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Early induction of c-Myc is associated with neuronal cell death

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ABSTRACT

Neuronal cell cycle activation has been implicated in neurodegenerative diseases such as Alzheimer's disease, while the initiating mechanism of cell cycle activation remains to be determined. Interestingly, our previous studies have shown that cell cycle activation by c-Myc (Myc) leads to neuronal cell death which suggests Myc might be a key regulator of cell cycle re-entry mediated neuronal cell death. However, the pattern of Myc expression in the process of neuronal cell death has not been addressed. To this end, we examined Myc induction by the neurotoxic agents camptothecin and amyloid-β peptide in a differentiated SH-SY5Y neuronal cell culture model. Myc expression was found to be significantly increased following either treatment and importantly, the induction of Myc preceded neuronal cell death suggesting it is an early event of neuronal cell death. Since ectopic expression of Myc in neurons causes the cell cycle activation and neurodegeneration *in vivo*, the current data suggest that induction of Myc by neurotoxic agents or other disease factors might be a key mediator in cell cycle activation and consequent cell death that is a feature of neurodegenerative diseases.

Introduction

A growing body of evidence indicates that aberrant cell cycle activation is closely involved in neurodegenerative diseases including Alzheimer's disease (AD). In mature neurons, the cell cycle is normally arrested at the G_0 phase, therefore it has been proposed that if neurons are forced to re-enter the cell cycle, they die rather than proliferate [11]. Consistently, the re-expression of cell cycle markers has been linked with cell death in certain types of neuronal cells such as sympathetic neurons deprived of nerve growth factor and cortical neurons exposed to amyloid-β peptides (Aβ) [3,5]. The proto-oncogene c-Myc (Myc) encodes a transcription factor that regulates cell size, proliferation and cell cycle, and overexpression of Myc is commonly associated with tumorigenesis in multiple types of cancer. Because of this undesirable effect, the expression of Myc is exquisitely regulated to normal conditions and ectopic overexpression of Myc has been shown to sensitize cells to apoptosis as well as cell cycle activation [1,4]. In fact, we previously demonstrated that ectopic expression of Myc induces neuronal cell cycle entry and neurodegeneration, supporting the causal role of Myc in neurodegeneration [11].

In this regard, it is of interest to note that Myc protein has been shown to be elevated in many neurological disorders including global ischemia, Pick's disease, traumatic brain injury, and AD [6,7,13,20] indicating the pathological role of Myc in those diseases. Myc might be an essential factor responsible for the cell cycle re-entry mediated neuronal cell loss in AD, however, the pattern of Myc expression in neuronal cell death has not been examined. Therefore, in this study, we investigated whether neurotoxic agents such as camptothecin and Aβ induce Myc in neuronal cells in an effort to understand the potential role of Myc in neuronal cell death.

Materials and methods

Chemicals and antibodies

Camptothecin was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethylsulfoxide to a concentration of 10 mM. It was further diluted in culture medium before use. $A\beta_{1-42}$ peptide, as a trifluoroacetate salt, was purchased from Bachem (Torrance, CA, USA). Lyophilized $A\beta_{1-42}$ peptide was stored as powder at −20 °C until use. To prepare amyloid fibrils, lyophilized $A\beta_{1-42}$ peptide was reconstituted in phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.2 at a concentration of 230 μM. Reconstituted $A\beta_{1-42}$ prefibrillar aggregates were obtained by diluting aliquots of 230 μM stock solution and incubating at 37 °C for 5 days. Fibrils were sonicated for 5 min to break up any cluster formation before adding to cells. For experiments using oligomeric Aβ, Aβ-derived diffusible ligands were prepared using $A\beta_{1-42}$ peptide as described before [10], except that phenol red free Opti-MEM

Abbreviations: RA, all-trans-retinoic acid; AD, Alzheimer's disease; Aβ, amyloid-β peptides; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.

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(Invitrogen, Carlsbad, CA, USA) was used instead of phenol red free F12 medium. Antibodies against phosphorylated p53 (Ser15) and cleaved PARP were purchased from Cell Signaling Technology (Beverly, MA, USA), the anti-Myc antibody was obtained from Epitomics (Burlingame, CA, USA), anti-actin antibody was obtained from Sigma, anti-tubulin antibody was purchased from Millipore (Bedford, MA, USA).

Cell culture and cytotoxicity assay

The human neuroblastoma cell line SH-SY5Y was cultured in Opti-MEM supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies, Inc., Carlsbad, CA, USA) and 1% penicillin–streptomycin (Life Technologies, Inc.). The cells were plated at an initial density of 2×10^5 cells/cm^2 in 6-well plates. All-trans-retinoic acid (RA) was added at a final concentration of 10 μM the second day after plating. Cells were differentiated for 5 days before each experiment. Differentiated cells were treated with camptothecin (Sigma) at a final concentration of 10 μM. For cytotoxicity in SH-SY5Y, the cells were seeded in a density of 1×10^4 cells/well onto 96 well plates and differentiated for LDH assay. The cytotoxicity of camptothecin was evaluated by the LDH assay kit (Roche Diagnostic, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, cell media were collected after each treatment and mixed with LDH substrate in a 96 well plate. After incubation for 20 min at room temperature, the optical density was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The measured optical density was converted after standardization with low (no treatment; 0% toxicity) and high controls (1% Triton X-100; 100% toxicity) by the following equation: cytotoxicity (%) = [(experimental value-low control)/(high control-low control)] × 100.

Western blot analysis

Cells were washed once with ice cold PBS without Ca^{2+} and Mg^{2+} and then suspended in a lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM sodium chloride, 1% Triton, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM PMSF). After centrifugation at 10,000 × *g* for 10 min, equal amounts of cellular protein lysates, determined using bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore). Following treatment with 10% nonfat milk at room temperature for 1 h, the membranes were probed with each antibody at 4 °C overnight followed by horseradish peroxidase-conjugated anti-rabbit or mouse IgG secondary antibodies (Cell Signaling Technology). Bound antibodies were visualized by chemiluminescence detection on autoradiographic film. Blots were stripped and reprobed with actin/tubulin. For quantitative analysis of the immunoblot bands, the densities of the bands were measured by scanning densitometry (BioRad, Hercules, CA, USA). The densitometric data were presented as mean ± SD of values obtained for four controls versus four experimental samples. Values were presented relative to tubulin or actin levels.

Results

SH-SY5Y human neuroblastoma cells were differentiated with RA for 5 days and showed neuron-like morphology with thin and long neurites and no significant proliferation. In these cells, the expression of Myc was highest at the undifferentiated states and was down-regulated during differentiation by RA (Fig. 1A and B), a finding which is consistent with the low level of Myc found in post-mitotic neurons *in vivo* [7,11]. Differentiated SH-SY5Y cells

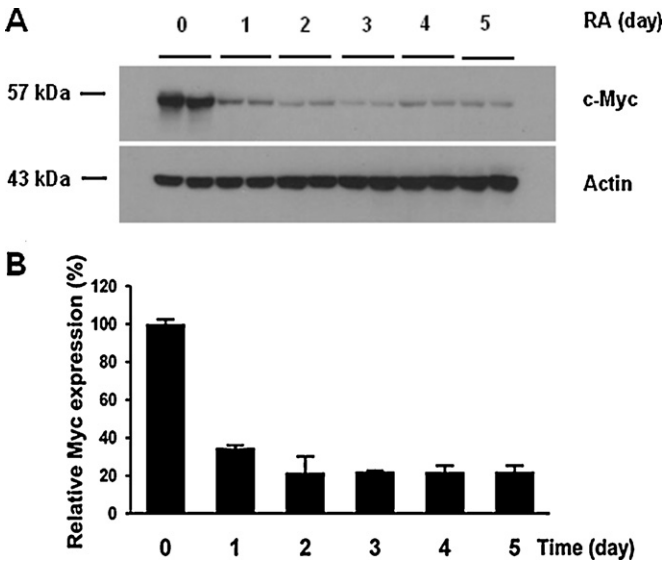

Fig. 1. Myc expression was down-regulated after differentiation with 10 μM RA (A). The cells were plated at an initial density of 2×10^5 cells/cm^2 in 6-well plates. RA was added at a final concentration of 10 μM the second day after plating. The densities of Myc were normalized with the level of actin (B). For all subsequent experiments, cells were used after 5 days differentiation with RA.

were then treated with 10 μM camptothecin, a DNA topoisomerase I inhibitor, which is known to elicit cell death via cell cycle re-entry in primary neurons [16], to determine whether the expression of Myc is induced by this DNA damaging agent. As shown in Fig. 2A and B, the expression of Myc was dramatically induced after 4 h treatment with camptothecin and reached even higher levels after 8 h. The expression pattern was parallel with the induction of phospho-p53 indicating the induction of DNA damage. Moreover, cleaved PARP, a marker for apoptosis, was not detected until 8 h after camptothecin treatment (Fig. 2A and B) suggesting apoptosis occurs after Myc induction. To confirm this temporal pattern of cell death, cells were treated with 10 μM camptothecin and cytotoxicity was determined at 0, 4, 8, 10, and 24 h by using the lactate dehydrogenase (LDH) assay kit (Roche Diagnostic, Indianapolis, IN). Consistent with PARP analysis data (Fig. 2A and B), the level of cytotoxicity reached significance after 8 h treatment (Fig. 2C) but no cytotoxicity was observed after 4 h treatment, the time-point which Myc induction occurs. Therefore, these data strongly suggest that Myc is an early response to camptothecin-mediated neurotoxicity and precedes p53-mediated apoptotic pathway.

We next examined whether the treatment of Aβ, a potential neurotoxic agent in AD, induces the expression of Myc. When differentiated SH-SY5Y cells were exposed to 20 μM fibrillar Aβ, increased Myc expression was significant at 8 h after Aβ treatment and the level of Myc was maintained higher than control through 16 h after Aβ treatment (Fig. 3A and B). Cleaved PARP was not detected until 12 h (Fig. 3A and B). In addition to the fibrillogenic form of Aβ, we also examined the effect of oligomeric Aβ, considered to be important in the pathogenesis of AD, and found that Myc was also significantly induced after treatment with 5 μM oligomeric Aβ (Fig. 4A and B). Interestingly, phospho-p53 protein expression peaked at 4 h and decreased by 8 h. These findings suggest that both forms of Aβ induce Myc expression and the induction of Myc either by camptothecin or Aβ is linked to p53-caspase dependent neuronal cell death.

Discussion

In this study, we examined the temporal expression pattern of Myc during the process of neuronal cell death induced by either

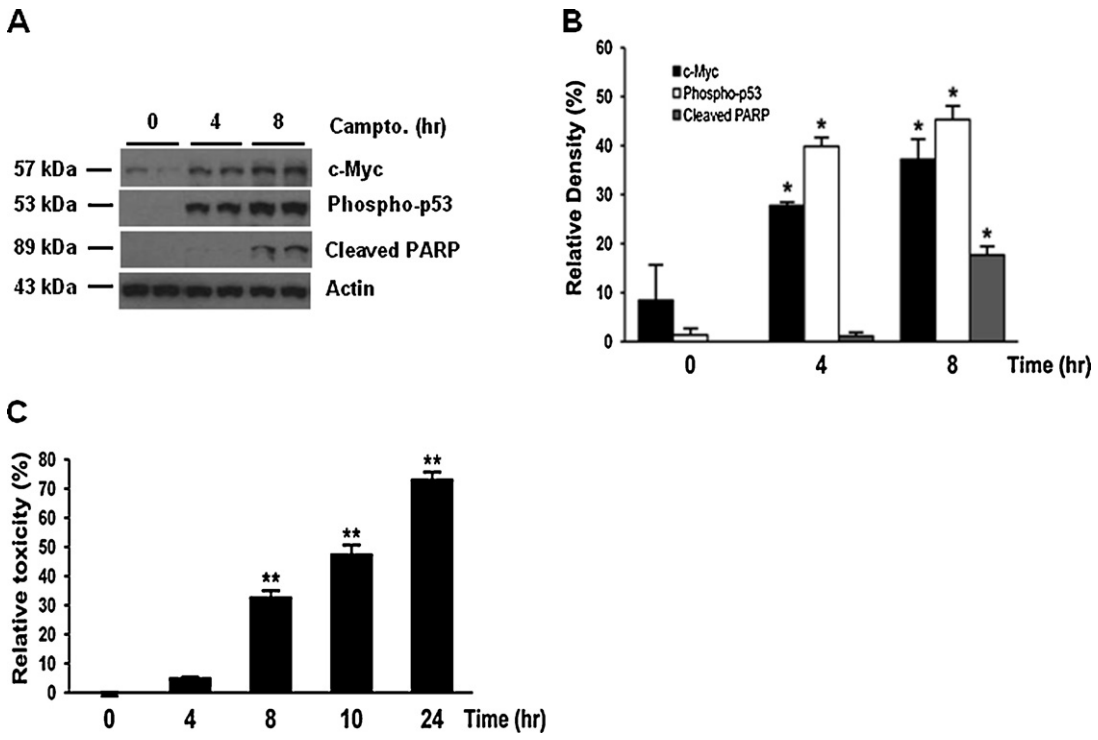

Fig. 2. Myc was an early response to camptothecin-mediated neuronal cell death. Differentiated SH-SY5Y cells were treated with 10 μM camptothecin and the expression of Myc and cell death markers were analyzed by western blot. Myc and phospho-p53 were induced after 4 h treatment followed by the increase of cleaved PARP at 8 h (A and B). $^{*}p < 0.01$ compared with the control group as assessed by Student's *t*-test ($n = 4$). Camptothecin-induced cytotoxicity was also analyzed with LDH assay in differentiated SH-SY5Y cells. The level of cytotoxicity reached the significant level after 8 h treatment. (C) Data were expressed as percent of control (mean ± SD) from four independent experiments. $^{**}p < 0.01$ compared with the 0 h treatment ($n = 4$).

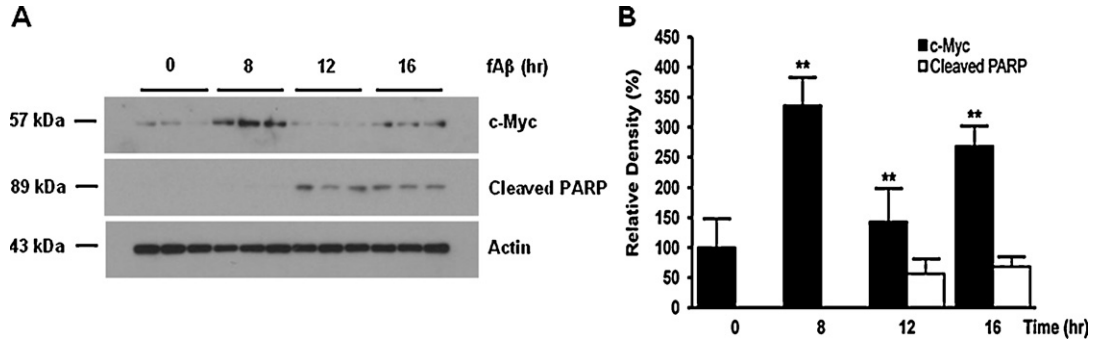

Fig. 3. Fibrillar Aβ (20 μM) induced Myc expression in differentiated SH-SY5Y cells. Myc expression peaked at 8 h after Aβ treatment followed by the increase of cleaved PARP at 12 h. Densities of Myc and cleaved PARP were normalized with actin (B). $^{**}p < 0.01$ ($n = 3$) respectively compared with 0 h.

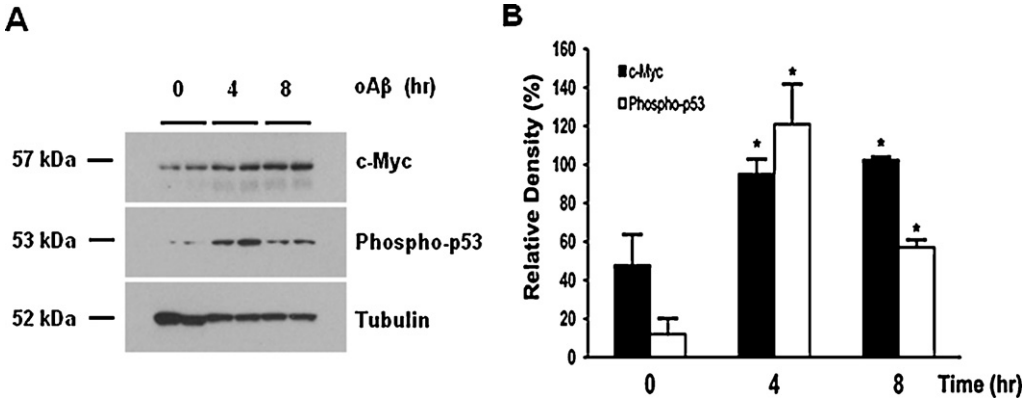

Fig. 4. Oligomeric Aβ (5 μM) induced Myc expression in differentiated SH-SY5Y cells. The level of Myc was also significantly elevated after 4 h treatment with 5 μM oligomeric Aβ. Phospho-p53 peaked at 4 h after Aβ treatment. The levels of Myc and phospho-p53 were normalized with tubulin (B). $^{*}p < 0.05$ compared with the control group as assessed by Student's *t*-test ($n = 4$).

camptothecin or Aβ. We found that both camptothecin and Aβ induced Myc expression in neuronal cells and the increase of Myc preceded neuronal cell death. From these studies, it is likely that Myc might regulate cell cycle re-entry in neuronal cell death since Myc has been tightly linked to both pathways. Supporting this notion, cell cycle re-entry has been found upstream of p53 mediated apoptotic pathways in camptothecin-treated neuronal cells [27]. Cell cycle re-entry has also been implicated in Aβ-mediated neuronal cell death [25].

Myc is pathophysiologically relevant to AD such that phosphorylated Myc is increased in dystrophic neurites and neurons with neurofibrillary tangles in AD [7]. In fact, overexpression of Myc induces neuronal cell cycle entry and neurodegeneration in an *in vivo* mouse model [11]. Consistently, ectopic expression of oncogenic SV40-T antigen in cortical neurons also induces neurodegeneration accompanied with DNA synthesis and cell cycle activation [18]. Furthermore, cell cycle proteins are increased in postmitotic neurons in response to neurotoxic stresses such as Aβ and kainic acid, an excitotoxic stressor [8,17], and are also induced in neurodegenerative diseases such as AD [15,26,28], Parkinson disease [9], and amyotrophic lateral sclerosis [19]. Here, we found Myc induction precedes camptothecin-mediated neuronal cell death and these data suggest Myc-mediated cell cycle activation might be a potential mechanism. Supporting this notion, it has been shown that inhibition of Myc is neuroprotective suggesting elevated levels of Myc makes neurons vulnerable [22]. Therefore, it is likely that the inhibition of Myc is also neuroprotective in campothecin and Aβ-induced neurotoxicity although this remains to be determined in future studies.

Alternately, the induction of DNA damage by camptothecin and Aβ might also be the precipitating mechanism of Myc-mediated neuronal cell death since both agents have been known to induce DNA damage [16,23] and induction of Myc by DNA damage has also been reported [12]. Interestingly, DNA damage in neurons has been observed during the progress of neurodegeneration in AD [14,21].

It is also interesting to note that fibrillar Aβ treatment induces Myc expression in a biphasic fashion (Fig. 3). The first wave of Myc induction precedes the occurrence of the apoptosis marker, cleaved PARP, suggesting this early Myc induction might play a key role in initiating apoptosis. A similar biphasic pattern of Myc expression associated with cell cycle and apoptosis has been reported in various conditions [2,24], thus the specific mechanism and pathological significance of this unique expression pattern of Myc induced by Aβ needs to be determined in future studies.

In conclusion, our data suggest that the induction of Myc is an early response in neuronal cell death and might represent a crucial pathogenic mechanism of neuronal cell loss in neurodegenerative diseases such as AD.

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