A modified version of galectin-9 suppresses cell growth and induces apoptosis of human T-cell leukemia virus type I-infected T-cell lines

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Abbreviations: AP-1, activator protein-1; ATL, adult T-cell leukemia; Cdk, cyclin-dependent kinase; CRD, carbohydrate recognition domain; EMSA, electorophoretic mobility shift assay; HTLV-I, human T-cell leukemia virus type I; IC_{50}, 50% inhibitory concentration; IL-2R, interleukin-2 receptor; NF-κB, nuclear factor-κB; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RT-PCR, reverse transcription-PCR; SCID, severe combined immunodeficiency; Tim-3, T-cell immunoglobulin- and mucin-domain-containing molecule-3; WST-8, water-soluble tetrazolium-8.

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This is the first report to demonstrate the therapeutic effect of a protease-resistant form of galectin-9 with a modified linker peptide structure, hG9NC(null), for adult T-cell leukemia and elucidate the molecular mechanism of hG9NC(null)-induced cell

cycle arrest and apoptosis. Our results show that hG9NC(null) might be a useful chemotherapeutic or chemopreventive agent in adult T-cell leukemia.

Abstract

ATL is a fatal malignancy of T lymphocytes caused by HTLV-I infection and remains incurable. Galectins are a family of animal lectins that function both extracellularly (by interacting with cell surface and extracellular matrix glycoproteins and glycolipids) and intracellularly (by interacting with cytoplasmic and nuclear proteins) to modulate signaling pathways. We found that protease-resistant galectin-9 by modification of its linker peptide, hG9NC(null), prevented cell growth of HTLV-I-infected T-cell lines and primary ATL cells. The suppression of cell growth was inhibited by lactose, but not by sucrose, indicating that β-galactoside binding is essential for hG9NC(null)-induced cell growth suppression. hG9NC(null) induced cell cycle arrest by reducing the expression of cyclin D1, cyclin D2, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C and c-Myc, and apoptosis by reducing the expression of XIAP, c-IAP2 and survivin. Most of these genes are regulated by NF-κB, which plays a critical role in oncogenesis by HTLV-I. hG9NC(null) suppressed IκBα phosphorylation, resulting in suppression of NF-κB. Most importantly, treatment with hG9NC(null) (6.7 mg/kg injected intraperitoneally every day) reduced tumor formation from an HTLV-I-infected T-cell line when these cells were inoculated subcutaneously into SCID mice. Our results suggest that hG9NC(null) could be a suitable agent for the management of ATL.

Introduction

ATL is a unique malignancy of mature CD4-positive T cells caused by HTLV-I.[1-3] ATL is subclassified into 4 subtypes: acute, lymphoma, chronic and smoldering. In the relatively indolent smoldering and chronic types, the median survival time is more than 2 years. However, at present, there is no accepted curative therapy for ATL and the condition often progresses to death with a median survival time of 13 months in aggressive ATL.[4] Chemotherapies that are specifically known to be active against other lymphoid malignancies are ineffective for treating aggressive forms of ATL. The death is usually due to severe infection or hypercalcemia. Therefore, the establishment of new therapeutic strategies for ATL is very important.

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.[5-9] The members can be classified into 3 subtypes according to their structures. The prototype (galectin-1, -2, -7, -10 and -13) and chimera type (galectin-3) galectins have a single CRD and they usually form a non-covalent homodimer resulting in homobifunctional sugar binding activity. 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.

Galectin-9 was first identified as a tumor antigen of unknown function in patients with Hodgkin's disease.[10] Recent studies suggested that galectin-9 is a modulator of immune functions; it induces chemotaxis of eosinophils[11] and apoptosis of thymocytes, suggesting a possible role in the process of negative selection occurring during T-cell development.[12] With the objective of finding newer agents for the treatment of ATL, the present study was designed to investigate the antitumor potential of galectin-9 on HTLV-I-infected T-cell lines and primary ATL cells *in vitro* and *in vivo*, and the possible mechanisms involved in such antitumor activities. Since the protease susceptibility of galectin-9 makes it difficult to efficiently carry out *in vivo* experiments with recombinant proteins, we used the protease-resistant galectin-9 by modification of its linker peptide, hG9NC(null), in this study.

Material and methods

Cell lines

Burkitt's lymphoma B-cell line, Ramos, HTLV-I-uninfected T-cell line, Jurkat and HTLV-I-infected T-cell lines, MT-2,[13] MT-4,[14] C5/MJ,[15] SLB-1,[16] HUT-102,[2] MT-1[17] and ED-40515(-)[18] were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (JRH Biosciences, Lenexa, KS), 50 U/ml penicillin and 50 μg/ml streptomycin. MT-2, MT-4, C5/MJ and SLB-1 are HTLV-I-transformed T-cell lines and constitutively express viral genes including Tax. MT-1 and ED-40515(-) are T-cell lines of leukemic cell origin established from ATL patients, confirmed by

chromosome analysis[17] or the same provirus integration site and T-cell receptor gene rearrangement pattern on Southern blot analysis as those of the original leukemic cells,[18] and do not express viral genes. HUT-102 was established from a patient with ATL and constitutively expresses viral genes, but it is unclear whether HUT-102 cells represent the tumor clone from the donor ATL patient.

Clinical samples

The diagnosis of ATL was based on clinical features, hematological findings and the presence of anti-HTLV-I antibodies in the sera. Monoclonal HTLV-I provirus integration into the DNA of leukemic cells was confirmed by Southern blot hybridization in all patients (data not shown). PBMCs from healthy volunteers and patients with acute and chronic types ATL were analyzed. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and washed with PBS. PBMCs from healthy volunteers were stimulated with PHA (10 μg/ml). All samples were obtained after informed consent.

Reagents

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 previously described.[19] Both mutant proteins are highly stable against proteolysis.[19] Lactose and sucrose were purchased from Wako chemicals (Osaka, Japan). Rabbit polyclonal antibodies to cyclin D2, c-IAP2, survivin, JunD, NF-κB subunits p65, p50, c-Rel and p52 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Rabbit polyclonal antibody to Bcl-x_L was purchased from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal antibodies to Bcl-2, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C, c-Myc, actin and phosphorylated form of the retinoblastoma protein (pRb) (Ser780) were purchased from NeoMarkers (Fremont, CA). 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) was purchased from Cell Signaling Technology (Beverly, MA).

Cell growth inhibition and apoptosis assays

The effect of modified versions of galectins on cell growth was examined by use of the cell proliferation reagent, WST-8 (Wako Chemicals). Briefly, 1×10^5 cells/ml (cell lines) or 1×10^6 cells/ml (PBMCs) 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 modified versions of galectins. After 24 hr of culture, WST-8 (5 μl) was added for the last 4 hr of incubation and the 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. The IC_{50} was extrapolated from trend line data. For detection of apoptosis, the Annexin V binding capacity of the treated cells was examined by flow cytometry (FACS Caliber, Becton Dickinson, San Jose, CA) 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). In brief, 1 x 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 a FACS Caliber using CellQuest. The population of cells in each cell cycle phase was determined with ModFit software.

In vitro *measurement of caspase activity*

Measurement of caspase activity was performed with the colorimetric caspase assay kits (MBL). Cell extracts were recovered with the use of the Cell Lysis buffer and assessed for caspase-3, -8 and -9 activities by means of colorimetric probes. Colorimetric caspase assay kits are based on detection of the 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.

Western blot analysis

Cells were lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% 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).

RT-PCR

Total cellular RNA was extracted with Trizol (Invitrogen Corp., Carlsbad, CA) 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 Shuzo, Kyoto, Japan) with random primers. Thereafter, cDNA was amplified for 30 cycles for galectin-8, galectin-9, Tim-3, cyclin D1, and cyclin D2 and 28 cycles for β-actin. The oligonucleotide primers used were as follows: for galectin-8, sense, 5’-TCCAGGTGGATCTGCAGAATGGCA-3’ and antisense, 5’-GATCCTGTGGCCATAGAGCAGAGT-3’; for galectin-9, sense, 5’-GATGAGAATGCTGTGGTCCG-3’ and antisense, 5’-GAAGCCGCCTATGTCTGCA-3’; for Tim-3, sense, 5’-ACAGAGCGGAGGTCGGTCAGAATG-3’ and antisense, 5’-AGCCAGAGCCAGCCCAGCACAGAT-3’; for cyclin D1, sense, 5’-CTGTCGCTGGAGCCCGTGAAAAAG-3’ and antisense, 5’-GAAGTTGTTGGGGCTCCTCAGGTT-3’; for cyclin D2, sense, 5’-TTGGCTGGGGTCCCGACTCCGAAG-3’ and antisense, 5’-TCAGAGACAGCTTCTCCCGCTGCT-3’; and for β-actin, sense, 5’-GTGGGGCGCCCCAGGCACCA-3’ and antisense, 5’-CTCCTTAATGTCACGCACGATTTC-3’. Product sizes were 257 bp for galectin-8, 260 bp for galectin-9, 572 bp for Tim-3, 405 bp for cyclin D1, 268 bp for

cyclin D2 and 548 bp for β-actin. Cycling conditions were as follows: denaturing at 94°C for 60 sec (for cyclin D1 and cyclin D2) or for 30 sec (for galectin-8, galectin-9, Tim-3, and β-actin), annealing at 60°C for 15 sec (for galectin-8 and galectin-9), for 60 sec (for cyclin D1) or for 30 sec (for β-actin), 62°C for 30 sec (for Tim-3) or 55°C for 60 sec (for cyclin D2) and extension at 72°C for 60 sec (for galectin-8 and galectin-9), for 45 sec (for Tim-3), for 120 sec (for cyclin D1 and cyclin D2) or for 90 sec (for β-actin). The PCR products were fractionated on 2% agarose gels and visualized by ethidium bromide attaining.

Preparation of nuclear extracts and EMSA

Cells were placed in culture and examined for inhibition of NF-κB after exposure to hG9NC(null) for 12 hr. Nuclear proteins were extracted and NF-κB binding activities to NF-κB element were examined by EMSA as described previously.[20] In brief, 5 μg of nuclear extracts were preincubated in a binding buffer containing 1 μg poly-deoxy-inosinic-deoxy-cytidylic acid) (Amersham Biosciences), followed by addition of [α-^{32}P]-labeled oligonucleotide probe containing NF-κB element (approximately 50,000 cpm). These 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 the NF-κB 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 *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 NF-κB proteins in the DNA-protein complex revealed by EMSA, we used antibodies specific for various NF-κB family proteins, including p65, p50, c-Rel and p52, 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.

In vivo *administration of hG9NC(null)*

Five-week-old female C.B-17/Icr-SCID mice obtained from Ryukyu Biotec Co. (Urasoe, Japan) were maintained in containment level 2 cabinets and provided with autoclaved food and water *ad libitum*. Mice were engrafted with 5 x 10^6 HUT-102 cells by subcutaneous injection in the post auricular region and were randomly placed into 2 cohorts of 5 mice each that received vehicle and hG9NC(null), respectively. Treatment was initiated on the day of cell injection. hG9NC(null) was dissolved in PBS at a concentration of 8.6 μM and 6.7 mg/kg body weight of hG9NC(null) was administered intraperitoneally every day for 21 days. Control mice received the same volume of the vehicle (PBS) only. Tumor size was monitored once a week. All mice were sacrificed on day 21, and then the tumors were dissected out and their weight was physically measured. This experiment was performed according to the guidelines for the Animal Experimentation of the University of the Ryukyus and was approved by the Animal Care and Use Committee of the University of the Ryukyus.

Statistical analysis

Data are expressed as mean ± SD. The significance of the difference between each experiment sample [treated with hG9NC(null)] and the control was determined using the Student's *t*-test. Volume and weight of tumors from hG9NC(null)-treated mice were compared to those of the vehicle-treated controls by the Mann-Whitney *U*-test.

Results

Modified version of galectin-9 inhibits growth of HTLV-I-infected T-cell lines and primary ATL cells

We first examined the effects of the recombinant mutant form of galectin-9 lacking the entire linker region, hG9NC(null), on the growth of HTLV-I-infected T-cell lines. Tax protein was detected by immunoblot analysis in the 5 HTLV-I-infected T-cell lines (MT-2, MT-4, C5/MJ, SLB-1 and HUT-102) but not in the 2 ATL-derived T-cell lines [MT-1 and ED-40515(-)]. Culture of cells with various concentrations (0 to 1 μM) of hG9NC(null) for 24 hr resulted in the suppression of cell growth in a dose-dependent manner in all 7 lines tested as assessed by the WST-8 assay (Fig. 1*a*, *left panel*). The concentrations of hG9NC(null) required to inhibit growth of HTLV-I-infected T-cell lines by 50% (IC_{50}) ranged from 0.012 to 0.095 μM (Table I). Maximum effect on most cell lines was observed at 0.1 μM. Although the sensitivity to hG9NC(null) varied among the cell lines studied, Tax did not influence the susceptibility to hG9NC(null) among the HTLV-I-infected T-cell lines. On the other hand, HTLV-I-negative T-cell lines, Jurkat, and Burkitt's lymphoma B-cell line, Ramos, were less susceptible to hG9NC(null) than HTLV-I-infected T-cell lines. IC_{50}

in Jurkat and Ramos was 0.197 and >1.0 μM, respectively (Table I). Recent studies reported that soluble galectin-8 inhibits growth of human non-small cell lung carcinoma cells.[21] Therefore, we studied in the next step the effect of recombinant mutant form of galectin-8 lacking the entire linker region, hG8NC(null), on the growth of HTLV-I-infected T-cell lines. However, 1 μM hG8NC(null) did not affect cellular growth (Fig. 1*a*, *right panel*). Three μM hG8NC(null) slightly inhibited growth of some cell lines (data not shown). We further examined the effects of hG9NC(null) on freshly isolated ATL cells from 7 acute type patients (ATL 1-7) and 2 chronic type patients (ATL 8 and 9). Tax protein was not detected in patient cells that were freshly isolated and lysed by immunoblot analysis (data not shown). ATL cells treated with hG9NC(null) showed reduced cell growth compared with PBMCs from normal healthy controls (Fig. 1*b*, *left panel*). IC_{50} of ATL cells ranged from 0.007 to 0.290 μM (Table I). The growth of PHA-stimulated PBMCs (PHA-blast) was also inhibited by hG9NC(null) in a dose-dependent manner (Fig. 1*c*, left panel). In contrast, hG8NC(null) did not affect the cell growth of ATL cells from 4 patients (ATL 6-9) and PBMCs from normal healthy controls (Fig. 1*b*, *right panel*) and PHA-stimulated PBMCs (Fig. 1*c*, right panel).

Expression of mRNA levels of galectin-9, galectin-8 and Tim-3 in HTLV-I-infected T-cell lines and primary ATL cells

HTLV-I-infected T-cell lines and primary ATL cells were highly sensitive to hG9NC(null)-induced growth suppression, whereas normal PBMCs were resistant. Therefore, we investigated the expression of the endogenous mRNA levels of

galectin-9 and galectin-8 in these cells by RT-PCR analysis. The results of this experiment are shown in Figure 1*d*. High levels of galectin-9 mRNA were observed in Jurkat cell line and normal PBMCs, whereas no expression of galectin-9 mRNA was detected in the sensitive MT-2 and MT-4 cell lines and all ATL cells. Low levels of galectin-9 mRNA were found in the residual HTLV-I-infected T-cell lines. Conversely, amplified bands for galectin-8 were highly detected in PBMCs from both normal controls and ATL patients. Among different T-cell lines, Jurkat, MT-2, C5/MJ, and SLB-1 cell lines highly expressed galectin-8 mRNA. These data indicated that there is an inverse correlation between the sensitivity to hG9NC(null) and the ability to express the endogenous *galectin-9* gene.

Recently, galectin-9 has been identified as the Tim-3 ligand.[22] Tim-3 is a Th1-specific cell surface molecule that seems to regulate Th1 responses and the induction of peripheral tolerance. We examined Tim-3 mRNA expression using RT-PCR. However, Tim-3 was expressed at relatively same or low levels in primary ATL cells compared with those in normal PBMCs (Fig. 1*d*). These results suggest that the degree of Tim-3 expression does not correlate with the sensitivity to hG9NC(null)-induced cell death.

β-galactoside binding is essential for hG9NC(null)-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 mM) in the presence of 0.3 μM hG9NC(null). Figure 1*e* shows that the cell growth inhibitory activity of hG9NC(null) is inhibited by lactose in a dose-dependent fashion, but not by sucrose,

indicating that β-galactoside binding activity is essentially required for hG9NC(null)-induced cell growth suppression.

hG9NC(null) induces apoptosis of HTLV-I-infected T-cell lines

To examine whether induction of apoptosis accounts for the cell growth inhibition observed in HTLV-I-infected T-cell lines, cells treated with hG9NC(null) 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 found in cells entering apoptosis. hG9NC(null) increases the proportion of cells positive for Annexin V in all cell lines and such an effect was observed in a dose-dependent fashion in HUT-102, SLB-1 and MT-1 cells (Fig. 2*a*). Furthermore, apoptosis was confirmed by immunostaining 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).[23,24] These results indicate increased proportion of apoptosis of hG9NC(null)-treated cells.

hG9NC(null)-induced apoptosis is caspase-dependent

We examined whether caspase activation is involved in hG9NC(null)-induced apoptosis. hG9NC(null) treatment resulted in activation of caspases-3, -8 and -9 in HUT-102 and MT-2 cells (Fig. 2*b*). These results demonstrate the involvement of caspase activation in hG9NC(null)-induced apoptosis in HTLV-I-infected T-cell lines.

hG9NC(null) induces cell cycle arrest

We investigated the effect of hG9NC(null) on the cell cycle progression in cell lines. The cells were incubated with hG9NC(null) for 12 hr and analyzed for cell cycle distribution by flow cytometry, since incubation for 24 hr induced cell death (Fig. 2*c*). hG9NC(null) inhibited cell cycle progression, as evidenced by an increasing proportion of cells in G_2-M phase in all cell lines with the exception of SLB-1. In addition, cultivation with hG9NC(null) increased the proportion of cells in the G_1 phase, with a reduction of cells in the S phase, indicating G_1 arrest in MT-4, C5/MJ and SLB-1 cells. The unchanged number of cells in G_1, despite the decrease of cells in the S phase in HUT-102 and MT-1 cells, can be explained by either no entry in the G_1 (due to G_2-M arrest) or no exit from G_1 (due to G_1 arrest). In the absence of G_1 arrest, cells accumulated exclusively in G_2-M phase. These results clearly show that hG9NC(null) induces G_1 and/or G_2-M arrest of the cells. The apparent increase of the proportion of cells in the sub G_0-G_1 phase was observed at 24 hr incubation, suggesting that cell cycle arrest is the cause of apoptosis.

hG9NC(null)-treated HTLV-I-infected T-cell lines express intracellular regulators of cell cycle and apoptosis

To clarify the molecular mechanisms of hG9NC(null)-induced inhibition of cell growth and apoptosis in HTLV-I-infected T-cell lines, 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, Bcl-2, Bcl-x_L, XIAP, c-IAP2 and survivin by Western blot analysis. As shown in Figure 3, hG9NC(null) did not alter Bcl-2 and Bcl-x_L levels. In contrast, hG9NC(null) significantly decreased the

expression of survivin, XIAP, c-IAP2, cyclin D1, cyclin D2, cyclin B1, Cdk1, Cdk4, Cdk6, Cdc25C and c-Myc in a dose- and time-dependent manner. Treatment with hG9NC(null) led to downregulation of the phosphorylated form of pRb. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin (Figs. 3*a* and 3*b*). We explored the effect of hG9NC(null) on expression of these proteins in freshly isolated ATL cells. hG9NC(null) decreased the expression of XIAP, cyclin D2, cyclin B1, Cdk1, Cdk4 and Cdk6 (Fig. 3*c*). To investigate the change of intracellular regulators expression at transcriptional level, we examined the expression of cyclin D1 and cyclin D2 mRNA by RT-PCR. The results demonstrated downregulation of both genes by hG9NC(null) treatment (Fig 3*d*). Thus, the downregulation of intracellular regulators expression by hG9NC(null) treatment was at transcriptional level.

hG9NC(null) 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.[25] Because NF-κB is constitutively active in Tax-expressing and HTLV-I-infected T-cell lines as well as primary ATL cells,[20] and the expression of survivin, XIAP, c-IAP2, cyclin D1, cyclin D2, Cdk4, Cdk6 and c-Myc are known to be regulated by NF-κB,[26-32] we examined whether hG9NC(null) 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 or hG9NC(null)-treated HTLV-I-infected T-cell lines and primary ATL cells. NF-κB oligonucleotide probe with nuclear extracts from untreated

HTLV-I-infected T-cell lines and primary ATL cells generated DNA-protein gel shift complexes (Fig. 4*a*). These complexes were due to specific bindings of nuclear proteins to the NF-κB sequence, because these binding activities were reduced by the addition of cold probe but not by an irrespective sequence (Fig. 4*b*). We also showed that NF-κB complexes contain p50, p65 and c-Rel in HTLV-I-infected T-cell lines. Constitutively activated NF-κB in primary ATL cells contains p50 and p65 (Fig. 4*b*). As shown in Figure 4*a*, nuclear extracts prepared from HTLV-I-infected T-cell lines and ATL cells treated with hG9NC(null) for 12 hr exhibited a decrease in the intensity of the NF-κB-containing gel shift complexes in a dose-dependent manner, suggesting that hG9NC(null) downregulates the DNA binding activities of NF-κB. Although reduced, there was still NF-κB activity detected in 0.3 μM hG9NC(null)-treated MT-1 cells, but at the same concentration the expression of NF-κB-inducible genes was switched off. These inconsistent results might be due to the difference of sensitivity between 2 assays.

Degradation of IκBα and subsequent release of NF-κB requires prior phosphorylation at Ser32 and Ser36 residues.[33] To investigate whether the inhibitory effect of hG9NC(null) is mediated through alteration of phosphorylation of IκBα, MT-1 cells were treated with hG9NC(null) and their protein extracts were checked for phospho-IκBα expression. Untreated MT-1 cells constitutively expressed Ser32- and Ser36-phosphorylated IκBα (Fig. 4*c*), while hG9NC(null) treatment decreased the phosphorylated IκBα in a dose-dependent manner. The increased expression of JunD, which composes the increased DNA-binding AP-1 protein, also plays a central role in the proliferation of HTLV-I-infected T-cell lines and primary ATL cells,[34] and

attempts to induce apoptosis by inhibition of JunD were succeful.[35] However, JunD expression level was not affected by treatment of cells with hG9NC(null) (Fig. 3*a*).

Antitumor effects of hG9NC(null) on subcutaneous HUT-102 tumors

Finally, we examined the effects of hG9NC(null) against ATL *in vivo*. SCID mice (n = 10) were inoculated with HUT-102 and then divided into 2 groups: untreated mice (n = 5) and hG9NC(null)-treated mice (n = 5). Treatment commenced on the day of the inoculation. At day 21 posttreatment, the mean tumor volume (Fig. 5*a*) and weight (Fig. 5*b*) were significantly lower than those of vehicle-treated mice ($p < 0.05$ by the Mann-Whitney *U*-test) (Fig. 5). These results suggest that hG9NC(null) also has *in vivo* anti-ATL effect.

Discussion

ATL follows an invariably fatal clinical course in spite of the introduction of various chemotherapeutic agents. Although many ATL patients initially respond to chemotherapy, drug resistance eventually develops, which prevents curative treatment. Currently, although allogenic hematopoietic stem cell transplantation produces better results, it often causes serious clinical side effects and entails the risk of graft-versus-host disease.[36] Therefore, a novel therapeutic approach based on new insights into the pathogenesis of ATL is strongly desired.

Like other mammalian lectins, galectins have been implicated in diverse biological processes, including modulation of cell-cell and cell-matrix interactions. Although galectin-1 and -3 have been well studied among the various galectins, we do

not have enough information on galectin-9. Wada *et al.*[12] showed that galectin-9 induces mouse thymocyte apoptosis. We have found that HTLV-I-infected T-cell lines and ATL cells from different patients are more susceptible to growth inhibition induced by treatment with hG9NC(null) than normal PBMCs. These data therefore demonstrate that hG9NC(null) is effectively cytotoxic in ATL cells without toxicity to normal PBMCs. Although all members of the galectin family contain β-galactoside binding domains, they do not consistently induce apoptosis. Indeed, hG8NC(null) did not induce apoptosis of these cells. However, hG9NC(null)-induced apoptosis was mediated *via* binding with specific galactosyl groups, since the effect could be competitively inhibited with lactose. These results suggest that cell surface hG9NC(null) binding molecules responsible for apoptosis are expressed in T cells susceptible to hG9NC(null). Recently, galectin-9 has been identified as the Tim-3 ligand.[22] We examined Tim-3 mRNA expression using RT-PCR. However, the degree of Tim-3 expression does not correlate with the sensitivity to galectin-9-induced cell death.

Galectins are widely distributed in various tissues with a variable degree of expression. Perillo *et al.*[37] demonstrated that galectin-1 is a mediator of T-cell apoptosis and that among T-cell leukemia cells some are sensitive and some are insensitive to treatment with galectin-1. It has been demonstrated that susceptible human T-cell leukemia cells do not express the endogenous *galectin-1* gene, whereas non-sensitive T-cell leukemia cells express high levels of *galectin-1*.[38] We examined galectin-8 and -9 expression in PBMCs from healthy volunteers and patients with ATL by RT-PCR. We determined galectin-8 expression in ATL cells and controls. In

addition, we have found that normal PBMCs express high levels of galectin-9, in contrast to primary ATL cells, which do not express galectin-9. These results suggest that non-sensitive PBMCs express a high level of galectin-9, whereas no detectable levels of galectin-9 mRNA were found in sensitive cells. Further experiments will be necessary to determine if the relationship between galectin expression and susceptibility is conserved in ATL cells and if ATL cells are selected for loss of production of apoptotic factors.

Our results showed that the growth-inhibitory potential of hG9NC(null) on HTLV-I-infected T-cell lines was mainly due to the induction of cell cycle arrest and apoptosis, because a significant population of cells remained in the G_1 and/or G_2-M phases of the cell cycle and underwent apoptosis in a dose-dependent manner after hG9NC(null) treatment. Cell cycle arrest in G_1 and G_2-M phases by hG9NC(null) treatment may be associated with the downregulation of expression of proteins involved in G_1-S transition (cyclin D1, cyclin D2, Cdk4, Cdk6 and c-Myc) and G_2-M transition (cyclin B1, Cdk1 and Cdc25C). Together, these changes caused a decrease in the phosphorylated pRb level in HTLV-I-infected T-cell lines.

hG9NC(null)-induced apoptosis in HTLV-I-infected T-cell lines 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,[39] 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,[40] 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, tumor necrosis factor receptor and tumor necrosis factor-related apoptosis-inducing ligand receptor, which HTLV-I-infected T cells express. 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. For the first time, 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 elevated expression levels 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, c-IAP2, survivin, cyclin D1, cyclin D2, Cdk4, Cdk6 and c-Myc, whose synthesis is known to be regulated by NF-κB,[26-32] was suppressed by hG9NC(null). Although Bcl-x_L and Bcl-2 are known as NF-κB targets, Stat3, which is constitutively activated in HTLV-I-infected T-cell lines and primary ATL cells, also regulates Bcl-x_L and Bcl-2 transcription.[41,42] Therefore, the expression of Bcl-x_L and Bcl-2 might not be affected by hG9NC(null) treatment. Decrease in the NF-κB activity, may be at least in part responsible for induction of cell cycle arrest and apoptosis by hG9NC(null) in HTLV-I-infected T-cell lines.

In HTLV-I-expressing cells, a virus-encoded regulatory protein, Tax, plays a critical role in the growth and survival of the infected T cells by perturbing normal regulatory mechanisms, including transcription, signal transduction and cell cycle progression, resulting in uncontrolled cell growth.[43] Tax activates NF-κB by stimulating the activity of the IκB kinase which in turn leads to phosphorylation and degradation of IκBα.[44] However, primary ATL cells are known to have very low or no expression of Tax and therefore, growth of ATL cells *in vivo* is believed to be Tax-independent.[45] hG9NC(null) could inhibit NF-κB, resulting in induction of cell death in Tax-negative HTLV-I-infected T-cell lines and primary ATL cells. In addition, hG9NC(null) did not inhibit the level of Tax expression in HTLV-I-infected T-cell lines (data not shown). Therefore, the growth inhibition of HTLV-I-infected T-cell lines and primary ATL cells induced by hG9NC(null) appears to be mediated by a Tax-independent pathway.

Several NF-κB inhibitors such as Bay 11-7082, dehydroxymethylepoxyquinomicin and NIK-333 were reported to block constitutive NF-κB activation and induce apoptosis in HTLV-I-infected T-cell lines.[46-48] The effect of Bay 11-7082, dehydroxymethylepoxyquinomicin and NIK-333 on growth suppression of HTLV-I-infected T-cell lines occurred at an IC_{50} of 2.5-5.0, 13-27 and 9.2-24.2 μM, respectively. On the other hand, IC_{50} of hG9NC(null) ranged from 0.012 to 0.095 μM. Thus, compared with the results described in previous reports, results of the present study suggest a more activity of hG9NC(null).

The potent and selective apoptotic effect of hG9NC(null) against HTLV-I-infected T-cell lines and primary ATL cells *in vitro* prompted us to evaluate

its *in vivo* anti-ATL effect in SCID mice bearing an HTLV-I-infected T-cell line, HUT-102. We used a protease-resistant form of galectin-9 with a modified linker peptide structure[19] and found that it not only inhibited tumor cell growth *in vitro* but also inhibited tumor formation *in vivo*. During the period from day 0 to 21, the control mice showed signs of severe disease, including piloerection. In contrast, mice treated with hG9NC(null) showed no significant adverse effects and tolerated this dose well. These results suggest that removal of the entire linker peptide region of galectin-9 greatly improved its stability against proteolysis without negative effects on its biological activities. We conclude that hG9NC(null) might be used as a chemotherapeutic or chemopreventive agent in ATL, but further clinical studies will be necessary to assess its potential in primary fresh ATL cells.

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TABLE I - IC_{50} FOR INHIBITION OF CELL GROWTH OF hG9NC(NULL)

Cell	HTLV-I	Tax status[1]	IC_{50} for inhibition of cell growth (μM)
MT-2	+	+	0.078
MT-4	+	+	0.023
C5/MJ	+	+	0.090
SLB-1	+	+	0.017
HUT-102	+	+	0.012
MT-1	+	-	0.095
ED-40515(-)	+	-	0.040
Jurkat	-	-	0.197
Ramos	-	-	>1.0
Normal 1	-	-	>1.0
Normal 2	-	-	>1.0
ATL 1	+	-	0.020
ATL 2	+	-	0.025
ATL 3	+	-	0.290
ATL 4	+	-	0.015
ATL 5	+	-	0.007
ATL 6	+	-	0.041
ATL 7	+	-	0.040
ATL 8	+	-	0.033

ATL 9	+	-	0.073
PHA-blast 1	-	-	0.013
PHA-blast 2	-	-	0.026

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[1]Tax expression can be detected by Western blotting.

Figure legends

FIGURE 1 - Inhibitory effects of hG9NC(null) on cell growth of HTLV-I-infected T-cell lines and primary ATL cells. Cells were incubated in the presence of various concentrations of hG9NC(null) or hG8NC(null) (0.01, 0.03, 0.1, 0.3 and 1 μM) for 24 hr and *in vitro* growth of the cultured cells was measured by WST-8 assay. Normal PBMCs were stimulated with PHA (10 μg/ml) in the designated wells. Relative growth of the cultured cells is presented as the mean determined on HTLV-I-infected T-cell lines (*a*), PBMCs from healthy controls and ATL patients (*b*) and PHA-stimulated PBMCs (*c*) from triplicate cultures. A relative growth of 100% was designated as the total number of cells that grew in the 24 hr cultures in the absence of modified versions of galectins. (*d*) Expression of galectin-9, galectin-8 and Tim-3 in HTLV-I-infected T-cell lines and primary ATL cells as assessed by RT-PCR. RNA was prepared from the indicated cells. β-actin expression served as the control. (*e*) β-galactoside binding is essential for hG9NC(null)-induced cell growth suppression. MT-1, SLB-1, MT-2 and HUT-102 cells were incubated with or without various concentrations of lactose or sucrose (10, 20 and 30 mM) in the presence of 0.3 μM hG9NC(null) and cell growth suppressive activities of hG9NC(null) were assessed by WST-8. A relative growth of 100% was designated as the total number of cells that grew in the 24 hr cultures in the absence of hG9NC(null). Data are mean ± SD of triplicate experiments.

FIGURE 2 - hG9NC(null) induces apoptosis and cell cycle arrest in HTLV-I-infected T-cell lines. (*a*) hG9NC(null) induces apoptosis in HTLV-I-infected T-cell lines. HTLV-I-infected T-cell lines were treated with or without hG9NC(null) (0.3 μM) for 24 hr. 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 hG9NC(null)-treated (solid bars) cells (*left panel*). HUT-102, SLB-1 and MT-1 cells were also incubated with various concentrations of hG9NC(null) (0.01, 0.03, 0.1, 0.3 and 1 μM) for 24 hr. The proapoptotic activity of hG9NC(null) was assessed by Annexin V staining (*right panel*). Data represent the mean ± SD of 3 experiments (*$p < 0.0005$, **$p < 0.0001$). (*b*) hG9NC(null) treatment activates caspase-3, -8 and -9 in HTLV-I-infected T-cell lines. HTLV-I-infected T-cell lines were treated with or without hG9NC(null) (0.3 μM) for 24 hr. Caspase activity was assayed as described in Material and methods and expressed relative to untreated cells, which were assigned a value of 1. Values represent the mean ± SD of 3 experiments. (*c*) Galectin-9 induces cell cycle arrest in HTLV-I-infected T-cell lines. HTLV-I-infected T-cell lines were incubated in the absence or presence of hG9NC(null) (0.3 μM) for 12 hr. The cells were then washed, fixed, stained with propidium iodide and analyzed for DNA content by flow cytometry. 3 independent experiments per cell line were performed and results are presented as mean percentage ± SD where $n = 3$ ($^{a}p < 0.05$; $^{b}p < 0.01$, $^{c}p < 0.001$; $^{d}p < 0.0005$; $^{e}p < 0.0001$).

FIGURE 3 - Western blot and RT-PCR analyses of MT-1 cells and primary ATL cells treated with hG9NC(null). MT-1 cells were treated with various concentrations

of hG9NC(null) (0.1, 0.3 and 1 μM) for 12 hr (*a*) and with 0.3 μM hG9NC(null) for the indicated periods (*b*). (*c*) PBMCs from an ATL patient (ATL 4) were also treated with or without 0.3 μM hG9NC(null) for 12 hr. 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. (*d*) Total RNA was extracted from MT-1 cells following treatment with 0.3 μM hG9NC(null) for the indicated periods. The mRNA expression of cyclin D1 and cyclin D2 was analyzed by RT-PCR analysis. β-actin served as an internal control in the RT-PCR procedure.

FIGURE 4 - hG9NC(null) suppresses nuclear NF-κB activity in HTLV-I-infected T-cell lines and primary ATL cells. (*a*) Effect of 12-hr treatment with various concentrations of hG9NC(null) in HTLV-I-infected T-cell lines and primary ATL cells on activation of the transcription factor NF-κB assessed by EMSA using oligonucleotide probe for NF-κB. (*b*) EMSA using nuclear extracts from untreated HTLV-I-infected T-cell lines and primary ATL cells, and radiolabeled NF-κB probe 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 using the radiolabeled NF-κB probe, untreated nuclear extracts and the indicated polyclonal antibodies to NF-κB components p50, p65, c-Rel and p52 showed that the NF-κB bands consisted of p50, p65 and c-Rel subunits. (*c*) Effect of hG9NC(null) on the level of phosphorylated IκBα by Western blot analysis. MT-1 cells were treated with various concentrations of hG9NC(null) for

12 hr, followed by protein extraction. Whole cell extracts (20 μg per lane) of treated cells were immunoblotted with specific antibodies against phospho-IκBα (Ser32 and Ser36) and actin.

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FIGURE 5 - hG9NC(null) inhibits growth of HUT-102 cells in SCID mice. (*a*) HUT-102 cells (5 x 10^6 per mouse) were inoculated subcutaneously into SCID mice. The mice (5 per group) received an intraperitoneal injection of either PBS or hG9NC(null) (6.7 mg/kg) every day. Treatment was initiated on the day of inoculation. 810 The mice were monitored for tumor volumes at 7, 14 and 21 days after cell inoculation. hG9NC(null) suppressed the growth of HUT-102 cells in contrast to the significant increase in tumor burden generated in PBS-treated control mice. (*b*) Weight of tumors removed from hG9NC(null)-treated mice and untreated mice on day 21 after cell inoculation. Data are expressed as mean ± SD of 5 mice.