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Running title: Galectin-9-Induced Apoptosis of BL and HL Cells

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Summary

Identification of galectin-9 as a ligand for T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3), expressed on Th1 cells, has established the Tim-3-galectin-9 pathway as a regulator of Th1 immunity. Whereas there is compelling evidence for the effects of galectin-9 on T-cell fate, limited information is available on the impact of galectin-9 on B lymphocytes. We found that protease-resistant galectin-9, hG9NC(null), but not galectin-1 or -8, prevented cell growth of malignant B cells such as Burkitt lymphoma (BL) and Hodgkin lymphoma (HL). β-galactoside binding was essential for galectin-9-induced cell growth suppression. hG9NC(null) induced cell cycle arrest by reducing the expression of cyclin D1, D2, B1, Cdk4, Cdc25C and c-Myc, and apoptosis by reducing the expression of XIAP, c-IAP2 and survivin. Most of these genes are regulated by nuclear factor-κB (NF-κB), and constitutive activation of NF-κB is a common characteristic of both types of malignancies. hG9NC(null) inhibited IκBα phosphorylation, resulting in suppression of NF-κB. AP-1 has also been implicated in the control of cell survival. hG9NC(null) inhibited the expression of JunD, resulting in the suppression of AP-1. Our results suggest that hG9NC(null) is a potentially suitable agent for the management of BL and HL.

Keywords: galectin-9, Burkitt lymphoma, Hodgkin lymphoma, NF-κB, AP-1.

Introduction

Galectins are a family of soluble β-galactoside-binding animal lectins. To date, 14 members of the galectin family have been identified. Each member exhibits diverse biological functions and many of them appear to function in cellular homeostasis through regulation of cell adhesion, cell proliferation, cell death and chemoattraction (Cooper & Barondes, 1999; Rabinovich, 1999; Liu, 2000; Hernandez & Baum, 2002; Liu *et al*, 2002). Galectins can be classified into three subtypes according to their structure; the prototype (galectin-1, -2, -7, -10 and -13) and chimera type (galectin-3) galectins have a single carbohydrate recognition domain (CRD) and they usually form a non-covalent homodimer resulting in homobifunctional sugar binding activity. The tandem-repeat type galectins (galectin-4, -8, -9 and -12) have two CRDs, which generally show different sugar binding specificities, joined by a linker peptide. This heterobifunctional property makes them capable of crosslinking a wide variety and combinations of glycoconjugates. Tandem-repeat type galectins, however, are more susceptible to proteolysis than other galectins due to the presence of the relatively long linker peptide.

Recent studies suggested that galectin-9 is a modulator of immune functions; it induces chemotaxis of eosinophils (Matsumoto *et al*, 1998) and apoptosis of thymocytes, suggesting a possible role in the process of negative selection occurring during T-cell development (Wada *et al*, 1997). The T-cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3; also known as HAVCR2) is a molecule expressed on terminally differentiated murine Th1 cells but not on Th2 cells (Monney

et al, 2002). Recently, galectin-9 has been identified as the Tim-3 ligand (Zhu *et al,* 2005). Galectin-9 triggering of Tim-3 on Th1 cells induces cell death. The Tim-3-galectin-9 pathway is thought to be an important regulator of Th1 immunity and tolerance induction. Whereas compelling evidence has accumulated regarding the effects of galectin-9 on T-cell fate, limited information is available on the impact of galectin-9 on B lymphocytes.

Burkitt lymphoma (BL) and Hodgkin lymphoma (HL) represent clonal malignant expansions of B cells. BL is a high-grade non-HL that occurs sporadically worldwide, but is endemic in Papua New Guinea and in the lymphoma belt of Africa (van den Bosch, 2004). Rates of BL have increased in low-incidence countries since the 1980s, preceding the advent of human immunodeficiency virus/acquired immunodeficiency syndrome. Patients with human immunodeficiency virus-associated lymphoma pose additional therapeutic challenges, particularly the risk of overwhelming opportunistic infection (Mounier *et al,* 2007).

Advances in chemotherapy and radiotherapy regimens for treatment of HL represent a significant breakthrough in clinical oncology and have increased the long-term survival rate. Today, problems of the late side-effects of chemotherapy such as secondary malignancies, myelodysplasia and cardiotoxicities, as well as resistance to chemotherapy, associated with poor prognosis have become important issues that need to be resolved (Diehl *et al,* 2004). Advances in molecular biology have provided many new insights into the biology and treatment options for BL and HL. Recently, a strategy that targets the molecules critical for maintenance and growth of tumor cells has been considered a key to the development of more effective treatment with less

undesirable effects (Griffin, 2001). This strategy should enhance the specificity of treatment against tumor cells and minimize undesirable effects on normal cells.

Galectin-1, -8 and -9 have been reported to trigger death of T-cell lines and various T-cell subsets (Stillman *et al,* 2006; Lu *et al,* 2007; Tribulatti *et al,* 2007). However, relatively little is known about the regulation of B-cell physiology. Constitutive NF-κB activation has recently been recognized as a critical pathogenic factor in BL and HL (Jost & Ruland, 2007). Inhibition of NF-κB signaling could potentially be effective as agents in BL and HL. We reported that NF-κB inhibition by galectin-9 resulted in a profound apoptosis induction and the killing of human T-cell leukemia virus type I-infected T cells (Okudaira *et al,* 2007). With the objective of finding newer agents for the treatment of B-cell lymphomas, the present study was designed to investigate the antitumor potential of galectin-1, -8 and -9 on BL and HL cell lines *in vitro,* and the possible mechanisms involved in such antitumor activities. Since galectin-8 and galectin-9 consist of two CRDs joined by a linker peptide, which is highly susceptible to proteolysis, we used in this study the protease-resistant galectin-8 and galectin-9 by modification of its linker peptide, hG8NC(null) or hG9NC(null).

Materials and methods

Lymphocyte purification, cell lines and culture

LCL-Ao, LCL-Ka, LCL-Ku and LCL-Ya are Epstein-Barr virus (EBV)-immortalized human B-cell lines generated from peripheral blood mononuclear cells (PBMC) of

healthy adults. Raji and Daudi are EBV-positive BL cell lines. BJAB and Ramos are EBV-negative BL cell lines. B95-8/BJAB and B95-8/Ramos are BJAB and Ramos infected with the B95-8 strain of EBV, respectively. L-428 and KM-H2 are HL cell lines. All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50 μg/ml streptomycin. $CD19^+$ B cells were purified from PBMC by positive selection with magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) after labeling with anti-CD19 microbeads. All biological samples were obtained after informed consent.

Reagents

Recombinant human galectin-1 was obtained as described previously (Matsushita *et al*, 2000). Recombinant mutant forms of human galectin-8 and galectin-9 lacking the entire linker region, hG8NC(null) and hG9NC(null), were expressed and purified as described previously (Nishi *et al*, 2005). Both mutant proteins are highly stable against proteolysis (Nishi *et al*, 2005). Lactose and sucrose were purchased from Wako Chemicals (Osaka, Japan). Rabbit polyclonal antibodies to cyclin D2, c-IAP2, survivin, IκBα, JunD, nuclear factor-κB (NF-κB) subunits p65, p50, c-Rel, p52 and RelB, and AP-1 subunits c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD, and goat polyclonal antibody to Tim-3, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody to Bcl-x_L was purchased from BD Transduction Laboratories (San Jose, CA, USA). Mouse monoclonal antibodies to Bcl-2, Bax, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C, c-Myc, p21, actin and

phosphorylated form of the retinoblastoma protein (pRb) (Ser780) were purchased from NeoMarkers (Fremont, CA, USA). Mouse monoclonal antibodies to XIAP and cyclin D1 were purchased from Medical & Biological Laboratories (MBL; Nagoya, Japan). Mouse monoclonal antibody to phospho-IκBα (Ser32 and Ser36), caspase-8 and caspase-9, and rabbit monoclonal antibody to cleaved caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell growth inhibition and apoptosis assays

The effect of galectins on cell growth was examined using the cell proliferation reagent, WST-8 (Wako). Briefly, 1×10^5 cells/ml (cell lines) or 1×10^6 cells/ml (PBMC and B cells) were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated FBS in a 96-well microculture plate in the absence or presence of various concentrations of galectins. After 24-h culture, WST-8 (5 μl) was added for the last 4 h of incubation and absorbance at 450 nm was measured using an automated microplate reader. Measurement of mitochondrial dehydrogenase cleavage of WST-8 to formazan dye provides an indication of the level of cell proliferation. For detection of apoptosis, the Annexin V binding capacity of the treated cells was examined by flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA, USA) using Annexin V-Fluos (Roche Diagnostics, Mannheim, Germany).

Cell cycle analysis

Cell cycle analysis was performed with the CycleTEST PLUS DNA reagent kit (Becton-Dickinson, Mountain View, CA, USA). In brief, 1×10^6 cells were washed

with a buffer solution containing sodium citrate, sucrose and dimethyl sulfoxide, suspended in a solution containing RNase A and stained with 125 μg/ml propidium iodide for 10 min. After passing the cells through a nylon mesh, cell suspensions were analyzed on an Epics XL flow cytometer. The population of cells in each cell cycle phase was determined.

In vitro measurement of caspase activity

Caspase activity was measured using the colorimetric caspase assay kits (MBL). Cell extracts were recovered using the Cell Lysis buffer and assessed for caspase-3, -8 and -9 activities using colorimetric probes. The colorimetric caspase assay kits are based on detection of chromophore *p*-nitroanilide after cleavage from caspase-specific-labeled substrates. Colorimetric readings were performed in an automated microplate reader at an optical density of 400 nm. Fold-increase in caspase activity was determined by comparing these results with the levels of the untreated control.

Western blot analysis

Cells were lysed in a buffer containing 62.5 mmol/l Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (20 μg) were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. The bands were

visualized with the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted with Trizol (Invitrogen Corp., Carlsbad, CA, USA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular RNA using an RNA PCR kit (Takara Bio Inc, Otsu, Japan) with random primers. Thereafter, cDNA was amplified for 35 cycles for *HAVCR2* and 28 cycles for *ACTB*. The oligonucleotide primers used were as follows: for *HAVCR2*, sense, 5'-ACAGAGCGGAGGTCGGTCAGAATG-3' and antisense, 5'-AGCCAGAGCCAGCCCAGCACAGAT-3'; and for *ACTB*, sense, 5'-GTGGGGCGCCCCAGGCACCA-3' and antisense, 5'-CTCCTTAATGTCACGCACGATTTC-3'. Product sizes were 572 bp for *HAVCR2* and 548 bp for *ACTB*. Cycling conditions were as follows: denaturing at 94°C for 30 sec, annealing at 60°C (for *ACTB*) or 62°C (for *HAVCR2*) for 30 sec and extension at 72°C for 45 sec (for *HAVCR2*) or for 90 sec (for *ACTB*). The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide staining.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were cultured and examined for inhibition of NF-κB and AP-1 after exposure to hG9NC(null) for 12 h. Nuclear proteins were extracted and NF-κB and AP-1 DNA-binding activities were examined by EMSA as described previously (Mori *et al*,

1999). In brief, 5 μg of nuclear extracts were preincubated in a binding buffer containing 1 μg poly(dI-dC) (Amersham Biosciences), followed by addition of [α-^{32}P]-labeled oligonucleotide probe containing NF-κB or AP-1 element (approximately 50 000 cpm). The mixtures were incubated for 15 min at room temperature. The DNA-protein complexes were separated on 4% polyacrylamide gels and visualized by autoradiography. To examine the specificity of each element probe, unlabeled competitor oligonucleotides were preincubated with nuclear extracts for 15 min before incubation with probes. The probes or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides; a typical NF-κB element from the *interleukin-2 receptor* (*IL-2R*) *α chain* gene (5'-gatcCGGCAGGGGAATCTCCCTCTC-3') and an AP-1 element of the *IL-8* gene (5'-gatcGTGATGACTCAGGTT-3'). Underlined sequences represent the NF-κB and AP-1 binding site, respectively. To identify nuclear proteins in the DNA-protein complex recognized by EMSA, we used antibodies specific for various NF-κB family proteins, including p65, p50, c-Rel, p52 and RelB and various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, junB and JunD, to elicit a supershift DNA-protein complex formation. These antibodies were incubated with the nuclear extracts for 45 min at room temperature before incubation with radiolabeled probes.

Statistical analysis

Data are expressed as mean ± SD. The significance of the difference between each experiment sample (treated with galectin-9) and the control was determined using the Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

A modified version of galectin-9 inhibits growth of EBV-immortalized human B-cell lines, BL and HL cell lines

We first examined the effects of the recombinant mutant form of galectin-9 lacking the entire linker region on the growth of human B-cell lines. Although the sensitivity to galectin-9 varied among the cell lines studied, culture of cells with various concentrations (0 to 1 μmol/l) of galectin-9 for 24 h resulted in the suppression of cell growth in a dose-dependent manner in all four EBV-immortalized human B-cell lines tested as assessed by the WST-8 assay (Fig 1A). On the other hand, PBMC from healthy subjects were less susceptible to galectin-9 than human B-cell lines. Galectin-9 had no effect in normal B cells. Recent studies reported that galectin-1 and galectin-8 inhibit growth of cancer cells, T-cell lines and various T-cell subsets (Arbel-Goren *et al,* 2005; Bremer *et al,* 2006; Stillman *et al,* 2006; Tribulatti *et al,* 2007). Therefore, we studied in the next step the effect of recombinant galectin-1 and recombinant mutant form of galectin-8 lacking the entire linker region on the growth of human B-cell lines. However, galectin-1 and galectin-8 had little effect on cell growth (Fig 1A). Treatment of EBV-positive and -negative BL cell lines and HL cell lines with galectin-9 also resulted in inhibition of cell growth. Galectin-1 and galectin-8 had little effect on the growth in these cell lines (Fig 1B and C). Galectin-1 and galectin-8 exhibited hemagglutination activity, indicating that both galectins used were indeed functional (data not shown).

Expression of mRNA levels of HAVCR2 *in BL and HL cell lines*

Next, we determined the expression levels of *HAVCR2* mRNA in BL and HL cell lines by RT-PCR analysis. Low levels of *HAVCR2* mRNA were found in all BL cell lines, whereas no expression was detected in L-428 and KM-H2 (Fig 2). Consistent with the results of RT-PCR analysis, Western blot analysis demonstrated that BL cell lines expressed low levels of Tim-3 protein, while HD cell lines did not. These results suggest that the degree of Tim-3 expression does not correlate with the sensitivity to galectin-9-induced growth suppression.

β-galactoside binding is essential for galectin-9-induced cell growth suppression

Next, to examine the requirement for β-galactoside binding, cells were exposed to various concentrations of lactose or sucrose (0, 10, 20 and 30 mmol/l) in the presence of 0.3 μmol/l galectin-9. Lactose and sucrose are β-galactoside and α-glucoside, respectively. Inhibition of binding of galectin-9 to the cell surfaces was observed in the thymocytes incubated in the presence of lactose, since lactose could bind to galectin-9 (Wada *et al*, 1997). Fig 3 shows that the cell growth inhibitory activity of galectin-9 was inhibited by lactose in a dose-dependent fashion, but not by sucrose, indicating that β-galactoside binding activity is required for galectin-9-induced cell growth suppression.

Galectin-9 induces apoptosis of BL and HL cell lines

To examine whether induction of apoptosis accounts for the cell growth inhibition observed in human B-cell lines, cells treated with galectin-9 were examined by the Annexin V method. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic finding in cells entering apoptosis. Galectin-9 increased the proportion of cells positive for Annexin V in all cell lines (Fig 4A). Furthermore, apoptosis was confirmed by immunostaining the cells with Apo2.7, which specifically detects the 38 kDa mitochondrial membrane antigen 7A6, which is expressed on the mitochondrial outer membrane during apoptosis (data not shown) (Zhang *et al*, 1996; Seth *et al*, 1997). These results indicate that galectin-9 induces apoptosis of BL and HL cells.

Galectin-9-induced apoptosis is caspase-dependent

We examined whether caspase activation is involved in galectin-9-induced apoptosis. Galectin-9 treatment resulted in activation of caspases-3, -8 and -9 in Raji cells (Fig 4B). In addition, we assessed levels of caspase-3, -8 and -9 in Raji cells after exposure to galectin-9. Cleavage of caspase-3, -8 and -9, indicating activation of these cystein proteases, was induced after exposure to galectin-9 (Fig 4C). These results indicate that galectin-9-induced apoptosis of BL cells is mediated through caspase activation.

Galectin-9 induces cell cycle arrest

We investigated the effect of galectin-9 on the cell cycle progression in BL and HL cell lines. The cells were incubated with galectin-9 for 12 h and analyzed for cell cycle distribution by flow cytometry, since incubation for 24 h induced cell death (Fig 5).

By 12 h after treatment with galectin-9, the percentage of cells in sub G0/G1 did not increase. Galectin-9 inhibited cell cycle progression, as evidenced by increased proportion of cells in G2/M phase in all cell lines tested. In addition, cultivation with galectin-9 increased the proportion of cells in the G1 phase, with a reduction of cells in the S phase, indicating G1 arrest in Ramos and Daudi cells. The unchanged number of cells in G1, despite the decrease in the proportion of cells in the S phase in BJAB and Raji cells, can be explained by either no entry in the G1 (due to G2/M arrest) or no exit from G1 (due to G1 arrest). In the absence of G1 arrest, cells accumulated exclusively in G2/M phase in L-428 and KM-H2 cells. These results indicate that galectin-9 induces G1 and/or G2/M arrest of the cells. The proportion of cells in the sub G0/G1 phase was increased at 24 h incubation (data not shown), suggesting that cell cycle arrest is the cause of apoptosis.

Effects of galectin-9 on cell cycle- and apoptosis-related proteins

To clarify the molecular mechanisms of galectin-9-induced inhibition of cell growth and apoptosis in Raji cells, we examined the expression of several intracellular regulators of cell cycle and apoptosis, including cyclin D1, cyclin D2, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C, c-Myc, p21, Bcl-2, Bcl-x_L, Bax, XIAP, c-IAP2 and survivin by Western blot analysis. As shown in Fig 6, galectin-9 did not alter Bcl-2, Bcl-x_L, Bax, Cdk1, Cdk6 and p21 levels, but significantly decreased the expression of survivin, XIAP, c-IAP2, cyclin D1, cyclin D2, cyclin B1, Cdk4, Cdc25C and c-Myc in dose- and time-dependent manners. Exposure to galectin-9 also downregulated the

levels of the phosphorylated form of pRb. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin (Fig 6).

Galectin-9 modulates activated NF-κB

NF-κB can act as a survival factor and is required for the proliferation of a variety of tumor cell types (Dolcet *et al,* 2005). Because NF-κB is constitutively active in BL (Jazirehi *et al,* 2005) and HL cell lines (Bargou *et al,* 1997), and the expression of survivin (Kawakami *et al,* 2005), XIAP (Stehlik *et al,* 1998), c-IAP2 (Chu *et al,* 1997), cyclin D1 (Hinz *et al,* 1999), cyclin D2 (Huang *et al,* 2001), Cdk4 (Iwanaga *et al,* 2001) and c-Myc (Duyao *et al,* 1990) are known to be regulated by NF-κB, we examined whether galectin-9 inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we performed EMSA with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from untreated and galectin-9-treated Raji cells. NF-κB oligonucleotide probe with nuclear extracts from untreated Raji cells generated DNA-protein gel shift complexes (Fig 7B). These complexes were due to specific bindings of nuclear proteins to the NF-κB sequence, because such activities were reduced by the addition of cold probe but not by an irrespective sequence (Fig 7B). We also showed that NF-κB complexes contain p50, p65, c-Rel and p52 in Raji cells. As shown in Fig 7A, nuclear extracts prepared from Raji cells treated with galectin-9 for 12 h exhibited a decrease in the intensity of the NF-κB-containing gel shift complexes in a dose-dependent manner, suggesting that galectin-9 downregulates the DNA-binding activities of NF-κB. Galectin-9 also inhibited the DNA-binding of NF-κB in KM-H2 cells (data not shown).

Degradation of IκBα and subsequent release of NF-κB require prior phosphorylation at Ser32 and Ser36 residues (Chen *et al,* 1996). To investigate whether the inhibitory effect of hG9NC(null) is mediated through alteration of phosphorylation of IκBα, Raji cells were treated with galectin-9 and their protein extracts were checked for phospho-IκBα expression. Untreated cells constitutively expressed Ser32- and Ser36-phosphorylated IκBα (Fig 7C), while galectin-9 treatment decreased the phosphorylated IκBα in a dose-dependent manner. Concomitantly, levels of IκBα accumulated, suggesting that galectin-9 inhibited phosphorylation of IκBα, followed by accumulation of this protein.

Galectin-9 modulates activated AP-1

Transcription factor AP-1 is also identified as a crucial mediator of both, cell cycle promoting and cell-death inhibiting pathways (Shaulian and Karin, 2001). Therefore, we focused on AP-1 inactivation after exposure to galectin-9. High constitutive AP-1 DNA-binding activities were detected in Raji cells (Fig 7E). Supershift analysis with antibodies indicated that the AP-1 complex in Raji cells contained JunD (Fig 7E). As shown in Fig 7D, AP-1 DNA-binding activity diminished in the presence of galectin-9 in a dose-dependent manner. Galectin-9 also inhibited the DNA-binding of AP-1 in KM-H2 cells (data not shown). In addition, galectin-9 also dose-dependently decreased the expression of JunD, which composes the increased DNA-binding AP-1 protein (Fig 7F). These findings suggest that galectin-9 depletes JunD, resulting in inactivation of AP-1.

Discussion

In this study, we showed that BL and HL cell lines are more susceptible to growth inhibition induced by a protease-resistant galectin-9, which was established by modification of its linker peptide, hG9NC(null) relative to normal PBMC and B cells. The data demonstrated that hG9NC(null) is effectively cytotoxic in BL and HL cells without toxicity to normal cells. Although all members of the galectin family contain β-galactoside binding domains, they do not consistently induce apoptosis. Indeed, galectin-1 and hG8NC(null) did not induce apoptosis of these cells. However, hG9NC(null)-induced apoptosis was mediated through binding with specific galactosyl groups, since the effects could be competitively inhibited with lactose. These results suggest that cell surface hG9NC(null) binding molecules responsible for apoptosis are expressed on BL and HL cells susceptible to hG9NC(null). Recently, galectin-9 has been identified as the Tim-3 ligand (Zhu *et al,* 2005). We therefore examined Tim-3 expression using RT-PCR and Western blotting. However, the degree of Tim-3 expression did not correlate with the sensitivity to galectin-9-induced cell death. Recently, it has been reported that Tim-3 expression was not universally required for galectin-9 death of T cells (Bi *et al*, 2008). The unknown cell surface glycoprotein receptors might participate in galectin-9-induced death. Galectin-9 was previously identified in HL (Türeci *et al*, 1997). Therefore, we investigated the expression of the endogenous mRNA levels of galectin-9 in L-428 and KM-H2 cells by RT-PCR. However, they were not so high (data not shown).

Our results showed that the growth-inhibitory potential of hG9NC(null) on BL and HL cell lines was mainly due to the induction of cell cycle arrest and apoptosis, because a significant population of cells remained in the G1 and/or G2/M phases of the cell cycle and underwent apoptosis after exposure to hG9NC(null). Cell cycle arrest in G1 and G2/M phases by hG9NC(null) treatment may be associated with the downregulation of expression of proteins involved in G1/S transition (cyclin D1, cyclin D2, Cdk4 and c-Myc) and G2/M transition (cyclin B1 and Cdc25C). Together, these changes caused a decrease in the level of phosphorylated pRb in Raji cells.

Our results showed that hG9NC(null)-induced apoptosis of BL and HL cells was associated with activation of caspase-3, -8 and -9. hG9NC(null) induced apoptosis in conjunction with downregulation of the antiapoptotic proteins, XIAP, c-IAP2 and survivin. Because XIAP and c-IAP2 inhibit caspase-3 and -9 activity (Deveraux *et al*, 1998), it appears that hG9NC(null) stimulates caspase-3- and -9-dependent apoptosis by downregulating XIAP and c-IAP2 expression. In our study, the expression of survivin, another member of the IAP family, was also downregulated by hG9NC(null). Because caspase-3 can be inhibited by survivin (Shin *et al*, 2001), it is possible that downregulation of survivin by hG9NC(null) could lead to activation of caspase-3. Caspase-8 is activated by death receptors, such as CD95 and tumor necrosis factor-related apoptosis-inducing ligand receptor, which are expressed on BL and HL cells (Nguyen *et al*, 1996; Hussain & Doucet, 2003; Mouzakiti & Packham, 2003). These death receptors may trigger signaling pathways in hG9NC(null)-treated cells.

Detailed mapping of intracellular molecules and signaling pathways might provide more efficient, less toxic treatment opportunities in which cellular components, critical for survival of the tumor, can be selectively targeted. We found that hG9NC(null) possessed anti-NF-κB activity. It inhibited the IκBα phosphorylation and NF-κB DNA-binding activity. Activation of NF-κB plays an important role in cell proliferation and prevention of apoptosis due to overexpression of several NF-κB-inducible molecules. We found that suppression of NF-κB by hG9NC(null) correlated with downregulation of the expression of several gene products regulated by NF-κB. The expression of XIAP (Stehlik *et al,* 1998), c-IAP2 (Chu *et al,* 1997), survivin (Kawakami *et al,* 2005), cyclin D1 (Hinz *et al,* 1999), cyclin D2 (Huang *et al,* 2001), Cdk4 (Iwanaga *et al,* 2001) and c-Myc (Duyao *et al,* 1990), whose synthesis is regulated by NF-κB, was suppressed by hG9NC(null). Although Bcl-x_L and Bcl-2 are known as NF-κB targets, signal transducer and activator of transcription 3, which is constitutively activated in BL and HL cells (Weber-Nordt *et al,* 1996; Kube *et al,* 2001), also regulates Bcl-x_L and Bcl-2 transcription (Sevilla *et al,* 2001; Jazirehi and Bonavida, 2005). Therefore, the expression of Bcl-x_L and Bcl-2 does not seem to be affected by hG9NC(null) treatment. The decrease in NF-κB activity, may be at least in part responsible for the induction of cell cycle arrest and apoptosis by hG9NC(null) in BL and HL cell lines.

Several NF-κB inhibitors, such as PS-341 (Zheng *et al,* 2004), dehydroxymethylepoxyquinomicin (Kimura *et al,* 2007; Watanabe *et al,* 2007), gliotoxin (Izban *et al,* 2001), MG132 (Izban *et al,* 2001), arsenic (Mathas *et al,* 2003) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Piva et al, 2005), were reported to block

constitutive NF-κB activation and induce apoptosis of HL and BL cell lines. Except for PS-341, the dose of NF-κB inhibitors used in these experiments is far higher than that of hG9NC(null). Thus, compared with the results described in previous reports, our findings suggest that hG9NC(null) may be more suitable for NF-κB inhibition in BL and HL cells.

The AP-1 is known to regulate cell proliferation, differentiation and apoptosis in various cell lines (Hess *et al,* 2004). In Raji cells, hG9NC(null) inhibited JunD expression, resulting in the suppression of AP-1 DNA-binding. The cyclin D2 promoter contains NF-κB and AP-1 sites (Brooks *et al,* 1996). Although cyclin D1 expression is regulated by NF-κB (Hinz *et al,* 1999), AP-1 proteins also bind directly to the cyclin D1 promoter and activate it (Shaulian & Karin, 2001). It is therefore likely that NF-κB and AP-1, in concert, support cell proliferation by activating cyclin D1 and cyclin D2. We speculate that galectin-9 inhibits cyclin D1 and cyclin D2 expression through the suppression of both NF-κB and AP-1, resulting in the induction of cell cycle arrest at the G1 phase. All EBV-immortalized human B-cell lines, but not PBMC from healthy subjects, exhibited constitutive activation of NF-κB and AP-1 (data not shown). This suggests a possible reason for the susceptibility to galectin-9 of EBV-immortalized human B-cell lines.

In conclusion, both NF-κB and AP-1 are potentially suitable molecular targets in the treatment of BL and HL. The protease-resistant galectin-9 is a suitable candidate for the development of new molecular-targeted therapies against BL and HL.

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Fig 1. Inhibitory effects of hG9NC(null) on cell growth of several human B-cell lines. Cells were incubated in the presence of various concentrations of hG9NC(null), hG8NC(null) or gelectin-1 (0.01, 0.03, 0.1, 0.3 and 1 μmol/l) for 24 h and *in vitro* growth of the cultured cells was measured by WST-8 assay. The relative growth of cultured cells is presented as the mean determined on PBMC or B cells from healthy controls and EBV-immortalized human B-cell lines (A), BL cell lines (B) and HL cell lines (C) from triplicate cultures. A relative growth of 100% was designated as the total number of cells that grew in the 24 h cultures in the absence of galectins.

Fig 2. Expression of Tim-3 in BL and HL cell lines as assessed by RT-PCR and Western blotting. RNA and cell lysates were prepared from the indicated cells. *ACTB* and actin expression served as the control. Normal PBMC stimulated with phytohaemagglutinin (PHA) (10 μg/ml) were used as positive control. Representative data of three experiments with similar results.

Fig 3. β-galactoside binding is essential for galectin-9-induced suppression of cell growth. Raji and KM-H2 cells were incubated with or without various concentrations of lactose or sucrose (10, 20 and 30 mmol/l) in the presence of 0.3 μmol/l galectin-9 and the cell growth suppressive activities of galectin-9 were assessed by WST-8. A relative growth of 100% was designated as the total number of cells that grew in the

24 h cultures in the absence of galectin-9. Data are mean ± SD of triplicate experiments.

Fig 4. Galectin-9 induces apoptosis of BL and HL cells. (A) BL and HL cell lines were treated with or without galectin-9 (1 μmol/l) for 24 h. Cells were harvested, then stained with Annexin V and analyzed by flow cytometry. Data represent the percentages of apoptotic cells for both untreated (open bars) and galectin-9-treated (solid bars) cells. (B) Galectin-9 activates caspase-3, -8 and -9. Raji cells were treated with or without galectin-9 (0.3 μmol/l) for 24 h. Caspase activity was assayed as described in Materials and Methods and expressed relative to untreated cells, which were assigned a value of 1. Values represent the mean ± SD of three experiments. (C) Raji cells were treated with galectin-9 (0.1, 0.3 and 1 μmol/l) for 12 h and were subjected to immunoblotting analyses with the indicated antibodies. Arrowheads and arrows denote procaspase-8 (57 kDa) and its processed fragments (43/41 and 18 kDa), and procaspase-9 (47 kDa) and its processed fragments (37 and 35 kDa), respectively. Representative data of three experiments with similar results.

Fig 5. Galectin-9 induces cell cycle arrest in BL and HL cell lines. The indicated cell lines were incubated in the absence or presence of galectin-9 (0.3 μmol/l) for 12 h. The cells were then washed, fixed, stained with propidium iodide and analyzed for DNA content by flow cytometry. Three independent experiments per cell line were performed and results are presented as mean percentage ± SD (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.005$; d, $P < 0.0005$).

Fig 6. Galectin-9 modulates levels of cell cycle- and apoptosis-regulating proteins. Raji cells were treated with various concentrations of galectin-9 (0.1, 0.3 and 1 μmol/l) for 12 h (A) and with 0.3 μmol/l galectin-9 for the indicated time periods (B). Total cellular proteins (20 μg per lane) were separated on SDS-polyacrylamide gels and transferred to the membrane. Protein levels were detected by Western blotting with antibodies directed against each protein. Representative data of three experiments with similar results.

Fig 7. Galectin-9 suppresses nuclear NF-κB and AP-1 activities. Effect of 12-h treatment with various concentrations of galectin-9 in Raji cells on activation of the transcription factors NF-κB (A) and AP-1 (D) assessed by EMSA using oligonucleotide probes for NF-κB and AP-1. EMSA using nuclear extracts from untreated Raji cells, and radiolabeled NF-κB (B) and AP-1 (E) probes generated DNA-protein complexes (arrows), which were eliminated by 100-fold molar excess of self-competitors but not by the same molar excess of unrelated oligonucleotides. Supershift assays were performed using the radiolabeled NF-κB (B) and AP-1 (E) probes, untreated nuclear extracts and the indicated polyclonal antibodies to NF-κB components p50, p65, c-Rel, p52 and RelB (B) and to AP-1 components c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD (E). Effects of galectin-9 on the levels of IκBα and phosphorylated IκBα (C) and JunD (F) by Western blot analysis. Raji cells were treated with various concentrations of galectin-9 for 12 h, followed by protein

extraction. Whole cell extracts (20 μg per lane) of treated cells were immunoblotted with specific antibodies. Representative data of three experiments with similar results.