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GADD34 gene restores virulence in viral vector used in experimental stroke study

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Running Title: Viral delivery of GADD34 in experimental stroke

Abstract

GADD34 is expressed in ischaemic brain and reverses protein synthesis shutdown. Consequently, GADD34 could have neuroprotective potential in stroke. BHK medium, a replication deficient HSV viral vector (HSV1716) with no insert or containing full-length GADD34, the amino terminal or a conserved portion of the gene, was injected into mouse brain prior to stroke. Infarct size was 1.0 ± 0.26 , 1.19 ± 0.36 , 1.5 ± 0.36 , 1.3 ± 0.36 & 1.1 ± 0.28 mm³, respectively. The increase in infarct size with full-length GADD34 was statistically significant ($p < 0.05$). Immunohistochemistry confirmed viral protein expression. Tissue culture studies revealed GADD34 gene restored virulence in HSV1716 suggesting HSV virulence, rather than increased GADD34, exacerbated ischaemic damage.

Keywords: GADD34, stroke, ET-1, HSV1716

Introduction

One of the major mechanisms influencing cell survival following stroke is inhibition of protein synthesis which can persist for long periods and may not recover in selectively vulnerable cells (Paschen, 2003). This is particularly pertinent for cells within potentially salvageable penumbral tissue, which if supported to maintain basic cellular processes including restoration of protein synthesis, could survive, limiting infarct expansion.

GADD34, one of five distinct GADD genes, is a cell cycle protein inducible by growth arrest and DNA damage (Hollander *et al.*, 1997). GADD34 is upregulated in rodent and human ischaemic brain (Imai *et al.*, 2002; White *et al.*, 2004) and is known to facilitate reversal of protein synthesis shutdown through the dephosphorylation of eukaryotic initiation factor 2 alpha (eIF2 α) (Novoa *et al.*, 2001). Since GADD34 mRNA levels correlate with cell survival during global ischaemia (Doutheil *et al.*, 1999) it is possible that GADD34 expression may be increased to counteract protein synthesis shutdown during cellular stress, thereby promoting cell survival.

The aims of the current study were to generate HSV viral vectors containing full-length, or GADD34 gene fragments, to investigate the consequences of increased GADD34 expression on ischaemic damage in a mouse stroke model. The hypothesis under test was that increased GADD34 expression would limit infarct size.

Materials & Methods

Viral Vectors

BHK cells were propagated in modified Eagle's medium with 10% newborn calf serum/10% tryptose phosphate broth and supplemented with glutamine and streptomycin/ penicillin. 3T6 cells were maintained in Dulbecco's modified Eagle's medium with 10% newborn calf serum and supplemented with glutamine and streptomycin/ penicillin.

The MyD116 sequence was pre-cloned into the pCMV-SPORT 6 plasmid (Invitrogen). Full-length, conserved 63 amino acid and amino terminal (full-length sequence minus conserved sequence) of MyD116 were PCR amplified including the CMV IE promoter and contained appropriate start and stop codons. The conserved sequence was amplified by PCR, cloned into the pcDNA 3.1 plasmid to gain a CMV IE promoter and excised by restriction digest. All three sequences were cloned into the RL1deliresGFP plasmid (provided by Dr P Dunn) which contained an inter-ribosomal entry site and a green fluorescent protein reporter gene (GFP). The RL1IRESGFP plasmid contains flanking sequences from either side of the herpes simplex viral gene RL1, which codes for ICP34.5.

Plasmid constructs were linearised, incubated with wild-type virus (HSV17⁺) DNA/calf thymus DNA and the mixture applied to confluent monolayers of BHK cells for incubation (37⁰C for 4 hours). The plates were then washed, incubated with dimethylsulphoxide (4mins), overlaid with medium and maintained at 37⁰C for 72 hours. Plaques formed from recombinant virus identified by GFP signal were harvested and multiple rounds of plaque purification performed until all plaques were fluorescent and pure stocks of recombinant virus were produced. Stocks of each recombinant virus were grown and titred.

Confluent monolayers of BHK and 3T6 cells were infected with HSV17⁺, HSV1716 (replication deficient vector lacking the RL1 gene encoding the virulence protein ICP34.5 (Brown *et al.*, 1994)) or one of the recombinant viruses at 0.1 p.f.u. per cell and absorbed at 37⁰C for 1 hour. The cells were overlaid with medium for incubation periods of 6, 24, 48 or 72 hours at 37⁰C and then scraped from the plate and stored at -70⁰C. The cells were thawed and sonicated for 5 mins to disrupt cells and release progeny virus. Confluent monolayers of BHK and 3T6 cells were infected with serial dilutions of each virus for 45mins and overlaid with methyl cellulose for 72 hours at 37⁰C. Finally, cells were Giemsa stained and the plaques counted on analogous plates.

Animal Procedures

Procedures were carried out under licence from the UK Home Office. Male C57BL/6 mice (22-30g) were anaesthetised with halothane (2-3%) in 70% N₂O:30% O₂ via a face mask and placed in a stereotaxic frame. Body temperature was maintained at 37⁰C±0.5⁰C and sub-cutaneous fluids given post-operatively to prevent dehydration.

Stereotaxic co-ordinates (2mm lateral, 0.5 mm anterior to bregma, 2mm ventral to dura) were used to deliver viral vector (1x10⁶p.f.u in 0.17-0.3ul) and (24 hours later) endothelin-1 (ET-1, 400 pmol, 0.5µl, Alexis Biochemicals, UK) into the striatum at 0.05µlmin⁻¹.

Mice were randomly assigned to 5 groups (n=8): Group 1: BHK Medium (vehicle for viral vectors); Group 2: HSV1716 (replication deficient vector). Group 3: HSVGADD34 (HSV1716 containing full-length GADD34 gene); Group 4:

HSV GADD34 conserved (HSV1716 containing GADD34 conserved sequence);
Group 5: HSV GADD34 aminoterminal (HSV1716 containing GADD34 amino terminal sequence). Groups 1 and 2 served as controls. The experiment was terminated by perfusion fixation (4% paraformaldehyde in PBS) 72hrs later.

Approximately 8 sections collected throughout the lesion were stained with H&E and examined by an investigator, blind to treatment allocation, to quantify lesion volume. Infarcted tissue was identified microscopically, transcribed onto line diagrams and measured by image analysis to generate infarct volume.

Immunohistochemistry: striatal sections were dewaxed, dehydrated and microwaved (10 min, 10 mM citric acid, pH 6.0). HSV rabbit polyclonal antibody (DAKO; 1:2000) or GADD34 (sc-8327, Santa Cruz; 1:100) incubated overnight at 4°C followed by a biotinylated goat anti-rabbit secondary antibody (1:100; Vector Laboratories) used with avidin/biotinylated horseradish peroxidase complex (ABC kit, Vector Laboratories) and 3,3'-diaminobenzidine (Vector Laboratories). GFP monoclonal antibody (Clontech, 1:100) was used with a biotinylated goat anti-mouse (1:100; Vector Laboratories) secondary antibody. HSV staining mapped onto line diagrams (Figure 1a).

Statistical analysis

Infarct volume in viral vector groups was compared to the vehicle group using repeated measures ANOVA with a Dunnett's post hoc test to determine statistical significance. All data are expressed as mean \pm SD.

Results

Viral protein expression *in vivo*

In vivo HSV antigen was detected in cell bodies within the infarct core and peri-infarct region in all but the vehicle group, confirming spread throughout the target zone (red shading in Figure 1a).

Increased GADD34 immunoreactivity was present in the peri-infarct region of all groups in both the cortex and striatum. However, ischaemia-induced endogenous GADD34 (vehicle group, left hand panel, Figure 1b) could not be differentiated from viral vector-derived GADD34. Therefore, GFP was used to confirm expression of new viral proteins. GFP was detected in cell bodies within infarct and peri-infarct regions in all recombinant virus groups, but not in the vehicle group, confirming cells containing the viral vector had expressed gene products (Figure 1b).

Effect GADD34 on infarct size

ET-1 causes local vasoconstriction resulting in a small, reproducible striatal infarct, extending along the needle tract into external capsule and cortex (Figure 1a).

HSV_{GADD34} caused a significant increase in infarct volume compared to vehicle (1.5 ± 0.36 versus $1.0 \pm 0.25 \text{ mm}^3$, $p < 0.05$). In contrast, HSV1716, HSV_{GADD34conserved} and HSV_{GADD34aminoterminal} (1.19 ± 0.36 , 1.3 ± 0.35 & $1.1 \pm 0.28 \text{ mm}^3$, respectively) had no significant influence on infarct volume.

The HSV_{GADD34} group displayed a greater spread of HSV immunostaining compared to other groups (Figure 1a) similar to a wild-type HSV17⁺ phenotype.

Additional animals, which did not undergo ischaemia, were subsequently injected with HSV17⁺ or the recombinant vectors to check for viral virulence and evidence of associated pathology. HSV 1716, HSVGADD34conserved and HSVGADD34aminoterminal constructs induced a mild inflammatory response around the needle tract (Figure 1c, bottom right hand panel) and mice showed no signs of ill health. As expected, 72 hours after HSV17⁺ injection, histology showed evidence of encephalitis characterised by perivascular inflammation, leptomenigeal inflammation, neuronophagia and necrotising encephalitis (Figure 1, top left panel). A similar pattern of pathology was observed with HSVGADD34 (Figure 1c top right & bottom left panels). HSV17⁺ and HSVGADD34 injected mice displayed behavioural signs of ill health such as hunching, shaking, inactivity and hypersensitivity, indicative of a wild-type HSV infection.

Virus replication

Multicycle growth curves provided a replication profile of the three recombinant viruses, wild-type HSV17⁺ and HSV1716. BHK cells were permissive for HSV17⁺ and HSV1716 and this was also true for the recombinant viruses (data not shown). 3T6 cells were permissive to HSV17⁺ but were non-permissive for HSV1716, HSVGADD34conserved and HSVGADD34aminoterminal. However, HSVGADD34 showed efficient growth in 3T6 cells where titres reached similar levels to HSV17⁺ (Figure 2).

Discussion

One of the most critical events induced by cerebral ischaemia is endoplasmic reticulum (ER) stress resulting in protein synthesis shutdown. The mechanism

involves upregulation of PKR-like ER kinase (PERK) resulting in phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α), inhibition of eIF2B and cessation of protein synthesis translation (Paschen et al., 2003). Although, generalised protein synthesis is shutdown, certain proteins are upregulated through open reading frame bypass scanning (Kojima et al., 2003). ATF4, an ER stress-induced protein increased following eIF2 α phosphorylation, induces several downstream genes including GADD34 (Blais et al., 2004) which dephosphorylates eIF2 α , restoring protein synthesis (Novoa et al., 2001).

We have reported upregulation of GADD34 in neurones and microglia in the peri-infarct region in rodent focal ischaemia (Imai *et al.*, 2002) and in human brain following cardiac arrest (White *et al.*, 2004). Since upregulation is within peri-infarct tissue and GADD34 is implicated in DNA repair, ischaemic tolerance and restoration of protein synthesis (Garcia *et al.*, 2004; Novoa *et al.*, 2001), GADD34 (or specific fragments) levels were increased by viral vector gene transfer to determine its influence on ischaemic damage.

HSV1716 successfully delivered and increased protein products of inserted genes which was confirmed by GFP immunohistochemistry, since viral GADD34 could not be distinguished from ischaemia-induced endogenous GADD34.

Unexpectedly, HSVGADD34 significantly increased infarct volume. The fact that HSV immunoreactivity spread further in the HSVGADD34 group (Figure 1a) alongside behavioural signs of ill health and growth curve data (Figure 2) suggested that the full-length GADD34 gene restored virulence to HSV1716.

Histology from animals which received HSVGADD34, without ischaemia confirmed evidence of encephalitis and comparable neuropathology to HSV17⁺ animals, both groups exhibiting behavioural signs indicative of HSV infection. In contrast, HSV1716, HSVGADD34conserved and HSVGADD34aminoterminal groups displayed only a mild inflammatory response around the injection tract with no signs of ill health.

HSV1716 lacks the virulence protein ICP34.5 (McKay et al., 1993) which prevents protein synthesis shutdown upon viral infection, allowing viral replication to occur (Chou & Roizman, 1994). By binding to protein phosphatase1 α (PP1 α), the carboxyl terminal of ICP34.5 removes phosphate from eIF2 α in response to a variety of eIF2 α kinases, including PKR and PERK (Cheng et al., 2005; He et al., 1997). The full-length GADD34 gene appears to compensate for the lack of ICP34.5 and restore virulence to HSV1716. Therefore, the increased infarct volume seen with HSVGADD34 is most likely due to GADD34 restoring virulence to HSV1716 and not to increased tissue levels of GADD34 *per se*. To avoid this problem, HSV amplicons or adenoviruses could be used as alternative delivery vehicles for GADD34 or studies conducted in GADD34 knockout mice (Kojima et al. 2003) used to provide further insights into the role of GADD34 in stroke.

Disclosure/Conflict of Interest

None

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Figure Legends

Figure 1.

a. Representative line diagrams of a coronal section from each of the five experimental groups showing location of the infarct (grey shading) and the spread of HSV immunostaining (red shading). Row 2, right-hand panel HSV immunostaining in the striatal peri-infarct region of a mouse injected with HSV1716 carrying full-length GADD(scale bar: 50 μ m).

b. Top left, endogenous GADD34 immunostaining in striatal peri-infarct region in a mouse injected with BHK Medium. Top right GFP immunostaining in striatal peri-infarct region in a mouse injected with HSV1716. Bottom left GFP immunostaining in striatal peri-infarct region of a mouse injected with HSV1716 carrying full-length GADD. Bottom right No evidence of GFP immunostaining in striatal peri-infarct region of mouse injected with BHK Medium (scale bars: 50 μ m).

c. Haematoxylin and Eosin stained sections. Top left, Photomicrograph of striatum from animal injected with wild-type HSV17⁺ (10⁶ pfu). Top right and bottom left, HSVGADD34 (10⁶ pfu) and bottom right, HSV1716 (10⁶ pfu). Infiltrating lymphocytes can be seen both in the striatum, top right, and in the subarachnoid space, bottom left, in mice injected with HSVGADD34 indicating acute HSV encephalitis. Bottom left panel also shows the presence of viral meningitis. Striatum from animal injected with HSV1716, bottom right, shows no evidence of HSV encephalitis. (Scale bars: 50 μ m).

Figure 2. Growth curves for full-length GADD34, amino terminal and 63 amino acid conserved sequence in RL1iresGFP viruses in 3T6 cells. 17⁺ refers to wild type HSV

virus while 1716 refers to the replication deficient HSV viral vector containing no gene insert.