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Beta-amyloid toxicity and reversal in embryonic rat septal neurons

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Abstract

Alzheimer's disease is characterized mainly by loss of neurons from the septal nucleus. In this study, neurons from the septal nucleus of the embryonic day 16 (E16) rat were grown in culture with a plane of astrocytes from the embryonic rat and in a defined medium in the absence of serum. Neurons were treated with beta-amyloid (A β : 0.1, 1 and 10 μ M) on day in vitro (DIV) 1 and DIV 4 and fluorescent microscopy was used to measure survival and apoptosis following exposure of the treated cells on DIV 7. Reversal of neurotoxicity was studied using the potentially neuroprotective agents nerve growth factor (NGF, 100 ng/ml), basic fibroblast growth factor (bFGF, 5 ng/ml), insulin-like growth factors (IGF1 and IGF2, 10 ng/ml) and estrogen (10 nM), administered on DIV 4 and DIV 5, that is, subsequent to the A β (10 μ M)-induced neurotoxicity. A β caused a significant decrease in survival at 10 μ M, and a significant increase in apoptosis at 0.1 and 10 μ M. IGF1, IGF2 and bFGF all caused a reversal of the A β -induced neurotoxic effect on survival while NGF and estrogen did not under these experimental conditions.

Keywords

Beta-amyloid; Neurotoxicity; Apoptosis; Septal neuron

Alzheimer's disease is caused primarily by neurotoxic deposition of the protein beta-amyloid (A β) within specific areas of the brain including the septum [30]. These deposits are known as senile plaques and are thought to cause pathological neurofibrillary tangles. A β 's toxicity appears to be due to activation of the apoptotic (programmed cellular death) pathway within brain neurons [11], possibly a cascading result of enzymatic cleavage by caspase of the amyloid precursor protein (A β PP) [25].

One therapeutic approach to AD has been to find ways to decrease the deposition of A β by inhibiting the proteases that produce it. Such inhibitory mechanisms function by preventing protease accumulation, inflammation and altering cholesterol homeostasis or amyloid aggregation by shifting ion content within the cells [15]. A second approach has been to replace the neurotrophins such as nerve growth factor (NGF) which are reduced or lost in AD. Neurotrophins are frequently neuroprotective and decreased levels are often associated

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with a number of brain disorders. Increasing the concentration of neurotrophins results in an increase in the survival of neurons at risk [20]. Other treatment pathways under investigation include other cellular growth factors and estrogens. Low levels of A β interact with the low affinity NGF receptor, p75, altering neurite development [36]. Nerve growth factor replacement therapy has been proposed for cognitive deficits due to a loss of cholinergic neurons of the basal forebrain [19]. In a mouse model of Down's syndrome, A β PP increases led to a decrease in retrograde transport of NGF [29]. Estrogens also protect cholinergic function in the basal forebrain [5], have a role in the maintenance of learning and memory in the rat [33], and appear to be neuroprotective in humans when administered in hormone replacement therapy prior to or shortly after menopause [13,40]. Insulin-like growth factor I (IGF1) and basic fibroblast growth factor (bFGF) have neuroprotective roles, upregulating gene expression in various protective pathways [42]. IGF1 acts via several pathways in the processing of the A β PP [1], and in prevention of vascular dysfunction [24]. Protection against stroke is conferred by bFGF in rats [39] and FGF protects against A β toxicity in endothelial cells [7]. Rat temporal cortex is protected against A β by IGF1 [2]. In fact, IGF1 has so many protective functions its decline appears to be related to senescence in the brain [37]. Increased insulin-like growth factor II (IGF2) also appears to slow the progression of A β induced neurodegeneration in mice [35].

Bilaminar cultures of neurons and astrocytes grown in apposition (about 1 mm apart) but not touching one another, allow for the study of effects of A β and various neuroprotective agents in the absence of serum and in the absence of the blood–brain barrier. The purposes of this study were two-fold: (1) to determine what effect A β has on survival and induction of apoptosis in septal neurons (particularly in response to varying concentrations of A β in this bilaminar model) and (2) to determine whether various agents which are neuroprotective might reverse the effects of A β on survival.

This study used a bilaminar model for the study of neurotoxic effects of A β and its reversal by various growth factors or hormones. Neurons and glial cells were grown in cell culture in close apposition to one another but not touching one another so that both direct and indirect (mediated by astrocytes) actions of A β and potential treatments might be studied in the absence of the blood–brain barrier.

Astrocytes were cultured from embryonic day 16 (E16) Sprague–Dawley rat brains (Charles River; Wilmington, MA, USA). Rats were euthanized by ether anesthesia. The care and use of, as well as all procedures involving, animals have been approved by Barry University's IACUC, in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Astrocytes from the cerebral cortex were microdissected and cultured as described previously [3] and plated in 100 mm culture dishes (Corning; Corning, NY, USA). Seven days before neuronal dissection, glial cells were transferred to 12-well cell culture cluster dishes. Twenty-four hours before neuronal dissection, medium was replaced with 1 ml N2.1 defined medium [3].

Sprague–Dawley rat brains were dissected on E16 and were microdissected and cultured as described previously [14]. Neurons were plated on silane treated poly-L-lysine coated glass coverslips before treatment [17]. After 2 h of incubation, cover-slips were inverted over the 40–60% confluent glial plane within the 12-well culture dishes. Glial and neuronal planes were separated by about 1 mm by the parafilm feet on the coverslips. Cytosine arabinofuranoside (GIBCO, 10^{-5} M) was added after 1 day in vitro (DIV) to prevent glial

proliferation. All cells were maintained in a humidified 37 °C, 95% air/5% CO₂ incubator (NUAIRE) until use.

Cells were treated on DIV 1 and on DIV 4 with three concentrations of A β (25–35 fragment, 0.1 μ M, 1 μ M and 10 μ M; Biosource Camarillo, CA, USA); this fragment is the biologically active part of the peptide [16,21]. Controls were treated with sterile water only. A β was added to sterile-filtered water and allowed to aggregate at 4 °C. On DIV 7 survival was visualized and quantified by fluorescence microscopy in random fields with 4.6 μ g/ml propidium iodide (PI) and 15 μ g/ml fluorescein diacetate (FDA) under a fluorescence microscope (Olympus BX-60) with a 450 nm excitation [18].

Cells were treated on DIV 1 and DIV 4 with A β (10 μ M). On DIV 4 and DIV 5 cells were treated with growth factors (IGF1, 10 ng/ml; IGF2, 10 ng/ml; Bfgf, 5 ng/ml and NGF, 100 ng/ml) (all from Upstate Biotechnology Incorporated, Lake Placid, NY, USA) and estrogen (EST, Sigma: 10 nM). Each of these factors was diluted in DMEM and added to the media with the exception of EST, which was prepared in ethanol at a concentration of 25 mM and subsequently diluted 2.5×10^6 times in DMEM. On DIV 7 the cells received no treatment and survival was quantified by fluorescence microscopy with PI and FDA as described previously.

Cells were treated as for the reversal experiment described previously. On DIV 7 silane-coated glass coverslips with adhered cells were placed in 12-well dishes and apoptosis was quantitated by ApopTag® Plus Fluorescein In Situ Apoptosis detection kit (Chemicon International, Temecula, CA, USA). Coverslips were placed on microscope slides and counterstained with 100 μ l 46 μ g/ml PI (Sigma, St. Louis, MO, USA) in HBSS. Cells were visualized under a fluorescence microscope (40 $\times$) with 450 nm excitation. Green and red fluorescent cells were counted in randomly selected fields from each slide.

All slides were assigned to treatment according to a randomized block design [43]; each slide was assumed to be independent of other slides, then assigned to treatment by lottery. Within a central area in each slide three to five randomly selected circular fields (area = 2.54 mm² per field) were selected, and within each circular field the number of surviving cells was counted. The same method (field and area) was used to count dead cells. Cells stained red died by necrosis, while cells stained green died by apoptosis. The proportion of cells dead from apoptosis per field was calculated as (number of green cells)/(number of red cells + number of green cells).

SigmaStat® 3.0 [31] was used for all statistical tests, with appropriate interpretation [34,43]. All tests used the $\alpha = 0.05$ level for statistical significance, and we defined P = probability that there is no significant pattern present.

Fig. 1 shows the photomicrographs of control and A β -treated neurons; survival of neurons is shown in Fig. 1A (control) and Fig. 1B (A β -treated), while apoptosis is shown in Fig. 1C (control) and Fig. 1D (A β -treated). These indicate an increase in dead cells (red nuclei) in the A β treatment relative to control, and an increase in apoptosis (green nuclei) in the A β treatment relative to control. We found significant effects due to A β in both percent survival of neurons exposed to A β , and in percent of dead cells killed by apoptosis.

Fig. 2 shows the mean percent survival of cells per microscopic field exposed to different concentrations of A β . One-way ANOVA indicated significant differences among treatment means ($F = 3.086$; d.f. = 3, 51; $P = 0.035$); the Holm–Sidak post hoc test indicated the A β 10.0 μ M treatment resulted in significantly reduced survival compared with control ($t = 2.93$; $P = 0.005$). The A β treatments for 0.1 μ M ($t = 1.94$; $P = 0.058$) and 1.0 μ M ($t = 0.93$; P

= 0.356) did not result in significantly lower mean survival compared with control, while the 10 μ M dose did result in significantly lower mean survival compared with control.

Fig. 3 shows the mean percent of dead cells killed by apoptosis per microscopic field exposed to different concentrations of A β . One-way ANOVA indicated significant differences among treatment means ($F = 4.315$; d.f. = 3, 74; $P = 0.007$); the Holm–Sidak post hoc test indicated the A β 0.1 μ M treatment ($t = 2.93$; $P = 0.005$) and the 10.0 μ M treatment ($t = 2.93$; $P = 0.005$) both resulted in significantly higher percent death by apoptosis compared with the control (0.0 μ M A β). The A β treatment for 1.0 μ M ($t = 1.94$; $P = 0.058$) did not result in significantly higher mean percent death by apoptosis compared with control, though its mean (24%) was over three times higher than the control mean (7.7%).

Fig. 4 shows the mean percent survival of cells per microscopic field exposed to different combinations of A β (10 μ M) with growth factors and estrogen. One-way ANOVA indicated significant differences among treatment means ($F = 16.234$; d.f. = 6, 121; $P < 0.001$); the Holm–Sidak post hoc test indicated significantly higher mean survival compared with A β -only treatment for the control treatment (no A β , $t = 6.57$; $P < 0.001$), the A β + bFGF treatment ($t = 6.81$; $P < 0.001$), the A β + IGF1 treatment ($t = 3.40$; $P = 0.001$), and the A β + IGF2 treatment ($t = 6.01$; $P < 0.001$). The treatments for A β + NGF ($t = 1.63$; $P = 0.101$) and A β + EST ($t = 0.58$; $P = 0.564$) did not result in significantly increased mean survival compared with A β -only treatment.

The important findings of this study were three-fold: (1) that A β significantly decreased the survival of septal neurons at 10 μ M, (2) that A β significantly increased apoptosis at doses of 0.1 and 10 μ M and (3) that bFGF, IGF1 and IGF2 significantly reversed the apoptotic effect due to A β . Some of these effects may be indirectly mediated by the glial cells as in another study [3] we have shown that A β decreases survival of glial cells at 0.1, 1.0 and 10 μ M concentrations under the same circumstances. The glial cell increase in apoptosis due to A β was reversed significantly by bFGF and IGF1. Differences between responsiveness of neurons and glial cells in our studies may be due to the shorter treatment of glial cells due to their propensity to divide. Astrocytes protect against the A β toxicity unless the astrocytes are themselves exposed to A β , in which case the protection is lost [28].

Studies in PC12 cells suggest that the increased cell death seen in AD may be due to apoptosis [12]. Neuroprotective factors appear to act by stabilizing Ca²⁺ homeostasis [26] or by interrupting apoptosis pathways. Both bFGF and IGF1 increase the number of cells containing the Ca²⁺-binding protein calbindin, while the other factors do not [32], a possible mechanism for their neuroprotection here. In addition, bFGF, but not NGF decreased Ca²⁺ disturbances in hippocampal neurons [27]. Estrogen alters expression of Bcl proteins involved in apoptosis pathways [41].

Mammals have decreased IGF1 with age [38]. Insulin-like growth factor stimulates neuronal release and clearance of A β and IGF1 levels are altered in AD [6]. The dose of IGF1 which is neuroprotective in hippocampal neurons is 10 μ M, the dose used in these studies [9]. In the same study, IGF2 was effective but less potent. Effects of IGF1 in hippocampal neurons are particularly encouraging as they occur (as in this study) when IGF1 is administered subsequent to the A β insult as well as when the IGF1 and A β are administered simultaneously [8]. In mice transgenic for a mutant A β PP, IGF2 is over-expressed and may provide some protection to hippocampal neurons [36].

Although nerve growth factor supports the cholinergic neurons of the septum [19,23], this treatment has limitations as a therapy. One approach to overcoming the broad effects of

nerve growth factor due to its interaction with the low-affinity p-75 receptor has been to develop molecules which mimic individual domains within the NGF structure [22,23]. These may be more effective than NGF itself. Estrogen is protective against A β in cell lines such as PC12 and human neuronal cells [4,10] but was ineffective on the differentiated cells in this study. The estrogen and NGF may be acting by a similar pathway as both alter phosphotyrosine content in septal neurons over a similar time period [14]. It may be that this pathway is ineffective in reversal of A β effects although it is clearly effective in other respects.

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Fig. 1. Photomicrographs of control and A β -treated neurons; survival of neurons is shown in (A) (control) and (B) (A β -treated), while apoptosis is shown in (C) (control) and (D) (A β -treated). Scale bar = 200 μ m.

Fig. 2. Mean percent survival of cells per microscopic field (± 1 S.E.) exposed to different concentrations of A β ; n = number of fields counted and '*' indicates treatment mean percent significantly lower than control mean (no A β).

Fig. 3.

Mean percent of dead cells killed by apoptosis per microscopic field (± 1 S.E.) exposed to different concentrations of A β ; n = number of fields counted and '*' indicates treatment mean percent significantly higher than control mean (no A β).

Fig. 4.

Mean percent survival of cells per microscopic field (± 1 S.E.) exposed to different combinations of A β (10 μ M) and growth factors (bFGF = 5 ng/ml, NGF = 100 ng/ml, IGF1 and IGF2 = 10 ng/ml, EST = 10 nM); n = number of fields counted and '*' indicates treatment mean percent significantly higher than mean for A β only.