

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 neurocytopathic 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

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Abbreviations: Bcl-2 = B-cell lymphoma-2 protein; CNS = central nervous system; GVT = monosodium luminol; HIV-1 = Human immunodeficiency virus 1; miRNA(s) = microRNA(s); MoMuLV = Moloney murine leukemia virus; ND = neurodegeneration; TB cells = thymus-borne marrow cell line; VEGF = vascular endothelial growth factor

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; Filipowicz *et al.*, 2008). Cellular miRNAs regulate a variety of cellular processes (Yeung *et al.*, 2005; Filipowicz *et al.*, 2008). In addition, a new evidence has shown that miRNA can serve in a defense mechanism against the virus infection (Lancellier *et al.*, 2005; Ding and Voinnet, 2007; Han and Siliciano, 2007; Yeung *et al.*, 2007; Gottwein and Cullen, 2008; Liu *et al.*, 2008; Chable-Bessia *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 against 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'-GACCCTGGTGGACAT CTTCCAGGA-3'; reverse, 5'-GGTGAGAGGTCTAG TTCCCGA-3' (Wang *et al.*, 1998) and β -actin primers: forward, 5'-ATGTACGTAAGCC AGGC-3'; reverse, 5'-AAGGAACTGGAAAAGAGC-3' (Mendes *et al.*, 2005).

For VEGF, one set of primers amplifying two different splice variants of VEGF mRNA (VEGF₁₆₄ and VEGF₁₈₈) 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 VEGF₁₆₄ and 514 bp for VEGF₁₈₈. 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 $P \leq 0.01$ was considered statistically significant.

Statistical analysis. Data were presented as mean \pm SD from 3–4 experiments and statistical comparisons were made using the Student's *t*-test. A difference at $P \leq 0.05$ 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.

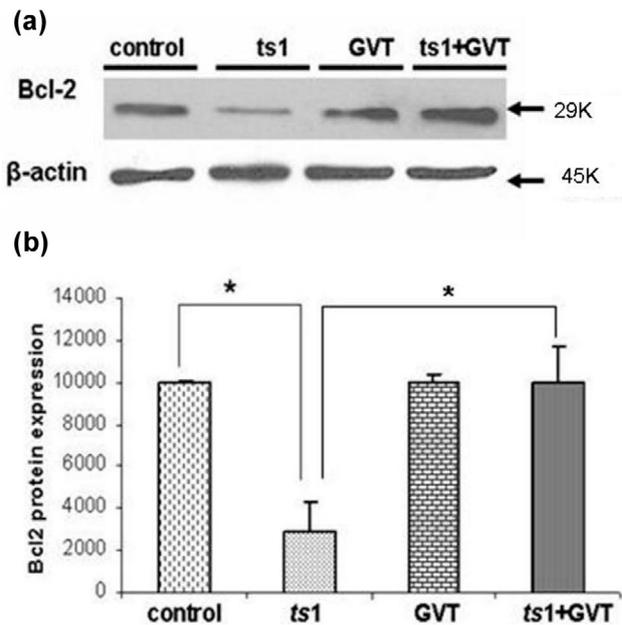


Fig. 1

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.

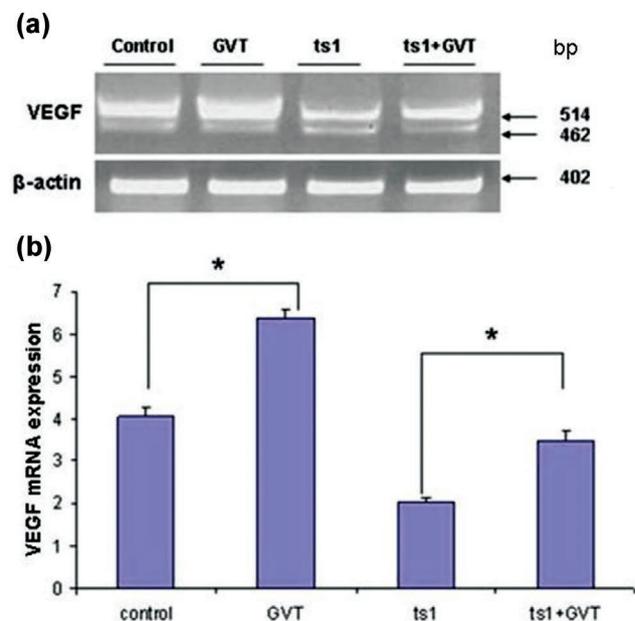


Fig. 2

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.

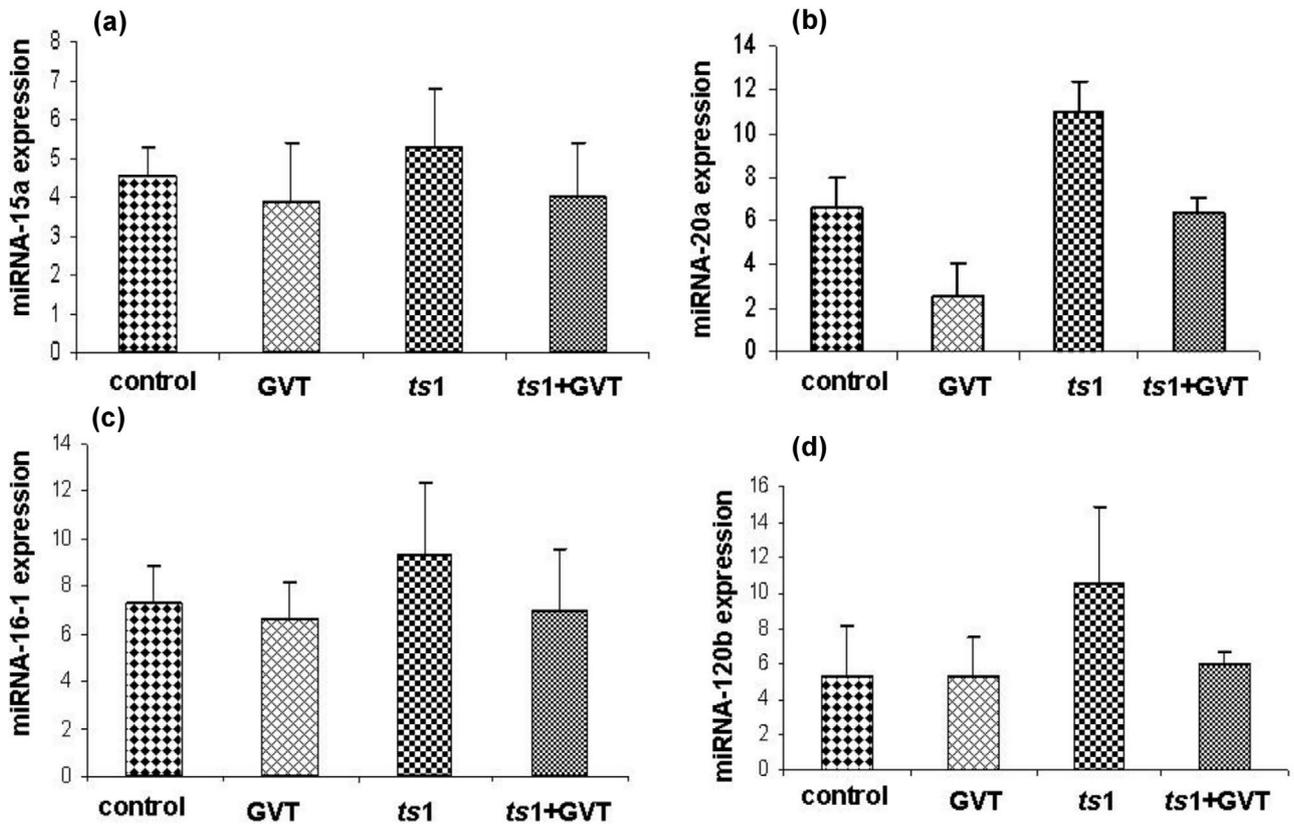


Fig. 3

Effects of GVT and *ts1* infection on the expression of miRNAs in the brainstem of mice
Microarray analysis, miRNA-15a (a), miRNA-16-1 (b), miRNA-20a (c), and miRNA-20b (d).

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 up-regulation 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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