Cellular Signalling 26 (2014) 2903–2911

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

TGF-β/NF1/Smad4-mediated suppression of ANT2 contributes to oxidative stress in cellular senescence

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ARTICLE INFO

Article history:
Received 17 July 2014
Accepted 15 August 2014
Available online 16 September 2014

Keywords:
Smad
Nuclear factor 1
Senescence
Adenine nucleotide translocase-2
Transforming growth factor-β
Oxidative stress

ABSTRACT

Oxidative stress and persistent activation of DNA damage response (DDR) are causally involved in the development of cellular senescence, a phenomenon implicated in fundamental (patho)physiological processes such as aging, fetal development and tumorigenesis. Here, we report that adenine nucleotide translocase-2 (ANT2) is consistently down-regulated in all three major forms of cellular senescence: replicative, oncogene-induced and drug-induced, in both normal and cancerous human cells. We previously reported formation of novel NF1/Smad transcription repressor complexes in growth-arrested fibroblasts. Here we show that such complexes form in senescent cells. Mechanistically, binding of the NF1/Smad complexes to the NF1-dependent repressor elements in the ANT2 gene promoter repressed ANT2 expression. Etoposide-induced formation of these complexes and repression of ANT2 were relatively late events co-incident with production and secretion of, and dependent on, TGF-β. siRNA-mediated knock-down of ANT2 in proliferating cells resulted in increased levels of reactive oxygen species (ROS) and activation of the DDR. Knock-down of ANT2, together with etoposide treatment, further intensified ROS production and DNA damage signaling, leading to enhanced apoptosis. Together, our data show that TGF-β-mediated suppression of ANT2 through NF1/Smad4 complexes contributes to oxidative stress and DNA damage during induction of cellular senescence.

1. Introduction

Smad proteins play a central role in regulating cell growth and differentiation in a wide variety of cell types that respond to the transforming growth factor-β (TGF-β) family of cytokines [1–3]. Several signaling pathways have been described for the TGF-β family, most of which trigger Smad binding to target genes and, subsequently, either activation or repression of such genes [4,5]. However, Smads bind weakly to DNA [6,7], and efficient targeting to responsive gene elements usually requires the aid of some additional DNA-binding transcription cofactor(s). Several such factors, and their roles in growth regulation, have been described [8,9]. We recently reported a new Smad transcription partner, nuclear factor 1 (NF1) [10]. The NF1 family consists of 4 genes (*nf1-a*, *nf1-b*, *nf1-c and nf1-x* [11]) that are widely expressed in mammalian cells [11] and play seminal roles in regulating cell growth [12–14] and differentiation [15–22]. In our studies, we observed induction of nuclear complexes of NF1/Smad4 in several cell types that undergo TGF-β-initiated growth-arrest [23], serum deprivation [10] or contact inhibition [10]. The NF1/Smad complexes formed in serum-deprived cells are dissociated upon serum-readdition, suggesting that, in this case, the process is reversible [24]. We have also demonstrated a direct physical interaction between Smad and NF1 proteins [24].

The function of the NF1/Smad complexes in growth arrested cells is not understood at present, though we have demonstrated that they play a central role in repression of one gene, the human adenine nucleotide translocase-2 (ANT2) [24]. ANT2 is widely expressed in tumor cells [25–27] and rapidly dividing nontransformed cells [4,5], and is responsible for exchanging cytosolic ADP for mitochondrial

Abbreviations: ANT, adenine nucleotide translocase; Go-1/Go-2, Go NF1-binding repressor elements 1 and 2; TGF-β RI, transforming growth factor-β type I receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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http://dx.doi.org/10.1016/j.cellsig.2014.08.029
0898-6568/

ATP, thus contributing to energy homeostasis of the cell. The human ANT2 promoter contains tandem NF1 binding sites [10] to which NF1/Smad4/Smad3 bind and repress ANT2 expression in all growth-arrested cells studied to date. However, the complex disassembles in growth-reactivated cells, and ANT2 expression is restored [24]. While the above studies show clearly that the NF1/Smad complexes repress transcription in growth-arrested fibroblasts, their general significance in initiation or maintenance of the growth-inhibited state, if any, remains to be documented.

Inspired by the above findings on ANT2 repression in growth-arrested cells and the formation of a novel type of NF1/Smad complexes implicated in ANT2 down-regulation [23,24], we wished to explore the possibility that such mechanism might be involved in induction and/or maintenance of cellular senescence. If proven, such evidence would implicate the NF1/Smad-ANT2 axis in biological processes that feature senescence, such as normal or premature aging, some aspects of fetal development, or pathological scenarios including tumorigenesis where cellular senescence represents one of the key intrinsic anti-cancer barriers (for a review, see e.g. [28]). Furthermore, given that cellular senescence is commonly fuelled by oxidative stress and DNA damage, we also examined potential links between the status of the emerging TGF-β–NF1/Smad–ANT2 pathway on one hand, and ROS and DNA damage signaling on the other. The results of these mechanistic analyses in human cells induced to undergo senescence are presented below, and discussed together with their conceptual implications.

2. Materials and methods

2.1. Cell culture

Human osteosarcoma U2OS, mammary carcinoma MCF-7 and human normal fibroblasts (BJ) at population doublings 30–35 (young) and 80 (senescent) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U of penicillin and 50 μg/ml streptomycin. TGF-β, 5 ng/ml, was added and cells were grown for 24 to 144 h. For serum starvation, cells were washed twice with PBS, serum-free media was added, and the incubation continued for 48 h. ANT2-Luc reporter plasmids with the wild type Go-2/Go-1 elements or the mutated Go elements used in stable transfection experiments (U2OS) were prepared as described [29]. Stable transfections were performed by transfection with GeneCellin (BioCellChallenge) according to the manufacturer's protocol. Resistant colonies (30–50) for each of the luciferase constructs were pooled and grown in the presence of Geneticin (0.4 mg/ml). Luciferase activity measurements were performed as described [29]. Protein concentration was measured by Bio-Rad Protein Assay (Bio-Rad).

2.2. Antibodies and reagents

Antiserum against NF1 (#8199) was kindly provided by Dr. Tanese, human anti-Smad4 and p21 from SantaCruz Biotechnology (sc-7154), GAPDH from GeneTex (Irvine, CA, USA), phosphothreonine 68 of Chk2, phosphoserine 465/467 of SMAD2 and cleaved form of PARP from Cell Signaling Technology (Danvers, MA, USA), and phosphoserine 139 of histone H2AX from Millipore (Billerica, MA, USA) were used for immunodetection. Etoposide and the inhibitor of TGF-β RI (SB431542) were from Sigma-Aldrich (St. Louis, MO, USA). SB431542 (10 μM) was added 30 min prior to the treatment with etoposide.

2.3. Preparation of nuclear extract

Cells were harvested, nuclei were prepared [30] and nuclear proteins were extracted as described in Ref. [10]. The protein was measured using the Bio-Rad Protein Assay (Bio-Rad). Nuclear extracts were stored at −70 °C.

2.4. Immunoprecipitation and Western blot analysis

Antibodies against NF1 were covalently linked to Protein A-Sepharose using the Seize X Protein Immunoprecipitation Kit (Pierce). Immunoprecipitation of NF1 was performed according to the manufacturer's manual. Eluted protein complexes were precipitated in the presence of 10% trichloroacetic acid, followed by two washes with ice-cold acetone. Samples were air-dried, dissolved in sample buffer and separated by 10% SDS-PAGE. After electrophoresis, samples were electroblotted onto the Hybond ECL membrane (Amersham Biosciences). Membranes were incubated with appropriate antibodies and developed with SuperSignal West Pico Chemiluminiscent Substrate (Pierce).

2.5. Determination of TGF-β1 in cultivation media

The conditioned medium from cells was collected 24 h after the fresh medium with treatment was changed and the numbers of cells per each dish were counted. The concentration of cytokines were estimated by 'FACS bead array' using FlowCytomix Human Simplex Kit (TGF-β1, BMS8249FF; Bender MedSystems, Wien, Austria) on flow cytometer LSRII (BD Biosciences, San Jose, USA) according to manufacturer's protocol.

2.6. RNA purification and RT PCR

RNA was isolated using the acidic guanidinium thioisocyanate/phenol/chloroform extraction [31]. RNA (1 μg) was reverse transcribed to generate the first strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Germany) following the manufacturer's instructions. cDNA (1/25) was used as template in PCR (25 μl) using primers for the ANT2 and *GAPDH* coding region. PCR reaction was performed for 28 cycles with 1 min of denaturation at 94 °C, 30 s of annealing at 60 °C and extension for 1 min at 72 °C. PCR products were separated on 2% agarose and stained with ethidium bromide. Quantitative real time PCR was performed with the Time PCR Detection system (Bio-Rad Laboratories, Inc., USA) and the Maxima SYBR Green qPCR Master Mix (2x) (Fermentas, Germany). cDNA (1/25) was used as the template in 20 μl PCR reaction mix. The PCR protocol applied consisted of 10 min 95 °C initial denaturation, followed by 39 repeats of 30 s 95 °C denaturation, 30 s 58 °C (NF1) or 60 °C (ANT2), annealing, 30 s 72 °C extension and 5 s 76 °C and 80 °C plate read. A gene expression analysis was calculated by Bio-Rad Software Manager, Version 1.5, provided by the manufacturer (Bio-Rad Laboratories, Inc., USA).

Primers used:

ANT2: forward: 5′GGGTCAAGCTGCTGCTGCAGG-3′
reverse: 5′-CGGAATTCCCTTTCAGCTCCAGC-3′
GAPDH: forward: 5′-GCCAAAAGGGTCATCATCTC-3′
reverse: 5′CTAAGCAGTTGGTGGTGCAG-3′
NF1-A: forward: 5′-GGAGGTCTTTACCCAGCACA-3′
reverse: 5′-TGACTGACTGCCACTTCCTG-3′
NF1-B: forward: 5′-TATCCAGAACGCCCATAACC-3′
reverse: 5′-TGCTTGGTGGAGAAGACAGA-3′
NF1-C: forward: 5′-TGGCGGCGATTACTACACTT-3′
reverse: 5′-GGGCTGTTGAATGGTGACTT-3′
NF1-X: forward: 5′-AGTTTGTGTGCTCGGATGG-3′
reverse: 5′-TGGAATCAGGCATAGGAAGG-3′
TGF-β1: forward: 5′-GCAGGGATAACACACTGCAA-3′
reverse: 5′-GCCATGAGAAGCAGGAAAGG-3′
Actin: forward: 5′-CCAACCGCGAGAAGATGA-3′
reverse: 5′-CCAGAGGCGTACAGGGATAG-3′