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Gene expression analysis of terminal differentiation of human melanoma cells highlights global reductions in cell cycle-associated genes

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ABSTRACT

Defects in differentiation are frequently observed in cancer cells. By appropriate treatment specific tumor cell types can be induced to terminally differentiate. Metastatic HO-1 human melanoma cells treated with IFN-β plus mezerein (MEZ) undergo irreversible growth arrest and terminal differentiation followed by apoptosis. In order to define the molecular changes associated with this process, changes in gene expression were analyzed by cDNA microarray hybridization and by semi-quantitative and quantitative RT-PCRs of representative 44 genes. The expression of 210 genes was changed more than two-fold at either 8 or 24 h post-treatment (166 up and 44 down). Major biological processes associated with the up-regulated genes were response to endogenous/exogenous stimuli (38%), cell proliferation (13%), cell death (16%) and development (30%). Approximately 34% of the down-regulated genes were associated with cell cycle, 9% in DNA replication and 11% in chromosome organization, respectively. Suppression of cell cycle associated genes appeared to directly correlate with growth arrest observed in the terminal differentiation process. Expression of Calpain 3 (CAPN3) variant 6 was suppressed by the combined treatment and maintained high in various melanoma cell lines. However, over-expression of the CAPN3 did not significantly affect growth kinetics and cell viability, suggesting that up-regulation of CAPN3 alone may not be a causative, but an associated change with melanoma development. This analysis provides further insights into the spectrum of up-regulated and the first detailed investigation of down-regulated gene changes associated with and potentially causative of induction of loss of proliferative capacity and terminal differentiation in human melanoma cells.

1. Introduction

Normal programs of differentiation are frequently altered in tumor cells. Depending on tumor type, specific cancer cells can be induced to terminally differentiate by appropriate pharmacological treatment(s), which, in some cases, is followed by apoptotic cell death (Fisher et al., 1985; Hass, 1994; Spira and Carducci, 2003; Kang et al., 2004). 'Differentiation therapy of cancer' is an attempt to eradicate tumor cells based on induction of irreversible growth suppression (and apoptosis) of tumor cells by treatment with cell-type specific differentiation agents (Leszczyniecka et al., 2001; Spira and Carducci, 2003).

Type I interferons, IFN-α and -β, are potent antiviral reagents that promote strong antiviral innate and adaptive immune responses (Stetson and Medzhitov, 2006). In conjunction with their antiviral activity, type I IFNs have pleiotropic actions on cell physiology including inhibition of cell proliferation, and induction of apoptosis and cell differentiation, which has been exploited in attempts to use these cytokines for therapeutic applications in cancer by themselves or in conjunction with conventional chemotherapeutic reagents (Vannucchi et al., 2007).

Mezerein (MEZ) is an antileukemic reagent that can induce differentiation of human promyelocytic leukemia cells into macrophage-like cells (Rovera et al., 1979). MEZ is a non-phorbol diterpene ester similar to 12-O-tetradecanoylphorbol-13-acetate (TPA) in chemical structure and biological activity (Klein-Szanto et al., 1980). MEZ can bind and activate certain PKC isoforms (PKCα and β) as does TPA and the biological activity of MEZ is mostly ascribed to the activation of PKC (Klein-Szanto et al., 1980; Miyake et al., 1984).

Treatment of HO-1 metastatic human melanoma cells with IFN-β induces a reversible differentiation phenotype that corresponds with

Abbreviations: EtBr, Ethidium bromide; IFN, interferon; MEZ, mezerein; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; RT-PCR, reverse transcription-polymerase chain reaction; PKC, protein kinase C; RACE, Rapid Amplification of cDNA Ends; SAM, Significance Analysis of Microarray; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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decreased cell proliferation and increased melanogenesis (Fisher and Grant, 1985; Fisher et al., 1985). Similarly, HO-1 cells treated with low-dose MEZ also develop a reversible differentiation phenotype, which also includes a reduction in growth rate and an increase in the formation of dendrite-like processes (not seen with IFN-β) (Fisher et al., 1985). In contrast to a single treatment, combined treatment with IFN-β and low-dose MEZ induces terminal differentiation of HO-1 cells resulting in irreversible growth arrest, development of multiple phenotypes characteristic of differentiated melanocytes and eventually apoptosis (Fisher et al., 1985; Kang et al., 2004).

Terminal differentiation of HO-1 cells by IFN-β plus MEZ correlates with significant alterations in gene expression (Jiang and Fisher, 1993; Huang et al., 1999a,b; Jiang et al., 2000). Identification and functional characterization of these altered genes provides an entry point for molecularly defining the process of terminal differentiation that could help identify novel targets for cancer therapy. Analysis of temporally-spaced subtractive cDNA libraries enriched for up-regulated genes during terminal differentiation identified numerous melanoma differentiation associated (*mda*) genes including *mda*-6, the p21 CDK inhibitor; *mda*-5, an intracellular dsRNA receptor; *mda*-7/IL-24, a novel cancer-specific apoptosis-inducing cytokine; and *hPNPase*, a *myc*-targeting RNase that induces senescence and apoptosis (Jiang et al., 1995a,b; Kang et al., 2002; Leszczyniecka et al., 2003; Fisher, 2005).

Even though previous experiments were successful in identifying up-regulated genes, systematic studies focusing on genes suppressed during terminal differentiation have not been described (Jiang and Fisher, 1993; Huang et al., 1999a,b; Jiang et al., 2000). In addition, gene expression analysis was limited to 1,400 cDNA clones, which is not exhaustive with respect to the proposed number of functional genes in the human genome. In order to more comprehensively identify genes down-regulated as well as up-regulated during the terminal differentiation of HO-1 cells, we compared gene expression profiles between untreated and 8 or 24 h IFN-β plus MEZ-treated cells by competitive hybridization of high density cDNA microarrays followed by confirmatory RT-PCR. We now report the expression pattern of additional up-regulated genes and for the first time newly identified down-regulated genes potentially involved in terminal differentiation of human melanoma cells.

2. Materials and methods

2.1. Cell culture

Culture conditions of FM-516SV immortalized melanocytes and human melanoma cell lines including HO-1 were described previously (Kang et al., 2004). Cells were either untreated or treated with IFN-β (2000 U/ml), mezerein (MEZ, 10 ng/ml, Sigma) or a combination of IFN-β+MEZ (2000 U/ml+10 ng/ml).

2.2. RNA preparation

RNA was extracted from cells using WelPrep™ Total RNA isolation reagent (JBI, Seoul, Korea) as described in the manufacturer's protocol. Quality of RNA was determined by A_{260}/A_{280} ratio and visual inspection of the integrity of 28S and 18S rRNA bands resolved by electrophoresis on 1% agarose gels containing 0.66 M formaldehyde.

2.3. cDNA microarray analysis

A 24K cDNA microarray containing 24,288 features derived from KUGI unigene library (http://kugi.kribb.re.kr) was used for global gene expression analysis as previously described (Lee et al., 2007). Total RNA (20 μg each) extracted from untreated HO-1 cells were labeled with Cy-5, while the RNA from 8 or 24 h treated cells were labeled with Cy-3 or vice versa using 3DNA Expression Array Detection Kit (Genisphere). Pairwise Cy-3 and Cy-5 labeled cDNAs were hybridized to cDNA microarray at 65 °C for 16 h in Array Chamber X (GenomicTree, Korea). Significantly changed genes were identified using Significance Analysis of Microarray (SAM) for average intensity $A>8$ spots in one class data option. Delta was set for zero false discovery rate and cutoff value of 2-fold change across the samples was applied (Tusher et al., 2001). Gene ontology analysis for SAM selected genes was performed on the web at Database for Annotation, Visualization and Integrated Discovery (DAVID) of National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH), USA (http://david.niaid.nih.gov/david/ease.htm). Additional information of individual gene function was obtained at GeneCards (http://www.genecards.org/index.shtml).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (5 μg) was reverse-transcribed with oligo dT primer using SuperScript® RT II (Invitrogen) as described in the manufacturer's protocol. An equal aliquot of cDNA was used in PCR with the following conditions: 94 °C for 2 min, 25–35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and final incubation for 5 min at 72 °C. The PCR products were separated by electrophoresis on 1% agarose gels, stained with EtBr and visualized with Gel Documentation System (BioRad). Real time RT-PCR was performed by incubation at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 56 °C for 5 s and 72 °C for 20 s with Light Cycler 2.0 (Roche) using QuantiFast SYBR Green PCR kit (Qiagen). Relative mRNA amount was calculated by $1/2^{CT}$ of a specific gene/$1/2^{CT}$ of GAPDH. Specificity of PCR products was determined by melting curve analysis, agarose gel electrophoresis and sequencing of the PCR products. Sequence of primers used in RT-PCR is described in Supplemental Table 1.

2.5. Northern blot hybridization

Northern blot hybridization with 10 μg total RNA resolved in 1% agarose gels containing 0.66M formaldehyde was performed as described in Kang et al. (2004). PCR products of CAPN3 (0.85 kb) and GAPDH (0.25 kb) were radiolabeled using Rediprime™ II Random Prime Labeling kit (Amersham Biosciences) with [α-^{32}P]dCTP and used as probes.

2.6. MTT assay

Cells were seeded in 96-well plates at 2000 cells/well one day before treatment and treated as specified in the figure legends. MTT assays were carried out as described in Kang et al. (2004) and absorbance at 595 nm was measured with VERSAMAX Tunable Microplate Reader (Molecular Devices).

2.7. Western blotting analysis

Western blot analysis with 40 μg protein per lane resolved in 12.5% SDS-PAGE was performed as in Kang et al. (2004). Antibodies used were anti-MYC antibody (1/1000 dilution, IG Therapy Co., Korea), anti-β-actin (1/1000 dilution, Santa Cruz Biotech) and HRP-conjugated anti-mouse antibody (1:5000, Amersham Biosciences).

2.8. Cloning CAPN3 cDNA.

CAPN3 cDNA was cloned with SMART® 5′RACE (ClonTech) as described in the manufacturer's protocol. Reverse transcribed cDNA was subjected to two successive PCRs with CAPN3 R1 (ccacagcagtagaacccagt)/universal primer mix (UPM) and CAPN3 R2 (cggatcctcaggcatacatggtgagctgca)/nested universal primer (NUP). The PCR product