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Invited Review

Identification and characterization of genes responsive to apoptosis: Application of DNA chip technology and mRNA differential display

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Summary. Apoptosis (programmed cell death) is a genetically programmed active cell death process for maintaining homeostasis under physiological conditions and for responding to various stimuli. Many human diseases have been associated with either increased apoptosis (such as AIDS and neurodegenerative disorders) or decreased apoptosis (such as cancer and autoimmune disorders). In an attempt to understand apoptosis signaling pathway and genes associated with apoptosis, we established two cell model systems on which apoptosis is induced either by DNA damaging agent, etoposide or by redox agent, 1,10-phenanthroline (OP). DNA chip profiling or mRNA differential display (DD) was utilized to identify genes responsive to apoptosis induced by these two agents. In etoposide model with chip hybridization, we defined signaling pathways that mediate apoptosis in p53 dependent manner (through activation of p53 target genes such as Waf-1/p21, PCNA, GPX, S100A2 and PTGF-ß) as well as in p53-independent manner (through activation of ODC and TGF-ß receptor, among others). In OP model with DD screening, we cloned and characterized two genes: glutathione synthetase, encoding an enzyme involved in glutathione synthesis and Sensitive to Apoptosis Gene (SAG), a novel evolutionarily conserved gene encoding a zinc RING finger protein. Both genes appear to protect cells from apoptosis induced by redox agents. Further characterization of SAG revealed that it is a growth essential gene in yeast and belongs to a newly identified gene family that promotes protein ubiquitination and degradation. Through this activity, SAG regulates cell cycle progression and many other key biological processes. Thus, SAG could be a valid drug target for anti-cancer and anti-inflammation therapies.

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Apoptosis - a general introduction

Apoptosis, also called programmed cell death, is a genetically programmed active process for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli (Vaux, 1993; Thompson, 1995). This form of cell death is characterized by cell membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation and DNA fragmentation (Wyllie et al., 1980). Apoptosis occurs in both embryonic cells during development as well as differentiated cells in adult tissues. Apoptotic cells usually commit suicide for the greater good of the body. Many human diseases have been associated with altered apoptosis. The common diseases associated with increased apoptosis include AIDS, ischemic injuries (such as myocardial infarction, strokes), and neurodegenerative disorders (such as Alzheimer's or Parkinson's diseases, amyotrophic lateral sclerosis, retinitis pigmentosa, and cerebellar degeneration). Diseases associated with decreased apoptosis include cancers, autoimmune disorders and viral infections (Thompson, 1995).

Apoptosis can be induced by a wide variety of physical, chemical and biological stimuli. They include ionizing radiation, UV, chemotherapeutic drugs that damage DNA, reactive oxygen species, cytokines and their receptors, and some oncogenes (myc, E1A) and tumor suppressor genes (p53), among others. On the other hand, apoptosis can be inhibited by some physiological inhibitors (such as growth factors, extracellular matrix), several viral genes (E1B, p35) and some pharmacological agents (calpain inhibitors, cysteine protease inhibitors) (Thompson, 1995).

The process of apoptosis can be divided into three

distinct phases: initiation, effector and degradation (Kroemer et al., 1995). Apoptosis initiators/inducers (both internal or external) trigger the effector molecules (e.g. caspases) in cells leading to apoptotic signal transduction and amplification, which eventually result in irreversible DNA and protein degradation and cell death (Fraser and Evan, 1996; Kroemer, 1997; Ashkenazi and Dixit, 1998; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). Many genes are involved in this well-coordinated but complicated apoptotic process. In general, they can be divided into two classes: pro-apoptosis genes that induce apoptosis and anti-apoptosis genes that inhibit apoptosis. The balance between the activities of these two classes of genes determines the life or death of the cell (Steller, 1995; Peter et al., 1997). Genes involved in the initiation phase of apoptosis, are those responsive to growth factor withdrawal (Bazenet et al., 1998) or DNA damage (e.g. p53), or the genes encoding ligands and their receptors (e.g. TNF/TNFR; Fas/Fas ligand) (Ko and Prives, 1996; Nagata, 1997; Ashkenazi and Dixit, 1998; Baker and Reddy, 1998; Dragovich et al., 1998; Evan and Littlewood, 1998). At the execution phase of apoptosis the genes encoding caspases, a group of cysteine proteases, play a key role (Nunez et al., 1998; Thornberry and Lazebnik, 1998). There are thus far 14 members of caspase family (Wolf and Green, 1999). During apoptosis signaling transduction, caspases can function as initiators (caspases-2, -8, -9, -10), amplifiers/processors (caspases-1, -4, -5, -11, -12, -13, -14) and executors (caspases-3, -6, -7) (Fraser and Evan, 1996; Salvesen and Dixit, 1997; Wolf and Green, 1999), leading to committed apoptosis. Also involved in this phase are the genes encoding Bcl-2 family members that regulate apoptosis both positively and negatively (Kroemer, 1997; Reed, 1997, 1998). At the degradation phase of apoptosis, proteins that serve as substrates of caspases are degraded, resulting in the apoptotic appearance of cell (Cryns and Yuan, 1998).

Significantly, the key players in apoptosis signaling pathways are highly conserved among different eukaryotic species (Vaux et al., 1994; Steller, 1995). Two genes in *C. elegans*, *ced-3* and *ced-4*, that are required for apoptosis during normal development (Hengartner and Horvitz, 1994) were found to be, respectively, the mammalian homologs of apoptotic protease activating factor-3 (Apaf-3 or caspase-9) (Li et al., 1997), or Apaf-1 (Zou et al., 1997). Also, the *C. elegans* death inhibitor ced-9 is a mammalian homolog of Bcl-2, which is able to prevent cell death in *C. elegans* (Vaux et al., 1992). Furthermore, the baculovirus p35 protein has been shown to suppress apoptosis in insects, nematodes, and mammals (Clem and Miller, 1994; Sugimoto et al., 1994), indicating that it interacts with a component of the cell death program that has been conserved in evolution (Steller, 1995). The conservation of these genes from nematode to man strongly indicates the essential necessity of apoptosis pathway in life maintenance.

p53, a tumor suppressor that induces apoptosis

A key molecule that mediates apoptosis is p53 protein. Human p53 is a 393-amino acid nuclear protein that acts biochemically as a transcription factor and biologically as a tumor suppressor. The p53 molecule consists of three major domains: the N-terminal transactivation domain that mediates transactivation of many downstream target genes; a DNA binding domain in the center portion of the molecule that specifically binds to its consensus binding site; and a C-terminal oligomerization domain that mediates homotetramer formation (Lane, 1994; Selivanova and Wiman, 1995; Ko and Prives, 1996). As a transcription factor, p53 specifically binds to its consensus DNA binding sequence, consisting of two copies of the 10 bp motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3'(el Deiry et al., 1992) and transactivates expression of the target genes. Two main biological functions of p53, induction of growth arrest and apoptosis, are mainly mediated through its downstream target genes (Baker et al., 1990; Kastan et al., 1995; Levine, 1997). Induction of G1 arrest is achieved mainly by transactivation of Waf-1/p21, a universal inhibitor of cyclin-dependent kinases (el Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), whereas G2 arrest is through activation of 14-3-3σ (Hermeking et al., 1997). On the other hand, p53-induced apoptosis is mediated by activation of *Bax* (Knudson et al., 1995; Miyashita and Reed, 1995), *KILLER/DR5* (Wu et al., 1997), and the genes involved in generation of reactive oxygen species (Polyak et al., 1997). p53 also regulates angiogenesis (Dameron et al., 1994a,b) and tumor metastasis (Sun et al., 1999a,b) by transcriptional regulation of the genes encoding EGFR (Ludes Meyers et al., 1996), thrombospondin (Dameron et al., 1994a,b), MMP-1 (Sun et al., 1999a,b), MMP-2 (Bian and Sun, 1997), cathepsin D (Wu et al., 1998), KAI1 (Mashimo et al., 1998), bFGF (Ueba et al., 1994), MDR1 (Chin et al., 1992), and maspin (Zou et al., 2000). Since p53 is such an important molecule in negatively regulation of cell growth, it is not surprised to find that mutational inactivation of p53 occurred in more than 50% of all human cancers (Greenblatt et al., 1994). These mutations were clustered in the specific DNA binding domain of the p53 molecule (Greenblatt et al., 1994), that leads to an inactivation of p53 function through abolishing p53 specific DNA binding and transactivation.

p53 is subjected to multiple-level controls, including transcriptional and translational regulation, post-translational modification (phosphorylation and acetylation), as well as cellular localization and protein-protein interactions (Giaccia and Kastan, 1998). p53 protein level and transcriptional activity are induced by many internal as well as external agents such as irradiation/UV/DNA damaging agents, redox, hypoxia, some oncogenes, adhesion and rNTP depletion (Giaccia and Kastan, 1998). Activation of p53 by these reagents would lead to either growth arrest that allows cell to

repair damaged DNA before entering the cell cycle or apoptosis to eliminate damaged cells when repair becomes impossible. Cancer is a disease associated with a decreased apoptosis. Identification and characterization of apoptosis-associated genes would ultimately lead to a mechanism-driven anti-cancer drug discovery. Since p53 is a key molecule in apoptosis regulation and dysfunctional p53 has been detected in many human cancer cells that become resistant to anticancer agents (Lowe et al., 1993, 1994; Baas et al., 1999), we would like to understand p53-mediated apoptosis signaling pathways. We have focused our attention to two major p53 regulators/inducers: DNA damaging agents and redox agents that activate p53 as well as induce apoptosis in our model systems.

Apoptosis models - DNA damage and redox disturbance

It is well known that p53 can be activated by DNA damaging agents, including ionizing radiation, UV radiation and many chemotherapeutical drugs (Kastan et al., 1991; Giaccia and Kastan, 1998). The DNA damaging agent we chose to use is etoposide (VP16). Etoposide is a DNA topoisomerase II inhibitor (Pratt et al., 1994). It has been shown to be active against a variety of tumor cells in both preclinical and clinical studies (Aisner and Lee, 1991). Etoposide is used as an antitumor agent in clinic, mainly for the treatment of testicular cancers and small cell lung carcinomas (Aisner and Lee, 1991; Belani et al., 1994). The main toxicity derived from the use of etoposide is bone marrow depression with leukopenia and thrombocytopenia. Other side-effects include nausea, diarrhea, mucositis, and hypotension (Aisner and Lee, 1991; Belani et al., 1994). As a DNA damaging reagent, etoposide has been shown to induce apoptosis in a variety of tumor cell lines harboring either wildtype or mutant p53 (Mizumoto et al., 1994; Martins et al., 1997). This provided us an opportunity to identify apoptosis-associated genes either dependent upon or independent upon p53. Although the entire signaling pathways mediating etoposide-induced apoptosis are not clear, one pathway could involve p53, since DNA damage induced by etoposide activates p53 activity (Bian and Sun, 1997).

The second choice of the p53 regulators is redox agents. In vitro test tube studies have initially shown that p53 DNA binding activity is inhibited by oxidant, metal ions and their chelators that disrupt p53 conformation, and p53 activity can be restored by reductants (Sun and Oberley, 1996). Later, it was found at the cellular level that p53 activity can be activated by nitric oxide (Calmels et al., 1997), thioredoxin reductase (Pearson and Merrill, 1998), and redox protein, Ref-1 (Jayaraman et al., 1997) and inhibited by metal ion copper, a zinc chelator, and antioxidant (Verhaegh et al., 1997, 1998). My laboratory is among the first to extend the observation on redox regulation of p53 from the test tube to the intact cells. We used luciferase-coupled transactivation assay to determine p53 activity. The luciferase reporters used are driven by a p53 specific binding site-containing intron or promoter sequence derived either from mouse MDM-2 gene (Juven et al., 1993), or from the Waf-1 gene (el Deiry et al., 1993), respectively. The recipient cells include L-RT101, a mouse JB6 tumor line, and H-Tx, a spontaneous transformed mouse liver line, both harboring endogenous wildtype p53 (Sun et al., 1993a,b). After transient transfection, cells were then exposed to the redox agents, including 1) the reducing or oxidizing agents; 2) metal ions or metal chelators; 3) the compounds that inhibit endogenous GSH synthesis; or 4) antioxidants (Sun et al., 1997). Attempts were made to identify compounds that regulate wildtype p53 activity (either up or down). We found that in our model, only 1,10-phenanthroline (OP), a metal chelating agent, induces p53 transactivation as well as p53 DNA binding activity. Activation of p53 is followed by induction of several known p53 target genes, including Waf-1, Mdm-2 and Bax and finally, apoptosis results (Sun et al., 1997).

We therefore established two models for apoptosis study. As shown in Figure 1, in the first model, apoptosis is induced by a DNA damaging agent, etoposide, and in the other, it is induced by a redox-sensitive agent, 1,10-phenanthroline (OP). Both agents are the p53 activators and induce apoptosis in p53-dependent and possibly in p53 independent manner. The cells chosen for etoposide experiment were U2-OS, a human osteogenic sarcoma line harboring endogenous wildtype p53 (Kastan et al., 1992) that is known to be activated by etoposide (Bian and Sun, 1997). Likewise, the cells for OP model is L-RT101, a mouse epidermal tumor line harboring again the endogenous wildtype p53 (Sun et al., 1993a,b), capable of being activated by OP (Sun et al., 1997). We have used these two models in an attempt to identify genes responsive to or responsible for apoptosis and to reveal potential apoptosis signaling pathways triggered by DNA damaging or redox agents. DNA chip hybridization and mRNA differential display, two methods for monitoring gene expression were employed for this task.

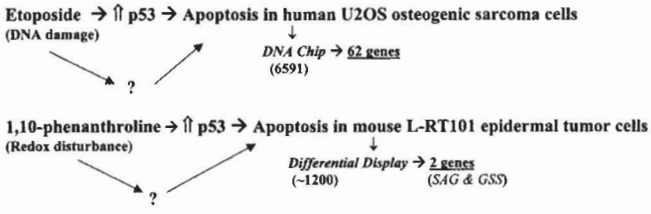

Fig. 1. Models used for identification of apoptosis-associated genes and signaling pathways that mediate apoptosis: Model 1: Human U2OS osteogenic sarcoma cells were exposed to etoposide for 6 hrs (without obvious signs of apoptosis) or 24 hrs (with obvious signs of apoptosis). RNAs were isolated and subjected to DNA chip profiling, capable of detecting of 6591 genes. Model 2: Mouse L-RT101 epidermal tumor cells were exposed to 1,10-phenanthroline for 6 hrs and subjected to mRNA differential display. Limited sets of primers, capable of detecting 1,200 genes were used.

Gene expression profiling - DNA chip technology and mRNA differential display

To profile the changes of gene expression during apoptosis induced by these two agents, we employed two methodologies: DNA chip technology (Ramsay, 1998, Eisen and Brown, 1999; Hacia, 1999; Hacia et al., 1998; Lipshutz et al., 1999) and mRNA differential display (Liang and Pardee, 1992, 1998; Sun et al., 1994).

DNA chip technology

The term "DNA chip" generally refers to a gridded array of oligonucleotides (25 mer) attached to a flat solid support. A single 1.28x1.28 cm array containing probe sets for approximately 40,000 human genes and ESTs has been developed (Lipshutz et al., 1999). Oligonucleotide probes in the high-density oligonucleotide arrays are chosen based upon uniqueness of sequence (to minimize cross-hybridization) and empirical rules derived to predict hybridization specificity and sensitivity (Lockhart et al., 1996). For each individual transcript, there are twenty pairs of 25 mer designed to hybridize to different regions of the same RNA that perfectly match with the cDNA sequenece (PM) as well as twenty pairs of corresponding 25 mer with one mismatch in the central position (MM). The use of multiple independent oligonucleotides for the same molecule greatly improves signal-to-noise ratios and the accuracy of RNA quantitation. The MM probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals and discrimination between the real signals and those derived from non-specific or semi-specific hybridization, thus increasing the reliability and reproducibility of the measurements. The ordered nature of the oligonucleotide arrangement on the chip surface allows for massively parallel analysis of a multitude of hybridization events occurring between the oligonucleotides on the chip and fluorescent-labelled cRNA applied in solution to the chip surface. Figure 2 showed how Affymetrix oligonucleotide arrays were used for mRNA expression profiling (Lockhart et al., 1996; Wodicka et al., 1997). Briefly, polyadenylated RNA was isolated and double stranded cDNA was prepared using Life Technologies Superscript Choice System and an oligo-$(dT)_{24}$ anchored T7 primer (Lockhart et al., 1996). Biotinylated RNA was synthesized using an Ambion T7 Megascript system with biotin-11-CTP and biotin-16-UTP. After purification using Qiagen RNAeasy columns and randomly fragmentation to minimize inter- and intramolecular structures, biotinylated RNA products were hybridized with Affymetrix GeneChip, arrays (HUM6000). Hybridized RNA was then detected using streptavidin-phycoerythrin staining. The DNA chips were scanned using a specially designed confocal scanner. Digitized image data were processed using the GeneChip software from Affymetrix (Lockhart et al., 1996). RNA abundance was determined based on the average of the differences between perfect match and mismatch intensities for each probe family. Gene induction or repression was considered significant if the change in average difference intensity was above twofold (de Saizieu et al., 1998; Wang et al., 1999). The method has been used successfully for a number of studies, addressing important biological questions (Lockhart et al., 1996; Wodicka et al., 1997; Cho et al., 1998; de Saizieu et al., 1998; Giaever et al., 1999; Lee et al., 1999; Wang et al., 1999).

mRNA differential display (DD)

The mRNA differential display is a RT-PCR (reverse transcription-polymerase chain reaction) based technique that favors rapid isolation of differentially expressed transcripts from two or more populations. Figure 3 showed how this technique works. Briefly, total RNA was isolated and subjected to RT using poly dT (T11) with either one nucleotide (G, A, or C) or two (shown in Figure as MG, MA, MC, or MT) anchored at its 3' end for specific transcription of a subset of mRNAs. The synthesized first stranded cDNA was then subjected to PCR amplification in the presence of [^{35}S] dATP using the correspoinding T_{12}MN (downstream) and one of the arbitrary 10 mers (upstream). PCR-amplified fragments

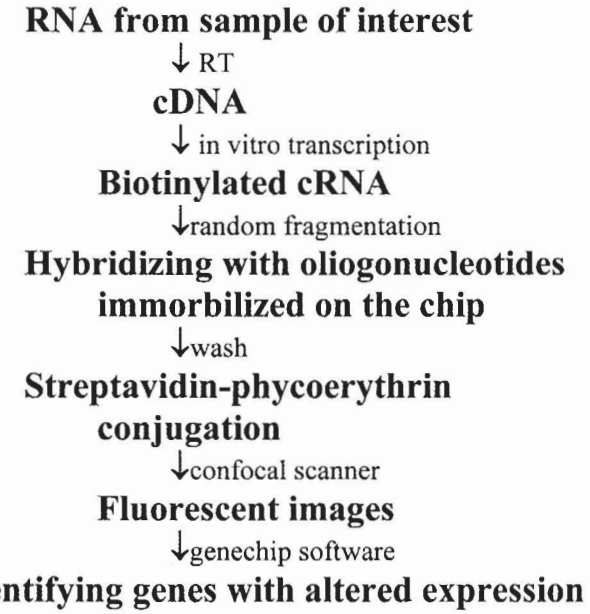

Fig. 2. Flow chart for DNA chip technology: RNAs were isolated from samples of interest and used to synthesized double stranded cDNA with oligo(dT) primer containing a T7 polymerase tail. cRNA target is generated by in vitro transcription using biotin-labelled ribonucleoside triphosphates, partially fragmented to disrupt secondary structures and subjected to hybridization with chip covered with millions of oligonucleotide probes representing different genes. After washing the arrays, hybridized RNA was conjugated to streptavidin-phycoerythrin and being detected by a specially designed confocal scanner as fluorescent images. The genes with altered expression (either increased or decreased) were scored using GeneChip software.

were loaded next to each other and run in a 6% denaturing polyacrylamide gel followed by autoradiography. The gel was visually examined and the reaction showing uniquely expressed fragments in one population was repeated. The PCR fragments reproducibly shown to be differentially expressed (detected only in one population, but not in the other) were excised from the gel, followed by PCR amplification of the eluted DNA. The PCR fragments were gel-purified and used as probes for Northern analysis to confirm the DD results. The clones detecting differential expression by Northern analysis were then subcloned into TA cloning vector and sequenced, followed by DNA database search and cDNA library screening for full-length cDNA, if necessary. The method has been widely used in many laboratories that leads to the cloning of many genes (Sun et al., 1994, 1995a,b; Liang and Pardee, 1997; Pennie et al., 1997; Sun, 1997; Goyns et al., 1998; Duan et al., 1999).

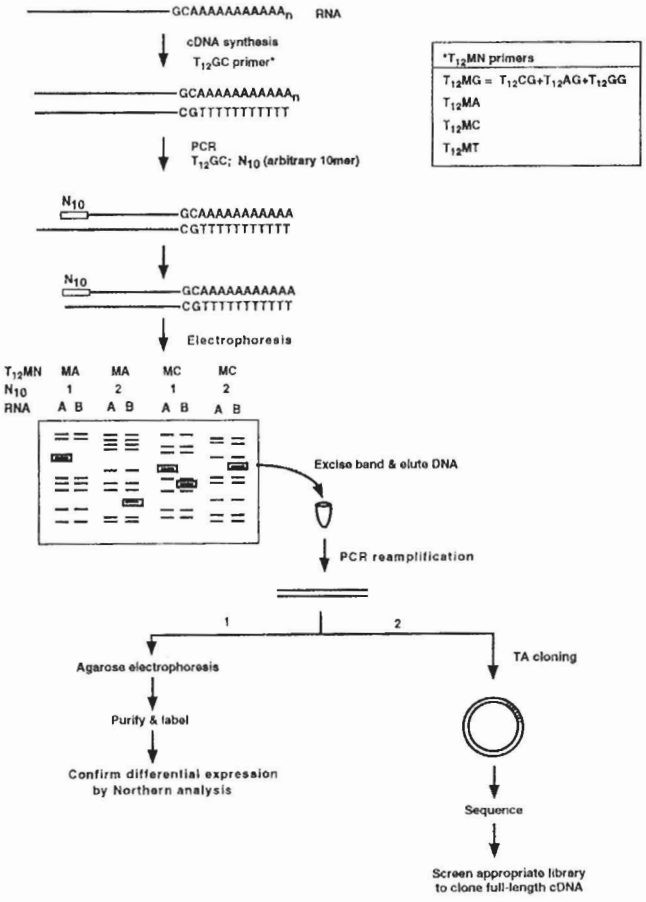

Fig. 3. Flow chart for mRNA differential display: Total RNAs were isolated from samples of interest and subjected to reverse transcription using poly d(T) with two nucleotides anchored in its 3'-end. The first stranded cDNAs were PCR amplified in the presence of ^{35}S-dATP with the corresponding primer used for RT and an arbitrary 10 mer. The PCR products were displayed in a sequencing gel. The bands reproducibly showing in one population, but not in the other were excised, PCR re-amplified and used as probes for Northern analysis to confirm the DD findings. Once confirmed, the PCR fragments were subcloned and sequenced to determine their identities. They may be used as probes for cDNA library screening, if necessary.

Signaling pathways mediating apoptosis induced by etoposide

Induction of apoptosis by etoposide in U2-OS cells

We have previously shown that etoposide, a topoisomerase II inhibitor, induces p53 DNA binding and transactivation in U2-OS, a human osteogenic sarcoma cell line harboring wildtype p53 (Bian and Sun, 1997). We have also observed that after etoposide treatment, cells undergo morphological changes such as cell shrinkage and detachment, reminiscent of apoptosis. To define the nature of cell toxicity, we performed a DNA fragmentation assay. Detection of 180 bp DNA laddering, a characteristic of apoptotic death would confirm cell death via apoptosis. Indeed, as shown in Figure 4, compared to the DMSO control (lane 1), etoposide treatment (25 μM) induces DNA laddering in a time dependent manner. No obvious DNA fragmentation was visualized 6 hrs post treatment (lane 2), but it is evident after 24 hrs treatment (lane 3). Induction of DNA laddering was dose-dependent. The higher dose (50 μM, lane 4) gave rise to more DNA fragmentation, as compared to a lower dose (25 μM, lane 3).

Identification of the genes responsive to apoptosis induced by etoposide

A set of four oligonucleotide arrays, representing

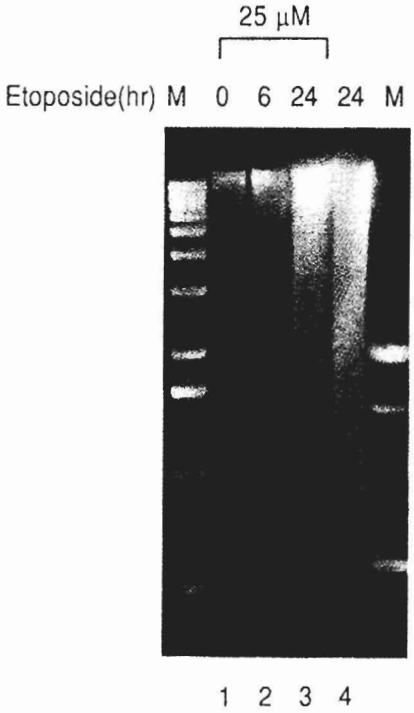

Fig. 4. Induction of DNA fragmentation in U2-OS cells by etoposide: Subconfluent U2-OS cells were treated with etoposide (25 μM or 50 μM) for 6 hrs or 24 hrs. The control cells were treated with DMSO for 24 hrs. After treatment, both detached and attached cells were harvested and subjected to DNA fragmentation assay as described (Sun et al., 1997). Lane 1: control; lane 2: etopside (25 μM) for 6 hrs; lane 3: etoposide (25 μM) for 24 hrs; and lane 4; etoposide (50 μM) for 24 hrs. The molecular markers (100 bp, on the right, and 1 kb, on the left) were included for size reference.

6,591 known genes, was screened for potential apoptosis-associated genes with poly (A+) mRNA isolated from cells treated with etoposide (25 μM) for 6 hrs (neither obvious morphological change, nor DNA fragmentation) and for 24 hrs (obvious morphological signs of apoptosis/DNA fragmentation). Cells treated with DMSO for 24 hrs were used as the control. Genes that displayed approximately two fold or greater changes were scored. Sixty-two mRNA species out of the 6,591 genes (~1%) were identified by these criteria. Of these sixty-two mRNA species, 41 were significantly induced and 21 were significantly repressed at either or both of the time points. These genes, based upon their potential functions, can be classified into 10 main functional groups, including 1) antioxidant enzymes/redox-related proteins; 2) cell cycle/growth regulators; 3) DNA binding proteins/transcription factors; 4) receptors/membrane proteins; 5) ribosomal proteins; 6) structural proteins; 7) enzymes and protein kinase; 8) metabolitic proteins; 9) proto-oncogenes; and 10) signal/stress proteins. Among these 62 genes, we characterized 12 genes by Northern analysis, including calcyclin/S100A6, ferritin LC, glutathione peroxidase, heterogeneous nuclear ribonucleoprotein core protein A1, metallothionein-2, ornithine decarboxylase, proliferaing cell nuclear antigen, S100 calcium-binding protein A2, transforming growth factor-ß type II receptor, thioredoxin, tubulin ß1, and Waf-1 (Wang et al., 1999). Although the fold changes were different between the chip and Northern methods, which varied by a number of factors including strengency of hybridization, variations in sample preparations, different ways in normalization for sample loading, and so on, an agreement of 92% was reached. This indicates that the DNA chip technology can be used as a rapid tool with a high accuracy to examine global changes of gene expression upon a particular stimulus (Wang et al., 1999).

An attempt to identify the genes possibly involved in a common signaling pathway of apoptosis triggered by various inducers

As one step further to identify which of these genes is the downstream molecule converging multiple apoptosis signals, we examined expression of all 12 genes after treatment with three different apoptosis inducers, etoposide, H_2O_2, or staurosporine. We reasoned that the gene(s) that is commonly activated by various apoptosis inducers or that converges various apoptosis signals would be an ideal one to study its function in apoptosis. Four cell lines, including osteogenic sarcoma lines, U2-OS (p53 positive), Saos-2 (p53 negative), lung carcinoma lines, NCI-H460 (p53 positive) and H1299 (p53 negative), were included in the study in an attempt to reveal p53-dependency as well as to minimize cell-line dependent variations. The results can be summarized as followings 1) none of these 12 genes was found to be induced or repressed by all three apoptosis inducers in all four cell lines; 2) none of the p53 responsive genes was induced by staurosporine, a universal protein kinase C inhibitor and apoptosis inducer; 3) the genes encoding Waf-1 and tubulin ß1 appeared to be induced or repressed, respectively, in

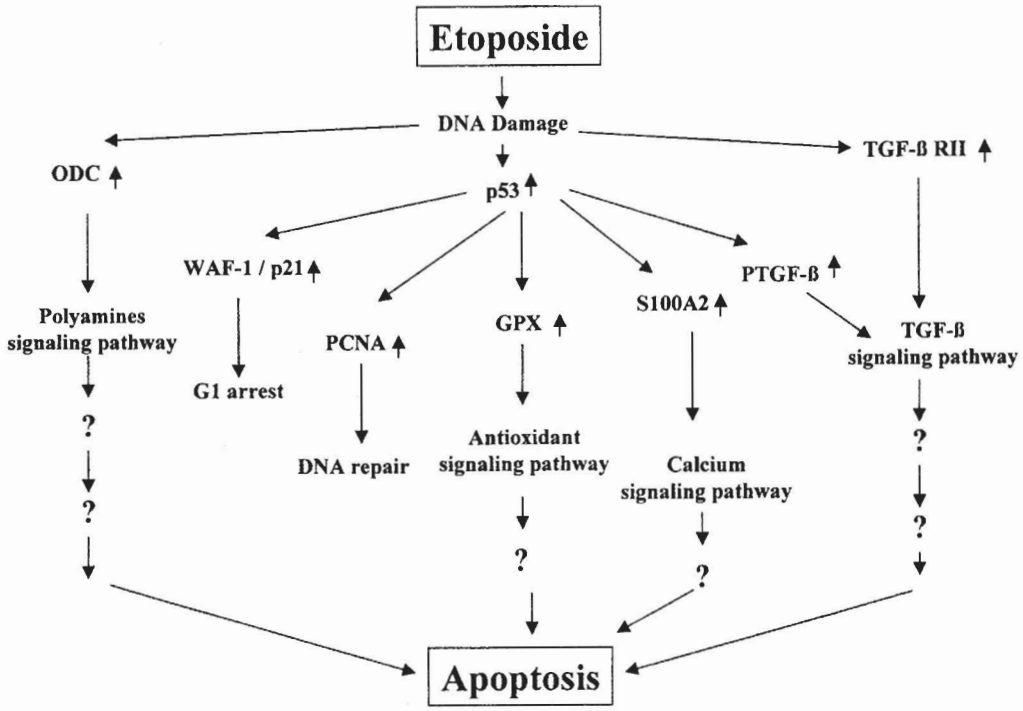

Fig. 5. Signaling pathways that mediate etoposide-induced apoptosis: DNA damage induced by etoposide triggers p53 signaling pathway as well as polyamines and TGF-ß signaling pathways via induction of expression of ODC (ornithine decarboxylase) and TGF-ß-RII (type II TGF-ß receptor). As shown, activated p53 induces expression of Waf-1/p21, PCNA (proliferating cell nuclear antigen), GPX (glutathione peroxidase), S100A2, and PTFG-ß, a novel TGF-ß family member. These p53 target genes, along with the genes encoding ODC and TGF-ß-RII, ultimately lead to apoptosis through uncharacterized details.

most of the cases; 4) there was cell line-dependent changes of gene expression even within the same apoptosis inducer (unpublished observation). Overall, none of 12 genes studied respond to all the inducers. The results indicate that different apoptosis stimuli could initiate unique signal pathways leading to apoptosis, and that different cell lines respond differently to various apoptosis inducers.

Signaling pathways that mediate etoposide-induced apoptosis

Among the 12 genes characterized, some are previously known as p53 responsive genes (Waf-1, PCNA), while others are the novel p53 target genes (GPX, S100A2, as well as PTGF-ß, a novel TGF-ß family member), and still others are probably involved in p53-independent signaling pathways (Tan et al., 1999a,b, 2000; Wang et al., 1999). A potential signaling pathways that mediate etoposide-induced apoptosis was proposed and shown in Figure 5. DNA damage induced by etoposide activates p53 and triggers p53 signaling pathway that in turn activates antioxidant pathway, calcium signaling pathway as well as TGFß pathway. On the other hand, DNA damage also triggers polyamine pathway by inducing ODC expression and TGFß signaling pathway by inducing expression of TGFß type II receptor. All these signal tranduction pathways converge and apoptosis results (Tan et al., 1999a,b, 2000; Wang et al., 1999).

Identification of two OP-inducible genes: glutathione synthetase (GSS) and sensitive to apoptotic gene (SAG)

The differential display (DD) technique was employed in an attempt to isolate genes responsible for or associated with OP-induced apoptosis in two murine tumor lines, both harboring wildtype p53 (Sun et al., 1993a,b, 1997). Since OP induced-apoptosis occurs obviously at 12 hrs post exposure (Sun et al., 1997), we reasoned that expression of the gene(s) responsible for apoptosis induction should be up- or down-regulated prior to the appearance of apoptosis. Six hrs of OP treatment was, therefore, conducted in one of these tumor lines, L-RT101, followed by the DD analysis. A total of 8 differential expressed fragments (four for up-regulation and four for down-regulation) was identified between DMSO control and OP treated cells after two independent DD analyses, using 8 upstream primers and 3 downstream primers. All eight fragments were PCR amplified and used as the probes for Northern analysis. Both OP-treated L-RT101 and H-Tx cells were examined. The gene(s) of interest will be those either being induced or repressed by OP in both cell lines since they both undergo apoptosis after exposure to OP. Only

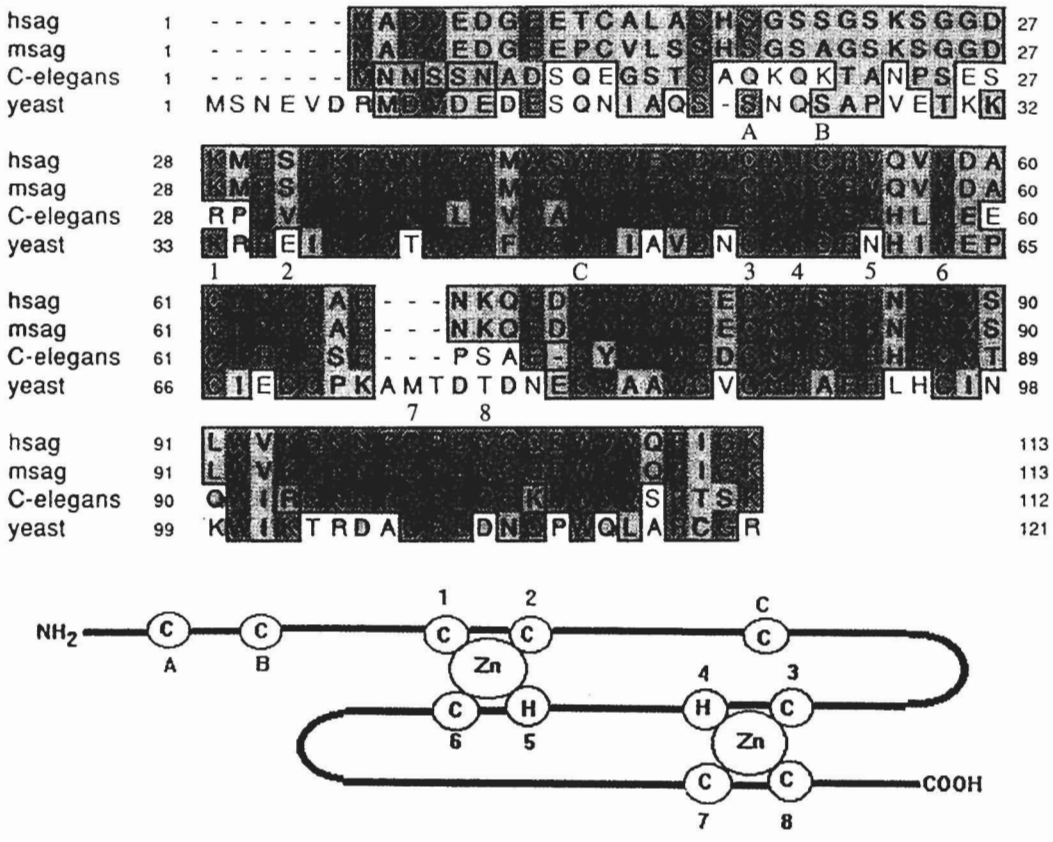

Fig. 6. SAG is evolutionarily conserved among different species: Comparative alignments of SAG coding sequence from human, mouse, *C. elegans* and *Saccharomyces cerevisiae* (yeast). Identity is shaded and similarity is boxed. The residues involved in the formation of the RING finger structure (C3H2C3) were labeled with Arabic numbers. Shown in the bottom panel is the putative zinc RING finger domain.

one of the eight fragments was confirmed as being induced by OP. Northern hybridization using this fragment as the probe detected two transcripts with the size of 1.2 kb and 2.1 kb, respectively. Both transcripts express at a very low basal level and are induced dramatically by the OP treatment (Sun, 1997). This fragment was then subcloned into a TA cloning vector and sequenced. The resulting clones fell into two different DNA sequences. The computer analysis using the GCG program revealed that one sequence was novel, named SAG for Sensitive to Apoptosis Gene which detected a single 1.2 kb transcript, while the second sequence had 98% identity to the 3' untranslated region of a cDNA encoding mouse glutathione synthetase (GSS) (Shi et al., 1996), detecting a 2.1 kb transcript. OP-induced GSS up-regulation occurred 6 hr post-treatment and reached the peak at 24 hrs. Induction is rather specific since it is not induced by DNA damaging agents such as adriamycin, camptothecin, or etoposide, indicating it is not induced through DNA damaging/p53 activation pathway (Sun, 1997). GSS is a rate-limiting enzyme catalyzing last step synthesis of glutathione (Meister and Larsson, 1995), an important intracellular thiol shown to scavenge reactive oxygen species (Sun, 1990). Induction of GSS by redox sensitive OP may reflect a cellular defense response against OP-induced redox disturbance. Overall, through DD analysis, we identified two OP-inducible genes, one is GSS, encoding a rate-limiting enzyme that catalyzes glutathione synthesis and the second is SAG, an evolutionarily conserved novel gene, encoding a zinc RING finger protein which is discussed below in much detail.

SAG inhibits apoptosis, stimulates cell growth and promotes protein ubiquitination and degradation

SAG protects cell from apoptosis induced by redox agents

The full-length SAG was cloned using DD-generated fragment as the probe to screen both mouse and human cDNA libraries. Computer analysis revealed that SAG encodes a novel zinc RING finger protein that consists of 113 amino acids with a calculated molecular mass of 12.6 kDa. SAG is highly conserved during evolution with a 96% sequence identity between human and mouse, 70% between human and *C. elegans* and 55% between human and yeast (see Fig. 6). In human tissues, SAG is ubiquitously expressed with a high level in skeletal muscles, heart and testis. SAG is localized in both the cytoplasm and the nucleus of cells and its gene was mapped to chromosome 3q22-24 (Duan et al., 1999). Bacterially expressed and purified human SAG is redox sensitive. Upon H_2O_2 exposure, SAG forms oligomers as well as monomer doublets due to the formation of the inter- or intra-molecular disulfide bonds, respectively. This process can be reversed by DTT or prevented by pre-treatment with an alkylating agent, N-ethylmaleimide (Swaroop et al., 1999). SAG was also found to bind to metal ions, zinc, copper as well as iron and prevents lipid peroxidation induced by copper or a free radical generator. When overexpressed in several human cell lines, SAG protects cells from apoptosis induced by redox agents (metal chelator OP and metal ions, zinc or copper) (Duan et al., 1999). Since apoptosis is mediated in many cases by cytochrome c release followed by caspase activation (Liu et al., 1996; Kluck et al., 1997; Li et al., 1997; Yang et al., 1997; Mignotte and Vayssiere, 1998), we examined this signaling pathway in metal ion-induced apoptosis models. SAG appears to inhibit and/or delay metal ions-induced cytochrome c release and caspase activation. Thus, SAG is a cellular protective molecule that appears to act as an antioxidant to inhibit metal ions- and ROS-induced apoptosis (Duan et al., 1999; Swaroop et al., 1999). Importantly, SAG was also induced during ischemia/reperfusion-induced brain injury in mice and injection of recombinant adenovirus expressing human SAG reduced infarct size of the ischemia-damaged brain (Yang et al., 2000).

It is noteworthy that although we do not know whether or not induction of GSS and SAG is p53-dependent at the present time, induced GSS and SAG do not mediate OP-induced apoptosis. Rather, they protect cells from apoptosis. Thus, OP-induced induction of GSS and SAG appears to reflect a cellular defense response against redox disturbance. When this disturbance overcomes the protective response, cells eventually undergo apoptosis.

Requirement of the RING finger domain for apoptosis protection

During the course of SAG cloning, we identified two forms of deletions of SAG mRNA in colon and testicular carcinoma cell lines. The first form (SAG-MU1) consists of 7 bp deletion that results in a frame shift and abolishes RING finger domain. The second (SAG-MU2) consists of in-frame 48 bp deletion that truncates 16-amino acids in protein but retains RING finger domain. Functional study using stably transfectant lines reveals that like wildtype SAG, the SAG-MU2 retains anti-apoptosis activity, whereas the SAG-MU1 showed no such a protection. The result indicates that the C-terminal zinc RING finger domain is required for anti-apoptosis activity of SAG (Sun, 1999).

Yeast homolog of human SAG is a growth essential gene

Since the sequence identity between human SAG and a yeast ORF (open reading frame, YOL133w) is 55%, and functional conservation exists between two species, we used yeast genetics approach to characterize SAG function in vivo. Targeted disruption of *ySAG*, yeast homologue of human SAG, and subsequent tetrad analysis revealed that *ySAG* is required for yeast viability. Complementation experiment showed that the lethal phenotype induced by the *ySAG* deletion is fully

rescued by wildtype SAG, but not by several hSAG mutants. The cell death induced by SAG deletion was accompanied by cell enlargement and abnormal cell cycle profiling. To determine the nature of yeast cell death induced by SAG-deletion, we employed again the DNA chip array, capably of detecting the entire ORF of the yeast genome (Ye6100). Changes in multiple genes involved in cell cycle progression were detected upon SAG withdrawal (Swaroop et al., 2000). Thus, SAG appears to be involved in cell cycle regulation.

SAG promotes cell growth/S-phase entry under serum-starved conditions

Having established in yeast that SAG is require for cell growth and regulates cell cycle progression, we next examined potential role of SAG in controlling mammalian cell cycle. We used several approaches to increase expression of SAG in mammalian cells and measured its effect. We found that SAG can promotes cell growth under serum-starved conditions. Micro-injection of SAG mRNA or protein, but not antisense mRNA or LacZ control into quiescent NIH3T3 cells induces S phase entry as determined by ^{3}H-thymidine incorporation. Likewise, infection of immortalized human epidermal keratinocytes (Rhek) with adenovirus SAG, but not Lac-Z control, stimulates cell growth. Moreover, SAG over-expressed cells, but not the vector control cells, continued to progress through cell cycle under serum starved conditions. Thus, SAG is a proliferating factor that promotes cell growth under stress conditions (Duan et al., 2000b).

SAG does not induce neoplastic transformation, but its antisense transfection inhibits growth of human colon carcinoma cells

Since SAG stimulates proliferation, we next examined its potential oncogenic activity by overexpressing SAG in mouse epidermal JB6 cells that is a well-established tumor promotion/progression model (Sun et al., 1993a,b, 1995a,b, 1996). Expression of SAG in transformation-resistant cells (P-variant) did not induce a promotion to transformation-sensitive phenotype. Likewise, SAG expression in transformation-sensitive cells did not cause cellular transformation. The results indicate that SAG is not a dominant oncogene to induce neoplastic transformation. We also found that the SAG protein level was elevated in human colon cancer tissues as compared to the adjacent normal tissues from the same patients. We then examined potential growth inhibitory activity of antisense SAG on human DLD-1 colon carcinoma cell line. Indeed, antisense transfection inhibits cell growth in culture plates. Stable clones that express antisense SAG mRNA showed a decreased ability to grow in soft agar (Duan et al., 2000a). Thus, although overexpression of SAG did not cause cellular transformation, certain level of SAG is important for the maintenance of tumor cell growth. Targeting SAG expression may, therefore, have a therapeutic value in cancer treatment.

SAG is a new component of E3 ubiquitin ligase - SAG stimulates cell growth probably by promoting p27 degradation

A RING finger protein, Rbx (RING box protein) or ROC (regulator of cullins) or Hrt was recently identified to be a new component of E3 ubiquitin ligase that promotes degradation of yeast cell cycle inhibitor, SIC1 and NF-κB inhibitor, IκBα (Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). Direct sequence comparison revealed that SAG is the second member of Rbx/ROC/Hrt family. Indeed, like ROC1/Rbx1/Hrt1, SAG has E3 ubiquitin ligase activity when complexed with cullin-1 (Swaroop et al., 2000).

Ubiquitin/proteasome system plays an important role in regulation of many key biological processes, including cell cycle progression, transcription, and signal transduction (King et al., 1996; Maniatis, 1999). The target proteins include cyclins (A, B, D1, E) and cyclin-dependent kinase inhibitors (p21, p27, p57), tumor suppressors (p53, ß-catenin), oncogenes (c-myc, c-Mos, EGFR, E1A), as well as transcription factors (c-Jun and c-Fos, DP1, E2F, DP1, NF-κB/IκB) (King et al., 1996; Rolfe et al., 1997; Maniatis, 1999). Ubiquitination of a target protein involves a multi-step, well-defined reactions catalyzed by several enzymes, including ubiquitin-activating enzyme, E1, ubiquitin-conjugating enzyme, E2, and ubiquitin ligase, E3, a multi-component protein complex (Ciechanover, 1998; Tyers and Willems, 1999). The polyubiquitinized target protein is recognized efficiently by the 26S proteasome followed by protein degradation (Ciechanover, 1998).

Cell cycle progression from G1 to S phase is precisely regulated by cyclin/cyclin dependent kinases (CDKs) and their inhibitors (CKIs) (Hunter and Pines, 1994). The CDK4,6/cyclin Ds promote early G1 progression that is inhibited by CKIs, p16, p15 and p21. The late G1 progression was mainly driven by CDK2/cyclin E and inhibited by p27 and p21 (Hartwell and Kastan, 1994; Hunter and Pines, 1994; Sherr, 1994). The CKIs fall into two classes: the Kip/Cip family that includes three structurally related proteins, p21, p27, p57 and INK4 family consisting of four related proteins, p16, p15, p18 and p19 (Clurman and Roberts, 1995; Elledge and Harper, 1994; Sherr and Roberts, 1995). It has been known that Kip/Cip family proteins were degraded through ubiquitin pathway (Pagano et al., 1995; Yu et al., 1998; Montagnoli et al., 1999; Rousseau et al., 1999; Shirane et al., 1999; Urano et al., 1999) and p27 is accumulated in quiescent cells (Sherr, 1994).

Since SAG/ROC2/Rbx2/Hrt2 is involved in ubiquitin pathway (Kamura et al., 1999; Ohta et al.,

1999; Skowyra et al., 1999; Tan et al., 1999), we, therefore, tested whether SAG-induced cell growth is achieved by promoting CKI degradation. Although no significant difference of p21 and p57 levels was seen between the vector controls and SAG over-expressed cells, serum starvation induces more than 10-fold accumulation of p27 in the vector control cells. Accumulation of p27 was remarkably inhibited in SAG-expressed cells. Thus, it appears that SAG induces S phase entry upon serum starvation at least in part by promoting p27 degradation (Duan et al., 2000b).

Conclusions and perspectives

Through the use of two expression profiling techniques, DNA chip hybridization and mRNA differential display, we have elucidated signaling pathways that mediate etoposide-induced apoptosis and have cloned a novel gene, SAG. Further characterization revealed that SAG is a redox sensitive protein and when over-expressed, it protects cell from apoptosis and stimulate cell growth. Sequencing comparison also defined SAG as a second member of Rbx/ROC/Hrt family that belongs to a new component of E3 ubiquitin ligase to promote degradation of many biological important molecules, such as p27. SAG functions and proposed mechanism of action were summarized in Figure 7. For apoptosis protection, SAG either a) inhibits/delays metal ion/ROS-induced cytochrome c release and caspase activation (Duan et al., 1999); or b) likely promotes degradation of IkBα (Ohta et al., 1999; Tan et al., 1999), leading to the activation of NF-κB, a transcription factor that inhibits apoptosis and mediates inflammation response (Baldwin, 1996; Beg and Baltimore, 1996; Mayo et al., 1997; Baeuerle, 1998; Ghosh et al., 1998). On the other hand, growth stimulation by SAG appears to involve at least in part its activity in promoting ubiquitination and degradation of p27, a cyclin dependent kinase inhibitor. Thus, SAG could be a valid therapeutic target for cancer (apoptosis inhibitor/growth promoter) as well as inflammation (NF-κB activator).

It is clear that the gene-profiling technology can contribute significantly to our understanding of complex cell responses to various stimuli. Once identified such information allows for precisely characterization of individual gene or set of genes in the same signaling pathway. Ultimate identification of a pathway or pathways in response to any given treatment of interest will have a profound influence in target identification and validation in future drug discovery.

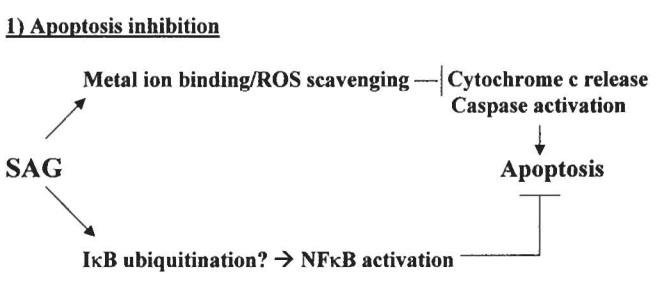

Fig. 7. SAG functions and proposed mechanism of action: SAG can inhibit apoptosis as well as promote cell growth. Shown are potential mechanisms of action. Some of them are subjected to experimental validation that is currently underway in my laboratory.

Acknowledgements. This work is dedicated to my wife, Hua Li and our children, Steven and Grace.

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