Toxicology in Vitro 29 (2015) 1240–1253

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Identification of pathway-based toxicity in the BALB/c 3T3 cell model

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ARTICLE INFO

Article history:
Received 11 June 2014
Accepted 2 October 2014
Available online 16 October 2014

Keywords:
Particulate matter
Pathway-based toxicity
Cell transformation assay
Gene expression
Environmental mixtures

ABSTRACT

The particulate matter represents one of the most complex environmental mixtures, whose effects on human health and environment vary according to particles characteristics and source of emissions. The present study describes an integrated approach, including *in vitro* tests and toxicogenomics, to highlight the effects of air particulate matter on toxicological relevant endpoints.

Air samples ($PM_{2.5}$) were collected in summer and winter at different sites, representative of different levels of air pollution. Samples organic extracts were tested in the BALB/c 3T3 CTA at a dose range 1–12 m^3. The effect of the exposure to the samples at a dose of 8 m^3 on the whole-genome transcriptomic profile was also assessed.

All the collected samples induced dose-related toxic effects in the exposed cells. The modulated gene pathways confirmed that toxicity was related to sampling season and sampling site. The analysis of the KEGG's pathways showed modulation of several gene networks related to oxidative stress and inflammation. Even if the samples did not induce cell transformation in the treated cells, gene pathways related to the onset of cancer were modulated as a consequence of the exposure.

This integrated approach could provide valuable information for predicting toxic risks in humans exposed to air pollution.

1. Introduction

The airborne particulate matter (PM) is responsible for relevant adverse effects on human health (Pope et al., 2009). The International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) have classified PM as a Group 1 carcinogen (Loomis et al., 2013; Hamra et al., 2014). The IARC classification was based on the evidence of lung tumors as a consequence of lifetime exposure to 10–30 $\mu g/m^3$ $PM_{2.5}$.

It has been also suggested that PM short and long-term effects are related to particles concentration, chemistry, and size (Valavanidis et al., 2008). However, predicting the toxicological risk associated with the exposure to environmental samples, such as PM and PM extracts, still shows some critical issues. The environmental samples are characterized by the simultaneous presence of a large number of pollutants, showing different mechanisms of action and toxicity profiles. The concentrations of the single components vary, according to the source. The current EU regulation establishes the list of chemicals that should be identified and characterized in the airborne PM and set the acceptable concentration levels for reference compounds, whose toxicological profile has been evaluated in standard tests (EU, 2008). The acceptable concentration levels

Abbreviations: ACE, absolute clonal efficiency; AEI, average exposure indicator; CTA, cell transformation assay; DEG, differentially expressed genes; DMSO, dimethyl sulfoxide; EEA, European Environment Agency; ECVAM, European Centre for the Validation of Alternative Methods; EU, European Union; FBS, Fetal Bovine Serum; GLM, Generalized Linear Model; IARC, International Agency for Research on Cancer; IF, impact factor; KEGG, KEGG Kyoto Encyclopedia of Genes and Genomes; 3-MCA, 3-methylcholanthrene; MEM, Minimum Essential Medium; PAH, polycyclic aromatic hydrocarbons; PCA, Principal Component Analysis; PM, particulate matter; RCE, relative clonal efficiency; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; ROS, reactive oxygen species; SHE, Syrian Hamster Embryo; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TF, transformation frequency; WHO, World Health Organization.

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http://dx.doi.org/10.1016/j.tiv.2014.10.002
0887-2333/

are derived from experimental studies. Below these levels the exposure is considered unlikely to be of concern for compounds that are not classified as carcinogens or not to increase the risk of cancer.

However, this approach may be inadequate to estimate the real risk from several environmental carcinogens co-present in a complex mixture at low doses.

Cancer may arise from the exposures to these environmental mixtures as the consequence of the interplay among single chemicals, each one affecting one (or more) cancer hallmarks. The adverse outcome may be reached at doses much lower than those at which the effect has been observed in traditional toxicological studies. Moreover, not all components in a complex mixture may be equally identified and characterized.

In recent years, a shift from *in vivo* costly and time-consuming animal studies to short term *in vitro* assays, supported by omics technologies, has been proposed to assess the hazard of single chemicals or complex mixtures (Collins et al., 2008; Kohonen et al., 2014). The EU current regulations on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) and on cosmetics are prompting the use of alternative test methods, including *in vitro* methodologies and the application of toxicogenomics technologies (EU, 2003, 2006).

Among *in vitro* alternative testing methods, the cell transformation assays (CTAs) may represent a good alternative to the rodent carcinogenicity bioassay, based on the scientific evidence that the cellular and molecular processes involved in *in vitro* cell transformation closely resemble *in vivo* carcinogenesis. They occur as a result of comprehensive cellular responses to direct and indirect damage to DNA, genes and cellular systems (Vanparys et al., 2012; Vasseur and Lasne, 2012).

CTAs could represent a valuable support for the identification of the transforming potential of chemicals and complex mixtures (Lilienblum et al., 2008; Mascolo et al., 2010; Corvi et al., 2012; Vanparys et al., 2012; Vasseur and Lasne, 2012).

BALB/c 3T3 CTA is one of the three available models to investigate the cell transformation *in vitro* as a consequence of the exposure to possible carcinogens. This model has been reported to show good predictability of mammalian carcinogenicity (IARC/NCI/EPA Working Group, 1985; Mascolo et al., 2010; Creton et al., 2012). Moreover, a modified protocol of the CTA on BALB/c 3T3 has been validated in the European Centre for the Validation of Alternative Methods (ECVAM) (Sasaki et al., 2012a,b).

BALB/c 3T3 cells are immortalized fibroblasts that retain enough metabolic activity to metabolize chemicals, are able to grow on a plastic substrate, are sensitive to contact inhibition and, when exposed to transforming agents, develop a full malignant phenotype. With respect to other appreciated CTA models, such as the Syrian Hamster Embryo (SHE) CTA, which is based on the use of hamster primary embryonic cells, BALB/c 3T3 is considered to be more entailed to 3R principles. Moreover, it had been used in several international laboratories for more than 40 years.

Transcriptomics is a powerful tool to assess the impact of exposure to complex mixtures. It allows the identification of the metabolic signaling and regulatory networks in cells exposed to the mixture, providing at the same time information for the mechanism of action and for predicting the final adverse outcome. The global gene expression changes caused by complex mixtures, such as cigarette smoking and its condensate, diesel exhaust and urban dust, have been intensively investigated, giving more insight into their effects on toxicological relevant endpoints (Mahadevan et al., 2005; Sen et al., 2007; Aung et al., 2011; Líbalová et al., 2012; DeMarini, 2013).

The approach of our study was to couple the BALB/c 3T3 CTA, which describes observable phenotypic outcomes, such as cell clonal efficiency and transformation, with the mechanistic endpoint offered by the gene modulation, which addresses the cell response at the gene level and highlights pathway-based toxicity as the consequence of the exposure.

By using this integrated approach, we evaluated the toxicological profile of $PM_{2.5}$, which represents the fraction that more easily enters the respiratory tract. $PM_{2.5}$ is considered more genotoxic than particles with larger aerodynamic diameters. It is also regarded as the best indicator for air quality in areas affected by sources of combustion pollution.

The combination of the BALB/c 3T3 CTA and of the microarray-based toxicogenomics allowed the identification of pathway-based toxicity profiles in $PM_{2.5}$ samples that may be considered early markers of adverse outcomes.

2. Materials and methods

2.1. Cells

The BALB/c 3T3 A31-1-1 cell line was purchased from the Health Science Research Resource Bank (Osaka, Japan). The cells were grown in Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS) and routinely maintained in a humidified incubator with an atmosphere of 5% CO_2 in air at 37 °C. The cell cultures were cryoconserved in MEM 10% FBS solution containing 5% dimethyl sulfoxide (DMSO) and used for the CTAs at passage 3–5 from the initial stock. For the transformation assays, only sub-confluent cells (about 70% confluence) were used. The target cells were not maintained in culture beyond the third passage after thawing.

2.2. Air samples collection

$PM_{2.5}$ samples were collected at several sites located in the surroundings of Bologna (Emilia Romagna, Italy). The sites were chosen on the basis of air dispersion models and were representative of different levels of environmental pollution. The chosen sites included: (1) a site typical of the urban background (GMA), (2) a rural site (SPC), (3) a site affected by the maximum fall-out of the emission of a waste-to-energy plant (MXW), which was chosen as a punctual source, (4) a site that was considered the minimum fall-out point of the punctual source (CTW). CTW was located upwind with respect to MXW. Both MXW and CTW were located in a downwind area, affected by the urban pollution. A brief description of the sampling sites is reported in Table 1.

$PM_{2.5}$ was collected daily on quartz-fiber filters (47 mm diameter) by low-volume air flow samplers (Skypost TCR TECORA), during 40-day collecting campaigns in the summer of 2008 and the winter of 2009. Each filter was weighed daily before and after $PM_{2.5}$ collection in order to obtain the gravimetric data.

2.3. Preparation of treatment solutions

All the filters collected in one season at each site were pooled to obtain a unique sample that was representative of the season. Each pooled sample was extracted with acetone, using a Soxhlet apparatus, then reduced to dryness and dissolved in DMSO at a final concentration of 800 m^3 equivalents/ml (stock solution). The treatment solutions were prepared by diluting the stock solutions in the culture media immediately before use, at concentrations ranging 1–12 m^3 equivalents. The concentration of the vehicle DMSO was 0.5% in each plate.

2.4. Cell transformation assay

2.4.1. Cytotoxicity test

The cytotoxicity assays were performed by seeding exponentially growing BALB/c 3T3 A31-1-1 cells at 250 cells/60-mm dish