Monosodium luminol upregulates the expression of Bcl-2 and VEGF in retrovirus-infected mice through downregulation of corresponding miRNAs

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Summary. – The retrovirus ts1 is a mutant of Moloney murine leukemia virus (MoMuLV) that causes neurodegeneration (ND) in susceptible mice. Our previous studies showed that the antioxidant drug monosodium luminol (GVT) prevented the development of ND in ts1-infected mice. In this study, we analyzed effect of GVT on the expression of B-cell lymphoma-2 protein (Bcl-2) and vascular endothelial growth factor (VEGF) in central nervous system (CNS) tissues of these animals. Our data showed that GVT treatment of ts1-infected mice significantly increased their expression of Bcl-2 and VEGF in brainstem compared with ts1-infected untreated mice. We also studied the expression of specific microRNAs (miRNAs) such as miRNA-15 and -16 (targeting Bcl-2), and miRNA-20 (targeting VEGF). We found that the expression of miRNAs inversely correlated with the upregulation of their target proteins in ts1-infected untreated as well as in GVT-treated-ts1-infected mice. The data showed that GVT treatment prevented ts1-induced ND at least in part by upregulating Bcl-2 and VEGF expression, what likely occurred as a consequence of downregulation of their corresponding miRNAs.

Keywords: ts1 virus; monosodium luminol; Bcl-2; VEGF; miRNAs

Introduction

The murine retrovirus MoMuLV-ts1 has been studied for many years in our laboratory. Its parental strain MoMuLV-TB induces T-cell lymphomas in susceptible strains of mice at 6–8 months of age (Yuen and Szurek 1989). However, ts1 mutant with a single point mutation in the envelope gene was able to cause a fulminant, acute disease in mice aged 4–6 weeks with T-cell and motor neuron loss (Wong et al., 1985, 1989, 1991; Prasad et al., 1989; Szurek et al., 1990). In CNS of ts1-infected mice, vacuolar degeneration of motor neurons and spongiform changes of white matter were present in the brainstem, cerebellum, and spinal cord (Stoica et al., 1993, 2000). Since glial cells are infected and neurons are not, neuronal death is likely to result from loss of glial support to neurons and toxic factors derived from glial and endothelial cells.

Among the several cytopathic murine retroviruses, ts1 mutant is most similar to the Human immunodeficiency virus 1 (HIV-1) concerning its selective infection of CD4+ cells, secondary neurocognitive effects and continuous viremia. These and other similarities indicate that ts1-mouse model is a valuable animal model for studying of HIV-AIDS dementia (Gonzales-Scarano et al., 1995; Clark et al., 2001).

GVT is a drug that suppresses reactive oxygen species accumulation and oxidative stress in ts1-infected primary astrocytes (Jiang et al., 2006). GVT administration to ts1-infected mice also delays hindlimb paralysis, body wasting, thymic atrophy, and development of spongiform and astrogliotic encephalopathy in the CNS. Our more recent studies showed that in ts1-infected thymus as well as in the small intestine, T-cells were killed by the
oxidative stress-mediated apoptosis. These events were prevented in T-cells by GVT administration (Scofield et al., 2009a,b).

MicroRNAs (miRNAs) are a newly discovered family of non-coding RNA molecules of about 22 nt in length. miRNAs regulate gene expression post-transcriptionally either by translational repression or by mRNA degradation in a sequence-specific manner (Bartel, 2004; Filippowicz et al., 2008). Cellular miRNAs regulate a variety of cellular processes (Yeung et al., 2005; Filippowicz et al., 2008). In addition, a new evidence has shown that miRNA can serve in a defense mechanism against the virus infection (Lancelier et al., 2005; Ding and Voinnet, 2007; Han and Siliciano, 2007; Yeung et al., 2007; Gottwein and Cullen, 2008; Liu et al., 2008; Chable-Bessa et al., 2009).

Bcl-2 is a central player in the inhibition of eukaryotic cells apoptosis under adverse conditions (Cimmino et al., 2005). The Bcl-2 gene protects various cell types including neurons from the apoptosis, even though apoptotic mechanisms are induced by very distinct stimuli in different cells (Allsopp et al., 1993; Davies et al., 1995). Overexpression of Bcl-2 in ts1-infected astrocytes prevents the apoptosis (Qiang et al., 2006). In addition, ts1-mediated ND is delayed in transgenic mice that overexpressed Bcl-2 in their neurons (Jolicœur et al., 2003).

VEGF, a neuroprotective factor secreted by glial and endothelial cells significantly contributes to the neuronal survival especially under hypoxic conditions. VEGF deficiency has also been implicated in other neurological disorders such as ischemic neuropathy, Parkinson’s disease, Alzheimer’s disease, and multiple sclerosis (Storkebaum et al., 2004).

The objective of this study was to determine the effect of ts1 infection and GVT treatment of ts1-infected mice on the expression levels of Bcl-2 and VEGF and the expression of specific miRNAs such as miRNA-15 and -16 (targeting Bcl-2), and miRNA-20 (targeting VEGF).

Materials and Methods

Virus. Mutant ts1 was propagated in thymus–bone marrow cell line (TB cells) and titrated on a non-transformed, sarcoma-positive, leukemia-negative cell line derived from the infection of TB cells with Moloney murine sarcoma virus (15F cells) as previously described (Wong et al., 1981).

Mice, virus inoculation, and GVT treatment. For these experiments we used FVB/N mice obtained from Taconic Farms (Germantown, USA). The mice were divided into four groups – control, GVT-treated, ts1-infected, and ts1-infected-GVT-treated. The control group received saline solution, while the ts1-infected group was inoculated intraperitoneally with 0.1 ml of ts1 viral suspension containing 10^6 to 10^7 IU/ml at day 2 post birth (Kim et al., 2002). GVT and ts1-infected-GVT-treated groups received freshly prepared GVT (200 mg/kg of body weight/day; kindly provided by Bach Pharma, Inc.) beginning at day 3 post infection and delivered intraperitoneally for 5 weekdays continually followed by 2 resting days. Mice from all groups were checked daily for clinical signs of disease and sacrificed at day 30 post infection For RT-PCR, microarray, and Western blot analysis, mice were sacrificed and the brainstems were removed, snap-frozen in liquid-nitrogen, and stored at -80°C.

Western blot analysis. Tissue lysates were prepared and Western blot analysis carried out as described previously (Lungu et al., 2008). The membranes were incubated at 4°C overnight with Bcl-2-antibody (1:200) (Santa Cruz Biotechnology) and monoclonal mouse IgG antibody targeting β-actin (Sigma-Aldrich). In the next step, the membranes were incubated in the peroxidase labeled anti-rabbit (1:10,000) and anti-mouse secondary (1:5,000) antibodies (Kirkegaard and Perry Laboratories). Further, after washing they were incubated with the Super Signal West Pico chemiluminescent substrate (Pierce). Densitometry determinations for blot bands density were performed using http://rsb.info.nih.gov/nih-image.

RT-PCR. Total RNA was extracted from brainstem tissue using a SV RNA extraction kit (Promega) and A_260 of the extracts was measured. Using a Super Script III First Strand Synthesis System (Invitrogen), 100 ng of total RNA was amplified by RT-PCR reaction and cDNAs were amplified. VEGF primers: forward, 5’-GACCCCTGGTGACATCTTCCAGGA-3’; reverse, 5’-GGTGAGAGGTCTTAGTTCCCGA-3’ (Wang et al., 1998) and β-actin primers: forward, 5’-ATGTAAGTAAAGGCAGGC-3’; reverse, 5’-AAGGAAGTGAAAGAGGC-3’ (Mendes et al., 2005).

For VEGF, one set of primers amplifying two different splice variants of VEGF miRNA (VEGF_s and VEGF_tst) was used. The PCR profile consisted of initial denaturation at 94°C for 7 mins, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 58°C for 30 secs, and extension at 72°C for 90 secs. A final extension at 72°C for 7 mins was carried out. The expected length of PCR products was 462 bp for VEGF_s and 514 bp for VEGF_tst. To confirm the integrity of the RNA samples used in the RT-PCR reactions, parallel amplifications with oligonucleotide primers for mouse β-actin were performed. The expected size for β-actin PCR product was 403 bp. Quantification of the RT-PCR bands density was performed using NIH Image program at http://rsb.info.nih.gov/nih-image.

Microarray analysis. miRNA microarray analysis was performed using a service provider (LC Sciences). The total RNA was extracted from brainstem tissue of 4 mice of each group – control, GVT-treated, ts1-infected and ts1-infected-GVT-treated using mirVana™ miRNA isolation kit (Ambion). Data were analyzed by subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two parallel experiments, the ratio of detected signals and P-values of Student’s t-test were calculated. P value of P ≤0.01 was considered statistically significant.

Statistical analysis. Data were presented as mean ± SD from 3-4 experiments and statistical comparisons were made using the Student’s t-test. A difference at P ≤0.05 was considered statistically significant.
Results

**GVT upregulated expression of Bcl-2 and VEGF in ts1-infected mice**

When transgenic mice overexpressing Bcl-2 in neurons were infected with ts1 mutant, they were largely protected from hindlimb paralysis and their brainstems displayed smaller spongiform lesions (Jolicoeur et al., 2003). Our previous studies with ts1-infected cultured astrocytes showed that Bcl-2 levels declined dramatically in these cells in comparison to uninfected astrocytes. This steep decline in Bcl-2 levels was correlated with cell death (Qiang et al., 2006). We showed in the same cells that induced overexpression of Bcl-2 mRNA and protein promoted cell survival after ts1 infection (Qiang et al., 2006). We therefore analyzed the effect of GVT treatment on the expression of Bcl-2 in brainstems of mice infected with ts1. These brainstems showed significantly decreased Bcl-2 protein expression in comparison to the brainstems of uninfected mice (Fig. 1a,b). Although GVT treatment had no effect on Bcl-2 levels in the brainstems of uninfected mice, it significantly upregulated Bcl-2 levels in the brainstems of ts1-infected GVT-treated mice.

Our previous studies demonstrated that VEGF protein and corresponding mRNA level were reduced in ts1-infected brains and in cultured ts1-infected cerebral vascular endothelial cells (Lungu et al., 2008). We therefore investigated the effects of GVT on VEGF levels in the brainstems of infected mice. GVT treatment significantly upregulated VEGF mRNA expression in the brainstems of infected and uninfected mice in association with neuroprotection (Fig. 2a,b).

**GVT downregulated expression of miRNAs targeting Bcl-2 and VEGF in ts1-infected mice**

Regulation mechanisms of Bcl-2 in hematopoietic cancer cells involved post-translational down-regulation by miRNA-15 and miRNA-16, what led to the activation of intrinsic apoptosis pathway (Cimmino et al., 2005). In the human genome, four members of the miRNA15/16 family showed the same 9-bp Bcl-2-complementarity sequence, what suggested that the complex and finely-tuned mechanisms that regulate Bcl-2 expression involve these miRNAs (Cimmino et al., 2005).

The miRNA-15a and miRNA-16-1 levels were upregulated in the brainstems of ts1-infected mice, as compared with the brainstems of uninfected controls (Fig. 3a,b). In uninfected GVT-treated and ts1-infected GVT-treated brainstems, however, clearly decreased levels of miRNA-15a and miRNA-16-1 were present (Fig. 3a,b). These results suggested that GVT downregulated miRNA15/16, what was correlated with upregulation of Bcl-2 expression and cell survival after ts1 infection.

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**Fig. 1**
Effects of GVT and ts1 infection on Bcl-2 protein expression in the brainstem of mice
Western blot analysis profiles (a) and their densitometry (b). Asterisks indicate statistical significance.

**Fig. 2**
Effects of GVT and ts1 infection on VEGF mRNA expression in the brainstem of mice
RT-PCR, agarose gel electrophoresis profiles (a) and their densitometry (b). Asterisks indicate statistical significance.
Like Bcl-2, VEGF was regulated by multiple miRNAs in the eukaryotic cells. Using human nasopharyngeal cell line, researchers have demonstrated that miRNA-15, -16, -20a, and -20b were involved in downregulation of VEGF levels (Hua et al., 2006). Using these data, we analyzed the expression of miRNA-20a and miRNA-20b in relation to VEGF expression levels in brainstems of the uninfected, ts1-infected, uninfected GVT-treated and ts1-infected GVT-treated mice. ts1-infected brainstems showed an upregulation of miRNA-20a and miRNA-20b compared with the brainstems of uninfected mice (Fig. 3c,d). The data also showed that the GVT treatment of uninfected GVT-treated and infected GVT-treated mice led to a significant reduction in their miRNA-20a levels in the brainstems.

Discussion

For many years, we have been studying the murine retrovirus ts1 as an animal virus model for research of HIV-1-associated dementia. We have gathered substantial amounts of data on the pathogenic mechanisms for ts1-induced ND in infected mice. The most important result has been an identification of the oxidative stress pathways as primary cause of ts1 cytopathology in the CNS.

Increasing evidence has shown that miRNAs participate in the host-virus interaction during virus infections (Ghosh et al., 2008). Some miRNAs are also known to be actively involved in the etiology or progression of ND (Bushati et al., 2008). In this setting, host miRNA exert both positive and negative regulatory effects on the viral replication, while viruses may use the host miRNA machinery to protect themselves against the cellular antiviral response. In light of this, miRNA-expression profiles could serve as a useful biomarker for the virus-infected cells as well as in the assessment of drug treatment.

We have already showed that expression of Bcl-2 and VEGF was downregulated in the cultured astrocytes and endothelial cells infected with ts1 (Qiang et al., 2006; Lungu et al., 2008). In addition, we found that ts1-induced ND...
could be significantly delayed, when the infected mice were treated with the antioxidant drug GVT (Jiang et al., 2006). Finally, it was found that overexpression of Bcl-2 in the cultured astrocytes prevented cell death after ts1 infection and ts1-mediated ND was delayed in Bcl-2 transgenic mice (Jolicoeur et al., 2003; Qiang et al., 2006).

The results obtained in this work confirmed our previous findings regarding the downregulation of Bcl-2 and VEGF expression in the brainstems of ts1-infected mice. Here, we showed for the first time that GVT treatment upregulated Bcl-2 and VEGF expression in the brainstem of ts1-infected mice. These new data confirmed the notion that GVT could be an important anti-apoptotic and neuroprotective factor. We also showed that levels of miRNA-15/16 targeting Bcl-2, and miRNA-20 targeting VEGF expression, were increased in ts1-infected brainstems. This event was associated with the decreased levels of Bcl-2 and VEGF mRNAs and corresponding proteins. By contrast, brainstems of ts1-infected mice treated with GVT had decreased levels of these miRNAs alongside with the normal or increased levels of their target proteins. Our new data showed that the regulation of miRNAs was correlated with the regulation of corresponding target proteins, but we did not detect a significant regulation of these miRNA between experimental groups, although these differences were detected in Bcl-2 and VEGF protein levels. These data suggested that besides miRNAs, there were also other regulation mechanisms for expression of these proteins.

However, our findings showed a direct correlation between the upregulation of specific miRNAs by ts1 and reversal of these effects by GVT. These data not only substantiate our previous findings, but also shed a light on the potential functional roles of miRNAs in the virus-mediated neurological disease and neuroprotective effects of GVT. To our knowledge, this is the first study that disclosed a causal link between miRNAs in the virus-mediated ND and pharmacological neuroprotection. Recently virus-encoded miRNAs have been identified in herpesviruses and are supposed to target host immune systems (Stern-Ginossar et al., 2007). However, lentiviral vectors were used for stable knockdown of miRNAs by overexpressing miRNA target sequences. These vectors effectively inhibited regulation of reporter constructs and natural miRNA targets (Gentner et al., 2009).

In conclusion, our study showed for the first time that GVT played an important role in regulation of Bcl-2 and VEGF levels in ts1 infection and that regulation of this protein inversely correlated with the regulation of miRNAs that target them. GVT might be an important neuroprotective and anti-apoptotic drug that could protect against ts1-mediated ND.

Now, we are expanding our investigation of miRNAs in ts1-infected and GVT-treated mice to determine how their levels are regulated and to learn more about their function and effects in miRNA-virus and miRNA-drug interactions. Since specific miRNA overexpression patterns are associated with pathologic condition of a number of diseases, specific knockdown of target miRNAs by drugs may hold promise for future therapeutics.

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References


Szurek PF, Yuen PH, Ball JK, Wong PK (1990): A Val-25-to-Ile substitution in the envelope precursor polyprotein, gpPr80env, is responsible for the temperature sensitivity, inefficient processing of gpPr80env, and neurovirulence of ts1, a mutant of Moloney murine leukemia virus TB. J. Virol. 64, 467–475.


