Acta Histochemica 117 (2015) 688–695

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Localization and distribution of neurons that co-express xeroderma pigmentosum-A and epidermal growth factor receptor within Rosenthal's canal

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ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form 6 October 2015
Accepted 10 October 2015

Keywords:
Stereology
Double-immunohistochemistry
DNA repair
Xeroderma pigmentosum

ABSTRACT

Xeroderma pigmentosum-A (XPA) is a C_4-type zinc-finger scaffolding protein that regulates the removal of bulky-helix distorting DNA damage products from the genome. Phosphorylation of serine residues within the XPA protein is associated with improved protection of genomic DNA and cell death resistance. Therefore, kinase signaling is one important mechanism for regulating the protective function of XPA. Previous experiments have shown that spiral ganglion neurons (SGNs) may mobilize XPA as a general stress response to chemical and physical ototoxicants. Therapeutic optimization of XPA via kinase signaling could serve as a means to improve DNA repair capacity within neurons following injury. The kinase signaling activity of the epidermal growth factor receptor (EGFR) has been shown in tumor cell lines to increase the repair of DNA damage products that are primarily repaired by XPA. Such observations suggest that EGFR may regulate the protective function of XPA. However, it is not known whether SGNs in particular or neurons in general could co-express XPA and EGFR. In the current study gene and protein expression of XPA and EGFR were determined from cochlear homogenates. Immunofluorescence assays were then employed to localize neurons expressing both EGFR and XPA within the ganglion. This work was then confirmed with double-immunohistochemistry. Rosenthal's canal served as the reference space in these experiments and design-based stereology was employed in first-order stereology quantification of immunoreactive neurons. The results confirmed that a population of SGNs that constitutively express XPA may also express the EGFR. These results provide the basis for future experiments designed to therapeutically manipulate the EGFR in order to regulate XPA activity and restore gene function in neurons following DNA damage.

1. Introduction

Nucleotide excision repair (NER) is a highly conserved and vital process that maintains the integrity of genomic DNA. Two genetically distinct NER pathways provide protection for transcriptionally active and inactive genes and thus safeguard the entire genome (Melis et al., 2013). NER proteins have been implicated in the repair of a large variety of structurally and chemically distinct types of DNA lesions (Brooks, 2007; Riedl et al., 2003; Thoma and Vasquez, 2003; Shell et al., 2013; Sakurai et al., 2015). In fact NER proteins have been shown to play significant roles in all the major mammalian DNA repair pathways (Melis et al., 2013; Sakurai et al., 2015; Zhang et al., 2009). There are over 30 different NER proteins and many of these proteins have cellular functions beyond repairing damaged genes. One critical protein, considered the rate-limiting factor for all NER pathways is a C_4-type zinc-finger scaffolding protein called xeroderma pigmentosum-A (XPA) (Köberle et al., 2006; Kang et al., 2011). This particular NER protein seems to be vital to neurons. For instance, the high metabolic activity of some neurons precipitate specific types of bulky-helix distorting DNA damage products that necessarily requires the repair function of XPA (Kuraoka et al., 2000; Brooks, 2007). Furthermore, human mutations in the XPA gene results in neurodegeneration (Blair et al., 1997; Rapin et al., 2000; Robbins et al., 1991). Therefore, under normal conditions neurons with high metabolic activity may constitutively express XPA as well as other types of cellular defenses.

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http://dx.doi.org/10.1016/j.acthis.2015.10.001
0065-1281/

Spiral ganglion neurons (SGNs) of Rosenthal's canal (Rc) exhibit high metabolic activity and it is known from human temporal bones that *xpa* gene mutations can result in what seems to be primary neurodegeneration in cochlear regions with apparently normal hair cells (Tylstedt et al., 1997; Viana et al., 2013; Guthrie and Xu, 2012; Banfi et al., 2004). A series of experiments have demonstrated that under normal conditions SGNs constitutively express XPA (Guthrie and Carrero-Martinez, 2010; Guthrie et al., 2008; Guthrie and Xu, 2012). Interestingly, SGNs appear to mobilize XPA as a general response to ototoxic stress from noise and cisplatin exposures (Guthrie et al., 2008; Guthrie, 2012; Guthrie and Xu, 2012). This is consistent with in vitro cell culture research that have shown that the optimization of a cells endogenous XPA response can increase the capacity of the cell to better protect its genome and thus resist cell death (Wu et al., 2006b; Shell et al., 2009). Therefore, it is conceivable that optimization of endogenous XPA functions within SGNs could serve as a future therapeutic approach against ototoxic stress. However, this prospect is limited by the lack of information on what cell signaling mechanisms may regulate XPA activity within neurons.

Kinase signaling that result in the phosphorylation of ser196 within the XPA protein is associated with increased DNA repair activity and resistance to cell death (Wu et al., 2006b; Nguyen et al., 2010). In vitro studies on cancer cell lines have also shown that kinase signaling mediated by the epidermal growth factor receptor (EGFR) is able to increase the capacity of cells to repair DNA damage products that can only be repaired via XPA (Hsu et al., 2009; Friedmann et al., 2004). Other studies have shown that EGFR signaling is able to increase the level of expression of the XPA interacting protein, excision repair cross-complementation group-1 (ERCC1) (Yacoub et al., 2001, 2003; Gandara et al., 2010). These combined observations are encouraging and suggest that manipulation of the EGFR might be a way to optimize XPA function among SGNs. This is particularly relevant because a previous study has revealed that EGFR expression is specifically localized to SGNs of the mature cochlea (Zine et al., 2000). However, no previous study has revealed whether or not SGNs or neurons in general could co-express EGFR and XPA. If SGNs co-express EGFR and XPA, then this would provide the basis for future studies to explore whether therapeutic manipulation of EGFR could optimize the protective function of endogenous XPA. The current study test the hypothesis that SGNs that are XPA positive will also express the EGFR.

2. Materials and methods

2.1. Animals

Twelve hooded male Long–Evans rats (250–300 g) served as subjects and were acquired from Harlan Laboratories, Inc. (Livermore, CA, USA). On arriving at the rat vivarium the animals were given one week to acclimatize to their new environment. They were housed in pairs in environmentally enriched cages (21° ± 1° C; 12-h light/dark cycle). For all procedures, the animals were anesthetized with ketamine/xylazine (87/13 mg/kg, im). Two types of euthanasia were conducted while the animals were anesthetized. In one type, the animals were decapitated in order to harvest the cochlea for reverse-transcription polymerase chain reaction and Western blots. In another type, the animals received intravascular perfusion with phosphate-buffered saline (PBS: 10 mM, pH 7.4) followed by periodate–lysine–paraformaldehyde fixative for immunolabeling (Guthrie, 2008). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Loma Linda VA Medical Center. The IACUC approval process certifies that all protocols are in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Animal Welfare Act.

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

After dissecting the cochlea, RNA was extracted using Qiagen Allprep DNA/RNA/Protein kit (Qiagen Inc.,Valencia, CA, USA) according to the protocol provided by the manufacturer. First-strand cDNA was then synthesized using random hexamers or oligo(dT) and the SuperScript® III First-Strand Synthesis System (Thermo Fisher Scientific Inc, Grand Island, NY, USA). Polymerase chain reaction (PCR) was then performed to detect the presence of EGFR and XPA in the cochlear samples. The annealing primers have been reported previously (Guthrie et al., 2008; Zine et al., 2000). Annealing temperature of 54 °C was employed and strands were amplified 40 cycles then run on 2% agarose gel. A 100 bp DNA ladder (Invitrogen) was used to identify the amplified bands.

2.3. Western blot

Cochlear tissues were extracted and homogenated in RIPA buffer (Sigma–Aldrich, St. Louis, MO, USA) with protease inhibitor cocktail at 4 °C for 10 min. The cochlear tissue homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C. Protein concentration in the supernatant was determined by the Bradford protein assay (Biorad, Hercules CA, USA). Approximately 30 and 50 μg of the supernatant was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated in a blocking buffer containing 5% nonfat dry milk in TBS for 1 h and then incubated with either EGFR (sc-31155) goat polyclonal IgG diluted 1:200 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or XPA (sc-853) rabbit polyclonal IgG diluted 1:200 (Santa Cruz) in TBS-T buffer containing 5% milk at 4 °C overnight. The membranes were washed three times with TBS-T and then incubated with donkey or goat anti-goat or anti-rabbit IgG (peroxidase conjugated, 1:200, Santa Cruz) in TBS-T buffer containing 5% milk for 1 h at room temperature. The membranes were washed again with TBS-T and visualized with the ECL western blotting detection reagent (Santa Cruz). Immunobloting for actin served as a loading control.

2.4. Immunofluorescence and immunohistochemistry

Cochlear sections were de-paraffinized in a grades series of xylene and alcohol, and then hydrated. Endogenous peroxidase activity was blocked with 0.9% H_2O_2 for 10 min at 22 °C. A high heat (90–98 °C) low pH (0.80–3.06) sodium citrate–citric acid antigen retrieval techniques was them employed (Guthrie, 2008). Pre-treatment with a blocking solution of normal goat or rabbit serum (Sigma, St. Louis, MO, USA) was performed in order to block non-specific binding. The primary antibodies were diluted in the blocking solution at a 1:100 concentration. The primary antibodies are commercially available and included goat anti-EGFR (sc-31155) and rabbit anti-XPA (sc-853) (Santa Cruz). We and others have previously characterized the specificity of these antibodies in pre-absorption experiments, immunolabeling and Western blots (Guthrie et al., 2008; Lai et al., 2009). For immunofluorescent staining the slides were treated with both the goat-EGFR antibody and the rabbit-XPA antibody for 48 h at 0 °C. They were then rinsed in PBS and incubated overnight with anti-goat IgG conjugated with Texas Red® fluorophore and anti-rabbit IgG conjugated with fluorescein isothiocyanate fluorophore (1:200 concentration). For immunohistochemistry the slides were incubated with only one primary antibody (either goat-EGFR or rabbit-XPA) for 48 h at 0 °C. The slides were then treated with either biotinylated anti-goat