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Comparison of gene expression profiles in BALB/c 3T3 transformed foci exposed to tumor promoting agents

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ABSTRACT

Identification of specific etiological carcinogens is one of the most important issues in environmental-toxicology studies. In this study, cDNA microarrays were used to analyze gene expression and discern chemical-associated profiles induced by a variety of tumor promoting agents in transformed cells. Two-stage transformation model of BALB/c 3T3 cells was established with MNNG as initiator, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), okadaic acid (OA), or cadmium chloride ($CdCl_2$) as tumor promoters. Nine morphologically transformed foci were isolated and the anchorage-independent growth of transformed cells was verified. The gene expression alterations in foci were evaluated using cDNA microarray with 1796 mouse genes. Unsupervised hierarchical clustering analysis revealed that the nine foci were classified into three groups in concordance with the promoters used to induce them and characteristic clusters of genes were identified. In these clusters, genes associated with oxidative stress were specially upregulated following distinct promoter exposure. Moreover, common gene expression alterations were also observed in foci, including upregulated genes associated with cell proliferation and downregulated genes associated with extracellular matrix. Our results demonstrate the presence of unique gene expression profiles in transformed cells which reflect the etiological chemicals and indicate the importance of characteristic molecular alterations as potential biomarkers of exposure to tumor promoters.

1. Introduction

While exposure to environmental carcinogens is often regarded as contributing to human cancer risk, identification of the specific etiological carcinogens and estimation of their roles are some of the most important issues in environmental-toxicology studies. Tumor promoters are a kind of non-genotoxic carcinogen. It has long been known that tumor promoters can induce tumor development in previously initiated cells by causing their selective expansion, and this effect is reversible early on in the tumor promoting process (DiGiovanni, 1992; Trosko, 2001; Trosko et al., 2005). Thus studies on the identification of potential tumor promoting chemicals have presaged modern chemoprevention in humans (Gills et al., 2006; Postema et al., 2005; Taji et al., 2008).

Traditionally, toxicologists employ a battery of tests to identify chemicals with potential carcinogenicity. However, these methods have proven to be both costly and labor intensive (Rivenbark and Coleman, 2007; Uehara et al., 2008). It is therefore valuable if the maximum amount of information can be acquired from each test. One approach to this issue has been cDNA microarray analysis, a high-content technology which can simultaneously monitor expression changes across the whole genome. Following statistical analysis of the underlying data, characteristic gene expression patterns of chemical action may be generated. These are called gene signatures, and they allow subsequent rapid identification of specific chemicals (Byoung et al., 2007; Plant, 2008; Aardema and MacGregor, 2002). Recent cDNA microarray analysis of chemically induced cancers has proven that the profile of gene expression may also be characteristic of the etiological carcinogen. Rat mammary cancers induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 7,12-dimethylbenz[*a*] anthracene (DMBA) could be distinguished by comparison of their gene expression profiles with clustering analysis (Kuramoto et al., 2002; Shan et al., 2002). Even when rat mammary cancers were induced by multiple chemical carcinogens, they were still classified into groups that exactly coincided with the carcinogens used to induce them irrespective of the cancer histopathology (Shan et al., 2005a,b). These reports revealed carcinogen-specific gene expression profiles in

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chemically induced cancers and suggested the possibility that distinct carcinogenic agents could be retrospectively estimated from their expression patterns.

Moreover, there are a growing number of reports on the clarification of cDNA microarray patterns induced by non-genotoxic carcinogen in short-term animal studies, where the gene expression alterations provided clues to filter the genomic-based signatures that may predict the mode of toxic action for specific chemicals at the early stage (Fielden et al., 2007; Nie et al., 2006). However, these predictive tools are not intended to replace the chronic and sub-chronic bioassays for carcinogenicity, because carcinogenic effects of non-genotoxic carcinogens are only manifested over a long period of exposure (Fielden et al., 2008). Thus the chemically induced tumor tissues or malignant cell populations become the reasonable objects of detecting the possible gene signatures that may reflect the distinct carcinogens. The etiology-specific profiles identified in cancers induced by genotoxic carcinogens therefore raised the question of whether non-genotoxic carcinogens, including tumor promoting agents, likewise induce characteristic gene expression profiles in produced tumor tissues or transformed cell lines that are resolvable by microarray analysis.

12-*O*-tetradecanoylphorbol-13-acetate (TPA), okadaic acid (OA), and cadmium (Cd) have been reported to promote carcinogenesis in experimental animals and/or enhance cell transformation *in vitro* (DiGiovanni, 1992; Sakai et al., 2003; Fang et al., 2001a, 2002; Isani et al., 2009). TPA has been widely used as a model compound for studying the mechanism of action of tumor promoter. It is a phorbol ester agent which activates protein kinase C (PKC) and exert numerous cellular effects (Ikuta et al., 2008). OA, which is a potent inhibitor of types 1, 2a and 3 serine/threonine protein phosphatases, is regarded as a non-TPA-type tumor-promoting compound (Fujiki et al., 2000; Boudreau and Hoskin, 2005; Messner et al., 2006). Different from TPA and OA, Cd is a poisonous transition metal with promoting capacity which partially caused by diverse cellular signal transduction (Fang et al., 2001b; Yang et al., 2008). Thus the mechanisms of tumor promoting effects differ among these three chemicals. In this study, we analyzed the expression profiles of nine transformed foci induced by TPA, OA or cadmium chloride ($CdCl_2$) in two-stage transformation of BALB/c 3T3 cells using cDNA microarrays, and we classified these foci based on their gene expression profiles to estimate whether the profile reflect the specific promoting agent. Meanwhile, common gene expression alterations for all three tumor promoters were determined by comparing the profiles in normal cells to TPA-, OA-, or $CdCl_2$-induced transformed foci to gain insight into the possible molecular basis associated with the cell transformation, a process which has been recognized as being directly relevant to carcinogenesis.

2. Materials and methods

2.1. Chemicals and cell culture

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and okadaic acid (OA) from Calbiochem–Novabiochem Co. (San Diego, CA, USA), cadmium chloride ($CdCl_2$) from Shanghai Chemical Co. (Shanghai, China). These chemicals were dissolved in dimethyl sulphoxide (DMSO, from Sigma Chemical Co.) before addition to the cultures. The final concentration of DMSO was 0.1% of the medium.

BALB/c 3T3 clone A31 cells were obtained from Cell Bank, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Frozen stock ampules of the cells were thawed and used for each experiment before four passages. DMEM medium (from Gibco/BRL Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% heat-inactivated bovine calf serum (BCS, from Gibco/BRL Life Technologies Inc.) was used throughout the assays.

2.2. Two-stage transformation assay

The two-stage transformation assay was carried out to examine the promoting activity of chemicals according to the previously described protocol (IARC/NCI/EPA Working Group, 1985; Dunkel et al., 1991). In brief, actively growing cells were seeded at a density of 10^4 cells per 60-mm dish (eight dishes for each treatment condition) in 5 ml of culture medium. Twenty-four hours after seeding, the cells were treated with 1 μg/ml MNNG or 0.1% DMSO for 4 h (initiating treatment), and then were grown in normal medium for 7 days. The cultures were then treated with medium containing a tumor promoter of test or 0.1% DMSO for 14 days (promoting treatment). Subsequently, the cells were cultured in normal medium for an additional 14 days. The medium was changed twice a week during the experiment. Four groups were designed: DMSO/DMSO (treated with DMSO plus DMSO); MNNG/DMSO (treated with MNNG plus DMSO); DMSO/tumor promoter (treated with DMSO plus tumor promoter); and MNNG/ tumor promoter (treated with MNNG plus tumor promoter). For transformation determination, the cells were fixed with methanol and stained with Giemsa solution. Type III transformed foci were identified and calculated according to the criteria recommended by Dunkel et al. (1991).

To estimate the cytotoxicity of each treatment, the colony-forming efficiency (CFE) was examined in parallel with the transformation assay. Briefly, cells were inoculated at 200 cells per 60 mm dish (three dishes for each treatment condition). After 24 h of incubation, the cells were treated with 1 μg/ml MNNG or DMSO for 4 h, then with a test promoter after removal of the initiator. The cultures were fixed and stained 9–11 days after seeding and colonies were counted. Only colonies comprised of more than 50 cells were scored. The CFE is the percentage of colonies with respect to seeded cells. Transformation frequency (TF) was finally calculated as the number of transformed foci among the number of cells at risk after chemical treatment. Statistical analysis was performed by the Student's *t*-test for transformed foci per dish.

2.3. Transformed foci selection and soft agar colony formation assay

At the end of incubation of the transformation test, a number of type III transformed foci produced in different cultures treated with MNNG plus TPA, MNNG plus OA, or MNNG plus $CdCl_2$ were trypsinized and removed from dishes using cloning rings for subculture. Non-transformed cells treated with DMSO plus DMSO were isolated at the same time as the control cells. All of the isolated cells were expanded for four passages, and then anchorage-independent growth of cells was evaluated by colony formation in soft agar. Briefly, transformed cells and control cells were trypsin-dispersed and suspended in DMEM medium containing 0.35% agar, and then seeded onto a basal layer of medium containing 0.7% agar in a 24-well microplate. For each population of cells, four wells were seeded at 500 cells per well and three independent experiments were carried out. Cells were incubated at 37 °C for up to 14 days and fed with fresh DMEM twice a week. Colonies formed in the soft agar were scored microscopically 14 days after seeding and CFE was calculated as a proportion of the original cell number seeded.

2.4. cDNA microarray analysis

Total RNA was extracted from cells subcultured from transformed foci, which were verified by anchorage-independent