Galectin-3: A Novel Antiapoptotic Molecule with A Functional BH1 (NWGR) Domain of Bcl-2 Family

Shiro Akahani, Pratima Nangia-Makker, Hidenori Inohara, Hyung-Reh Choi Kim, and Avraham Raz

Tumor Progression and Metastasis Program [S. A., P. N.-M., H. I., A. R.], Karmanos Cancer Institute, and Departments of Pathology [H-R. C. K.] and Radiation Oncology, Wayne State University School of Medicine [A. R.], Detroit, Michigan 48201

Abstract

Galectin-3, a β-galactoside-binding protein, has been shown to be involved in tumor progression and metastasis. Here, we demonstrate that expression of galectin-3 in human breast carcinoma BT549 cells inhibits cis-diaminedichloroplatinum (cisplatin)-induced poly(ADP-ribose) polymerase degradation and apoptosis, without altering Bcl-2, Bcl-xL, or Bax expressions. Galectin-3 contains the NWGR amino acid sequence highly conserved in the BH1 domain of the bcl-2 gene family, and a substitution of glycine to alanine in this motif abrogates its antiapoptotic activity. Our findings demonstrate that galectin-3 inhibits apoptosis through a cysteine protease pathway and highlight the functional significance of the NWGR motif in apoptosis resistance of a non-Bcl-2 protein.

Introduction

A number of antineoplastic agents have been developed for the eradication of malignancies, but successful chemotherapy still depends on the control of multidrug resistance, because failure may lead to mortality. For example, CDDP, a potent anticancer compound, which functions through interstrand DNA cross-links and the induction of apoptosis (1), has improved the outcome of many cancer patients, but the mechanism(s) of CDDP resistance remains to be defined. Expression of galectin-3, a Mr 31,000 carbohydrate-binding protein (2), correlates with neoplastic progression in head and neck cancer (3), thyroid cancer (4), gastric cancer (5), and colon cancer (6). In several experimental tumor systems, galectin-3 expression is related to the metastatic potential (7, 8). Recently, it has been suggested that galectin-3 may inhibit apoptosis through interactions with complementary carbohydrates (9) or with the antiapoptotic protein Bcl-2 (10). Galectin-1 and -9 have been reported to induce apoptosis (11-13), suggesting that some members of the galectin family are involved in the regulation of apoptosis. Moreover, a domain in the COOH terminus of galectin-3 was found to have a significant sequence similarity with the BH1 domain of the Bcl-2 family of proteins containing the NWGR motif (10), which is responsible for the antiapoptotic activity of Bcl-2. Further investigation was thus prompted to establish a possible role of galectin-3 in the mechanism of drug-induced apoptosis. To this end, we have used the recently identified galectin-3-null cells, i.e., the human breast carcinoma BT549 cells. In this study, we demonstrate that expression of galectin-3 in human breast carcinoma BT549 cells inhibits CDDP-induced PARP degradation and apoptosis, and that the NWGR motif of galectin-3 is required for its antiapoptotic activity, as determined by analysis of a mutagenesis study showing that an amino acid substitution of glycine to alanine at position 182 abrogates its function.

Materials and Methods

Cells and Culture Conditions. The human breast cancer cell line BT549 was obtained from Dr. E. W. Thompson, Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center (Washington, D.C.). BT549 transfectants with plasmid DNA accompanying inserts in either the sense (11811, 11913, and 11914) or the antisense (41421) orientation encoding the human galectin-3 cDNA were established as reported previously (8). These cell lines were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, nonessential amino acids, and antibiotics (Life Technologies, Inc.). The cultures were maintained at 37°C in a humidified atmosphere of 7% CO2 and 93% air. Cultures were used for the experiments within three passages after recovery from frozen stocks.

Drugs. CDDP (Sigma Chemical Co., St. Louis, MO) was dissolved in the filtered normal saline and stored at 4°C until use.

Galectin-3 and Bcl-2 Expressions in Untreated BT549 Cells and Transfectants. Cells (5×106) were pelleted by centrifugation, washed with CMF-PBS (pH 7.4), and subsequently lysed in 1 ml of reducing SDS-PAGE sample buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 5% β-mercaptoethanol. Then, cell lysates were agitated overnight at room temperature and stored at -70°C until use. For Western blot analyses, approximately 20 μg of total proteins were boiled for 5 min, separated on 10% SDS-PAGE, and blotted onto a nitrocellulose membrane. Blots were probed with rat anti-galectin-3 (American Type Culture Collection, Rockville, MD) or mouse anti-Bcl-2 antibody (DAKO Corp., Carpinteria, CA) according to the enhanced chemiluminescence (Amersham, Buckinghamshire, England)-Western blot procedure.

Cell Viability. Cell viability was assessed by a trypan blue dye exclusion test. Cells were cultured with or without CDDP and were collected as described by Huddart et al. (14). Briefly, at the indicated times, cells were trypsinized and centrifuged at 2000 rpm for 5 min. Viability was evaluated on the hemacytometer (Baxter, McGaw Park, IL) following the addition of 0.4% trypan blue (Matheson, Coleman, and Bell, Norwood, OH) dissolved in 0.85% saline.

DNA Degradation. DNA fragmentation induced by CDDP was detected as an increased fluorescence in the hypoploid DNA region of a flow cytometric cell cycle histogram according to the method described by Telford et al. (15). Briefly, cells with or without CDDP treatment were fixed with 80% ethanol at 4°C for 30 min, washed with CMF-PBS (pH 7.4), and resuspended with 50 μg/ml propidium iodide (Coulter Immunology, Hialeah, FL) diluted with CMF-PBS (pH 7.4) containing 0.1% Triton X-100, 0.1 mM EDTA (pH 8.0), and 50 μg/ml RNase A. Then, samples were agitated at 4°C for not less than 15 min prior to analysis with flow cytometry.

Cell Morphology. The change of cell morphology with CDDP treatment was assessed by fluorescence microscopy using the DNA-binding fluorochrome bis-(benzimidazole)-triiodohycridone ( Hoechst 33258; Sigma) as described previously by Oberhammer et al. (16). Briefly, 1×106 CDDP-treated cell pellets were collected by centrifugation at 2000 rpm for 5 min and fixed with 3% paraformaldehyde at room temperature for 10 min. Then, cells were...
washed with CMF-PBS (pH 7.4) and stained in the dark with 8 μg/ml Hoechst 33258 in CMF-PBS (pH 7.4) at room temperature for 15 min. Samples were loaded on the glass slide coated with 3-aminopropyl-trimethoxysilane (Sigma), and chromatin condensation and nuclear fragmentation in apoptotic cells were observed with a fluorescence microscope equipped with a UV filter.

**Activation of CPP32/Yama Protease and PARP Degradation.** Before and after the treatment with 25 μM CDDP for 72 h, cell lysates were electrophoresed on reducing or 15% SDS-PAGE, transferred to nitrocellulose, and probed with mouse anti-PARP antibody or goat anti-CPP32/Yama antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

**Site-directed Mutagenesis of Galectin-3.** Mutated galectin-3 (glycine-182-alanine) was designed and obtained using the in vitro site-directed mutagenesis kit (Stratagene, La Jolla, CA) and cloned into the EcoRI site of the pCNC10 vector. For transfection, 2 μg of plasmid DNA were isolated and incubated with 10 μl of lipofectamine (Life Technologies, Inc.), and this mixture was added onto the cells grown to 70% confluence. Clones were selected at random using 800 μg/ml G418 (Life Technologies, Inc.) and maintained in 10% complete DMEM containing 400 μg/ml G418. Oligonucleotide primers for PCR were sense (5'-TGGATAATAACTGGGCAAGG-GAAGAAAG-TT) and antisense (5'-CTTTCCTCCCTGCGCCAGTTAT-TATCCA-3'), encoding 548–575 nucleotides in human galectin-3 cDNA.

**Results and Discussion**

Human breast carcinoma BT549 cell clones transfected with plasmid DNA containing inserts in either the sense (11811, 11913, 11914) or the antisense control (41421) orientation encoding human galectin-3 were established from the galectin-3-null parental cells as reported previously (8). We have confirmed galectin-3 expression in the parental BT549 cells and its clones 41421 and 11914 by Western blot analysis, and only sense-transfected cell clones expressed galectin-3, whereas the other two cell types remained galectin-3 null (Fig. 1A).

To test the effect of galectin-3 expression in CDDP-induced cytotoxicity, we treated all clones with CDDP and subsequently evaluated cell viabilities by a trypan blue dye exclusion test. After an exposure to 25 μM CDDP, cell death was observed predominantly in parental BT549 and 41421 cells when compared with 11811, 11913, and 11914 cells (Fig. 1B). Parental BT549 and 41421 cells showed similar sensitivity to CDDP treatment; a 72-h exposure to CDDP exhibited a decrease in viability to less than 30% of the parental BT549 and 41421 cells, whereas more than 60% of all galectin-3-expressing clones remained viable even after a 72-h exposure with the same CDDP concentration.

To assess whether CDDP-treated cells underwent apoptosis, cells were stained with the DNA-binding dye (propidium iodide) and subjected to flow cytometric DNA analysis. The apoptotic subpopulation can be detected as the appearance of a discrete peak and increased fluorescence in the sub-G1 cell cycle region in the DNA histogram. It has been shown that the sub-G1 peak was absent from apoptotic cells in G0/G1 region (15). The sub-G1 population of the galectin-3 null parental BT549 and 41421 cells occupied more than 30% of the DNA histogram after a 72-h exposure to 25 μM of CDDP, whereas that of 11914 cells occupied not more than 20% (Fig. 2, D–F). To further investigate the apoptotic features of CDDP-treated cells, including nuclear condensation and fragmentation, cells were visualized under fluorescence microscopy after paraformaldehyde fixation and staining with the DNA-binding fluorochrome bis-(benzimide)-trihydrochloride (Hoechst 33258). As shown in Fig. 2, G–I, CDDP-induced cell death in all three types of cells involved apoptosis. More than 40% of CDDP-treated parental BT549 cells exhibited nuclear morphologies that are indicative of apoptosis, i.e., DNA fragmentation and condensed chromatin (Fig. 2G). Similar patterns of chromatin condensation and nuclear fragmentation were seen in CDDP-treated 41421 cells (Fig. 2H). However, 11914 cells responded differently to CDDP with more visible nuclei, mostly without altered chromatin structure (Fig. 2I). When considered together with the result of flow cytometry, apoptosis was observed in galectin-3-null parental BT-549 and 41421 cells, yet it was largely absent in galectin-3-expressing 11914 cells.

We further evaluated whether the expression of galectin-3 in BT549 cells resulted in altered expression of the Bcl-2 family of proteins, because previous studies had indicated that an elevated Bcl-2 level influenced the cell survival effect (17, 18). Galectin-3-null and -positive cell clones showed no difference in the level of Bcl-2 protein expressions (Fig. 1A); no significant difference in the level of the Bcl-XL and Bax expressions among these three cell clones was observed (data not shown), and their expressions were not altered by a 72-h exposure to 25 μM CDDP (data not shown). We then investi-

---

5 Biomed Research Meeting, Plymouth Meeting, PA.
Fig. 2. Flow cytometric cell cycle histograms of ethanol-fixed parental BT549 and transfectants stained with 50 μg/ml propidium iodide before [parental BT549 (A), 41421 (B), and 11914 (C) cells] and after [parental BT549 (D), 41421 (E), and 11914 (F) cells] treatment with 25 μM CDDP for 72 h. * cells in the sub-G1 region that have undergone chromatin degradation associated with apoptosis. G-I. Morphological changes associated with apoptosis in parental BT549 cells (G) and transfectants [41421 (H) and 11914 (I)] treated with 25 μM CDDP for 72 h. Cells were paraformaldehyde fixed, stained dark with Hoechst 33258, and visualized through fluorescence microscopy with a UV filter. Magnification, ×400.

Fig. 3. Cleavage of the DNA repair enzyme PARP and precursor CPP32/Yama expressions in parental BT549 cells and transfectants exposed to CDDP. Before and after the treatment with 25 μM CDDP for 72 h, proteins from cell lysates were electrophoresed on reducing 8 or 15% SDS-PAGE, transferred to nitrocellulose, and probed with mouse anti-PARP antibody5 or goat anti-CPP32/Yama antibody (Santa Cruz Biotech, Santa Cruz, CA), respectively. The amount of protein used was the same as that described in the Fig. 1A legend.

GALACTIN-3 IN APOPTOSIS

Gated the possibility that galectin-3 might regulate apoptosis through interactions with members of the Bcl-2 family of proteins, but a complex formation between galectin-3 and Bcl-2 or Bax in 11914 cells could not be established by coimmunoprecipitation assay (data not shown).

PARP is a substrate for mammalian cell death proteases and is degraded by activated cysteine proteases of the interleukin-1β converting enzyme/ced-3 family, including CPP32/Yama (caspase-3; Refs. 19 and 20). Degradation of PARP is a characteristic feature of the early stage of apoptosis and leads to inhibition of DNA repair; activation of an endonuclease; and, eventually, cell death (21). To further study the involvement of galectin-3 in the apoptosis pathway, we examined expressions of intact and cleaved PARP before and after a 72-h exposure to CDDP, showing that CDDP induced the cleavage of intact PARP (M, 116,000) into the inactive fragment (M, 85,000) in galectin-3-null parental BT549 and 41421 cells but not in galectin-3-expressing 11914 cells (Fig. 3). Interestingly, the levels of precursor CPP32/Yama in parental BT549 and 41421 cells attenuated after a 72-h exposure to CDDP, when compared to expression in 11914 cells.

The human proto-oncogene bcl-2 and the Caenorhabditis elegans gene ced-9, both of which protect cells from apoptosis (22–24), and a substitution of glycine in the NWGR motif of Bcl-2 BH1 domain and its highly conserved motif of ced-9 lead to a loss of the antiapoptotic activity of Bcl-2 and its binding activity to Bax (25–26). The BH1 domain in the Bcl-2 family of proteins and its partial homology with the COOH terminus of galectin-3 suggest that the highly conserved NWGR motif might be also critical to the apoptotic function of galectin-3 (Fig. 4A). To determine the significance of the NWGR motif in galectin-3, mutant galectin-3 (glycine 182 to alanine) was generated and cloned into the expression vector, and the parental BT-549 cells were transfected with it. Three stable transfectants showing differences in expression levels of the mutant galectin-3 [clone 5 expressed mutant galectin-3 at the highest level (the most among the three), and its expression was as strong as that of the wild-type 11914 cells (Fig. 4B)] were established in culture. Simultaneously, we examined in them the expression of Bcl-2, but no alteration in Bcl-2 expression was detected following the introduction of mutant galectin-3 (data not shown). To evaluate its function in CDDP-induced apoptosis, we have treated the clones with 25 μM CDDP for up to 72 h, and a time-dependent cell viability was assessed and compared to that of the parental and the galectin-3 wild-type transfected BT-549 cells (Fig. 4C). The three clones containing the mutated galectin-3 and the parental BT-549 cells showed a similar sensitivity to CDDP, with clone 5 being the most susceptible to CDDP among the clones. Next, we stained CDDP-treated cell clones with propidium iodide for the analysis of sub-G1 content in a DNA histo-
GALECTIN-3 IN APOPTOSIS

A

<table>
<thead>
<tr>
<th></th>
<th>ELFRDGV-NWGRIVAFFEFGG</th>
<th>DMFSGNFNWGRVVALFYFAS</th>
<th>ELFRDGV-NWGRIVAFFFSFGG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>136</td>
<td>98</td>
<td>150</td>
</tr>
<tr>
<td>Bax</td>
<td>174</td>
<td>150</td>
<td>118</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>174</td>
<td>150</td>
<td>169</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>174</td>
<td>NTKLD-N-NWGRE-ERQS</td>
<td></td>
</tr>
</tbody>
</table>

Galectin-3/G182A

Fig. 4. Stable clones expressing mutant galectin-3 and comparison with parental BT549 cells and transfectants of galectin-3 cDNA. A, schematic representation of the BH1 domain and its similarity with the carbohydrate-binding domain of galectin-3. Bold, the NWGR motif, which is responsible for the anti-apoptotic function of Bcl-2. An amino acid substitution of glycine 182 to alanine is shown in the bottom row (arrow). B, Western blot analysis: galectin-3 with an amino acid substitution of 182 glycine for alanine was established by following the protocol of an in vitro site-directed mutagenesis kit (Stratagene, La Jolla, CA) and transfected into parental BT549 cells. Mutant galectin-3 in stable clones was detected using rat anti-galectin-3, as shown in Fig. 1A. C, cell viability of clones expressing mutant galectin-3 (○, clone 1; □, clone 3; and △, clone 5) with 25 μM CDDP treatment in a time-dependent manner and comparison with parental BT549 (■) and wild-type galectin-3 transfectant 11914 cells. Viability was determined as described in the Fig. 2 legend.

gram and with Hoechst 33258 for the assessment of chromatin condensation and fragmentation. All galectin-3 mutant clones displayed similar features of apoptosis, as observed in parental BT-549 and 41421 cells (data not shown). In addition, all of the galectin-3 mutant clones underwent activation of CPP32/Yama and proteolytic cleavage of PARP after 72 h of CDDP exposure (Fig. 5). Taken together, these findings indicate that the expression of the mutant galectin-3 resulted in failure to inhibit CDDP-induced apoptosis in BT-549 cells and that the NWGR motif of galectin-3 is critical for its antiapoptotic function similar to that of Bcl-2 protein. It should be noted that the mutant galectin-3 maintained activity similar to that of the wild-type as determined by homotypic aggregation process and similar cellular distribution, as visualized by indirect immunofluorescence.

The results also indicate that galectin-3 confers apoptosis resistance to CDDP in a cysteine protease-dependent manner, similarly to Bcl-2 (20). It remains possible that galectin-3 can replace or mimic Bcl-2, leading to the inhibition of CDDP-induced apoptosis involving CPP32/Yama protease activation and PARP degradation.

Mutagenesis studies revealed that like Bcl-2 or ced-9, galectin-3 regulates its antiapoptotic activity through the glycine residue of the NWGR motif. It has been demonstrated previously that synthetic glycoamines or glycosylated molecules, such as Mac-2 binding protein, could regulate homotypic aggregation through their interactions with galectin-3 (27, 28), and it was suggested that the disruption of galectin-3-mediated cell-cell interactions on the cell surface resulted in induction of apoptosis in murine melanoma B16-F10 (9). The results from the COOH terminus mutation raises the possibility that substitution of the glycine residue may change the affinity to carbohydrates on the cell surface, block galectin-3 mediated cell-cell interaction, and cause the loss of antiapoptotic function of galectin-3.
However, our preliminary study showed that in parental BT549 cells, CDDP activity was not inhibited by exogenously added recombinant wild-type galectin-3 with a concentration of 10 μg/ml. This finding indicates that the role of galectin-3 in apoptosis is more likely to involve a mechanism in the intracellular compartment rather than on the cell surface. It is now well accepted that Bcl-2 localized at the mitochondrial membrane regulates apoptosis by blocking cytochrome c translocation (29, 30), whereas galectin-3 can be found at the nucleus, the cytoplasm, the cell surface, or in a secreted form, but not at the Golgi apparatus or the mitochondria. This suggests that the two proteins regulate their antiapoptotic activities at different cellular compartments.

It should be emphasized that galectin-3 does not belong to the Bcl-2 gene family, because they share less than a 30% homology (10). However, whether they have merged from a common ancestral gene containing the NWGR motif is unknown; it should also be noted that among the galectin family of proteins, only galectin-3 contains the Bcl-2 NWGR motif. In summary, we show here that galectin-3 is a novel antiapoptotic molecule, which like Bcl-2 acts through cysteine protease pathways; this finding may lead to the development of new reagents to induce apoptosis of tumor cells and contribute to the understanding of the role of galectin-3 in metastasis.

Acknowledgments

We thank E. W. Thompson for providing us with the BT549 cell line; F. G. Kern for the pCNC10 vector; E. Van Buren, R. Johnson, and L. Tait for the flow cytometry analyses; V. Powell for typing and editing; and Drs. W. Wei and R. Bright for their critical evaluation of the manuscript.

References


5276

Downloaded from cancerres.aacrjournals.org on April 19, 2017. © 1997 American Association for Cancer Research.
Galectin-3: A Novel Antiapoptotic Molecule with A Functional BH1 (NWGR) Domain of Bcl-2 Family

Shiro Akahani, Pratima Nangia-Makker, Hidenori Inohara, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/23/5272