Apoptosis-Induced Gene Profiles of a Myeloma Cell P3-X63-Ag8.653

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ABSTRACT

Background: Apoptosis is a physiologic phenomenon involved in development, elimination of damaged cells, and maintenance of cell homeostasis. Deregulation of apoptosis may cause diseases, such as cancers, immune diseases, and neurodegenerative disorders. The mouse myeloma cell P3-X63-Ag8.653 (v653) is an HGPRT deficient (HGPRT$^-$) mutant strain. High dependency on de novo transcription and translation of aminopterin induced apoptosis of this cell seems to be an ideal experimental system for searching apoptosis-induced genes. **Methods & Results:** For searching apoptosis-related genes we carried out GE-array (dot blot), Affymetrix GeneChip analysis, Northern analysis and differential display-PCR techniques. The chip data were analyzed with three different programs. 66 genes were selected through Affymetrix GeneChip analyses. All genes selected were classified into 8 groups according to their known functions. They were Genes of 1) Cell growth/maintenance/death/ enzyme, 2) Cell cycle, 3) Chaperone, 4) Cancer/disease-related genes, 5) Mitochondria, 6) Membrane protein/signal transduction, 7) Nuclear protein/nucleic acid binding/ transcription binding and 8) Translation factor. Among these groups number of genes were the largest in the genes of cell growth/maintenance/death/enzyme. Expression signals of most of all groups were peaked at 3 hour of apoptosis except genes of Nuclear protein/nucleic acid binding/ transcription factor which showed maximum signal at 1 hour. **Conclusion:** This study showed induction of wide range of proapoptotic factors which accelerate cell death at various stage of cell death. In addition apoptosis studied in this research can be classified as a type 2 which involves cytochrome c and caspase 9 especially in early stages of death. But It also has progressed to type 1 in late stage of the death process. **(Immune Network 2006;6(3):128-137)**

Key Words: Myeloma, P3-X63-Ag8.653, apoptosis, GE-array, GeneChip analysis, Northern analysis, cytochrome c, caspase 9

Introduction

Apoptosis is a morphological phenomenon. As viewed with microscope, the characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation/pyknosis, plasma membrane blebbing, and cell shrinkage. Eventually, the cells breaks into small membrane-surrounded fragments/ apoptotic bodies, which are cleared by phagocytosis without inflammations (1). The mouse myeloma cell line P3-X63-Ag8.653 (v653) is an HGPRT deficient (HGPRT) mutant strain. This feature, along with a high fusion frequency, provides this cell with a benefit to be used as a fusion partner to generate hybridoma cells (2). While HGPRT+ cells are resistant to aminopterin in the presence of hypoxanthine and thymidine, HGPRT− cells are selectively cytotoxic in response to aminopterin (3).

It is evident that in many cases the process of apoptosis is dependent on RNA and protein synthesis in the dying cell, leading to speculation on the potential role of proapoptotic factors that are responsible for the phenomenon (4). In previous study, we demonstrated that aminopterin induced cell death through an apoptotic pathway in the cell line (5). This apoptotic pathway is shown to be dependent on de novo RNA and protein synthesis. The results have delineated possible mechanisms of aminopterin-induced negative selection occurred in HGPRT cells during the process of hybridoma generation. Apoptosis was drastically reduced by addition of actino-

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mycin D or cycloheximide, indicating that active RNA and protein synthesis is required for the apoptotic effect of aminopterin. Interestingly, the induction of c-myc gene expression preceded the activity of DNA fragmentation in aminopterin-treated cells (6). The results suggest that cells deficient in the salvage pathway of purine biosynthesis are susceptible to aminopterin-induced apoptosis that requires de novo synthesis of proapoptotic factors.

The present study was aimed to elucidate expression profiles and role of genes which were known to be involved in this apoptosis.

Materials and Methods

Cell culture. P3-X63-Ag8.653 HGPRT- mouse myeloma cells (ATCC CRL1580) were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin [all from Life Technologies, Gaithersberg, MD]. HAT media (1×10^{2} M hypoxznthine, 4×10^{3} M methotrexate, 1.6×10^{3} M thymidine) [Sigma, St. Louis, MO] were treated at different time points (0, 22.5, 60, 180, 300 min) (2).

Differential Display-PCR. Double-stranded cDNA was

Table I. Primers used for differential display-PCR

Kind	Primer
Adaptor-L	5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT-3'
Adaptor-S	5'-ACCGCCCTCCGC-3'
OU	5'-TGTAGCGTGAAGACGACAGAA-3'
T	5'-CGCAGTCGACCGTTTTTTTTTTTTT-3'
In	5'-AGGGCGTGGTGCGGAGGGCGGT-3'
InExx	5'-AGGGCGTGGTGCGGAGGGCGGTCCxx-3'
TExx	5'-CGCAGTCGACCGTTTTTTTTTTTTTxx-3'

Table II. Relative intensities of dot blots

Gene name	Time point (hour)				Remark (vs GeneChip)
	0	1	3	5	
bad	33.48	—	—	—	(1A)(1B)
bax	39.7	2.29	159.14	—	(1C)(1D)
bcl-2	20.6	—	56.26	—	(1E)(1F)
bcl-w	—	—	45.99	18.96	(2A)(2B)
bcl-x	78.49	39.13	148.2	23.55	(2C)(2D)
caspase-1	—	—	38.04	24.61	**(2E)(2F)**
caspase-2	199.37	0.78	422.07	305.1	(3A)(3B)
caspase-3	—	—	70.88	65.34	**(3C)(3D)**
caspase-7	—	—	150.79	114.69	(3E)(3F)
caspase-8	—	—	144.64	135.82	**(4A)(4B)**
c-myc	—	—	159.61	176.67	(4C)(4D)
DR-5	—	—	68.22	131.06	(4E)(4F)
E2F1	—	—	108.47	143.15	(5A)(5B)
EI24	—	—	163.68	111.64	(5C)(5D)
Fas L	—	—	11.95	136.91	(5E)(5F)
GADD45	—	—	—	19.81	(6A)(6B)
iNOS	—	—	—	19.28	**(6C)(6D)**
mdm2	2,375.42	2,245.71	2,260.48	1,954.11	(6E)(6F)
NFkb1	35.43	—	—	—	(7A)(7B)
p21Wafl	—	—	—	—	(7C)(7D)
p53	—	—	—	—	(7E)(7F)
Rb	—	—	—	—	(8A)(8B)
Trail	—	—	—	—	(8C)(8D)

synthesized with the T-primer using cDNA Great Length Synthesis Kit (Table I, Clontech Laboratories Inc., USA). The cDNA samples were further digested by *RsaI*. The cDNA was then ethanol-precipitated and half was taken for ligation with Apator-L and Apator-S primers. Ligation was performed overnight at 16°C in a 10 ml volume with 2 μm adapter primer. For A-tail pool preparation, 1 μl of 1/50 dilution of the ligation mixture was used for PCR with OU primer and T-primer. Amplification was carried out in 1× buffer (50 mM Tricine-KOH pH 8.7, 16 mM $(NH4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg/ml BSA), with 250 μm dNTPs, 0.3 μm primers, 150 μm ammonium sulfate and 5 U KlenTaq polymerase (Ab Peptides), in a volume of 20 μm. Polymerase was added to the PCR mixtures at 72°C before the first denaturation stage. The amplification profile was: 95°C for 4 s, 65°C for 20 s, 72°C for 1 min (tube control mode), 20 cycles. After amplification, 1 μl of 1/20 dilution in water of this PCR product served as initial material for amplifying simplified subsets. For differential display, first, respective InExx primers were ^{32}P-labelled by T_4 polynucleotide kinase. Labelling reactions contained 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM spermidine, 1 mM EDTA, 1 μm primer, 1 μm [γ-^{32}P] ATP and 2.5 U T_4 polynucleotide kinase, in a 10 μl. The reaction was carried out for 30 min at 37°C and was stopped by heating the tube for 1 min at 100°C. Then 2 μl of this mixture was added to 18 μl of PCR mixture, containing 20 mM Tricine-HCl pH 8.7, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg/ml BSA, TaqStart antibodies (Clontech Laboratories Inc., USA), 2.5 U KlenTaq polymerase, 2 pM non-labelled TE primer, 250 mM dNTPs and 1 ng of the third cDNA fragment sample under investigation. PCR was carried out according to the following profile: 95°C for 30 s, 69°C for 30 s, 72°C for 1.5 min, 23 cycles. To discriminate PCR products, 2 μl of each reaction was analyzed on 5% polyacrylamide sequencing gel. As a running buffer 2× TBE (0.1 M Tris, 0.89 M boric acid, 2 mM EDTA, pH 8.5) was used in gel and lower chamber, while 1× TBE was used in the upper chamber. Wells were washed and filled with 0.4× TBE prior to the pre-run (for 5 min at 600 V) and again before sample loading. After the run, the gel was dried at 70°C and exposed to X-ray film for 1-2 days. The bands excised for further analysis were used a northern blot and sequencing analysis. Primer sequences used are shown in Table I (7).

Gene Expression array analysis. To determine the expression of pathway-specific gene, four samples per time point (0, 60, 180 and 300min) were analyzed by using the GE-array™ kit [superarray Bioscience, Frederick, MD]. Total RNA was isolated from HAT media treated cell by time points at 0, 60, 180, and 300min. RNA samples were reverse transcribed to produce ^{32}P-labeled probes following the manufacture's protocol, respectively. Pathway specific genes in the nylon membranes were hybridized to heat-denatured radiolabeled probes at 68°C for 16 h in a hybridization buffer provided by the manufacturer, and after washing twice with 2× SSC/1% SDS, followed by two additional washes in 0.1× SSC/1% SDS at 68°C for 20 min each, the membranes were exposed to X-ray film. The spots were detected by autoradiography, and the intensities of the corresponding spots in the four membranes were compared for four RNA population used to generated the probes (8).

Affymetrix GeneChip probe array. Affymetrix Mu74Av2 GeneChips, including about 12,000 genes and ESTs on one array were processed according to the manufacturer's recommendations (9).

To evaluate the efficiency of the protocol, we carried out experiments with 100, 50, 20, 10 and 1 ng of total RNA from murine myeloma cell line using the procedure described below. Targets were then hybridized to GeneChip® Mu11KsubA arrays. For comparison and concordance studies, 10 μg of the same sample was also labeled using the standard assay protocol as detailed in the GeneChip® Expression Analysis Technical Manual. For experiments described in this Technical Note, we used aliquots of a single source of total RNA purified in bulk. Since GeneChip® expression analysis requires high-quality and high-purity RNA as starting materials, some general guidelines for RNA purification and quality assessment are provided in the GeneChip® Expression Analysis Technical Manual (10).

GenMAPP. Signal transduction pathways, metabolic pathways, and other functional groupings of genes were evaluated for differential regulation using the visualisation tool GenMAPP (UCSF) (11). GenMAPP is a recently reported tool for visualising expression data in the context of biological pathways. We imported the statistical results of our data set into the program and used GenMAPP to illustrate pathways containing differentially expressed genes. Differential gene expression was based on prenatal versus postnatal expression change (two-fold and $p < 0.05$, *t* test).

Northern blot analysis. Total RNA was prepared from cell suspensions stimulated under the time points according to the manufacturer's protocol after harvest and lysis in TRI Reagent LS [Molecular Research Center, Inc., Cincinnati, OH]. Fifty micrograms of RNA samples were fractionated in a 1% agarose gels in the presence of formaldehyde, transferred to blotted on Hybond N^+ membrane [Amersham, Arlington Heights, IL, USA]. The blot was subsequently hybridized in the presence of 50% formamide at 42°C

with column purified ODD PCR product probes labeled by a random-primer technique [Life Technologies] (7).

Results

Differential display PCR. Using method described in Materials and Methods we cloned upto 900 clones showing distinctive induction patterns in differential display PCR (Fig. 1A). Consequently the same-sized bands were isolated by re-PCR (Fig. 1B). Re-PCR products were cloned and inserted genes showed various sizes (Fig. 1C). Cloned genes were sequenced and the data were compared with the result of GeneChip assay later on.

GE-Array. Except 22.5min group RNA from four groups, 0, 60, 180, and 300 min were reverse transcribed to produce ^{32}P-labeled probes and hybridized commercial dot blot membrane described in Materials and Methods (Table II, and Fig. 2). Among 23 genes

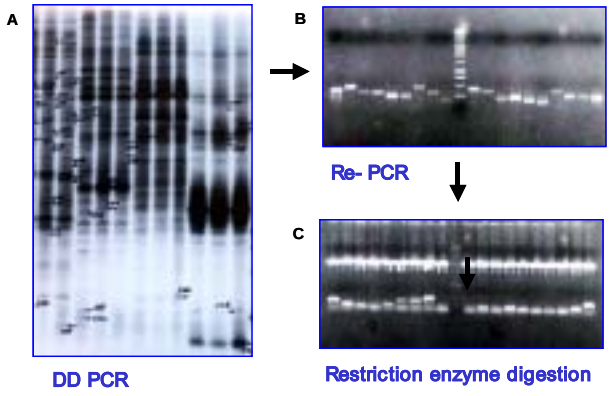

Figure 1. Experimental procedure of DD-PCR and some genes cloned thereby. (A) Differential display PCR was carried out by using primers (Table I) and PCR products were analyzed on 5% polyacrylamide gel. (B) Bands were excised and used as templates for re-PCR. PCR products with the same-sized bands were isolated. (C) Re-PCR products were cloned and inserted genes showed various sizes.

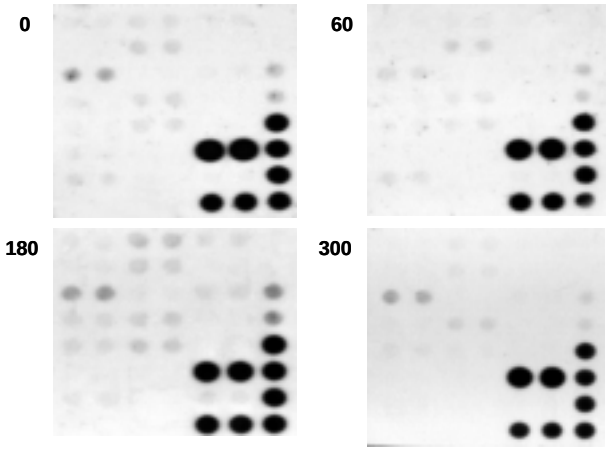

Figure 2. Dot blot patterns GE array. RNA samples from 0 min (0), 60 min (60), 180 min (180), and 300 min (300) of apoptosis-induction were analyzed by using the GEarray^{TM} kit. RNA samples were reverse transcribed to produce ^{32}P-labeled probes. Pathway specific genes in the nylon membranes were hybridized to heat- denatured radiolabeled probes and then the membranes were exposed to X-ray film.

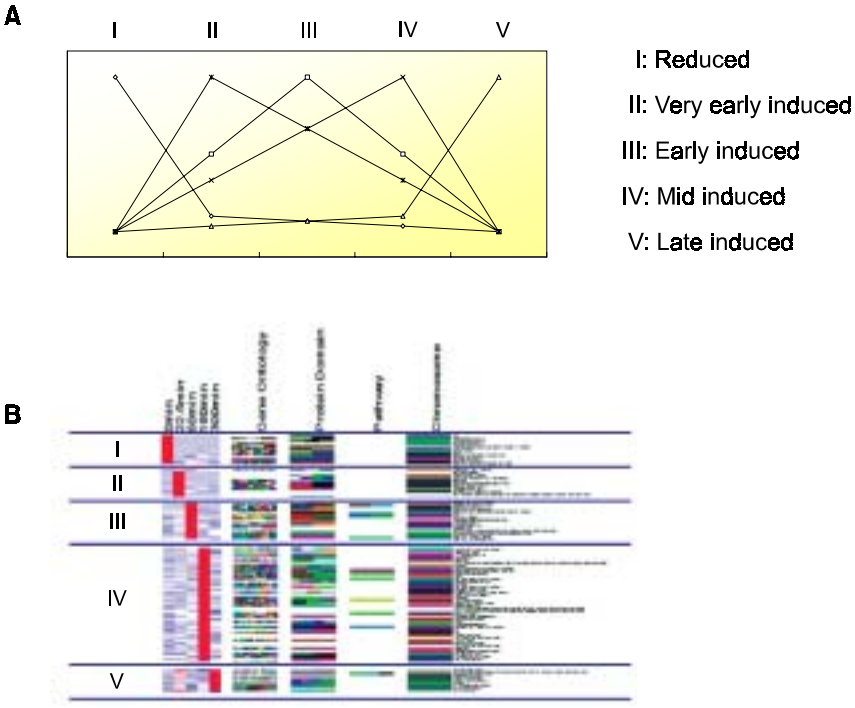

Figure 3. Five induction patterns of apoptosis-induced genes. (A) Data from Affymetrix GeneChips array were selcted 66 gense and wer classified into 5 groups according to their induction patterns. (B) Hierachial clustering using dCHIP showed 5 different induction patterns of genes filtered.

p21, p53, *Rb* and *Trail* showed no signal in 4 experimental groups, and *mdm*2, caspase 1 and caspase 2 were all positive at every group. Signal of *mdm*2 were decreased as apoptosis progress while signals of *bcl-x* and caspase 2 were increased to peak at 3 h. Expression of NFk*B*1 and *bad* could only be detected at 0 which meant deduction on apoptosis. And *bax* and *bcl*-2 were undetectable at 5 h while *gadd*45 and iNOS could only detected at 5 h. Rest of genes including *bcl-w*, caspase-1, caspase-3, caspase-3, caspase-7, caspase-8, *c-myc*, DR5 E2F1, EI24, and FasL were induced up since 3 h. Among these genes signal of caspase-2 was the strongest.

Affymetrix GeneChip assay. Through extensive Affymetrix GeneChip analysis 66 genes which have shown at least 2 fold increase of signals were selected. These genes were classified into 5 types according to time course patterns (Fig. 3A). Among these 5 groups

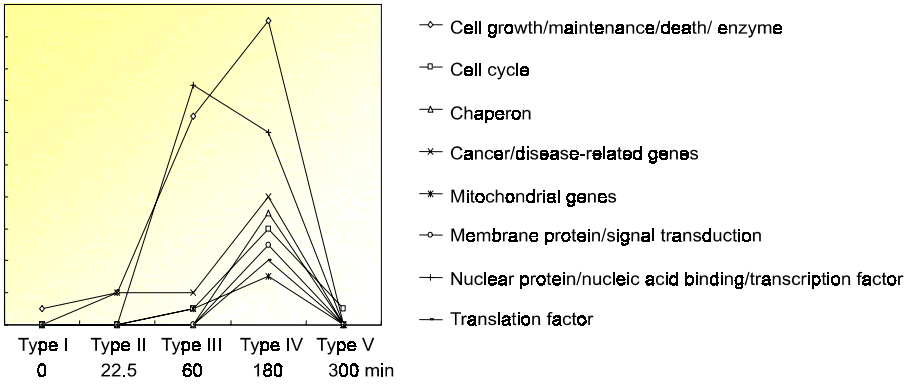

Figure 4. Time courses of induction of 8 functional groups. The graphs show induction time course of 8 different groups of genes, 1) Cell growth/maintenance/death/enzyme, 2) Cell cycle, 3) Chaperone, 4) Cancer/disease-related genes, 5) Mitochondria, 6) Membrane protein/signal transduction, 7) Nuclear protein/nucleic acid binding/transcription binding and 8) Translation factor.

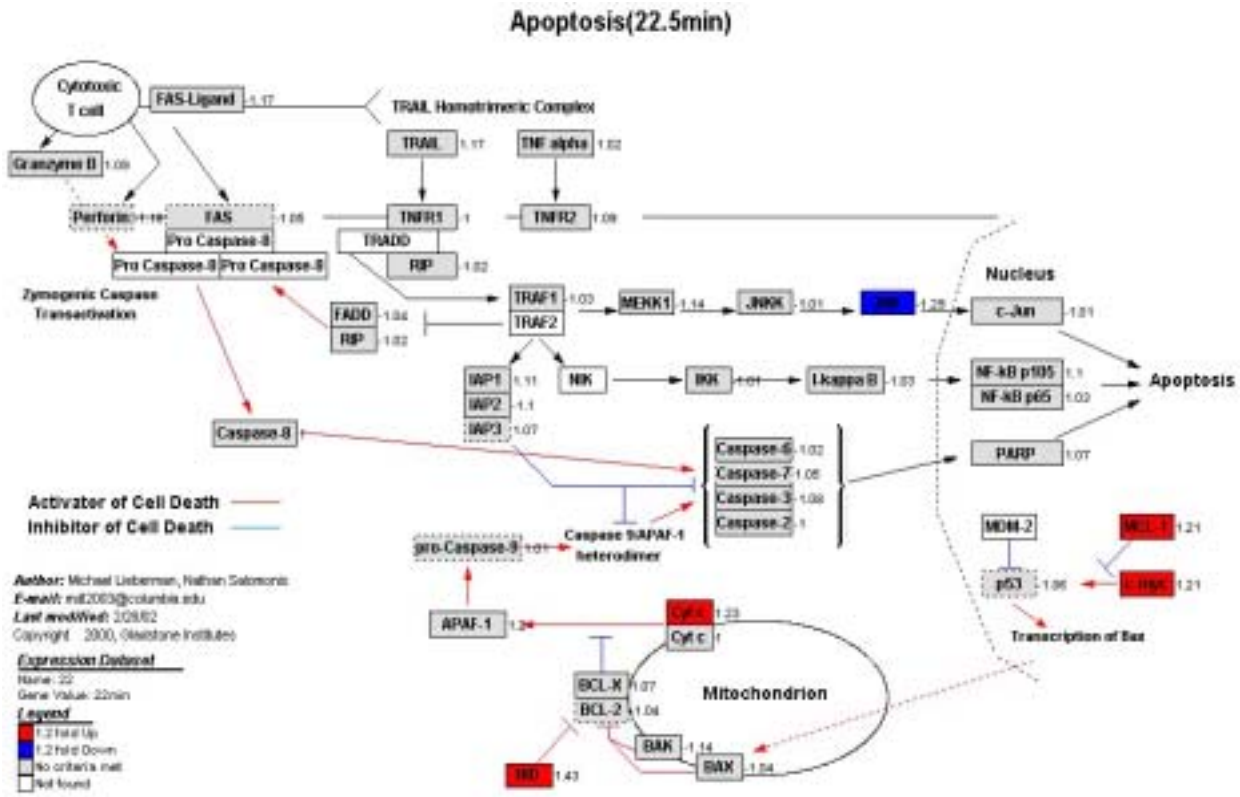

Figure 5. GenMAPP finding of apoptosis related genes induced at 22.5 min. Apoptosis pathway of genes induced at 22.5 min of death induction was analyzed by using the visualisation tool GenMAPP (UCSF). Differential gene expression was based on pre-apoptosis versus post-apoptosis expression change (two-fold and $p<0.05$, *t* test). Red colored boxes represent significant increase (1.2-fold and $p<0.05$, *t* test) of gene expression while blue colored boxes represent significant decrease (1.2-fold and $p<0.05$, *t* test).

most of genes belong to type 5 showed a tendency to induced slightly up at 22.5min (Fig. 3B).

All genes selected were classified into 8 groups according to their known functions. They were genes of 1) Cell growth/maintenance/death/enzyme, 2) Cell cycle, 3) Chaperone, 4) Cancer/disease-related genes, 5) Mitochondria, 6) Membrane protein/signal transduction, 7) Nuclear protein/nucleic acid binding/transcription binding and 8) Translation factor. Among these groups number of genes were the largest in the genes of cell growth/maintenance/death/enzyme. The second and the third largest were genes of Nuclear protein/nucleic acid binding/transcription binding and genes of cancer/disease respectively. Expression signals of most of all groups were peaked at 3 h of apoptosis. Only exception was genes of Nuclear protein/nucleic acid binding/transcription factor which showed maximum signal at 1 h (Fig. 4).

GenMAPP analysis. Gene induction patterns of apoptosis were analyzed by using GenMAPP® program. At 22.5 min *c-myc*, MCL-1, BID and cytochrome-c were shown to be increased (Fig. 5). Among these apoptosis BID was shown the highest increase (FC, 1.43). JNK was the only gene reduced in expression.

At 1 h (Fig. 6) *c-myc* and BID were turned down to reduced in expression. FAS was identified to reduced newly. Both MCL-1 and cytochrome-c were continued to be induced. Caspase-9, caspase-3, PARP, and TNFR1 were newly induced to be expressed.

At 3 h (Fig. 7) MCL-1 and cytochrome-c induced up more while perforin, TNFR2 and *c-myc* were reduced. It was observed that caspase-3 together with caspase-8 induced up. Also induced were RIP, IAP3.

At 5 h (Fig. 8) only cytochrome-c and caspase-3 were shown sustaining induction pattern. And *c-myc,* BID, caspase-7, IAP-1 JNK, *c-jun* were observed to reduced. Among these *c-myc* was the most drastically reduced gene and BID was the next.

Northern blot analysis. Some of genes identified by differential display-PCR and Affymetrix GeneChip analysis were selected and their transcription pattern were analyzed by using northern blot analysis. Mdm-2 and growth arrest specific 2 gene were shown type I induction pattern. Caspase-9, *c-myc,* E2F1, and peroxiredoxin gene were observed as type II pattern. Caspase-8 and Tumor rejection antigen gp96 were shown type III. Caspase-1 was revealed as type IV while CDK inhibitor p21 and fatty acid binding

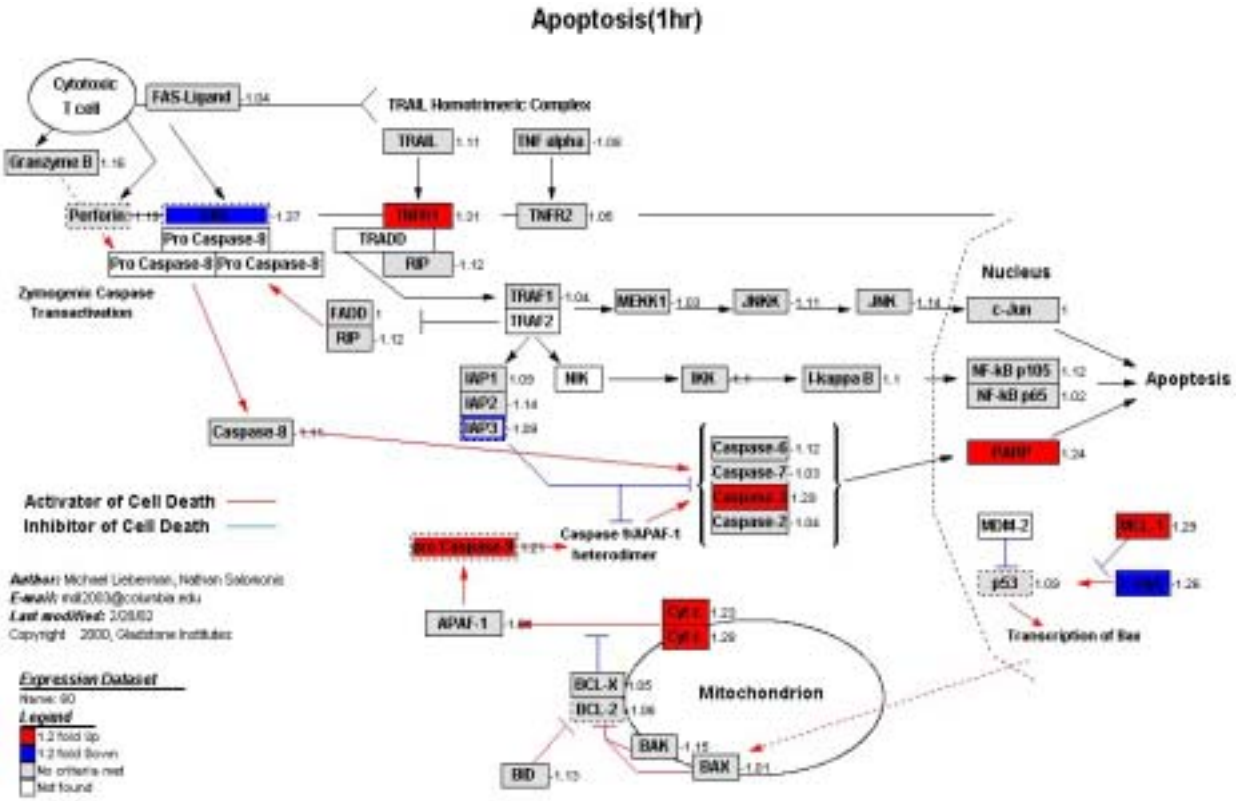

Figure 6. GenMAPP finding of apoptosis related genes induced at 1 hour. Apoptosis pathway of genes induced at 1 hour of death induction was analyzed by using the visualisation tool GenMAPP (UCSF). Differential gene expression was based on pre-apoptosis versus post-apoptosis expression change (two-fold and $p<0.05$, *t* test). Red colored boxes represent significant increase(1.2-fold and $p<0.05$, *t* test) of gene expression while blue colored boxes represent significant decrease (1.2-fold and $p<0.05$, *t* test).

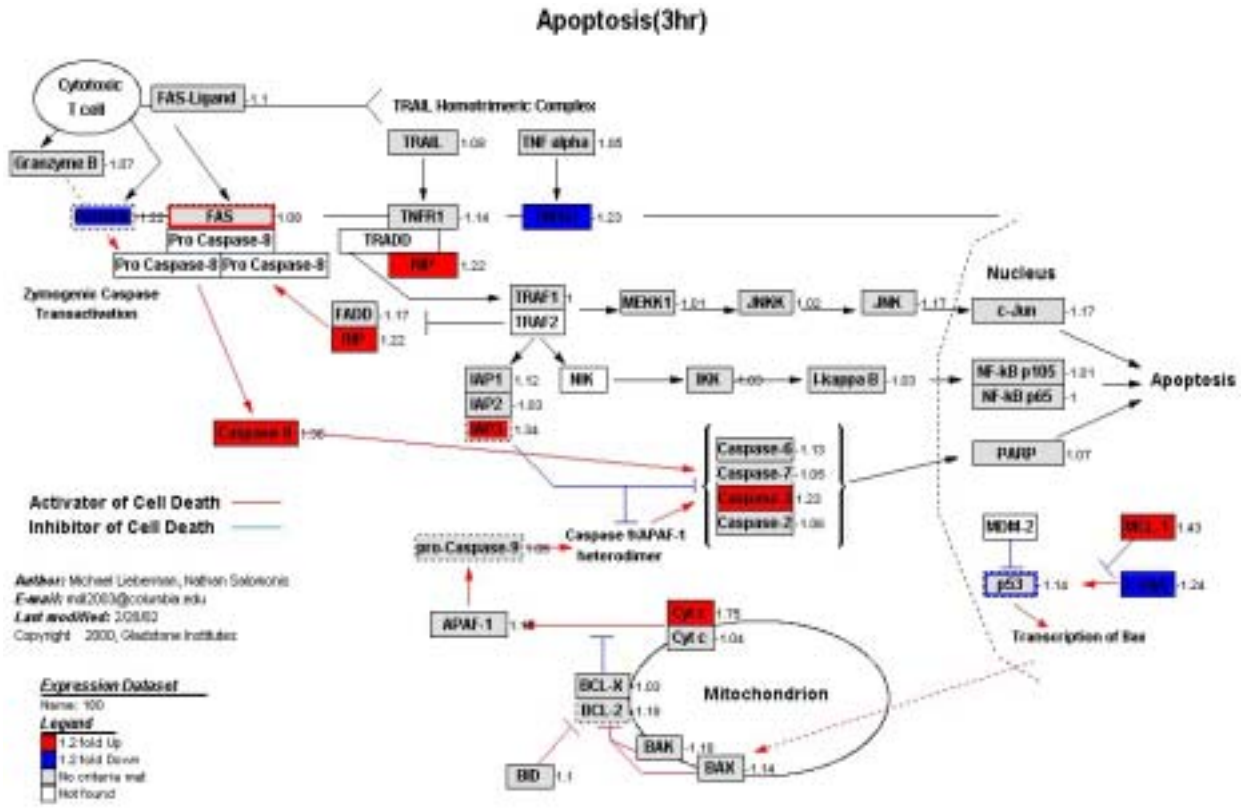

Figure 7. GenMAPP finding of apoptosis related genes induced at 3 hours. Apoptosis pathway of genes induced at 3 hours of death induction was analyzed by using the visualisation tool GenMAPP (UCSF). Differential gene expression was based on pre-apoptosis versus post-apoptosis expression change (two-fold and $p<0.05$, *t* test). Red colored boxes represent significant increase (1.2-fold and $p<0.05$, *t* test) of gene expression while blue colored boxes represent significant decrease (1.2-fold and $p<0.05$, *t* test).

protein 4 were shown as type V (Fig. 9).

Discussion

Inhibition of transcription for example with Actinomycin-D or inhibition of translation with cycloheximide has been widely used and studied for strong apoptosis inducer in various types of cells (12,13). Contrary to these findings aminopterin-induced cell death has revealed unique features of remarkable inhibitions by either one of these strong apoptosis inducers. This study has confirmed the effects of transcriptional inhibitor, actinomycin-D and translational inhibitor, cycloheximide on viability and DNA fragmentation. The result showed that this cell death is highly dependent on *de novo* transcription and translation. This feature has made this cell death very attractive experimental model for searching apoptosis-related genes.

GE-array dot blot analysis found that the inductions of various genes involved in apoptosis though the patterns are not exactly coincided with that found in GeneChip analysis, northern blot analysis.

Affymetrix GeneChip analysis showed majority of genes induced were peaked up at 3 h of death induction. While only genes of Nuclear protein/nucleic acid binding/transcription factor which showed maximum signal at 1 h (Fig. 4). This finding shows that for de novo macromolecular synthesis cell must precede to turn on major gene transcription by inducing transcription factors of necessity.

Apoptotic cell death can occur by two different pathways. Type 1 is initiated by the activation of death receptors (Fas, TNF-receptor-family) on the plasma membrane followed by activation of caspase 8. Type 2 involves changes in mitochondrial integrity initiated by various effectors like Ca (2+), reactive oxygen species (ROS), bax, or ceramide, leading to the release of cytochrome c and activation of caspase 9. The release of cytochrome c is followed by a decrease of the mitochondrial membrane potential (14). The release of cytochrome c from mitochondria during apoptosis results in the enhanced production of superoxide radicals, which are converted to H_2O_2 by Mn-superoxide dismutase. Cytochrome c in participating in apoptosome formation and in triggering the caspase cascade (15). Apoptosis is executed by a subfamily of cysteine proteases known as caspases. In mammalian cells, a major caspase activation pathway

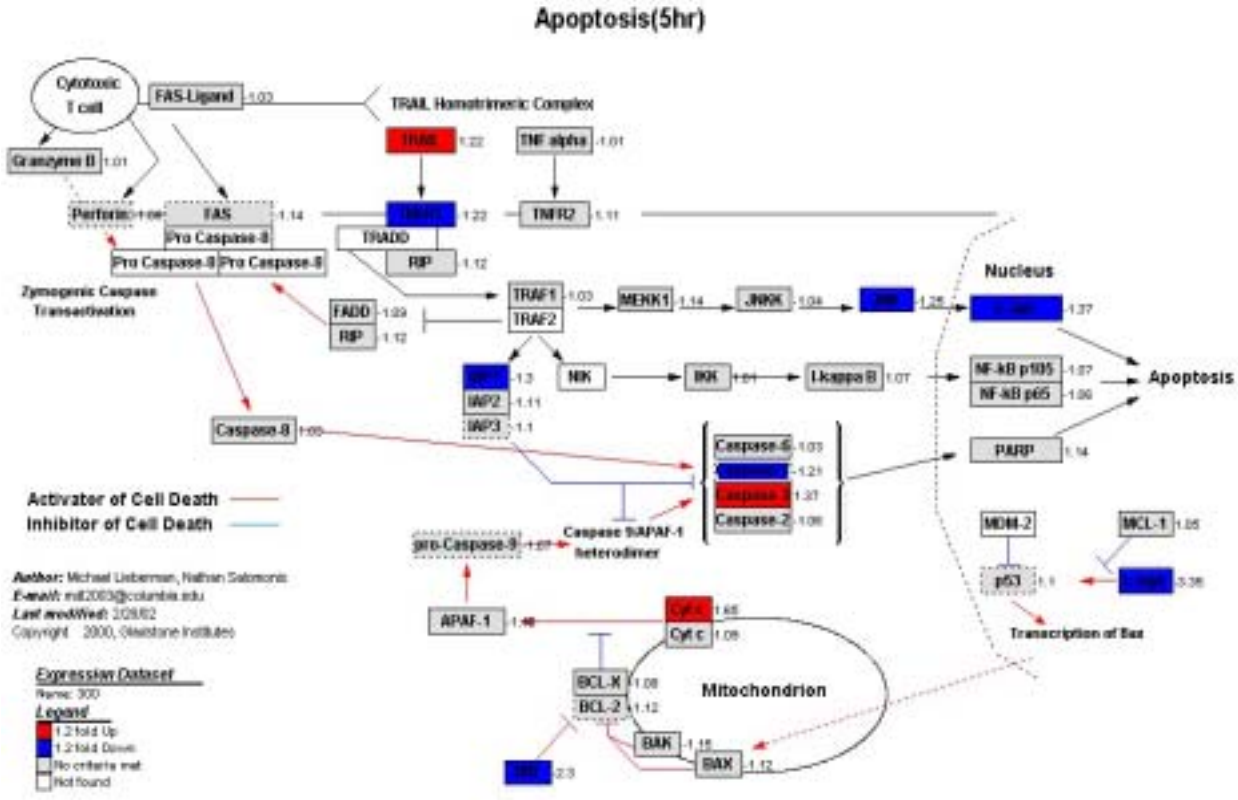

Figure 8. GenMAPP finding of apoptosis related genes induced at 5 hours. Apoptosis pathway of genes induced at 22.5 min of death induction was analyzed by using the visualization tool GenMAPP (UCSF). Differential gene expression was based on pre-apoptosis versus post-apoptosis expression change (two-fold and $p<0.05$, *t* test). Red colored boxes represent significant increase (1.2-fold and $p<0.05$, *t* test) of gene expression while blue colored boxes represent significant decrease (1.2-fold and $p<0.05$, *t* test).

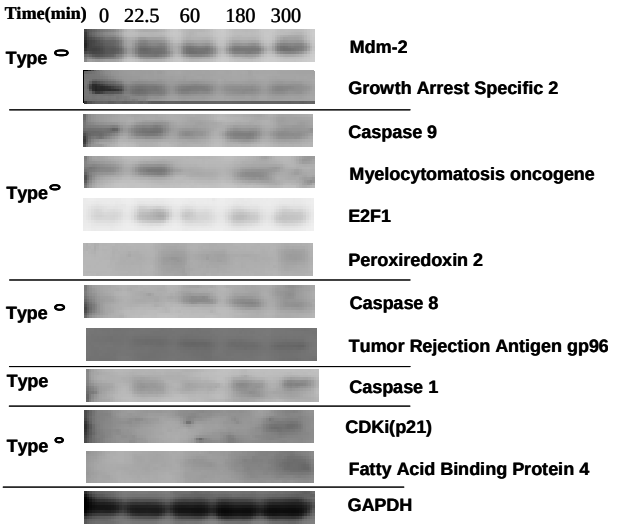

Figure 9. Northern blot analysis of some genes induced on apoptosis. Transcription patterns of 1~4 representative genes of 5 different induction types were analyzed by using northern blot analysis. Total RNA was prepared from cell suspensions were hybridized with column purified ODD PCR product probes of each genes on right side labeled by a random-primer technique.

is the cytochrome c-initiated pathway. In this pathway, a variety of apoptotic stimuli cause cytochrome c release from mitochondria, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death (16). GenMAPP finding showed that aminopterin induced apoptosis is type 2 which involves cytochrome c and activation of caspase 9 early stages of death. However if one looking mid to late stage it also involves type 1 apoptosis.

A dramatic increase in mitochondrial-derived reactive oxygen species (ROS) occurs during the apoptotic death of both of these cell types. These ROS lie downstream from the proapoptotic protein, Bax. normally resides in the cytoplasm, but translocates to the outer mitochondrial membrane during apoptosis. Once associated with mitochondria, Bax causes release of proapoptotic factors from the mitochondria into the cytoplasm, thus inducing or augmenting the apoptotic cascade (17).

The caspases are known to play a crucial role in the triggering and execution of apoptosis in a variety of cell types. Pancreatic cancer tissue showed a clear cytoplasmic overexpression of caspase-1 in tumor cells

in 71% of the tumors, whereas normal pancreatic tissue showed only occasional immunoreactivity (18).

The cyclin-dependent kinase inhibitor p21 is known to be induced by both p53-dependent and -independent mechanisms following stress, and induction of p21 may cause cell cycle arrest. As a proliferation inhibitor, p21 is poised to play an important role in preventing tumor development. This notion is supported by data indicating that p21-null mice are more prone to spontaneous and induced tumorigenesis, and p21 synergizes with other tumor suppressors to protect against tumor progression in mice. A number of recent studies have pointed out that in addition to being an inhibitor of cell proliferation, p21 acts as an inhibitor of apoptosis in a number of systems, and this may counteract its tumor- suppressive functions as a growth inhibitor (19). It was found that disruption of fatty acid binding protein in mouse leads to changes in the following two major metabolic processes: 1) decreased adipose NEFA efflux and 2) preferential utilization of glucose relative to fatty acids (20). The adipocyte fatty acid-binding protein, aP2, has important effects on insulin resistance, lipid metabolism, and atherosclerosis. Its expression in macrophages enhances early foam cell formation and atherosclerosis in vivo (21).

Induction of *gadd*45 was only detected in GE array analysis. This gene has been known to promote G2/M arrest via nuclear export and kinase activity of Cdc2, increases global genomic DNA repair and inhibits cell death in keratinocytes (22). As a p53-effector genes, *gadd*45a is working as a negative regulator of T cell proliferation. It was reported that *gadd*45a(-/-) mice develop an autoimmune disease, similar to human systemic lupus erythematosus (SLE) (23).

Taken together It was found that wide range of proapoptotic factors which accelerate cell death are found to be induced in this cell death system. Some of them were characterized their role in apoptosis very well though some are not yet. More importantly there are many anti-apoptotic factors being induced at various stage of this cell death. So for further future studies of genes induced with unknown function experiment should be designed with care to clarify each of their function as proapoptotic or antiapoptotic factor. In addition apoptosis studied in this research can be classified as a type 2 which involves cytochrome c and caspase 9 especially in early stages of death. But It also has progressed to type 1 in late stage of death process.

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