Acta Scientiae Veterinariae. 34(1): 83-88, 2006.

ORIGINAL ARTICLE
Pub. 657

ISSN 1678-0345 (Print)
ISSN 1679-9216 (Online)

Immunohistochemical detection of GFAP and TGF β1 in C57Bl6 mice during acute vesicular stomatitis virus encephalitis

Detecção imunoistoquímica de TGF-BETA e GFAP em camundongos C57Bl6 com encefalite aguda induzida pelo vírus da estomatite vesicular

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ABSTRACT

Activation of astrocytes or astrogliosis is a prominent component of the inflammatory response and an indicator of injury in the brain. These astrocytes produce a large array of inflammatory mediators, growth and neuroprotective factors. This study was an investigation from astrocyte (GFAP) response and TGF-β1 involvement during VSV acute encephalitis using immunohistochemistry to verify relation between astrocytes and TGF-β1. Animals developed symptoms around 6th day after VSV inoculation. Viral proteins were mainly detected at olfactory bulb, ventricular cell layer and disseminated to hippocampus, mesencephalon and diecephalon areas and brain stem. Also at 6th day post inoculation GFAP and TGF-β1 staining was observed in good association with virus-detected areas of brain. However, in mice with severe symptoms we observed reduction in the intensity of GFAP labeling at the same areas where TGF-β1 upregulation was observed. These areas show correlation with areas of necrosis and where are astrocytes with degenerative aspect. We observed TGF-β1 staining in damaged astrocytes, suggesting an effort of those cells in controlling inflammation in acute phase of VSV encephalitis.

Key words: astrocytes, gliosis, TGF-β, vesicular stomatitis virus.

RESUMO

Ativação de astrócitos ou astrogliose é um componente evidente da resposta inflamatória e um indicador de injúria no sistema nervoso. Estes astrócitos produzem uma grande combinação de mediadores inflamatórios, fatores de crescimento e neuroprotetores. Este trabalho investiga a resposta de astrócitos (GFAP) e a participação de TGF-β1 durante a fase aguda da encefalite por VSV usando imunoistoquímica para verificar a relação entre astrócitos e TGF-β1. Os camundongos desenvolveram sintomas cerca de 6 dias após a inoculação (pi) do VSV. As proteínas virais foram detectadas principalmente no bulbo olfatório, células ventriculares e disseminadas em áreas do hipocampo, mesencéfalo e diencéfalo. Também no sexto dia pi, a marcação para GFAP e TGF-β1 foi observada associada com áreas onde o vírus foi detectado. Entretanto, em camundongos com sintomas severos, nós observamos áreas onde a marcação para TGF-β1 foi mais intensa. Estas áreas apresentaram correlação com áreas de necrose, onde astrócitos degenerados foram observados. A marcação para TGF-β1 em astrócitos lesados, sugeriu um esforço destas células em controlar a inflamação na fase aguda da encefalite por VEV.

Descritores: astrócitos, Gliose, TGF-β1, vesicular estomatite vírus.

Received: December 2005 www.ufrgs.br/favet/revista Accepted: March 2006

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INTRODUCTION

Activation of astrocytes or astrogliosis is a prominent component of the inflammatory response and an indicator of injury in the brain. These astrocytes produce a large array of inflammatory mediators, growth and neuroprotective factors [29,30]. Communication between astrocytes and astrocytes or others cells in CNS occurs by gap junctions and it could result in a favorable metabolic co-operation [2]. Whereas some of these effects are clearly beneficial, astrocyte communication could mediate additional death of the adjacent cells by the bystander effect [2].

Mice with moderate or low levels of TGF-β1 expression had a less pronounced astrogliosis, whereas GFAP expression was consistently increased [28]. Indeed, TGF-β1 directly increases GFAP transcription in cultured astrocytes [22]. Studies on cultured neurons show a protective effect of TGF-1 against various toxins and injurious agents. Little is known about TGF-β1 rules during acute phase of CNS injuries in an animal model [reviewed in 25].

Mice experimental infection of vesicular stomatitis virus by the intranasal route results in spread by retrograde transport in neurons and ventricular surfaces [12,20]. Current data suggests that acute encephalitis induces apoptosis, tissue injury, and mortality in VSV-infected mice [24].

The aim of the present study was to investigate the possible correlation between astrocytes (GFAP) and TGFβ1 during acute phase of vesicular stomatitis virus encephalitis comparing the localization of both proteins in encephalon of symptomatic and assymptomatic mice.

MATERIALS AND METHODS

Virus

The sample of vesicular stomatitis virus, strain Indiana type II was maintained at -80ºC in BHK. Mouse brain suspension used for inoculation was obtained from intracerebral inoculation of mice as previous described [20]. The VSV titration was determined as infecting dose per tissue culture ($TCID_{50}$/0.1 ml) expressed in decimal logarithms was 10^5 virus/0,1ml.

Experimental infection of mice

Thirty male C57BI6 mice, 5 to 7 weeks old (CEMIB-UNICAMP-SP-Brazil), were used for viral inoculation. Mice were mildly anesthetized in a closed container with ether followed by intranasal inoculation with VSV suspension (10^5 virus/0.1ml) in a total volume of 0.03ml administered equally between each nostril according to [11]. Five mice got sterile PBS into the nostril were used as control (Group 1). Animals were housed with food and water *ad libidum* and were randomly sacrificed for histological and immunohistochemical analysis at day 6, symptomatic mice comprised Group 2, and assymptomatic mice, Group 3. Each group was composed by at least 6 mice. All the protocols were approved and experiments were carried out in agreement with UNESP- Animal Ethic in Animal Experimentation Committee for animal care.

Histological and Immunohistochemical analysis

Mice were anesthetized with ether and sacrificed with intraperitoneal lethal dose of 5mg of pentobarbital sodium (Nembutal®[1] - Abbott Laboratories) in normal saline solution. Each mouse was perfused transcardially with 30-40ml of phosphate-buffered saline (PBS pH 7.4). After perfusion whole brains were removed and fixed during 8 hours in freshly prepared 4% buffered paraformaldehyde (pH 7.4) and embedded in paraffin according to standard procedures. Selected areas were based on viral dissemination route described by [12,20].

Five-micrometer brain longitudinal sections were stained with hematoxylin and eosin in order to detect inflammatory, degenerative or reactive features of neurons. For immunohistochemistry, endogenous peroxidase activity was blocked by incubating sections in 1% H_2O_2. Sections were treated with Tripsin 1% at 37ºC before blocking of nonspecific binding with powdered skimmed milk (3% in phosphate-buffered saline) for 30 minutes. Incubation with primary antibodies was performed overnight at at 4ºC. For demonstration of astrocytes morphology polyclonal anti-GFAP was used (1:400, Dako[2]) and for characterization of TGF-β1 distribution we used anti-TGF-β1 (Santa Cruz[3], SC-146). Biotinylated anti-rabbit (1:100, Dako[2]) and anti-goat (Santa-Cruz, 1:100) was applied as secondary antibodies and avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories, USA) served as the third reagent. Positive antigen-antibody reaction was visualized by incubation with 3.3-diaminobenzidine-tetrahydrocloride-(DAB)-H_2O_2 followed by counterstain with Mayer hematoxilin. Negative control sections were incubated in the absence of the primary antibody.

RESULTS

Clinical signs

Six inoculated mice showed signs of disease at 6th day post inoculation (pi). Typical clinical signs included ruffled fur, conjunctivitis, reduced mobility and progressive posterior paralysis. To minimize suffering to the animals they were sacrificed at start of the paralysis symptoms.

Histopathology

Brains observed at 6th day post inoculation showed intense inflammation in the olfactory bulb. Leptomeningitis, which the intensity varied from mild to intense, was observed at olfactory bulb and extended to encephalic ventral surface. Most affected mice showed necrosis at olfactory bulb and also ventriculitis at laterals, third and fourth ventricles affecting subependymal layer (Figure 1A). Perivascular infiltrates and necrosis was also observed at thalamus, hypothalamus and brain stem. Inflammatory infiltrates were composed by neutrophils and lymphocytes.

Immunohistochemistry for viral antigen

Six days after nasal instillation, in symptomatic mice (Group 3), VSV proteins were detected at olfactory bulb, ependymal and subependymal layers, hyppocampal, hypothalamic and thalamic areas, some nucleus at mesencephalon and brain stem. Cerebellum and cortical areas were less affected (Figure 1D).

Immunohistochemistry for GFAP

Astrocytes positive for GFAP were easily identified in animals of all groups (Table 1). Reactive astrocytes were characterized mainly for having large and clear nucleus, clumped chromatin located peripherally, and large nucleoli. The cytoplasm was evident and the thick processes were intensively stained. Cells with those characteristics were observed mainly in the glomerular and mitral cell layers of olfactory bulb, even in assymptomatic mouse sacrificed at day 6-post inoculation. Mice presenting symptoms sacrificed at day 6th showed astrogliosis in other encephalic areas as glia limitans externa, glia limitans interna, hippocampus, diencephalon and mesencephalon.

Strong to intense GFAP reactivity was characteristic on symptomatic mouse (Table 1), apart from reduction in the intensity of the reaction in some areas of the encephalon that corresponds to necrotic areas of olfactory bulb and around ventricular areas. GFAP-positive cells, with large swollen nuclei, evident nucleoli and processes with a fragmented feature were observed in areas where inflammation was intense and necrosis could also observed (Figure 1C and F). Surrounding necrotic tissue there was a transitional area where astrocytes showed less marked degenerative morphological aspects, i.e., the processes were short, thick and showed only a mild fragmentation.

Immunohistochemistry for TGF-β

TGF β1 immunorreactivity was observed at leptomeninges, at ependymal and subependymal cells and choroid plexus in control group (Group 1). Six days post VSV inoculation TGF-β upregulation was observed in encephalon associated to inflammation and VSV dissemination. Immunostaining was positive in neurons and astrocytes at olfactory bulb, in cells at subependymal area, in neurons and astrocytes of hippocampus and brain stem. Leptomeninges and choroid plexus epithelium showed positive staining, as some inflammatory cell at subpial and perivascular space. At olfactory bulb (Figure 1E) and periventricular ependymal and subependymal layer (Figure 1B) some structures similar to astrocytes processes also were marked. Positive extra cellular diffuse staining was also observed.

Table 1. General immunohistochemical staining patterns distribution for GFAP and TGF-β on brain of mice inoculated and sacrificed at 6 days post inoculation and controls.

Immunoreactivity	GFAP			TGF-β		
Groups	G1 (n=4)	G2 (n=6)	G2 (n=9)	G1 (n=4)	G2 (n=6)	G2 (n=9)
Cortex	+	++	+	-	+++	-
Olfactory bulb	++	++	++	-	++++	+++
Ependymal and Subependymal layer	-	++	-	-	+++	-
Hippocampus	++	++++	++	-	+++	-
Diencephalon	++	++++	++	-	++	-
Mesencephalon	++	+++	++	+	+++	+
Brain Stem	++	+++	++	-	+++	-

(-) no staining; (+) weak staining; (+ +) moderate staining; (+ + +) strong staining; (+ + + +) intense staining. Group 1: Control; Group 2 : with symptoms at 6th day; Group 3: without symptoms at 6th day.

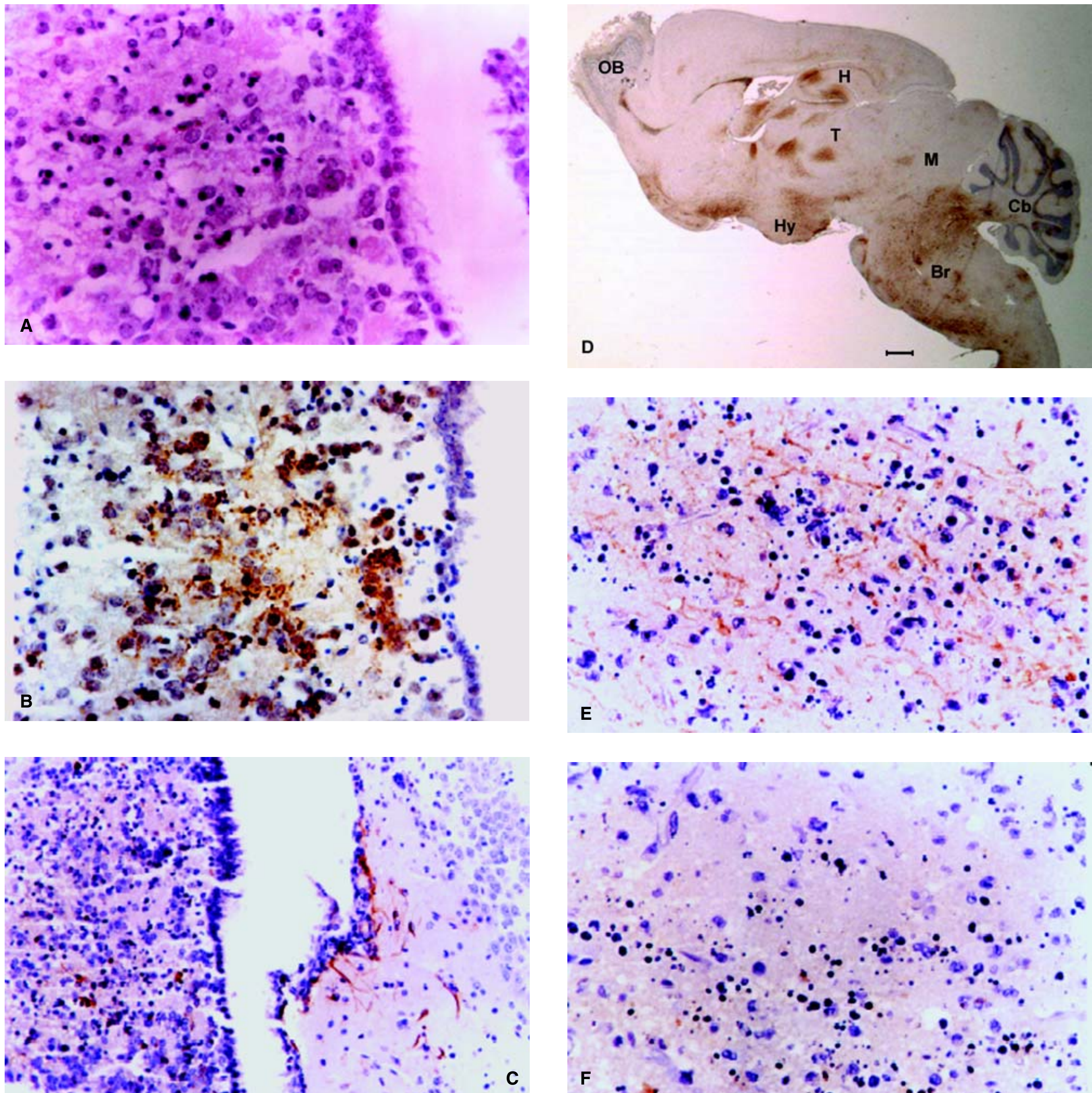

Figure 1. A, B and C - Serial sections of an infected brain removed at day six. **A -** Cells at subependymal layer showing signs of cell death (HE, bar = 25µm). **B -** TGF-β immunohistochemistry at subependyaml layer of lateral venticle (bar = 25µm). **C -** GFAP staining. Note attenuation of GFAP positivity inside square demarcated area (bar = 50µm). **D -** Distribution of VSV antigen in sagital seciton of brain 6 days post inoculation. (OB) Olfactory bulb; (T) thalamus; (H) hypocampus formation; (Hy) hypothalamus; (M) mesencephalum; (Cb) cerebellum; (Br) brainstem (bar = 1mm). **E and F -** Serial sections of olfactory bulb. **E -** TGF-β intense staining at astrocytes (arrows) (bar = 25µm). **F -** Decreasing in GFAP detection. Only few structures are marked (arrows) (bar = 25µm).

DISCUSSION

Similar to the rabies virus, VSV presents neurotropism and the neuronal infection results in cell death. Acute CNS major lesion in VSV infection includes leptomeningitis and ventriculitis, followed by neuronal damage and a parallel glial activation. Viral migration in the course of infection occurs via olfactory tracts and ventricles surface, involving hippocampus, diencephalon, mesencephalon, and brain stem [12, 20].

In mice sacrificed on day 6 without symptoms, the tissue injury was less intense and mainly limited to the olfactory bulb, where GFAP-positive astrocytes with activated morphology were observed. In those mice viral proteins were detected essentially in olfac-

tory bulb. In symptomatic mice at 6th day after virus inoculation reactive astrocytes were detected in the olfactory bulb and other areas such as hippocampus, mesencephalic and diencephalic areas and brain stem, thus confirming the observations of de [5,20,26], which described rapid response to VSV infection, both from astrocytes and also microglia activation. Areas where the down regulation of GFAP labeling was detected corresponded to sites where there was tissue necrosis, intense viral and TGFβ1 detection.

Astrocytes are important cells contributing to immune response, and also for healing and/or regeneration of nervous tissue. Down regulation of GFAP associated with morphological changes are a strong indicative of astrocyte degeneration. Morphological changes observed in nervous tissue, including those observed in astrocytes at 6th day pi may be caused by virus multiplication and beginning of cell death triggered by apoptosis [24]. However, neuron and glial cells are very likely to suffer injury secondary to the inflammatory process, as a bystander effect. Astrocyte degenerative changes might be associated with functional changes, which might contribute to the aggravation of CNS lesions [4, 21,23].

TGF-β1 has been found in glial cells, Schwann cells and in certain populations of neurons [9]. Comparing with control (Group 1), all groups had stronger staining, specially those mice with symptoms of Group 3. We observed neuronal cells stained positively for TGF-β1 at brain steam nucleus at day 6 pi. TGF-β1 expression in neurons is related with neurodegeneration [13]. So, in VSV model, TGF-β1 expression in neurons seems to be related to a response to viral neurotropism.

At olfactory bulb and ependymal and subependimal layers, crucial areas for virus dissemination, structures very similar to astrocytes processes presented TGF-β1 positivity (Figure 1E and F). On sequential sections, GFAP detection was almost inexpressive and suggested astrocyte damage. TGF-β1 has been found mainly in glial cells and is detected in low quantities at mature CNS. After different injuries TGF-β1 is synthesized by microglia and, at low levels, by astrocytes [1, 8,19]. In particular, astrocytic expression of TGF-β is a powerful suppressor of microglial cells [10,14,15,18].

Comparing both protein expressions on day six-post inoculation we noticed a commitment of astrocytes in synthesizes TGF-β1 during acute VSV infection. As general rule, reports with reference to TGFβ1 suggest the importance of this cytokine in chronic phase of diseases as modulator molecule witch interfere in cellular differentiation, extracellular matrix production and immunological response [3,7,16,17,27]. Recent research shows the importance of TGF-β1 and its signaling pathways as putative modulator of astrocyte biology and its implication as a possible novel mediator of cellular interactions in the CNS [6,25]. We observed TGFβ1 staining in damaged astrocytes, suggesting an effort of those cells in controlling inflammation in acute phase of VSV model of viral encephalitis.

Acknowledgements. We thanks financial support provided by FAPESP and FUNDUNESP.

NOTAS INFORMATIVAS

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Acta Scientiae Veterinariae
Pub. 657