REGULATION OF CELL PLASTICITY BY THE ARYL HYDROCARBON RECEPTOR (AHR) AND ITS CELLULAR TARGET, NEDD9/HEF1/CAS-L.

Coumoul X*, Bui L-C*, Tomkiewicz C*, Chevallier A*, Pierre S*, Bats A-S*, Mota S*, Raingeaud J[$], Pierre J[$], Diry M[§], Transy C[§ ¶], Garlatti M*, Barouki R.*

*** INSERM UMR-S 747 & Université Paris Descartes, Centre universitaire des Saints-Pères, 45 rue des Saints Pères, 75006 Paris France**
§ INSERM U490, 45 rue des Saints Pères, 75006 Paris France
[$] INSERM U461, Faculté de Pharmacie, 5 Rue Jean Baptiste Clément, 92296 Chatenay-Malabry, France.
[¶] Present address: Institut Cochin, Inserm, U567, 27 rue du faubourg St Jacques, 75014 Paris France

Introduction

Populations of industrialized and third world countries are commonly exposed to numerous organic contaminants or pollutants; several of them are persistent in the environment and accumulate in the adipose tissue of animals and humans. These Persistent Organic Pollutants (POPs) are characterized by a long half-life and are slowly metabolized. They have various toxicological manifestations among which are carcinogenesis, teratogenicity and immunotoxicity. They are also classical endocrine disrupters. The toxicity of a POP depends upon several factors: its molecular structure, its half-life, the contamination dose and the mechanism of action. Carcinogenesis has been subdivided into distinct steps: initiation, promotion and progression. The influence of various POPs has been demonstrated, clearly, in the initiation and the promotion steps. Several polyaromatic hydrocarbons are metabolized into highly reactive intermediates which display potent genotoxicity. Other POPs are not directly genotoxic but can promote cell division and alter the balance between cell apoptosis and proliferation. Surprisingly, few studies have been undertaken to establish a relationship between POP exposure and tumor progression, particularly metastasis development.

At the cellular level, tumor invasion and metastasis appear to require the transition of a cancer cell from an epithelial to a mesenchymal phenotype (EMT for Epithelial Mesenchymal Transition). This transition leads to a change in the anchorage and the migration properties of the cell. EMT and cellular plasticity also are involved in a number of physiological or pathological processes during development such as wound repair or immune maturation in addition to their likely contribution to tumor progression. Further, very few studies have addressed the relationship between POP exposure and cellular plasticity or EMT in contrast to the wealth of studies on the cell cycle, apoptosis or tumor initiation. We have shown, using the human carcinoma cell lines (MCF-7 and HepG2), that dioxins (including 2,3,7,8-TetraChloroDibenzoDioxin or TCDD) and related polycyclic aromatic hydrocarbons alter cellular morphology, anchorage, E-Cadherin expression and Jun N-terminal Kinases (JNK) activation in a manner reminiscent of EMT (1).

TCDD is a chlorinated aromatic hydrocarbon, which is produced during combustion processes. It accumulates in the food chains and in the adipose tissues of contaminated organisms. This molecule is very stable and difficult to eliminate (half-life of 8 years). Remarkably, it is non-genotoxic. However, it activates an intracellular receptor, the aryl hydrocarbon receptor (AhR) which is, also, a promiscuous hydrocarbon sensor (ex: benzo(a)pyrene from tobacco smoke). Upon ligand binding, the AhR associates with a partner called ARNT (AhR Nuclear Translocator) in the nucleus and, thus, constitutes a functional transcription factor. For a long period of time, only XMEs (Xenobiotic Metabolism Enzymes) such as cytochrome P450 1A1, were believed to be AhR target genes. Large-scale genomic studies of several groups, including ours, have shown since that a variety of other genes are regulated also by dioxin (1). In a recent study, we demonstrated that one of these genes, called NEDD9/CAS-L/HEF1 (Human Enhancer of Filamentation 1), is involved in TCDD-regulated cell plasticity or motility (2, 3).

Material and methods

Cell Culture. Human hepatoblastoma HepG2 and mammary MDA-MB-231 and MCF-7 cells were cultured in a humidified atmosphere in 5% CO2 at 37°C in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, 200 U/ml penicillin, 50 µg/ml streptomycin and 0.5 mg/ml amphotericin B.

siRNA transfection. One day before transfection, HepG2 cells (4 x 10^5 cells/well) were seeded into 6-well plates. 24h later, the medium was replaced by DMEM without phenol red supplemented with 3% charcoal-treated calf serum and cells were then transfected with 5 nM siRNA, using the HiPerfect transfection reagent (Qiagen) according to the manufacturer's protocol. For Hef1 experiments, after 8 h of incubation at 37 °C, the medium was replaced and the cells were treated or not with 25nM TCDD. 72 h post-transfection, RNA and proteins were purified or cell immunofluorescence were performed. For AhR experiments, cells were treated with 25nM TCDD 16h post-transfection and extraction or immunostaining was performed 48h post-transfection.

Reporter assays: the -850/+4 human Hef1 promoter (NM_182966.2) was ligated in the polylinker sequence of a pGL3 basic vector (Promega, Madison, WI). Cells were seeded into 6 well plates (3 x 10^5 cells/well); twenty four hours later, the medium was replaced with DMEM without phenol red supplemented with 3% charcoal-treated calf serum and transfection was performed with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were treated 24h post-transfection with 25 nM dioxin (TCDD). After an overnight incubation, cells were homogenized with phosphate lysis buffer (Promega) for enzymatic assays. A dual luciferase assay was performed with a Promega kit and a luminometer.

Wound healing Assay: HepG2 cells (1 x 10^6 cells/well) were seeded into 6-well plates. Twenty four hours later, the cells were cultivated in DMEM without phenol red supplemented with 3% charcoal-treated calf serum overnight and transfected with siHef1 528 or a control, as previously described. Six hours later, cells were treated with 25 nM dioxin. Twenty four hours post-transfection, HepG2 were wounded in serum-free medium, 1% BSA, with a sterile 200 µl pipet tip to remove cells. After washing away suspended cells, treatment with 25nM TCDD was continued. The progress of migration was photographed with an inverted microscope (Nikon TMS-F, 301655) equipped with a digital camera (Nikon Digital shot DS-L1) (after identification of each wounded zone) in 6 regions, immediately and then for 2 days after wounding (0h-24h-48h),.

RNA extraction, reverse transcription and quantitative RT-PCR. Total RNAs were extracted using the RNeasy mini kit (Qiagen) as described by the manufacturer and quantified with a spectrophotometer (ND-1000 Nanodrop). Reverse transcription was performed on each RNA sample (2 µg) using the cDNA High-Capacity Archive kit from Applied Biosystems (Courtaboeuf) in a final reaction volume of 50 µl according to the manufacturer's instructions. Quantitative RT-PCR was performed with 40 ng of the cDNA, 300 nM of each primer, and ABsolute™ QPCR SYBR® Green (Abgene) to a final volume of 10 µl. Quantitative RT-PCR measurements were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR cycles were as follows: Taq activation (15 min), denaturation (15 s, 95°C), and annealing and extension (1 min, 60°C). The melting-curve analysis showed the specificity of the amplifications. The relative mRNA levels were estimated using the delta-delta Ct method with RPL13A as the reference gene.

Immunoblotting. Whole-cell lysates were prepared from HepG2 cells in M-PER® Mammalian Protein Extraction Reagent containing protease and phosphatase inhibitor cocktails (Sigma). Equal amounts of total protein were separated by SDS–PAGE and transferred onto nitrocellulose membranes. Blots were probed with primary antibodies. Signals from immunopositive bands were visualized on X-ray film using alkaline phosphatase-linked secondary antibody (Applied Biosystems, T2191 or T2192, 1:20000) and an enhanced chemiluminescence system CDP-Star® (Applied Biosystems). After scanning of the blots, quantification was performed with the ImageJ freeware (http://rsb.info.nih.gov/ij/).

Immunofluorescence. HepG2 cells were seeded onto glass coverslips 72h before being processed for immunofluorescence (approximately $5x10^5$ cells per well in 6-well plates). Treatments with TCDD (25nM) were performed for 48h in DMEM without phenol red and supplemented with 3% charcoal-treated calf serum. Coverslips were washed twice in 1X PBS and then fixed with 4% paraformadehyde for 10 min. The cells were permeabilized for 10 min in PBS-0.5% Triton then incubated in PBS-1% BSA for 10 min. Incubation with the primary antibody was done for 1h at room temperature. The secondary antibody (Alexa Fluor 488 dye-conjugated anti-mouse antibody) was diluted in PBS-1% BSA and incubated with the slides for 1 hour at room temperature. For actin staining, TRITC-conjugated phalloidin was included during incubation with the secondary antibody. The coverslips were sealed with Vectashield mounting medium for fluorescence with Dapi (Vector Laboratories) and images were acquired using a Nikon Eclipse TE-2000 E microscope. Deconvolution and 3D reconstitution was performed using Autoquant imaging and Autodeblur version X 1.4.1 (AutoDebur & Autovisualize, Mediacybernetics).

Matrigel invasion assay. Cell invasion was measured using matrigel coated film inserts (8µm pore size) fitted into 24-well invasion chambers (Beckon-Dickinson Bioscience)

Results and discussion

Using the human carcinoma cell lines (MCF-7 and HepG2), we have previously shown that dioxins (including 2,3,7,8-TetraChloroDibenzoDioxin or TCDD) and related polycyclic aromatic hydrocarbons alter cellular morphology, anchorage, E-Cadherin expression and Jun N-terminal Kinases (JNK) activation in a manner reminiscent of EMT (1).

Using siRNA-based knockdowns, we have shown also that the AhR is involved in dioxin-related cell plasticity (1). Large-scale genomic studies of several groups, including ours, have shown since that a variety of other genes are regulated also by dioxin. Among these new targets, we identified several that could potentially regulate cell plasticity (1). We found that one of these genes, called NEDD9/CAS-L/HEF1 (Human Enhancer of Filamentation 1), is involved in these phenomenona (2, 3). HEF1 is a direct transcriptional target of the AhR and its ligands. Further, dioxin-mediated HEF1 induction resulting from cell exposure to AhR ligands was repressed using RNA interference and inhibited EMT-related processes. Considering that HEF1 has been characterized as a metastatic marker, these studies suggest that exposure to certain POPs plays a role in tumor progression (2).

The demonstration of an effect of POPs on tumor progression and EMT enhances our knowledge of the mechanisms of action of POPS and suggests numerous applications (2). For example, it will be possible to identify new mechanisms and target genes involved in AhR-related and POP-related plasticity in the search for therapeutic agents. Antagonists of the AhR pathway are already known and can be used in this respect.

Acknowledgements

This work was supported by AFSSET (Agence Française de Sécurité Sanitaire de l'Environnement et du Travail; all authors); ANR (Agence Nationale de la Recherche, 06SEST26, Oncopop; all authors); ARC (Association pour la Recherche sur le Cancer, 3927; all authors); Fondation pour la Recherche Medicale (Bourse post-doctorale, Linh-Chi Bui); INSERM (Institut National de la Santé et de la Recherche Médicale; all authors); Ligue contre le Cancer (Bourse post-doctorale, Linh-Chi Bui); Ministère de l'enseignement supérieur et de la recherche (Bourse doctorale, Aline Chevallier); Région Ile de France (bourse doctorale, Stéphane Pierre); Université Paris Descartes .

References

1 - Diry M, Tomkiewicz C, Koehle C, Coumoul X, Bock KW, Barouki R, Transy C. (2006); *Oncogene*. 25(40):5570-4.

2 - Barouki R, Coumoul X. (2010) *Cell Adhesion Migration*. (1):72-6.

3 - Bui LC, Tomkiewicz C, Chevallier A, Pierre S, Bats AS, Mota S, Raingeaud J, Pierre J, Diry M, Transy C, Garlatti M, Barouki R, Coumoul X. (2009). *Oncogene*. 28(41):3642-51.