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Research paper

HEI-OC1 cells as a model for investigating drug cytotoxicity

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ABSTRACT

The House Ear Institute-Organ of Corti 1 (HEI-OC1) is one of the few, and arguable the most used, mouse auditory cell line available for research purposes. Originally proposed as an in vitro system for screening of ototoxic drugs, it has been used to investigate, among other topics, apoptotic pathways, autophagy and senescence, mechanism of cell protection, inflammatory responses, cell differentiation, effects of hypoxia, oxidative and endoplasmic reticulum stress, and expression of molecular channels and receptors. However, the use of different techniques with different goals resulted in apparent contradictions on the actual response of these cells to some specific treatments. We have now performed studies to characterize the actual response of HEI-OC1 cells to a battery of commonly used pharmacological drugs. We evaluated cell toxicity, apoptosis, viability, proliferation, senescence and autophagy in response to APAP (acetaminophen), cisplatin, dexamethasone, gentamicin, penicillin, neomycin, streptomycin, and tobramycin, at five different doses and two time-points (24 and 48 h), by flow cytometry techniques and caspase 3/7, MTT, Cytotoxicity, BrdU, Beclin1, LC3 and SA-β-galactosidase assays. We also used HEK-293 and HeLa cells to compare some of the responses of these cells to those of HEI-OC1. Our results indicate that every cell line responds to the each drug in a different way, with HEI-OC1 cells showing a distinctive sensitivity to at least one of the mechanisms under study. Altogether, our results suggest that the HEI-OC1 might be a useful model to investigate biological responses associated with auditory cells, including auditory sensory cells, but a careful approach would be necessary at the time of evaluating drug effects.

1. Introduction

House Ear Institute-Organ of Corti 1 (HEI-OC1) cells derive from the auditory organ of the transgenic mouse Immortomouse™, which harbors a temperature-sensitive mutant of the SV40 large T antigen gene under the control of an interferon-γ-inducible promoter element (Jat et al., 1991; Kalinec et al., 2003). For expression of the immortalizing gene, Immortomouse™-derived cells must be cultured at 33 °C (permissive conditions), resulting in de-differentiation of the cells and accelerated proliferation; moving the cells to 39 °C (non-permissive conditions) results in denaturation of the protein encoded by the gene, leading to decreased proliferation, cell differentiation and, at least in the case of HEI-OC1, cell death (Devarajan et al., 2002; Kalinec et al., 2003).

HEI-OC1 cells were cloned and characterized in our laboratory over a decade ago (Devarajan et al., 2002; Kalinec et al., 2003). Initial studies indicated that HEI-OC1 expressed specific markers of cochlear hair cells, such as prestin, myosin 7a, Atoh1, BDNF, calbindin and calmodulin, but also markers of supporting cells like connexin 26 and fibroblast growth factor receptor (FGF-R) (Kalinec et al., 2003). Therefore, it was suggested that HEI-OC1 could represent a common progenitor for sensory and supporting cells of the organ of Corti (Kalinec et al., 2003). Parallel studies provided strong evidence that archetypal ototoxic drugs like cisplatin, gentamicin and streptomycin induced caspase-3 activation in these cells, while drugs considered non-ototoxic, like penicillin, did not. Therefore, the cell line was proposed as in vitro system to investigate the cellular and molecular mechanisms involved in ototoxicity and for screening of the potential ototoxicity or otoprotective properties of new pharmacological drugs. Since cell death induced by incubating the cells at non-permissive conditions is a potential confounding factor in the evaluation of the mechanism/s of drug-induced cell death, this type of study is usually performed with HEI-OC1 cells grown at permissive conditions (33 °C). It is

Abbreviations: HEI-OC1, House Ear Institute-Organ of Corti 1; APAP, N-acetyl-para-aminophenol/acetaminophen; BrdU, 5-Bromo-2′-deoxyuridine

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estimated that HEI-OC1 cells have been used in more than one hundred and fifty studies published in the last ten years.

Whereas important cell processes like autophagy and senescence have just started to be investigated in HEI-OC1 cells (e.g., (Chen et al., 2012; Hayashi et al., 2015; Tsuchihashi et al., 2015; Youn et al., 2015)), looking at the potential pro-apoptotic effect of different drugs was the major goal of most of the studies involving this cell line. Interestingly, even though we included in the original publication the statement *"Although caspase-3 activation is a signal associated with earlier stages of apoptosis, we cannot equate it with apoptotic cell death"* (Kalinec et al., 2003), we did not perform additional experiments to demonstrate actual cell death because, at the time of the first studies with these cells, activation of the "executioner" caspase-3 was considered a definitive signal of irreversible apoptotic cell death. Thus, the finding that some drugs that indeed activated caspase-3 in HEI-OC1 cells were actually not killing them (Cederroth, 2012; Chen et al., 2012) was a surprise for us as well as many other researchers. Since those drugs were known to kill cochlear outer hair cells (OHCs) *in vivo* and *in situ*, this result shed doubts about the usefulness of this cell line as an in vitro system for the screening of the potential ototoxicity of pharmacological drugs. It is worth mentioning, however, that the idea that every ototoxic drug kills OHCs "per se", that is, in absence of any factor/s and/or conditions produced by other cochlear cell populations, has never been proved.

The purpose of this study is to provide a comprehensive set of data about cell death, survival, proliferation, senescence and autophagy induced by different clinically used drugs in HEI-OC1 cells, looking for a better understanding of the response of these cells to cytotoxic and presumably non-cytotoxic agents. We selected eight pharmacological drugs frequently used in the clinic, some of them considered ototoxic and others safe for the auditory organ, but all of them already investigated for us or other laboratories in HEI-OC1 cells. Our results indicate that HEI-OC1 cells respond to the each drug in a different way, with a distinctive dose- and time-dependent sensitivity to at least one of the mechanisms under study.

2. Materials and methods

2.1. Cell culture

HEI-OC1 cells were cultured under permissive conditions (33 °C, 10% CO_2) in high-glucose Dulbecco's Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS; Gibco BRL) without antibiotics as described in the literature (Kalinec et al., 2003). HeLa and HEK-293 cells were obtained from ATCC (Manassas, VA), and cultured at 37 °C, 5% CO_2 following standard protocols.

2.2. Viability (MTT assay)

HEI-OC1, HeLa and HEK-293 cells were collected, counted using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA), and the concentration adjusted to 2.0×10^5 cells/mL. The cells were then seeded on 96-well flat bottom plates (100 μL per well), incubated overnight for attachment to the substrate, and then treated with eight different pharmacological drugs: N-acetyl-para-aminophenol (*aka* APAP or acetaminophen), cisplatin, dexamethasone, gentamicin, neomycin, penicillin, streptomycin, or tobramycin (all from Sigma–Aldrich, St. Louis, MO) at the concentrations listed in Table 1. After either 24 or 48 h of treatment at 33 °C, TACS® MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was performed following the manufacturer's protocol. Absorbance was measured with Spectra Max 5 Plate Reader with Soft Max Pro 5.2 Software (Molecular Devices, Sunnyvale, CA), and average OD in control cells was taken as 100% of viability.

2.3. Caspase assay

HEI-OC1 cells were collected and counted using Cellometer Auto T4. Concentration was adjusted to 2.0×10^5 cells/mL, and the cells plated on white 96-well clear bottom plates and treated as described before. At the time points, Caspase-Glo® 3/7 Assay (Promega, Madison, WI) was performed following the manufacturer's protocol. Luminescence was measured using Spectra Max 5 Plate Reader with Soft Max Pro 5.2 Software.

2.4. Flow cytometry

HEI-OC1, HeLa and HEK-293 cells were collected and counted using Cellometer Auto T4. Concentration of the cells was adjusted to 1.0×10^5 cells/well in 6-well plates, and incubated at 33 °C overnight. After overnight incubation, cells were treated with the drugs listed in Table 1, and incubated again at 33 °C for either 24 or 48 h. At the end of the treatment cells were washed with phosphate buffer saline (PBS, pH 7.4. Gibco-Life Technologies, Grand Island, NY), detached with a non-enzymatic cell dissociation solution (Sigma–Aldrich), and stained with Alexa 488 (Molecular Probes-Invitrogen, Eugene, OR) diluted 1:500 in PBS. Flow cytometry experiments were performed using a FACSAriaIII® instrument (BD Biosciences, Erembodegem, Belgium) with 488 nm excitation (blue laser) and FL1: 530 ± 15 nm. In all cases, 10,000 cells were analyzed per experiment by using FACS Diva software (BD Biosciences).

2.5. Cell toxicity

HEI-OC1, HeLa and HEK-293 cells were incubated in cell culture medium alone (negative control) or medium plus the drugs (Table 1). At the end of the treatment cells were washed three times with PBS, and detached using a non-enzymatic cell dissociation solution. After detaching, cells were spun down, the supernatant was removed, and the cells were stained for 15 min in the dark with Cell-Tox™ Green Cytotoxicity Assay (Promega, Madison, WI) at a concentration of 2 μL of 1X CellTox™ per mL of medium. The number of green positive cells was determined using flow cytometry as described above.

2.6. Cell division

HEI-OC1, HeLa and HEK-293 cells were collected, counted, seeded on 96-well clear flat bottom plates, and then treated with the pharmacological drugs as mentioned above. One hour after treatment, 1 μL of prepared 100X BrdU (5-Bromo-2′-deoxyuridine) solution was added to each well and plates were returned to the incubators. At time points, existing medium was removed; each well was washed once with PBS then the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) was used to perform proliferation studies by following the manufacturer's protocol with the exception that the STOP solution was added 10 min after incubation with the TMB Substrate. Absorbance was measured with Spectra Max 5 Plate Reader with Soft Max Pro 5.2 Software (Sunnyvale, CA).

2.7. Senescence

HEI-OC1 cells were incubated with medium plus drugs for each experimental condition (Table 1) or medium alone for untreated control condition. At the end of the treatment, the protocol for flow cytometry described in the literature (Debacq-Chainiaux et al., 2009) was followed. Briefly, at the end of the experimental