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Inhibition of the neuronal NFκB pathway attenuates bortezomib-induced neuropathy in a mouse model

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ABSTRACT

Bortezomib is a proteasome inhibitor with a remarkable antitumor activity, used in the clinic as first line treatment for multiple myeloma. One hallmark of bortezomib mechanism of action in neoplastic cells is the inhibition of nuclear factor kappa B (NFκB), a transcription factor involved in cell survival and proliferation. Bortezomib-induced peripheral neuropathy is a dose-limiting toxicity that often requires adjustment of treatment and affects patient's prognosis and quality of life. Since disruption of NFκB pathway can also affect neuronal survival, we assessed the role of NFκB in bortezomib-induced neuropathy by using a transgenic mouse that selectively provides blockage of the NFκB pathway in neurons. Interestingly, we observed that animals with impaired NFκB activation developed significantly less severe neuropathy than wild type animals, with particular preservation of large myelinated fibers, thus suggesting that neuronal NFκB activation plays a positive role in bortezomib induced neuropathy and that bortezomib treatment might induce neuropathy by inhibiting NFκB in non-neuronal cell types or by targeting other signaling pathways. Therefore, inhibition of NFκB might be a promising strategy for the cotreatment of cancer and neuropathy.

1. Introduction

In the last 10 years, proteasome inhibitors have become a good strategy to treat some malignant neoplasms. Bortezomib is the first proteasome inhibitor approved to treat multiple myeloma and mantle cell lymphoma, and other solid neoplasms (Richardson et al., 2006). The blockade of protein degradation, mediated by the ubiquitin-proteasome system, inhibits several cell signaling pathways that lead to cell cycle arrest, apoptosis and inhibition of angiogenesis. It is widely accepted that the antineoplastic effect of bortezomib is partly mediated by the inhibition of nuclear factor kappa B (NFκB) (Demchenko and Kuehl, 2010; Goldberg, 2012; McConkey and Zhu, 2008), although NFκB can remain active in some cell types after bortezomib administration (Hideshima et al., 2009; Li et al., 2010). In non-stressed cells, NFκB remains in the cytoplasm bound to its inhibitor IκBα. After stress or inflammatory stimuli, IκBα protein is phosphorylated at two N-terminal serine residues, and ubiquitinated and degraded by the proteasome. The degradation of IκBα allows NFκB to be translocated to the nucleus, where it will bind to DNA sites of NFκB responsive genes (Hideshima et al., 2009) to mediate, among other responses, cell proliferation, antiapoptosis, and cytokine secretion (Karin and Greten, 2005). In fact, the NFκB pathway is considered a prototypical proinflammatory signaling pathway, closely related with proinflammatory genes including cytokines, chemokines and adhesion molecules.

Like other types of antineoplastic agents, bortezomib cause toxic peripheral neuropathy, which indeed is one of the limiting side effects of these treatments, and several mechanisms have been implicated in this neurotoxicity (reviewed in Alé et al., 2014a). In a previous study, we observed that bortezomib administration in mice induced the translocation of NFκB to the nucleus in primary sensory neurons, together with an increase of several cytokines (Alé et al., 2014b). However, NFκB is described as an ambiguous transcription factor in neurons. It may mediate neuronal survival but also can lead to cellular activation and inflammation (Hunot et al., 1997; Drive and Francisco, 1997).

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Therefore, in this study we evaluated the role of NFκB in our mouse model of bortezomib-induced peripheral neuropathy (BIPN). Firstly, we studied how bortezomib modulates expression of IκBα in dorsal root ganglia (DRG). Secondly, to specifically study the selective role of neuronal NFκB in BIPN, we evaluated the evolution of the neuropathy in mice with a transgenic dominant negative form of IκBα, which prevents degradation of endogenous IκBα and thereby prevents NFκB translocation to the nucleus, inhibiting its activity.

2. Material and methods

2.1. Animals and study design

OF1, C57BJL6 and TgNFL-dnIκBα mice aged 2.5 months were used in the study. In a first experiment, 20 mice per group (control and transgenic strain) were used to assess the role played by cytokines and IκBα after bortezomib exposure on DRG neurons. Subsequently, a second experiment was performed evaluating nerve conductions, functional status and histological outcome in the same bortezomib-induced peripheral neuropathy model with the aim to validate the role of NFκB. The experimental procedures were approved by the ethics committee of the Universitat Autònoma de Barcelona, and were carried out in accordance with the European Community Council Directives.

2.2. Generation and characterization of NFL-dnIκBα-IRES-EGFP transgenic mice

FM131 [TgN(act-EGFP)OsbC14-Y01-FM131] green transgenic mice (kindly provided by Masaru Okabe, Osaka University, Japan) were used for isolation of genomic DNA which was used as a positive control for RT-PCR analyses of TgNFL-dnIκBα mice. For the generation of TgNFL-dnIκBα mice, we used a mutant murine IκBα in which phosphorylation sites Ser 32, Ser 36, Ser 288, Ser 293 (important for the constitutive phosphorylation and rapid degradation of IκBα) Thr 296, Ser 298 and Thr 301 were converted to Ala (Schwarz et al., 1996) (kindly provided by Edward M. Schwarz, University of Rochester Medical Center, NY, USA). The dnIκBα was cloned downstream of a 1.7 kb 5′ flanking sequence of the murine neurofilament gene (NFL) that confers neuronal specificity. EGFP fused to the internal ribosome entry site (pIRES2-EGFP) (Clontech, UK) was cloned downstream of dnIκB to facilitate screening of the transgenic mice. The two independent transgenic lines produced, Tg4472 and Tg4617, showed CNS-specific expression of the dnIκBα-IRES-EGFP transgene. The Tg4472 line, which showed higher expression of the transgene, was used for this study. Mice were backcrossed for at least 12 generations into the C57BL/6 background and were bred under specific-pathogen free conditions in the experimental animal unit of Hellenic Pasteur Institute.

Total RNA was extracted with TRIzol (InVitrogen, UK) according to the manufacturer's instructions. DNase-treated (Promega, UK) RNA was reverse transcribed with M-MLV Reverse Transcriptase (Promega, UK) and random hexamers (Roche, Germany). The NFL-dnIκB-IRES-EGFP transgene was amplified using the following primers on the EGFP sequence (forward: 5′-TGA ACC GCA TCG AGC TGA AGG C-3′ and reverse: 5′- TCC AGC AGG ACC ATG TGA TCG C-3′). Genomic DNA from FM131 transgenic green mice expressing EGFP cDNA was used as positive control for transgene expression. Mouse β-actin was amplified using primers (forward: 5′- CAT CAC TAT TGG CAA CGA GC-3′ and reverse: 5′- ACG CAG CTC AGT AAC AGT CC- 3′) as a loading control. Analysis of expression of the dnIκBα-IRES-EGFP transgene was performed by RT-PCR of total RNA from tissues isolated from transgenic mice. Transgene expression was detected in brain and spinal cord, but not other tissues, isolated from adult progeny of the founder mice indicating specificity for the CNS (Fig. 1).

2.3. mRNA analysis

A group of OF1 mice (n = 20) treated with bortezomib at 1 mg/kg subcutaneously twice per week and a group of untreated mice (n = 20) were used to analyze the mRNA expression of cytokines during the treatment. Four animals per group were sacrificed by decapitation after deep anesthesia at 1, 2, 4, 6 and 8 weeks of treatment. DRG, spinal cord and sciatic nerves were rapidly dissected, maintained in RNA-later solution (Qiagen, Barcelona, Spain) and processed for mRNA analyses. Total RNA was extracted using RNeasy Mini kit (Qiagen) including a DNase step (RNase free DNase set, Qiagen). One microgram of RNA per sample was reverse-transcribed using 10 μmol/L DTT, 200 U M-MuLV reverse transcriptase (New England BioLabs, Barcelona, Spain), 10 U RNase Out Ribonuclease Inhibitor (Invitrogen) and l μmol/L oligo (dT), 1 μmol/L of random hexamers (BioLabs, Beverly, MA, USA). The reverse transcription cycle conditions were 25 °C for 10 min, 42 °C for 1 h and 72 °C for 10 min. We analyzed the mRNA expression of the inhibitor of nuclear factor kappa B alpha (IκBα). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used to normalize the expression levels.

Gene-specific mRNA analyses were performed by SYBR-green real-time PCR using the MyiQ5 real-time PCR detection system (BioRad Laboratories, Barcelona, Spain). We previously fixed the optimal concentration of the cDNA to be used as template for each gene analysis to obtain reliable CT (threshold cycle) values for quantification. Realtime PCR amplification reactions contained the same amount of RT product, 10 μl of 2X QuantiMix EASY SYG KIT (Biotools, Madrid, Spain), 300 nM of forward and reverse primers, and completed with nanopure water to obtain a final volume of 20 μl. The thermal cycling conditions comprised 3 min polymerase activation at 95 °C, 40 cycles of 10 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension, followed by a DNA melting curve for the determination of amplicon specificity. All experiments were performed in duplicate. CT values were obtained and analyzed with the BioRad Software. Fold change in gene expression was estimated using the CT comparative method ($2^{-\Delta\Delta T}$) normalizing to GAPDH CT values and relative to control samples. The fold-increase expression of samples during bortezomib treatment with respect to basal samples was calculated for each transcript, and was based on real-time PCR threshold values. The mean value was obtained from three pools of each tissue per condition in each group.

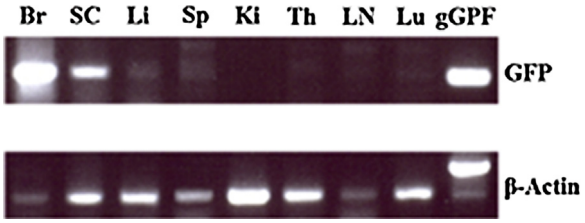

Fig. 1. TgNFL-dnIκB mice show CNS-specific expression of the dnIκBα-IRES-EGFP transgene. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of RNA from tissues isolated from transgenic mice using primers for GFP showed expression in brain (Br) and spinal cord (SC), but not in liver (Li), spleen (Sp), kidney (Ki), thymus (Th), lymph node (LN) or lung (Lu) (upper panel). Genomic DNA from FM131 transgenic green mice expressing GFP cDNA is shown as positive control (last lane). RT-PCR for β-actin shows the quality and quantity of mRNA in each sample. Note that the β-actin PCR product from genomic DNA isolated from FM131 transgenic mice (last lane) is larger than the product from cDNAs derived from RNA isolated from TgNFL-dnIκB tissues.