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Oxidative stress causes DNA triplet expansion in Huntington's disease mouse embryonic stem cells

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Abstract Huntington's disease (HD) is a neurodegenerative disorder caused by an expanded trinucleotide CAG repeat in the *Huntingtin* (*Htt*) gene. The molecular basis for the development and progression of HD is currently poorly understood. However, different DNA repair pathways have been implicated in both somatic expansion and disease progression. Embryonic stem cells provide a remarkable in vitro system to study HD and could have implications for understanding disease development and for therapeutic treatment. Here, we derive pluripotent stem cells from the mouse R6/1 HD model and demonstrate that repeated exposure to genotoxic agents inducing oxidative DNA damage gave a significant and dose dependent increase in somatic triplet expansion. Further investigation into specific steps of DNA repair revealed impaired double stranded break repair in exposed R6/1 cells, accompanied by the induction of apoptosis. We also found that differentiation status, and consequently DNA repair efficiency influenced somatic expansion. Our data underscore the importance of DNA damage and repair for the stability of the HD triplet in pluripotent stem cells.

Introduction

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder characterized by progressive loss of motor and cognitive function. The clinical course of HD typically progresses over 10–20 years from a presymptomatic state to complete disability and death. There are no disease altering treatments and symptomatic therapy has limited benefit. HD is caused by an expansion of an unstable trinucleotide CAG repeat in the gene encoding Huntingtin (*Htt*), resulting in an extended polyglutamine tract in the expressed protein (Anon., 1993). Healthy individuals have 6–35 CAG repeats, and affected individuals have more than 36 repeats (Langbehn et al., 2004). Individuals with longer expansions have an earlier onset, increased severity and progression of the disease (Langbehn et al., 2004).

Involvement of DNA damage and DNA repair pathways in disease progression and CAG expansion in HD is underscored by a number of recent studies (Kovtun et al., 2007; Dragileva et al., 2009; Wheeler et al., 2003; Goula et al., 2009). Increased oxidative stress is found in HD individuals, and oxidative DNA damage preferentially accumulates at CAG

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repeats in a length dependent manner in a HD mouse model (Goula et al., 2009; Bogdanov et al., 2001; Browne et al., 1997). Importantly, the change of age-dependent somatic instability by the BER enzyme OGG1 clearly links oxidative DNA damage to CAG repeat instability (Kovtun et al., 2007). Other DNA repair proteins, such as Ku70, a component of the non-homologous end joining (NHEJ) machinery, also seem to be involved in disease progression. The exogenous expression of Ku70 rescues pathological phenotypes in a mouse model of HD (Enokido et al., 2010).

Pluripotent stem cells have great potential in terms of regenerative medicine as well as disease modeling. They have been used to extensively model development; differentiation and some disease state in vitro (Ben-David et al., 2012). Many human HD pluripotent stem cell lines have been generated to date, including some induced pluripotent stem cell (iPS) lines which give valuable insight into the pathology of HD (reviewed in Jonson et al., 2013). However, no HD-related phenotypes have been reported with these human embryonic stem cell (ESC) lines (reviewed in Kaye and Finkbeiner, 2013). On the other hand, murine ESC-based models can reveal HD-related phenotypes. ESCs generated from mice with either 150 or 77 repeats showed similar neural proliferative properties to human neural cells in HD brains (Curtis et al., 2003, 2005).

As a step towards understanding the role of oxidative DNA damage and somatic instability of the CAG triplet in early differentiation and development, we have derived embryonic stem cells from the R6/1 HD mouse model carrying the expanded HD CAG repeat. We found that oxidative stress enhanced somatic instability. Furthermore, we found impairment in double stranded break repair which was accompanied by the induction of apoptosis.

Materials and methods

Cell culture

ESCs were derived as described by Bryja et al. (2006). ESCs were maintained on a feeder layer of irradiated MEF cells (GlobalStem) in stem cell medium consisting of Knockout Dulbecco's modified Eagle's medium (KO-DMEM) (Life Technologies) supplemented with 20% Knockout Serum Replacement (Life Technologies, 10828-028), 50 μg/ml Pen/Strep (Medprobe), 1 mM L-glutamine (Life Technologies), 0.1 mM Non-Essential Amino Acids (Medprobe, BE13-114E), 0.1 mM β-mercaptoethanol (Sigma Aldrich) and 1000 units/ml Leukemia Inhibitory Factor (LIF) (Millipore). For differentiation, stem cell medium without LIF was used and 1 μM retinoic acid was added fresh daily. For all experiments, passages between 10 and 15 were used (the long term experiments start at passage 10 and end at passage 22).

Genotoxic treatment

H_2O_2 (Sigma Aldrich) was added to KO-DMEM to make either a 150 μM or a 50 μM solution. Cells were left with the H_2O_2 solution at 37 °C with 5% CO for 30 min. $KBrO_3$ (Sigma Aldrich) was added to KO-DMEM to make a 7.5 mM solution. Cells were left with the $KBrO_3$ solution at 37 °C with 5% CO for 20 min. MMS (Sigma Aldrich) was added to KO-DMEM to make a 3 mM solution. Cells were left with the MMS solution at 37 °C with 5% CO for 20 min.

Immunocytochemistry

Cells were either grown on coverslips or cytospun onto slides (2000 rpm for 10 min). Cells were fixed in 4% paraformaldehyde (Sigma Aldrich) for 15 min at RT and washed with PBS. The cells were permeabilized with 0.5% Triton X-100 (Sigma) and blocked with 5% goat or donkey serum (Sigma) and 5% bovine serum albumin (Sigma) for 1 h at RT. Cells were incubated overnight at 4 °C with primary antibodies. After incubation, the cells were washed with PBS and incubated with secondary antibodies 1 h at RT. Cells were then washed with PBS, incubated with 4′-6-diamidino-2-phenylindole (DAPI) (Life Technologies) for 5 min, and analyzed under a fluorescence microscope. Primary antibodies included: NANOG (Abcam, 1:200), SOX2 (Millipore, 1:200), OCT4 (Abcam, 1:100) and γH2A.X (Millipore, 1:200). Secondary antibodies: Alexa488- or Alexa594-conjugated (Life Technologies, 1:200-500).

Alkaline Phosphatase staining

Alkaline Phosphatase staining was performed with Alkaline Phosphatase Detection Kit (Millipore) according to manufacturer's instructions.

TUNEL apoptosis assay

Cells were cytospun onto slides (2000 rpm for 10 min) before the TUNEL assay (Roche) was performed according to the manufacturer's instructions. The cells were then counterstained with DAPI for 5 min and washed three times with PBS for 5 min before mounting in Mowiol.

$DiIC_1(5)$ survival assay

The MitoProbe $DiIC_1$ (Wheeler et al., 2003) Assay (Life Technologies) was performed according to the manufacturer's instructions. The cells were then analyzed with an Accuri flow cytometer in PBS. A minimum of 10,000 cells were measured per data point.

Image analysis

Fluorescent staining intensity of γH2A.X staining was quantified using the ImageJ software.

Conventional and quantitative PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) and cDNA was generated from 2 μg of total purified RNA using the High capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer's instructions. All conventional polymerase chain reactions (PCRs) were performed with Pfu DNA polymerase (Stratagene). Quantitative PCR (qPCR) was performed with SYBR Green qPCR kit (Applied Biosystems) using 10 μl of total reaction and analyzed on the