DNA-Ligase IV and DNA-Protein Kinase Play a Critical Role in Deficient Caspases Activation in Apoptosis-resistant Cancer Cells by Using Doxorubicin

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Resistance toward cytotoxic drugs is one of the primary causes for therapeutic failure in cancer therapy. DNA repair mechanisms as well as deficient caspases activation play a critical role in apoptosis resistance of tumor cells toward anticancer drug treatment. Here, we discovered that deficient caspases activation in apoptosis-resistant cancer cells depends on DNA-ligase IV and DNA-protein kinase (DNA-PK), playing crucial roles in the nonhomologous end joining (NHEJ) pathway, which is the predominant pathway for DNA double-strand break repair (DNA-DSB-repair) in mammalian cells. DNA-PK(+/+) as well as DNA-ligase IV (+/+) cancer cells were apoptosis resistant and deficient in activation of caspase-3, caspase-9, and caspase-8 and in cleavage of poly(ADP-ribose) polymerase after doxorubicin treatment. Inhibition of NHEJ by knocking out DNA-PK or DNA-ligase IV restored caspases activation and apoptosis sensitivity after doxorubicin treatment. In addition, inhibition of caspases activation prevented doxorubicin-induced apoptosis but could not prevent doxorubicin-induced DNA damage, indicating that induction of DNA damage is independent of caspases activation. However, caspases activation depends on induction of DNA damage left unrepaired by NHEJ-DNA-DSB-repair. We conclude that DNA damage left unrepaired by DNA-ligase IV or DNA-PK might be the initiator for caspases activation by doxorubicin in cancer cells. Failure in caspases activation using doxorubicin depends on loss of DNA damage and is due to higher rates of NHEJ-DNA-DBS-repair.

INTRODUCTION

DNA damage and DNA repair mechanisms play a critical role in sensitivity and resistance of tumor cells during and after anticancer drug treatment and irradiation (Christmann *et al.*, 2003; Kaina, 2003; Willmore *et al.*, 2004; Deriano *et al.*, 2005).

DNA repair mechanisms such as mismatch repair, base excision repair, nucleotide excision repair, direct damage reversal, and DNA double-strand break repair (DNA-DSB-repair) are involved in the integrity of DNA. Defects in DNA repair give rise to hypersensitivity to DNA-damaging agents, accumulation of mutations in the genome, and finally to the development of cancer and various metabolic disorders (Christmann *et al.*, 2003).

Doxorubicin, cisplatin, cyclophosphamide, and etoposide induce DNA damage, which generates a complex cascade of events leading to cell cycle arrest, transcriptional and post-transcriptional activation of a subset of genes including those associated with DNA repair, and triggering of apoptosis (Friesen *et al.*, 1996, 1999a; Christmann *et al.*, 2003; Kaina, 2003).

The genotoxic effects of DNA double-strand breaks (DNA-DSBs) are highly potent inducers of cell death (Christmann *et al.*, 2003). In higher eukaryotes a single nonrepaired DNA-DSB inactivating an essential gene can be sufficient for the induction of cell death via apoptosis (Rich *et al.*, 2000). The repair of DNA-DSBs is critical for the survival of cells exposed to DNA-damaging agents (Christmann *et al.*, 2003). Homologous recombination (HR) and nonhomologous end joining (NHEJ) are the two principal pathways repairing DNA-DSBs (Christmann *et al.*, 2003). In simple eukaryotes, HR is the main pathway, whereas in mammalian cells, NHEJ predominantly repairs DNA-DSBs, resulting from DNA-damaging agents (Jeggo, 1998a,b; Haber, 2000; Cromie *et al.*, 2001). NHEJ does not depend on the presence of homologous DNA sequences and requires the DNA-dependent protein kinase (DNA-PK) complex and the XRCC4/DNA-ligase IV complex (Jeggo, 1998a,b; Lieber, 1999). DNA-PK is a member of the phosphatidylinositol-3 kinase family that includes ataxia-telangiectasia-mutated (ATM) and ATM-Rad3-related (ATR) (Durocher and Jackson, 2001). It consists of a heterotrimeric complex consisting of proteins Ku70 and Ku80 (Ku70/80) and the catalytic DNA-PK subunit (Christmann *et al.*, 2003). DNA-PK exhibits protein kinase activity only when bound to DNA (Christmann *et al.*, 2003). The first step in DNA-DSB-repair by NHEJ is the binding of Ku70/80 to the damaged DNA followed by the recruitment of the catalytic subunit DNA-PK, thereby forming the active DNA-PK holoenzyme (Smith and Jackson, 1999; Christmann *et al.*, 2003). One of the targets of DNA-PK is XRCC4, which

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forms a stable complex with DNA-ligase IV, joining the ends of broken DNA strands (Grawunder *et al.*, 1998a,b).

Doxorubicin, cisplatin, cyclophosphamide, and etoposide not only induce DNA damage but also induce apoptosis and activate caspases (Friesen *et al.*, 1996, 1999a,b, 2004; Kaufmann and Earnshaw, 2000; Christmann *et al.*, 2003; Deriano *et al.*, 2005). Caspases play a critical role in apoptosis induction (Kaufmann and Earnshaw, 2000). The apoptotic caspases are divided into two classes, effector caspases and initiator caspases. Effector caspases are responsible for the cleavage that disassemble the cell, and initiator caspases initiate a proteolytic caspase-activating cascade (Kaufmann and Earnshaw, 2000). Caspase-3, -6, and -7 are the effector caspases, and caspase-8 and -9 are the major initiator caspases (Kaufmann and Earnshaw, 2000). Caspase-8 or -9 is activated in a multimeric complex. Caspase-8 is activated in the death-inducing signaling complex, and caspase-9 is within the apoptosome (Kaufmann and Earnshaw, 2000). On activation of initiator caspases, caspase-8 and -9 acquire the ability to cleave and activate effector caspases such as caspase-3 (Kaufmann and Earnshaw, 2000). Caspases activation can be triggered by two different mechanisms, the death receptor pathway and the mitochondrial pathway, and it is negatively regulated at the receptor level by Flice-inhibitory protein that blocks caspase-8 activation, at the mitochondria level by Bcl-2 family proteins, and by inhibitor of apoptosis proteins (Kroemer, 1997; Deveraux *et al.*, 1999; Srinivasula *et al.*, 2001).

In the present study, we investigate the role of NHEJ-DNA-DSB-repair, DNA-ligase IV, and DNA-PK in deficient caspases activation by doxorubicin. A greater understanding of the links between deficient caspases activation and DNA repair in apoptosis-, chemo-, as well as radioresistance will lead to the development of more effective cancer prevention and treatment strategies.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The human pre-B leukemia cell lines Nalm6 and Nalm6 (DNA-ligase IV +/+ , +/- , -/-) obtained from M. Lieber (University of Southern California, Los Angeles, CA) were grown in RPMI 1640 medium (Invitrogen, Eggenstein, Germany) containing 10% fetal calf serum (Biochrom, Berlin, Germany), 10 mM HEPES, pH 7.3 (Biochrom), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Biochrom). Nalm6^{DoxoR}, a variant of Nalm6 that is resistant to a concentration of 0.1 µg/ml doxorubicin was generated for >12 mo. To preserve the resistance of these cell lines, Nalm6^{DoxoR} was treated with doxorubicin every 4 wk with a concentration of 0.1 µg/ml doxorubicin. All cell lines were mycoplasma free.

DNA-PK-deficient (-/-) and Proficient (+/+) Cells

The human glioblastoma cell lines MO59K [DNA-PK (+/+)] and MO59J [DNA-PK (-/-)] were obtained from American Type Culture Collection (Manassas, VA) and grown in complete DMEM (Invitrogen), which contained 10% heat-inactivated fetal bovine serum (Biochrom), 5 mM HEPES (Biochrom), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Biochrom) at 37°C in an atmosphere of 5% CO₂.

Treatment of Adherent Cells

DNA-PK (+/+) and DNA-PK (-/-) glioblastoma cells were trypsinized and replated on 75-cm² tissue culture flask at a concentration of 8000 cells/cm². Cells were allowed to attached to the bottom of the wells, and then cells were treated with different concentrations of doxorubicin. At different time points, the Comet assay, the Nicoletti analysis, flow cytometry analysis, and Western Blot analysis were performed.

Treatment of Nonadherent Cells

Cells (2 × 10⁵ cells/ml; Nalm6, Nalm6^{DoxoR}, Nalm6 [DNA-ligase IV +/+ , +/- , -/-]), were treated with different concentrations of doxorubicin. At different time points, the Comet assay, the Nicoletti analysis, flow cytometry analysis, and Western Blot analysis were performed.

Determination of Induction of Apoptosis and Cell Cycle

Apoptosis was determined by the Nicoletti method (Nicoletti *et al.*, 1991) or forward light scatter/ side scatter (FSC/SSC) method (Carbonari *et al.*, 1994). The percentage of apoptotic cells was measured by hypodiploid DNA (sub-G₁) by the Nicoletti method or FSC/SSC analysis by flow cytometry (FACS-Calibur; BD Biosciences, Heidelberg, Germany).

Inhibition of Caspases Activation by Benzoylcarbonyl-Val-Ala-Asp-fluoromethyl Ketone (z-VAD-fmk)

The broad-spectrum tripeptide inhibitor of caspases z-VAD-fmk (Enzyme Systems Products, Dublin, CA) was used at a concentration of 50 µM. Cells were treated 1 h before doxorubicin treatment.

Western Blot Analysis

Western blot analysis was done as described previously (Friesen *et al.*, 2003). Immunodetection of poly(ADP-ribose) polymerase (PARP), caspase-3, active caspase-3, caspase-9, active caspase-9, and β-actin was done using rabbit anti-PARP polyclonal-antibody (1:5000; Enzyme Systems Products), mouse anti-caspase-3 monoclonal antibody (1:500; Cell Signaling Technology, Danvers, MA), rabbit anti-active-caspase-3 polyclonal-antibody (1:200; Millipore Bioscience Research Reagents, Temecula, CA), rabbit anti-caspase-9 polyclonal-antibody (1:5000; BD Biosciences Transduction Laboratories, Lexington, KY), rabbit anti-active caspase-9 polyclonal antibody (1:200; Millipore Bioscience Research Reagents), and mouse anti-β-actin monoclonal-antibody (Sigma Chemie, Deisenhofen, Germany). Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or peroxidase-conjugated goat anti-rabbit IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibody was used for the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Equal protein loading was controlled by β-actin analysis.

Measurement of DNA Damage (Comet Assay)

DNA damage (DNA breaks) were measured by the alkaline Comet assay (Hartmann *et al.*, 2003). Analysis was performed with a fluorescence microscope using a charge-coupled device camera connected to a personal computer and analysis software. Relative DNA breakage was expressed as olive tail moment (OTM), which was determined by measuring the fluorescence intensity of tail and nucleus using Kinetic Imaging Comet 5.0 Software (BFI Optilas, Puchheim, Germany).

RESULTS

Caspases Activation Depends on Induction of DNA Damage in Doxorubicin-sensitive and Doxorubicin-resistant Cancer Cells, Which Were Apoptosis Resistant

First, we analyzed doxorubicin-induced apoptosis, caspases activation, and DNA damage in doxorubicin-sensitive Nalm6 and doxorubicin-resistant Nalm6 (Nalm6^{DoxoR}) leukemia cells. Nalm6 and Nalm6^{DoxoR} cells were incubated with 0.03 µg/ml doxorubicin. After different time points of doxorubicin treatment, we found that doxorubicin-resistant Nalm6 cells were apoptosis resistant (Figure 1A), and they were deficient in caspases activation (Figure 1B), in contrast to the parental doxorubicin-sensitive Nalm6 cells (Figure 1, A and B), which were apoptosis-sensitive and proficient in caspases activation. In addition, we found a strong induction of DNA damage (DNA-DSBs and DNA single-strand breaks) in sensitive Nalm6 cells after doxorubicin treatment (Figure 1C). In Nalm6^{DoxoR} cells, DNA damage could not be detected after doxorubicin treatment (Figure 1C). Furthermore, DNA damage occurred before caspases activation and apoptosis induction (unpublished data).

To examine whether DNA damage induced caspases activation resulting in apoptosis or caspases activation induced DNA damage resulting in apoptosis, we incubated Nalm6 cells with doxorubicin alone or in combination with 50 µM z-VAD-fmk, a specific inhibitor of caspases. After different time points, we measured apoptosis induction (Figure 1D) and DNA damage (Figure 1D). We found that z-VAD-fmk inhibited apoptosis through inhibition of caspases in chemosensitive and apoptosis-sensitive Nalm6 cells after doxorubicin treatment (Figure 1D). In contrast, induction of

Figure 1. Caspases activation depends on induction of DNA damage in apoptosis-sensitive and apoptosis-resistant cancer cells after doxorubicin treatment. (A) Doxorubicin induces apoptosis in doxorubicin-sensitive Nalm6 and doxorubicin-resistant Nalm6^{DoxoR} cells. Doxorubicin-sensitive Nalm6 (Nalm6; black bars) and doxorubicin-resistant Nalm6 (Nalm6^{DoxoR}; white bars) cells were incubated with 0.03 µg/ml doxorubicin (Doxo). The percentages of apoptotic cells were measured after 24, 48, and 72 h. The percentages of apoptotic cells were measured by hypodiploid DNA. Percentage of specific cell death was calculated as follows: $100 \times (\text{experimental dead cells} [\%] - \text{spontaneous dead cells in medium} [\%]) / 100\% - \text{spontaneous dead cells in medium} (\%)$. Data are given as mean of triplicates, with a SD of <10%. Similar results were obtained in three independent experiments. (B) Doxorubicin induces activation of caspase-3, caspase-8, caspase-9, and PARP cleavage in doxorubicin-sensitive Nalm6 and doxorubicin-resistant Nalm6^{DoxoR} cells. Doxorubicin-sensitive Nalm6 (Nalm6) and doxorubicin-resistant Nalm6 (Nalm6^{DoxoR}) cells were incubated with 0.03 µg/ml Doxo or left untreated (control) for 24 and 48 h. Western blot analysis for caspase-3, caspase-9, caspase-8, and PARP was performed. The active fragment of caspase-8 was detected at ~18 kDa, the active fragment of caspase-9 at ~37 kDa, the active fragment of caspase-3 at ~17 kDa, and the cleaved product of PARP at ~85 kDa. Equal protein loading was controlled using a β-actin antibody. (C) Doxorubicin induces DNA damage in doxorubicin-sensitive Nalm6 (Nalm6) and doxorubicin-resistant Nalm6 (Nalm6^{DoxoR}) cells. Nalm6 and Nalm6^{DoxoR} cells were incubated with 0.03 µg/ml doxorubicin (Doxo) or left untreated (control) for 3 h. DNA damage (DNA-DSBs and DNA single-strand-breaks) was measured by the Comet assay. (D) Inhibition of caspases blocks doxorubicin-induced apoptosis, but it cannot prevent doxorubicin-induced DNA damage. Nalm6 cells were treated with different concentrations of Doxo as indicated in the absence (white bars) or presence (black bars) of z-VAD-fmk. The percentages of apoptotic cells were measured by hypodiploid DNA (apoptosis; left). The percentage of specific cell death was calculated as described in A. DNA damage (DNA-DSBs and DNA single-strand-breaks) was measured by the Comet assay and quantified by DNA strand break frequency (OTM) (DNA damage; right). (E) Inhibition of DNA-PK by wortmannin enabled doxorubicin-induced apoptosis in doxorubicin-resistant Nalm6 cells (Nalm6^{DoxoR}). Nalm6^{DoxoR} were incubated with 0.03 µg/ml Doxo or left untreated (control) in the absence (medium; white bars) or in addition of 20 µM wortmannin (black bars) for 48 h. The percentages of apoptotic cells were measured by hypodiploid DNA. Percentage of specific cell death was calculated as described in A. (F) Inhibition of DNA-PK by wortmannin enabled doxorubicin-induced DNA damage in doxorubicin-resistant Nalm6 cells (Nalm6^{DoxoR}). Nalm6^{DoxoR} were incubated with 0.03 µg/ml Doxo or left untreated (control) in the absence (medium; white bars) or in addition of 20 µM wortmannin (black bars) for 12 and 16 h. DNA damage (DNA-DSBs and DNA single-strand breaks) was measured by the Comet assay and quantified by DNA strand break frequency (OTM).

DNA damage was not inhibited by inhibition of caspases activation with z-VAD-fmk (Figure 1D). In doxorubicin-sensitive as well as in doxorubicin-resistant HL-60 and CEM leukemia cell lines, we found comparable results (unpublished data). This indicates that the induction of DNA damage is independent of caspases activation, but caspases ac-

tivation and induction of apoptosis are dependent on the induction of DNA damage. To analyze which DNA repair factors may contribute to the doxorubicin resistance, we treated doxorubicin-resistant Nalm6 cells, with 0.03 µg/ml doxorubicin alone or in combination with 20 µM wortmannin, a specific inhibitor of the phosphatidylinositol-3-kinase,

including DNA-PK, ATM, and ATR (Durocher and Jackson, 2001). We found that wortmannin enabled doxorubicin-induced apoptosis (Figure 1E) and DNA damage (Figure 1F) in doxorubicin-resistant Nalm6 cells.

DNA-Ligase IV and DNA-PK Play a Critical Role in Deficient Caspases Activation after Doxorubicin Treatment in Cancer Cells

NHEJ repairs predominant DNA-DSBs in mammalian cells (Jeggo, 1998a). DNA-PK as well as DNA-ligase IV play crucial roles in DNA-DSB-repair by NHEJ (Collis *et al.*, 2005).

To analyze whether NHEJ plays an important role in deficient caspases activation in cancer cells, we measured first the role of DNA-PK in induction of DNA damage (DNA-DSBs and DNA single-strand breaks) and apoptosis after doxorubicin treatment in DNA-DSB-repair-proficient DNA-PK (+/+) and DNA-DSB-repair-deficient DNA-PK (-/-) glioblastoma cells lines (Figure 2A). After 6, 11, and 17 h, we found a strong induction of DNA damage in a DNA-PK (-/-) cells after treatment with doxorubicin (Figure 2A). In DNA-PK (+/+) cells, less DNA damage was detected after doxorubicin treatment, indicating that doxorubicin-induced DNA damage were repaired in DNA-PK (+/+) cells (Figure 2A). In parallel, we measured doxorubicin-induced apoptosis (Figure 2A). At 6, 11, and 17 h, we could not find an induction of apoptosis in DNA-PK (-/-) cells in contrast to induction of DNA damage. Induction of

apoptosis was found after doxorubicin treatment in DNA-PK (-/-) cells at later time points (48 and 72 h) (Figure 2A), indicating that DNA damage, left unrepaired by DNA-PK, occurred before apoptosis was detected. In addition, in contrast to DNA-PK (-/-) cells, apoptosis could not be detected after doxorubicin treatment from 3 h until 24 h (Figure 2A) in DNA-PK (+/+) cells, suggesting that DNA-PK is involved in doxorubicin and apoptosis resistance. Minimal apoptosis induction was detected at 48 and 72 h. We next investigated whether DNA-PK is involved in deficient caspases activation in cancer cell (Figure 2B). We treated DNA-DSB-repair-proficient DNA-PK (+/+) and DNA-DSB-repair-deficient DNA-PK (-/-) cells with 0.1 and 0.3 $\mu\text{g}/\text{ml}$ doxorubicin (Figure 2B). After 24 and 48 h, Western blot analysis was performed. We found caspase-3 activation, caspase-8 activation and PARP cleavage in DNA-PK (-/-) cells lines after doxorubicin treatment (Figure 2B). In DNA-PK (+/+) cells lines, caspases activation could not be detected after doxorubicin treatment (Figure 2B). In addition, inhibition of DNA-PK by wortmannin, a specific inhibitor of the phosphatidylinositol-3-kinase, including DNA-PK, ATM, and ATR (Durocher and Jackson, 2001), reversed apoptosis resistance (Figure 2C) and deficient activation of caspase-3, and caspase-8 after doxorubicin treatment in DNA-PK (+/+) cells (unpublished data). These findings indicate that DNA-PK plays an important role in doxorubicin-induced caspases activation and apoptosis.

Figure 2. DNA-PK plays a critical role in deficient caspases activation by doxorubicin in cancer cells. (A) Doxorubicin induces DNA damage and apoptosis in DNA-PK (-/-) and DNA-PK (+/+) cells. Doxorubicin induces DNA damage (left) in DNA-PK (-/-) and DNA-PK (+/+) cells. DNA-PK (-/-) (white bars) and DNA-PK (+/+) (black bars) cells were incubated with 0.1 $\mu\text{g}/\text{ml}$ doxorubicin or left untreated (control [Co]) for 3, 6, 11, and 17 h. DNA damage (DNA-DSBs and DNA single-strand-breaks) was quantified by DNA strand break frequency (OTM). Doxorubicin induces apoptosis (middle and right) in DNA-PK (-/-) and DNA-PK (+/+) cells. DNA-PK (-/-) (white bars) and DNA-PK (+/+) (black bars) cells were incubated with 0.1 $\mu\text{g}/\text{ml}$ doxorubicin for 6, 11, and 17 h and for 24, 48, and 72 h. The percentages of apoptotic cells were measured by hypodiploid DNA. Percentage of specific cell death was calculated as described in Figure 1A. (B) Doxorubicin induces activation of caspase-3, caspase-8, and PARP cleavage in DNA-PK (-/-) and DNA-PK (+/+) cells. DNA-PK (-/-) and DNA-PK (+/+) cells were incubated with 0.3 or 0.1 $\mu\text{g}/\text{ml}$ doxorubicin (Doxo) or left untreated (control) for 24 and 48 h. Western blot analysis for caspase-3, caspase-8, and PARP was performed. The active fragment of caspase-8 was detected at ~18 kDa, the active fragment of caspase-3 was detected at ~17 kDa, and the cleaved product of PARP at ~85 kDa. Equal protein loading was controlled using a β -actin antibody. (C) Inhibition of DNA-PK by wortmannin reverses deficient doxorubicin-induced apoptosis.

in DNA-repair proficient DNA-PK (+/+) cells. DNA-PK (+/+) cells were incubated with 0.1 $\mu\text{g}/\text{ml}$ Doxo (white bars) or left untreated (control) or in addition of 20 μM wortmannin (black bars) for 48 h. The percentages of apoptotic cells were measured by hypodiploid DNA. Percentage of specific cell death was calculated as described in Figure 1A.

DNA-PK plays an important role in NHEJ. However, DNA-PK is a molecular sensor for DNA damage that enhances the signal via phosphorylation of many downstream targets, not exclusively NHEJ (Collis *et al.*, 2005). DNA-PK as well as DNA-ligase IV play crucial roles in DNA-DSB-repair by NHEJ (Collis *et al.*, 2005). We next examined whether DNA-PK exclusively or DNA-ligase IV as a key enzyme for NHEJ also plays an important role in deficient caspases activation in cancer cells. Therefore, we incubated DNA-ligase IV (+/+), DNA-ligase IV (+/-), and DNA-ligase IV (-/-) Nalm6 cells with different concentrations of doxorubicin (Figure 3A). After 24, 48, and 72 h, we found a strong induction of apoptosis in DNA-ligase IV (-/-) cells in contrast to DNA-ligase IV (+/-) and DNA-ligase IV (+/+) cells (Figure 3A). In addition, we measured caspases activation after doxorubicin treatment in DNA-ligase IV (+/+), DNA-ligase IV (+/-), and DNA-ligase IV (-/-) Nalm6 cells by Western blot analysis (Figure 3B). After 48 h, we could detect activation of caspase-3, caspase-9, caspase-8, and PARP cleavage in DNA-ligase IV (-/-) Nalm6 cells lines (Figure 3B). In DNA-ligase IV (+/-) and DNA-ligase IV (+/+) Nalm6 cells, caspases activation could not be found at 48 h (Figure 3B). Furthermore, we measured induction of DNA damage (DNA-DSBs and DNA single-strand breaks) (Figure 3C). After 12, 16, 20, 24 h, we found a strong induction of DNA damage in DNA-ligase IV (-/-) Nalm6 cells after treatment with doxorubicin. Induction of DNA damage was barely detected in DNA-ligase IV (+/+) and DNA-ligase IV (+/-) Nalm6 cells at identical doxorubicin concentrations. This demonstrates that DNA-ligase IV as well as DNA-PK play a critical role in caspases activation, suggesting that DNA damage left unrepaired by NHEJ is crucial for caspases activation in cancer cells.

DISCUSSION

DNA damage and DNA repair mechanisms play a critical role in sensitivity and resistance of tumor cells during and after anticancer drug treatment and irradiation (Christmann *et al.*, 2003; Kaina, 2003; Willmore *et al.*, 2004; Deriano *et al.*, 2005). If left unrepaired, DNA damage can result in permanent cell cycle arrest, induction of apoptosis, and mitotic cell death caused by a loss of genomic material (Olive, 1998). Caspases such as caspase-3, caspase-8, and caspase-9 play a critical role in anticancer drug-induced apoptosis, in apoptosis-resistance and anticancer drug resistance (Friesen *et al.*, 1999a; Christmann *et al.*, 2003; Kaina, 2003). Anticancer drugs interact with DNA-activate caspases and induce DNA damage in anticancer drug-sensitive tumor cells (Friesen *et al.*, 1999a; Kaufmann and Earnshaw, 2000; Christmann *et al.*, 2003; Kaina, 2003). In anticancer drug-resistant tumor cells, caspases activation is blocked and DNA repair mechanisms are up-regulated (Friesen *et al.*, 1997, 1999a; Los *et al.*, 1997; Christmann *et al.*, 2003; Kaina, 2003).

In our study, we provide the evidence that DNA damage left unrepaired by NHEJ-DNA-DSB-repair initiates activation of caspase-8, caspase-9, and caspase-3 and that DNA-DSB-repair by NHEJ is critical for deficient caspases activation in cancer cells after doxorubicin treatment.

Doxorubicin activates caspases and induces DNA damage in cancer cells (Friesen *et al.*, 1999a; Kaufmann and Earnshaw, 2000; Willmore *et al.*, 2004). In chemosensitive Nalm6 cells, we found a strong activation of caspase-3, caspase-8, and caspase-9, cleavage of PARP, induction of apoptosis, as well as induction of DNA damage (DNA-DSBs and DNA single-strand breaks) after doxorubicin treatment in a time course. Induction of DNA damage was found before caspases

Figure 3. DNA-ligase IV play a critical role in deficient caspases activation by doxorubicin in cancer cells. (A) Doxorubicin induced apoptosis in DNA-ligase IV (-/-), DNA-ligase IV (+/-) and DNA-ligase IV (+/+) Nalm6 cells. DNA-ligase IV (+/+) (Lig.IV +/+; white bars), DNA-ligase IV (+/-) (Lig.IV +/-; gray bars), and DNA-ligase IV (-/-) (Lig.IV -/-; black bars) Nalm6 cells were incubated with 0.01 or 0.003 µg/ml doxorubicin for 24, 48, and 72 h. The percentages of apoptotic cells were measured by hypodiploid DNA. Percentage of specific cell death was calculated as described in Figure 1A. (B) Doxorubicin induced apoptosis and activation of caspase-8, caspase-9, caspase-3, and PARP cleavage in DNA-ligase IV (-/-), DNA-ligase IV (+/-), and DNA-ligase IV (+/+) Nalm6 cells. DNA-ligase IV (+/+) (Lig.IV +/+; white bars), DNA-ligase IV (+/-) (Lig.IV +/-; gray bars), and DNA-ligase IV (-/-) (Lig.IV -/-; black bars) Nalm6 cells were incubated with 0.003 µg/ml doxorubicin (Doxo) or left untreated (control) for 48 h. Western blot analysis for caspase-8, caspase-9, caspase-3, and PARP was performed. The active fragment of caspase-8 was detected at ~18 kDa, the active fragment of caspase-9 at ~37 kDa, the active fragment of caspase-3 at ~17 kDa, and the cleaved product of PARP at ~85 kDa. Equal protein loading was controlled using a β-actin antibody. (C) Doxorubicin induced DNA damage in DNA-ligase IV (-/-), DNA-ligase IV (+/-), and DNA-ligase IV (+/+) Nalm6 cells. DNA-ligase IV (+/+) (+/+ Lig.IV; white bars), DNA-ligase IV (+/-) (+/- Lig.IV; gray bars), and DNA-ligase IV (-/-) (-/- Lig.IV; black bars) Nalm6 cells were incubated with 0.01 µg/ml doxorubicin at time points as indicated. DNA damage (DNA-DSBs and DNA single-strand breaks) were measured by the Comet assay and quantified by DNA strand break frequency (OTM).

activation and induction of apoptosis. This suggests that DNA damage was induced first and caspases activation and apoptosis was followed at later times after doxorubicin treatment. Doxorubicin-resistant cell lines are apoptosis-resistant, and they show a defect in caspases activation (Friesen *et al.*, 1996, 1997, 1999a,b, 2004; Los *et al.*, 1997; Posovszky *et al.*, 1999). Consistent with these data, we found that doxorubicin could not induce apoptosis and activation of caspase-3, caspase-9, and caspase-8 in doxorubicin-resistant Nalm6 cells. DNA repair is critical for cell survival and plays an important role in chemo- and radioresistance

(Christmann *et al.*, 2003). Induction of DNA damage was not measurable after treatment with identical concentrations of doxorubicin in doxorubicin-resistant Nalm6 cells, in contrast to doxorubicin-sensitive Nalm6 cells. This suggests that caspases activation is blocked and DNA damage was repaired after doxorubicin treatment in doxorubicin-resistant Nalm6 cells. In doxorubicin-sensitive as well as in doxorubicin-resistant HL-60 and CEM leukemia cell lines, we found comparable results (unpublished data).

p53 accumulates in response to DNA damage and p53 plays an important role in the cellular choice between life and death (Friesen *et al.*, 2003, 2004). p53 can transactivate numerous genes coding for proteins acting either at the receptor level of the apoptotic signals or at downstream stages of the apoptotic process. However, p53-deficient HL-60 cells and p53-mutant CEM cells harbor nonfunctional p53, suggesting that caspases activation and DNA damage may be independent of p53 in these cells lines (Friesen *et al.*, 2003, 2004).

Antracyclines such as doxorubicin could be subject to a multidrug-resistant (MDR) phenotype with decreasing drug uptake in anthracycline-resistant tumor cells (Slapak *et al.*, 1990). Doxorubicin resistance in Nalm6 as well as CEM and HL-60 cells is not due to an MDR phenotype, because we found that doxorubicin resistance is independent of MDR in leukemia cell lines (Friesen *et al.*, 1997, 1999b).

DNA-PK is involved in DNA repair, and it is a molecular sensor for DNA damage that enhances the signal via phosphorylation of many downstream targets (Collis *et al.*, 2005). Inhibition of DNA-PK seems to be a valid approach to enhance tumor cell-killing effects of treatments such as irradiation (Collis *et al.*, 2005). Inhibition of DNA-PK sensitized doxorubicin-resistant Nalm6 cells to doxorubicin and reversed deficient doxorubicin-triggered apoptosis, deficient induction of DNA damage, and deficient caspases activation after doxorubicin in doxorubicin-resistant Nalm6, suggesting that DNA-PK is involved in insufficient tumor cell kill and in deficient caspases activation in doxorubicin- and apoptosis-resistant leukemia cells.

DNA-PK interacts with NHEJ mechanism (Collis *et al.*, 2005). NHEJ is the predominant pathway for DNA-DSB-repair in mammalian cells (Lieber, 1999; Smith and Jackson, 1999; Durocher and Jackson, 2001; Christmann *et al.*, 2003). NHEJ involves the XRCC4-DNA-ligase IV complex and the DNA-PK holoenzyme, consisting of the DNA binding heterodimer KU70/KU80 and the catalytic subunit DNA-PK (Grawunder *et al.*, 1998a,b; Lieber, 1999; Smith and Jackson, 1999; Durocher and Jackson, 2001; Christmann *et al.*, 2003). Cell lines deficient in any of these genes are generally highly sensitive to ionizing radiation and have marked deficiencies in DNA-DSB-repair (Riballo *et al.*, 1999; Belenkov *et al.*, 2002; Adachi *et al.*, 2003; Holgersson *et al.*, 2003; Collis *et al.*, 2005; Deriano *et al.*, 2005). Doxorubicin induced DNA damage in the cell line deficient in DNA-PK. Induction of DNA damage was strongly reduced after doxorubicin treatment in DNA-DSB-repair-proficient DNA-PK (+/+) cells at identical concentrations and at same time points, suggesting that doxorubicin-induced DNA damage was repaired in cell lines with intact DNA-PK. DNA-PK (-/-) cells are highly apoptosis-sensitive and DNA-PK (+/+) cells are apoptosis and doxorubicin resistant. In addition, doxorubicin induced activation of caspase-3 and caspase-8 in DNA-PK (-/-)-deficient cells, and caspases activation is lacking after doxorubicin treatment in DNA-PK (+/+) cells. Inhibition of DNA-PK reversed deficient caspases activation in DNA-PK (+/+) cells. These data suggest that caspases activation by doxorubicin depends on DNA-PK in cancer cells.

DNA-ligase IV is an key enzyme in NHEJ-DNA-DSB-repair (Lieber, 1999). Doxorubicin strongly induced apoptosis and caspases activation in DNA-ligase IV (-/-) cells similar to DNA-PK (-/-) cells. DNA-ligase IV (+/+) cells are apoptosis resistant to doxorubicin, and caspases activation is lacking after doxorubicin treatment similar to DNA-PK (+/+) cells, suggesting that not only DNA-PK exclusively but also ligase IV as a key enzyme for NHEJ-DNA-DSB-repair plays an important role in deficient caspases activation in cancer cells. On the basis of these results, we have developed a radioactive antibody that breaks chemo- and radioresistance by overcoming NHEJ-DNA-DSB-repair (Friesen *et al.*, 2007).

NHEJ is involved in surviving topoisomerase II-mediated DNA damage (Malik *et al.*, 2006). Doxorubicin intercalates with DNA and interacts with DNA-topoisomerase-II (topo-II) (Bodley *et al.*, 1998). Mutated topo-II plays a critical role in anthracycline resistance such as doxorubicin resistance. In doxorubicin-resistant Nalm6 cells, doxorubicin resistance, and deficient caspases activation are highly unlikely attribute to topo-II mutation because wortmannin, a specific inhibitor of the phosphatidylinositol 3-kinase including DNA-PK, ATM, and ATR (Durocher and Jackson, 2001), reversed the phenotype in doxorubicin-resistant Nalm6 cells. This suggests that NHEJ plays a critical role in deficient caspases activation by doxorubicin in doxorubicin- and apoptosis-resistant cancer cells independently of topo-II mutation.

Caspases activation was inhibited by z-VAD-fmk a known inhibitor for caspases activation (Friesen *et al.*, 2003). z-VAD-fmk blocked doxorubicin-induced apoptosis and caspases activation in doxorubicin and apoptosis-sensitive Nalm6 cells. Caspases activation and induction of apoptosis by doxorubicin were also inhibited by z-VAD-fmk in DNA-PK (-/-) and DNA-ligase IV (-/-)-deficient cell lines (unpublished data). This demonstrates that doxorubicin-induced apoptosis depends on caspases activation in anticancer drug-sensitive as well as in repair-deficient cell lines. DNA damage was induced by doxorubicin in doxorubicin-sensitive cells as well as in DSB-repair deficient DNA-PK (-/-) cells and DNA-ligase IV (-/-) cells before caspases were activated and apoptosis was detected. In contrast to induction of apoptosis, induction of DNA damage was not inhibited by blockade of caspases activation with z-VAD-fmk in doxorubicin-sensitive Nalm6 cells as well as in DNA-DSB-repair-deficient DNA-PK (-/-) cells and DNA-ligase IV (-/-) cells, indicating that induction of DNA damage is independent of caspases activation, but it depends on the NHEJ-DNA-DSB-repair. In addition, this suggests that caspases activation depends on induction of DNA damage left unrepaired by NHEJ-DNA-DSB-repair, and DNA-DSBs might be the initiator for caspases activation.

Collectively, we found that caspases activation depends on induction of DNA damage. DNA damage, which is left unrepaired in the NHEJ-DNA-DSB-repair pathway, seems to be the initiator for caspases activation and induction of apoptosis. Increasing NHEJ-DNA-DSB-repair leads to failure of induction of DNA damage, and it is responsible for deficient caspases activation in apoptosis-resistant and doxorubicin-resistant cancer cells. Modulation of NHEJ-DNA-DSB-repair restores caspases activation by doxorubicin in previously doxorubicin-resistant cells. Overcoming NHEJ-DNA-DSB-repair might offer promising strategies to enhance caspases dependent apoptosis-mediated tumor cell kill.

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