Communication

Reactive Oxygen Intermediates Are Involved in the Induction of CD95 Ligand mRNA Expression by Cytotoxic Drugs in Hepatoma Cells*

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Oxidative stress has been associated with the induction of programmed cell death. The CD95 ligand/receptor system is a specific mediator of apoptosis. We have used the model of drug-induced apoptosis to assess whether the CD95 ligand mRNA is induced by reactive oxygen intermediates. Treatment of HepG2 hepatoma cells with bleomycin induced the production of reactive oxygen intermediates and, as an additional parameter of oxidative stress, resulted in glutathione (GSH) depletion. In parallel, CD95 ligand mRNA expression was induced. In a similar fashion CD95 ligand mRNA expression increased after treatment with H2O2. Additional treatment with the antioxidant and GSH precursor N-acetylcysteine resulted in partial restoration of intracellular GSH levels and in reduced induction of CD95 ligand mRNA. Induction of CD95 ligand mRNA by bleomycin was further reduced by combined treatment with N-acetylcysteine and deferoxamine. These data suggest a direct role of oxygen radicals in the induction of the CD95 ligand.

CD95 (APO-1/Fas) is a 45-kDa glycosylated transmembrane protein belonging to the tumor necrosis factor receptor family of type I membrane proteins (1, 2). The CD95 ligand (CD95L) is a 40-kDa Type II transmembrane protein and a member of the tumor necrosis factor family of cytokines (1, 3). In addition to the transmembrane form, a soluble form of the CD95L exists.

1 The abbreviations used are: ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline.
Generation was detected by a chemiluminescence scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 μmol/L) treated HepG2 cells. Cells were treated with 3 mg/ml bleomycin for the indicated times and analyzed for CD95L mRNA by RT-PCR, yielding a DNA fragment of 467 base pairs. Human β-actin (661-base pair fragment) served as control for equal loading.

for 30 s, at 56 °C for 30 s, and at 72 °C for 2 min in a volume of 100 μL. 10 μL of the PCR sample were analyzed on 1.5% agarose gels. In all cases at least three independent sets of experiments were performed.

FACS Analysis—Floating cells from the tissue culture supernatant were collected by centrifugation at 200 × g. Adherent cells were harvested by incubation with 1% trypsin. HepG2 cells were collected by centrifugation at 200 × g, washed with PBS, and fixed in 70% ethanol. This was followed by staining with propidium iodide (50 μg/ml PBS). DNA fluorescence was measured in a Becton Dickinson FACSscan according to the method of Nicoletti et al. (24). A minimum of 10,000 events was measured per sample. Data analysis was performed with Lysis II software.

Detection of Intracellular Glutathione—HepG2 cells were maintained on 35-mm plates. After bleomycin treatment cells were harvested with a cell scraper, washed in PBS, and finally taken up in 300 μL of the PCR sample were analyzed on 1.5% agarose gels. All cases at least three independent sets of experiments were performed.

Detection of Reactive Oxygen Species—O2− generation was detected by a chemiluminescence reaction as described previously (26). HepG2 cells were subconfluently seeded in sterile scintillation vials and treated with 3 mg/ml bleomycin for 24 h. Thereafter, medium was discarded and replaced by the scintillation solution containing 0.25 mmol/L lucigenin (Sigma) dissolved in 2 ml of Krebs-HEPES buffer. Counts were obtained at 1-min intervals at room temperature. To determine the specificity of the reaction the O2− scavenger 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 mmol/L, Sigma) was added.

RESULTS AND DISCUSSION

Bleomycin-Induced Apoptosis and Activation of the CD95 System in Hepatoma Cells—Treatment with bleomycin in concentrations between 10 μg/ml and 3 mg/ml induces apoptosis as demonstrated by the appearance of a sub-G1 fraction of apoptotic nuclei. FACS analysis according to Nicoletti et al. (24) (Fig. 1A; see also Ref. 15) and by morphological and DNA fragmentation analysis (data not shown). This is accompanied by an induction of CD95 mRNA (15) and also of CD95L mRNA (Fig. 1B). The functional relevance of this observation has been demonstrated by blocking access of CD95L to the CD95 receptor using F(ab′)2 antagonistic antibody fragments which largely inhibited induction of apoptosis (15). Here, HepG2 cells were treated with 3 mg/ml bleomycin for 0, 5, 10, 24, 32, and 48 h, and expression of CD95L mRNA was assessed by RT-PCR. CD95L mRNA expression was detectable after 5 h, showed its highest level between 24 and 32 h, and decreased around 48 h (Fig. 1B).

Recently we demonstrated dependence of CD95 induction on the presence of the tumor suppressor gene product p53 (15). CD95 expression took place only in hepatoma cell lines with p53 wild type configuration (HepG2) but not with p53 mutant configuration (HuH7) or in the absence of p53 (Hep3b). In contrast, CD95L mRNA was found to be inducible independently of the presence of p53 wild type and thus, seems to be regulated in a different manner (15). Since bleomycin treatment has been described to result in oxidative stress (21, 22), we posed the question whether CD95L mRNA activation is correlated to the generation of ROS in response to bleomycin treatment.

Bleomycin Treatment Results in Oxidative Stress—In initial experiments we investigated the induction of oxidative stress following bleomycin treatment in hepatoma cells. As a parameter of oxidative stress intracellular glutathione (GSH) levels were assessed. Bleomycin treatment resulted in a rapid depletion of total GSH from initial values of 18 nmol/mg of protein in untreated controls to 0.7 nmol/mg of protein after 48 h (Fig. 2A) indicating disturbances in the cellular redox status. Additional treatment with N-acetylcysteine partially prevented GSH depletion of bleomycin-treated cells, with GSH levels of 14.8 nmol/mg of protein after 48 h.

As a direct measure of induction of reactive oxygen species in
bleomycin-treated cells we investigated generation of superoxide (O$_2^-$) using a chemiluminescence assay (26). We observed a strong induction in chemiluminescence following treatment with 3 mg/ml bleomycin for 24 h. This signal could be completely blunted by addition of the O$_2^-$ scavenger Tiron, demonstrating specificity of the reaction.

**Induction of CD95L mRNA by H$_2$O$_2$ and Inhibition by Antioxidants**—To establish a causative relationship between the observed induction of CD95L mRNA and presence of reactive oxygen species we treated HepG2 cells with H$_2$O$_2$. In a manner similar to bleomycin treatment H$_2$O$_2$ at concentrations between 0.1 and 10 μM induced expression of CD95L mRNA (Fig. 3A). Higher concentrations proved to be cytotoxic as demonstrated by a decrease in cell number and a consecutive decrease in β-actin and CD95L mRNA expression (data not shown).

Further evidence for ROS-mediated CD95L mRNA expression was obtained from experiments with the antioxidants deferoxamine and N-acetylcysteine. Deferoxamine is an iron chelator. It prevents the formation of the hydroxyl radical from hydrogen peroxide via the Fenton reaction (27). N-Acetylcysteine interferes with the generation of ROS and is a glutathione precursor. A direct effect of glutathione depletion on the induction of apoptosis seems unlikely, because glutathione depletion alone (using buthionine sulfoximine treatment of human leukocytes) failed to induce apoptosis (28). HepG2 cells were treated with bleomycin (3 mg/ml) for up to 48 h in the absence or presence of antioxidants as shown in Fig. 3B. At -32 h maximum expression of CD95L mRNA was reached. This expression was reduced in the presence of N-acetylcysteine and deferoxamine (Fig. 3B, lower panel). Deferoxamine alone did not result in a reproducible decrease of CD95L mRNA expression (data not shown). In a similar fashion, H$_2$O$_2$-induced CD95L expression was reduced in the presence of N-acetylcysteine and deferoxamine (data not shown).

Taken together the above data point to an important role of ROS in the transcriptional regulation of CD95L expression. Interestingly and in agreement with our experimental evidence in hepatoma cells, a positive correlation between the intracellular levels of ROS and CD95L expression has been observed recently in activation-induced death of mature T lymphocytes and hybridomas (29). The exact mechanism of CD95L mRNA induction via ROS remains to be clarified. Potentially involved regulatory proteins include redox-dependent transcription factors such as NF-κB or AP-1.

Our data suggest a coordinated activation of the CD95 system in induction of apoptosis and thus elimination of injured cells, which involves p53-mediated expression of the CD95 receptor in response to DNA damage and ROS-mediated expression of the CD95 ligand. This might result in autocrine suicide of damaged cells and might add to the concept of maintenance of genomic integrity as it has been suggested as an important functional role of p53. Any alteration of the cellular capability to respond to cytostatic agents both on the level of CD95 or CD95L might result in a decreased sensitivity toward chemotherapeutic drug action and could thus add to primary or secondary resistance to anti-cancer therapy.

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