Targeting Activating Transcription Factor 3 by Galectin-9 Induces Apoptosis and Overcomes Various Types of Treatment Resistance in Chronic Myelogenous Leukemia

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Abstract

Tyrosine kinase inhibitors (TKI) against Bcr-Abl are the first-line therapeutics for chronic myelogenous leukemia (CML). However, the resistance to Bcr-Abl TKIs is induced in leukemic cells not only by loss of sensitivity to TKIs through Bcr-Abl–related molecular mechanisms but also by loss of addiction to Bcr-Abl TK activity by acquiring Bcr-Abl–unrelated additional oncogenic mutations. Therefore, the identification of an additional therapeutic target has been anticipated for achievement of a complete cure and to overcome resistance to treatment. We here showed that modified human Galectin-9 (hGal9), a lectin that show specific affinity for β-galactosides, inhibits the proliferation of five CML-derived cell lines by inducing apoptosis at their IC50s from 17.5 to 164.9 nmol/L. Our study revealed that activating transcription factor 3 (ATF3), a member of the ATF/cAMP-responsive element binding protein family transcription factors, is the critical mediator for cell killing by hGal9, and that Noxa is one of the downstream effector molecules of ATF3. Bim, on the other hand, the BH3-only protein essential for apoptosis by Bcr-Abl TKIs, was not associated with hGal9-induced cell death. ATF3-mediated cell death by hGal9 was not hampered by the absence of p53, the presence of mutant AblT315I, or by P-glycoprotein overexpression. In addition, hGal9 showed the additive growth-inhibitory effect with imatinib on CML cell lines. Collectively, hGal9 is a candidate agent that may overcome various kinds of resistance to treatment for CML and may suggest that ATF3 may be a new target molecule for the development of new treatment modalities that can overcome resistance to currently available chemotherapeutics. Mol Cancer Res; 8(7); 994–1001. ©2010 AACR.

Introduction

The constitutive active fusion tyrosine kinase (TK) Bcr-Abl is responsible for deregulated cell proliferation and resistance to cytotoxic insults in chronic myelogenous leukemia (CML) cells, whereas the blockade of the Bcr-Abl signaling pathway by TK inhibitors (TKI) leads to the inhibition of cell proliferation and induces apoptosis mediated by Bim, a proapoptotic BH3-only protein, in CML cells (1-3). TKIs against Bcr-Abl such as imatinib mesylate (IM) have greatly improved the therapeutic outcome of CML patients, but treatment failure by resistance or intolerance to IM may occur in ~40% of patients with CML in the chronic phase (4), and even the more potent second generation Bcr-Abl TKIs, such as nilotinib and dasatinib, are not always promising for IM-refractory CML (5, 6). Resistance to Bcr-Abl TKIs is induced in leukemic cells not only by loss of sensitivity to TKIs through the acquisition of Ab mutation or the overexpression of Bcr-Abl but also by loss of addiction to Bcr-Abl TK activity by acquiring Bcr-Abl–unrelated additional oncogenic mutations (7). Therefore, a new treatment approach that induces an antileukemic effect through Bcr-Abl–unrelated molecular pathways is urgently needed for the achievement of a complete cure and to overcome TKI resistance.

Galectins are a family of animal lectins that show specific affinity for β-galactosides. Among 14 mammalian galectins, galectin-9 (Ga9) has been shown to possess the anticancer properties by regulating various cellular functions, such as cell adhesion, cell proliferation, or...
apoptosis (8-12). These prompted us to investigate whether Gal9 can have an anti-CML effect through signaling cascades distinct from the pathway used by Bcr-Abl TKIs or by other commonly used anticancer agents.

**Materials and Methods**

**Cell lines and reagents, and gene trasfection**

CML-derived BV173, KT-1, KCL22, K562 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), KBM5 (13) and MYL (14) cell lines, Jurkat T cell, an immortalized T lymphocyte cell line, and HL60, Bcr-Abl–negative myelogenous leukemia cell line were maintained in RPMI 1640 with 10% FCS, 2 mmol/L l-glutamate, and penicillin/streptomycin. IM-resistant KBM5/IMR with Abl T315I mutation was generated by continuous culture in medium containing 1.0 μmol/L IM (15). Generation of K562 subclones artificially overexpressing either FLAG-tagged Bcl-2 (K562/Bcl-2), Bcl-XL (K562/Bcl-XL), Mcl-1 (K562/Mcl-1), or dominant-negative FADD (K562/FADD.DN) has been described elsewhere (1, 2); multidrug-resistant K562/D1-9 is a P-glycoprotein overexpressing the subline of K562 (16-18). K562 cells were transfected with commercial SureSilencing short hairpin RNA (shRNA) plasmids (pGeneClip vector containing puromycin-resistant gene) for human activating transcription factor-3 (ATF3) and Noxa (SABiosciences) by means of an Amaxa Nucleofector (Nucleofector kit V, protocol T-16; Amaxa AG), and three transfected clones for ATF3 (K562/shATF3#1, #2, and #3) and for Noxa (K562/shNoxa #1, #2, and #3) were selected with 5.0 μg/mL puromycin (Sigma Aldrich), respectively. SureSilencing shRNA plasmids kit either for ATF3 or Noxa consisted of the mixture of four shRNA vectors targeted toward four different sequences within the target gene (Supplementary Table S1). K562 cells were also transfected with negative control shRNA plasmids for ATF3 or Noxa, supplied in SureSilencing shRNA plasmids kits. The proteolysis-resistant recombinant mutant form of human Gal9 (hGal9) was synthesized by the Galpharma Research Institute (19).

**Assays for growth inhibition, apoptosis, cell viability, and mitochondrial outer membrane permeabilization**

The growth-inhibitory effect of hGal9 was analyzed either with a modified MTT assay using Cell Counting Kit-8 (Dojindo) or by direct cell counting with trypan...
blue dye staining under a light microscope. To determine cell viability, the cells were stained with propidium iodine (PI) and subjected to flow cytometric analysis. Cells were stained with JC-1 (Becton Dickinson) and their mitochondrial outer membrane permeabilization (MOMP) was assayed by means of flow cytometry.

Western blotting
Protein samples were separated by means of SDS-PAGE and then electroblotted onto a Hybond-PDVF membrane (Amersham). The membranes were saturated with 5% (wt/vol) nonfat dry milk in PBS with 0.1% (vol/vol) Tween 20 (Sigma). We used antibodies against caspase-3, cleaved caspase-3, caspase-8, caspase-9 (Cell Signaling Technology), caspase-4, ATF3, p53 (Santa Cruz Biotechnology), Actin, FLAG (Sigma), Bcl-2 (Upstate Biotechnology), Bcl-X<sub>L</sub> (Stressgen), Noxa (Enzo Life Sciences), and Bim (clone 3C5, a gift from Dr. A. Strasser, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia).

Combinatory growth-inhibitory effect between hGal9 and IM
Cells were treated with hGal9 or IM alone, or their combination at various concentrations, and the combination index (CI) for hGal9 and IM was calculated with CalcuSyn (Biosoft; ref. 17). This method provides the quantification of synergism (CI < 1) and antagonism (CI > 1) at different dose and effect levels. CI calculations were made under the assumption that the mechanisms of the drugs were not mutually exclusive.

Statistical analysis
All data were analyzed with two-sided unpaired *t* tests, and a *P* value of 0.05 was considered statistically significant. Values are expressed as mean ± SD of triplicate independent experiments for the cell death assay and modified MTT.

Results
hGal9 inhibits the growth of CML cells by inducing apoptosis
hGal9 displayed a growth-inhibitory effect for 48 hours on five CML cell lines, BV173, K562, KBM5, KCL22, and MYL, in a concentration-dependent manner. The effects at the IC<sub>50</sub>s were 17.5, 156.7, 50.8, 164.9, and 73.1 nmol/L, and at their IC<sub>80</sub>s were 26.5, 355.3, 116.6, 241.4, and 104.7 nmol/L, respectively (Fig. 1A).

FIGURE 2. A, Western blot for Bcl-2 family proteins, p53, ATF3, and ATF4. K562 and MYL cells were treated with hGal9 at their IC<sub>80</sub>s for the indicated periods. p53-independent Noxa induction by hGal9 was accompanied by ATF3 induction in CML cell lines. B, expression of Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, and FLAG-protein tagged with dominant-negative mutant FADD in nontreated parental K562 and K562 subclones. C, cell death inducing effects of hGal9 and IM on parental K562 (♦), K562/Bcl-2 (▲), K562/Bcl-x<sub>L</sub> (●), K562/Mcl-1 (×), and K562/FADD.DN (□). Cells were treated with either hGal9 (left) or IM (right) for 48 h, and cell viability was assessed by incorporating propidium iodide.
also inhibited the growth of Jurkat T cells and HL60 cells with their IC50 39.0 and 68.9 nmol/L, respectively (data not shown), indicating that the effect of hGal9 was not restricted to leukemic cells driven by Bcr-Abl signaling. The addition of 25 mmol/L lactose prevented the growth-inhibitory effect by hGal9 in K562 (Fig. 1B), indicating the essential role of β-galactoside binding activity in the anti-CML activity of hGal9. Within 24 hours, hGal9 treatment increased the number of cells undergoing apoptosis (Fig. 1C and D). hGal9 treatment was accompanied by the loss of MOMP, as was shown by a decrease in JC-1-positive cells (Fig. 1E), and also caused the processing of caspase-3, caspase-8, and caspase-9 within 3 hours. The induction of caspase-4 processing was also detected after 12 hours of treatment with hGal9 in MYL, indicating the involvement of endoplasmic reticulum (ER) stress, whereas caspase-4 was absent in K562 cells (Fig. 1F).

**The relationship between the effect of hGal9 and regulators in intrinsic and extrinsic apoptosis pathways in CML cell lines**

Bcl-2 family proteins are crucial regulators of cell death machinery as part of mitochondrial involvement of intrinsic apoptosis pathway, whereas death receptor pathway regulates the extrinsic apoptosis pathway (20, 21). Within 3 hours, treatment with hGal9 induced Noxa, a proapoptotic BH3-only protein. As for antiapoptotic relatives of Bcl-2 proteins, hGal9 treatment caused an increase of Mcl-1, but did not affect the expression levels of Bcl-2 or Bcl-XL (Fig. 2A). K562 sublines overexpressing Bcl-2, Bcl-XL, or Mcl-1 showed resistance to cell death induced by IM, but were as sensitive to hGal9-induced cell death as the parental cells (Fig. 2B and C), suggesting the involvement of a pathway that is independent of Bcl-2 family proteins. These results also indicate that the accumulation of Mcl-1 following hGal9 treatment does not hamper apoptotic induction by hGal9. Besides, the expression of FADD.DN did not hamper the effect of hGal9 (Fig. 2C), also indicating that the death receptor pathway was not responsible for apoptosis induced by hGal9.

**Function of ATF3 in Noxa induction and cell death by hGal9 in CML cells**

To identify the molecule responsible for cell death by hGal9, we focused on Noxa upregulation by hGal9. Noxa was initially identified as a p53 tumor suppressor–induced proapoptotic effector, whereas several other molecules, including ATF3, also induce Noxa in a p53-independent fashion (22, 23). hGal9 induced Noxa in MYL cells with wild-type p53 as well as in K562, which is deficient in a functional p53 gene (24). In addition, hGal9 did not cause p53 accumulation in MYL cells, indicating that hGal9 induces Noxa through a p53-independent pathway. In contrast, ATF3 was clearly induced by hGal9 in K562 and MYL (Fig. 2A). To determine the role of ATF3 in Noxa induction, we used the RNA interference technique to generate subclones of K562 cells with stable expression of ATF3 levels. The basal expression level of ATF3 was less in nontreated K562/shATF3#1 than in parental K562, but this suppression was not complete (Fig. 3A). Indeed, ATF3 was induced by hGal9 in K562/shATF3#1, although...
to a lesser extent than in parental K562. Importantly, this lesser induction of ATF3 was accompanied by lesser induction of Noxa by hGal9 in K562/shATF3#1 than in parental K562, indicating that Noxa induction by hGal9 was mediated by ATF3.

We next investigated the role of ATF3 in cell death induced by hGal9 in CML cells. The degree of ATF3 suppression differed among three subclones of K562 cells with ATF3 knockdown expression. Because the knockdown of ATF3 was partial, ATF3 was still induced by hGal9 in those subclones, although less than that induced in parental K562 (Fig. 3B). The subclones were less susceptible to hGal9-induced killing than the parental cells, and importantly, the extent of ATF3 suppression correlated with both the extent of ATF3 induction by hGal9 and that of protection from hGal9-induced cell killing in K562/shATF3 subclones (Fig. 3C). Expression of a control RNA interference construct had no effect on the response to hGal9 (data not shown). These show that ATF3 is at least one of the critical mediators of cell death by hGal9 in CML cells.

Noxa is partly involved in cell death induced by hGal9 in CML cells

Given the results described above, we then investigated the role of Noxa in cell death induced by hGal9 in CML cells. The knockdown of Noxa was partial in three subclones generated; therefore, Noxa was still induced by hGal9, although less than that induced in parental K562 (Fig. 4A). Those Noxa knockdown K562 subclones were less susceptible to hGal9-induced killing than the parental cells, especially at low concentration of hGal9 (Fig. 4B). Expression of a control RNA interference construct had no effect on the response to hGal9 (data not shown). These show that Noxa is also at least one of the critical mediators of cell death by hGal9 in CML cells.

Utility of hGal9 for the treatment of CML

Finally, we assessed the utility of hGal9 for CML treatment. In IM-sensitive K562 and MYL cells, the combination of IM and hGal9 showed an additive effect on both cell lines at all concentrations examined (Fig. 5A). We also investigated whether hGal9 exerts its anti-CML effect on IM-resistant cells. KBM5/IMR cells harboring Abl mutation T315I were resistant to IM compared with the parental IM-sensitive KBM5, and as sensitive to hGal9 as the parental KBM5 (Fig. 5B). In addition, although K562/D1-9 was completely resistant to IM, the cells were as sensitive to hGal9 as the parental K562 (Fig. 5C).

Discussion

Our study showed that the cell death inducing mechanism by hGal9 uses ATF3, a member of the ATF/CREB family of transcription factors that functions as a tumor suppressor (25-29), but not p53 or Bim, the major proapoptotic factors in cell death induced by conventional genotoxic agents or Bcr-Abl TKIs. This implies that hGal9 is effective against CML cells that have lost their addiction to Bcr-Abl TK activity or their functional p53. Indeed, hGal9 was effective against CML cells with T315I Abl mutation, which completely impedes the binding of Bcr-Abl TKIs to drug-binding sites in Abl and K562 without functional p53. In addition, hGal9 was effective against K562 expressing P-glycoprotein, the causative for multidrug resistance due to drug efflux at the cellular membrane, perhaps because the effect of hGal9 depends on binding to the cellular surface and does not require the cellular incorporation (10). Overexpression of antiapoptotic Bcl-2, Bcl-XL, or Mcl-1 has been shown to affect the efficacy of Bcr-Abl TKIs (1, 2), whereas the effect of hGal9 was not diminished by Bcl-2, Bcl-XL, or Mcl-1, perhaps because hGal9 also activates a Bcl-2 family-independent pathway, which eventually activates caspase-4 and caspase-8. Considering that the activation of caspase-4 and caspase-8 is involved
in ER stress–induced apoptosis (30, 31), and that Noxa induction by ATF3 is crucial in the cell death induced by inhibitors for ER-associated protein degradation (22), we suggest that hGal9-induced cell death may at least partly involve ER stress.

Importantly, hGal9 had an additive effect in combination with IM in CML cells. IM induces apoptosis through the activation of Bim and Bad, BH3-only proteins (1, 2). In this context, Bim inhibits all antiapoptotic Bcl-2 proteins, that is, Bcl-2, Bcl-XL, and Mcl-1, whereas Bad inhibits only Bcl-2 and Bcl-XL, but not Mcl-1 (20, 21, 32). Considering that hGal9 induces Noxa, which inhibits Mcl-1, but not Bcl-2 or Bcl-XL, and that the concomitant activation of Bad and Noxa is as potent as that of Bim in inducing apoptosis (30), the combination of IM and hGal9 may further enhance the combined effects of the BH3-only proteins Bim, Bad, and Noxa in CML cells. Moreover, hGal9 also activates caspase-4 and caspase-8, which may further enhance the apoptotic effect of IM, which is unrelated with the activation of caspase-4 or caspase-8 (Fig. 6).

In conclusion, hGal9-induced apoptosis is mediated by the ATF-Noxa pathway, but is independent of p53 or Bcr-Abl. Therefore, hGal9 is a potent killer of Bcr-Abl–positive CML cells, including TKI-resistant cells. Furthermore, ATF3 is a candidate molecular target for future developments of antileukemia agents.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.
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