Identification of Potential Biomarkers of Human Peripheral Blood Mononuclear Cell intoxication by dioxins

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Introduction

The dioxins-type toxic compounds polychlorodibenzo-*p*-dioxins (PCDDs) and polychlorodibenzofurans (PCDFs) can accumulate through the food chain, reaching high concentrations in top entities like marine mammals and humans. Recently, DNA microarray successfully identified dioxin-responsive genes expressed after exposure to AhR ligands[1]. The complementary analysis of the entire proteome could reveal the impact of toxics after exposure of cells in well defined conditions, integrating all the effects[2,3]. If a correlation can be found between the level of expression of unambiguous protein biomarkers and the intoxication dose, this could be the basis of a bio-analytical methods to evaluate and compare the toxicity of different mixtures of dioxin-like chemicals. Many studies show that dioxin-type compounds are immunotoxic and various immunological assays based on functional tests and on cellular morphology have been developed[4]. The goal of the present study is to identify specific early biomarkers expressed by human lymphocytes after *in vitro* treatment with a mixture of 17 toxic congeners of PCDD/Fs.

Material and methods

Peripheral blood mononuclear cells (PBMCs). 20ml of fresh human blood obtained from good health donors was diluted with 20 ml of phosphate buffered saline solution. PBMCs were isolated by Ficoll-Paque gradient 1077 (Amersham Biosciences). 100,000 cells were seeded in each well of 96 wells multiplate with 100µl RPMI medium with 10% foetal calf serum, penicillin-streptomycin, L-glutamine and 1µg phytohaemagglutinin (PHA, Amersham Biosciences) stimulating specifically the T lymphocytes.

Treatments. PBMCs were exposed to different concentrations (0.1, 1, 10, 100, 1000 pgTEQ/ml) of EPA^{Ò} mixture (stock solution of EPA: 2000pg TEQ/µl DMSO, containing the 17 toxic congeners of PCDD/Fs, CamproScientific) during 24h and 72h.

Biological assays. The cellular viability was quantified by CellTiter 96Ò Aqueous cell proliferation assay (Promega). DNA and protein synthesis were evaluated by incorporation of [methyl-^{3}H]-thymidine or [^{3}H]-leucine (Amersham Life Science; 0.5 µCi/100µl medium).

Proteomic analysis. 50,000,000 human PBMCs were seeded in T175 with 50 ml culture medium. Control and treated PBMCs (1pgTEQ/ml during 24h) were lysed to recover the cytoplasmic proteins as previously described[6]. Protein concentrations were determined with the PlusOne 2D Quant Kit^{Ò}(Amersham Pharmacia Biotech). After purification with 2D-Clean-up kit^{Ò}(Amersham Pharmacia Biotech), an internal standard of proteins was constituted by pooling equal amounts of control and treated samples. 25µg of proteins of each sample were labeled with 200 pmol of Cy2 (control), Cy3 (treated) or Cy5 (standard), according to the manufacturer's instructions (Amersham Biosciences). Proteomic analysis was performed by two-dimension polyacrylamide gel electrophoresis, in which proteins are separated according to charge (pI) by isoelectric focusing (IEF) and according to size in the second dimension. Gels were digitalized in three colors with a Molecular Dynamics Typhoon 9400 laser scanner and analyzed with DeCyder software (Amersham Biosciences). After spots matching between all the gels of the experiment, significant differences between each sample were determined using a one-way analysis of variance test (ANOVA) with a value equal to 0.05. Image analysis was realized on each sample and a same threshold was applied (max volume is 10^5 and volume ratio is ± 1.5). The modified spots were excised, and digested with

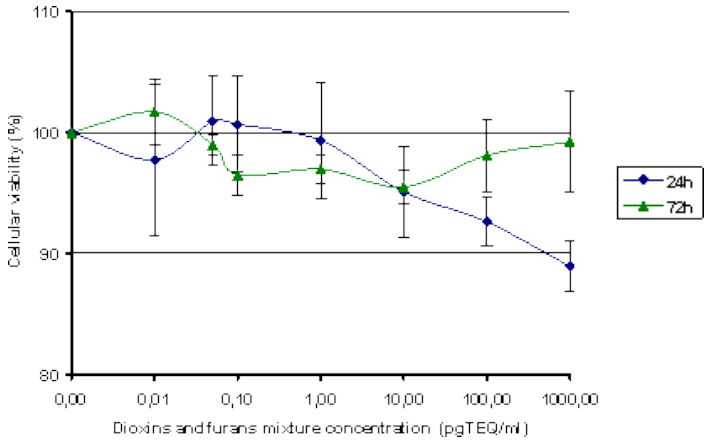
12.5ng/µl trypsin to produce a tryptic digest. Peptides were then separated by liquid chromatography on a C18 column with water-acetonitrile gradient (50/50) during 80 min. The chromatography was coupled with electrospray source ionization mass spectrometer (nanoLC-ESI-MS/MS, Bruker). The proteins were identified using Mascot (Matrix Science) online search.

Results and Discussion

Biological tests

Cellular viability. A slight decrease (less than 10%) was observed from 10 to 1000 pgTEQ/ml after 24h or from 0.1 to 10 pgTEQ/ml after 72h treatment. Viability was slightly decreased at 24h and became back to normal after 72h, at 100 or 1000 pgTEQ/ml (Figure 1).

Figure 1: Cellular viability of human PBMCs in function of EPA-Ò concentration, after 24h or 72h. Means ± SD from 5 human samples, each in triplicate. Control values were taken as 100%.



The assay reflects the growth/proliferation and/or the activation of PBMC population, in particular the T lymphocytes population. So, on the basis of this endpoint assay, no clear effect could be detected. We used then the assay using radioactive precursor uptake, more specific to cell proliferation.

DNA and protein syntheses. Precursors uptake during last four hours of treatment was analyzed. The DNA synthesis rates fluctuated sligthly in function of concentration and time of treatment (30% maximum). An increased rate was observed at 1 pg and 100 pgTEQ/ml at 72h or at 100 pgTEQ/ml at 24h. Protein synthesis was significantly decreased at 100 pgTEQ/ml but was increased at 1000pgTEQ/ml at 24 h. At 72h, protein synthesis was clearly inhibited at all concentrations (30%).

We previously observed that DNA and protein syntheses were drastically inhibited already at 1 pgTEQ/ml and after 24h with bovine or seal PBMCs[5], bovine PBMCs being among those the most susceptible ones.

Figure 3 :H^3Leucine uptake by human PBMCs in function of EPA-Ò concentration and incubation time. Means ± SD from 2 human samples, each in

triplicate. Control values were taken as 100%.

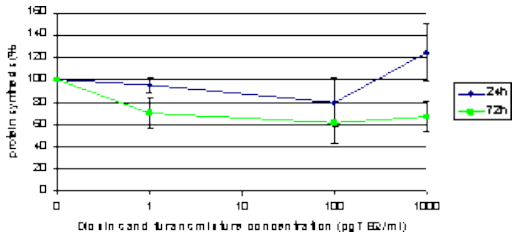
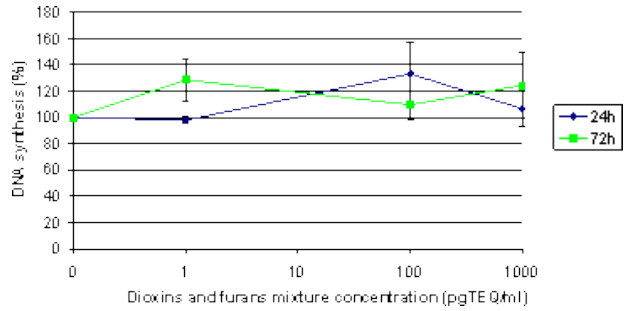


Figure 2: H^3Thymidine uptake by human PBMCs in function of EPA^{Ò}concentration and incubation time. Means ± SD from 2 human samples, each in triplicate. Control values were taken as 100%.



Proteomic analysis

As a first proteomic approach, 2D DIGE analysis was realized with cytoplasmic proteins of PBMCs from three human donors exposed to 1 pgTEQ/ml during 24h. These conditions are at background level of environmental intoxication.

Image analysis detects 4000 spots and shows differential expression (43 modified spots) between control and treated samples from one individual (figure 4) but these modifications were not observed in two others individuals nor in pooled gel from the three individuals. The identification of modified spots in the first individual was made difficult because of to their low intensity. Two spots were however identified: MIF protein (macrophage migration inhibitory factor), a cytokine that has the potential to inhibit or activate monocytes/macrophages[7] and calgranulin A, a calcium binding protein of S100 family involved in Ca^{++} signal transduction, cytoskeletal-membrane interaction, cell differentiation and cell-cycle progression[8]. The toxicity of dioxins is mediated by their binding with the aryl hydrocarbon receptor (AhR) and the translocation of the dimer bound to dioxin in the nucleus. With that respect, the specific analysis of the nuclei fraction is under investigation.

Recent papers report significant difference in the genes[1] and the proteins expression[9,10,11] of human, rat and mouse cell types (Hepatocytes, Sertoli cells and thymus extracts) in vivo at high doses of pure 2,3,7,8 TCDD. These strong effects are not observed at the proteome level in cell culture of PBMCs at low doses. Our preliminary results suggest that more abundant differences should be observed at higher exposition (concentrations and times). In order to identify a higher number of potential biomarkers we will have modify our strategy. We will increase the exposition dose (time and concentration) and try to identify proteins for which the gene(s) are affected. We will trace those proteins by targeted proteomic methods at lower doses to determine their limit of quantification. Pre-concentration using specific affinity methods will help to further lower those limits.

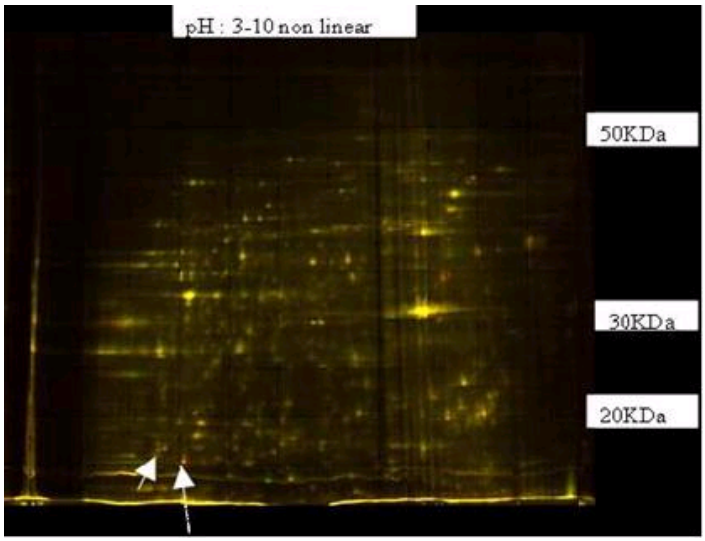


Figure 4: Analysis of control and treated human PBMCs cytoplasmic fractions by 2D fluorescence difference gel electrophoresis (2D-DIGE). Proteins were separated in a non-linear pH gradient 3-10 followed by second dimension in 12% acrylamide gel and were detected by fluorescence scanning (proteins from control PBMCs in green, proteins from treated PBMcs in red). Yellow spots when proteins equally expressed in both conditions. Molecular masses of standard proteins are given to the right.

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