J. M., and Osheroff, N. (2000) *Prog. Nucleic Acid Res. Mol. Biol. 64*, 221−253.
14. Li, T. K., and Liu, L. F. (2001) *Annu. Rev. Pharmacol. Toxicol. 41*, 53−77.
15. Walker, J. V., and Nitiss, J. L. (2002) *Cancer Invest. 20*, 570−589.
16. Wilstermann, A. M., and Osheroff, N. (2003) *Curr. Top. Med. Chem. 3*, 321−338.
17. Wang, J. C. (1996) *Annu. Rev. Biochem. 65*, 635−692.
18. Sabourin, M., and Osheroff, N. (2002) in *Encyclopedia of Molecular Medicine* (Creighton, T. E., Ed.) pp 3192−3197, John Wiley & Sons, New York.
19. Wang, J. C. (1998) *Q. Rev. Biophys. 31*, 107−144.
20. Nitiss, J. L. (1998) *Biochim. Biophys. Acta 1400*, 63−81.
21. Wang, J. C. (2002) *Nat. Rev. Mol. Cell Biol. 3*, 430−440.
22. Goto, T., and Wang, J. C. (1984) *Cell 36*, 1073−1080.
23. Wyckoff, E., Natalie, D., Nolan, J. M., Lee, M., and Hsieh, T. (1989) *J. Mol. Biol. 205*, 1−13.
24. Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. M., Ross, W. E., Mattern, M. R., Johnson, R. K., and Crooke, S. T. (1987) *J. Biol. Chem. 262*, 16739−16747.
25. Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T., and Mirabelli, C. K. (1989) *Biochemistry 28*, 8154−8160.
26. Heck, M. M., and Earnshaw, W. C. (1986) *J. Cell Biol. 103*, 2569−2581.
27. Heck, M. M., Hittelman, W. N., and Earnshaw, W. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1086−1090.
28. Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K., and Drake, F. H. (1991) *Cell Growth Differ. 2*, 209−214.
29. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) *Nature 379*, 225−232.
30. Burden, D. A., and Osheroff, N. (1998) *Biochim. Biophys. Acta 1400*, 139−154.
31. Sander, M., and Hsieh, T. (1983) *J. Biol. Chem. 258*, 8421−8428.
32. Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., and Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365−15370.
33. Zechiedrich, E. L., Christiansen, K., Andersen, A. H., Westergaard, O., and Osheroff, N. (1989) *Biochemistry 28*, 6229−6236.
34. Baguley, B. C., and Ferguson, L. R. (1998) *Biochim. Biophys. Acta 1400*, 213−222.
35. Kaufmann, S. H. (1998) *Biochim. Biophys. Acta 1400*, 195−211.
36. Hande, K. R. (1998) *Biochim. Biophys. Acta 1400*, 173−184.
37. Ross, J. A., Potter, J. D., and Robison, L. L. (1994) *J. Natl. Cancer Inst. 86*, 1678−1680.
38. Felix, C. A. (1998) *Biochim. Biophys. Acta 1400*, 233−255.
39. Rowley, J. D. (1998) *Annu. Rev. Genet. 32*, 495−519.
40. Strick, R., Strissel, P. L., Borgers, S., Smith, S. L., and Rowley, J. D. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4790−4795.
41. Felix, C. A. (2001) *Med. Pediatr. Oncol. 36*, 525−535.
42. Kingma, P. S., and Osheroff, N. (1998) *Biochim. Biophys. Acta 1400*, 223−232.
43. Kingma, P. S., Corbett, A. H., Burcham, P. C., Marnett, L. J., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 21441−21444.
44. Kingma, P. S., and Osheroff, N. (1997) *J. Biol. Chem. 272*, 1148−1155.
45. Sabourin, M., and Osheroff, N. (2000) *Nucleic Acids Res. 28*, 1947−1954.
46. Osheroff, N. (1987) *Biochemistry 26*, 6402−6406.
47. Osheroff, N., and Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303−4309.
48. Worland, S. T., and Wang, J. C. (1989) *J. Biol. Chem. 264*, 4412−4416.
49. Elsea, S. H., Hsiung, Y., Nitiss, J. L., and Osheroff, N. (1995) *J. Biol. Chem. 270*, 1913−1920.
50. Kingma, P. S., Greider, C. A., and Osheroff, N. (1997) *Biochemistry 36*, 5934−5939.
51. Fortune, J. M., and Osheroff, N. (1998) *J. Biol. Chem. 273*, 17643−17650.
52. O'Reilly, E. K., and Kreuzer, K. N. (2002) *Biochemistry 41*, 7989−7997.
53. Burden, D. A., Kingma, P. S., Froelich-Ammon, S. J., Bjornsti, M.-A., Patchan, M. W., Thompson, R. B., and Osheroff, N. (1996) *J. Biol. Chem. 271*, 29238−29244.
54. Byl, J. A., Cline, S. D., Utsugi, T., Kobunai, T., Yamada, Y., and Osheroff, N. (2001) *Biochemistry 40*, 712−718.
55. Miller, K. G., Liu, L. F., and Englund, P. T. (1981) *J. Biol. Chem. 256*, 9334−9339.
56. Subramanian, D., Furbee, C. S., and Muller, M. T. (2001) *Methods Mol. Biol. 95*, 137−147.
57. Nackerdien, Z., Kasprzak, K. S., Rao, G., Halliwell, B., and Dizdaroglu, M. (1991) *Cancer Res. 51*, 5837−5842.
58. Lloyd, D. R., Phillips, D. H., and Carmichael, P. L. (1997) *Chem. Res. Toxicol. 10*, 393−400.
59. De Boeck, M., Lison, D., and Kirsch-Volders, M. (1998) *Carcinogenesis 19*, 2021−2029.
60. Bauman, M. E., Holden, J. A., Brown, K. A., Harker, W. G., and Perkins, S. L. (1997) *Mod. Pathol. 10*, 168−175.
61. Austin, C. A., and Marsh, K. L. (1998) *BioEssays 20*, 215−226.
62. Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, N. J., and Hickson, I. D. (1998) *Biochim. Biophys. Acta 1400*, 121−137.

63. Capranico, G., and Binaschi, M. (1998) *Biochim. Biophys. Acta 1400*, 185−194.
64. Bromberg, K. D., Burgin, A. B., and Osheroff, N. (2003) *J. Biol. Chem. 278*, 7406−7412.
65. Bromberg, K. D., Burgin, A. B., and Osheroff, N. (2003) *Biochemistry 42*, 3393−3398.
66. Xu, Y. C., and Bremer, H. (1997) *Nucleic Acids Res. 25*, 4067−4071.
67. Deng, H., and Bloomfield, V. A. (1999) *Biophys. J. 77*, 1556−1561.
68. Gueron, M., Demaret, J., and Filoche, M. (2000) *Biophys. J. 78*, 1070−1083.
69. Miyoshi, D., Nakao, A., and Sugimoto, N. (2001) *Nucleic Acids Res. Suppl.*, 259−260.
70. Thorpe, J. H., Teixeira, S. C., Gale, B. C., and Cardin, C. J. (2003) *Nucleic Acids Res. 31*, 844−849.
71. McCullough, A. K., Dodson, M. L., and Lloyd, R. S. (1999) *Annu. Rev. Biochem. 68*, 255−285.
72. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, 2nd ed., American Society for Microbiology Press, Washington, DC.
73. Wilson, S. H. (1998) *Mutat. Res. 407*, 203−215.

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