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Up-regulation of Galectin-3 in HIV-1 tat-transfected Cells

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Previous studies have demonstrated that expression of galectin-3, a member of family of beta-galactoside-binding animal lectin, is associated with pathological conditions including cancer, atherosclerosis, and viral infection. An increase of this lectin has been observed after infection by Kirsten murine sarcoma, human T lymphotropic virus-1 (HTLV-1), and human immunodeficiency virus-1 (HIV-1). Viral transactivation protein Tax of HTLV-1 mediates the increase in the lectin. In case of HIV-1, there are evidences that Tat would be related with increase in galectin-3. We investigated whether Tat directly induced galectin-3 expression in cells. We found that HIV-1 tat gene activated galectin-3 promoter in RAW264.7 cells. To demonstrate direct induction of galectin-3 by HIV-1 tat, we transfected the tat into a rabbit smooth muscle cell line (Rb1) and obtained Rb1TatCl-2, a clone of cell stably transfected with tat gene. The Rb1TatCl-2 cells exhibited activation of LTR promoter and up-regulation of galectin-3 transcript as well as protein. Our results indicate that HIV-1 tat alone is sufficient to induce the expression of galectin-3. The Rb1TatCl-2 cells could be valuable for study of the effect of HIV-1 tat on expression of cellular genes.

Key words – galectin-3, smooth muscle cell, tat

The galectin families which have shared characteristic amino acid sequences in the carbohydrate binding domain and affinity for galactoside sugars in a Ca2+-dependent manner are widely distributed from lower invertebrates to mammalian in animal kingdom. Galectin-3 is one of the best characterized members[4, for review]. The wide distribution in tissues and existencein various types of cells suggest pleotropic effects of this lectin. Galectin-3 was reported to play a role in inflammation[16], cell-cell adhesion[19], and apoptosis[2,32]. This lectin activated mast cells[9], monocyte/macrophage[17], and lymphocytes[32] and amplified inflammatory reaction. Expression of this lectin modulated proliferation and apoptosis of T cells[32]. The absence of galectin-3 changed inflammation reaction and survival of macrophages[14]. In addition, this lectin was identified as a RNA splicing factor in the component of hnRNP[7].

The expression of galectin-3 is altered in certain pathological conditions including atherosclerosis and cancer. Upregulation of this lectin has been observed in human atherosclerotic lesions[22]. Galectin-3 is also expressed in some types of cancer of which normal parental cells do not express. The examples include specific types of lymphoma[15], thyroid carcinoma[31], and hepatocellular carcinoma[12]. The transfection studies support a role of this lectin in tumor tranformation and metastasis[25]. Down-regulation of this lectin, however, has been observed in other kinds of neoplasm, including colon[18], breast[6], and uterine carcinoma[29].

The expression of this lectin is correlated with differentiation and proliferation of cells. Proliferating fibroblasts had a higher level of galectin-3 than did quiescent cultures of the same cells[21]. Differentiation of monocytes to macrophages markedly increased the level of galectin-3[24]. Viral infection also influenced expression of galectin-3. Much higher amount of galectin-3 transcript was detected in 3T3 cells transformed with Kirsten murine sarcoma virus than in normal 3T3 cells[1]. Human T lymphotropic virus-1 (HTLV-1) markedly increased this lectin in human thymocytes and T cell lines which resulted from the activation of galectin-3 promoter by viral transactivation protein Tax[13]. Infection of Molt-3 cells with human immunodeficiency virus-1 (HIV-1) increased expression of galectin-3, concomitantly with the onset of the expression of the viral regulatory gene tat [27]. The correlation between galectin-3 and tat gene after viral infection prompted us to investigate the role of Tat in galectin-3 expression. In the present study, we showed that tat regulatory gene induced up-regulation of galectin-3 transcript as well as protein through activation of galectin-3 promoter.

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Materials and Methods

Cells culture

Spontaneously transformed rabbit vascular smooth muscle cells (Rb1)[23] and RAW264.7 (ATCC TIB 71) cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum.

Plasmids

Expression plasmid for HIV-1 tat (pPL-12) was obtained from Dr. F. Wong-Staal, NIH, Bethesda, MD. The human galectin-3 cDNA clone, rabbit galectin-3 promoter clone pPG3-5, and HIV-1 LTR clone (pLTRX-Luc) were provided by Dr. A. Legrand, University of Orleans, Orleans, France. pPG3.5 is a construct into which the 4.5 kb promoter region of the rabbit galectin-3 was inserted upstream of the luciferase gene in the promoterless plasmid pMCS-Luc[11].

Transient transfection

An equal number of cells was plated onto 60 mm tissue culture plates at 60% confluence and transfected by the calcium phosphate precipitate procedure. For each transfection 1 ug of pCMV beta plasmid was mixed with 3 μg of pPG3-5 and 3 μg of either pRSV-neo, pRSV-r161, for a total of 7 μg of DNA per tissue culture plate. The cells were incubated with the DNA-calcium phosphate precipitate for 8 hours and shocked with cold 10~20% dimethylsulfoxide (DMSO) solution in phosphate-buffered saline (PBS) for 2~3.5 minutes. After washing three times with cold PBS, the cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% FBS. After 48 hours, the luciferase activity was determined using the Luciferase Assay System (Promega, Madison, WI). The luciferase activity was normalized to beta-galactosidase activity.

Stable transfection

Rb1 cells were transfected with plasmids containing neomycin resistant gene and pPL-12 by calcium phosphate precipitate method as described below. At 48 hour post the transfection, the cells were selected in the presence of 400 μg/ml of geneticin for 2 weeks. Clone of cell resistant to neomycin was expanded and maintained in DMEM medium with 10% FBS supplemented with 100 μg/ml geneticin.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA was copied into cDNA using a 25 μl reaction mix containing 2 μg purified total cellular RNA, 1 μg olio (dT)15, 100U Moloney murine virus reverse transcriptase, 40U RNAsin, 800 μM dATP, dCTP, dGTP, and dTTT, 2 mM dithiothreitol (DTT), 4 mM MgCl2, 50 mM KCl, and 10 mM TrisHCl (pH7.3). The cellular RNA and oligo (dT) 15 in a volume of 10 μl were heated at 65℃ for 3 minutes and cooled on ice. Remaining components prepared in a master mix were added. The mix was overlaid with mineral oil and incubated at 45℃ for an hour. Each reaction mix was then diluted with 150 μl glass distilled water and placed in a boiling water bath for 10 minutes to inactivate the reverse transcriptase. PCR was performed with hot start technique. The lower phase contained 10 mM TrisHCl (pH 8.3), 6 mM $MgCl_2$, 800 uM dATP, dCTP, dGTP and dTTP, and 40 μM of each primer. Liquid wax was added, and the tube was heated briefly to melt wax and cool to allow the wax to set. The upper phase was then added. This contained 67 mM KCl, 13 mM TrisHCl (pH 8.3), 2.5 U Taq polymerase and 10 μl of the diluted reverse transcript mix. This was subjected to a 2.5 minutes time delay step at 95℃ in a DNA thermal cycler 480 (Perkin Elmer, Norwalk, CT), followed by 30 cycles of PCR (95℃ for 1 minute, 58℃ for 1 minute, 72℃ for 1 minute). The products were separated on a 2% agarose gel, stained with ethidium bromide and photographed. The amplification primers for HIV-1 tat were 5'-GAAGCATCCAGGAAGTCAGC and 5'-GGAGGTGGGTT-GCTTTGATA.

Northern blot analysis

Cells were lysed by vigorous vortexing in Ultraspec RNA™ (Biotex Laboratories, Inc., Houston, TX) at 1 ml/5×10^6 cells. Total RNA extracted according to manufacturer's instructions was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. For each sample 20 μg of total RNA was loaded on the gel. RNA was transferred to Hybond N+ membrane (Amersham) by capillary action. The membrane was washed, air dried, and crosslinked under UV light (Stratalinker UV Crosslinker, Stratagene, La Jolla, CA). The membrane was prehybridized for 1 hour at 65°C and hybridized overnight at 65°C with ^{32}P-labeled probe prepared by random priming. After washing, the membrane was exposed to a Kodak XAR-5 film, and kept at -70°C for exposure. To monitor the amount of RNA loaded and to normalize these values, the blot was stripped and probed with the ^{32}P-labeled cDNA probe for 18S rRNA. The intensity of the band on the film was analyzed with an ULTROSCAN

XL laser densitometer (LKB BIOCHROM Ltd., Cambridge, England).

Western blot analysis

The cells were lysed in a lysis buffer (50 mM TrisHCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was passed through a 23-gauge needle to shear chromosomal DNA and centrifuged to 12,000 xg for 5 minutes. The protein concentration in the supernatant was determined with the Bio-Rad DC Protein Assay kit. Proteins were separated on SDS-PAGE gels and transferred to polyvinylidine difluoride membranes (Millipore Corp., Bedford, MA). For each sample of cells, an equal amount of cell extract containing 20 μg of total protein was mixed with loading buffer, boiled and loaded into the separating gel. Following the transfer, nonspecific binding sites on the membrane were blocked in T-TBST (50 mM TrisHCl, pH 7,4, 150 mM NaCl, 0.1% Triton X-100) containing 3% bovine serum albumin. The membranes were incubated for 2 hours with the monoclonal antibody in T-TBST. After 3 washes with T-TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rat IgG (1:8,000 dilution). After washing 3 times, the membranes were incubated with ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Inc. Piscataway, NJ). The chemiluminescence signal was imaged on X-ray film.

Results

Activation of galectin-3 promoter by HIV-1 tat

It was examined whether tat could activate galectin-3 promoter. The pPG3-5 plasmid was transiently co-transfected with expression plasmid for Tat (pPL-12), or control plasmid into RAW264.7 cells known to express galectin-3. Forty eight hours later, luciferase activity in the transfected cells was determined (Fig. 1). Co-transfection with Tat expression plasmid increased the luciferase activity more than two fold (217%) compared with pRSVneo, suggesting tat encoded in plasmid pPL-12 activated galectin-3 promoter in cells.

Up-regulation of galectin-3 in cells stably transfected with Tat expression vector

To study further the effect of tat on galectin-3 expression, Rb1 cells were stably co-transfected with tat-encoding plasmid pPL-12 and neomycin resistant gene pRSVneo. The Rb1-TatCl-2 clone resistant to geneticin was selected and expanded. The expression of tat from the clone was examined by amplifying tat transcript by RT-PCR (Fig. 2). A band of 198 bp was visible by ethidium bromide staining only from Rb1TatCl-2, indicating the presence of tat transcript in the clone. Since Tat transactivates retroviral 5′ long-terminal repeat (LTR) promoter, the activity of Tat in the clone was investigated using the plasmid pLTRX-Luc[28]. The Rb1 TatCl-2 and parental Rb1 cells were transiently transfected

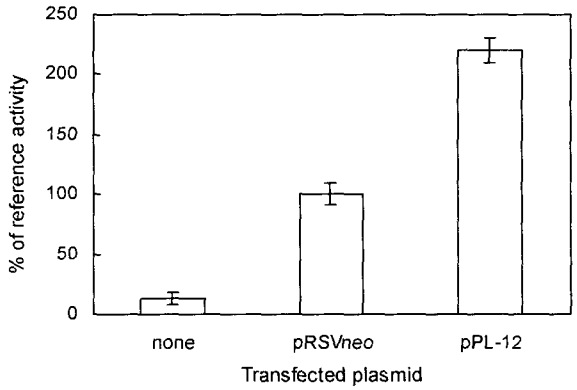

Fig. 1. Effect of tat on galectin-3 promoter activity. pPG3-5 was transiently transfected into RAW264.7 cells with expression plasmid for tat (pPL-12) or neomycin resistant gene (pRSVneo). Luciferase activity in the cell lysates obtained 48 hr post transfection was determined and normalized to galactosidase activity. The luciferase activity of the cells transfected with pPL-12 was expressed as a percentage of cells transfected with pRSVneo.

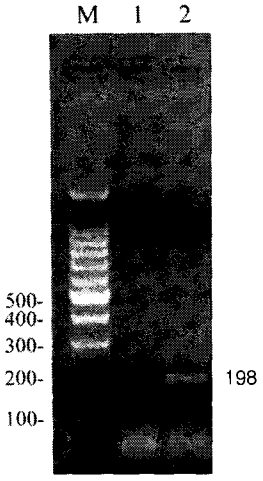

Fig. 2. Ethidium bromide visualization of RT-PCR product of tat RNA from Rb1TatCl-2 cells. Total RNA was extracted from Rb1 (lane 1) and Rb1TatCl-2 (lane 2) cells, and mRNA for tat was amplified by RT-PCR. The PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide. Lane M shows size markers of a 100 bp ladder. The migration position for tat RT-PCR product (product size, 198 bp) is indicated.

with pLTRX-Luc and then the luciferase activity was determined (Fig. 3). Compared with Rb1 cells, Rb1TatCl-2 cells showed remarkably increased luciferase activity. The result indicates that Rb1TatCl-2 contains biologically active Tat.

Next, the level of galectin-3 expression in Rb1 and Rb1-TatCl-2 was investigated by Western blot analysis and Northern blot analysis (Fig. 4). Up-regulation of galectin-3 transcript was observed in Rb1TatCl-2. Compared with Rb1, Rb1TatCl-2 showed approximately a two-fold increase of galectin-3 mRNA. When the density of galectin-3 protein band detected by Western blot analysis from Rb1 cells was counted 100%, the band density from Rb1TatCl-2 cells was 186% of that of Rb1.

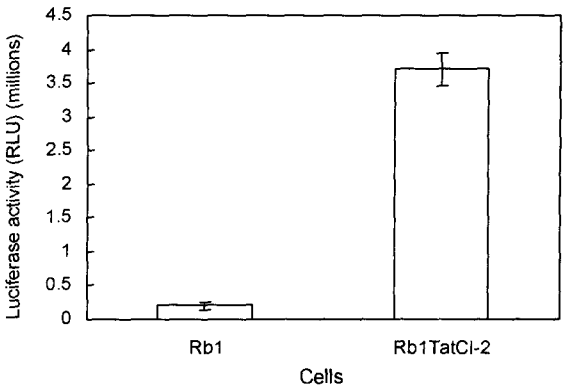

Fig. 3. Activation of LTR promoter in Rb1TatCl-2 cells. Rb1 and Rb1TatCl-2 cells were transiently co-transfected with plasmid pLTRX-Luc. Forty eight hours later, the transfected cells were lysed. Luciferase activity in the cell lysates was determined and normalized to galactosidase activity.

Discussion

The present study showed that HIV-1 tat up-regulated galectin-3 by transactivating galectin-3 promoter. These findings are consistent with previous studies suggesting a role of tat in galectin-3 expression. The increase of galectin-3 in HIV-1 infected Molt-3 cells depended on the existence of Tat in the cell[27]. In transient co-transfection experiments, the activity of 5'-regulatory sequence of the galectin-3 gene was up-regulated by the tat-encoding vector of pNAF[8]. Full-length Tat is essential for viral replication and is encoded by two exons on a spliced transcript and is 86-101 amino acids in length, depending on the viral isolate. Tat is directed to the nucleus by a basic nucleus localization domain and transactivates transcription from the viral promoter located in the LTR. In this study, HIV-1 tat-transfected Rb1TatCl-2 cells showed remarkably increased activity of luciferase located downstream of LTR promoter, indicating transactivation of LTR promoter in the cell. In addition to viral promoter, it has been reported that tat could transactivate cellular genes such as tumor necrosis factor (TNF)-alpha[5,26] and interleukin-2[30]. Even though the ways Tat regulates galectin-3 expression has not been investigated, we showed that galectin-3 is one of the cellular genes whose expression is enhanced by HIV-1 Tat.

It is possible that elevated galectin-3 expression can be related to cell proliferation and cell viability, and thereby might be involved in progression of certain diseases. T cells transfected with galectin-3 cDNA displayed higher growth rate than the control transfectants[8]. Expression of this lectin in leukemia cell line Jurkat E6-1[32] and in human breast carcinoma BT549 cells[2] conferred resistance

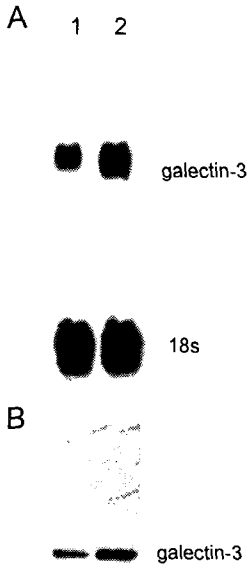

Fig. 4. Comparison of galectin-3 mRNA and protein in Rb1 and Rb1TatCl-2. A) Total RNA isolated from Rb1 (lane 1) and Rb1TatCl-2 cells (lane 2) was fractionated and transferred onto a Hybond N+ membrane. The membrane was probed with ^{32}P-labelled cDNA probe for human galectin-3. The blot was then stripped and probed with ^{32}P-labelled cDNA for 18s RNA. B) Equal amounts of total protein extracted from the Rb1 (lane 1) and RblTatCl2 (lane 2) cells were separated on an SDS-polyacrylamide gel, and transferred onto a PVDF membrane. The band of galectin-3 was identified with the monoclonal antibody B2C10.

to cispaltin- and anti-Fas antibody-induced apoptosis, respectively. Overexpressing of this lectin significantly enhanced

adhesion to laminin, fibronectin and vitronectin and survival upon exposure to different apoptotic stimuli, such as cytokine and radiation in human breast carcinoma cell lines [19]. The beneficial effects of galectin-3 to cancer cells suggest that induction of galectin-3 might contribute to the pathogenesis post-viral infection.

The fact that HIV-1 tat induced the activation of some kinases suggests that Tat might up-regulate cellular genes through various signaling pathways. In neurons and glial cells, Tat stimulated tyrosine phosphorylation of one isoform of Shc, an adaptor protein linking activation of growth factor receptors and transmembrane receptors to Ras signaling and MAPK activation[20]. Tat also activated MAPK, c-Jun amino terminal kinase (JNK), and Src kinase in Kaposi sarcoma cells[10]. We have data demonstrating that Ras and MAPK pathways are involved in the expression of galectin-3 when monocytes differentiate into macrophages by phorbol ester (unpublished data). Tat protein contains a basic domain that can interact with growth factor tyrosine kinase receptor. It is speculated that tat is able to mimic other growth factors and might interact with and activate growth factor tyrosine kinase receptors present in cells. Tat induced tyrosine phosphorylation of FIK-1/KDR receptor in endothelial cells[3]. Further investigation is required to elucidate the mechanisms by which HIV-1 tat induces galectin-3 expression.

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