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GAPDH-targeted therapy – A new approach for secondary damage after traumatic brain injury on rats

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ABSTRACT

Massive neuronal death caused by a neurodegenerative pathology or damage due to ischaemia or traumatic brain injury leads to the appearance of cytosolic proteins in the extracellular space. We found that one of the most abundant cellular polypeptides, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), appearing in the medium of dying cells or body fluids is able to form aggregates that are cytotoxic to adjacent cells. Since we previously showed that the hydrocortisone derivative RX624 can inhibit the ability of GAPDH to transport the enzyme complex with polyglutamine and reduce the cytotoxicity of the complex, we explored the effects of GAPDH on SH-SY5Y neuroblastoma cells. We found that the latter treated with particular forms of GAPDH molecules die with a high efficiency, suggesting that the exogenous enzyme does kill adjacent cells. RX624 prevented the interaction of exogenous GAPDH with the cell membrane and reduced the level of death by more than 10%. We also demonstrated the efficiency of RX624 treatment in a rat model of traumatic brain injury. The chemical blocked the formation of GAPDH aggregates in the brain, inhibited the cytotoxic effects of cerebrospinal fluid and rescued the motor function of injured rats. Importantly, RX624 treatment of rats had a similar effect as the intracranial injection of anti-GAPDH antibodies.

1. Introduction

Oxidative stress, ischaemic tissue injury, and trauma may lead to the release of cytosolic proteins into the extracellular space, blood and cerebrospinal fluid (CSF) [1,2]. Since there is no system for maintaining proteostasis in the extracellular space, such proteins that can be in oxidised, nitrosylated or denatured states may form aggregating complexes and damage neighbouring cells. An example of the above stressors, traumatic brain injury (TBI), initiates from the death of a part of cell population and is followed by secondary damage, which causes the complications of post-trauma recovery. This long period during which neurons are dying provides time and opportunity for therapeutic intervention.

One of the major cell proteins that is frequently found in the intercellular space, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is well known as an oxidative stress sensor. The oxidation and nitrosylation of endogenous GAPDH can cause its translocation into the nucleus and apoptosis [3]. Another mechanism of denatured GAPDH toxicity is mitochondrial damage via permeability transition pore opening [4]. An important property of GAPDH is its ability to form aggregates in response to certain types of stress, particularly oxidative stress; the degree of aggregation correlates well with mortality [5–7]. GAPDH can form aggregating complexes with different mutant proteins with an irregular conformation, such as beta-amyloid [8], and superoxide dismutase 1 [9], and a variety of polypeptides which cause polyglutamine pathologies, in particular mutant huntingtin [10,11]. Recently, we showed that GAPDH can form migrating extracellular complexes with mutant huntingtin; the enzyme significantly increases toxicity [12].

The notion that denatured GAPDH tends to form cytotoxic oligomers and aggregates allowed us to search for small molecules able to prevent those deleterious effects. Several such substances were described, including hydrocortisone 21-hemisuccinate (RX624), which specifically binds GAPDH and prevents the formation of toxic complexes consisting of the oxidized enzyme [13], or

Abbreviations: Cell-ELISA, cellular enzyme linked immunosorbent assay; CSF, cerebrospinal fluid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; TBI, traumatic brain injury; TBS, tris-buffered saline.

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those linked to polyglutamine [14]. The most valuable property of RX624 is that it demonstrates anti-aggregating activity outside cells and therefore does not affect any intracellular signalling processes.

In the current study, we first analysed the effects of different forms of GAPDH by fractionating the enzyme preparation; the aim of this section was to imitate the conformations that the enzyme molecules possibly adopt outside a cell. In experiments performed with the use of human neuroblastoma cells, we proved that aggregating GAPDH is the species with maximal toxic activity, while RX624 reduces that by 80–90%. In the second part, we employed the TBI model in which trauma causes massive cell death and the corresponding release of cellular polypeptides. GAPDH aggregates were found in the intercellular space (CSF) in the brain and the process of aggregation coincided with the loss of important behavioural characteristics. RX624 was found to ameliorate these pathological manifestations.

2. Materials and methods

2.1. Proteins and chemicals

GAPDH from rabbit muscle was kindly provided by Professor V.I. Muronetz (Lomonosov Moscow State University, Russia). For confocal microscopy, we prepared fluorescently-labelled GAPDH (Alexa-555, Invitrogen, USA) according to the manufacturer's protocol. For the experiments on cells, GAPDH fractions were prepared as follows. To denature GAPDH (5 mg/ml) it was dialysed against tris-buffered saline (TBS) containing 20 mM Tris HCl pH 7.5, and 50 mM $NaCl_2$, followed by a freeze/thaw cycle that led to denaturation and even aggregation of the enzyme [15]. The GAPDH-T fraction is a 100 μg/ml solution of pre-denatured GAPDH in cell culture medium. The GAPDH-S fraction is the supernatant produced following the centrifugation of GAPDH-T (13,000 rpm, 15 min). The GAPDH-P fraction is the precipitate obtained from a GAPDH-T by centrifugation (13,000 rpm, 15 min) and resuspension of the precipitate in an equivalent volume of the cell culture medium. The RX624 compound was received from Dr A. Shavarda (Komarov Botanical Institute, Russia).

2.2. Cell culture

Human neuroblastoma SY-SH5Y cells (ATCC, USA) were grown in Dulbecco's modified Eagle's medium supplemented with Nutrient Mixture F-12, L-glutamine (Gibco, USA), 10% foetal calf serum (PAA Laboratories GE, Austria), and 50 mg/mL gentamycin (Biolot, Russia) in 5% CO_2 at 37 °C. To analyse the amount of GAPDH-containing aggregates in cells, we employed the filter trap assay, as described previously [16]. Anti-GAPDH monoclonal 6C5 antibodies were used (Abcam, UK).

To explore the dynamics of necrosis and apoptosis, the cells were placed in a 96-well microtitre plate, and ethidium bromide and acridine orange in phosphate-buffered saline (PBS) were added to give a concentration of 5 μg/mL for each dye. Stained cells were analysed with the aid of a Zeiss Axioscope (Zeiss, Jena, Germany). Cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide) assay.

For the cellular enzyme-linked immunosorbent assay (Cell-ELISA), we used protocol as described earlier [12]. Briefly, after washing with PBS, the cells were fixed in 4% formaldehyde solution for 30 min, permeabilised in methanol for 30 min and stained with 6C5 anti-GAPDH antibodies. An important addition was that we excluded the permeabilisation step to measure the amount of GAPDH bound to the cell membrane.

2.3. Animals

Wistar rats weighing 200–250 g were divided into 5 groups: not injured (Control, n = 4); injured (TBI, n = 5); injured and fed RX624 (RX624, n = 5); injured and injected with 2 μg (in 5 μl) of 6C5 antibody against GAPDH (6C5, n = 4), and injured and injected with 2 μg (in 5 μl) of antibody against green fluorescence protein (GFP) as a control (GFP, n = 4).

TBI was performed according to the protocol of Mychasiuk et al. [17] with a minor difference: the height of the drop was 120 cm (instead of 100 cm). Musculoskeletal deficit in the front and hind legs was evaluated by the Beam Walking test (OpenScience, Russia) on the 60th day after TBI. The testing procedure was recorded on a video with subsequent analysis of the number of slippages of the legs, as described earlier [13]. The injection of antibodies was performed with the aid of a stereotaxic device (SR-5R Narishige Scientific Instrument Laboratory, Japan), according to the coordinates: AP = −4 mm, L = 0 mm, DV = 4.5 mm.

CSF was collected through foramen magnum immediately after the Beam Walking test. To estimate the aggregate amount, we mixed 50 μl of CSF sample with 150 μl of TBS including sodium dodecyl sulphate (SDS) to a concentration of 2% (w/w) and then analysed the results using the filter trap assay with subsequent staining with 6C5 antibodies against GAPDH. To analyse CSF toxicity, we mixed 50 μl of CSF sample with 50 μl of cell culture medium and incubated this with SH-SY5Y cells for 24 h. Cell viability was measured using the MTT assay. 6C5 antibodies against GAPDH were used for the immunodepletion of CSF samples.

All animal experiments were carried out in accordance with the guidelines for the welfare of animals of the Institute of Cytology, Russian Academy of Science.

3. Results

3.1. Exogenous GAPDH interacts with plasma membrane and penetrates SH-SY5Y cells

The critical property of GAPDH is its readiness to adapt to alien conditions or be oxidised into different forms, some of which can exist in the medium conditioned by dying cells and possess distinct aggregation ability or toxicity. To imitate these forms, we prepared GAPDH fractions (GAPDH-T, GAPDH-S, and GAPDH-P) and fluorescently labelled them. The verification of GAPDH aggregates content in each fractions is shown on Fig. 1 (Ref. [18]). These fractions were added to human neuroblastoma cells SH-SY5Y and incubated for 5, 12, or 24 h. The analysis of micrographs show that the pool of aggregated GAPDH (GAPDH-P) was located predominantly along the cell membrane without passing inside the cytoplasm, while the soluble protein GAPDH-S, on the contrary, penetrated the cells (Fig. 1A). Next, we analysed GAPDH-containing aggregates in the cells and on their surface after incubation with various fractions of the GAPDH using the filter trap assay. GAPDH-T, GAPDH-S or GAPDH-P samples were added to the cells, and after 5, 12 or 24 h, the cells were lysed in solution containing SDS; the lysates were subjected to ultrafiltration (Fig. 1B and C). GAPDH-containing aggregates could be detected 5 h after the initiation of cell incubation with GAPDH-T or GAPDH-P. Moreover, the number of detectable aggregates for all fractions except GAPDH-S increases with an extension of the incubation time. The incubation of cells with the GAPDH-S fraction did not result in the appearance of cell-associated GAPDH aggregates. To prove the results, we employed two versions of Cell-ELISA: one with permeabilisation of the plasma membrane, when antibodies recognised GAPDH both on the membrane and in the cytoplasm (Fig. 1D, upper panel), and the second one, without permeabilisation, i.e. antibodies interacted

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