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Modulation of mitochondrial function by stem cell-derived cellular components

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ABSTRACT

Huntington's disease (HD) is the most common hereditary neurodegenerative diseases, in which the loss of striatal neuron caused by the aggregation of mutant huntingtin protein (mHtt) is the main pathological feature. Our previous studies have demonstrated that human adipose stem cells (hASC) and its extracts can slow down the progression of HD *in vitro* and *in vivo*. hASC are readily accessible adult stem cells, and the cytosolic extracts contain a number of neurotrophic factors. Here, we further explored the role of the hASC extracts in neuronal death and mitochondrial function in HD. Our results showed that the hASC extracts prevent mHtt-induced cell toxicity and cell apoptosis. Moreover, the hASC extracts recovered mHtt-induced mitochondrial oxidative stress and reduced mitochondrial membrane potential. The hASC extracts blocked the interaction between p53 and mHtt, and decreased the endogenous p53 levels at both transcriptional and post-translational levels, resulting in the instability of p53 and increased neuronal survival. Taken together, these findings implicate protective roles of hASC extracts in mHtt-induced mitochondrial apoptosis, providing insights into the molecular mechanism of the hASC in the therapeutic strategy of HD.

1. Introduction

Huntington's disease (HD) is an incurable progressive autosomal dominant neurological disorder caused by expanded CAG repeats coding for glutamine in the *Huntingtin* (*Htt*) gene. Many studies have shown that aggregation of mutant Htt (mHtt) caused cell death *in vivo* [1–3] and *in vitro* [4,5]. Specially, mHtt contributes to neurodegenerative disorders through the activation of intrinsic apoptosis pathway involving mitochondria [6–8]. The lower level of mitochondrial membrane potential has been detected from both human HD patient and full-length Htt-transgenic HD mice brains compared to the normal human and wild-type mice [9]. More interestingly, N-terminal mHtt is identified on the mitochondrial membrane from YAC72 mice brain [9] and mHtt is present in mitochondrial fraction from HD *in vitro* cell model [10]. Mitochondrial dysfunction is a causative factor in most of neurodegenerative diseases [11–13]. Many events can occur during the process of mitochondria-mediated cell death including loss of mitochondrial membrane potential, imbalance of anti-apoptotic proteins and pro-apoptotic proteins. As a result, the levels of mitochondrial reactive oxygen species (ROS) are increased and other apoptotic inducing factors are released to the cytosol, which lead to the activation of pro-caspase to induce apoptosis[14,15].

p53, a tumor suppressor protein, promotes apoptosis during the development of central nervous system in response to injury and in neurodegenerative disorders [16]. In response to injury and cellular stress, p53 induces DNA damage and cell death through the transcriptional transactivation of its target genes including Bax, puma and p21 [17,18]. In addition, p53 protein levels also can be regulated through its stabilization by stress signals and its destabi-

Abbreviations: EGFP, enhanced green fluorescent protein; hASC, human adipose stem cells; HD, Huntington's disease; RT–PCR, reverse transcription polymerase chain reaction; ROS, reactive oxygen species; mHtt, mutant huntingtin protein.

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lization by protein degradation system [16,19]. Current studies have shown that p53 induces apoptosis both in transcription-dependent pathway and transcription-independent action at the mitochondria [20]. p53 plays a key role in the mitochondria-associated cellular dysfunction and behavioral abnormalities of HD mice [21]. In HD mice, mHtt binds to p53 and upregulates p53 transcriptional activity [21]. Behavior abnormalities and neurodegeneration in mHtt-transgenic mice are suppressed by the genetic deletion of p53 [21]. It has also reported that p53 is stabilized by mHtt, resulting in accumulation of p53 and increase the activity of p53 [21,22].

Human adipose stem cells (hASC) have been shown to be multipotent and possessed the ability to differentiate into multiple cell lineages. hASC are feasible source for stem cell-based therapy due to their abundance, multipotency, and ethical consideration [23–25]. Current study shows that the hASC can produce many important neurotrophic factors that are essential for neuronal growth, differentiation and survival [26]. Similarly, a recent study has shown that hASC protect the brain from traumatic brain injury-induced neurodegeneration and motor and cognitive impairments [27]. Our previous studies have demonstrated that both the hASC and the hASC extracts could slow down the progression of HD through reducing mHtt aggregation [28,29]. However, the detailed molecular mechanism whether the hASC extracts can prevent the neuronal death and mitochondrial dysfunction has not been well studied. In this study, we investigated the protective role of the hASC extracts in cell death and mitochondrial dysfunction in HD, and further explored the p53 involved mechanism.

2. Materials and methods

2.1. Cell culture

Mouse neuroblastoma cell line (N2a) were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Cell apoptosis assay and mitochondrial dysfunction assay

For LDH release assays, 200 μl of culture medium were collected and used. The LDH level was measured with *in vitro* Toxicology assay kit (Sigma–Aldrich, MO, USA) by spectrophotometer following the manufacturer's instructions. For CCK8 cell viability assay, cell culture medium was replaced with new medium containing 10% CCK-8. Two hours later, the culture medium was collected and the absorbance was measured at 450 nm using a microplate reader. For Annexin V/PI cell death assays, the Annexin-V-FITC and PI Apoptosis Detection Kit (BD Bisosciences) was used followed by FACS analysis (FACS Calibur, BD, CA, USA) according to the manufacturer's instructions. For JC-1 staining, after harvesting and washing with PBS, cells were stained with JC-1 (Invitrogen), MitoSoxRed (Invitrogen) or Mitotracker (Invitrogen) Red for 20 min. Cells were washed once with binding buffer and measured by flow cytometry and (or) captured by fluorescence microscopy.

2.3. Immunoblotting and immunoprecipitation

Cell lysate was lysed with RIPA lysis buffer (Biosesang Inc., Seoul, Korea) and the total protein concentration was quantified by a colorimetric detection assay (BCA Protein Assay, Pierce, USA). Equal amounts of protein lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Interested proteins were probed by primary antibodies and corresponding peroxidase-labeled secondary antibodies, followed with detection by ECL (Millipore Corporation).

For immunoprecipitation experiments, amount of 500 μg of protein lysate from N2a cells transfected with appropriate plasmids including Htt and mHtt alone were precipitated with 1.5 μg of mouse anti-mHtt antibody. Amount of 30 μl protein A/G-Sepharose beads (American Qualex, CA, USA) were added and the immune complexes were pulled down overnight at 4 °C under rotation. Beads were washed extensively with cool lysis buffer and lysed by boiling in the presence of Laemmli buffer. Immunoprecipitates were subjected to SDS–PAGE.

2.4. Statistical analysis

Data were analyzed using Student's *t*-test. Data were expressed as mean ± S.E.M. Differences were deemed significant when $P < 0.05$. Image intensity was quantified using Nikon NIS-Elements-AR software.

3. Results

3.1. The hASC extracts alter pro- and anti-apoptotic proteins in vitro and in vivo

To investigate whether there is a protective role of the hASC extracts in HD *in vivo* and *in vitro*, we determined the cell death and the mitochondria related proteins using immunoblotting. We found that there were higher expression levels of p53, Bax and cleaved caspase-3 protein and lower expression level of Bcl2 protein in the region of striatum in R6/2 mice brain compared to the wild type mice. However, the injection of the hASC extracts to the R6/2 mice normalized these protein expressions, shown in Fig. 1A.

In addition, we also determined the above proteins in mHtt-transfected N2a cells and found that there were higher p53, Bax and cleaved caspase-3 protein expression levels in mHtt-transfected cells compared to the control (normal Htt was used as vector control here and below), but, with the treatment of the hASC extracts to mHtt-transfected cells significantly rescued these proteins to the normal levels except that of Bcl2 protein, which did not show a significant change in total lysates (Fig. 1B).

3.2. The hASC extracts prevent mHtt-induced cell toxicity and mitochondrial dysfunction

Since we observed the alterations of cell death related proteins by mHtt and the hASC extracts, we hypothesized that the hASC extracts may have a protective role in cell death and mitochondrial dysfunction. To test this hypothesis, we evaluated the cell viability using CCK8. Our result revealed that the cell viability was significantly reduced by the overexpression of mHtt compared to the control, but it was significantly rescued after the treatment of the hASC extracts (Fig. 2A). Then, in LDH release assay cells that transfected with mHtt were significantly increased LDH release compared to the control. However, this was decreased to the normal level with the treatment of the hASC extracts (Fig. 2B). We also detected the cell death using PI & Annexin V staining. The analysis graph showed the increase of apoptotic proportion due to the overexpression of mHtt, and the decrease by the treatment of the hASC extracts (Fig. 2C and S1A).

In order to determine the hASC extracts might have a protective role in mHtt-induced mitochondrial dysfunctio, MitoSoxRed was