Hypersensitivity to DNA Cross-linking Agents Associated with Up-Regulation of Glucose-regulated Stress Protein GRP78

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ABSTRACT

We have previously shown that NAD/poly(ADP-ribose) polymerase-deficient cells that overexpress M. 78,000 glucose-regulated stress protein (GRP78) are resistant to topoisomerase II inhibitors, such as etoposide, m-amsacrine, and doxorubicin. However, these cells have been found to be hypersensitive to DNA cross-linking agents, including melphanal, cisplatin, and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). These observations prompted us to examine whether overexpression of GRP78 is associated with modulation of cytotoxicity of clinically useful DNA-cross-linking agents such as melphanal, BCNU, and cisplatin. We up-regulated GRP78 in V79 Chinese hamster cells by 2-5-fold using two independent approaches that include exposure to 6-aminonicotinamide, or 2-deoxyglucose. Subsequently, these GRP78-overexpressing cells were trypsinized, plated in regular medium without GRP78-inducing agents, and allowed a 5-h attachment time before being treated with melphanal, BCNU, or cisplatin for 1 h to determine clonogenic survivals. In addition, repair of DNA cross-links induced by those agents were determined by alkaline elution assay. Our results show that the GRP78-overexpressing V79 cells are hypersensitive to DNA cross-linking agents compared to the control V79 cells. Furthermore, repair of drug-induced DNA cross-links appears to be considerably slower in these cells relative to that found in control V79 cells. Thus, our results suggest that (a) up-regulation of GRP78 is associated with an impairment of DNA cross-link repair, (b) up-regulation of GRP78 is associated with potentiation of cytotoxicity induced by alkylating and platining agents, and (c) up-regulation of GRP78 can be considered as a potentially useful tool to modulate the cytotoxicity of clinically useful alkylating and platining agents.

INTRODUCTION

We previously reported the development of V79 Chinese hamster derived mutant cell lines defective in poly(ADP-ribose) synthesis due to deficiency in the enzyme PARP or its substrate NAD (1, 2). These cell lines have been shown to be hypersensitive to a variety of bifunctional DNA cross-linking agents compared to their parental V79 cells (3–5). In contrast, these cell lines are significantly resistant to topo II inhibitors such as etoposide, m-amsacrine, and Adriamycin (6, 7).

Because previous studies had shown that elevated levels of glucose-regulated stress protein, GRP78, are associated with resistance to topo II-active agents (7–10), we evaluated these mutant cells and demonstrated that GRP78 levels are elevated constitutively at both the transcriptional and translational levels (7, 8). Thus, our studies show that the up-regulation of GRP78 in poly(ADP-ribose) synthesis-deficient cell lines is associated with resistance to topo II inhibitors (7). GRP78, a stress protein located in the ER of virtually all mammalian cells, associates transiently with nascent proteins, facilitates their translocation into the ER and aids their folding and transport through ER (11). For example, GRP78 associates with nascent heavy and light chains in B lymphocytes, until they assemble to form complete immunoglobulin molecules (12–16). Thus, GRP78 is thought to function as a catalyst of protein assembly, binding and hydrolyzing ATP as it associates and dissociates from peptides and proteins (17–19). However, various stress situations, such as glucose deprivation, anoxia, and so forth, up-regulate GRP78 levels (11).

In subsequent studies, we showed that treatment of cells with 6-AN, an agent that interferes with NAD and PARP metabolism, resulted in up-regulation of GRP78 and subsequent development of resistance to topo II inhibitors (8). Furthermore, the degree of 6-AN-induced over-expression of GRP78 correlates with the degree of resistance to topo II inhibitors. Because our initial studies demonstrated that NAD/PARP-deficient cells are hypersensitive to the cytotoxic effects of a variety of DNA adduct-forming agents, the present study was conducted to determine whether the pharmacological up-regulation of GRP78 would be associated with the development of hypersensitivity to alkylating and platining agents. These studies were conducted using either 6-AN or 2-dG to up-regulate GRP78 in V79 Chinese hamster cells. Studies were also carried out to investigate the effects of GRP78 overexpression on alkylating and platining agent-induced DNA cross-link repair.

MATERIALS AND METHODS

Materials. 6-AN, 2-dG, cisplatin, and melphanal were obtained from Sigma Chemical Co. (St. Louis, MO). BCNU (NSC 409962) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 6-AN and 2-dG were dissolved in distilled water to prepare stock solution of 10 mm and 1 m, and aliquots were kept frozen at −20°C. Cisplatin was prepared fresh in DMSO (10 mm) and diluted in PBS prior to use. Melphanal was prepared fresh by dissolving it in acid/alcohol (95% alcohol and 5% 1 N HCl) at a concentration of 10 mm and diluted in PBS before treatment. Prior to each experiment, BCNU solutions were prepared fresh at a concentration of 10 mm in 100% ethanol and diluted subsequently in PBS.

Cell Culture Maintenance and Clonogenic Survival Assays. V79 Chinese hamster cells were maintained in α-modified MEM buffered to pH 7.2 with 25 mm HEPES and supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% heat-inactivated FCS. To determine the effect of over-expression of GRP78 on the cytotoxicity of alkylating and platining agents by clonogenic survival assays, V79 cells were grown in 100 µm 6-AN or 10 mm 2-dG for 48 h to up-regulate GRP78. Subsequently, these cells were washed twice with PBS, trypsinized, diluted in regular growth medium, and allowed to attach in 60 mm plates for 5 h. This attachment period is necessary to allow NAD/PARP metabolism to become normal, because 6-AN treatment interferes with NAD/PARP metabolism. Following attachment, cells were subjected to drug treatment for 1 h at 37°C in growth medium to determine clonogenic cytotoxicity. Procedures for clonogenic survival assays were described previously (3–6, 20–22). Thus, this strategy provides us with the opportunity to associate overexpression of GRP78 with clonogenic cytotoxicity.

Determination of DNA Cross-Links. Cells grown in medium, containing [14C]thymidine under identical conditions as outlined above, were exposed to the drugs following a similar treatment protocol. These cells were subjected to an alkaline elution procedure to determine the amount of DNA cross-links, as
Fig. 1. V79 cells were grown in 100 μM 6-AN or 10 mM 2-dG for 48 h, washed twice with PBS, and allowed to further grow in regular medium for 5 or 24 h. Cytosolic extracts were then prepared from these cells and analyzed by SDS-PAGE and Western blotting with anti-GRP78 monoclonal antibody, as described in “Materials and Methods.” Lane 1, V79 untreated control; Lane 2, cells were grown in 2-dG and then grown in regular medium for 5 h; Lane 3, same as Lane 2, except that the cells were grown in 2-dG and then grown in regular medium for 24 h; Lane 4, cells grown in 6-AN and then grown in regular medium for 5 h; Lane 5, same as Lane 4, except that the cells were grown in regular medium for 24 h after 6-AN treatment.

described previously in detail (20, 21, 23). V79 cells labeled with [3H]thymidine and irradiated with 6 Gy γ-rays served as internal controls.

Cytoplasmic Preparation, Gel Electrophoresis, and Western Blotting. Cells were trypsinized, suspended in medium, and collected by centrifugation. The cell pellets were washed twice with cold PBS and resuspended finally at 4°C for 15 min in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM glucose, 10 mM iodoacetamide, 1% Triton, 1 mM phenylmethylsulfonyl fluoride (freshly added from a 1 M stock in anhydrous methanol), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. The samples were then centrifuged at 14,000 × g for 20 min at 4°C, and the clear supernatant cytosol was collected. Aliquots were removed for protein determination by the method of Bradford (24), using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). As described previously (7, 8), 22 μg of cytosolic protein samples were separated by SDS-PAGE (gel consisted of 10% separating and 5% stacking gel) according to the method of Laemmli (25). The gel was then transferred electrophoretically at room temperature onto Immobilon-P membrane (Millipore, Bedford, MA) for 1 h at 24 V, as described by Towbin et al. (26). The membrane was then blocked for 10 min in a 5% solution of nonfat dry milk in 0.1% T-PBS. Subsequently, the blot was incubated for 1 h in a 1:500 dilution of a monoclonal anti-GRP78 (StressGen Biotechnology, Victoria, British Columbia, Canada) and then washed three times in T-PBS. The blot was incubated in 1:1000 horseradish peroxidase-conjugated secondary antibody (antimouse immunoglobulin; Amersham Corp., Arlington Heights, IL). After drying three times in T-PBS, the blot was placed in chemiluminescence detection reagent (Boehringer Mannheim, Indianapolis, IN) and exposed on Hyperfilm (Amersham Corp., Buckinghamshire, England) at room temperature. The level of actin for each lane was obtained by replotting the blot with a monoclonal antibody to actin (Amersham Corp., Arlington Height, IL). GRP78 protein band intensity were quantitated by densitometric scanning (SC1scan 5000, United States Biochemical Corp., Cleveland, OH) and normalized to the intensity of the corresponding actin band that served as an internal control.

RESULTS

Effects of 6-AN or 2-dG Treatment on GRP78 Levels. To confirm the agent’s ability to induce GRP78, the V79 cells were exposed to 100 μM 6-AN or 10 mM 2-dG under two different conditions. In one case, cells were treated with 6-AN or 2-dG for 48 h, trypsinized, and collected by centrifugation. The cell pellet was then used to prepare cytosolic fractions, as described in “Materials and Methods,” and subsequently, proteins in the cytosolic fractions were analyzed by SDS-PAGE and Western blotting with anti-GRP78 and antiactin antibody. In the second case, to analyze the decay of elevated GRP78, protein, cells were grown in 6-AN or 2-dG for 48 h and then washed with PBS and incubated further in regular medium without 6-AN or 2-dG for another 24 h. Following this incubation, the cytosolic fractions of these cells were analyzed as outlined above. As shown in Fig. 1, treatment of cells with either 6-AN or 2-dG caused substantial elevation of GRP78 levels compared to the control V79 cells. The levels of GRP78 remained elevated significantly even in cells that were further grown for 24 h without 6-AN or 2-dG in the medium. These studies demonstrate that treatment of cells with 6-AN or 2-dG significantly up-regulates GRP78, and these elevated levels of GRP78 remain high for at least another 24 h following removal of 2-dG or 6-AN.

Effects of 6-AN Treatment on the Cytotoxicity of BCNU, Cisplatin, and Melphalan. To examine the association of elevated GRP78 levels with alkylating agent and platinating agent cytotoxicity, V79 cells were treated with 100 μM 6-AN for 48 h, following which cells were incubated in regular medium without 6-AN for 5 h to allow clearance of 6-AN and return of NAD levels while maintaining high levels of GRP78. These cells were then exposed to various concentrations of BCNU, cisplatin, or melphalan for 1 h and evaluated subsequently for clonogenic survival. Fig. 2, A–C, shows clearly that pretreatment with 6-AN causes a significant potentiation of cytotoxicity of BCNU, cisplatin, and melphalan, respectively, compared to the control V79 cells. This sensitization is much more pronounced in cells that were further grown for 24 h without 6-AN or 2-dG in the medium. These studies demonstrate that treatment of cells with 6-AN or 2-dG significantly up-regulates GRP78, and these elevated levels of GRP78 remain high for at least another 24 h following removal of 2-dG or 6-AN.

Effects of 2-dG Treatment on the Cytotoxicity of BCNU, Cisplatin, and Melphalan. Similar experiments were performed as outlined above, except V79 cells were treated with 10 mM 2-dG for 48 h
to induce GRP78, and then cells were incubated in the absence of 2-dG for 5 h before exposure to cytotoxic agents. 2-dG is a well-established inducer of GRP78 that does not interfere with NAD/PARP metabolism. This approach of inducing GRP78 with 2-dG provides us the opportunity to evaluate the effect of GRP78 up-regulation on alkylating agent sensitivity without interfering with NAD/PARP metabolism. Fig. 3, A–C, shows that pretreatment with 2-dG results in sensitization of V79 cells to BCNU, cisplatin, and melphalan similar to that induced by 6-AN. Sensitization was again noted to be greater for melphalan and cisplatin compared to that for BCNU. Thus, these results provide independent confirmation that up-regulation of GRP78 is associated with significant sensitization to alkylating and platinating agents.

Effects of 6-AN Treatment on DNA Cross-Link Repair Induced by BCNU, Cisplatin, and Melphalan. We subsequently used alkaline elution to analyze the kinetics of formation and disappearance of DNA cross-links induced by alkylating and platinating agents following the 6-AN treatment protocol outlined above. Our results (Fig. 4, A–C) show clearly that, during the experimental time frame, the levels of DNA cross-links induced by BCNU, cisplatin, or melphalan appear to be consistently higher in 6-AN-pretreated cells compared to the control V79 cells. These results suggest that up-regulation of GRP78 resulting from 6-AN treatment is associated with a significant reduction in the rate of DNA cross-link repair and that the increased levels of unrepaired cross-links are associated with the hypersensitivity demonstrated by these cells.

DISCUSSION

We have shown previously that the use of 6-AN to interfere with cellular NAD and PARP metabolism results in up-regulation of GRP78 and subsequent development of resistance to topo II-active agents (8). In this study, we report that cells overexpressing GRP78 induced by 6-AN treatment are hypersensitive to clinically useful DNA-damaging agents, such as BCNU, melphalan, and cisplatin compared to control V79 cells. We have also shown that similar pretreatment with 2-dG, a known inducer of GRP78 that has no effect on NAD/PARP metabolism, potentiates the cytotoxicity of BCNU, melphalan, and cisplatin to a similar extent as does 6-AN. Because 6-AN and 2-dG affect different cellular metabolic pathways, these results with two different inducers of GRP78 indicate that hypersensitivity to alkylating and platinating agents is associated with the up-regulation of GRP78 rather than with the direct presence of the agents responsible for inducing its overexpression.

Our results show clearly that pretreatment of V79 cells with 6-AN or 2-dG significantly up-regulates GRP78 and potentiates the cytotoxic action of BCNU, cisplatin, and melphalan. Furthermore, we have shown that DNA cross-link frequencies induced by the DNA adduct-forming agents are considerably higher in 6-AN-treated cells compared to V79 cells. These differences were most prominent at 12 h following withdrawal of the cross-linking agents, whereas at earlier time points, there was less difference in alkylating agent-induced cross-links. These results suggest that elevated GRP78 is associated
with slower DNA cross-link removal in 6-AN-pretreated cells compared to untreated cells. Although inhibitors of PARP metabolism have been shown to sensitize cells to alkylating agents, the present study shows that interference with NAD/PARP metabolism may also sensitize cells to alkylating and platinating agents indirectly through up-regulation of GRP78. Thus, interference with NAD/PARP metabolism appears to be one stimulus to initiate up-regulation of GRP78, which is then associated with acquisition of hypersensitivity to alkylating and platinating agents. Our original studies showed that cells with defective NAD and PARP metabolism had a constitutive increase in GRP78 and were hypersensitive to alkylating and platinating agents (4, 5, 7). We showed subsequently that use of 6-AN to interfere pharmacologically with NAD and PARP metabolism also results in up-regulation of GRP78 and subsequent induction of alkylating agent hypersensitivity (8, 27). In the present study, we have shown that cells with elevated GRP78 retain their hypersensitivity to alkylating and platinating agents despite removal of 6-AN. Thus, although interference with NAD/PARP metabolism may be a primary stimulus through which 6-AN treatment leads to elevated GRP78 levels, this interference is no longer necessary for potentiation of alkylating agent cytotoxicity once GRP78 levels are up-regulated. This proposition is supported further by the fact that treatment of cells with 2-dG, an agent that does not interfere with NAD/PARP metabolism but clearly serves as a strong inducer of GRP78, results in similar sensitization to alkylating and platinating agents. In fact, cells retain elevated GRP78 levels and remain hypersensitive to alkylating agents even 24 h after removal of 6-AN or 2-dG.

The cytotoxicities of cisplatin, melphalan, and BCNU are each mediated, in part, by formation of DNA cross-links. Cisplatin causes DNA interstrand and intrastrand cross-links; a majority of the lesions are repaired through the excision repair pathway (28). Melphalan (L-phenylalanine mustard) belongs to the nitrogen mustard family of agents, which predominantly exert their effects when the lesions are repaired through the excision repair pathway (28). These cross-links are also repaired by the excision repair process (28). Treatment of cells with BCNU results in the formation of a variety of DNA adducts, including interstrand cross-links between guanine and cytosine, which are presumed to be the major cause of cytotoxicity (29). In many cell types, the primary repair mechanism for BCNU-induced adducts is mediated through a specific DNA repair protein, O6-alkylguanine DNA alkyltransferase (29). Because V79 cells have essentially undetectable levels of O6-alkylguanine DNA alkyltransferase (30, 31), it is probable that BCNU-induced adducts are also repaired in V79 cells by the excision repair process. Thus, excision repair appears to be important in correction of cisplatin-, melphalan-, and BCNU-induced damage in these cells. These observations, in conjunction with our results, suggest that GRP78 up-regulation is associated with impaired excision repair of DNA adducts resulting in a delay in the disappearance of cross-links and increased cytotoxicity.

In summary, our studies indicate that (a) up-regulation of GRP78 is associated with sensitization to DNA cross-linking agents, (b) the sensitization is independent of the nature or presence of the agents used to up-regulate GRP78, and (c) the sensitization is associated with impairment of DNA cross-link repair. These studies indicate that it will be useful to study the effects of GRP78 up-regulation on various DNA excision repair processes to further elucidate the molecular mechanism of the sensitization process. More importantly, these studies suggest a new strategy for enhancing the clinical effectiveness of DNA alkylating and platinating agents by up-regulating tumor GRP78. This mechanism may explain our earlier observation that 6-AN pretreatment potentiated the antileukemic effect of BCNU in L1210 cells in vitro and in vivo (32). Thus, we showed previously that treatment with varying combinations of BCNU and 6-AN produced a synergistic increase in life span and cures of mice bearing L1210 leukemia (32).

REFERENCES

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