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Atrazine represses *S100A4* gene expression and TPA-induced motility in HepG2 cells

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ABSTRACT

Atrazine (ATZ) is probably the most widely used herbicide in the world. However there are still many controversies regarding its impacts on human health. Our investigations on the role of pesticides in liver dysfunctions have led us to detect an inhibition of FSP1 expression of 70% at 50 μm and around 95% at 500 μM of ATZ ($p < 0.01$). This gene encodes the protein S100a4 and is a clinical biomarker of epithelial–mesenchymal transition (EMT), a key step in the metastatic process. Here we investigated the possible effect of ATZ on cell migration and noticed that it prevents the EMT and motility of the HepG2 cells induced by the phorbol ester TPA. ATZ decreases Fak pathway activation but has no effect on the Erk1/2 pathway known to be involved in metastasis in this cell line. These results suggest that ATZ could be involved in cell homeostasis perturbation, potentially through a S100a4-dependant mechanism.

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1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, ATZ) is a selective triazine herbicide that is slightly soluble in water. In the USA, this xenobiotic is used mainly in corn and sorghum farms to protect crops from broadleaf and grassy weeds. ATZ is the most heavily used pesticide in this nation and can be found at high concentrations in surface and ground water sources as well as in the soil of many agricultural lands (Powell et al., 2011). ATZ is stable at pH 7 in aqueous media and volatilization from water is minimal. In addition, it is poorly absorbed by suspended solids and sediment. Such physical and chemical properties make ATZ a serious contaminant of surface and ground water. ATZ can be absorbed into the blood stream through oral, dermal and inhalation exposure. It is considered as a possible human carcinogen (group C) based on evidence of induction of mammary gland tumor growth in laboratory animals, however data on its carcinogenic potential in humans is lacking (Tchounwou et al., 2001). *In vitro* studies have shown an increase in DNA damage induced by atrazine-based herbicide in lymphocytes (Zeljezic et al., 2006), endocrine disruption resulting in an increased aromatase expression (Laville et al., 2006; Sanderson et al., 2001), arsenic-dependant potentiation of cytotoxicity and transcriptional activation of stress genes (Tchounwou et al., 2001), and growth inhibition of the HepG2 cells at low doses (Powell et al., 2011). ATZ also induces reproductive effects such as decreased sperm motility and birth defects (Betancourt et al., 2006; Winchester et al., 2009), as well as developmental (Gammon et al., 2005) and immune perturbations (Whalen et al., 2003). Some epidemiological studies have linked ATZ to ovarian, prostate, brain, testicular and breast cancer as well as to leukemia and non-Hodgkin's lymphoma (Alavanja et al., 2003; Donna et al., 1989; Engel et al., 2005; De Roos et al., 2003; Mills, 1998; Schroeder et al., 2001) however other authors believe that more consistent and scientifically convincing evidence is required to support such a causal relationship between exposure to ATZ and human cancers (Freeman et al., 2011; Hessel et al., 2004; Rusiecki et al., 2004; Sathiakumar et al., 2011; Simpkins et al., 2011).

Cell migration is a multistep process that requires stimuli such as cytokines or growth factors to enhance cell movement. Transduction of such a message from the cell receptors inwards increases the activity of the cellular machinery leading to a succession of changes including the cyclic formation of cytoplasmic protrusions and focal adhesions disruptions, and induction of contractions mainly orchestrated by the RhoGTPases Cdc42, Rac and Rho (Entschladen et al., 2011). The Src/Fak pathway is known to be involved in the transduction of the signal from the extracellular matrix to the cytoskeleton. Indeed, it allows the phosphorylation of the PKC proteins and the activation of the RhoGTPases downstream (Schaller, 2010). Focal adhesion kinase (FAK) also plays a role in adhesion, invasion, proliferation, apoptosis and epithelial-to-mesenchymal transition (EMT) through the activation of

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ERK/MAPK and the AKT pathway (Yam et al., 2009). To allow EMT and facilitate cell movement, an increase in the recycling of integrins occurs (Pinzani, 2011) as well as the repression of E-cadherin and the loss of cell–cell adhesions. In such cases, migration is individual and is called amoeboid or mesenchymal (Yilmaz and Christofori, 2009).

S100a4 is a calcium-dependent protein that is used as a clinical marker of fibrosis and metastasis. Increases in expression of this protein are strongly correlated with an aggressive malignant phenotype but also with inflammatory disease. S100a4 has a large number of partners including cytoskeletal proteins (NMMHC, actin, tropomyosin) or other proteins (S100a1, P53, septins, CCN3, liprin β1, MetAP2, P37) with which it interacts by heterodimerization. It affects motility through its direct regulation of myosin IIA assembly. Indeed, S100a4 contributes to the dismantling of myosin filaments at the ends of cytoplasmic protrusions which results in the local enrichment of myosin monomers required for the formation of neo protrusions during migration (Sack and Stein, 2009). S100a4 is also known to affect the function of the LAR family of transmembrane protein-tyrosine phosphatases by binding to liprin β1 and thereby modulating its cellular adhesion leading to the establishment of a migratory phenotype. It also plays a role in invasion by regulating metalloproteases (MMPs) positively and TIMPs (MMPs inhibitors) negatively both of which involve extracellular matrix (ECM) remodeling. Finally, S100A4 also regulates apoptosis through its effects on p53, a protein involved in modulating the expression of genes such as *Bax* and $P21^{Waf1}$ (Helfman et al., 2005; Garrett et al., 2006). S100a4 can inhibit the activation by phosphorylation of p53 through PKC as well as physically interact with p53 thereby modulating its regulatory activity (Grigorian et al., 2001). On cancer cells, suppression of *S100A4* can however lead to the arrest of cell growth (Grum-Schwensen et al., 2005) or to anoikis initiation (Shen et al., 2011). S100a4 can also play the role of extracellular paracrine factor thus participating in invasion and angiogenesis. It has an affinity for various unidentified receptors, including the receptor RAGE. Once linked to this receptor, S100a4 induces the activation of NFκB and the MAPK pathway, leading to the regulation of target genes involved in angiogenesis and tumor progression (Boye and Maelandsmo, 2010). S100a4 expression can be controlled by activation of the TGFβ pathway during EMT and by FGF-2 and its transcription level is modulated by β-catenin/Tcf complex activation (Schneider et al., 2008).

In this study, high concentrations of atrazine (250 and 500 μM) have been used to highlight the major molecular events whereas 25 and 50 μM have been used to demonstrate effects of atrazine on the cell phenotype even at lower doses. However, for clear mechanistic aspects, those concentrations are not relevant of those found in environment and in human body. Indeed, atrazine does not bioaccumulate in fatty tissue or in liver (ATSDR, 2003). For these reasons, such high concentrations can only be found after poisoning and accidental ingestion of the compound (Pommery et al., 1993). The aim of this study was to improve our understanding of the molecular events involved and provide evidence to support the link between atrazine and tumorigenesis. Among all EMT biomarkers tested, S100A4 was the only one that was strongly modulated by ATZ and that presented an interest in terms of its role in migration, invasion, angiogenesis and metastasis. To work in metastable conditions, we used the HepG2 cell line and the phorbol ester TPA which has been described as a potent EMT inducer able to initiate migration of HCC cells (Hu et al., 2008; Murata et al., 2009; Wu et al., 2006). In this way, we hypothesized that ATZ could affect the TPA-induced motility of the HepG2 cells by inhibiting S100A4 gene expression. We found that it could prevent TPA-induced migration without affecting MAPK/ERK signaling. ATZ seems to modulate only the FAK pathway as well as the expression of fibronectin and its receptor Itga5.

2. Materials and methods

2.1. Materials

The human hepatocellular carcinoma cells HepG2 were obtained from ATCC (American Type Culture Collection, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin solution, sodium pyruvate and Eagle's non-essential amino acids were from BioWhittaker (Cambrex Company, Walkersville, USA). DMSO (dimethylsulfoxide) and chemicals were from Sigma–Aldrich (L'Isle d'Abeau Chesne, Saint Quentin Fallavier, France). Protein assay materials were from Bio-Rad. All fluorescence reagents were from Molecular Probes (Eugene, OR). The herbicide atrazine was from Sigma Aldrich. The antibodies FAK, p-FAK, Erk2 and p-Erk1/2 used for western blotting experiments were from Cell Signaling. Cells were visualized with a Nikon Eclipse TE2000 phase contrast microscope.

2.2. Cell culture and drug treatments

HepG2 cells were maintained in DMEM with 1% penicillin/streptomycin, 1% non essential amino acids and sodium pyruvate, and 10% FBS, in humidified atmosphere at 37 °C containing 95% O_2 and 5% CO_2. After washing with sterile phosphate buffer saline (PBS), cells were detached by trypsinization (trypsin/EDTA) and plated at a concentration of $0.5–2 \times 10^6$ cells in 6-well plates depending on the experiment. For all experimental conditions, FBS was reduced to 5% in DMEM medium.

2.3. Western blot

HepG2 cells were scraped into a hypotonic buffer (20 mM HEPES ph7.5, 10 mM KCl, 15 mM $MgCL_2$, 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and a phosphatase inhibitory cocktail – PhosphoSTOP, Roche-). The protein concentration in each cell lysate was measured with a BCA Protein Assay Kit (Pierce), with bovine serum albumin (BSA) as a standard. Equal protein amounts were separated by SDS–polyacrylamide gel electrophoresis on 10% gels before being transferred to PVDF membranes. The membranes were then immunoblotted with antibodies for 1 h at room temperature or overnight at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit immunoglobulin G; Promega, Madison, WI, USA) for 2 h at room temperature. After washing, the blot was reacted using an ECL detection kit. A signal was acquired using a CDD camera (ChemiGenius2, Syngene) and semi-quantitative analysis was then performed using GeneTools software.

2.4. Reverse transcription-quantitative polymerase chain reaction

Total RNA was isolated by acid phenol extraction. One microgram of total RNA was reverse transcribed using a kit (SuperScript II; Invitrogen Corp, Carlsbad, California) following the manufacturer's instructions. The resultant complementary DNA was diluted 100-fold (conditions set to obtain 98% efficacy), and for each gene (target genes *S100A4, ITGA5, FN1,* $P21^{waf1}$ and *ALBUMIN,* or reference gene *GAPDH*) and each condition (DMSO, atrazine, TPA, TPA + atrazine), a mixture of Taq polymerase, 6.4 mmol/L of magnesium chloride, deoxynucleotide triphosphate, primer, and the probe (https://www.roche-applied-science.com) was added. The cDNA was then amplified in a thermocycler (LightCycler 480; Roche Applied Science, Penzburg, Upper Bavaria, Germany) for 45 cycles using conditions of 95 °C and 60 °C for 10 s each.

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