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TWO MOLECULAR MECHANISMS OF APOPTOSIS RESISTANCE

by

Brent Daniel Butts

A Dissertation Submitted to the Faculty of the

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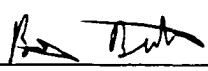
SIGNED: 

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Abstract:

Many cancer cells develop resistance to apoptosis. It is important to understand how this phenotype develops, so that these cancers can be effectively treated. The results presented in this dissertation describe two molecular mechanisms of apoptosis resistance.

A mouse keratinocyte model system consisting of the benign 308 parental cell line and two malignantly progressed variants (6M90 and 6R90 cells) were used to explore the relationship between reactive oxygen species (ROS) and apoptosis resistance. Previous work showed elevated basal levels of ROS in 6M90 and 6R90 cells. The results shown here demonstrate increased resistance to UV-induced apoptosis of the variants compared to the parental line. Pharmacological and genetic approaches were used to decrease the steady-state levels of ROS in the two malignant cell lines. This increased their sensitivity to apoptosis. ROS are implicated in the activation of the anti-apoptotic Akt kinase. 6M90 and 6R90 cells had higher levels of activated Akt. Modulation of ROS levels in the 6M90 and 6R90 cells decreased the levels of activated Akt. These studies provide a molecular mechanism to explain the chronically elevated ROS and apoptosis resistance seen in many tumors.

Another mechanism by which tumor cells resist apoptosis is to upregulate the anti-apoptotic protein Bcl-2. A putative response element (PPRE) for the peroxisome proliferator activated nuclear receptor (PPAR) was found in the 3' UTR of *bcl-2*. Further experiments indicated that the γ subtype of PPAR bound the putative PPRE and could activate transcription. In cells transfected with PPARγ, increased levels of *bcl-2* mRNA

and Bcl-2 protein were seen as compared to empty vector-transfected cells. When treated with bile acids to induce apoptosis, the PPARγ-transfected cells were twice as resistant as empty vector-transfected cells. These studies show, for the first time, that a sequence within the 3' end of the *bcl-2* gene can regulate transcription of the gene through interactions with PPARγ. These findings may be particularly relevant in colon cancer, where PPARγ and Bcl-2 are often overexpressed.

Chapter I. Introduction

Programmed cell death, or apoptosis, was described over 20 years ago by Wyllie et al. [1] and has become a major area of research. Much progress has been made towards understanding the molecular mechanisms of apoptosis and how this important process is regulated. Once cell death was recognized as a potentially regulated process, it quickly became evident that dysregulation of apoptosis occurs in many human diseases.

Three Stages of Apoptosis :

The molecular events of apoptosis have been grouped into three general stages: signaling, commitment and execution. In the signaling stage, either an internal or external signal is transmitted to cells which causes them to evaluate whether apoptosis should commence. The committed step primarily involves the release of proapoptotic factors from the mitochondria. These factors can initiate a chain of events from which there is no rescinding of the death command. The final stage is the "execution" of the cell, largely carried about by a specific group of proteases known as caspases. This stage also includes cleanup of the "cell corpse" by neighboring healthy cells. The different stages of apoptosis are regulated by the Bcl-2 family of proteins which contains both pro- and antiapoptotic members. How this family works to promote or prevent apoptosis will be discussed in detail further below. First, an overview of the three stages of apoptosis will be discussed.

Signaling Stage

In the signaling stage, the cell senses an apoptotic stimulus and conveys this message so that it can be evaluated in light of other intra- and extracellular signals and events. Most apoptotic signals may be classified as either receptor-mediated or non-receptor mediated, based on how they are received by the cell.

One of the more extensively studied signaling cascades is through the Fas receptor [2]. Cell surface Fas receptor (Fas) molecules trimerize with other Fas receptors upon binding to their ligands (FasL). A critical domain of the Fas intracellular region is a stretch of approximately 80 amino acids known as the death domain (DD) [3]. Cytoplasmic proteins are able to bind the DD and initiate the apoptotic signal. Upon aggregation, the internal domain of FasR recruits the Fas-associated death domain protein (FADD). In addition to containing a DD, FADD contains another essential region, the death effector domain (DED). As the name implies, this region is important in bringing about apoptosis. The DED enables FADD to recruit caspase 8, which similarly contains a DED [4, 5]. When caspase 8 is recruited to the membrane, an autoproteolytic activation occurs, converting the pro-form of the caspase to the active form. Additionally, this autoproteolytic step releases the caspase from the FasR/FADD complex and once freed, it moves to the cytosol and activates other unbound caspase 8 proenzymes [6] and other "downstream" caspases. In addition to Fas, there are several other receptors which can induce apoptosis upon ligand binding, such as the TNFα receptor [7], DR3,DR4, DR5 and CAR1. (For reviews on receptor-mediated apoptosis, see [8, 9].)

Many apoptotic signals are not receptor-mediated. For instance, DNA damage is known to signal apoptosis in many cells, often through upregulation of p53 [10]. Loss of attachment to extracellular matrix is another signal for apoptosis for many cell types [11]. This process of apoptosis induced by loss of attachment is known as anoikis.

The fact that a diverse array of signals can cause cells to undergo apoptosis highlights the importance of apoptosis in maintaining homeostasis in healthy tissues. By assimilating external and internal signals, cells are able to kill themselves in response to different stimuli. This cellular suicide is necessary in order to prevent the continued growth of cells which are potentially "dangerous"—such as those with massive DNA damage, or those which have detached from their surrounding neighbors.

Committed Stage

Research indicates that the mitochondria are critical to the pivotal committed stage of apoptosis (reviewed in [12-17]). The ultimate commitment point seems to be the release of cytochrome *c* from the inner membrane of the mitochondria to the cytosol. Early experiments with cell-free extracts hinted at the importance of cytochrome *c* in apoptosis [18]. Release of cytochrome *c* has since been shown to be an almost universal phenomenon in mammalian cells undergoing apoptosis [19-21].

Cytochrome c release: two theories

Mechanistic details of how cytochrome *c* is released are still not fully elucidated. Two prevalent theories attempt to explain this event. One theory is that the opening of the permeability transition pore (PTP) enables cytochrome *c* to be released. The PTP is a large protein complex within mitochondrial membranes. It consists in part of the adenine

nucleotide translocator (ANT) in the inner membrane, the voltage dependent anion channel (VDAC) in the outer membrane and cyclophilin D in the matrix. When opened, the PTP allows solutes with a molecular weight of 1500 Da or below to freely diffuse from the matrix to the cytosol [22]. A transient opening of the PTP is proposed to be mediated by proapoptotic members of the Bcl-2 family, such as Bax [23], through their direct interactions with VDAC. Conversely, the antiapoptotic Bcl-2 family members were shown to close the VDAC channel. Additional studies demonstrated the interaction of proapoptotic Bcl-2 proteins with ANT. As with the VDAC/Bax interaction study, an *in vitro* lipid bilayer containing the ANT was created and addition of Bax caused an opening of a channel through the bilayer. Interestingly, this channel had a different electrophysiological profile from those formed by Bax or ANT alone [24]. Similar to the results with VDAC, the antiapoptotic protein Bcl-2 was able to inhibit channel formation by ANT and Bax.

Structural studies of the antiapoptotic protein Bcl-x_L led to the idea that Bcl-2 family members regulate apoptosis by forming channels in the mitochondrial membrane. The tertiary structure of Bcl-x_L consists of 7 helices—two layers of amphipathic helices enclosing two central helices. The two central helices are hydrophobic and are each about 20 amino acids long, which has been calculated to be of sufficient size to penetrate the hydrophobic cross-section of a typical lipid bilayer [25]. These two helices by themselves are not enough to form a channel, but the ability of Bcl-2 family members to dimerize or oligomerize would enable the helices from multiple proteins to form a

channel [26]. This structure was found to be similar to some pore-forming domains of bacterial toxin proteins [27].

Using the Bcl-x_L structure as a "scaffold", computer modeling has determined that proapoptotic members of the Bcl-2 family also may have channel-forming abilities [26]. *In vitro* experiments with isolated mitochondria and recombinant Bax showed that over 20% of the total cytochrome *c* (greater than 5-fold the background amount) was released into the cytosol after 1 hr incubation with Bax. Similar results were seen when Bax was overexpressed in cells [28].

The paradox that antiapoptotic members such as Bcl-x_L are capable of forming channels (a seemingly proapoptotic characteristic) may be resolved by noting that the channel-forming abilities of the antiapoptotic members may merely be an *in vitro* artifact, or that the conformational changes necessary for the correct helices to be exposed may not occur *in vivo*, given the different pH and physiologically relevant concentrations of proteins. Conversely, this hypothesis of pore-formation or inhibition of pore-formation by pro- and antiapoptotic members of the Bcl-2 family members, respectively, is the basis for the rheostat model of Bcl-2 action. This model will be discussed in detail below.

Role of cytochrome c in apoptosis

Despite the uncertainty in exactly *how* cytochrome *c* is released from the mitochondria, once it enters the cytosol, its role in apoptosis is fairly well defined. A complex consisting of cytochrome *c*, dATP, the apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 aggregate together in what has been termed the apoptosome ([29] and reviewed in [30]). The function of Apaf-1 in the apoptosome is to act as a

scaffold protein, as it has both oligimerization domains (in order to form homo-oligomers) as well as caspase associated recruitment domain (CARD) domains for the recruitment of caspase 9. In addition, Walker A and B boxes are present, enabling Apaf-1 to bind nucleotides. The hydrolysis of ATP to ADP by Apaf-1 is a necessary step in the activation of caspase 9 [21]. Similarly, cytochrome *c* binding appears to be indispensable for normal Apaf-1 to activate caspase 9. When all members of the apoptosome are present, caspase 9 is activated by the proximity of other caspases within the apoptosome. Thus, the regulation of the apoptosome is largely a spatial regulation, in that a key component of its assembly (cytochrome *c*) is kept sequestered within the mitochondria until the cell has committed to undergo apoptosis.

Additional mitochondrially sequestered factors

Before discussing the execution stage of apoptosis, it should be noted that other molecules sequestered in the mitochondria appear to have roles in apoptosis. This further demonstrates the central role the mitochondria play in committing the cell to undergo apoptosis. It is also intellectually appealing that certain "killer factors" are kept safely sequestered within a double-membraned organelle until the decision has been made for the cell to die by apoptosis.

One mitochondrially-sequestered molecule was found independently by two different research groups [31, 32] and hence has acquired two names: second mitochondrially-derived activator of caspases (Smac) and direct IAP binding protein with low pH (Diablo). Its mode of action is to inhibit an inhibitor. A class of molecules known as inhibitors of apoptosis (IAP) block apoptosis at the execution stage by

inhibiting caspase activation (reviewed in [33]). Smac works to induce apoptosis by interfering with the interaction between IAPs and caspases [34]. By removing this inhibition, Smac allows apoptosis to proceed.

Another mitochondrially-sequestered, proapoptotic protein is apoptosis-inducing factor (AIF) [35, 36]. It is a flavoprotein with significant homology to bacterial oxidoreductases. Flavin adenine dinucleotide (FAD) is incorporated into the protein through interactions with specific amino acids. Interestingly, while FAD incorporation is not necessary for AIF's apoptogenic activity, mutation of amino acids within the oxidoreductase domain abolishes the ability of AIF to induce apoptosis [37]. After AIF is released from the mitochondria, it translocates to the nucleus, where it causes chromatin condensation and high molecular weight DNA cleavage. AIF's mechanism of action is not known, but in cellular and cell-free systems its activity has been shown to be caspase-independent. (These results and other aspects of AIF are reviewed in [38].)

Execution stage

The execution stage of apoptosis is generally due to the action of a family of proteins called caspases. These are proteases which recognize an essential aspartic acid residue in the cleavage site of their substrates, making them one of the more specific types of proteases. Caspases have an N-terminal pro-domain, a large subunit containing the conserved active site cysteine and a smaller C-terminal subunit. Within the cell, caspases exist in an inactive state. They require two rounds of cleavage to become active. The first cut separates the small and large caspase subunits from each other, while the second removes the N-terminal prodomain. The region between the small and large

subunits of caspases contain an aspartic acid. This is consistent with the observations that, at a high enough concentration, caspases can autoactivate and that they mediate and amplify the apoptotic signal through activation of other caspases. Once the two cleavage events occur, caspases assemble as tetramers of two large and two small subunits, containing two active sites [39-42].

To date, fourteen mammalian caspases have been described, twelve of which are present in humans. Based on their preferred substrate cleavage sequence [43], they have been grouped into three different categories. Category I caspases are prominent in cytokine processing and typically are not involved in apoptosis. Category II caspases are the effector caspases. Category III caspases are the initiator caspases and they have longer N-terminal domains, containing motifs such as DED and CARD which are crucial for protein interaction/localization. Once initiating caspases (e.g., 8 or 9) are activated, they cleave and thereby activate effector caspases (e.g., 3, 6 and 7). It is the targets of the latter caspases that are thought to bring about the gross morphological changes observed in apoptosis.

Caspase Targets

Well over one hundred proteins have been identified as caspase substrates [44, 45]. These include structural proteins, signaling proteins and regulators of transcription, translation, cell cycle, DNA repair and cell-cell interactions. Thus, caspase activity renders many cellular proteins nonfunctional and disrupts important cellular functions which helps lead ultimately to cellular demise. Some proteins, however, are activated by caspase cleavage. Usually these proteins are capable of bringing about further

destruction. For instance, caspase activated DNAse (CAD, also known as DFF40) is a nuclear DNAse that is kept inactive by association with an inhibitory subunit iCAD (DFF45). Cleavage by caspase 3 [18, 45, 46] releases the inhibitory subunit, allowing the DNAse to become active and initiate DNA fragmentation, a common hallmark of apoptosis.

Caspase Regulation

Given the irreversible nature of caspase activity, it is imperative that these proteins are kept under strict control until the cell is fully committed to apoptosis. Several levels of caspase regulation are known. The first, noted above, is that caspases are zymogens and two rounds of cleavage must occur for their full activation. Once caspases are in an active conformation, their activity can be inhibited by a class of proteins called the inhibitor of apoptosis (IAP). Eight IAP family members have been discovered in humans (reviewed in [33]). The prototypical human family member, XIAP, inhibits caspases 3, 7 and 9. Additional family members inhibit other caspases (reviewed in [47]). IAPs function by impeding access to the active site of a caspase and thus preventing it from interacting with substrates.

Another recently described mode of caspase regulation, at the post-translational level, is through modification of the active site cysteine. S-nitrosylation of this cysteine renders the caspase inactive [48, 49]. When MCF-7 breast cancer cells (which do not express caspase 3) were transfected with caspase 3, it was shown that the caspase was S-nitrosylated. This was reversible upon stimulation of the cells to undergo apoptosis [50].

This additional level of regulation further ensures that caspases are not activated until necessary.

"Cellular Cleanup"

A vital, recently explored facet of apoptosis is the final step wherein the membrane-enclosed cell fragments (called apoptotic bodies) are phagocytosed by surrounding cells. Apoptotic cells undergo cell surface changes so that 'eat me' signals are displayed to neighboring cells. Phosphatidylserine (PS) is normally found in the inner leaflet of the cell membrane and its movement to the outer leaflet during apoptosis is a well-documented phenomenon. The exposure of PS is one "eat me" signal that an apoptotic cell transmits. Externalized PS binds to a receptor on phagocytic cells [51]. Intriguingly, it appears that not only does the apoptotic cell send 'eat me' signals to phagocytes, but in some cases phagocytes send signals that accelerate (and sometimes even appear to cause) apoptosis ([52] and citations therein). Upon receipt of the 'eat me' signals, the engulfment machinery of the phagocyte signals back to the execution machinery of apoptotic cells to ensure complete apoptosis.

Typically, membrane integrity is maintained throughout apoptosis such that cytosolic components are not released interstitially. In contrast, necrotic cells do not maintain membrane integrity but rather swell and burst. Hence, necrotic death results in an inflammatory response. The absence of an inflammatory response is an important part of apoptosis. The specific interactions between the phagocytic cell and the apoptotic cell allows for cell removal without inflammation. Interactions between the apoptotic cell and phagocytes may in fact further reduce inflammatory signals. For instance, macrophages

are suppressed from releasing pro-inflammatory factors, such as TNF–α, when they phagocytose apoptotic cells [53, 54].

Bcl-2 Family and Apoptosis

Bcl-2 was the first oncogene discovered which functions to prevent cancer cells from dying rather than increasing their proliferative capacity [55]. Since that time, the family of Bcl-2-related genes has grown to a current count of over a dozen. Despite intense research efforts, the precise manner in which Bcl-2 is able to prevent apoptosis is still not fully understood.

Bcl-2 structure

In order to be considered part of the Bcl-2 family, significant homology with at least one of four regions termed Bcl-2 homology domains (BH) must be present. The BH3 and BH4 regions may be the most functionally important parts of these proteins. Most proapoptotic members seem to have BH3 but not BH4 regions; some only have a BH3 region. Conversely, most antiapoptotic members contain BH4 and may or may not possess a BH3 domain. Several studies have shown that mutating or eliminating the BH4 region of Bcl-2 increases apoptosis or decreases resistance to apoptotic signals [56-58]. Studies by Cheng et al. [59], demonstrating that caspases can cleave Bcl-2 and cause it to become proapoptotic, further demonstrate the importance of the BH3 and BH4 regions. The site of cleavage is predicted to remove the BH4 region, causing Bcl-2 to contain only BH1-3 regions, much like Bax and other proapoptotic members.

The BH regions tend to form alpha helices and structural studies with Bcl-x_L have revealed that BH1, 2 and 3 form a hydrophobic cleft on one side of the molecule [60]. A peptide corresponding to the BH3 region of the proapoptotic family member, Bak, was shown to bind at this cleft. While this peptide was a random coil in solution, it formed an alpha helix upon binding to Bcl-x_L [60]. More recently, the 3D structure of monomeric Bax was published ([61] and reviewed in [62]). An intriguing finding is that the BH3 domain and the putative transmembrane domains are masked inside a hydrophobic core. Given that this is the conformation of inactive Bax, it follows that one or both of these regions must be exposed upon "activation" of Bax to a proapoptotic form. Similar to the Bcl-x_L/Bak interaction described above, another BH3-only protein, Bid, is able to interact with Bax and induce a conformational change. This allows Bax to insert into the mitochondrial membrane. Bid binding has been shown to induce Bax oligomerization *in vitro* [63]. Because Bax oligomerization is thought to occur via the BH3 domains [64, 65], this would indicate that BH3 exposure (unmasking) is accomplished by Bid binding. This cannot be the sole mechanism for its activation, however, as Bax has been found to insert into the mitochondrial membrane in $Bid^{-/-}$ cells [66]. Given the 10 or more currently known BH3-only proteins, there is likely to be some functional redundancy between those members.

Models for apoptosis regulation by Bcl-2 family members

Rheostat model

Implicit in the above descriptions of domain interactions between pro- and antiapoptotic members is the assumption that these interactions are what ultimately determines whether apoptosis will occur. This "rheostat model" of apoptosis regulation by Bcl-2 family members [67] essentially states that the ratio of pro- to antiapoptotic proteins will determine the outcome of the cell. It is hypothesized that Bcl-2 family members are capable of making pores in membranes. Proapoptotic Bcl-2 family members cause release of cytochrome *c* while antiapoptotic members strive to prevent this release, presumably by binding and inactivating the proapoptotic members.

Evidence in support of the rheostat model includes studies showing that overexpression of antiapoptotic members prevents or delays the release of cytochrome *c* while overexpression of proapoptotic members hastens its release [18, 20, 68]. Release of cytochrome *c* by proapoptotic Bcl-2 members may occur through oligomerization at the mitochondrial membrane to form pores, through which cytochrome *c* is released. The inhibition of this release by antiapoptotic members would occur by binding to the proapoptotic members and inhibiting their pore-forming abilities. Bcl-2 members are capable of forming ion channels or releasing cytochrome *c* from artificial lipid bilayer micelles [69-71] or isolated mitochondria [28]. The physiological relevance of these results with cell free models is supported by studies showing that enforced expression or dimerization of Bax alters mitochondrial membrane potential and in some cases leads to

release of cytochrome *c* [72-74]. In many cases, Bcl-2 overexpression reverses these effects.

The mitochondria is a double-membraned organelle, with the inner membrane having a larger surface area. Since cytochrome *c* is localized to the outer surface of the inner membrane, only where the inner and outer membrane abut each other can the pores formed by Bax (or other proapoptotic members) result in its release. When more Bax/Bax oligomers are present, there is a greater chance that some will insert at a junction point of the inner and outer membranes. A greater ratio of Bcl-x_L/Bax oligomers could suppress apoptosis by either forming complexes which cannot effectively form pores or by titrating Bax away from other Bax complexes, thus inhibiting the formation of pore-forming oligomers.

While the rheostat theory is attractive, it is still not proven and enough exceptions exist that this may not be a universal mode of Bcl-2 action. The observation that Bcl-2 overexpression is not always capable of inhibiting the release of cytochrome *c* is one such exception. Another is the necessity of localization at the mitochondrial membrane. Given that cytochrome *c* release via pore formation or interference with mitochondrial channels is critical, it is reasonable to assume that Bcl-2 members must act at the mitochondrial surface. However, E1B is a viral homolog to Bcl-2 which localizes to the nuclear envelope or the endoplasmic reticulum and is able to inhibit apoptosis as effectively as Bcl-2 [75, 76]. Another study that seems to contradict the rheostat model describes Bcl-x_L mutants which maintain their antiapoptotic properties but are unable to interact with proapoptotic members [77]. Clearly this finding is hard to reconcile with a rheostat

model, which would require the Bcl-x_L to directly interact with proapoptotic members. Nonetheless, there is no reason to reject wholesale this rheostat model, as clearly certain aspects of it do seem to hold true in many cell types.

Prion-like model

A slightly different model for the mechanism of action of pro- and antiapoptotic Bcl-2 family members is one reminiscent of prion activation. The proponents of this model note that it is speculative, but point out that it does help answer some of the findings which do not "fit" with the rheostat model. The similarity to prion activation is that a threshold level of a conformation switch is required. As explained in their review of apoptosis signaling, Strasser et al. [78] posit that antiapoptotic members such as Bcl-2 may form a lattice or network across the mitochondrial membrane. Nearby Bcl-2 neighbors influence the conformation of each other. Upon receipt of death signals, BH3-only members may interact with the antiapoptotic members and cause them to switch to a proapoptotic state. These converted molecules now resemble the other proapoptotic members (ones containing more than just the BH3 regions) and the entire lattice is now converted to a proapoptotic state. From this point, release of cytochrome *c* may occur. Alternatively, an as yet undescribed Apaf-1-like molecule may bind this proapoptotic "lattice" and commence further downstream apoptotic signaling [78, 79].

Evidence for the prion model is derived from studies demonstrating that some BH3-only members (Bim, Bad and Noxa) exclusively interact with antiapoptotic family members [80]. Despite this interaction with only antiapoptotic members, it was

found that Bim could not cause apoptosis in cells which were defective for both Bax and Bak, two proapoptotic members which contain more than the BH3 region [81]. From this study it was concluded that it was not sufficient for Bim to interact with and potentially neutralize antiapoptotic members, but also that proapoptotic family members need to be present.

Although the prion model is highly speculative, it helps explain a perplexing finding in *C. elegans*. It has been observed that the cell death machinery of *C. elegans* does not contain a Bax-like protein. The three most studied *C. elegans* apoptosis proteins are Ced 9, 4 and 3. Ced-9 is homologous to Bcl-2, Ced-4 is homologous to Apaf-1 and Ced-3 is a caspase. Some proapoptotic BH3-only proteins have been described in *C. elegans*, yet proapoptotic proteins containing more than a BH3 domain have not been observed. However, it has been noted that under certain conditions Ced-9 may promote rather than inhibit apoptosis [82]. Given that there are no Bax homologs within *C. elegans* (nor any proapoptotic Bcl-2 family members save BH3-only types, such as Egl-1) it is possible that Ced-9 can exist in two conformations. When unbound to Egl-1, it is antiapoptotic, but by binding Egl-1, CED-9 would switch to a proapoptotic conformation. In mammalian cells, these two conformation states are mirrored by either antiapoptotic members or the proapoptotic members such as Bax. Thus an intriguing possibility is that BH3 only members do not merely neutralize the antiapoptotic activity of other family members but rather actively convert them into a proapoptotic configuration.

Another puzzling finding which may be resolved with the prion-like model is the observation that specifically mutated Bcl-x_L proteins may inhibit apoptosis even though

they do not interact with proapoptotic members [77] and thus, cannot be switched to a proapoptotic state. Impervious to the influence of the proapoptotic members, these mutants may continue to stabilize the lattice into an antiapoptotic conformation.

The prion-like model could also explain different potencies of proapoptotic Bcl-2 family members. BH3-only members are often more potent at inducing apoptosis. The prion model would argue that this is because they are able to influence the switch of other members from anti- to proapoptotic. This is in contrast to the larger proapoptotic members containing more than the BH3 region, which cannot switch other molecules, but only 'mimic' that conformation. In this manner, enough units of the lattice are in the proper conformation required to convert the lattice to a proapoptotic conformation.

Similarities of Rheostat and Prion-like Model: Spatial regulation

Features common to the rheostat and prion models are the interactions between Bcl-2 family members and the idea that these interactions function as a switch to initiate apoptosis. This is an attractive idea because the proapoptotic activity is regulated spatially; by being localized at certain outposts, proapoptotic members are distal to other Bcl-2 members and unable to activate an apoptotic response until needed. Moreover, the localization at other locales within the cell may in some cases allow the Bcl-2 members to act as "sentinels" for perturbations, such that under appropriate conditions these sentinels will commence apoptosis. For example, the BH3-only member Bim is normally bound to the dynein motor complex through interactions with the LC8 dynein light chain [83]. Upon apoptotic stimuli such as UV-irradiation or staurosporine treatment, LC8 and

Bim disassociate from the motor complex and relocalize to the cytosol, presumably to migrate to the mitochondria and interact with other Bcl-2 members to induce apoptosis. Two attractive hypotheses are that Bim monitors the microtubule cytoskeleton, such that severe damage may trigger displacement of Bim to commence apoptosis. Alternatively, the dissociation of Bim after an apoptotic signal may help further hasten cell death by destabilizing the microtubule network. Whether or not Bim serves to monitor microtubule integrity, its localization to the dynein motor complex safely sequesters it until apoptosis is required. A very similar mode of regulation has been described for another BH3-only member, Bmf. This Bcl-2 family member has been found to interact with dynein light chain 2 (DLC), which is part of the actin-based myosin V motor [84]. Thus, while Bim may monitor the microtubule component of cytoskeletal integrity, Bmf appears to be sequestered by and perhaps monitors the actin cytoskeleton.

Another example of spatial sequestration is found in the BH3-only member, Bad. Under normal conditions, it is sequestered in the cytosol by binding to 14-3-3 adaptor proteins. This interaction only occurs if Bad is phosphorylated [85]. Upon an apoptotic signal (such as IL-3 withdrawal), Bad is dephosphorylated, released from 14-3-3 and moves to the mitochondria, where it can act to hasten apoptosis. As a potential mechanism to rescind a death order, it has been observed that the kinase PKA which can phosphorylate (and thus inactivate) Bad is mitochondrially localized. Upon exposure to survival factor, mitochondrially anchored Bad can be phosphorylated and released to the cytosol [86].

As a final example of spatial sequestration and potential monitoring capabilities of Bcl-2 members, the activation of another BH3-only member, Bid, requires caspase activation. Bid is normally cytosolic. When it is cleaved at its N-terminus by caspase 8 [87, 88], the resulting carboxy-terminal fragment translocates to the mitochondria where it can help release cytochrome *c*. Recent work by Werner et al. has revealed that the Bcl-2 family member, Bfl, binds to Bid and prevents it from releasing cytochrome *c* [89]. Bfl does not interfere with Bid's activation by caspase cleavage, but translocates with it to the mitochondria and there exerts its antiapoptotic effect. Given Bid's potent proapoptotic ability it is likely that interaction with yet another member is required to release Bfl's inhibitory effect. With regards to being a "monitor", Bid may act as a sensor of unregulated caspase activity. Recalling that caspase 8 is activated early in the signaling phase of apoptosis, it is possible that if this caspase is unduly activated in the absence of receptor-mediated aggregation, the cleavage and subsequent activation of Bid may then help kill this cell which has inappropriately regulated caspase activity.

Cellular redox, apoptosis and Bcl-2 regulation of redox and lipid interactions

Early attempts to characterize the way in which Bcl-2 inhibits apoptosis led some to postulate that it protected cells through redox-related properties. One of these initial reports found that Bcl-2 appeared to act as an antioxidant [90], protecting cells from hydrogen peroxide-induced apoptosis. This was a rather timely finding, as reviews [91] at that time suggested that reactive oxygen species (ROS) play an important role in the signaling phase of apoptosis, based on evidence that antioxidants could often block

apoptotic signaling. Moreover, it was found that Bcl-2 overexpression could occasionally mimic the protection seen by antioxidants [92]. *In vivo* studies seemed to support this idea, in that Bcl-2 knockout mice displayed severe polycystic kidney disease and an early graying of the hair [93]. These phenotypes are associated with chronically elevated levels of ROS. The finding that Bcl-2 seems to act at the mitochondria [90, 94, 95] provided additional support, since mitochondria are prodigious ROS-generators. Bcl-2 would be expected to be present at the site of ROS generation, if it prevents apoptosis by acting as an antioxidant.

Subsequent experiments challenged the idea that Bcl-2 was acting as an antioxidant. Jacobson et al. [96] demonstrated that cells lacking a functional mitochondrial respiratory chain underwent apoptosis and Bcl-2 still served to inhibit this apoptosis. Further work by Jacobson, studying cells in virtually anaerobic conditions [97], showed similar results, in that apoptosis occurred and Bcl-2 protected the cells. Since both situations were assumed to lead to minimal production of ROS, the authors concluded that Bcl-2 prevents apoptosis in other ways than acting as an antioxidant.

Recent studies by Esposti and McLennan have documented ROS production in cells undergoing apoptosis in virtual anaerobiosis [98]. These investigators employed fluorescent dyes and two different methods of determining ROS levels. Therefore, even under anaerobiosis, the antioxidant properties of Bcl-2 could be a valid mechanism of protection. Additional work has reconciled more seemingly disparate findings about Bcl-2's mode of action. In 1995, a group reported that Bcl-2 seems to *produce* rather than inhibit ROS [99]. Re-examining this issue with more sensitive methodologies, Esposti et

al. concluded that overexpression of Bcl-2 results in elevated *basal* levels of ROS (in agreement with Steinman's work) but this chronic elevation causes a concomitant strengthening of antioxidant defenses, such that an otherwise apoptosis-inducing burst of ROS may be successfully thwarted [100].

These aforementioned results implicate Bcl-2 as having an antioxidant activity, but do not reveal mechanistically *how* Bcl-2 members achieve this function. Since structural studies of the family have not revealed any known antioxidant motif or domain within the protein, Bcl-2 members must act indirectly as antioxidants. In fact, it appears that they may act in several places to indirectly affect the redox status of cells, which in turn confers resistance to apoptosis.

Bcl-2 members and glutathione

Glutathione acts as a major intracellular redox buffer and concentrations as high as 1 to 10 mM have been observed within cells [101]. It is a tripeptide of glutamate, glycine and cysteine. The free sulfhydryl group within cysteine is an important aspect of this antioxidant, as it may detoxify hydrogen peroxide or organic peroxides through the following reaction :

2 GSH + R—O—OH → GSSG + H_2O + ROH. Alternatively, glutathione can help to reduce an oxidatively damaged protein by being conjugated to it, after which it may be released again as free GSH through enzymatic reactions [102].

Ellerby et al. [103] have used two different neural cell lines, engineered to overexpress Bcl-2, to examine the ratio of oxidized (GSSG) to total glutathione. Basal GSH levels were increased in Bcl-2 overexpressing cells. Both cell types which

overexpressed Bcl-2 had a lower GSSG/GSH ratio compared to the controls, indicating that the cells were probably in a more reduced state. This report extended the idea that Bcl-2 seems to have antiapoptotic activity through affecting the cells' ability to deal with ROS.

Other investigators have employed radiation sensitive (AS) and resistant (AR) murine lymphoma cell lines to study the relationship between Bcl-2 and GSH [104]. AR cells overexpress Bcl-2 and have elevated GSH levels. Buthionine sulfoximine (BSO) is a specific inhibitor of γ-glutamyl cysteine synthetase, the rate limiting step of GSH synthesis [105]. Bcl-2 overexpressing AR cells remain resistant to death after BSO treatment, while AS cells die after treatment. BSO depletes cytoplasmic but not mitochondrial or nuclear GSH pools. In contrast, compounds which deplete cytoplasmic, mitochondrial and nuclear GSH pools are able to sensitize Bcl-2 overexpressing cells to radiation-induced apoptosis. Similar studies by Kane et al. showed that depletion of only cytoplasmic GSH pools in neural cells which overexpress Bcl-2 is insufficient to affect their resistance to apoptosis. The results of these studies argue that sequestered pools of GSH may be important in apoptosis resistance and that Bcl-2 can maintain critical GSH levels in the mitochondria or nucleus.

Voehringer, et al. [106] have used a regulatable system of expression in HeLa cells to further explore the role of Bcl-2 in maintaining GSH pools. By overexpressing Bcl-2 with the Tet-off system, they observed that the overexpression of Bcl-2 resulted in a redistribution of GSH. Almost 75% of the total GSH was found within the nucleus of Bcl-2 overexpressing cells, while suppression of Bcl-2 expression led to only 30%

nuclear localization of GSH. Importantly, this correlation held across several cell lines: AR and AS cells; and thymocytes derived from Bcl-2 transgenic or knockout mice. The relevance of the nuclear pool to apoptosis protection was seen in that DNA fragmentation was reduced in isolated nuclei which were pre-incubated with GSH.

Further experiments showed that addition of GSH to isolated nuclei appeared to inhibit caspase 3. Caspases are present in the nucleus [107] and Bcl-2 is found in nuclear membranes [108]. As mentioned previously, a known target of caspases is the iCAD, the cleavage of which allows a DNAse to become active. Thus, by sequestering GSH within the nuclei, Bcl-2 may prevent activation of caspases that cause DNA fragmentation.

Bcl-2 may increase the amount of GSH by inhibiting its export from cells. Studies of Bcl-2 overexpressing HeLa cells have shown that while the rate of GSH synthesis is not altered by Bcl-2 overexpression, GSH efflux is inhibited [109]. Previous reports have noted that extrusion of GSH occurs early in apoptosis and inhibition of this extrusion seems to rescue cells from undergoing apoptosis [110-112]. Thus, the findings of Meredith et al. that Bcl-2 blocks efflux offers some insight into this area of Bcl-2 activity as it relates to glutathione pools. More work will be required to elucidate the molecular mechanisms of this inhibition.

Bcl-2 members and lipids

Another redox-related model stems from the observation that overexpression of Bcl-2 is correlated with decreased lipid oxidation [90, 113]. Recent studies indicate more efficient engulfment of cells when cell membranes are enriched with oxidized PS compared to enrichment with non-oxidized PS [114]. Bcl-2 may regulate this PS

oxidation [115]. Apoptosis of the squamous non-small cell lung cancer line NCI-H226 was induced by treatment with anti-sense oligonucleotides directed against Bcl-2. Using a technique which measures oxidation of externalized PS, it was found that the Bcl-2 antisense treatment caused a significant increase in oxidized PS, even at an early timepoint (5 hr), before morphological characteristics of apoptosis were seen. This implied that the presence of Bcl-2 may help prevent oxidation of PS. In human leukemia HL-60 cells [116, 117], keratinocytes [118] and PC12 neural cells [119] the externalization of PS is an early event, indicating it is not merely a consequence of apoptosis but, as noted above, a signaling event between the dying cell and the surrounding cells to hasten engulfment. Thus, the experiments of Koty et al. demonstrate the Bcl-2 may prevent oxidation of a signaling molecule that triggers engulfment.

A proposed mechanism for the oxidation of PS implicates cytoplasmic cytochrome *c* [120]. Within the electron transport chain, cytochrome *c* acts as an antioxidant [121, 122], but it can function as a pro-oxidant in other situations. It may form free radicals upon interaction with hydrogen peroxide [123] and cytochrome C^{2+} catalyzes the formation of the hydroxyl radical more efficiently than iron ([120] and references therein). Since cytochrome *c* is a basic protein [124], Kagan et al. have proposed that when released from the mitochondria it could readily interact with and oxidize the negatively-charged PS located in the inner-leaflet of the cell membrane [120].

Another lipid with which Bcl-2 members have been found to interact is cardiolipin. This lipid is found in the mitochondrial membrane. The BH3-only member, Bid, is cleaved by caspase 8 and translocates to the mitochondria, where it affects

cytochrome *c* release [87, 88]. Earlier work pointed to a possible interaction between Bid and cardiolipin [125]. Esposti et al. have demonstrated that Bid and the caspase-cleaved tBid are able to transfer fluorescent lipids from a donor to acceptor liposome as well as from a donor liposome to isolated mitochondria [126]. These authors postulate that Bid may be induced to migrate to the mitochondrial membrane by trafficking lipids to this organelle.

Bid seems to interact with other proapoptotic members of the Bcl-2 family through its BH3 domain and may help to enhance the proapoptotic activities of those members. Bid may also affect cytochrome *c* release through a direct interaction which alters the curvature of the mitochondrial membrane. Lipid bilayers have a certain intrinsic curvature. Changes in lipid components or addition of proteins with certain properties can increase this curvature, to the point where leakage can occur. This highly curved lipid state is called the hexagonal phase. Studies by Epand et al. [127] have shown that tBid can induce leakage from cardiolipin-containing liposomes, there is an increased rate of phase change to hexagonal in liposomes incubated with tBid, and tBid-induced cytochrome *c* release from isolated mitochondria is inhibited by co-incubation with tritrpticin, a peptide which causes positive membrane curvature [128]. Taken together, these studies suggest that by acting as a lipid transporter, Bid is directed to the mitochondrial membrane, where it causes negative curvature, leading to cytochrome *c* release.

Bcl-2 and "global" redox changes

As a final note, microarray-based comparisons of apoptosis resistant and sensitive cells support a connection between Bcl-2's antiapoptotic functions and the cellular redox state [129]. Murine lymphoma cells which are radiation sensitive (LY-AS) or resistant (LY-AR) were used in this study. Caution must be taken to not overinterpret the results, given that LY-AT cells are spontaneously resistant and may have other changes in addition to the observed high level of Bcl-2 expression. Nonetheless, it is interesting to note that many redox-related genes are differentially expressed between the two cell types. These include the VDAC and UCP-2 proteins, which increase after irradiation of LY-AS cells but not LY-AR cells. As previously discussed, VDAC is part of the mitochondrial electron transport chain and has been postulated to be part of a channel through which cytochrome *c* and other mitochondrially-sequestered proteins are released during apoptosis. UCP-2 is a protein which functions to uncouple electron transport from oxidative phosphorylation. This further highlights the possibility that a genetic program is enacted in sensitive cells to cause mitochondrial dysfunction, which commits the cell to undergo apoptosis. Two of the more differentially expressed genes, seen to be overexpressed in the LY-AR cell line, are fructose-1,6-bisphosphatase and glutathione-S-transferase. A downstream effect of increased fructose-1,6-bisphosphatase is an increase in NADPH, which provides reducing equivalents needed to maintain GSH pools. Similarly, glutathione-S-transferase helps to maintain a reduced environment by conjugating glutathione to toxic metabolites and in this way may act as a chain terminator of oxidation events. Thus, apoptosis resistant cells may achieve resistance in part through

a global upregulation of genes that regulate the redox state of the cell, potentially as a result of Bcl-2 overexpression.

Overlap and harmony of the theories of action

The various theories for the mechanism by which Bcl-2 family members function have some overlap. For example: (1) the rheostat model posits that Bcl-2 works on the mitochondrial membrane preventing cytochrome *c* release by proapoptotic members; (2) the prion-like model, although mechanistically different, still recognizes that mitochondrial factors are released and prevented from release by pro- and antiapoptotic Bcl-2 members, respectively; and (3) the redox model of Bcl-2 function further clarifies that the effect of preventing cytochrome *c* release from the mitochondria is the inhibition of oxidation of PS. It is probable that all three theories are facets to the overall mechanism(s) by which Bcl-2 and its family members work.

As emphasized in a review of Bcl-2 and glutathione, [130], many of the antiapoptotic affects of Bcl-2 members relate to controlling where specific proteins are localized. This introduction has emphasized the importance of the mitochondria and nucleus in apoptosis and Bcl-2 members localize to the membranes of these organelles. Thus, it is possible that Bcl-2 and other members act as gatekeepers, allowing the passage of factors into and out of the mitochondria and nucleus. This overall view encompasses both the rheostat and redox models of action, in that the direct interaction of Bcl-2 members with each other (rheostat model) determines the passage of potentially oxidizing or reducing agents such as cytochrome *c* or GSH, respectively (redox model).

Irrespective of how Bcl-2 members work to promote or inhibit apoptosis, their central role in the apoptotic process is undeniable.

Transcriptional regulation of *bcl-2*

The promoter region of the human *bcl-2* gene has been characterized [131] and it appears that *bcl-2* can be regulated at the transcriptional level [131-133]. There are numerous reports of Bcl-2 protein or RNA levels changing in response to various stimuli (see [134] and references therein), but the underlying mechanisms of *bcl-2* regulation have not been established. While RNA stability is one mode of regulation, it appears that this is not extensively used to regulate *bcl-2* [131].

Several mechanisms for the negative regulation of *bcl-2* have been elucidated. First, a negative regulatory element has been found between the two transcriptional start sites of *bcl-2* [134, 135]. Second, Wilms tumor suppressor gene WT1 appears able to repress *bcl-2* transcription [136]. Third, p53 has also been reported to repress transcription of *bcl-2* [137, 138].

Mechanisms for the positive regulation of *bcl-2* are scarce. The chromosomal translocation event which led to the initial identification of *bcl-2* [139, 140] is not present in many cancer cells known to have elevated Bcl-2 levels. Several reports implicate integrins in the upregulation of *bcl-2* [141, 142] but these studies do not identify the transcription factors that upregulate *bcl-2* transcription. The transcription factor PAX8 has been shown to activate *bcl-2* transcription [143]. PAX8 seems to be expressed only in certain tissue types [143] and thus, other mechanisms must exist for transcriptionally

upregulating *bcl-2*. A review by Otsuki found correlations in the expression patterns of c-Jun and Sp-3 with Bcl-2 in endometrial glandular cells, implicating these as potential transcriptional upregulators of *bcl-2* [144]. Overall, however, transcriptional regulation of *bcl-2* has not been well-defined, especially in cancer cells where Bcl-2 is often overexpressed.

Apoptosis in Disease

Increased Apoptosis

Some diseases occur because there is a deregulation of apoptosis and inappropriately increased levels of apoptosis are seen in the pathological state. AIDS, myocardial infarctions and many neurodegenerative disorders are examples of diseases where increased apoptosis is observed [145]. In Alzheimer disease, the β-amyloid precursor protein is cleaved by caspase 3. The cleaved membrane-bound β-amyloid is proapoptotic. Thus, a vicious circle may ensue, wherein apoptotic cells (with activated caspases) cause more proapoptotic proteins to be created, leading to further apoptosis [146]. In Huntington disease, another neurodegenerative disease marked by increased apoptosis, it is hypothesized that the normal huntingtin protein confers resistance to apoptosis [147]. This protein is mutated in Huntington disease and a gain-of-function is seen in addition to the loss-of-function of its antiapoptotic ability (reviewed in [148]). For these diseases, apoptosis research may provide strategies for preventing excessive cell death.

Decreased Apoptosis

Conversely, the ability to resist apoptosis is thought to be an important and necessary step in carcinogenesis [149]. In addition, apoptosis resistance is a vital step for the continued viability of cancer cells. Only cells which maintain an upregulated apoptotic-resistant phenotype are able to resist chemotherapy (reviewed in [150]). This resistance to treatment regimens is the basis for the more aggressive and often fatal drug-resistant phenotype seen in late stage, drug-refractory cancers. Glucocortacoid-resistant lymphomas are one such example of this phenomena. Lymphomas are often treated with synthetic hormone analogs (such as dexamethasone) which normally induces apoptosis. This is an effective "first line" form of treatment for lymphomas. Unfortunately, almost invariably, some fraction of the lymphoma cells acquire a resistance to hormone-induced-apoptosis. This fraction of cells, having a survival advantage, soon constitutes the majority of the cancer and such treatment is no longer effective. Hence, it is important to understand the molecular mechanisms of apoptotic resistance in order to effectively inhibit this resistance and thereby succeed in rendering the cancer susceptible to death signals.

The role of reactive oxygen species (ROS) in cancer

Tumor cells often have elevated levels of ROS compared to normal cells [151-153]. Elevated levels of ROS are seen in all stages of tumorigenesis and likely play a functional role [154]. Free radicals have been shown to generate mutations in DNA through oxidative damage, which may lead to initiation [155, 156]. Many tumor promoters act through ROS-mediated mechanisms [157], including the well-studied

promoter 12-O-tetradecanoylphorbol-13-acetate [158-160]. Many tumor progressors are free radical generators [161]. Finally, antioxidant treatment often inhibits or reverses the effects of tumor promoters or progressors (reviewed in [162, 163]), further implicating the role of ROS in tumorigenesis.

The observation that cancer cells often have decreased antioxidant enzyme levels (reviewed in [164]) illustrates that after a tumor has formed it maintains an elevated level of ROS. Besides loss of antioxidant enzymes, tumor cells can also maintain elevated ROS through constitutive activation of ROS-producing genes (see [165]) or regulators of such genes. A common example of the latter is the *ras* oncogene which is mutated in approximately 30% of all human tumors ([166] and references therein). Constitutive activation of *ras* leads to the production of superoxide radicals and mitogenic signaling which may be inhibited by antioxidants [167, 168].

Advantages of maintaining elevated ROS within tumor cells include an increased growth potential. Certain transcription factors such as NF-κB and AP-1 have elevated activity upon exposure to ROS or an oxidative environment [169, 170]. Upstream from transcription factors, kinase signaling cascades are also positively effected by ROS [171]. MAPK, [172], protein kinase *c* signaling [173] and various tyrosine kinases [174, 175] are all upregulated by ROS and inactivation of phosphatases by ROS leads to the same endpoint of elevated kinase activity (reviewed in [176]).

More recent research has focused on the role of ROS in apoptosis resistance. This field is relatively understudied compared to that of ROS in increased growth. One mechanism by which ROS may inhibit apoptosis is through inhibition of caspase

activation. Caspases all contain a cysteine residue in their active site, rendering them susceptible to oxidation reactions on the sulfhydryl group of cysteine [177, 178]. It has recently been suggested that the oxidized environment created by chronically elevated basal levels of ROS in cancer cells may contribute to resistance to apoptosis since apoptosis is more favorable under reduced conditions [179]. Given that cancer cells have elevated ROS and increased resistance to apoptosis, it will be important to discern the molecular mechanisms of how chronic ROS may confer apoptosis resistance.

Statement of the problem

Due to the prevalence of resistance to apoptosis in cancer, both at an early stage during carcinogenesis and continued tumor survival, much research is devoted to understanding the regulation of apoptosis. The goal of the research presented in this dissertation was to examine two distinct mechanisms of apoptosis resistance: one mediated by chronic ROS; and the other through transcriptional upregulation of the apoptosis resistance gene *bcl-2*.

The first hypothesis examined in this dissertation is that chronically elevated ROS observed in cancer cells contribute to an apoptotic resistant phenotype. The role of the chronically elevated ROS seen in tumorigenesis has not been well-defined. Many studies have resorted to artificially and often transiently upregulating ROS in model systems to evaluate their effects on apoptosis. The research presented in Chapter 2 of this dissertation describes further characterization of a model system of keratinocyte cell lines which are ideal for the study of ROS in carcinogenesis. With this model system, a

molecular mechanism is described which implicates the chronically elevated ROS levels observed in cancer cells as contributing to the apoptosis resistant phenotype of many tumors.

The second hypothesis addressed in this dissertation is that *bcl-2* transcription is mediated by PPARs, contributing to an apoptosis-resistant phenotype. While much research is devoted to understanding the mechanism by which Bcl-2 acts to inhibit apoptosis, there is very little research published about the transcriptional regulation of *bcl-2*. Activation of the peroxisome proliferator activated nuclear receptor (PPAR) has been associated with tumorigenesis and apoptosis resistance, but the exact mechanism has not been elucidated. The research presented in Chapter 4 demonstrates that *bcl-2* transcription may be mediated by PPARs, which has implications for apoptosis resistance and cancer.

Chapter II. Murine keratinocytes with elevated basal reactive oxygen species and phospho-Akt are resistant to UV-induced apoptosis

Introduction

A mouse model for skin cancer has been developed to study the molecular events in the progression to a malignant state in skin cancer. The 308 mouse keratinocyte cell line was derived from 4,12 dimethyl benz[a]anthracene-treated mouse skin [180]. Subcutaneous injection of 308 cells into athymic nude mice results in no tumor formation. However, transplantation of 308 cells to the skin of athymic nude mice results in the formation of benign papillomas. Thus 308 cells are said to be initiated, in that they form papillomas upon transplantation, but they are not yet fully transformed into a malignant phenotype. Two malignant variants (6M90 and 6R90 cells) were created from the benign 308 mouse keratinocyte through repeated exposures to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine or γ-irradiation, respectively. Injection of 6M90 and 6R90 cells into athymic nude mice results in rapidly growing malignant tumors [172], indicating that malignant transformation has occurred.

The malignant 6M90 and 6R90 cell lines were known to have elevated ROS [172]. These cell lines have upregulated certain signal transduction pathways that lead to increased proliferative capacity. Activity of the mitogenic MAP kinases Erk-1/2 and p38 is elevated in the malignant variants compared to 308 cells. Besides these increased MAPK activities, transcription factor activity of NFκB and ATF-2 are increased in both lines and AP-1 activity is additionally increased in the 6M90 cells [172]. Attenuation of

ROS affects the growth component of the malignant phenotype [172]. This was shown by using the antioxidants N-acetyl cysteine (NAC) and the water soluble vitamin E analog Trolox to reduce ROS levels. Upon reduction of ROS, decreases in DNA synthesis, transcription factor activity and MAP kinase activity were all observed to some extent in the malignant variants. Taken together, these data indicate that the malignant mouse skin keratinocyte cell lines have perturbations in signaling pathways which result in increased growth rates.

Apoptosis and the role of ROS in apoptosis have not been examined in 6M90 and 6R90 cells. A key mediator in decreasing apoptosis is the threonine/serine kinase Akt. Activation of the Akt (also known as PKB) signaling pathway reduces apoptosis in many cell types (reviewed in [181]). Akt is activated when upstream kinases, including PDK-1 and PDK-2, phosphorylate threonine 308 and/or serine 473. Akt activation leads to increased phosphorylation of downstream targets, the net result of which is decreased apoptosis. Akt both inactivates pro-apoptotic proteins [182-184] and activates antiapoptotic proteins [185] through phosphorylation, leading to cell survival. ROS have been implicated in the regulation of Akt signaling in mouse Swiss 3T3 cells, human embryonic kidney cell line [186], muscle cells [187, 188], human keratinocyte [189] and mouse epidermal cells [190]. Importantly, in these studies, the outcome of apoptosis was not examined after modification of ROS levels. We provide evidence in malignant mouse keratinocyte cell lines that chronically elevated levels of ROS, as a result of decreased catalase activity, result in increased Akt phosphorylation. This contributes to their

apoptotic-resistant phenotype, since resistance may be reversed by attenuating ROS levels.

Materials and Methods

Cells and transfections

The murine keratinocyte-derived 308 cell line and 6M90 and 6R90 [191] cells were cultured in MEM (Invitrogen, Carlsbad, CA) supplemented with 7.5 % fetal bovine serum (Gemini Bioproducts, Woodland , CA), 2.5% fetal calf serum (Invitrogen), 500 U/ml penicillin and 5000 U/ml streptomycin (Invitrogen) at 37° C in a 7% CO_2 humidified environment. A catalase expression plasmid (2 μg) containing a neomycin selection marker [192] or a vector control plasmid containing only the neomycin selection marker were transfected into 0.5 $\times 10^6$ 6M90 and 6R90 cells with the DOTAP liposomal transfection reagent (Avanti Polar Lipids, Inc., Alabaster, AL), according to manufacturer's instructions. The transfected cells were cultured in 400 μg/ml G418 for stable selection of clones. Individual clones were selected from each cell line and tested for increased catalase activities.

Antioxidant Enzyme Assays

Total SOD activity was measured from subconfluent cultures using a spectrophotometric assay, based on the inhibition of a superoxide-driven NADPH oxidation, as described in [193]. Catalase activity was determined by using a kinetic spectrophotometric assay which measures a decrease in the absorbance of hydrogen peroxide [193]. Briefly, cells from subconfluent cultures were lysed and assayed for their

ability to reduce the absorbance of hydrogen peroxide at 240 nm over a period of 2 min. Seventy micrograms of the lysate were added to the assay buffer (35 mM phosphate buffer, pH 7.2 and 0.02% Triton X-100) in a final volume of 970 µl. The reaction was started by adding 30 µl of 1% hydrogen peroxide and observing a decrease in absorbance at 240 nm as a function of time. DT-diaphorase activity was measured as described previously [194].

Northern Analysis

Cells were removed from the plate with trypsin, pelleted and rinsed twice with PBS. Cell pellets were snap frozen in a dry ice/ethanol bath. RNA was harvested using the RNAid Plus kit (Quantum Biotechnologies, Inc., Carlsbad, CA), except that the cells were lysed by sonication in the lysis buffer rather than by homogenization. Once lysed, the manufacturer's protocol was followed. Standard hybridization procedures were followed [191] for ^{32}P-labeled probes and images were quantified on a phosphorimager (ImageQuant, Molecular Dynamics, Sunnyvale, CA).

UV-B Treatment

Cells were grown to approximately 90-95% confluency, during which time, if necessary, they were pretreated with antioxidants or H_2O_2 as described below. The media was then removed and saved. Cells were rinsed with PBS and after removal of PBS, exposed to a 300 J/m^2 dose of ultraviolet-B (UV-B) light in a sterile hood. A bank of two SF20 UVB bulbs with a peak emission at 313 nm (National Biological Corporation, Twinsburg, OH) was used to irradiate. After irradiation, cells were replated with the same media until harvested 13 hours after irradiation.

Antioxidant/Oxidant treatment

To test the effects of antioxidants or oxidants, cells were treated with either 200 μM hydrogen peroxide, 20 mM NAC, or 2 mM Trolox (all from Sigma Chemical Company, St. Louis, MO) approximately 30 hours before harvesting for western blots or exposure to UV. For inhibition of Akt phosphorylation, cells were pretreated with 5 μM wortmannin (Sigma Chemical Company, St. Louis, MO) for one half hour before harvesting or exposure to UV-B.

Apoptosis Measurements

Cells were harvested by trypsinization, adjusted to 5 X 10^5 cells/ml and deposited onto glass slides by cytocentrifugation (Shandon, Inc., Pittsburgh, PA). The slides were stained using the Diff-Quik staining kit (Dade Behring Inc., Newark, DE) according to the manufacturer's protocol. Percent apoptosis was determined by blinded morphological examination of 200 cells per slide.

Flow Cytometry Analysis

The intracellular hydrogen peroxide level was determined using the oxidation-sensitive fluorescent probe 5,6-carboxy-2',7'- dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is oxidized to a highly fluorescent compound, DCF. Cells were detached from the plate by trypsinization, after which the trypsin was neutralized by addition of equal amounts of serum-containing media. Cells were spun down and washed with serum-free MEM (sfMEM) and incubated with 10 mM DCFH-DA (Molecular Probes, Eugene, OR) in sfMEM for 0.5-1 h. The fluorescence intensity was measured with a FAC Scan flow cytometer (Becton Dickson, San Jose, CA) using the data

acquisition program CELLQuest. DCF data were collected using an argon laser emitted light of 488 nm and collected 10,000 events using a 530 +/- 30 bandpass filter.

Western Analysis

After appropriate treatments, cells grown in 60X15 mm dishes were rinsed once with PBS and then lysed by scraping with 100 µL of lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 2.5 mM Na_4PO_7, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM ß-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/mL pepstatin). After brief incubation on ice, the lysate was centrifuged at 14,000 rpm for 10 min. Protein concentration of the supernatant was determined by Pierce BCA Protein Assay reagent (Pierce, Rockford, IL). For Western analysis, 30–40 µg of protein were resolved on a 12 % SDS-polyacrylamide gel. The protein was transferred to a polyvinylidene difluoride membrane by electroblotting for 1 hr at 250 mA. The membrane was then blocked in 5% nonfat dry milk TBST (10 mM Tris pH 8.0, 150 mM NaCl and 0.05% Tween 20) at room temperature for 1 h. Primary antibody (against phospho-Akt (ser 473 specific) or total Akt (New England Biolabs, Inc., Beverly, MA)) was diluted at 1:1000 in 5% milk / TBST and incubated with the membrane overnight at 4°C. The membrane was then washed three times for 5 min each with TBST. The secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit) (New England Biolabs, Inc.) was added at 1:2000 in 5% nonfat dry milk/TBST at room temperature for 1 h and washed three times for 5 min each with TBST. Chemiluminescence was performed using Western Lightning ™ (Perkin Elmer Life Sciences, Inc. Boston, MA) according to manufacturer's instructions. Stripping of

membranes was accomplished by incubating membranes at 45°C in stripping solution (62.5 mM Tris pH 6.7, 2 % SDS, 114.4 mM β-mercaptoethanol) for 15 min. Membranes were then rinsed in TBST before blocking and reprobing.

Results

6M90 and 6R90 cells have chronically elevated ROS due to several genetic changes

6M90 and 6R90 cells are malignantly progressed variants of the papilloma-forming 308 cell line [172, 191]. To ascertain the cause for the previously observed basal increase in ROS levels within the two malignant variants, we examined the RNA levels of some antioxidant and prooxidant genes within the three cell types. RNA message levels for catalase, the μ class of glutathione-S-transferases (GSTμ) and cyclooxygenase 2 were examined (Figure 1). A marked reduction in catalase (20-30% of the levels seen 308 cells) message was observed in 6M90 and 6R90 cells (Figure 1A). The message levels of the μ class of GST, another antioxidant enzyme, were also found to be decreased (~50% of that seen in 308 cells) in the malignant variants (Figure 1B). Finally, mRNA levels of the prooxidant cyclooxygenase-2 (COX-2) were approximately 11-fold higher in the 6R90 cells than 308 or 6M90 cells (Figure 1C.) No detectable differences between the three cell types were observed in message levels of the antioxidant enzymes Cu,Zn SOD, MnSOD, thioredoxin or DT-diaphorase. Similarly, message levels of the GP91 subunit of the prooxidant NADPH oxidase were unchanged in the malignant

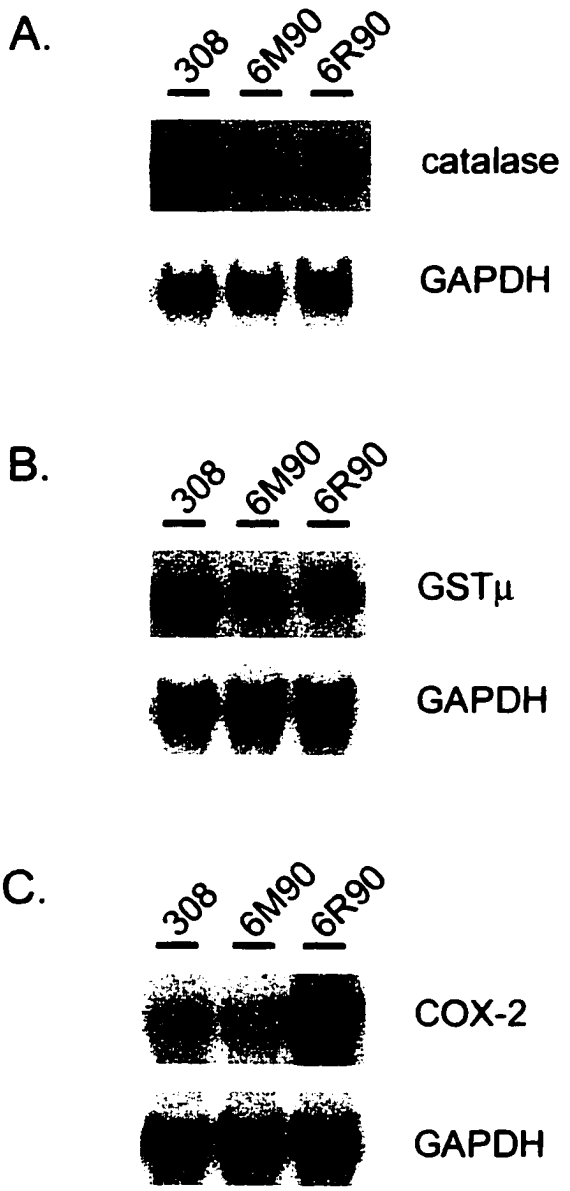


Figure 1: Northern analysis of selected anti- or pro-oxidant genes. mRNA was harvested from untreated 308, 6M90 and 6R90 cells and probed for (A) catalase, (B) GSTμ and (C) COX-2 levels. A decrease in levels of catalase and GSTμ were observed in both 6M90 and 6R90 cells, while 6R90 cells had increased COX-2 message levels compared to 308 and 6M90 cells. Equal loading was ensured by probing for GAPDH. Representative blots are shown.

variants (data not shown).

Because catalase RNA levels were seen to be decreased in both malignant cell lines, catalase protein (Figure 2A) and activity levels (Figure 2B) in the 308 cells and malignant variants were examined. As predicted, 308 cells had greater amounts of catalase protein and concomitantly higher activity levels of catalase than the two malignant cell lines. Total SOD activity was also measured (Figure 3A) but no significant difference was seen between the three cell types. DT-diaphorase enzyme assays similarly showed no significant difference between the three cell types, though 6M90 cells showed a trend towards an increased activity ($p = 0.069$) (Figure 3B).

Malignant keratinocytes show increased resistance to UVB induced apoptosis

I chose UV exposure as a relevant apoptotic stimulus to determine whether the malignant phenotype of the 6M90 and 6R90 cells includes resistance to apoptosis. Based on the results of initial dose-response and timecourse experiments to determine the optimal conditions for irradiation (data not shown), cells were exposed to a 300 J/m^2 dose of UVB and harvested 13 hr later. Quantitation of apoptosis by morphological examination revealed that 6M90 and 6R90 cells are more resistant to UVB-induced apoptosis (Figure 4) than the 308 cell line.

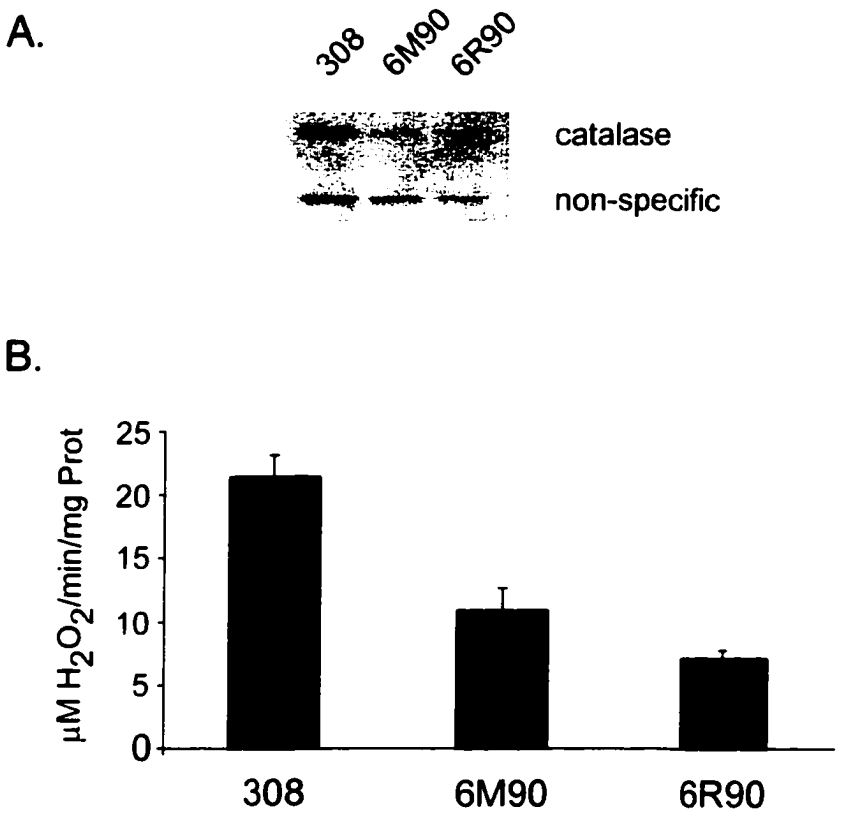


Figure 2: (A) Western analysis and (B) activity measurement of catalase in 308 and malignant variant lines. Decreased levels of protein (A) and activity (B) of catalase are observed in 6M90 and 6R90 cells compared to parental 308 cells. A representative blot is shown; activity assay is the mean ± S.E.M., n = 3.

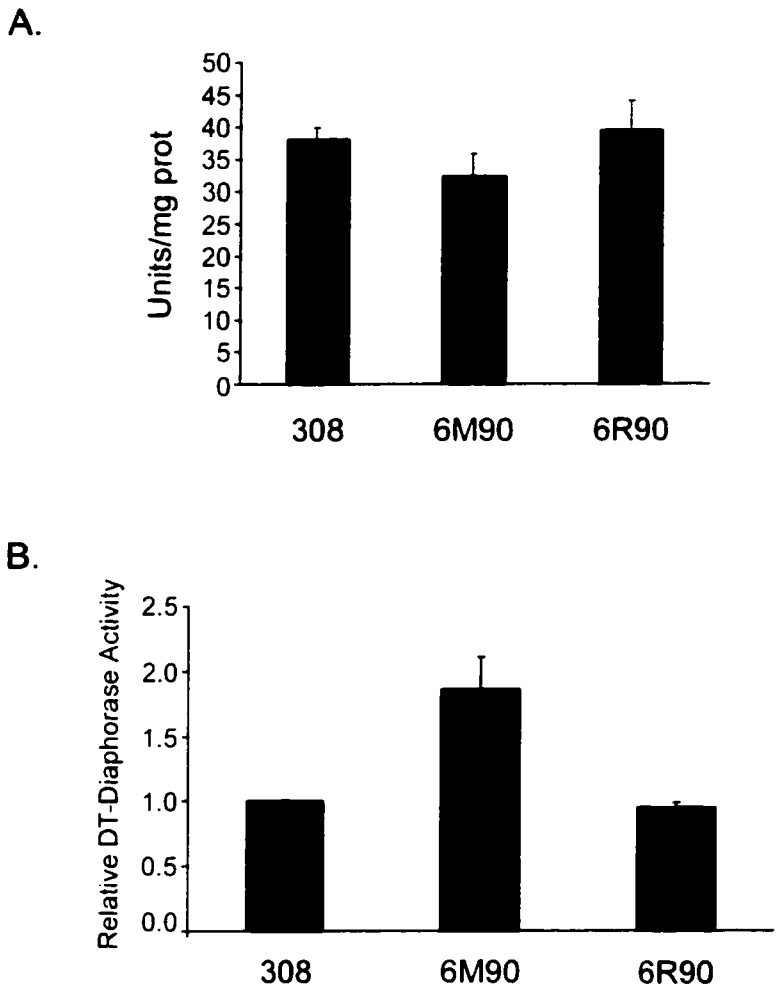


Figure 3: (A) Total SOD and (B) DT-diaphorase activity analysis of 308, 6M90 and 6R90 cells. SOD and DT-diaphorase activities are not statistically significant in either 6M90 or 6R90 cells compared to the 308 line ($p > 0.05$) though the diaphorase activity in 6M90 cells approached significance ($p = 0.069$). The results of the diaphorase assay are expressed relative to 308 activity. (mean ± S.E.M., $n = 3$)

Stable transfection of catalase in malignant variants results in increased catalase activity

6M90 and 6R90 cells have decreased catalase message, protein and activity compared to the 308 parental cells. One implication from previous work correlating loss of catalase activity to increased malignancy is that elevated steady state levels of ROS are the contributing factor in increasing malignancy, given the ability of catalase to remove ROS. We therefore stably transfected rat catalase cDNA into the two malignant variants. The amounts of catalase activity observed in our stable transfections were 1.69 fold and 1.39 fold higher in 6M90 cat69 and 6R90 cat25 cells, respectively, as compared to the neo-transfected 6M90 clone neo2 (Figure 5). While not large, these were still statistically significant differences from all the neo clones ($P < 0.0001$). These low but significant increases in levels of catalase activity via transfection have been shown to produce profound biological effects in another cell type [192].

Decreasing ROS levels in 6M90 and 6R90 cells results in increased sensitivity to UVB-induced apoptosis

It is predicted that decreasing steady-state ROS levels will be able to decrease malignancy. In addition to re-expressing catalase, a complementary approach to decrease ROS would be to add antioxidants exogenously to the culture media. In both cases, it is necessary to determine the effectiveness of these manipulations in reducing ROS levels before testing an attenuation of apoptosis.

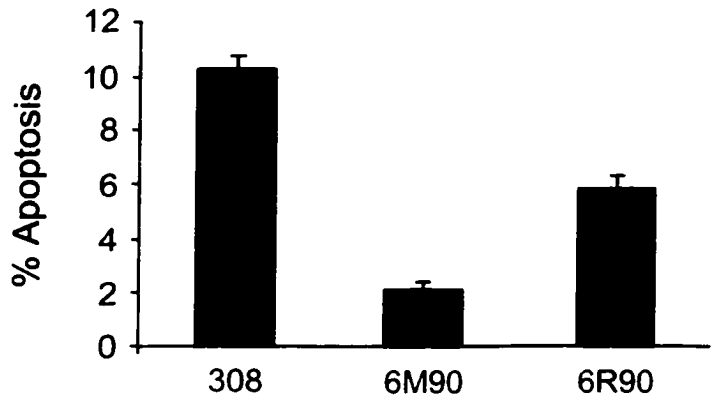


Figure 4: Percentage of UVB-induced apoptosis in the three keratinocyte cell lines. Cells were exposed to a 300 J/m^2 dose of UV-B and harvested 13 hours later. Percent apoptosis was determined by morphological assessment of 200 cells per slide. (mean ± S.E.M., n = 6) The malignant variants are more resistant to apoptosis than the parental cell line.

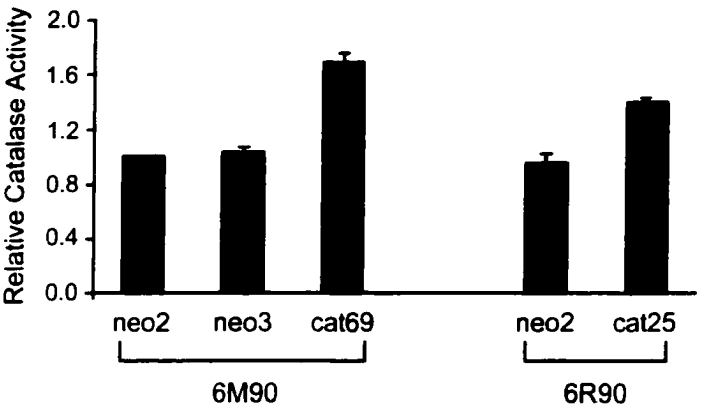


Figure 5: Elevated catalase activity in stable transfectants. Catalase activity was measured in 6M90 and 6R90 cells stably overexpressing rat catalase cDNA (or the empty neo vector) using a hydrogen peroxide clearance assay. The results are expressed relative to the catalase activity of the neo2 clone of 6M90. (mean ± S.E.M., $n \geq 3$)

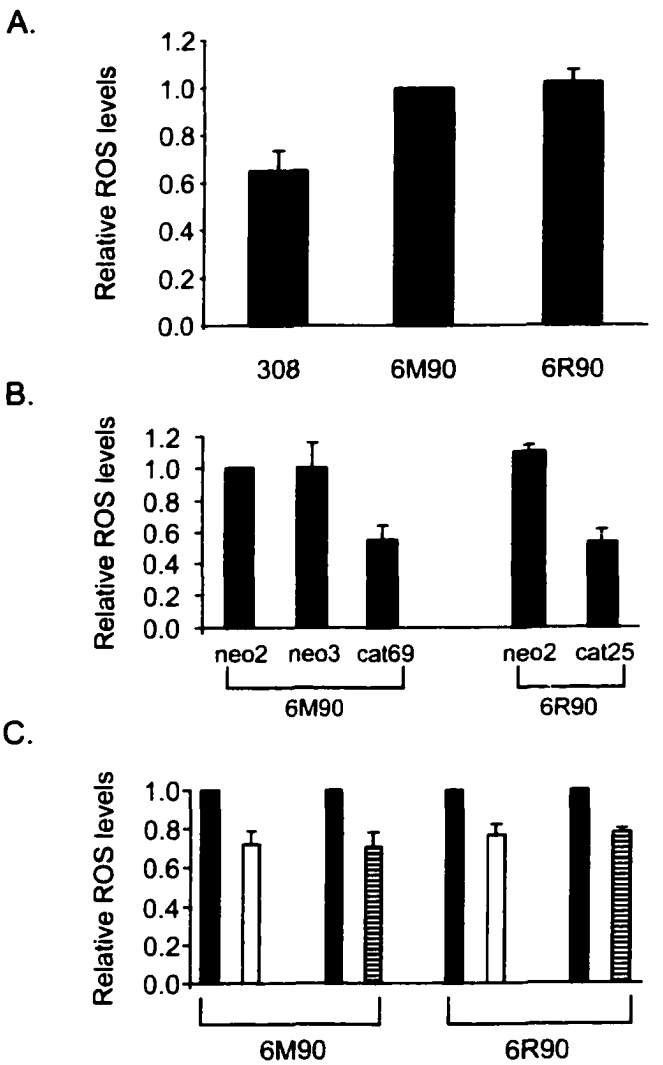
A standard approach to measuring intracellular ROS levels relies on luminescent or fluorescent detection of the oxidation reaction of a target compound which is taken up and oxidized within the cell. One common reagent is dichlorodihydrofluorescein diacetate (DCFH-DA). This particular reagent is advantageous in that it is reported to have some specificity for detecting hydrogen peroxide [195]. Additionally, because flow cytometry is used for detection of the oxidized species, the same number of cells may be measured in each sample, aiding in reproducibility.

Previous studies showed that 6M90 and 6R90 cells have higher levels of ROS compared to their parental 308 cell line [172]; other methods than DCFH-DA were used in those analyses. Using DCFH-DA, we observed that 6M90 and 6R90 cells have significantly ($P < 0.05$) higher levels of ROS compared to the 308 parental line (Figure 6A), consistent with the earlier study.

We next determined the biological ramifications of catalase overexpression by measuring the steady-state levels of ROS in the catalase and neo control clones. As seen in Figure 6B, the relative fluorescence of oxidized DCFH-DA was lower by nearly half (0.51 and 0.53 for 6M90 cat69 and 6R90 cat25 clones, respectively) in catalase overexpressing clones compared to a neo clone of 6M90 cells.

To further test the idea that increased steady-state levels of ROS are key in the development of an apoptotic-resistant phenotype, a 30 hr pretreatment regimen with antioxidants was used as additional means of lowering ROS in 6M90 and 6R90 cells. Two different antioxidants, NAC and the water soluble vitamin E analog, Trolox, were used. Figure 6C shows that both NAC and Trolox pretreatment significantly lowered

Figure 6: Measurements of ROS levels in the 308 keratinocyte lines and variants. (A) 6M90 and 6R90 cells have increased levels of ROS compared to the benign parental 308 cells. ROS levels were measured by flow cytometry analysis of oxidized DCFH. The results are expressed relative to the DCFH fluorescence of 6M90. (B) Stable catalase clones have decreased levels of ROS compared to the neo controls. The results are expressed relative to the DCFH fluorescence of 6M90 neo2. (C) Antioxidant pretreatment of cells decreases ROS levels. 6M90 and 6R90 cells treated with vehicle (sfMEM or ethanol for NAC or Trolox, respectively) (black bars) have higher levels of ROS compared to cells pre-treated for approximately 30 hours with 20 mM NAC, (white bars) or 2 mM Trolox (striped bars). The results are expressed relative to the DCFH fluorescence of the appropriate vehicle control. All values are the mean ± S.E.M., $n \geq 3$.


A.
Relative ROS levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
308
6M90
6R90
B.
Relative ROS levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
neo2
neo3
cat69
neo2
cat25
6M90
6R90
C.
Relative ROS levels
1.0
0.8
0.6
0.4
0.2
0.0
6M90
6R90

ROS levels in 6M90 and 6R90 cells, compared to vehicle controls ($P < 0.05$). It is noteworthy that the reduction in ROS between the antioxidant pretreatments compared to their vehicle controls (~0.71-0.77) is similar to that seen with parental line compared to the variants (~0.65).

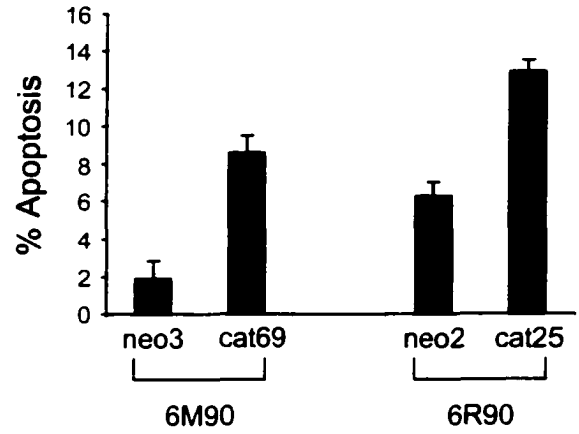
Having demonstrated that catalase overexpression or 30 hr antioxidant pretreatments were sufficient to lower steady-state ROS levels, I tested whether these manipulations reversed the resistance to apoptosis. Similar amounts of apoptosis were seen in the neo control transfectants (Figure 7A) compared to non-transfected 6M90 and 6R90 cells. In contrast, the catalase clones were more sensitive to UVB-induced apoptosis. Similarly, cells pretreated for 30 hr with either NAC or Trolox had greater levels of apoptosis after exposure to UVB compared to the respective vehicle controls (Figure 7B). No toxicity was observed with the pretreatments alone (data not shown). Finally, the converse experiment was performed wherein the 308 cells, normally having low steady-state ROS levels, were pretreated for 30 hours with a low dose of hydrogen peroxide (200 μM) before exposure to UVB. This treatment was sufficient to decrease the amount of apoptosis to a level similar to that seen in the malignant variants (Figure 7C).

Phospho-Akt levels are attenuated with changes in ROS levels

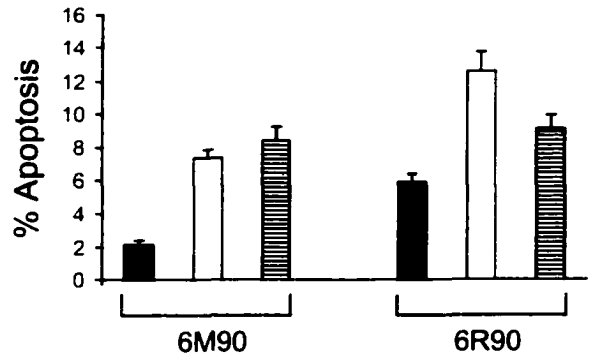
Akt is a serine-threonine kinase involved in the regulation of apoptosis. In many cell lines activation of Akt by phosphorylation has been associated with elevated ROS levels. Therefore western blots were used to determine the extent of phosphorylation of Akt. Figure 8A shows that untreated 6M90 and 6R90 cells have increased levels of

Figure 7: Measurements of apoptosis levels in the 308 keratinocyte line and variants after ROS attenuation. (A) Stable catalase clones have increased levels of apoptosis compared to their neo controls. Cells were exposed to a 300 J/m^2 dose of UV-B and harvested 13 hours later. Apoptosis was determined by morphological assessment of 200 cells per slide. (B) 6M90 and 6R90 cells treated with vehicle (sfMEM or ethanol for NAC or Trolox, respectively) (black bars) have decreased levels of apoptosis compared to cells pre-treated for approximately 30 hours with 20 mM NAC, (white bars) or 2 mM Trolox (striped bars). (C) 308 cells pretreated for approximately 30 hours with 200 μM hydrogen peroxide have decreased levels of apoptosis compared to vehicle (water) treated cells. All values are the mean ± S.E.M., $n \geq 3$

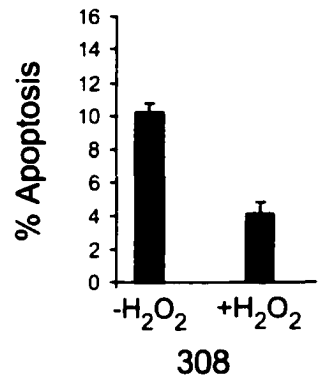
A.


% Apoptosis
16
14
12
10
8
6
4
2
0
neo3
cat69
neo2
cat25
6M90
6R90

B.


% Apoptosis
16
14
12
10
8
6
4
2
0
6M90
6R90

C.


% Apoptosis
16
14
12
10
8
6
4
2
0
-H2O2
+H2O2
308

phospho-Akt compared to 308 cells. The 30 hr pretreatment with NAC or Trolox decreased phospho-Akt levels of 6M90 and 6R90 cells. Conversely, a 30 hr pretreatment of the benign parental line (308) with hydrogen peroxide resulted in an increase in phospho-Akt levels. Examination of the catalase and neo clones (Figure 8B) revealed a similar trend. The catalase overexpressing clones (6M90 cat69 and 6R90 cat25) which have lower ROS levels and lower resistance to UVB-induced apoptosis also displayed decreased phospho-Akt levels as compared to the neo controls which contained high ROS levels and increased apoptosis resistance. Importantly, the observed differences in the level of phosphorylated Akt cannot be explained by changes in the total amount of Akt protein (see lower panels of Figure 8A and B).

The importance of Akt phosphorylation in conferring resistance to apoptosis was tested using an inhibitor of PI-3 kinase, wortmannin, which is known to inhibit Akt activation [196]. Pretreating 6M90 and 6R90 cells with a dose of wortmannin which abolishes Akt phosphorylation (but is non-toxic when administered alone) resulted in an increase in apoptosis after UV irradiation (data not shown). The amount of apoptosis was comparable or slightly exceeding that seen with irradiation of antioxidant pre-treated or catalase overexpressing 6M90 and 6R90 cells.

Discussion

The ability to evade apoptosis may be particularly critical to the development of skin cancer following sun exposure. Normal keratinocytes which sustain too much damage following ultraviolet irradiation undergo apoptosis. Cells that are resistant to UV-induced apoptosis may become tumorigenic, possibly having DNA damage, as well as

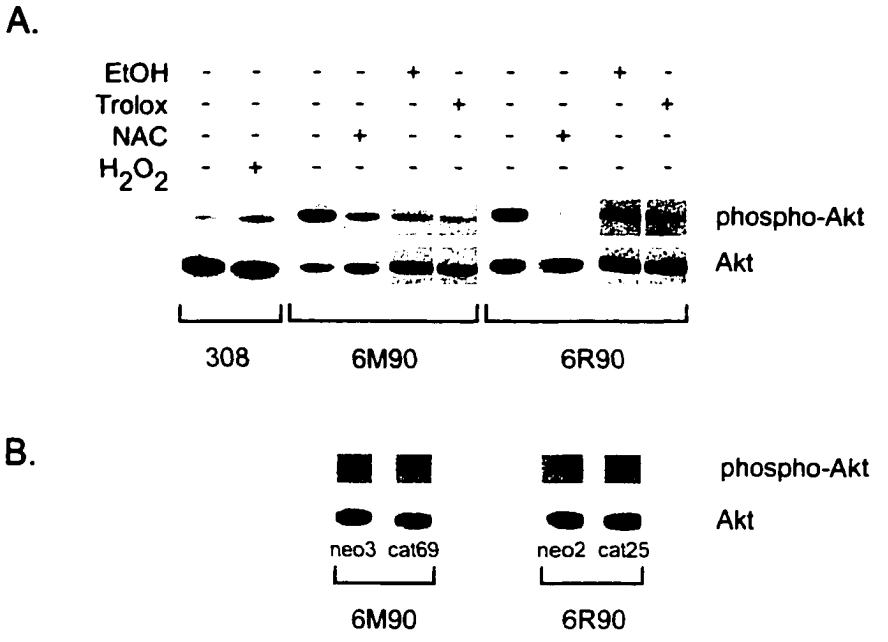


Figure 8: Western blot analysis for phosphorylated and total Akt in 308 keratinocytes and variants. (A) 308 cells pretreated for approximately 30 hours with 200 μM hydrogen peroxide have higher levels of phospho-Akt compared to vehicle (water) treated cells. 6M90 and 6R90 cells treated with vehicle (sfMEM or ethanol for NAC or Trolox, respectively) have higher levels of phospho-Akt compared to cells pre-treated for approximately 30 hours with 20 mM NAC or 2 mM Trolox. (B) Stable catalase clones have lower levels of phospho-Akt compared to the neo controls. Note that total levels of Akt remain unchanged despite treatments.

being vulnerable to future "hits" to their DNA [197-199]. Using an *in vitro* model for skin carcinogenesis, I found that in the conversion from a benign to malignant state, mouse keratinocyte cell lines acquired resistance to UVB-induced apoptosis.

There is increasing evidence for the role of ROS in maintaining a malignant phenotype. This "persistent oxidative stress" hypothesis ([151] and reviewed in [152, 200]) is supported by two broad areas of research. One is the observation that oxidatively modified DNA base products and proteins are increased within many tumors ([201], see Table 1 of [151]). Secondly, antioxidant defenses are decreased in many tumors (reviewed in [164]). One such example is decreased catalase activity seen in mesotheliomas [202], skin cancers ([203] and reviewed in [162, 163]), oral squamous cell carcinomas [204] and lung cancers [205]. Studies which report a decrease in any one antioxidant system or enzyme must be interpreted carefully, since cells may adopt compensatory mechanisms in order to maintain a particular redox environment. Yet a decrease in one antioxidant component can impact malignancy, as shown by studies demonstrating a decrease in tumorigenicity when that antioxidant is augmented [206-208].

The results of the studies described here provide additional evidence in support of the persistent oxidative stress hypothesis. Examination of antioxidant defenses revealed that both 6M90 and 6R90 cells had decreased catalase message, protein and activity levels. This finding is consistent with previous work showing that the malignant variants have increased basal levels of ROS compared to 308 cells. Thus, in this mouse skin keratinocyte model system, the observed persistent oxidative stress is in part due to a loss

of the antioxidant catalase enzyme. As mentioned previously, loss of one antioxidant can be compensated by other antioxidant defenses. Glutathione peroxidases are also capable of removing hydrogen peroxide. No differences in glutathione peroxidase activity were observed between the three cell types however (data not shown). The DCFH data further suggests that full compensation of antioxidant defenses was not achieved, since the 6M90 and 6R90 cells do have increased DCFH fluoresence. Additional work will be necessary to completely characterize the antioxidant defenses to ascertain if other defenses are also downregulated.

I also found that 6R90 cells have elevated levels of COX-2 RNA. Further examination by Thompson et al. showed that the non-inducible cyclooxygenase-1 (COX-1) message is increased in 6R90 cells and both COX-1 and -2 protein levels are similarly increased in 6R90 compared to 308 cells (6M90 cells were not examined) [209]. Increased COX activity is observed in many cancers, notably colon [210, 211] but also skin [212, 213]. While much focus has been on the prostaglandins produced by COXs, it should be noted that reactive oxygen byproducts may also be formed during prostaglandin synthesis [286]. These may be important for carcinogenesis, since inhibition of COX-2 activity results in decreased oxidative DNA damage [214, 215]. Thus, in addition to decreased catalase, overexpression of COX enzymes could contribute to chronic oxidative stress in 6R90 cells.

In addition to changes in catalase and COX enzymes, it was found that 6M90 and 6R90 cells have approximately half as much RNA message for the μ class of glutathione-S-transferases (GSTμ).While not directly responsible for preventing the formation of

oxygen radicals, glutathione-S-transferases (GSTs) are nonetheless important antioxidant enzymes [216, 217]. GST conjugates glutathione to xenobiotics and internally-derived oxidative products. The lipid peroxidation product 4-hydroxynonenal can cause oxidative damage and the GST-catalyzed conjugation of glutathione to this compound is an important antioxidant defense [218]. A decrease in GSTμ may be yet another compromise in the antioxidant defense system of the two malignant variants, contributing to persistent oxidative stress.

The basal elevation of ROS in 6M90 and 6R90 cells was associated with resistance to UV-induced apoptosis. I used both pharmacological and genetic approaches to test whether the basal elevation in ROS was critical for resistance to apoptosis. Addition of the water-soluble vitamin E analog Trolox or N-acetyl cysteine attenuated this resistance, implicating a functional role of ROS in apoptosis resistance. Similarly, the critical contribution of decreased catalase was demonstrated in experiments where enforced catalase overexpression rendered 6M90 and 6R90 cells sensitive to apoptosis. Transient transfections of catalase or treatment of 6M90 and 6R90 cells with Trolox or NAC was previously shown to attenuate their increased proliferative capacity [172]. Cell cycle and cell death are tightly coupled processes, such that perturbations in one may affect the other [219, 220]. For instance, it has been shown that some apoptosis-regulatory genes are regulated in a cell-cycle-dependent manner (reviewed in [221]). It is possible that the increased apoptosis sensitivity obtained by modulating ROS is an indirect effect of the decreased proliferative capacity previously reported to occur by ROS modulation. More research will be needed to determine if this possibility is correct.

Alternatively, ROS may directly mediate apoptosis resistance by modulating an apoptotic signaling pathway. Previous studies with human keratinocyte and mouse epidermal cell lines addressed the question of whether Akt activity is modulated by ROS. Following exposure to UVC irradiation, increased levels of ROS and phosphorylated Akt were seen in JB6 mouse epidermal cells [190]. In that study, Akt was also found to be activated following exposure of the cells to UVB; whether this treatment altered ROS levels was not examined. Using the HaCaT human keratinocyte cell line, Zhang et al. [189] have found that UVB irradiation leads to an increase in Akt phosphorylation. Since pretreatment with NAC before exposure of the cells to UVB inhibits the increase seen in the level of Akt phosphorylation, these investigators conclude that the UV treatment results in the generation of ROS. Therefore, Akt phosphorylation appears to be affected by ROS levels.

The results presented here show that basally elevated levels of ROS results in increased Akt phosphorylation. In addition, these studies addressed the important question of whether this Akt phosphorylation resulted in increased resistance to apoptosis. In contrast to JB6 or HaCaT cells, exposure of 308 mouse keratinocyte cells to UVB irradiation did not result in an increase Akt phosphorylation. As might be predicted, therefore, 308 cells underwent apoptosis after irradiation.

Differences between cell types could explain the observed differences between my study and the previous ones. The 308 cells were derived from DMBA-treated mouse skin and selected for resistance to Ca^{2+}-induced differentiation [180]. It is possible that they are more 'progressed' than JB6 and HaCaT cells and are more resistant to stressors

such as UV, although less than the two malignant variants. Perhaps 308 cells have sufficient antioxidant defenses to remove any acute burst of ROS triggered by irradiation before it can signal an increase in Akt. An alternative possibility is that compared to JB6 or HaCaT cells, 308 cells have a sufficiently high steady state level of ROS so that the additional amount generated by UV irradiation is not enough to stimulate Akt phosphorylation. Addition of exogenous H_2O_2 to 308 cells did increase Akt phosphorylation (see Figure 5A), however, indicating that induction of Akt phosphorylation through ROS is possible in these cells.

A fundamental difference between the previous studies and mine is the issue of acute versus chronically increased ROS levels. While both acute and chronic doses of ROS result in an upregulation of Akt phosphorylation, the reasons and hence the consequences of these exposures may differ. The acute exposure to ROS used in the previous studies models a "one time" cellular insult, wherein the insult itself is the burst of ROS. It is advantageous for a cell to attempt to overcome this acute insult by elevating survival signals such as phosphorylation of Akt. These can allow the cell to recover from the insult, unless the damage is too great, in which case death signals will override and the cell will die. The addition of antioxidants in this situation attenuates Akt phosphorylation because the "insult" (ROS) itself is lessened; that is, fewer ROS are available to damage the cell and induce a stress response. Short term incubation with the antioxidant NAC [189, 190] or catalase protein [190] before UV or peroxide treatment did attenuate Akt phosphorylation, presumably because the burst of ROS was blocked by the available antioxidants. Rather than modeling transient increases in ROS associated

with acute cell injury, the malignant 308 variants have chronically elevated ROS levels, which have altered the cellular redox environment. We think that this system more accurately models a critical event in tumorigenesis. Perhaps through the same signaling pathway as in acute ROS doses, Akt activity is elevated but remains elevated because of the chronically elevated ROS. The role of antioxidants in attenuation of this signal differs slightly. Short term pretreatment of the malignant variants with NAC did not reverse their UV resistance (data not shown), presumably because Akt phosphorylation was unchanged. Instead, a chronic "environmental" change must occur with antioxidant treatment. I chose a duration of pretreatment that was longer than the cells' doubling time to accomplish this and this effectively decreased Akt phosphorylation and apoptotic resistance. It was concluded that the longer pretreatment continually dampened the chronic production of ROS and ensuing signals. In doing so, the "environment" of the cell was changed and the stimulus for Akt phosphorylation was reduced. Further experiments are still required to delineate the signaling pathway between ROS and Akt phosphorylation, especially in those chronic situations which promote tumorigenesis.

The model that steady state levels of increased ROS contribute to malignancy through an upregulation of Akt activity has some correlative support from a recent report of Akt in mouse skin tumorigenesis [222]. This *in vivo* study found that Akt activity increased during the development of squamous cell carcinoma. Although the authors did not measure ROS levels, it is well established that the DMBA/TPA initiation and promotion protocol gives rise to elevated ROS [159, 160, 223]. It is therefore possible that in the early stages, increased Akt activity was due in part to elevated ROS levels.

When the mode of Akt upregulation was examined, the negative regulator PTEN phosphatase was found to decrease in activity (but not amount), but only in the later stage of squamous cell carcinoma. Recalling the persistent oxidative stress model, this decrease in phosphatase activity levels at a late stage could also be explained by chronically elevated ROS within the tumor cells, given that hydrogen peroxide is able to decrease PTEN activity [224]. Finally, subcutaneous injection of a poorly tumorigenic keratinocyte cell line overexpressing wild type Akt revealed an increase in tumorigenicity and a decrease in apoptosis within those tumors. This experiment highlights the role that Akt plays in bringing about an apoptotic-resistant phenotype within tumors. The experiments reported here point to a potential molecular mechanism for increased Akt activity seen through various stages of malignant transformation in mouse skin [222]. A state of chronically elevated ROS within cells leads to chronically increased levels of phosphorylated Akt. This in turn leads to an apoptotic-resistant phenotype, which may be reversed by lowering ROS levels.

Chapter III. Additional Characterization of 308, 6M90 and 6R90 cells: Differential sensitivity to DNA damaging drugs

Introduction:

The observation that 6M90 and 6R90 cells were more resistant to UV-induced apoptosis led to further characterization of their resistance to other death-inducing stimuli. Given that 6M90 cells had slightly higher levels of DT-diaphorase compared to 308 or 6R90 cells, it was hypothesized that they would be more sensitive to death induced by EO9. EO9 is activated by DT-diaphorase [225], enabling it to form DNA crosslinks [226]. Cells with higher diaphorase activity have been shown to be more sensitive to EO9 [194]. Melphalan [227, 228] and cisplatin [229] also kill cells by forming crosslinks but they are not activated by DT-diaphorase. Transplatin was also tested, though it forms crosslinks much more slowly and hence is not an efficient chemotherapeutic agent [229]. Cross-resistance was tested by examining sensitivity to mitoxantrone, which does not kill by cross-linking but through intercalation of DNA [230].

Materials and methods:

308, 6M90 and 6R90 cells (2000 per well) were grown in 96-well microtiter dishes. After an overnight incubation, vehicle alone or drugs were added. EO9 was a gift of Dr. Hans R. Hendriks at the European Organization for Research and Treatment of Cancer New Drug Development Office (Amsterdam, the Netherlands). All other drugs were

supplied by Dr. William Bellamy at the University of Arizona (Tucson, AZ). In EO9 experiments, the medium was removed after 4 h and cells were rinsed once with PBS and refed with fresh MEM (all other drugs were continuously incubated with cells). Three days later, the number of attached (viable) cells was measured using a Sulforhodamine B (SRB) assay as previously described [231] with some modifications. Cells were washed twice with PBS, fixed in cold 10% trichloroacetic acid for 30 min at 4°C, washed four times with tap water and air dried before staining with SRB for 15–20 min. Absorbances of vehicle controls were set at one hundred percent and E.C. 50 values were determined based on plots of percent growth vs. drug dose. Quadruplicate wells were used for each drug concentration and experiments were done in duplicate (transplatin or mitoxantrone), triplicate (cisplatin or melphalan) or quadruplicate (E09).

Results

Diaphorase activity not correlated to EO9 sensitivity

Enzyme activity assays showed that 6M90 cells had elevated DT-diaphorase activity compared to 308 and 6R90 cells (See figure 3B). Although the increased activity did not reach statistical significance, we reasoned that 6M90 cells could be more sensitive to EO9-induced death than 6R90 cells and 308 cells. Figure 9 shows that 6M90 cells are only slightly more sensitive to EO9 than 6R90 cells, which are approximately 2-fold more sensitive than the parental 308 cells.

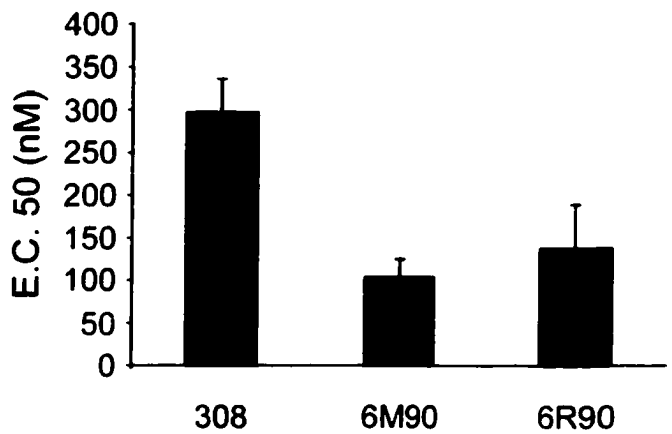


Figure 9: E.C. 50 values of the DT-diaphorase-activated DNA crosslinker EO9. 308 cells are more resistant to E09 cell killing than 6M90 and 6R90 cells. SRB assays were performed after three days on cells treated for 4 hr with EO9. Values are the mean S.E.M., n = 4.

Malignant variants are more sensitive to other DNA crosslinking agents but not a DNA intercalating agent

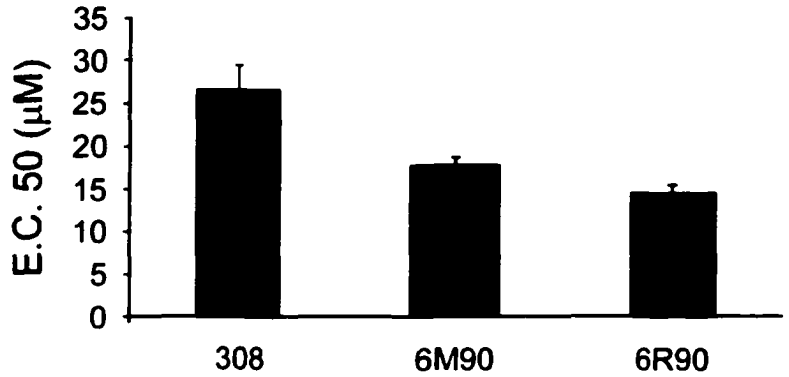
After it has been bioactivated by DT-diaphorase, EO9 kills cells by causing DNA crosslinks. Since diaphorase activity was not correlated to sensitivity to EO9, other DNA crosslinking agents were tested for differential sensitivity between the progressed variants and the parental cell line. 6M90 and 6R90 cells are more sensitive to melphalan (Figure 10 A), cisplatin (Figure 10 B) and transplatin (Figure 10 C). To test whether the sensitivity observed in the malignant variants was specific to DNA crosslinking damage only, cells were tested for sensitivity to mitoxantrone, a DNA intercalating agent. Figure 11 shows that both the malignant variants and the parental 308 cell line have similar sensitivity to mitoxantrone.

Discussion

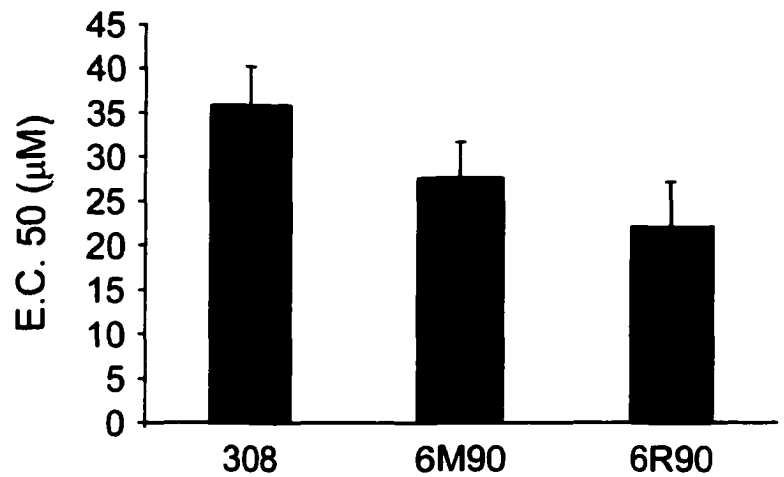
These studies were undertaken to assess the resistance of the 6M90 and 6R90 cells to other means of cell killing besides UV. Such studies could provide additional information on the molecular changes which occurred during the transition of the cells to a malignant phenotype. Testing for sensitivity to EO9 could determine whether the slightly increased DT-diaphorase activity observed in 6M90 cells was biologically relevant. However, no correlation was seen between DT-diaphorase activity and sensitivity to EO9. Both malignant variants were more sensitive to EO9 than 308 cells, despite the similarity in DT-diaphorase activity between 308 and 6R90 cells. In other studies, DT-diaphorase activity was artificially elevated from tens to hundred-fold or greater levels after which marked EO9 sensitivity was observed [232, 194].

Figure 10: E.C. 50 values of melphalan (A), cisplatin (B) and transplatin (C). 6M90 and 6R90 cells are more sensitive to cell killing induced by other DNA crosslinking agents. SRB analysis was performed on cells continuously exposed to the drugs for 72 hours. Results are the mean ± S.E.M., n = 3 in A and B; in C, n = 2.

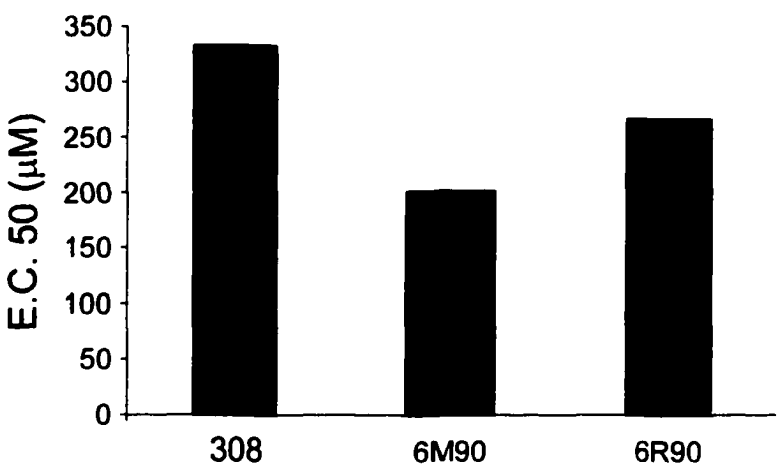
A.


E.C. 50 (μM)
35
30
25
20
15
10
5
0
308
6M90
6R90

B.


E.C. 50 (μM)
45
40
35
30
25
20
15
10
5
0
308
6M90
6R90

C.


E.C. 50 (μM)
350
300
250
200
150
100
50
0
308
6M90
6R90

The near two-fold change in diaphorase activity was not sufficient to sensitize 6M90 cells to a greater extent than 6R90 cells, though both were more sensitive than the parental 308 cells. A likely explanation is that a threshold level of diaphorase activity is needed to bioactivate EO9 in all three cell types, yet the 6M90 and 6R90 cells are unable to efficiently repair the DNA damage caused by the bioactivated EO9. This hypothesis was tested by comparing the sensitivity of the cells to other DNA crosslinking agents which do not rely on DT-diaphorase for activation. The clinically relevant drugs cisplatin and melphalan were shown to similarly cause decreased cell survival in 6M90 and 6R90 cells. Nucleotide excision repair [233] and recombination strategies [234] are thought to be important for survival of cells after DNA crosslinkage. Further work will be necessary to determine if either of these pathways are deficient in the malignant variants, which could account for their increased sensitivity.

Transplatin is considered a less active compound than the cis-isomer, as reflected in E.C. 50 doses that are almost ten-fold higher than those for cisplatin. The decreased efficiency is thought to be due to its much slower ability to form the second adduct required for crosslinking [235, 236]. This slow conversion presumably allows for transplatin compounds to be recognized as monofunctional adducts by DNA repair enzymes. Plasmids which had been exposed to transplatin were repaired in an *in vitro* nucleotide excision repair assay using cell extracts from HeLa cells [237], indicating that pathways exist to repair the damage of transplatin. Moreover, because certain DNA-

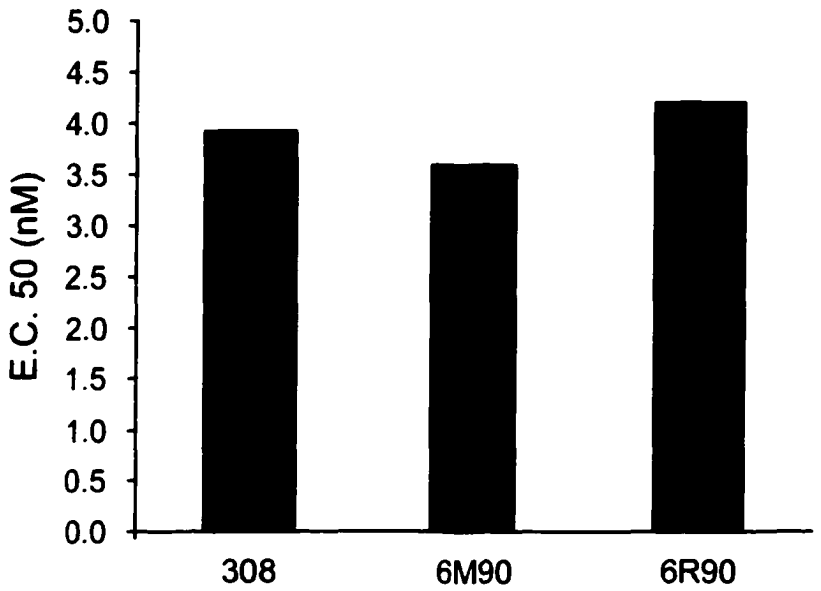


Figure 11: E.C. 50 values of mitoxantrone, a DNA intercalating agent. 308, 6M90 and 6R90 cells are similarly sensitive to mitoxantrone. SRB analysis was performed on 2000 cells/well after continuous three-day exposure to mitoxantrone. Results are the average of duplicate experiments.

binding proteins selectively bind cisplatin- but not transplatin-induced adducts, the repair pathway for transplatin may be distinct [233]. Thus another repair mechanism may additionally sensitize 6M90 and 6R90 cells to such DNA damaging agents.

An alternative explanation for the increased sensitivity of the malignant variants to DNA cross-linking agents is a difference in drug metabolism. Different classes of GST [238], including the μ class can conjugate glutathione to both cisplatin and melphalan. As noted previously, 6M90 and 6R90 cells have decreased GSTμ mRNA. It is possible that this has led to decreased protein levels. HeLa cells grown in suspension have lower levels of GSTs and are more sensitive to cisplatin and melphalan than attached cultures [239]. Moreover, in PC3 prostatic cells [240] upregulation of μ class GST is correlated with resistance to cisplatin and melphalan. Therefore, the malignant variants may be deficient in their ability to detoxify these drugs given the requirement of GSTs for detoxification. Further experiments with the variants, such as overexpressing GSTμ before exposure to drugs, will be needed to determine whether this pathway is the cause for increased sensitivity.

Cross-resistance to mitoxantrone was not observed in the malignant variants. Mitoxantrone is a DNA intercalator and is thought to act primarily as a topo-isomerase II inhibitor, though other modes of action have been described (reviewed in [241]). Mitoxantrone, but not cisplatin and melphalan, is susceptible to excretion by the membrane-bound P-glycoprotien, an enzyme partially responsible for the so-called multi-drug resistant phenotype [242]. Since 308, 6M90 and 6R90 cells were uniformly susceptible to mitoxantrone killing it may be argued that no alterations have occurred in

P-glycoprotein expression. Finally, loss of DNA mismatch repair is reported to be important for resistance to both cisplatin and mitoxantrone [243]. Given the uniform sensitivity to mitoxantrone but increased sensitivity to cisplatin, it appears that mismatch repair enzymes are also unaltered in the three cell types. This lack of differential sensitivity to mitoxantrone is useful in characterizing the drug-sensitive phenotype of the malignant variants. However, further research will be required to better elucidate the mechanism behind the differential sensitivity of 6M90 and 6R90 cells to DNA crosslinking agents.

Chapter IV. Identification of a Functional PPAR Response Element in the 3' UTR of the Human *bcl-2* Gene

Introduction

A crucial step in carcinogenesis is the development of resistance to apoptosis [149]. One mechanism by which cells can develop resistance to apoptosis involves peroxisome proliferator activated receptors (PPARs). These proteins are part of the superfamily of ligand-activated steroid/thyroid nuclear receptors (reviewed in [244-246]). Three PPAR subtypes, α, δ (also known as β) and γ, have been identified and characterized in mammals and amphibians. Chronic treatment of rats and mice with PPAR ligands has been shown to cause liver cancer [247, 248]. Studies by Lefebvre et al. [249] and Saez et al.[250] have demonstrated that treatment of Min+ mice with PPARγ ligands increases the number of colon polyps and tumors which form. In *in vitro* studies, pre-treatment of mouse hepatocytes with a PPAR ligand decreases the amount of apoptosis seen following treatment with TGF-β or bleomycin [251]. The same pre-treatment has no effect on the amount of apoptosis seen, however, when hepatocytes from PPARα-null mice are exposed to TGF-β or bleomycin. Similar work in rat hepatocytes has demonstrated that apoptosis induced by TGF-β can be inhibited by pretreatment with PPAR ligands and this effect can be blocked by expression of a naturally occurring dominant negative form of human PPARα [252].

The mechanism by which PPARs mediate resistance to apoptosis appears to involve the antiapoptotic protein, Bcl-2. Treatment of primary rat hepatocytes with a PPARα ligand increases Bcl-2 levels [251]. Bcl-2 levels in colon cancer cells are increased following exposure of the cells to prostaglandin E [253]. This is significant because prostaglandin metabolites are thought to act as endogenous PPAR ligands [254, 255]. In the studies mentioned above, the molecular mechanism by which PPARs alter Bcl-2 levels was not addressed. Given their known function as transcription factors, I investigated whether PPARs directly regulate *bcl-2* gene expression. Evidence is provided that a putative PPAR response element (PPRE) within the 3' untranslated region of the *bcl-2* gene is functional and can mediate resistance to apoptosis.

Materials and Methods

Plasmids

The human RXR α expression vector (pCMX-hRXRα (Kpn)) was provided by Dr. Ron Evans (Salk Institute, La Jolla, CA). The *Xenopus* PPAR α, γ and β expression vectors (pSG5-xPPARα, -γ and -β, respectively) were provided by Dr. Liliane Michalik (Université de Lausanne, Switzerland). The empty parental vector (pSG5) was created by excising the xPPARβ cDNA from pSG5-xPPARβ with EcoRI and religating. The *bcl-2* cDNA plasmid was provided by Dr. Roger Meisfeld (University of Arizona).

The luciferase reporter plasmids, pPPRE-luc and pPPREmut-luc, were created by a triple ligation as follows. The promoterless pGL-3-Basic plasmid (Promega, Madison,

WI) was digested with NheI/HindIII and gel purified. A ClaI/HindIII-digested and gel-purified fragment of the promoter region of the GADD153 (-34 to +91) gene was used as the minimal promoter [256] Oligonucleotides containing either the putative PPRE from human *bcl-2* or a mutated version were synthesized (Integrated DNA Technologies, Inc. Coralville, IA). The sequences were as follows (sequence from the human *bcl-2* gene underlined, mutations in lowercase):

Upper strand bcl-2PPRE oligonucleotide:
5' CTAGCAGAAACAAGGTCAAAGGGACA 3'
Lower strand bcl-2PPRE oligonucleotide:
5' GATCTGTCCCTTTGACCTTGTTTCTG 3'
Upper strand bcl-2PPREmut oligonucleotide:
5' CTAGCAGctcCAAGGTCggtGGGACA 3'
Lower strand bcl-2PPREmut oligonucleotide:
5' GATCTGTCCCaccGACCTTGgagCTG 3'.

The respective upper and lower strand oligonucleotides were annealed according to the manufacturer's protocol, creating a 5' NheI overhang and 3' ClaI overhang. The three fragments (pGL-3-Basic, GADD153 promoter region, oligonucleotides) were ligated using T4 DNA Ligase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The plasmid constructs were verified by sequencing.

In Vitro Transcription/Translation

The expression vectors for hRXRα, xPPARα, β and γ were transcribed and translated *in vitro* using the TnT Quick Coupled kit (Promega). ^{35}S-methionine-labeled translation products were run in parallel on an SDS-polyacrylamide gel to verify molecular weights and equal translation efficiency, according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay

Annealed bcl-2PPRE and bcl-2PPREmut oligonucleotides were ^{32}P-labeled using Klenow Fragment (Invitrogen) in a fill-in reaction according to the manufacturer's protocol. EMSAs were performed essentially as described previously [257], except that 50,000-65,000 cpm were added to the 15 µl reaction. In some reactions, 20- or 100-fold molar excesses of non-labeled double-stranded bcl-2PPRE oligonucleotides or bcl-2PPREmut oligonucleotides were added immediately after the labeled probe for competition analysis.

Cell Culture and Transfection

HCA-7 colon cancer cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen) with 10% fetal bovine serum (Invitrogen) and 500 U/ml penicillin and 5000 U/ml streptomycin (Invitrogen) at 37° C in a 7% CO_2 humidified environment. For luciferase assays, cells were plated on 60 mm tissue culture plates and grown to a density of approximately 40-60% confluency before transfection. Cells were triply transfected in serum-free DMEM (sfDMEM) with the transfection agent Cellfectin (Invitrogen), according to the manufacturer's protocol. The following amounts of plasmids were used per transfection: 1.495 µg of pSG5-xPPARα,-β, -γ or the empty vector pSG5, 1.495 µg of the reporter plasmids pPPRE-luc or pPPREmut-luc and 10 ng of the transfection control plasmid, pRL-TK (Promega). Briefly, 200 µl of sfDMEM containing 15 µl Cellfectin was combined with 200 µl of sfDMEM containing the various plasmids and

allowed to incubate at room temperature for 20 min. The plates were then rinsed once with PBS and overlaid with the approximately 425 µl sfDMEM/Cellfectin/DNA mix. An additional 1.2 ml of sfDMEM was added to each plate. The media was replaced with serum-containing media 24 h after transfection and cells were allowed to recover an additional 24 h before harvesting in 1X Passive Lysis Buffer (Promega) according to the manufacturer's instructions.

For RNA samples, the same transfection procedure was followed with the exceptions that 100 mm plates were used, 6 µg of pSG5-xPPARγ or pSG5 were transfected and sfDMEM and Cellfectin volumes were doubled.

RNA collection

RNA was harvested from transiently transfected HCA-7 cells, 24 h after replacement of sfDMEM with DMEM. Cells were removed from the plate with trypsin, pelleted and rinsed twice with PBS. Cell pellets were snap frozen in a dry ice/ethanol bath. RNA was harvested using the RNAid Plus kit (Quantum Biotechnologies, Inc., Carlsbad, CA), except that the cells were lysed by sonication in the lysis buffer rather than by homogenization. Once lysed, the manufacturer's protocol was followed.

Northern Blot Analysis

Standard procedures [258] were used for northern blot analysis, with 25 µg of RNA per lane. The blots were probed with a ^{32}P-labeled, 600 bp BamHI/EcoRI fragment from

human *bcl-2* cDNA and membranes were prehybridized as described previously [258]. After exposure to film, blots were stripped and reprobed with a ^{32}P-labeled probe for human *GAPDH* in order to verify equal loading.

Dual Luciferase Assay

Luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Firefly luciferase activities from the pPPRE-luc and pPPREmut-luc plasmids were normalized to the sea pansy luciferase activities of pRL-TK to adjust for transfection efficiency. Activities were further normalized to the activities of cells which had been triply transfected with the empty vector construct (pSG5), pPPRE-luc and pRL-TK. In some experiments, GW2331 (a PPARα and PPARγ-activating ligand) was added at final concentrations of 50 nM (for PPARα activation) or 300 nM (for PPARγ activation) in DMSO [254]. GW2331 was synthesized by Dr. Yushun Li of the SWEHSC Synthetic Core Facility (University of Arizona, Tucson AZ). In other experiments, GI251929X (a PPARγ-specific ligand) or GW327647X (a PPARα-specific ligand) were added at final concentrations of 5 nM in DMSO. Both GI251929X and GW327647X were a generous gift from Drs. Tim Willson and Steve Kliewer, Glaxo-Wellcome, Research Triangle Park, NC.

Confocal Analysis

HCA-7 cells were transiently transfected in 100 mm dishes with 6 μg of pSG5 or pSG5-xPPARγ as described above. The serum-free media containing Cellfectin and DNA was replaced with serum-containing media 24 h after transfection and cells were allowed to recover an additional 24 h before harvesting by trypsinization. Cell samples were deposited onto glass slides by cytocentrifugation (Shandon, Inc., Pittsburg, PA). The slides were air dried for 1 h and then fixed for 20 min in 4% formaldehyde/PBS at room temperature, followed by three washes with PBS and a permeabilization with 100% methanol at -20° C for 6 min.

To measure Bcl-2 levels by confocal microscopy, the fixed slides were first incubated with 5% BSA/PBS for 5 min and then blocked for 5 min in 10% goat serum/PBS. The slides were incubated for 1 h with a 1:50 dilution of anti-Bcl-2 antibody (SC-509, Santa Cruz Antibodies, Santa Cruz, CA), washed in PBS and incubated for 1 h with a 1:100 dilution of biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Both antibodies were diluted in 1% BSA/PBS. The slides were washed and incubated for 1 h at a 1:50 dilution of Cy-5-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA). The slides were then subjected to an hour-long RNAse digestion step, after which the nuclear counterstain YOYO-1 (Molecular Probes, Inc. Eugene, OR) was applied for 10 min at a final concentration of 0.5 μM. Coverslips were then mounted using fluorescent mounting media (DAKO Corp., Carpinteria, CA) and slides were stored at 4° C until analysis. A laser scanning

confocal microscope (LEICA TSD-4D, Heidelberg, Germany) equipped with an argon-krypton laser was used to obtain images. Semi-quantitative digital image analysis of the images was performed as described previously [259]. Briefly, all settings of the microscope were kept constant (photomultiplier tube voltage, number of line scans, laser power) so that images could be accurately compared to each other. All images to be compared to each other were obtained during the same session on the confocal microscope. An average grayscale intensity per cell was calculated using Image-Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD). Each transfection was carried out in duplicate and two slides were made from each transfection, one of which was stained for Bcl-2 and the other used as a control for background fluorescence by omitting the primary (Bcl-2) antibody incubation step. Images of at least three random fields per slide were taken at 40X magnification and at least 15 cells from each field were analyzed. The final results are averaged from three separate experiments such that a total of 266 cells from all pSG5 transfections and 267 cells from all PPARγ transfections were analyzed.

Apoptosis measurements

HCA-7 cells were transiently transfected with 2.3 μg of pSG5 or pSG5-xPPARγ in 60 mm plates as described above. The sfDMEM/Cellfectin/DNA mixture was replaced with serum-containing media and 625 μM deoxycholic acid (sodium salt, monohydrate, ICN Biochemicals, Inc. Aurora, OH) 24 h after transfection. After 5.5 h, the cells were harvested by trypsinization, adjusted to 5 X 10^5 cells/ml and deposited onto glass slides

by cytocentrifugation (Shandon, Inc.). The slides were stained using the Diff-Quik staining kit (Dade Behring Inc., Newark, DE) according to the manufacturer's protocol. Percent apoptosis was determined by morphological examination of 200 cells per slide.

Results

The human *bcl-2* gene contains a putative PPRE

The peroxisome proliferator response element (PPRE), to which PPARs bind and activate transcription, consists of both a core element of two direct repeats of six nucleotides separated by a single nucleotide and seven basepairs immediately 5' to this 13 basepair core (Table 1). These seven basepairs are reportedly important in determining the specificity of PPAR binding and activation [260]. The genomic sequence of human *bcl-2* as reported by Tsujimoto [261] was examined for a possible PPRE. The inverse sequence of basepairs 2790-2809 was found to be highly homologous to the consensus PPRE [260]. This corresponds to the inverse sequence of basepairs 4248-4267 in the human *bcl-2* Genbank sequence, accession number M13994. As shown in Table 1, the murine *bcl-2* gene and the nematode homolog, *ced-9*, have similar PPRE-like sequences.

Table 1: The human and murine *bcl-2*, and nematode *ced-9*, contain sequences with homology to the consensus PPRE.

Organism/Gene	Genomic Location	Basepairs (ref)	Sequence*
human *bcl-2*	3' UTR	2790-2809 (261)	AGAAACAAGGTCAAAGGGAC
murine *bcl-2*	intron 1	2468-2487 (284)	GGATTCTGGGTCAAAATGGG
nematode *ced-9*	intron 2	2810-2829 (285)	CAAAACTTGGTCAAAAATAG
Consensus PPRE	--	(260)	CAAAACTAGGTCAAAGGTCA GG T T

*Underlined nucleotides are exact matches to the consensus.

The human *bcl-2* PPRE binds PPAR and RXR heterodimers

For binding to DNA, PPARs form a heterodimer with another class of nuclear receptors, the retinoid X receptors (RXRs). Electrophoretic mobility shift assays (EMSAs) were used to determine whether the putative PPRE in the human *bcl-2* gene binds to PPAR/RXR heterodimers. Addition of equal amounts of *in vitro* translated PPARα (Figure 12A), PPARβ (Figure 12B) and PPARγ (Figure 12C) showed that all three PPAR subtypes bound the PPRE in the presence of RXR, albeit with different affinities. PPARγ exhibited the strongest binding of the three PPAR subtypes. Neither RXR nor any of the PPARs by themselves were able to bind the oligonucleotide (lanes 1 and 4). The mutated PPRE oligonucleotide was not able to bind the PPAR/RXR complexes, as seen in lane 3. Specificity of binding was further analyzed by competition experiments with unlabeled oligonucleotides. Excess amounts of the unlabeled PPRE oligonucleotides were able to decrease the binding of the PPAR/RXR complexes to the labeled oligonucleotides (lanes 5 and 6). Unlabeled mutant oligonucleotides, however, were unable to compete with the labeled PPRE oligonucleotides, even at 100-fold higher concentrations (lane 7).

Transcriptional activation of a reporter gene is mediated by the human *bcl-2* PPRE

To determine whether the PPRE-like sequence found in the human *bcl-2* gene can

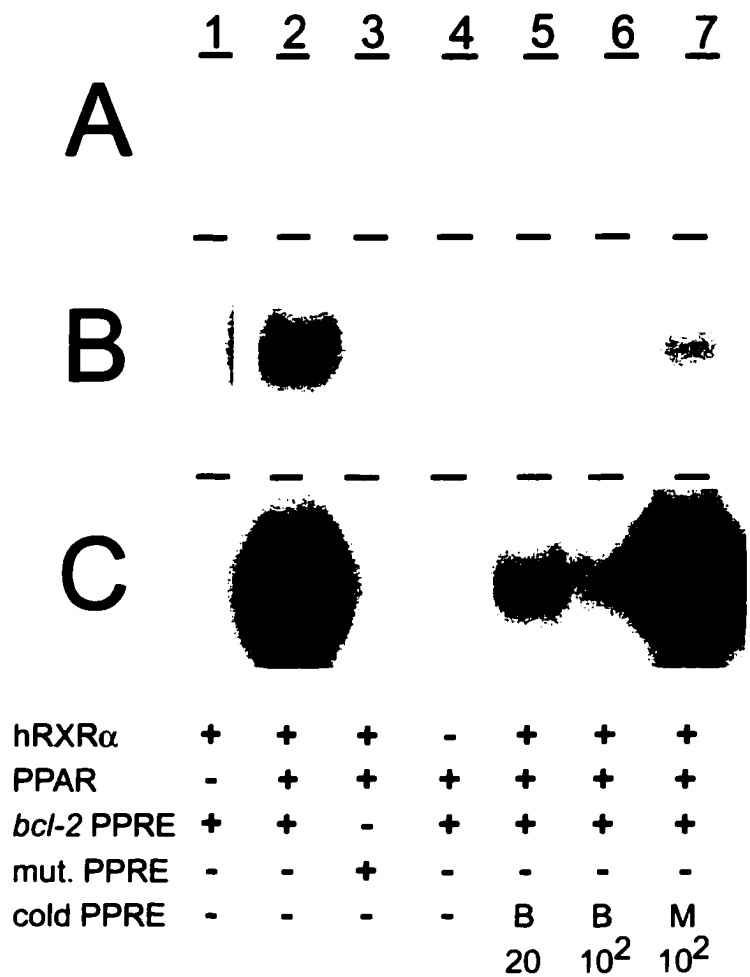


Figure 12: PPAR/RXR heterodimers bind an oligonucleotide containing the PPRE from human *bcl-2*. EMSAs with a ^{32}P-labeled oligonucleotide containing the human *bcl-2* PPRE were carried out with *in vitro*-translated *Xenopus* PPARα (A), β (B), γ (C) and human RXRα. Competitor unlabeled oligonucleotides of regular ("B") or mutated ("M") *bcl-2* PPRE were used at a 20- or 100-fold molar excess. The experiments have been replicated and representative gels are shown.

mediate transcriptional regulation by interaction with PPARs, luciferase activity was measured using reporter plasmids. These contained the *bcl-2* PPRE (pPPRE-luc) or a mutated version (pPPREmut-luc) upstream of a minimal promoter driving the luciferase gene. Cotransfection of PPARγ with pPPRE-luc gave the strongest induction of luciferase (Figure 13). PPARα and PPARβ were not able to appreciably increase luciferase activity higher than that seen with the empty expression vector pSG5. Also, luciferase expression in cells transfected with the PPAR expression vectors and the pPPREmut-luc plasmid was decreased in comparison to pPPRE-luc. HCA-7 cells transfected with only a luciferase plasmid containing two contiguous *bcl-2* PPREs but not PPAR expression vectors gave luciferase values comparable to background luminescence (data not shown), indicating low endogenous levels of PPARs in HCA-7 cells.

Like many nuclear receptors, PPARs have ligand dependent and independent activities (reviewed in [262, 263]). Addition of GW2331 (a PPARα and PPARγ-activating ligand), GI251929X (PPARγ-specific ligand) or GW327647X (PPARα-specific ligand) to cells in the presence of the PPAR expression vectors, however, resulted in no difference in levels of luciferase activity compared to DMSO alone (data not shown). This has been reported in other cell types [260, 264] and is most likely due to the presence of an endogenous ligand within HCA-7 cells. It has been reported that HCA-7 cells have measurable cyclooxygenase-2 (COX-2) activity [253, 260, 264, 265]. Therefore, it is possible that the lack of increased luciferase activity upon addition of

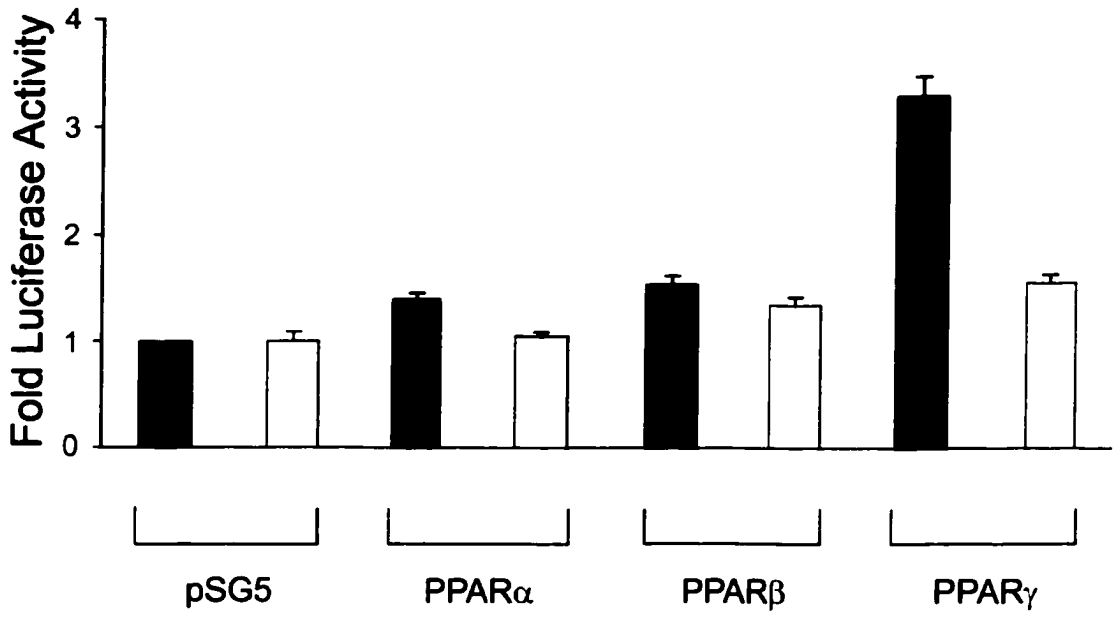


Figure 13: PPARγ increases luciferase reporter gene activity driven by a minimal promoter and the bcl-2 PPRE. Cells were triply transfected with PPAR expression vectors (or the empty pSG5 vector), pPPRE-luc or pPPREmut-luc and a *Renilla* luciferase plasmid for transfection controls. Filled bars represent pPPRE-luc and clear bars represent pPPREmut-luc. The results are expressed as fold pSG5 + PPRE-luc (mean ± S.E.M.; n = 3).

PPAR ligands was due to the fact that HCA-7 cells already produce saturating amounts of a PPARγ-specific ligand, possibly downstream metabolites of the prostaglandins produced by COX-2 (see discussion).

bcl-2 RNA transcript levels are elevated with PPARγ transfection

Both EMSA and luciferase experiments measured the activity of the putative *bcl-2* PPRE out of the broader context of the surrounding sequences within the *bcl-2* gene. Therefore, while the EMSA and luciferase data point to an interaction of PPARs with the 20 basepair sequence found in the 3' UTR of *bcl-2*, I needed to confirm that PPARs directly regulate *bcl-2* in the natural context of the gene in whole cells. Because PPARγ displayed the strongest binding in the EMSA experiments and the highest level of luciferase induction, HCA-7 cells were transfected with pSG5-xPPARγ or the empty expression vector pSG5. RNA was collected and *bcl-2* message was examined by northern blots. Our results confirmed that PPARs regulate *bcl-2* in the context of the whole cell; cells transfected with pSG5-xPPARγ had higher levels of the *bcl-2* message (Figure 14), compared to pSG5-transfected HCA-7 cells. Equal loading was ensured by reprobing the blot for GAPDH.

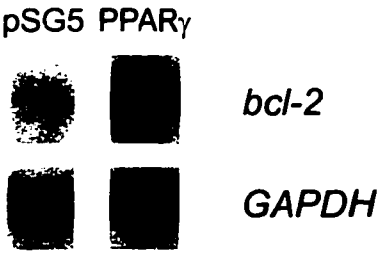


Figure 14: Transfection of PPARγ increases the 5.5 kB bcl-2 mRNA band. Cells were transiently transfected with the empty vector pSG5 or an expression vector for PPARγ. After 24 hours of transfection and 24 hours of recovery, total RNA was collected and *bcl-2* message levels were examined by northern analysis (25 μg/lane). Equal loading was ensured by stripping the blot and reprobing for *GAPDH*. The experiments have been replicated and a representative blot is shown.

Bcl-2 protein levels are increased with PPARγ transfection

Having demonstrated that PPARs can directly regulate *bcl-2* gene transcription, it was necessary to confirm that this led to increased Bcl-2 protein levels in the cells. Using the same timecourse as described for RNA collection, cells were transiently transfected with pSG5 or PPARγ expression vectors and Bcl-2 protein detected by immunofluorescence. Visualization of the cells by confocal microscopy showed a more intense staining for Bcl-2 protein in the cells which were pSG5-xPPARγ-transfected, compared to pSG5-transfected cells. Quantitation of the images confirmed that pSG5-xPPARγ-transfected cells had increased levels of Bcl-2 protein compared to pSG5-transfected cells (Figure 15).

Cells transfected with PPARγ are more resistant to apoptosis

Given that Bcl-2 is an antiapoptotic protein, it is predicted that the increased Bcl-2 protein levels in the PPARγ-transfected cells would increase their resistance to apoptosis. HCA-7 cells that had been transiently transfected with pSG5-xPPARγ or the empty vector pSG5 were treated with deoxycholic acid, an agent known to cause apoptosis in colon cancer cells [266]. Based on a morphological assessment, cells which had been transfected with PPARγ underwent approximately half as much deoxycholic acid-induced apoptosis as control transfected cells (Figure 16).

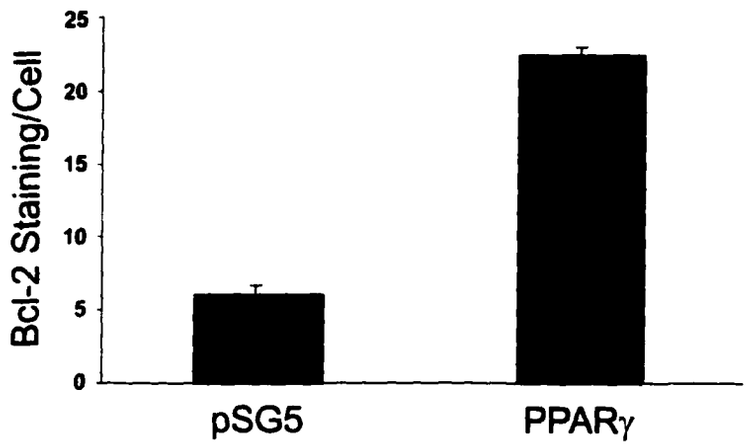


Figure 15: HCA-7 cells transiently transfected with PPARγ have higher levels of Bcl-2 protein per cell than the empty-vector-transfected cells. Bcl-2 levels were analyzed by confocal analysis of cells after 24 hours of transfection and 24 hours of recovery. Grayscale quantitation of multiple confocal images was performed on three separate experiments (see materials and methods). The results are expressed as mean ± S.E.M.

Discussion

The results shown here are the first direct evidence of PPARγ-mediated transcriptional regulation of an apoptosis-related gene, *bcl-2*. Although the promoter region of *bcl-2* is characterized [131, 135], very few response elements for specific transcription factors are described as capable of modulating the transcription of *bcl-2*. Likewise, the role of PPARs in carcinogenesis has not been clearly defined. Some reports demonstrate that PPARs induce differentiation [267] and apoptosis [268], while others report a decrease in apoptosis [251, 252, 269]. The data reported here provide a direct link between PPARs and apoptosis that may help to define the role of PPARs in carcinogenesis.

Genes that are regulated by PPARs contain a sequence for binding PPAR/RXR heterodimers. A sequence in the 3' untranslated region of the human *bcl-2* gene was identified which had 75% (15/20 basepairs) homology to the consensus PPAR response element. It is noteworthy that similar sequences are found in the murine *bcl-2* and the nematode homolog, *ced-9*; such conservation across species suggests functional significance. EMSA experiments demonstrated that PPAR/RXR heterodimers were able to bind the *bcl-2* PPRE-containing oligonucleotides. Of the three subtypes, PPARγ exhibited the strongest binding. When the *bcl-2* PPRE was used to modulate expression of a luciferase reporter gene, co-transfection of PPARγ caused 3.6-fold increase in luciferase activity compared to co-transfection of the empty expression vector. This

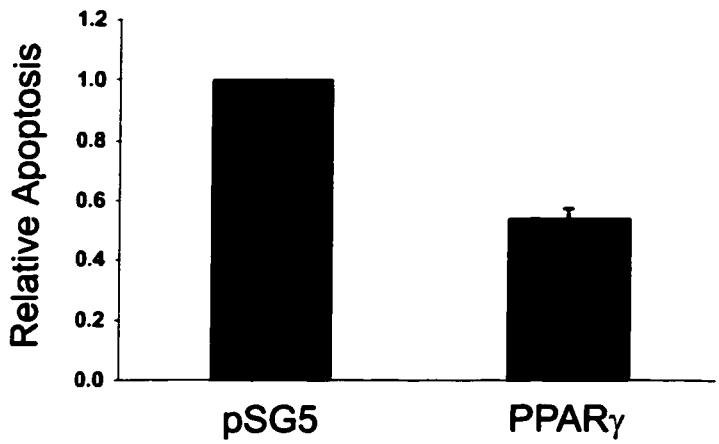


Figure 16: HCA-7 cells expressing PPARγ are more resistant to deoxycholic acid-induced apoptosis than cells transfected with the empty vector pSG5. 24 h after transient transfection of PPARγ or pSG5, cells were exposed for 5.5 h to deoxycholic acid to induce apoptosis. Morphological assessment of apoptosis was performed on fixed and stained cells. The results are expressed relative to apoptosis in pSG5-transfected cells (mean ± S.E.M.; n = 3).

correlated well with the EMSA data in confirming that PPARγ is the subtype most likely to enhance transcription from the *bcl-2* PPRE.

PPAR subtype specificity is determined by several variables. These include the specific sequence of the PPRE, surrounding sequences and the availability of ligands, cofactors and repressors. Previous studies by Juge-Aubry et al. [260] compared the PPAR subtypes for their ability to bind the PPREs in sixteen different genes. Similar to our results, these authors found that PPARγ showed stronger binding than the other subtypes in EMSA experiments. Although PPARα and PPARβ could bind the *bcl-2* PPRE as seen by the EMSAs, they did not increase luciferase transcription when the *bcl-2* PPRE was placed next to a minimal promoter driving luciferase expression. Since increased luciferase activity was not observed with the addition of an exogenous PPARα ligand, ligand availability does not appear to explain the observed specificity for PPARγ in these assays. One recently discovered protein, PPARγ coactivtor-1 (PGC-1), has been proposed to regulate PPAR subtype specificity. Interactions between PGC-1 and an as yet uncharacterized repressor [270] inhibits PPARα transactivation, presumably by interfering with PPARα/PGC-1 interactions. It is therefore possible that these or similar proteins exist in HCA-7 cells or colonic tissue *in vivo*, enabling PPAR subtypes to preferentially upregulate transcription in a tissue- or cell-specific manner, depending on the availability of such repressors or coactivators.

Bile acids can induce apoptosis in colon cells [266] and it has been postulated that resistance to bile acid-induced apoptosis plays a causal role in colon carcinogenesis [271]. In this model, high fat diets, which have been correlated to increased risk of colon

cancer [272], cause an increase in bile acid production [273, 274]. Increased exposure of the colonic mucosa to bile acids increases the probability that apoptosis-resistant cells are selected for survival. Since bile acid is thought to induce apoptosis partially through DNA damage [266], any apoptosis-resistant cell risks accumulating additional DNA damage which may result in further progression to malignancy. Our results indicate that colon cells may develop resistance to bile acid-induced apoptosis as a consequence of PPARγ upregulating *bcl-2* transcription.

Our findings suggest a mechanism for how elevated COX-2 expression as seen in colorectal cancers [211, 275] promotes carcinogenesis. The function of COX-2 is to convert arachadonic acid to prostaglandin H, from which all other prostaglandins are synthesized. Importantly, prostaglandin metabolites appear to be PPAR ligands [254, 255, 276, 277]. Human colon tissue [278] and colon cancers [279] express PPARγ. The observation of Bcl-2 overexpression in colon cancers [280] could be explained by downstream metabolites of COX-2 acting as PPARγ ligands and enabling the upregulation of *bcl-2* through its PPRE by PPARγ. Sheng et al. [253] have demonstrated that the addition of prostaglandin E_2 to colon cancer cells causes an increase in Bcl-2 protein levels and a resistance to apoptosis. Moreover, when rat intestinal epithelial cells overexpressing COX-2 are induced to differentiate with a physiologically relevant agent (sodium butyrate), higher levels of Bcl-2 protein and less apoptosis are observed, compared to cells transfected with antisense COX-2 [281]. Upregulation of Bcl-2 in colon cells *in vivo* may make the cells resistant to bile acid-induced apoptosis. Additional new research points to the intriguing possibility that bile acid exposure itself

may increase COX-2 activity [282]. Thus, bile acids may trigger a cycle of resistance in colon cells which contain sufficient levels of PPARγ and necessary coactivators. Increased COX-2 activity in the cells could produce PPARγ ligand(s), that enable increased Bcl-2 production. From this point, resistance to death could allow for further genetic changes that would ultimately lead to a carcinoma.

Concluding Statements

Apoptosis is a necessary safeguard which helps to prevent cancer formation. Should severe DNA damage occur without subsequent death of the damaged cell, mutations in key genes may enable the apoptosis-resistant cell to further propagate. In later stages of tumorigenesis, resistance to apoptosis continues to benefit the cancer cell. For example, loss of attachment from extracellular matrix should trigger apoptosis. Ignoring such a signal allows cells to migrate and invade to distant sites. The emergence of chemotherapy-resistant cells is often accomplished by overcoming the apoptotic signals induced by treatment. Understanding the molecular mechanisms employed by cancer cells to overcome apoptotic stimuli is necessary to develop effective prevention and treatment strategies. The overall goal of the research presented in this dissertation was to investigate mechanisms of apoptosis resistance.

The elevated levels of ROS and increased resistance to apoptosis observed in many tumors led us to the hypothesis that increased basal levels of ROS may affect apoptosis resistance. The research presented in Chapter 2 supports this hypothesis by demonstrating that the two malignant keratinocyte cell lines are more resistant to UV-induced apoptosis than the benign parental cell line, which also had lower basal ROS. One goal of the research presented here was to determine the cause of elevated ROS which led to increased resistance in the malignant variants. A common defect identified in both cell lines was a decrease in catalase message and enzyme activity. Given the differing methods by which 6M90 and 6R90 cells were created from 308 cells, the fact that both had a diminution of catalase activity is strong circumstantial evidence for a

central role of catalase in prevention of tumorigenesis. Kevin Kwei, a graduate student in Dr. Tim Bowden's lab, has measured catalase levels in during progression of mouse skin tumors. His experiments have demonstrated a correlation between decreased catalase protein and progression from papillomas to squamous cell carcinomas. This *in vivo* work further supports the idea that catalase may be an important tumor suppressor gene.

A decrease in message of the μ class of glutathione-S-transferase was also observed in both malignant cell lines compared to the benign parental line. This enzyme detoxifies xenobiotic compounds and repairs oxidatively damaged molecules in the cell. Because some oxidatively damaged lipids can cause further oxidative stress, a reduction in μ class GST levels may contribute to elevated levels of ROS. Additional research examining levels of the α and π GST isoforms may be informative, since they detoxify other endogenous oxidation products. This may also uncover the molecular mechanisms to account for the observations in Chapter 3 that the malignant variants were more sensitive to certain DNA-damaging drugs, given the role of GSTs in detoxifying xenobiotic compounds.

The research presented in Chapter 2 demonstrates that attenuating ROS levels either pharmacologically or genetically increases sensitivity to apoptosis. Further experiments implicated Akt signaling as a molecular mechanism for this resistance pathway. Elevated ROS have previously been shown to increase Akt signaling, but much of the previous work employed acute doses of ROS to stimulate Akt. Recent data from in vivo studies of tumorigenesis in mouse skin [222] further supports the role of Akt in tumorigenesis and indirectly points to the role of ROS in upregulating Akt. By examining

Akt phosphorylation (indicative of activation) in a model system which mimics the chronically elevated ROS observed in cancer, the research presented here is unique and perhaps more relevant to tumorigenesis than previous studies of Akt during acute oxidative stress. Figure 17 shows a schematic of the pathway elucidated in this research which implicates chronic ROS mediating survival.

Because attenuation of ROS decreased Akt phosphorylation and reversed apoptosis resistance, it is concluded that this signaling pathway is active in the malignant variants. Further research will be needed to examine the downstream targets of Akt phosphorylation which ultimately lead to apoptosis resistance. Preliminary data not presented here indicates that Bad, which is a known target of Akt phosphorylation, is not differentially phosphorylated in the 6M90 and 6R90 cells. Other known substrates of Akt phosphorylation such as members of the Forkhead transcription factor family or caspase 9 should be examined for differential phosphorylation within the variants. Additionally, more research will be needed to uncover how elevated ROS are sensed and how cells upregulate Akt in response to elevated ROS. Akt is activated by phosphorylation and the tumor suppressor phosphatase PTEN negatively regulates Akt activation. Examination of these upstream regulators of Akt may help to further elucidate the mechanism by which ROS affects Akt, leading to apoptosis resistance.

A second mechanism of apoptosis resistance, that of transcriptional regulation of the antiapoptotic *bcl-2* gene was also examined. Bcl-2 is overexpressed in many cancers, but in many cases the mechanism of overexpression is not known. Additionally, PPAR nuclear receptors have been implicated in apoptosis resistance and are observed to be

Figure 17: Proposed model for the contribution of chronic ROS to increased survival. A chronically elevated level of ROS within cells may be obtained through a loss of anti-oxidant defenses, an increase in pro-oxidant enzyme activities or a combination thereof. In addition to previously described elevated proliferative capacity afforded by chronic ROS levels, the research presented here indicates that chronic ROS causes the pro-survival Akt kinase to be constitutively phosphorylated. This continued survival signaling allows cells to resist apoptosis. Therefore, reducing steady state levels of ROS (by antioxidant treatment or catalase re-expression) inhibits this survival pathway, causing diminished Akt phosphorylation ultimately leading to decreased survival.

elevated in some malignancies, but the means by which they prevent apoptosis was not yet fully delineated. These observations led to the hypothesis that PPAR regulates *bcl-2* expression. The research presented in Chapter 4 indicates that an element found in 3' UTR of *bcl-2* is responsive to PPAR regulation, leading to elevated Bcl-2 protein levels and ultimately increased apoptosis resistance.

PPAR-mediated resistance to apoptosis may be particularly relevant in colon cancers. Literature suggests that prostaglandins may act as ligands for PPARγ; this is the subunit which I found to be most effective in binding the *bcl-2* response element. The findings presented in Chapter 4 provide a mechanism to explain the observations that many colon cancers have elevated levels of Bcl-2, PPARs and COX-2 (see Figure 18). It is possible that elevated COX-2 activity produces prostaglandins which act as ligands for the PPARs in colon cancer cells, leading to transcriptional upregulation of *bcl-2*. Further research will be needed to determine if prostaglandins specifically enhance *bcl-2* regulation through PPARs. The benefit of NSAIDs or other COX-2 inhibitors in preventing colon cancer have previously focused on decreased inflammation as a result of COX-2 inhibition [283]. The results of the research reported here suggest that decreased apoptosis resistance may be an additional benefit of COX-2 inhibition. Our study is the first to demonstrate that PPARs regulate an anti-apoptotic gene. Previously-characterized PPAR-responsive genes have not been related to apoptosis. Because PPARs are transcription factors, additional research may reveal that other apoptotic-related genes are coordinately regulated by this nuclear receptor, in a manner analogous to the simultaneous regulation of many genes by the glucocorticoid receptor.

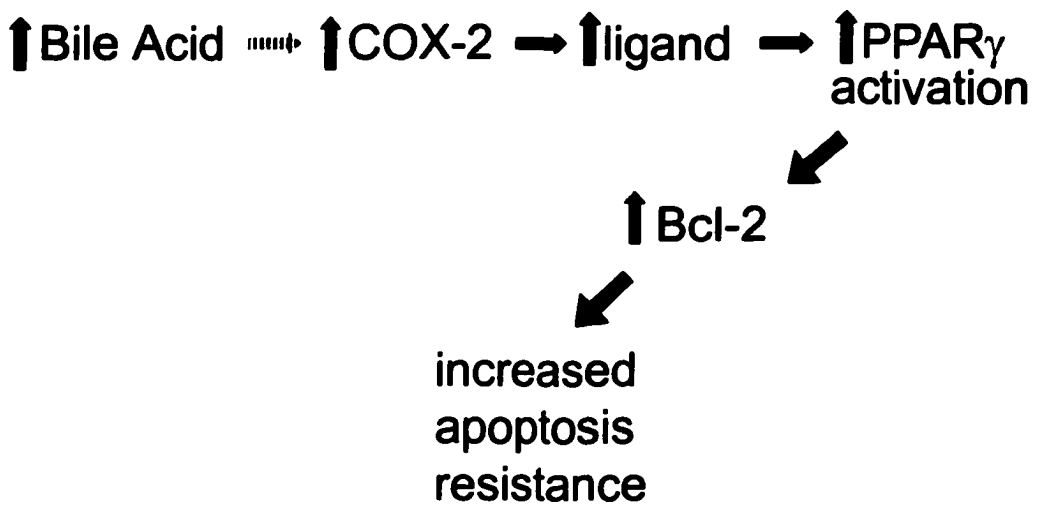


Figure 18: Proposed model of apoptosis resistance in colon cells. Previous research has implicated increased COX-2 activity in survival. Other studies demonstrate that a downstream metabolite of prostaglandin may be a ligand for PPARγ. The research presented here shows that a PPRE is present within the Bcl-2 gene which is responsive to PPARγ. Thus, if a subset of colon cells have elevations in both PPARγ and COX-2, *bcl-2* transcription can increase because both the nuclear receptor and its ligand are present. Constitutively elevated COX-2 may not be necessary, since exposure of colon cells to apoptosis-inducing bile acid causes an elevation in COX-2 activity. This would lead to increases in the PPAR ligand at a time when cells most require it, in order to mount a survival response to the bile acid. These studies may provide a molecular explanation for the observation that some cells chronically exposed to bile acid become resistant to apoptosis; an important step in colon carcinogenesis.

Taken together, the body of work presented in this dissertation describes two mechanisms by which cancer cells may increase their resistance to apoptosis. In addition to expanding our knowledge of how cancer cells can evade apoptosis, these findings could lead to more successful and directed approaches for treating apoptosis-resistant tumors.

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