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Reactive nucleolar and Cajal body responses to proteasome inhibition in sensory ganglion neurons ☆

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ABSTRACT

The dysfunction of the ubiquitin proteasome system has been related to a broad array of neurodegenerative disorders in which the accumulation of misfolded protein aggregates causes proteotoxicity. The ability of proteasome inhibitors to induce cell cycle arrest and apoptosis has emerged as a powerful strategy for cancer therapy. Bortezomib is a proteasome inhibitor used as an antineoplastic drug, although its neurotoxicity frequently causes a severe sensory peripheral neuropathy. In this study we used a rat model of bortezomib treatment to study the nucleolar and Cajal body responses to the proteasome inhibition in sensory ganglion neurons that are major targets of bortezomib-induced neurotoxicity. Treatment with bortezomib induced dose-dependent dissociation of protein synthesis machinery (chromatolysis) and nuclear retention of poly(A) RNA granules resulting in neuronal dysfunction. However, as a compensatory response to the proteotoxic stress, both nucleoli and Cajal bodies exhibited reactive changes. These include an increase in the number and size of nucleoli, strong nucleolar incorporation of the RNA precursor 5′-fluorouridine, and increased expression of both 45S rRNA and genes encoding nucleolar proteins UBF, fibrillarin and B23. Taken together, these findings appear to reflect the activation of the nucleolar transcription in response to proteotoxic stress Furthermore, the number of Cajal bodies, a parameter related to transcriptional activity, increases upon proteasome inhibition. We propose that nucleoli and Cajal bodies are important targets in the signaling pathways that are activated by the proteotoxic stress response to proteasome inhibition. The coordinating activity of these two organelles in the production of snRNA, snoRNA and rRNA may contribute to neuronal survival after proteasome inhibition. This article is part of a Special Issue entitled: Role of the Nucleolus in Human Disease.

1. Introduction

The ubiquitin-dependent proteasome system (UPS) is the major pathway responsible for cellular protein degradation in eukaryotic cells. Proteasomes degrade essential proteins for cellular homeostasis including those involved in transcription, DNA repair, cell cycle progression and apoptosis [1,2]. Proteolysis is performed by the 26S proteasome, a multi-subunit complex formed by the 20S proteolytic core and one or two flanking 19S regulatory particles that recognize polyubiquitinated protein substrates [3,2]. In the cell nucleus, proteasomes are abundant in euchromatin domains, nuclear speckles of pre-mRNA splicing factors, PML (promyelocytic leukemia) nuclear bodies and clastosomes [4,5]. Proteasomal activity has been detected with fluorogenic protein substrates in nucleoplasmic foci, but not in the nucleolus [6,7]. Nuclear proteolysis seems to be involved in quality-control mechanisms and in the turnover of short-lived proteins, particularly certain transcription regulators and splicing factors [4,5]. In the nervous system, dysfunction of proteasome is an age-related process and also occurs in several neurodegenerative diseases [8].

The ability of proteasome inhibitors such as bortezomib to induce cell death has emerged as a powerful strategy for cancer therapy [9]. Bortezomib is a reversible inhibitor that primarily targets the chymotrypsin-like activity of the proteasome β5-subunit and to a somewhat lesser extent the caspase-like activity of the β1 proteasome subunit [3]. Bortezomib cytotoxicity in cancer cells is mediated by several mechanisms, including inhibition of NF-kB, up-regulation of genes involved in pro-apoptotic pathways and induction of endoplasmic reticulum stress [10]. However, peripheral neurotoxicity is the most relevant dose-limiting side effect of bortezomib treatment, and frequently leads to a severe sensory peripheral neuropathy [11,12].

The nucleolus is the nuclear factory for rRNA synthesis, processing of the maturing rRNA transcripts and preribosome subunit assembly. These processes are reflected in the structural organization of the main nucleolar components, fibrillar centers (FCs), dense fibrillar component (DFC) and granular component (GC) [13–16]. In addition, several studies support that the nucleolus is a central hub for sensing and coordinating cellular stress response [17,18]. Furthermore, recent studies indicate a functional link between the nucleolus and the UPS, suggesting

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the participation of the former in cellular proteostasis [19]. In fact, treatments with proteasome inhibitors can alter the nucleolar morphology and function [20–22].

Mammalian projection neurons exhibit prominent nucleoli with a high rate of ribosome biogenesis for the maintenance of active protein synthesis and cellular proteostasis. Neurons accommodate, at least in part, changes in the demand for protein synthesis by regulating the number and size of nucleoli, as well as the number of fibrillar centers [23–26]. In this way, a recent study has shown that the postsynaptic density protein AIDA-1 mediates a link between synaptic activity and protein synthesis by regulating neuronal nucleologenesis [27]. Moreover, nucleolar activity may influence the potential for neurorepair and nerve regeneration [25,28]. Conversely, morphological and biochemical manifestations of nucleolar dysfunction are associated with certain neurological disorders [29,28,30,31].

Cajal bodies (CBs) are nuclear structures discovered by Cajal in mammalian neurons [32]. They concentrate many molecular components, including coilin, the survival motor neuron (SMN) protein, the nucleolar protein fibrillarin and small nuclear (snRNPs) and nucleolar ribonucleoproteins (snoRNPs) required for pre-mRNA and pre-rRNA processing [33–36]. In neurons, CB number positively correlates with neuronal activity [24,37]. Moreover, an activity-dependent association of CBs with nucleoli has been reported in several neuronal populations [38,27].

In rat and mouse models of bortezomib treatment, we and others have reported that sensory ganglion neurons are primary targets of the peripheral neurotoxicity when using doses equivalent to those used in clinical patients [39,40]. In particular, we demonstrated that the bortezomib-induced inhibition of proteasome activity, measured by cleavage of a fluorogenic substrate, caused a proteotoxic stress characterized by the accumulation of ubiquitylated proteins, dissolution of the rough endoplasmic reticulum (chromatolysis) and nuclear retention of polyadenylated RNAs in nuclear bodies called poly(A) granules [39]. Here we have analyzed the effects of the bortezomib-induced proteasome inhibition on the structure, organization and function of the nucleoli, as well as on the organization of CBs, in rat sensory ganglion neurons. We show that proteasome inhibition induces dose-dependent nucleolar hypertrophy and nucleologenesis, activates rRNA synthesis, and increases the number of CBs. This nucleolar response supports the participation of the nucleolus in a compensatory response to the proteotoxic stress induced proteasome inhibition, which may be important for neuronal survival.

2. Material and methods

2.1. Rats

Experiments were designed and performed to minimize the use of animals using a total of 60 young male Sprague–Dawley young rats of 125–130 g, distributed into one control and two experimental groups treated with low or high doses of bortezomib ($n = 20$ per group). Animal care and handling was in accordance with Spanish legislation (Spanish Royal Decree 53/2013 BOE) and the guidelines of the European Commission for the accommodation and care of laboratory animals (revised in Appendix A of the Council Directive 2010/63/UE). The experimental plan was preliminarily examined and approved by the Ethics Committee of the University of Cantabria. All the animals were housed in a limited access environment with a 12-h light/dark cycle, where room temperature and relative humidity were set at 22 ± 2 °C and $55 \pm 10\%$ respectively and there was free access to food and water. Animal sacrifice was performed under deep pentobarbital anesthesia (50 mg/kg).

2.2. Drug administration and sacrifice

For the study of the nuclear effects of proteasome inhibition, we used the proteasome inhibitor bortezomib (Velcade, provided by Millennium Pharmaceuticals Inc., Cambridge, MA). The animals received two doses of bortezomib 0.2 mg/kg (low doses, Btz-LD) or 0.5 mg/kg (high doses, Btz-HD), dissolved in sterile saline and administered intravenously via the tail vein, on days 1 and 3 and were sacrificed 1d post-administration of the second dose. Moreover, the administration of two doses (0.5 mg/kg twice a week) was well tolerated by the Sprague Dawley rats and no animals died during the experiment. Saline-treated rats were used as controls. At the end of the treatment period the rats were sacrificed. The general condition of the animals was assessed daily.

2.3. Immunofluorescence and confocal microscopy

For immunofluorescence and in situ hybridization for poly(A) RNA the animals were perfused under deep anesthesia with pentobarbital (50 mg/kg) with 3.7% paraformaldehyde (freshly prepared) in PBS. As a model of sensory ganglion neurons we used trigeminal ganglia. After fixation, the ganglia were removed and washed in PBS. Tissue fragments from trigeminal ganglia were transferred to a drop of PBS on a siliconized slide and squash preparations of dissociated neurons were performed following the procedure previously reported [38].

For immunofluorescence, the samples were, then, sequentially treated with 0.5% Triton X-100 in PBS for 45 min, 0.1 M glycine in PBS containing 1% bovine serum albumin (BSA) for 30 min and 0.05% Tween 20 in PBS for 5 min. The samples were incubated for 3 h with the primary antibody containing 1% BSA at room temperature, washed with 0.05% Tween 20 in PBS, incubated for 45 min in the specific secondary antibody conjugated with FITC or TexasRed (Jackson, USA), washed in PBS and mounted with the ProLong anti-fading medium (Invitrogen).

Confocal images were obtained with a LSM510 (Zeiss, Germany) laser scanning microscope and using a 63× oil (1.4 NA) objective. In order to avoid overlapping signals, images were obtained by sequential excitation at 488 nm and 543 nm, to detect FITC and TexasRed, respectively. Images were processed using Photoshop software.

The morphometric and quantitative analysis of the nucleolar size and nucleoli and CB numbers per neuron were performed in the population of large neurons (≥40 μm) [38]. Dissociated neurons were immunostained for B23 for nucleolar parameters, and coimmunolabeled for coilin and SMN to estimate CB number. Nucleolar diameters (only of mononucleolated neurons) and nucleoli and CB numbers were estimated on confocal images using a 63× oil objective and the LSM510 software for morphometric and quantitative analysis. Data were analyzed using one-way ANOVA followed by Bonferroni tests for comparisons. Statistical significance was set at $p < 0.05$. All the analyses were carried out using GraphPad software for Windows.

2.4. Antibodies

The following primary antibodies were used in this study. Mouse monoclonal antibodies anti-UBF (F-9) (Santa Cruz Lab. La Jolla, CA, USA, dilution 1:100 IF), anti-SMN (BD transduction laboratories, dilution 1:50 IF), anti-fibrillarin (Abcam, dilution 1:500 IF), anti-p53 (Pab 246) (Santa Cruz Lab. La Jolla, CA, USA, dilution 1:500 WB) and anti-B23 (Abcam, dilution 1:200 IF). Rabbit polyclonal antibodies anti-coilin (210.4) (dilution 1:200 IF), anti-fibrillarin ([41], dilution 1:100, immunogold), anti-Pp53 (Ser15) (Cell Signaling, dilution 1:1000 WB) and anti-Myc (Santa Cruz Lab. La Jolla, CA, USA, dilution 1:1000 WB). Goat polyclonal antibody anti-actin (Santa Cruz Lab. La Jolla, CA, USA, dilution 1:2000 WB).

2.5. In situ hybridization for poly(A) RNAs

Sensory ganglion neurons fixed and dissociated as previously described were used for fluorescence in situ hybridization. Preparations of ganglion neurons were permeabilized with TBS-E-SDS for 15 min at 37 °C, washed three times in 6× SSPE-Tween 20 0.1% for 15 min, and incubated with the probe containing tRNA for 3 h, at 42ªC in a humidified chamber. An oligo dT $_{(50)}$-mer, 5′-end labeled with biotin