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The RPTEC/TERT1 cell line as an improved tool for in vitro nephrotoxicity assessments

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Abstract

In earlier studies, we have characterized a newly developed cell line derived from the renal proximal tubule epithelial cells (RPTEC) of a healthy human male donor in order to provide an improved *in vitro* model with which to investigate human diseases, such as cancer, that may be promoted by toxicant exposure. The RPTEC/TERT1 cell line has been immortalized using the human telomerase reverse transcriptase (hTERT) catalytic subunit and does not exhibit chromosomal abnormalities (Evercyte Laboratories). We have previously conducted single-compound and binary mixture experiments with the common environmental carcinogens, cadmium (Cd) and benzo[a]pyrene (B[a]P). Cells exhibited cytotoxic and compound-specific responses to low concentrations of B[a]P and Cd. We detected responses after exposure consistent with what is known regarding these cells in a normal, healthy kidney including significant gene expression changes, BPDE-DNA adducts in the presence of B[a]P, and indications of oxidative stress in the presence of Cd. The RPTEC/TERT1 cell line was also amenable to co-exposure studies due to its sensitivity and compound-specific properties. Here, we review our earlier work, compare our findings with commonly used renal cell lines, and suggest directions for future experiments. We conclude that the RPTEC/TERT1 cell line can provide a useful tool for future toxicological and mixture studies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Keywords

Cadmium; Benzo[a]pyrene; Mixtures toxicology; RPTEC/TERT1; DNA damage; Oxidative stress; Nephrotoxicity

Kidney and Renal Pelvis Cancer

Kidney and renal pelvis cancer is one of only five cancers with an increasing incidence of 3–4% or more per year over the last several decades [1]. Additionally, approximately 90% of kidney cancer diagnoses are a classified as specific type of kidney cancer, Renal Cell Carcinoma (RCC) [1, 2]. Risk factors for RCC are largely environmental and include obesity, cigarette smoking, and hypertension. Only 2% of RCC cases can be directly attributed to genetic factors such as von-Hippel Lindau (VHL) disease in which patients exhibit specific mutations in the *VHL* gene [3–5]. However, the majority of RCC cases are found in patients with no familial history. Thus, environmental risk factors play a significant role in the development of RCC.

RCC originates in the renal proximal tubule epithelial cells of the kidney. These cells perform vital tasks in the body such as reabsorption of essential nutrients including glucose, small amino acids, sodium, and water from filtrate, as well as aid in pH regulation of the body. However, because the renal system primarily filters and concentrates many substances, the kidney is potentially exposed to a variety of DNA damaging agents. In particular, the renal proximal tubule epithelial cells are sensitive to DNA damage because they are the first cells to receive the filtrate after the glomerulus [6, 7]. Additionally, the high metabolic capacity and detoxification properties of renal proximal tubule epithelial cells in comparison to other renal cell types increases their probability of exposure to xenobiotics and xenobiotic metabolites [8].

Environmental Contamination and Exposure to Renal Genotoxicants

Two environmental toxicants strongly implicated in the development of RCC are the heavy metal, cadmium (Cd), and the polycyclic aromatic hydrocarbon, benzo[a]pyrene (B[a]P). Each of these toxicants works through distinctly different mechanisms to cause DNA damage in the kidney.

Cadmium

Cd is a divalent heavy metal, a hazardous environmental contaminant, a group I carcinogen, and a known renal toxicant [1, 9]. Because Cd is neither metabolized nor rapidly excreted by the body, it bioaccumulates in target organs with a half-life of 10–30 years. The kidney, a primary target organ of Cd toxicity, filters and reabsorbs Cd in the proximal tubules as a complex bound to the metallothionein (MT-Cd) protein. Here, it increases in concentration over time until the cellular burden is exceeded and Cd is released due to progressive renal cell destruction and injury [1, 10, 11]. An increased body burden of Cd correlates with an elevated risk of developing RCC. Accordingly, kidney tissue sections of renal cancer patients show greater levels of Cd than those from patients who died of other causes [2, 11, 12].

Currently, Cd is ranked 7th out of 275 priority hazardous substances assessed by the Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency (EPA) because of its toxicity and potential for human exposure [3–5, 13]. Dietary intake constitutes the majority of Cd exposure in non-smokers with approximately 30 μg consumed daily, and smokers have approximately twice the body burden of Cd [6, 7, 14–16]. Although acute Cd exposure is not known to be responsible for carcinogenic effects, chronic low dose Cd exposure is suspected to act through multiple mechanisms to initiate and promote cancer development at several sites in both humans and animals. This includes cancers of the urinary bladder, breast, kidney, lung, pancreas, and prostate [8, 16–25].

Primarily, because of its structural similarity to the essential element zinc (Zn), Cd can substitute for Zn with up to a 1,000-fold higher binding constant for some proteins such as Xeroderma Pigmentosum A (XPA), a protein responsible for recognizing structure-distorting DNA damage in the Nucleotide Excision Repair (NER) process [24]. Once substitution occurs, Cd stabilizes these proteins through thiol binding of cysteine residues resulting in decreased functionality and DNA binding ability [24, 26]. Because many DNA damage recognition and repair proteins rely on an active Zn-finger domain for proper DNA binding as well as recruitment of co-factors, Cd exposure may significantly and deleteriously affect genome maintenance and integrity.

In addition to impaired processing of genetic damage, low doses of Cd are known to promote cellular proliferation and inhibit apoptosis [19]. Cd influences cellular proliferation by increasing Reactive Oxygen Species (ROS) production. ROS increase cellular oxidative stress and act as second messengers to stimulate cell growth receptor mediated pathways [10, 27]. Studies conducted in primary rat proximal tubular cells have confirmed Cd's role in ROS production and an altered glutathione homeostasis at low concentrations of Cd exposure [28]. It is suspected that MAPK is activated in response to cellular stress, namely ROS, induced by Cd, which leads to aberrant cell growth signaling [14, 19, 27, 29]. In accordance with carcinogenic hallmarks, Cd inhibits apoptosis by decreasing functionality of the Zn-finger dependent apoptotic regulator protein, p53 [20].

The genetic insult due to Cd alone provides a simplistic explanation for tumorigenesis. However, in a biological system, these factors become part of a complex process. In addition to environmental Cd contamination, the human body encounters other exogenous carcinogens every day, both man-made and natural.

Benzo[a]pyrene

B[a]P is a representative compound of the polycyclic aromatic hydrocarbon (PAH) class and, like Cd, is a group I carcinogen [30]. B[a]P is found ubiquitously in the environment as a byproduct of incomplete combustion. Exposure routes include cigarette smoke, chargrilled meats, especially red meat, coal, wood fires, and automobile exhaust among many others [31]. Because of its toxicity and frequency of human exposure, B[a]P is currently ranked near Cd as number 8 out of 275 priority hazardous substances monitored by the ATSDR [32].

Previous studies have found that as B[a]P intake increased through consumption of chargrilled meat, the risk of RCC increased more than twofold [33, 34]. In humans, B[a]P is metabolized by the phase I metabolic enzymes Cytochrome P450s, CYP1A1 and CYP1B1, and microsomal epoxide hydrolase (EPHX1) to the ultimate carcinogen (+)-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) [35, 36]. This epoxide reacts with the N7 position of guanine and the N7 and N3 positions of adenine forming bulky DNA adducts [36]. Therefore, after biotransformation, B[a]P metabolites act directly as genotoxicants. Under normal cellular conditions, the majority of adducts formed after B[a]P exposure and metabolism are detected and repaired by the NER pathway. However, when DNA repair is functioning at a decreased capacity, mutagenic potential increases as unrepaired damage is more likely to become permanent in the genome [37].

Characterizing a New Tool for Exposure Science and Cancer Research

In order to provide the most appropriate information regarding human health and risk assessment, it is important to conduct experiments that are representative of biologically relevant conditions. Until now, the majority of human *in vitro* models have relied on cells which retain the ability to proliferate due to a tumorigenic phenotype or those which have been immortalized with a viral vector. Although these cell lines have provided tools for experimentation, their altered genotype does not truly represent healthy, normal cells within the body. Additionally, transformation of normal cells with viral vectors has been shown to increase genetic instability and alter DNA repair mechanisms [38]. These studies demonstrate the need for improved *in vitro* models that more accurately reflect mutagenic effects that xenobiotics may have on normal cells.

Recently, Evercyte Laboratories (Vienna, Austria) successfully isolated and immortalized renal proximal tubule epithelial cells (RPTEC) from a normal, healthy male donor [39]. By using only the catalytic subunit of the endogenous human telomerase reverse transcriptase (hTERT) enzyme for immortalization, telomere length is stabilized, and normal cells are prevented from senescing without viral transformation [40]. Evercyte Laboratories has characterized the functional properties of this hTERT immortalized renal cell line, RPTEC/TERT1. The cells retain normal renal proximal tubule functionality, characteristics, and genomic stability for over 90 population doublings. Although the RPTEC/TERT1 cell line has been immortalized and functionally well characterized, studies using these cells as a toxicological model for exposure science have been limited. Therefore, we aimed to develop a system that better models the mutagenic effects that occur in normal cells following exposure to DNA damaging agents. Our goal was to use this cell line to study an environmentally relevant chemical mixture exposure scenario, a binary mixture of B[a]P and Cd, and to evaluate cellular responses, DNA damage, and mutagenesis.

Discussion

In previous studies, we demonstrated the sensitivity of the RPTEC/TERT1 cell line to concentrations of B[a]P and Cd as low as 1 nM and 3 μM, respectively [41, 42]. Cells showed significant increases in the expression of genes encoding B[a]P metabolizing enzymes (*CYP1A1, CYP1B1*) after B[a]P exposure and heavy metal responsive genes (*MTIA*

and *MTIIA*) after Cd exposure. Increased gene expression and activity of CYP enzymes in response to B[a]P exposure indicates that these cells appear to have active Aryl Hydrocarbon Receptor (AhR) signaling. The detection of BPDE-DNA adducts after B[a]P exposure also strongly indicates involvement of the AhR bioactivation pathway. AhR is known to activate a large number of downstream effectors after ligand binding, many of which are associated with bioactivation of carcinogens [43].

Interestingly, in the presence of Cd and B[a]P as a binary mixture, BPDE-DNA adduct levels were lower at the highest concentrations studied, 1 μM Cd × 1 μM B[a]P, than adduct levels observed following treatment with 1 μM B[a]P alone. Corresponding activation of the NRF2-antioxidant response in 1 μM Cd × 1 μM B[a]P groups supports the hypothesis that the activation of compensatory or detoxification mechanisms led to either an increase in DNA damage repair or a reduction/detoxification of DNA damaging metabolites [44]. Specifically, increased expression of NRF2 targets, including the antioxidant responsive genes, glutamate-cysteine ligase, catalytic subunit (*GCLC*), heme oxygenase 1 (*HMOX1*), and NAD(P)H dehydrogenase, quinone 1 (*NQO1*) was detected in RPTEC/TERT1 cells after 24 hours. When total glutathione (GSH) was examined as an indicator of oxidative stress, all co-exposure conditions containing 1 μM Cd resulted in significantly increased levels of GSH in comparison to untreated controls [42].

Studies examining responses of healthy primary human kidney epithelial cells to Cd exposure and other nephrotoxicants demonstrate findings similar to our results with the RPTEC/TERT1 cell line [45, 46]. In healthy primary human kidney cells, Cucu *et al.* (2011) found that exposure to concentrations as low as 0.5 μM Cd significantly increased *MTIA* gene expression after 3 hours followed by a subsequent decline after 24 hours. Additionally, significant increases in *HMOX1* gene expression were demonstrated after exposure to 0.5 μM Cd indicating an oxidative stress response [45]. A study by Li *et al.* (2013) compared healthy primary human kidney cells to two commonly used virally immortalized kidney cell lines, Human Kidney 2 (HK-2) and Lewis Lung Cancer-Porcine Kidney 1 (LLC-PK1). Pro-inflammatory responses were measured after cells were exposed to ranges of nephrotoxic and non-nephrotoxic chemicals, drugs, and heavy metals. Results showed that primary human kidney cells more accurately predicted renal injury than the virally immortalized cell lines [46]. In our own studies, using HK-2 cells as a comparative model, we found low level constitutive gene expression of drug metabolizing enzymes, *CYP1A1* and *CYP1B1*, which were not induced after exposure to concentrations of B[a]P [41]. We hypothesize that viral transformation may have disrupted the expression of these and other genes. Other studies confirm a lack of canonical metallothionein expression in HK-2 cells in comparison to primary human proximal tubule cells [47].

Ideally, primary human renal cells would be used more frequently to assess human renal pathogenesis and toxicity; however, some challenges prevent such studies from becoming more common. Primary human cells are difficult to obtain directly from donors especially if the cells should be healthy and free of disease. Although there are commercially available primary human cells, the finite number of growth cycles of any primary cell presents a challenge for ongoing research and reproducibility. Lastly, the properties of the same cell type sampled from different individuals may vary greatly potentially reducing the reliability

of repeated experiments. The RPTEC/TERT1 cell line may provide an improved model and suitable alternative to the experimental and biological difficulties of virally transformed and primary renal cell lines.

Limitations and Future Directions

While the RPTEC/TERT1 cell line has been appropriate for the studies presented here, we acknowledge its intrinsic limitations. First, as with any *in vitro* cell line, the accuracy with which the cell line represents the tissue of origin is questionable. Although the RPTEC/TERT1 cell line was isolated from a healthy, normal donor and its renal properties were very well characterized by Wieser *et al* (2008), it would be desirable to also characterize TERT1 with respect to copy number, expression level, and insertion location in the genome. Future studies may focus on characterization of the cell line regarding TERT1 immortalization. This information would more fully describe the cell line and address possibilities of insertional mutagenesis. Second, based on the scope of our experiments, the RPTEC/TERT1 cell line functioned as we would expect renal proximal tubule cells in the body. However, it is important to note that the kidneys are comprised of many different cell types that work together to perform renal functions [8]. While RPTECs are important for studies pertaining to renal metabolism, biotransformation, pH balance, nutrient reabsorption, heavy metal uptake, and renal cancer, the totality of cells in the kidney may need to be taken into consideration when extrapolating to *in vivo* comparisons. Third, while we proposed to ultimately determine the mutagenic potential of adducts alone and those formed in the presence of Cd, the RPTEC/TERT1 cell line proved difficult to adapt to the well-established *HPRT1* gene mutation assay. Using this cell line for gene mutation analysis requires additional optimization. Because the cell line was isolated from a normal, healthy donor, it would be ideal to be able to use it as a model to study the genotoxic progression from toxicant exposure to gene mutation and potentially tumorigenesis. Thus, alternative methods and additional analyses are currently being explored to establish this cell line as a mutational model.

Additionally, potential studies could examine in greater detail the DNA damage detected due to BPDE-DNA adducts. We did not expect the reduction of adducts under co-exposure conditions with Cd. While we hypothesize that this may be due to a protective antioxidant and detoxification response due to the presence of both compounds, it would be suitable to confirm the mutagenicity of the adducts under B[a]P alone and in combination with Cd. Although there were fewer adducts detected at the highest concentration of B[a]P under co-exposure in comparison to B[a]P alone, those adducts that remained may have proportionally increased mutagenic potential resulting from Cd inhibition of DNA repair that could initiate carcinogenesis. Future experiments should therefore characterize Cd's role in affecting XPA's ability to repair BPDE-DNA adducts. Studies have shown Cd's ability to substitute for Zn in XPA thereby decreasing cellular DNA repair capacity [24, 26]. Testing DNA repair capacity and XPA's interaction with Cd would identify potential consequences that may promote carcinogenesis under these exposure scenarios. Likewise, defining the amounts of intracellular Cd after exposure could indicate Cd's affects on cellular health. Cd is known to offset the oxidative balance within cells leading to ROS and oxidative DNA

damage. Closer examination of Cd's role in oxidative stress in the RPTEC/TERT1 cell line and RCC development may explain effects seen under co-exposure.

Our results demonstrated canonical responses to one heavy metal, Cd, and one PAH, B[a]P. Based on the cellular responsiveness to both of these compounds, it is possible that this cell line would exhibit canonical responses to similar classes of toxicants. Future experiments may be directed to examine other metals known to cause renal toxicity such as chromium, mercury, lead, platinum, and uranium as well as other carcinogenic PAHs [8, 9, 31, 32, 48]. The RPTEC/TERT1 cell line would be useful for exploring these and other nephroxtoxicants in binary and increasingly complex environmentally relevant mixtures. Lastly, as stated previously, we have found the cell line to behave as we would expect in the body. However, it would be ideal to conduct comparative experiments between the RPTEC/TERT1 cell line and/or primary human kidney cells and/or animal models.

Conclusions

In vitro systems which retain inherent functional properties and mirror canonical responses in tissues are invaluable tools with which to more accurately assess environmentally relevant exposure conditions. Our studies have demonstrated initial properties of the RPTEC/TERT1 immortalized cell line as a suitable model for experimentation involving toxicant response, metabolic activation, and DNA damage. We conclude that the RPTEC/TERT1 cell line is amenable to numerous applications and treatment conditions for further study of nephrotoxicity.

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