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A novel *in vitro* pancreatic carcinogenesis model

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ABSTRACT

Environmental factors (e.g., BaP) have been pointed out as one of the etiologies of pancreatic cancer. However, very limited experimental assays are available to identify pancreatic specific environmental mutagens or susceptibility genes. In this study, we have developed a simple *in vitro* cell culture model system that can be used to study the molecular and biochemical aspects of carcinogenesis in a near-normal immortalized pancreatic ductal epithelial cell lines. In order to demonstrate that xenobiotic stress response is intact in these cells, we employed standard molecular biology techniques. For examples, luciferase reporter and/or real-time quantitative PCR assays were used to determine stress-induced CYP1A1 and CYP1B1 gene expression. Western blotting and immunocytochemistry assays were used to demonstrate that TCDD or BaP could activate AhR signaling. For exploring the carcinogenesis mechanism, we incubated cells with [^{3}H]BaP and determined BaP–DNA binding activity by measuring its radioactivity. BaP–DNA adduct formation was further confirmed by [^{32}P]-postlabeling assay. Finally, we demonstrated the effects of endogenous AhR or BRCA1 in BaP–DNA adduct accumulation in our cell system. As results, no apparent BaP–DNA adduct accumulation by [^{32}P]-postlabeling assay was found in either control-siRNA or AhR-siRNA pretreated cells. On the other hand, a significant increase of BaP–DNA adduct accumulation was found in BRCA1 knockdown cells. In conclusion, we suggest that this *in vitro* model may provide the feasibility for future studies on the molecular basis of pancreatic ductal cell carcinogenesis caused by dietary mutagens.

1. Introduction

Pancreatic cancer has the highest lethality among human malignancies. In 2008, estimated deaths from pancreatic cancers (34,290) were approximately equal to its incidence (37,680) in the United States (Jemal et al., 2008). Most pancreatic cancer patients have been diagnosed at unrespectable stages (Kim and Saif, 2007). Thus, they usually receive palliative chemotherapy (Kang and Saif, 2008), which show poor results due to drug resistance (Kim and Saif, 2007; Kang and Saif, 2008). The cause of pancreatic cancer is not clearly demonstrated as other cancers. Up to 10% of patients have family history of pancreatic cancer (Koorstra et al., 2008). A subset of these patients harbors germline mutations harboring in K-ras (Almoguera et al., 1988), CDKN2A/p16 (Cyclin-dependent kinase inhibitor 2A) (Caldas et al., 1994), p53 (Redston et al., 1994), and BRCA2 (Breast Cancer Type 2 susceptibility protein) (Goggins et al., 1996). However, the vast majority of onsets in pancreatic cancer has been attributed to environmental factors such as age, cigarette smoking (Silverman et al., 1994; Lin et al., 2002), diet pattern and obesity (Michaud et al., 2001, 2002), and diabetes mellitus type II (Huxley et al., 2005).

So far, smoking and dietary mutagens are well characterized and consistently implicated in epidemiological studies (Silverman et al., 1994). Lowenfels and Maisonneuve (2006) estimated that smoking causes about 25% of all pancreatic cancer. Cigarette smoke contains lots of carcinogenic chemicals including dioxins (e.g., polychlorinated dibenzodioxins (PCDDs) and benzo(a)pyrene (BaP)), which are present in a wide range of materials including plastics, resins, or bleaches (Agency for Toxic Substances and Disease Registry, 1998). The most toxic dioxin, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), became well known as a contaminant of Agent Orange (2,4,5-trichlorophenoxyacetic acid) used in the Vietnam War. In addition,

Abbreviations: CYP1A1, cytochrome P450, family 1, subfamily A, polypeptide 1; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; AhR, arylhydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzodioxin; BaP, benzo(a)pyrene; AhRR, arylhydrocarbon receptor repressor; BRCA1, breast cancer susceptibility gene 1.

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high consumption of smoked meat is a source of carcinogenic heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) (Skog, 1993; Sugimura et al., 1994).

Presumably, humans have evolved to be able to detoxify a wide range of xenobiotic chemicals. Biotransformation and elimination of xenobiotics are processed through the set of metabolic pathways such as phase I, II and III (Xu et al., 2005). In phase I, enzymes such as cytochrome P450 oxidases (CYPs) introduce reactive or polar groups into xenobiotics. These modified compounds are then conjugated to polar compounds in phase II and excreted by phase III enzymes (Denison and Nagy, 2003).

Aryl hydrocarbon receptor (AhR) is a main transcription factor that regulates phase I gene expression. It resides in cytosol and normally inactive, bound to several co-chaperones. Upon AhR binding to chemicals such as TCDD and BaP, the chaperones dissociate resulting in dimerization of AhR with AhR nuclear translocator (ARNT) (Hankinson, 1995). The AhR/ARNT heterodimeric complex, then translocates into the nucleus, where it interacts with DNA by binding to recognition sequences, referred to xenobiotic-responsive element (XRE), in the promoter region of AhR responsive genes. The activation of AhR leads to expression of various genes encoding phase I enzymes such as CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1), and CYP1B1, and phase II enzymes such as NQO1 (NAD(P)H dehydrogenase, quinone 1), and UGT1A2 (UDP glucuronosyltransferase 1 family, polypeptide A2) (Hankinson, 1995).

AhR is expressed ubiquitously in human and rodent systemic organs including the pancreas (Dolwick et al., 1993; Yamamoto et al., 2004). There are several lines of evidence showing that overexpression of AhR promotes tumorigenesis. Transgenic mice, which express constitutively active AhR, spontaneously develop hepatocarcinoma (Moennikes et al., 2004) and stomach cancer (Andersson et al., 2002). Whereas AhR-knockout has increased resistance to carcinogens (Shimada et al., 2002) and knockdown of AhRR, AhR repressor, promotes tumor cell growth (Zudaire et al., 2008). Therefore, identification of environmental risk factors and understanding the mechanism of AhR activation in the pancreas is essential for elucidating etiology of pancreatic cancer.

Immortalized but not transformed human pancreatic ductal epithelial (HPDE) cell lines were developed by expressing human papilloma virus (HPV)-16 E6E7 genes in normal human pancreatic epithelial cells as an *in vitro* model system (Furukawa et al., 1996). Although these cells have been altered by viral gene products, subsequently isolated clones such as HPDE6-C7 and HPDE6-C11 showed near normal genotypic and phenotypic characteristics of normal human pancreatic cells including anchorage-dependent growth requirement and nontumorigenic in immune-deficient mice (Ouyang et al., 2000). Moreover, tumorigenicity of HPDE6-C7 cells in SCID mice requires further transformation by oncogenic K-ras protein (Qian et al., 2005).

In this study, we are reporting these two non-transformed human pancreatic cell lines as *in vitro* models that are useful tools in identifying genes that are susceptible to environmental stress. In addition, these cell lines will be useful in identifying environmental factors and susceptible genes in normal pancreas.

2. Materials and methods

2.1. Cell culture and chemicals

Human pancreatic ductal epithelial cells, HPDE6-C7 and HPDE6-C11 from Dr. Tsao (Furukawa et al., 1996), were cultured in keratinocyte serum-free (KSF) medium supplemented by an epidermal growth factor and bovine pituitary extract (Invitrogen) and 1× Antibiotic-Antimycotic (Invitrogen). 3-Methylcholanthrene (3MC), indole-3-carbinol (I3C) and BaP were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). TCDD was obtained from Ultra Scientific (N. Kingstown, RI). Radio-labeled [^{3}H]BaP was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and [γ-^{32}P] ATP was obtained from Perkin Elmer, Inc. (Waltham, MA).

2.2. Reporter gene assay

Cells (5×10^4 cells/well) were seeded in 24-well plates and transfected overnight with reporter plasmids, p(XRE.1A1)-Luc (125 ng/well) (Kang et al., 2006a), with or without human AhRR expression plasmid (pCDNA3-AhRR Δexon) (Karchner et al., 2009) or fish AhRR expression plasmid (pCDNA3-FAhRR) (Watanabe et al., 2001) using Lipofectamine Plus (Invitrogen). Next, cells were further incubated for 24 h with various chemicals as indicated and lysed in a reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luminometer and the luminescence signals were normalized for relative transfection efficiency by measuring β-galactosidase activity of a co-transfected reporter plasmid (Kang et al., 2006a,b).

2.3. Real-time PCR

HPDE6 cells incubated with 10 nM of TCDD or 5 μM of BaP for indicated times were harvested and RNAs were purified with Trizol solution (Invitrogen). Reverse transcription (RT) was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed as previously described (Kang et al., 2006a). Reactions were performed in tetraplicate using the 1× TaqMan Universal PCR Master Mix (Roche Applied Science), on an Applied Biosystems-Prism Sequence Detector System 7700 and analyzed with SDS software. The mRNA level of GAPDH was also determined for each RNA sample and was used for normalization. The following sequences were used for primer: 5′-ctt gga cct ctt tgg agc tg-3′ (forward) and 5′-cga agg aag agt gtc gga ag-3′ (reverse) for CYP1A1 (NM_000499, +933 to +1144); 5′-cac caa ggc tga gac agt ga-3′ (forward) and 5′-gat gac gac tgg gcc tac at-3′ (reverse) for CYP1B1 (NM_000104, +3837 to+4000); 5′-gta tga caa cga att tgg cta cag-3′ (forward) and 5′-agc aca ggg tac ttt att gat ggt-3′ (reverse) for GAPDH.

2.4. Western blotting

Cells were cultured for 24 h, treated with TCDD or BaP, lysed and centrifuged for total protein analysis. Cytosolic/nuclear fraction and standard Western blots were prepared and analyzed as described previously (Kang et al., 2006b). Primary antibodies used in this experiment are as follows: rabbit polyclonal anti-BRCA1 (C-20, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-AhR (H-211, Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-CYP1A1 (Affinity BioReagents, Golden, CO), rabbit polyclonal anti-Lamin B1 (Abcam, Cambridge, MA), mouse monoclonal anti-β-tubulin (Sigma), mouse monoclonal anti-β-actin (Sigma). Anti-mouse and -rabbit IgG-peroxidase antibody produced in goat (Sigma) were used for secondary antibody and ECL solution (Santa Cruz Biotechnology, Inc.) was used for detection.

2.5. Immunocytochemistry assay

Immunofluorescence staining was used to identify the changes of cellular localization of AhR. Cells cultured on coverslips were incubated with 10 nM TCDD for indicated times then fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with incubation of 0.1% Triton X-100 for 5 min and rinsed three times with PBS containing 0.5% BSA. Then they were incubated with rabbit anti-AhR antibody (H-211, Santa Cruz Biotechnology, Inc.) for 3 h and washed with PBS three times and further incubated with Alexa-fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h. Fluorescence images were taken with a fluorescence microscope (Carl Zeiss, Berlin, Germany).

2.6. Ethoxyresorufin-O-deethylation activity

CYP1A1 enzymatic activity was measured using a CYP1A1 ethoxyresorufin-O-deethylation (EROD) activity kit (IKZUS Environment, Italy) according to the procedures provided by manufacturer. Briefly, HPDE6 cells treated with various doses of BaP and TCDD for 24 h, and then cells were washed twice with PBS and incubated with reaction buffers, containing 5 μM of 7-ethoxyresorufin and 10 μM of dicumarol for 30 min at 37 °C. Fluorescence was measured every 10 min for 60 min at 37 °C with an Ultra 384 fluorometry (Tecan, Männedorf, Switzerland) using 535 nm excitation and 590 nm emission filter as previously described (Burke and Mayer, 1974; Kang et al., 2008b).

2.7. [^{3}H]BaP–DNA binding assay

Cells (HPDE6) were transfected with 100 nM of each siRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen) for 72 h (Bae et al., 2004). The sequence of each siRNAs are as follows: control, 5′-GAC GAG CGG CAC GUG CAC A-3′; AhR, 5′-UAC UCC CAC CUC AGU UGG C-3′; BRCA1, 5′-UGA UAA AGC UCC AGC AGG A-3′. Then cells were treated with [^{3}H]BaP for 24 h. Genomic DNA was isolated using Wizard SV Genomic DNA purification system (Promega, Madison, WI).