B-Cell Translocation Gene 2 Is Over-Expressed in Peri-Infarct Neurons after Ischaemic Stroke

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Key Words

B-cell translocation gene 2 • Neuronal survival • Ischaemic stroke

Abstract

Objectives: Recovery from stroke is dependent on the survival of neurons in the dynamic peri-infarcted region. Although several markers of neuronal injury and apoptotic cell death have been described, administration of neuroprotective drugs directed at specific molecules has had limited success. A complete understanding of deregulated genes associated with neuronal death would be beneficial. Our previous microarray studies identified increased expression of a novel protein, the B-cell translocation gene 2 (BTG2), in infarcted regions. **Methods:** We have used immunohistochemistry and Western blotting to examine the expression and localization of BTG2 in stroked brain tissue and immunofluorescent staining of human fetal brain neurons to determine if oxygen-glucose deprivation affected its expression. **Results:** We show that BTG2 is strongly expressed in peri-infarcted and infarcted regions of brain tissue, localizing in neuronal nuclei and cytoplasm, whilst being absent or very

weakly expressed in normal looking contralateral tissue. Exposure of human fetal brain neurons to oxygen-glucose deprivation also induced BTG2 expression in the cytoplasm and perinuclear regions of cells staining positive for propidium iodide (a marker of nuclear damage). **Conclusions:** BTG2 may be a modulator of cell survival and differentiation and could help to protect against cell death by inhibition of necrosis and/or apoptotic signalling pathways.

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Introduction

The extent of recovery from stroke is dependent on the survival of neurons, particularly in peri-infarcted regions, which are subject to the risk of permanent damage but are potentially salvageable. Neuronal survival is a complex process, requiring a fine balance between numerous protective and deleterious factors. The brain activates neuroprotective mechanisms in an attempt to counteract neurodestructive pathways. Recent studies have tried to identify novel markers which may impact upon neuronal survival after ischaemic stroke. Several

Table 1. Patient data

Patient No.	Age/sex	Survival after stroke, days	SSS on admission	SSS final	Hypercholesterolaemia ¹	Obesity ²	Hyper-tension ³	Coronary disease	Atrial fibrillation	History of TIA	Smoking
1	84/M	3	18	0	no	no	yes	yes	no	no	yes
2	85/F	5	8	0	no	yes	yes	yes	no	no	no
3	51/M	14	4	0	no	no	yes	no	no	yes	no
4	86/M	15	40	0	yes	no	yes	yes	no	no	no
5	73/M	26	35	0	yes	yes	yes	yes	no	no	no
6	84/F	29	34	0	yes	no	yes	yes	no	no	no
7	73/M	29	29	18	no	no	no	yes	yes	no	yes
8	60/F	31	16	16	yes	no	yes	yes	no	no	yes

SSS = Scandinavian stroke scale; TIA = transient ischaemic attack.

¹ Yes = hypercholesterolaemia >5.2 mmol/l. ² Body mass index >24. ³ Diastolic >90 mm Hg; systolic >140 mm Hg.

members of fibroblast growth factor and hypoxia-inducible factor families, as well as leptin hormone and interleukin-6, are involved in neuroprotective brain mechanisms [for a review, see ref. 1]. There are currently no clinically proven neuroprotective drugs, and several drugs have failed in clinical trials because of toxicity [2]. Glutamate (N-methyl-D-aspartate) and amino-methyl propionic acid antagonists are in development, and one compound traxoprodil, an antagonist of the NR2B subtype of the N-methyl-D-aspartate receptor, appears promising. The identification of new molecules involved in neuroprotection might reduce patient cognitive impairment and mortality after stroke.

Using cDNA microarray, our previous studies have identified increased B-cell translocation gene 2 (BTG2) expression in peri-infarcted and infarcted regions of patients following acute ischaemic stroke [3]. BTG2 is a member of a newly identified family of anti-proliferative genes that has emerged as an important regulator of cell growth, differentiation and DNA damage repair by functioning as both an effector of pRb and p53 action [4]. BTG2 was first described as an immediate early gene whose expression is induced in response to mitogenic as well as differentiating and anti-proliferative factors [5]. Although the biological functions of the BTG2 protein remain to be elucidated, several studies have attempted to identify the different cues capable of inducing its expression [6, 7]. Of relevance to this work, nerve growth factor (NGF) and brain-derived neurotrophic factor, which are released after ischaemia, can activate a signalling pathway leading to Bcl-2 activation and concomitantly inhibit p53, resulting in inhibition of neuronal apoptosis [8].

Since BTG2 may play a role as a protective factor in stroked brain tissue, here, we have examined its expression patterns in human brain in more detail.

Materials and Methods

Human Brain Autopsy Specimens

Human brain tissue samples were obtained from 9 patients who died from acute ischaemic stroke, with the approval of the local ethics committee. All patients were admitted with large middle cerebral artery strokes confirmed by CT scan or MRI (NIH Stroke Scale on admission 18 ± 6). The patients, 8 males and 1 female, were aged between 51 and 86 years and had survived between 3 and 37 days following ischaemic stroke (table 1). Full clinical examinations and routine blood parameters were determined on admission. Immediately after death, the body was put in a cold chamber and tissue was collected within 6 h of death. Tissue samples were taken from infarct and peri-infarcted zones, whilst controls were obtained from the contralateral hemisphere at the same time. The peri-infarcted areas were defined in tissue sections as the tissue immediately surrounding the infarcted core which contained some necrotic cells and showed evidence of tissue disorganization and disruption of neuronal cell layers which was confirmed by histology. Sections were stained with 2,3,5-triphenyltetrazolium chloride which stains active mitochondria pink; therefore, non-stained areas represented stroke-affected cortical regions (data not included). Tissue specimens were immediately frozen in liquid nitrogen, kept at -70°C , and a portion of each sample was fixed in 10% formaldehyde and processed for histology, where subsequently haematoxylin and eosin was used to determine tissue morphology [9].

Human Cerebral Cortical Neurons

Primary cultures enriched in cerebral cortical neurons were established from fetal brain tissue, following the procedure described previously [3]. Brain tissue was obtained from fetal specimens of 14–19 weeks gestational age, legally aborted and after

written informed consent from donors. Permission for use of human tissue was obtained from the ethics committee of the Consejo Superior de Investigaciones Científicas. Briefly, tissues were diced into small cubes and dissociated by incubation at 37°C with 0.025% trypsin and 40 µg/ml DNase in PBS for 10 min. Dissociated cells were resuspended in minimum essential medium supplemented with 5% horse serum, 2 mM glutamine, 25 mM D-glucose, 0.25 µg/ml fungizone, 50 µg/ml gentamycin and 1% non-essential amino acids. To obtain neuron-enriched cultures, cells were seeded at a density of 3×10^5 cells/cm² in multiwell plates previously coated with 10 µg/ml poly-L-lysine, maintained in a humidified CO₂ incubator and fed weekly. Experimental procedures were performed after 12–14 days in vitro. Selected experiments were done after 2 days in vitro. The neuronal cultures consisted of 70–85% neurons, 15–30% astrocytes, 1–2% microglia and 0.1–1% oligodendroglia.

In vitro Oxygen-Glucose Deprivation

For oxygen-glucose deprivation (OGD) experiments, the above culture medium was replaced by glucose-free medium containing 2% fetal bovine serum (TCS CellWorks, Buckingham, UK), and cells were cultured at 37°C in a humidified chamber with 95% N₂, 1% O₂ and 5% CO₂ for 14 h followed by 24 h reperfusion in fresh medium containing 4.5 g/l glucose. This resulted in approximately 30% of cells undergoing apoptosis after OGD and 60% following re-oxygenation, as determined from our pilot studies. Cells cultured in normoxic conditions without glucose deprivation were used as controls. The fluorescent exclusion dye propidium iodide is widely used as a vital dye in tissue culture systems and labels the nuclear DNA and, in particular, changes in DNA structure caused by strand breaks in dying and apoptotic cells which lack an intact plasma membrane. In some experiments, propidium iodide (10 µg/ml) was added to the cultures 1 h before the end of the experiment to stain dead and dying cells, as described in our previous studies [10].

Protein Extraction and Western Blotting

Proteins were extracted from tissues, and the protein concentration of each sample was determined using the BioRad assay. For Western blotting, 10 µg of protein lysate in RIPA buffer was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (13% w/v), and the proteins were electroblotted onto nitrocellulose filters as described previously [10]. Filters were blocked in 1% w/v bovine serum albumin in Tris-buffered saline Tween and stained overnight at 4°C with antibodies to BTG2 (sc-30342; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) diluted in 1% bovine serum albumin: goat anti-BTG2 (1:100) and α-actin (1:1,000; Sigma, Dorset, UK) in 5% w/v milk in Tris-buffered saline Tween for 1 h. Blots were developed with the ECL detection system (Amersham, UK). The relative intensities of the bands were measured in an LKB densitometer (Victoria, Australia). Results are semi-quantitative and are given as a numerical (fold) change compared with the control (contralateral tissue) which was given an arbitrary value of 1.0. All experiments were performed twice and a representative example is shown.

Immunohistochemistry

Paraffin-embedded tissue samples were processed and serial 5-µm sections were cut. The avidin-biotin peroxidase (ABC Vectastin kit; Vector Laboratories, Peterborough, UK) method was

used, and goat anti-BTG2 antibody was used at a dilution of 1:50. Paraffin-embedded sections were deparaffinized, rehydrated and boiled for 10 min in an antigen unmasking solution of concentrated citric acid, pH 6.0. Slides were incubated in 0.5% v/v H₂O₂ in methanol for 30 min, with normal 5% v/v rabbit serum for 20 min and then with a primary antibody (1:50 diluted in 1% v/v rabbit serum) for 1 h, followed by 1 h incubation with biotinylated secondary antibody made in rabbit serum (diluted 1:200 in 1% v/v rabbit serum) and finally with ABC complex (diluted 1:50) for 30 min at room temperature. Staining was completed after incubation with diaminobenzidine substrate chromogen solution for 3–10 min. Slides were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX. Negative control slides were performed in parallel, where the primary antibody was replaced with PBS, and sections were subsequently processed as above (data not included).

Immunofluorescence

Cultured cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 10 min, blocked with normal serum and stained with the primary antibody as above, followed by 1 h incubation with FITC-conjugated secondary antibodies (1:200) at room temperature. Negative control cells were performed in parallel, where the primary antibody was replaced with PBS and sections processed as above (data not included).

Results

Western Blotting Showed Increased Expression of BTG2 in Stroke-Affected Tissue of Seven out of Eight Patients Examined

A comparison between contralateral and peri-infarcted tissue showed that 5 out of 8 patients had increases of at least 1.5 fold in damaged tissue (range 1.5–2.0; fig. 1). Similarly, in infarcted tissue, 6 out of 8 patients had increased BTG2 expression (range 1.5–4.0).

BTG2 Protein Expression Was Increased in Neurons in Peri-Infarcted and Infarcted Regions of Human Stroke Tissue

Immunohistochemistry demonstrated a strong staining of BTG2 in peri-infarcted (fig. 2a, b) and infarcted (fig. 2c, d) areas with specific localization to neuronal cytoplasm and nuclei (arrows). Occasionally, we found BTG2 staining in microvessels (fig. 2b; arrows), but the majority of vessels were negative. Figures 2f and 2g show examples of neuronal nuclei in peri-infarcted and infarcted regions, respectively, which stained strongly for BTG2. Only very weak staining was seen in normal looking neurons from the contralateral hemisphere (fig. 2e; arrows point to negatively stained neurons).

Fig. 1. Western blot analysis of BTG2 in contralateral hemisphere, peri-infarcted and infarcted regions of patients following acute ischaemic stroke. **a** Control areas from stroke patients had lower expression of BTG2 than peri-infarcted and stroke areas; representative Western blots for patients 1 and 3 are shown. C = Contralateral area; PI = peri-infarcted area; S = stroke or infarcted area. α -Actin was used as a control to demonstrate equality of protein loading. **b** Table showing fold differences in BTG2 expression obtained by scanning densitometry.

Immunofluorescence Showed Increased Protein Expression of BTG2 in Neurons following OGD

Immunofluorescence demonstrated an increase in staining of BTG2 in human fetal brain neurons following 8 h of OGD (fig. 3). BTG2 upregulation was most pronounced in the cytoplasm of propidium iodide-positive neurons, suggesting an association with cell apoptosis.

Discussion

BTG2 is a member of a novel anti-proliferative gene family and plays a role in transcription regulation. BTG genes are considered immediate early genes whose expression is induced in response to mitogenic as well as differentiating and anti-proliferative factors [11]. In this study, we have seen a strong upregulation of BTG2 protein expression in stroke-affected regions. In these areas, we found both cytoplasmic and nuclear BTG2-positive

staining in neurons. In contrast, only weak or no staining was seen in normal looking neurons from the contralateral hemisphere. In vitro studies showed increased expression of BTG2 in neurons following OGD. This suggests that upregulation of BTG2 expression in neurons is induced following ischaemic events.

There are few previous publications referring to the involvement of BTG2 in ischaemic diseases or stroke. BTG2 was induced following brief spinal ischaemia using a rat balloon occlusion model [12], and increased expression of BTG2 mRNA was also observed in the kidney after 30-min ischaemia [13]. Previous studies have shown that oxidative DNA damage precedes DNA fragmentation in neurons after experimental stroke [14], and that these events are followed by the triggering of different cellular processes including apoptosis [15].

In this respect, it is interesting to note that several previous studies have demonstrated an anti-apoptotic role for BTG2 in neuronal cell cultures. For example, BTG2 protected terminally differentiated PC12 cultures against apoptosis elicited by NGF deprivation [16], and also prevented apoptosis of a terminally differentiated rat pheochromocytoma cell line (PC12) [17]. In response to DNA damage, eukaryotic cells can delay cell cycle progression at both G1 into S, and G2 into M phases by induction of anti-proliferative genes, such as BTG2, which may be one mechanism through which it helps to protect against neuronal cell death.

Our results suggest that BTG2 upregulation in the brain following ischaemic stroke might form part of a protective mechanism against apoptosis. BTG2 expression is normally induced through a p53-dependent mechanism, and its function may be relevant to cell cycle control, through regulation of the G1/S transition of the cell cycle, and to cellular response to DNA damage [5]. The study by Rouault et al. [5] showed that differentiated embryonic stem cells from which BTG2 has been ablated underwent apoptosis following DNA damage because of a failure in growth arrest. Similarly, el-Ghissassi et al. [17] showed that inhibition of BTG2 mRNA with specific antisense oligonucleotide promoted programmed cell death in NGF differentiated cells. This suggests that BTG2 triggers a physiological role in the control of cell cycle arrest following DNA damage and other types of cellular stress.

BTG2 appears to trigger inhibition of the cell cycle through arrest of cells in the G1 phase via inhibition of cyclin D1 transcription and, in this way, contributes to neuron differentiation [18]. Cell cycle arrest is known to be an essential preliminary requirement for terminal dif-

Fig. 2. Immunohistochemistry of BTG2. **a** Strong expression of BTG2 in neurons from the peri-infarcted region. **b** Occasional staining of microvessels (patient 4). **a, b** $\times 100$. **c, d** Infarcted region with more intense nuclear staining (patient 1; arrows). $\times 100$.

e Portion from the contralateral hemisphere of patient 3 and weak BTG2 staining of neurons (arrows). $\times 100$. **f, g** Magnified images showing nuclear staining of a neuron of patient 4 (**f**) and patient 1 (**g**). $\times 200$.

ferentiation and, in this context, previous studies have highlighted a potential role for BTG2 in the control of neurogenesis through induction of Math1 which is a transcription factor required for neuronal differentiation [19].

BTG2 was originally isolated as a gene induced by NGF during neuronal differentiation of rat PC12 cells, and it is a marker of neuronal birth in vivo. The localiza-

tion of BTG2 mRNA in the adult central nervous system in specialized structures, such as the ventricular zone, suggests it could be important in maintaining the brain stem cell population [20], and therefore, directly influence this process under pathological conditions, such as in brain ischaemia accompanying a stroke [21–24]. Our in vivo studies showed that BTG2 was present in both the cytoplasm and the nucleus of stroke-affected neurons,

Fig. 3. Human fetal brain neurons showed increased protein expression of BTG2 following OGD. **a** Untreated neurons showed almost no BTG2 expression (control). **b** Neurons exposed to OGD showed upregulation of BTG2 protein (BTG2, FITC, green; prop-

idium iodide, red, showing cell apoptosis). **c** Magnified portion of the image showing cytoplasmic expression. $\times 100$. The colours refer to the online version of the figure.

whilst *in vitro* following OGD, the expression was also increased but limited to the cytoplasm. BTG2 is able to behave as a transcriptional coregulator in different model systems, although the molecular mechanisms of this action are probably multiple and often unclear. Previous studies showed that following treatment with the cell-differentiating agent retinoic acid, BTG2 detached from its transcriptional nuclear receptor RAR α and translocated from the nucleus to the cytoplasm. These events prime histone H4 demethylation and acetylation, increase cell differentiation and could be events similar to those which occur following OGD and hypoxia *in vitro* in our human fetal neurons [25]. The presence of the immediate early gene BTG2 simultaneously in neuronal nuclei after stroke may be an indication of the complexity and greater sever-

ity of signalling processes in the local microenvironment including expression of growth factors such as NGF, and be associated with its function as a p53 transcriptional target following DNA damage [26].

In conclusion, the role of BTG2 in neuronal survival following ischaemic stroke should be examined in greater detail to identify its importance in mediating signalling pathways associated with stroke repair.

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