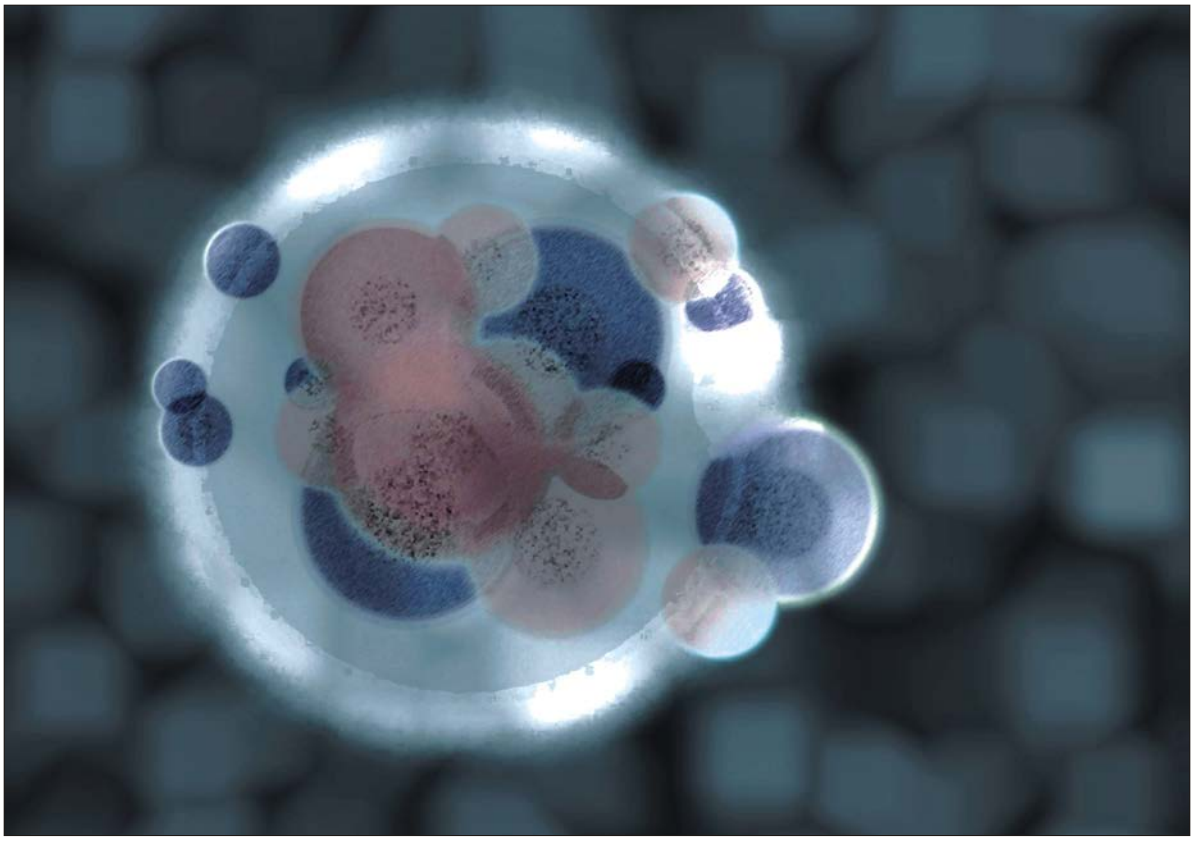

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Induction of Gene Expression Alterations by Culture Medium from Trypsinized Cells

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Abstract: This study hypothesized that trypsin treatment itself could be a stress inducer before any other physical or chemical mediated stress is introduced in a clonogenic assay performed on cells grown in culture as a monolayer. To further understand the role of trypsin treatment, we incubated adherent cells with conditioned growth medium isolated from trypsinized cells after several hours of trypsin action and examined global gene expression profile with microarray technology. Microarray data identified large-scale gene expression alterations in cells receiving conditioned medium from trypsin treated cells compared to control cells that did not receive such medium. Twenty eight genes were found to be upregulated with at least two-fold change in the expression level, while 70 genes were downregulated. Gene expression signature clearly identified stress response. Taken together this data cautions the contribution of background stress while assessing the effects of radiation, certain drugs or environmental mutagens. Further attention is required while determining the role of conditioned medium in elucidating radiobiological phenomenon such as bystander effect.

Key words: Clonogenic assay, cell survival assay, microarrays, gene expression, stress response

INTRODUCTION

Clonogenic or cell survival assay technique is an effective way of determining the effects of physical agents like radiation or chemical agents like drugs, mutagens etc. on the proliferation of cells grown in culture. The use of cell culture based assays began over 50 years ago. These assays can be divided into two categories: those that measure cell survival and those that measure cytotoxicity (Plumb, 2004). The clonogenic cell survival assays have been described for studying the effects of chemical mutagens and ionizing radiation on cells (Chaudhry and Fox, 1990). A typical experiment involves plating of the cells, application of the treatment, a time window to observe visible cell colonies (usually 7-10 days) followed by fixing, staining with crystal violet and counting of cell colonies containing >50 cells and normalizing to the plating efficiency of control and treatment dishes. Mathematical calculations are done to determine the percentage of cells that survived the treatment. Cell survival data are generally plotted as logarithm of the surviving fraction versus dose. A graphical representation of survival versus drug concentration or dose of radiation is called a cell survival curve and defines relationship between the dose to produce an effect and the fraction of cells retaining their ability to reproduce (Munshi *et al*., 2005).

Modern developments in the application of clonogenic assay include drug development. The cytotoxic effect of different concentrations of a new drug at different exposure durations is assessed by a clonogenic assay (Subramanian *et al*., 2006). Clonogenic assay has been used in high throughput screening of new anticancer agents (Zips *et al*., 2005) and to predict the adverse effects by granulocyte-macrophage colony-forming unit (CFU-GM) clonogenic assay (Negro *et al*., 2001), reviewed in (Parent-Massin, 2001). The classical evaluation of a drug hematotoxic effect is monitored with cell survival assays (Pessina *et al*., 2005). The use of metabolically competent cells allows to assess potential drug side effect. Simple cytotoxicity assays in HepG2 cells (Xu *et al*., 2004) are indicative of hepatotoxicity and provide simple solution to predict adverse events. Clonogenic survival assays have been used to select stem cells for transplantation (Michejda, 2004) and to determine the effects of radiation exposure (Olive, 1998; Skov, 1999).

To monitor effects on adherent cells grown as monolayer, the cells are first trypsinized and counted. Single cell suspension is then made and cells are plated at required densities. From this point there are two ways the cells are proceeded with the experimental treatment. Either the cells are directly treated with radiation or drugs or the cells are allowed to attach with the culture dish for one or more hours followed by radiation or drug treatment. We

argued that trypsinization of cells itself poses a stress in addition to the stress imposed by radiation or drug treatment. In the present investigation we examined the gene expression profile of cells incubated with growth medium from trypisinized cells to understand the effect of stress induced by trypsin incubation.

MATERIALS AND METHODS

Human normal lung fibroblast cell line HFL1 was purchased from American Type Culture Collection (ATCC) (Manassas, VA) and was cultured according to ATCC recommended conditions. Gene Chips (U133A) were purchased from Affymetrix, Santa Clara, CA. Target preparation, microarray processing and data analysis were done as previously described (Chaudhry *et al*., 2002). Exponentially growing HFL1 cells were trypsinized and centrifuged to remove previous medium. The cells were suspended in fresh medium with serum, plated and incubated at 37°C for 4 h. By that time the cells were attached with the culture dish as examined under the microscope. The medium from these cells was collected and was used to replace a parallel HFL1 culture growing as a monolayer and that had not been trypisinized. The cells receiving the conditioned medium were further incubated at 37°C for additional 4 h. After that time total RNA was harvested from these cells by directly adding Trizol reagent (Invitrogen Inc.) to the culture dish without any trypsin treatment. The control cells in this experiment were a parallel culture growing as a monolayer that did not receive any conditioned medium. The RNA from control and conditioned medium incubated cells was first evaluated on Bioanalyzer 2100 (Agilant) for quality control before subjecting to microarray based gene expression profiling. The targets for probe array hybridization on Affymetrix GeneChips were prepared with high quality RNA. The quality of the target was first evaluated with the Affymetrix Test 3 chip prior to hybridization on Affymetrix U133A GeneChips.

The statistical algorithms of Affymetrix microarray suite 5 (MAS 5) were used to determine the presence, absence and fold change of each transcript. The resultant absolute data files were used for comparison analysis between control and conditioned medium receiving cells. The absolute analysis uses probe pair intensities to generate a detection p-value to determine the presence or absence call. In comparison analysis, two sets of algorithms were used to generate change significance and change quantity metrics for every probe set. The change algorithms compare each probe set on the experimental array to the baseline array and a change p-value is calculated (Wilcoxon's signed-rank test), indicating an increase, decrease, or no change in gene expression. The signal-log ratio estimates the magnitude and direction of change of a transcript, which is computed using the one-step Tukey's Biweight method. The fold change was calculated from the signal-log ratio. The arrays were normalized before conducting comparisons to correct for variation between arrays using MAS 5.0. All the altered genes were selected based on the detection and the change calls in three experiments. The experiments were independently run in triplicates and the genes observed in all the experiments were catalogued.

The probe set ID was used to annotate all the genes using the Affymetrix Netaffx Analysis Center Website (http://www.affymetrix.com/analysis/ index.affx).

RESULTS AND DISCUSSION

Altered gene expression using the criteria of at least 2-fold change resulted in the identification of a number of upregulated and downregulated genes in cells receiving conditioned culture medium. More genes were downregulated compared to the upregulated genes monitored for expression analysis. The expression of 70 genes was downregulated (Table 1) and 28 genes were upregulated (Table 2). The categories of modulated genes belong to stress response pathways, metabolism, cell signaling, growth factors, transcription, cell communication and unknown biological functions.

Gene expression signature clearly identified stress response in the cells receiving conditioned medium from trypisinized cells. Many genes belonging to signal transduction pathways were modulated. STAT-induced STAT inhibitor 2 was downregulated (Table 1). The signal transducers and activators of transcription (STAT) factors function as modulators of cytokine signaling and act as sensors responding to cellular stress (Stephanou, 2004). It has been shown that oxidative stress-evoked signal transduction pathways leads to the activation of STATs (Burova *et al*., 2003). Alterations in the expression of genes belonging to the Ras signaling pathway were observed. Ras homolog gene family, member I, Ras-related associated with diabetes and GTP-binding protein were downregulated (Table 1). Rho family GTPases are key signal transducers that regulate a variety of cellular responses, including changes in gene expression (Cernuda-Morollon and Ridley, 2006) and are induced by proinflammatory mediators, hypoxia, or shear stress. Rho family GTPases (Rho, Cdc42 and Rac) and also STAT act as mediators of cytokine and growth factor signaling in a variety of cellular functions involved in inflammation, tumorigenesis and development (Debidda *et al*., 2005). TGF-beta-induced membrane ruffles occur via Rho

Table 1: Downregulated genes in cells incubated with growth medium from trypisinized cells

Gene identifier	Gene name	Ratio	Function
BE965369	IMAGE: 3895653	10.07	Unknown
AL556409	cDNA clone CS0DK004YA08	5.38	Cell communication
U46768	Stanniocalcin 1	5.16	Cell communication
AI300520	IMAGE: 1902178	4.63	Cell communication
NM_004267	N-acetylglucosamine sulfotransferase 2	4.40	Catalytic activity
AU118882	cDNA clone HEMBA1004569	4.15	G-protein signaling
AB004903	STAT induced STAT inhibitor-2	4.01	Regulation of growth
M13436	Inhibin, beta A (activin A, activin AB)	3.92	Response to biotic stimulus
AK021882	Ras homolog gene family, member I	3.69	Cell communication
NM_001657	Amphiregulin	3.28	Cell communication
NM_005110	Glutamine-fructose-6-phosphate transaminase 2	3.23	Amino acid metabolism
NM_005242	Coagulation factor II receptor-like 1	3.02	G-protein signaling
NM_000963	Prostaglandin-endoperoxide synthase 2	3.01	Response to biotic stimulus
NM_000270	Nucleoside phosphorylase	2.92	DNA metabolism
AK026420	Desmuslin	2.91	Binding
NM_005627	Serum/glucocorticoid regulated kinase	2.87	Physiological process
NM_005261	GTP-binding protein	2.83	Response to biotic stimulus
BC000669	Hypothetical protein	2.83	Physiological process
NM_004165	Ras-related associated with diabetes	2.77	Intracellular signaling cascade
NM_001724	2,3-bisphosphoglycerate mutase	2.77	Hexose catabolis
AI189753	Tumor-Associated Antigen L6	2.77	Unknown
NM_003670	Basic helix-loop-helix domain	2.73	Transcription
NM_001957	Endothelin receptor type A	2.72	G-protein signaling
NM_025195	Phosphoprotein	2.71	Protein metabolism
AF022375	Vascular endothelial growth factor	2.64	Regulation of biological process
BF514079	cDNA clone IMAGE:3071198	2.62	Transcription
N74607	cDNA clone IMAGE:296424	2.61	Physiological process
NM_006997	Transforming, acidic coiled-coil 2	2.59	Cellular component
NM_005904	MAD 7	2.55	Serine/threonine kinase signaling
NM_000877	Interleukin 1 receptor, type I	2.54	Response to biotic stimulus
NM_016109	Angiopoietin-like 4	2.54	Response to biotic stimulus
NM_014918	Chondroitin synthase	2.53	Unknown
NM_005842	Sprouty (Drosophila) homolog 2	2.53	Cell communication
NM_002422	Matrix metalloproteinase 3	2.51	Proteolysis and peptidolysis
M90657	Transmembrane 4 superfamily member 1	2.51	Unknown
NM_016651	Heptacellular carcinoma novel gene-3	2.51	Cellular component
AF119835	KIT ligand	2.48	Cell communication
NM_004973	Jumonji (mouse) homolog	2.41	Morphogenesis
AA293502	IMAGE: 726107	2.38	Cell communication
NM_018057	Homolog of rat orphan transporter v7-3	2.35	Physiological process
NM_003155	Stanniocalcin 1	2.34	Cell communication
U77914	Jagged 1 (Alagille syndrome)	2.33	Regulation of biological process
NM_002421	Matrix metalloproteinase 1	2.28	Proteolysis and peptidolysis
NM_006732	FBJ murine osteosarcoma viral oncogene	2.25	Physiological process
NM_024616	Hypothetical protein FLJ23186	2.25	Unknown
NM_005737	ADP-ribosylation factor-like 7	2.23	Intracellular signaling cascade
AA583044	cDNA clone IMAGE:1090221	2.21	Cell communication
AI346835	Tumor-Associated Antigen L6	2.19	Unknown
NM_000641	Interleukin 11	2.18	Response to biotic stimulus
NM_002006	Fibroblast growth factor 2 (basic)	2.18	Cell communication
NM_021972	Sphingosine kinase 1	2.17	Cell communication
NM_006724	Mitogen-activated protein kinase kinase kinase 4	2.17	Cell communication
L03203	Peripheral myelin protein 22	2.15	Cell communication
AF133207	Protein kinase H11	2.14	Response to stress
NM_004694	Solute carrier family 16, member 6	2.14	Organic acid transport
NM_005655	TGFB inducible early growth response	2.13	Cell communication
J04621	Syndecan 2	2.12	Unknown
AF247704	NK homeobox (*Drosophila*), family 3, A	2.11	Transcription
BF445047	cDNA clone IMAGE:3366330	2.08	Physiological process
U88964	Low level estrogen modulated HEM45	2.05	Physiological process
BC000006	ATPase, Na+/K+ transporting, beta 1	2.05	Physiological process
NM_005502	ATP-binding cassette, sub-family A	2.00	Sterol metabolism
AW972765	EST 384859	2.00	Unknown
NM_003247	Thrombospondin 2	2.00	Cell communication

Table 1: Continued

Gene identifier	Gene name	Ratio	Function
NM_004415	Desmoplakin (DPI, DPII)	1.99	Epidermis development
NM_017664	Hypothetical protein FLJ20093	1.98	Unknown
NM_004566	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1.97	Hexose metabolism
NM_015675	Growth arrest and DNA-damage-inducible, beta	1.97	Cell communication
AB029551	RING1 and YY1 binding protein	1.97	Transcription
NM_001423	Epithelial membrane protein 1	1.97	Physiological process
BG029530	cDNA clone IMAGE: 4391375	1.95	Morphogenesis
J03241	Transforming growth factor, beta 3	1.95	Cell communication

Table 2: Upregulated genes in cells incubated with growth medium from trypisinized cells

Gene identifier	Gene name	Ratio	Function
AW592266	IMAGE: 2935134	4.75	Transcription
NM_001078	Vascular cell adhesion molecule 1	3.06	Cell adhesion
NM_014890	Downregulated in ovarian cancer 1	3.05	Unknown
NM_002852	Pentaxin-related gene, induced by IL-1 β	2.93	Response to biotic stimulus
AL136842	Cdc42 effector protein 3	2.93	Cell communication
NM_001709	Brain-derived neurotrophic factor	2.90	Morphogenesis
AI961231	IMAGE: 2507536	2.73	Transcription
AI754416	Jia bone marrow stroma HBMSC	2.53	Cell communication
NM_005195	CCAAT/enhancer binding protein (C/EBP), delta	2.50	Unknown
AF247168	Hypothetical protein dJ465N24.2.1	2.38	Unknown
S69738	Cytokine A2 (monocyte chemotactic 1)	2.35	Response to biotic stimulus
BF791738	IMAGE: 4344389	2.26	Unknown
AF095192	BCL2-associated athanogene 2	2.17	Protein metabolism
AB016517	Fibroblast growth factor 5	2.14	Cell communication
AY014180	E3 ubiquitin ligase SMURF2	2.13	Proteolysis and peptidolysis
NM_000104	Cytochrome P450, subfamily I	2.08	Physiological process
NM_002742	Protein kinase C, mu	2.05	Cell communication
AW269335	IMAGE: 2772777	2.04	Cell communication
NM_005757	C3H-type zinc finger protein	2.04	Nucleic acid binding
AK025843	Palladin	2.03	Unknown
NM_006350	Follistatin	2.03	Cell-cell signaling
AK023621	KIAA0878 protein	2.03	Unknown
NM_006079	Cbp/p300-interacting transactivator	2.02	Physiological process
NM_014350	TNF-induced protein	2.01	Apoptosis
AL136139	Enhancer of filamentation (HEF1)	2.01	Unknown
NM_017709	Hypothetical protein FLJ20202	1.99	Unknown
NM_001290	LIM domain binding 2	1.96	Development
NM_002053	Guanylate binding protein 1	1.96	Response to biotic stimulus

GTPase-dependent pathways, whereas long-term effects require cooperation between Smad and Rho GTPase signaling pathways (Edlund *et al*., 2002). Small GTPase Rho and its downstream effector ROCK (Rho-associated coiled-coil-containing protein kinase) mediate vascular endothelial growth factor (VEGF) induced hyperpermeability (Sun *et al*., 2006). VEGF was also found to be downregulated (Table 1). Recent studies have shown that stress induced by reactive oxygen species modulates the expression of VEGF (Lee *et al*., 2006). Mitogen activated protein kinase 4 was downregulated (Table 1). The dual specificity kinases Mitogen-Activated Protein Kinase (MAPK), kinase MKK7 and MKK4 are known to directly activate stress-activated protein kinases (SAPKs)/c-Jun N-terminal kinases (JNKs) in response to environmental or mitogenic stimuli.

The upregulation of protein kinase C (PKC) was identified in the present study (Table 2). Increased activity of membranous protein kinase C (PKC) and increased oxidative stress have been proposed to explain the adverse effects of hyperglycemia which can induce the stimulation of L-type Ca_2+ channel via G protein-coupled adenylyl cyclase/cAMP and phospholipase C/PKC pathways (Hashim *et al*., 2006). Oxidative stress induced by H_2O_2 activates protein kinase D (PKD) via a protein kinase C (PKC)-dependent signal transduction pathway (Waldron and Rozengurt, 2000). Protein kinase D protects against oxidative stress via Rho/ROK/PKC pathway activation (Song *et al*., 2006). Oxidative stress induces PKD activation and upstream PKC-delta and Rho/ROK pathways regulate this activation. It has been suggested that PKD activation protects from oxidative stress-induced apoptosis (Song *et al*., 2006).

The expression level of a number of growth factors was affected. Transforming growth factor beta and its family member activin were downregulated (Table 1). Activins cause the activation of RhoA and CDC42, leading to MEKK1-dependent phosphorylation of JNK and transcription factor c-Jun (Zhang *et al*., 2005). Recently, it was reported that activin may have cell

survival function (Fukuda *et al*., 1998). Activin receptor-like kinase (ALK) 7 can induce apoptosis by activating traditional TGF-beta pathway components Smad, MAPKs JNK and p38 that initiates cross-talk with the cellular stress death pathway (Kim *et al*., 2004). ALK is also implicated in angiogenesis (Lamouille *et al*., 2002). The expression of amphiregulin was altered (Table 1). Recently it was shown that Epidermal Growth Factor Receptor (EGFR) ligand amphiregulin is induced by diesel exhaust particles (Blanchet *et al*., 2004). The basic fibroblast growth factor (bFGF) was also modulated (Table 1). It has been reported that excessive stress inhibits the production of bFGF (Fujiwara *et al*., 2005).

The alteration in the expression of various transcription factors was also seen in the present study (Table 2). Inflammatory cytokines such as interleukin-1 and tumor necrosis factor-alpha modulate a transcription factor cascade to induce and sustain a stress response (Yang *et al*., 2006). The transcription factors involved were STAT, CCAAT/enhancer-binding protein (C/EBP) and Cbp/p300. Inflammatory cytokines become a trigger for the production of phospholipase A2, eicosnoids, NO, endothelin-1, thrombomodulin, polymorphonuclear leukocyte and adhesion molecules. These mediators can cause complex pathophysiologic conditions. The expression of Basic-Helix-Loop-Helix-Leucine Zipper transcription factor was altered (Table 1). This family of transcription factors participate in distinct transcriptional processes and are key regulators of a wide number of gene regulation networks, including the stress (Corre and Galibert, 2005).

The expression of matrix metalloproteinase 1 and 3 was altered in the cells incubated with conditioned media (Table 1). The expression of MMP-1 and MMP-3 has shown to be induced by heat shock (Park *et al*., 2004). We also identified alteration in the expression of endothelial adhesion molecules (Table 1). Other studies have shown alterations in the expression of endothelial adhesion molecules as result of superoxide dismutase treatment (Segui *et al*., 2005) It has been suggested that oxidative stress and endothelial dysfunction could play a role in the pathological processes (Couillard *et al*., 2005). Brain-derived Neurotrophic Factor (BDNF) was found to be modulated (Table 1). Exposure to stress decreases levels of BDNF and the upregulation of BDNF with other neurotrophic/growth factors could reverse or block the atrophy and cell loss (Duman, 2004). The upregulation of Bcl-associated athanogene (Table 2) indicates the involvement of this pathway. The Bcl-2-associated athanogene (BAG) proteins are a family of chaperone regulators that modulate a number of diverse processes ranging from proliferation to growth arrest and cell death. BAG-1 interact with the HSC70 and HSP70 heat shock proteins and have been proposed to promote cell survival by coordinating the function of these chaperones with the proteasome to facilitate protein degradation. Alterations in inducible heat shock protein and BAG-1 has been shown as a stress response in injured brain (Seidberg *et al*., 2003).

The alterations in the expression of a number of genes belonging to several cellular pathways involved in stress response indicates that trypsin treatment induces stress in the cells. These cells release factor(s) in the culture medium that in turn impacts the overall cellular physiology. Standard established methods for clonogenic survival assay are employed for many applications (Lu and Wong, 2005). If adherent cells are under investigation and trypsin treatment is needed to prepare cells, then one must be careful in determining the effects of a certain treatment, considering there could be a background stress due to trypsin incubation. The results reported in the present study have clearly indicated that the process of trypsin application itself can lead to alterations in gene expression. Majority of the genes with alterations in the expression levels are implicated in stress response. The other concern is for the studies where conditioned medium is employed to investigate radiobiological phenomenon such as bystander effect. The treatment with irradiated conditioned medium, i.e., feeding of unexposed cells with medium taken from irradiated cultures (Ballarini *et al*., 2002) has been a popular experimental choice for such studies. Caution should be undertaken and rigorous controls must be included at each stage of the experiment to allow the observation of treatment related biological effects.

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