Implication of Radical Oxygen Species in Ceramide Generation, c-Jun N-Terminal Kinase Activation and Apoptosis Induced by Daunorubicin

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ABSTRACT

Anthracyclines such as daunorubicin (DNR) generate radical oxygen species (ROS), which account, at least in part, for their cytotoxic effect. We observed that early ceramide generation (within 6–10 min) through neutral sphingomyelinase stimulation was inhibitable by the antioxidants N-acetylcysteine and pyrrolidine dithiocarbamate, which led to a decrease in apoptosis (>95% decrease in DNA fragmentation after 6 h). Furthermore, we observed that DNR triggers the c-Jun N-terminal kinase (JNK) and the transcription factor activated protein-1 through an antioxidant-inhibitable mechanism. Treatment of U937 cells with cell-permeant ceramides induced both an increase in ROS generation and JNK activation, and apoptosis, all of which were antioxidant-sensitive. In conclusion, DNR-triggered apoptosis implicates a ceramide-mediated, ROS-dependent JNK and activated protein-1 activation.

The anthracycline daunorubicin (DNR) is one of the major antitumor agents widely used in the treatment of acute myeloid leukemias. However, its mechanism of action is still not fully understood. It is generally postulated that most of DNR-induced cytotoxicity is related to DNA intercalation of the drug and its interaction with nuclear topoisomerase II (for a review, see Cummings et al., 1991). It has also been shown that DNR induces apoptosis in myeloid leukemia cell lines (U937 and HL-60; Quillet-Mary et al., 1996). However, present knowledge does not allow us to determine whether apoptosis simply reflects DNA lesions or represents an independent cytotoxic mechanism triggered by a specific signaling pathway (for a review, see Hannun, 1996).

In previous studies, we demonstrated the involvement of the sphingomyelin (SM)-ceramide (CER) cycle in DNR-induced apoptosis. Indeed, exposure at concentrations that induced apoptosis (0.5–4 μM) also stimulated SM hydrolysis due to the stimulation of a neutral magnesium-dependent sphingomyelinase (N-SMase) and subsequent CER generation in both U937 and HL-60 cells (Jaffrézou et al., 1996). The observation that cell-permeant ceramides, as well as endogenous CER (generated by treating cells with bacterial sphingomyelinase (SMase)), induce apoptosis strongly supported that CER was a mediator of DNR-induced apoptosis. The role of CER in DNR-induced apoptosis was substantiated by additional studies that showed that pharmacological manipulations, which led to the inhibition of SMase stimulation, resulted not only in the blockage of SM hydrolysis and CER production, but also in the inhibition of apoptosis in DNR-treated U937 cells (Mansat et al., 1997a,b). Indeed, it is now well established that modulation of these initial signaling events can have significant repercussions on pathways farther downstream. Such an apoptotic signaling pathway has also been described in vincristine-, ionizing radiation-, anti-Fas-, and tumor necrosis factor α (TNFα)-induced apoptosis (for a review, see Hannun, 1996). In turn, CER activates several downstream signaling pathways among which the stress-activated protein kinase cascade (MEKK1-SEK1-SAP/JNK) plays a critical role for apoptosis (Verheij et al., 1996).

The SM-CER pathway appears to be efficiently regulated downstream of CER generation. Among different factors including PKC activity (Jarvis et al., 1994), we and others also found that the oxidative balance plays a major role in the regulation of CER-induced apoptosis (Quillet-Mary et al.,...
1997). In addition, it should be noted that Bcl-2, a potent regulator of apoptosis, which may act by suppressing the formation or effects of radical oxygen species (ROS), inhibits apoptosis induced by CER (Martin et al., 1995). However, whether or not ROS may influence DNR-CER production is currently unknown.

Anthracyclics, including DNR, generate ROS due to electron transfer from the semiquinone ring and disturb mitochondrial oxidative metabolism (Sinha and Mimmaghu, 1990). Moreover, we have reported that ROS scavengers such as pyrrolidine dithiocarbamates (PDTCs) and N-acetylcysteine (N-Ac) (a thiol antioxidant and a GSH precursor) inhibited, whereas buthionine-sulfoximine (BSO), which depletes glutathione store, enhanced DNR-induced apoptosis (Quillet-Mary et al., 1996). Therefore, we speculated that DNR-induced ROS generation could contribute to the regulation of the SM-CER-c-Jun-N terminal kinase (JNK) apoptotic pathway.

Materials and Methods

Drugs and Chemicals. DNR (Cerubidine) was supplied by Laboratoire Roger Bellon (Neuilly-sur-Seine, France). N-Ac was purchased from Sigma Chemical Co. (St. Louis, MO). All other drugs and reagents were obtained from Sigma, Carlo Erba (Rueil-Malmaison, France), or Prolabo (Paris, France).

Cell Line and Culture. The human leukemic cell line U937 (monocytic) purchased from the American Type Culture Collection (Rockville, MD), was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 μg/ml penicillin (all obtained from Eurobio, les Ulis, France) at 37°C and 5% CO2. Cell stocks were screened routinely for Mycoplasma (Stratagene, La Jolla, CA).

Cytotoxic Chemical Staining. Changes in cellular chromatin were evaluated by fluorescence microscopy by DAPI (4’,6-diamidino 2-phenylindol) staining as described previously (Jaffrézou et al., 1996).

DNA Fragmentation. The specific DNA fragmentation was evaluated as described previously (Quillet-Mary et al., 1996). Briefly, exponentially growing cells were labeled with 1 μCi/10⁶ cells of methyl-[3H]thymidine for 24 h and washed 3 times with fresh medium. Radiolabeled cells were then incubated overnight in RPMI containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 μg/ml penicillin (all obtained from Eurobio, les Ulis, France) at 37°C and 5% CO2. Cell stocks were screened routinely for Mycoplasma (Stratagene Mycoplasma PCR kit; Stratagene, La Jolla, CA).

Effect of N-Ac and PDTC on DNR-Induced N-SMase Activation. To ascertain the potential effect of antioxidants on DNR-induced N-SMase stimulation, U937 cells were preincubated with 25 mM N-Ac for 2 h or 10 μM PDTC for 30 min and then treated with DNR. At several time points, cells were washed and N-SMase activity was measured. As shown in Fig. 1, treatment with 1.0 μM DNR led to a >40% increase in N-SMase activity, which peaked at 8 min. However, pretreatment of cells with N-Ac or PDTC completely inhibited N-SMase stimulation but had no effect on basal levels (approximately 43 pmol/h/mg of protein).

Electrophoretic Mobility Shift Assay. The gel was then dried and autoradiographed with intensifying film. Electrophoresis was conducted using 0.75% agarose gels containing 62.5 mM Tris–HCl, 250 mM LiCl, and 10 mM EDTA at pH 6.8. The DNA probe was excised and purified using a QIAEX II agarose gel extraction kit (Qiagen). The gel was stained with ethidium bromide to confirm the migration of the DNA samples. The gel was then dried and autoradiographed with intensifying film.

Effect of N-Ac and PDTC on DNR-Induced CER Generation. To determine whether the inhibition of DNR-induced N-SMase stimulation may influence CER generation, we measured CER levels in U937 cells. As expected, a significant increase in intracellular CER levels (about 25%) was observed in DNR-treated cells (1.0 μM) within 5 to 8 min. Similar results were observed at 4 μM but no CER production was seen at 0.1 μM. Pretreatment of cells with N-Ac or PDTC completely abolished CER generation (Fig. 2).

Effect of N-Ac on DNR- and C6-CER-Induced JNK Activation. We evaluated the effects of DNR (1.0 μM), cell-permeant CER (C6-CER) (25 μM), and bacterial SMase on a CER target: JNK. We showed that these stimulated JNK activity as early as 5 min, increasing substrate phosphorylation...
tion ([32P-GST-Jun]) between 200 and 280% within 30 min (Fig. 3 A-C).

To determine whether the inhibition of DNR-induced N-SMase stimulation and CER generation may influence JNK activation, we measured JNK activation in DNR- and C6-CER-treated cells in the presence of N-Ac. As shown in Fig. 3D, pretreatment with N-Ac completely inhibited JNK activation by DNR. Moreover, in C6-CER-treated cells, preincubation with N-Ac completely abolished JNK activation (Fig. 3E). Basal JNK activity was only slightly affected by N-Ac treatment (<15%) (data not shown). Similar results were obtained with PDTC (data not shown). Interestingly, 0.1 μM DNR, which does not induce CER generation, failed to activate JNK (data not shown). These results confirm that ROS are implicated in both CER generation and CER effects.

**Effect of N-Ac on DNR- and CER-Induced Activation.** C-Jun, a transcriptional factor subunit of AP-1, is a target of JNK. Therefore, we tested the effect of the antioxidant, N-Ac, on AP-1 activation induced by DNR or C6-CER using a gel shift assay. As shown in Fig. 4A, within 1 h DNR presented a dose-dependent activation of AP-1, which was significant at 0.5 (1.6-fold) and 1 μM (2.7-fold). Specificity was evaluated by adding 100-fold excess unlabelled AP-1 consensus oligonucleotide (<1.2-fold increase), and TPA was used as a positive control (6.2-fold increase). Kinetic studies revealed AP-1 activation as early as 30 min. Preincubation of cells with N-Ac blocked 1 μM DNR-induced AP-1 activation (<0.8-fold). Treatment of cells with C6-CER (25 μM) similarly induced a 3.2-fold increase in AP-1, and this was similarly blocked by N-Ac (<0.6-fold) (Fig. 4B).

**Effect of N-Ac and PDTC on DNR-Triggered Apoptosis.** To evaluate the effects of the inhibition of CER generation on DNR-induced apoptosis, U937 cells were preincubated for 2 h with N-Ac and further incubated with DNR for 1 h, washed, and resuspended in drug-free medium. After 5 h, cells were analyzed for apoptosis by DAPI staining (Fig. 5A-D). DNR treatment (1.0 μM) led to cell shrinkage and chromatin condensation in more than 80% of cells as observed by fluorescence microscopy. Quantitative analysis revealed that DNR induced about 50% DNA fragmentation (Fig. 6). Preincubation with N-Ac or PDTC potently inhibited DNA fragmentation (>95%) as well as the morphological features of DNR-triggered apoptosis (Figs. 5 and 6). Similar results were observed with cells treated by C6-CER (data not shown).

**Effect of C6-CER on Initial H2O2 Production.** The fluorescence distribution of the C2938 dye, which reveals the presence of hydrogen peroxide, was measured by flow cytometry in the viable cell population. Figure 7 shows the increase in the mean C2938 fluorescence in 25 μM C6-CER-treated cells compared with untreated cells. The mean fluorescence increased as a function of time at 5 to 20 min and returned to the baseline at 30 min, reflecting H2O2 generation in U937 cells induced by the cell-permeant CER. We could not detect H2O2 generation before 5 min (data not shown). To determine the effect of antioxidant on CER-induced H2O2 production, we used a ROS scavenger PDTC. We did not use N-Ac in this assay because of fluorescence interference by C2938. As shown in Fig. 7, preincubation with 10 μM PDTC for 30 min completely inhibited H2O2 generation.

Although we could not detect H2O2 generation by DNR with our assay, preincubation of cells with 50 μM BSO for 24 h, which depletes 70% glutathione store, enhanced DNR-induced H2O2 generation within 5 min (Fig. 7, inset). This result argues for the implication of DNR-generated H2O2 within the initial steps of apoptosis signaling.

**Discussion**

During the treatment of cells with anthracyclines, NADPH-dependent flavin reductase reduces the drug to a semiquinone radical, which can donate its free electron to molecular oxygen and generate the superoxide radical (O2\*). At neutral pH, the main reaction of O2\* is a relatively slow spontaneous dismutation to H2O2 and O2, but this reaction can be accelerated by superoxide dismutase. Superoxide anion and hydrogen peroxide may interact (with metal ions such as iron or copper as catalyst) by the Haber-Weiss reaction to generate hydroxyl radicals (‘OH). By using electron...
spin resonance together with a spin-trap such as 5,5-dimethyl pyrroline-N-oxide, anthracycline-induced \( \cdot \text{OH} \) has been detected in several types of cancer cells (for a review, see Sinha and Mimmaugh, 1990). This result suggests that anthracycline-induced free radical production may occur independently of the interaction of the drug with internal membranes or even DNA. Although there is strong evidence that ROS play a role in the cytotoxicity of anthracyclines, the mechanism by which ROS influence cell viability remains unclear.

Our study shows that DNR induces, within 5 to 10 min, an N-SMase stimulation through an N-Ac inhibitable mechanism, evoking a role for ROS in this process. However, using a flow cytometry analysis technique, we were unable to detect \( \text{H}_2\text{O}_2 \) production in DNR-treated cells before 60 min. Furthermore, reports using electron spin resonance, includ-
ing the most recent studies, only detected anthracycline-produced \( \cdot \)OH at 1 to 2 h, and at much higher drug concentrations (Yang et al., 1996). This could be due to the relatively low sensitivity of the techniques used. Indeed, when cells were pretreated with BSO, which depletes GSH store, DNR induced a weak but significant \( \text{H}_2\text{O}_2 \) production within 5 to 10 min, whereas BSO alone did not influence the intracellular \( \text{H}_2\text{O}_2 \) level. These observations suggest that DNR does indeed lead to early ROS production, which in turn results in N-SMase stimulation. This hypothesis is supported

Fig. 4. Inhibition by N-Ac of DNR- or C6-CER-triggered AP-1 activation. U937 cells were preincubated or not with N-Ac (25 mM for 2 h) followed by exposure to DNR (0.1, 0.5, 1.0 \( \mu \text{M} \)) (A) or C6-CER (25 \( \mu \text{M} \)) (B). Nuclear extracts were isolated and gel shift assays were performed as described in Materials and Methods. Specificity was confirmed by adding 100- and 1000-fold excess cold AP-1 consensus oligonucleotide to 1 \( \mu \text{M} \) DNR-treated cells. 12-O-tetradecanoyl phorbol-13-acetate (phorbol ester; 100 nM) was used as a positive control.

Fig. 5. Inhibition by N-Ac of DNR-triggered apoptosis in U937 cells. Cells were preincubated in the absence (A and B) or the presence (C and D) of 25 mM N-Ac for 2 h followed by a 60-min incubation with (B and D) or without (A and C) 1.0 \( \mu \text{M} \) DNR. These cells were then washed and incubated for another 5 h in drug-free medium. Morphological alterations of chromatin were evaluated by DAPI staining and viewed at an original magnification of 50×.
by two recent studies that showed that ROS influence N-SMase activity. It has been reported that GSH inhibits both in vitro and in vivo N-SMase in both leukemic and epithelial tumor cells; in addition, pretreatment with GSH inhibits TNFα-induced SM hydrolysis and CER generation as well as cell death (Liu et al., 1998). In a separate report, it has been described that H2O2 stimulates SM hydrolysis and CER generation, and that N-Ac and PDTC, another antioxidant, were potent inhibitors of TNFα-induced SM degradation to CER (Singh et al., 1998).

More studies are needed to determine the mechanism by which ROS generated by DNR stimulates SMase. Because on one hand, phosphorylation events appear to play a central role in the regulation of SMase activity (Mansat-De Mas et al., 1997b) and on the other hand, there is mounting evidence that ROS modulate both PKC and tyrosine kinase activities (Chen et al., 1996a; Ohmori et al., 1998), one could speculate that DNR-induced ROS interfere with SMase activity by influencing the subcellular localization or the activity of one or several critical protein kinase(s). Alternatively, it could be possible that DNR-induced ROS trigger the generation of second messengers, which in turn may influence SMase activity. For example, anthracyclines may stimulate PLA2 activity (Mustonen and Kinnunnen, 1991), and PLA2 products are possibly involved in the regulation of SMase activity (Jayadev et al., 1994); perhaps DNR-induced ROS stimulate PLA2, which in turn triggers SMase activity. Whatever the mechanism by which ROS exert their regulatory function on SMase, our study may have important clinical implications. Indeed, if oxidative stress does represent a critical early event in the DNR-induced apoptosis signaling pathway, it is conceivable that overexpression and/or activity of some antioxidant systems may explain the lack of apoptosis inducibility in nonresponsive leukemic cells. This hypothesis may account for the facilitating effect of BSO, a pro-oxidant agent, on DNR-induced DNA fragmentation in resistant acute myeloid leukemia cells (Quillet-Mary et al., 1996).

Our study shows that DNR activates the JNK 1 through an N-Ac-inhibitable mechanism. Anthracycline-induced JNK activation has been reported previously (Osborn and Chambers, 1996). In fact, most genotoxic agents, including ionizing radiation (Kharbanda et al., 1995a; Chen et al., 1996b), cytosine arabinoside (araC) (Kharbanda et al., 1995b; Bradshaw et al., 1996), cis-platinum, mitomycin C (Kharbanda et al., 1995c), and etoposide (Osborn and Chambers, 1996) activate JNK. One potential function of JNK may be the initiation of programmed cell death through c-Jun phosphorylation. Indeed, it was shown that overexpression of MEKK1, a potent and specific in situ activator of SEK1 and JNKs, had a lethal effect on fibroblasts (Lassignal Johnson et al., 1996), and enhanced the apoptotic response to UV irradiation (Lassignal Johnson et al., 1996). More recently, it has been shown that overexpression of ASK1, another SEK1 regulator, induced apoptotic cell death (Ichijo et al., 1997). Conversely, the expression of dominant negative mutants of JNK regulator proteins prevented the UV-C-, γ radiation-, and cis-platinum-induced cell death (Chen et al., 1996c). Therefore it is admitted that JNK-c-Jun activation by antitumor agents contributes to apoptosis. However, the role of ROS in cytotoxic agent-activated JNK signaling pathway is still debated. Indeed, although ionizing radiation and most DNA-damaging agents that activate JNK can generate free radicals, it is generally believed that DNA damage is the dominant stimulus for JNK activation. As far as anthracyclines are concerned, and based on our experiments with N-Ac, it can be concluded that ROS are most critical for DNR-activated JNK signaling pathway and apoptosis.

Our study also shows that permeant ceramides induce JNK1 activation through an N-Ac-inhibitable mechanism. The fact that CER activates the JNK signaling pathway was expected because it has been extensively documented that exposure to permeant CER analogs, as well as treatment with exogenous SMase, results in a rapid (within 10–15 min) stimulation of JNK in a number of cellular models (Westwick et al., 1995; Verheij et al., 1996; Huang et al., 1997; Hartfield et al., 1998). Furthermore, the role of JNK in ceramide-induced

Fig. 6. Inhibition by N-Ac and PDTC of DNR-triggered apoptosis in U937 cells. Cells were preincubated in the absence or presence of 25 mM N-Ac for 2 h or 10 μM PDTC for 30 min, followed by a 60-min incubation with or without 1.0 μM DNR. These cells were then washed and incubated for another 5 h in drug-free medium. DNA fragmentation was assayed as described in Materials and Methods. Results are representative of four independent experiments (data are means; bars are S.E.). Open column, control; hatched column, N-Ac; filled column, PDTC.

Fig. 7. Kinetics of ROS production induced by C6-CER in U937 cells. U937 cells were incubated with C2938 fluorescent probe for 1 h followed by incubation in the absence or presence of 10 μM PDTC for 30 min then C6-CER (25 μM) for the time intervals indicated. The cells were washed and cell fluorescence was determined using flow cytometry. DFL1 was determined by comparing mean fluorescence of treated cells with that of untreated cells. Results are representative of three experiments. Inset: cells were preincubated or not 24 h with BSO (50 mM) then 1 h with fluorescent probe C2938, then exposed to 1.0 μM DNR for 5 min.
apoptosis was directly evidenced by using defective SEK mutants (Verhoij et al., 1996). However, to the best of our knowledge, the role of ROS in CER-induced JNK activation has not been previously investigated. We and others have reported that permeant ceramides induced significant H2O2 production, which was detected at 60 min, then increased up to 150 min in U937 cells, and that this H2O2 production resulted from mitochondrial oxidative metabolism disturbance (Quillet-Mary et al., 1997). The present study shows that CER exposure caused an earlier and more transient H2O2 burst production. The temporal relationship between CER-induced early ROS production and CER-induced JNK activation as well as the fact that antioxidants blocked both ROS production and JNK activation strongly suggests that ROS are involved in CER-induced JNK activation.

Because, on one hand, exogenous CER activates the JNK signaling pathway, and on the other hand, most genotoxic agents, TNFα, or Fas agonists may trigger the SM cycle (for a review, see Hannun 1996), it has been proposed that JNK activation by these cytotoxic molecules could be mediated by endogenous CER produced by SM hydrolysis (for a review, see Peña et al., 1997). In fact, the role of the SM cycle in JNK activation and apoptosis was evidenced in UV-irradiated human lymphoblasts (Huang et al., 1997). However, as far as cytokines or drugs are concerned, there is still limited direct evidence of a link between endogenous CER production and JNK activation. Moreover, in a recent study using p55 TNFα receptor deletion mutants, Kronke’s group demonstrated that TNFα can activate JNK without direct participation of SMase or CER (Adam et al., 1998). Similarly, it has been recently reported that there is no temporal relationship between N-SMase stimulation and JNK activation in AraC-treated cells (Bradshaw et al., 1996). Conflicting with these results, our study shows a temporal relationship between DNR-induced CER generation and JNK activation; furthermore, the lack of CER generation in N-Ac-treated cells correlates with the lack of JNK activation. These results strongly suggest that DNR-triggered SM cycle activation is responsible for JNK activation. However, because N-Ac inhibits CER-induced JNK activation, we cannot rule out that DNR activates JNK and apoptosis through a CER-independent but ROS-dependent mechanism. However, the observation that serine protease inhibitors and PKC activators block both DNR-triggered CER production and apoptosis strongly argues for the role of CER-generated ROS in apoptosis signaling (Mansat et al., 1997a,b).

We also observed that DNR activates AP-1 transcription factor through an N-Ac inhibitable mechanism. Because N-Ac inhibits DNR-induced JNK activation, it is not surprising that N-Ac also blocks DNR-induced AP-1 activation. Activation of c-jun/AP-1 has been described in cells treated with a number of cytotoxic compounds, including etoposide (Rubin et al., 1991), AraC (Kharbanda et al., 1990), cis-platinum (Rubin et al., 1992), and CER (Sawai et al., 1995), and it has been suggested that AP-1 activation plays a direct role in apoptosis (Bullock et al., 1995). However, whether or not anthracyclines may also induce c-jun/AP-1 activation was less documented and remains controversial. Indeed, whereas doxorubicin was found to stimulate c-jun phosphorylation or AP-1 binding activity (Osborn and Chambers, 1996), it has been recently reported that both doxorubicin and DNR were unable to induce AP-1 activation in A549 adenoma carcinoma cells (Das and White, 1997). However, it should be noted that in the latter study, DNR was used at 15 μM, a suprapharmacological dose that we found to be unable to trigger apoptosis in U937 cells, but rather necrosis (Quillet-Mary et al., 1996). In addition, previous reports have implicated PKC activation as a step leading to c-jun activation in the molecular cascade leading to apoptosis induced by antileukemic drugs. For example, in separate reports, the modulation of PKC activity has been shown to affect AraC-mediated c-jun induction as well as apoptosis (Kharbanda et al., 1991; Jarvis et al., 1994). Based on our findings, we propose, at least for anthracyclines (and perhaps for any drug that can trigger CER generation, including AraC), another mechanism according to which drug-induced AP-1 activation is mediated by ROS produced directly by the drug itself and/or indirectly by the endogenous CER issued from SM hydrolysis.

Finally, our study shows that early ROS production by DNR is a critical event for the stimulation of N-SMase, SM hydrolysis, CER generation, and apoptosis. Moreover, ROS appear to be involved in both DNR- and CER-induced JNK/c-jun signaling activation. These results suggest that ROS play an important role in the SM-CER apoptotic pathway triggered by DNR at two different levels: upstream CER generation by stimulating N-SMase activity and downstream CER production by mediating CER-induced JNK activation. They also suggest a novel function of ROS in the cytotoxicity mechanism of anthracyclines and may explain that cellular oxidative defenses can greatly influence the clinical efficacy of this class of drugs.

References


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