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Review Article

"Prognostic Significance of *Bax* and *Bcl-2* Gene Expression and Their Ratio in Carcinogenesis"

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Abstract

The ability of malignant cells to escape from apoptosis is a hallmark of cancer; cancer cells show several characteristics that would readily stimulate apoptosis in normal cells-such as, they violate checkpoints of cell cycle as well as withstanding the exposure to cytotoxic agents; Because of these characteristics, cancer cells tend to survive. Apoptosis is an effective barrier to developing cancer; avoiding apoptosis is important to development of tumor and resistance to cancer therapy. Members of bcl-2 family regarded as important mediators of apoptosis in health and disease, often shown to be deregulated in tumor and lead to the survival of malignant clones. Bax and Bcl-2 are the main members of bcl-2 family that have critical role in cancer progression and inhibition of intrinsic apoptotic pathway stimulated by mitochondrial dysfunction. The balance of pro- (Bax) and anti-apoptotic (Bcl-2) genes can determine the fate of malignant cell. Bcl-2 protein family is the hallmark of apoptosis regulation and disturbance of apoptosis signaling pathways plays critical role in carcinogenesis. Members of Bcl-2 genes were found to be differentially expressed in many types of malignancies. The Bcl-2 protein family is possibly correlated to cancer pathophysiology and resistance to conventional chemotherapy, through its role in regulation of apoptosis. The aim

of study was to explain the role of Bax/Bcl-2 Ratio in tumor progression and the effect of its disturbance in carcinogenesis.

Introduction

Apoptosis is a process through which eukaryotic cells commit suicide. It leads to elimination of unwanted and defective cells through an orderly process of cellular disintegration that has the advantage of not inducing undesirable inflammation [1]. Elimination of apoptotic cells can occur during normal development and turnover, in addition to pathological conditions; moreover; improper regulation of apoptosis leads to disorders such as cancer, autoimmune diseases, and neurodegenerative disorders [2].

The word "apoptosis" is of Greek origin, meaning "dropping off" or "falling off" of petals from flowers or leaves from trees. The term "apoptosis" was first introduced by Kerr *et al.* [3]. It is an active, programmed cell death that can be stimulated or inhibited by group of environmental stimuli, both physiological and pathological. This phenomenon was first described by Carl Vogt, more than 100 years ago in 1842 [4].

Apoptosis is typically affected through a family of cysteine proteases known as caspases [5]. There are two major known pathways of apoptosis induction; the extrinsic pathway and the intrinsic pathway. The extrinsic pathway is dependent on the interaction of a death ligand (such as FasL or TNFα) with a corresponding 'death receptor' on the cell surface, whereas the intrinsic pathway can be activated by various stress or damage stimuli to the cells. Once activated, the pathways lead to the activation of caspase-8 or -9 (extrinsic or intrinsic pathway, respectively), which in turn process and activate the effector caspases-3, 6 and 7 [6].

Effector caspases are responsible for initiating the hallmarks of the degradation phase of apoptosis; including DNA fragmentation, cell shrinkage and membrane blebbing [7,8]. Other characteristics of apoptosis include, mitochondrial remodeling, production of ROS and breakage of several proteins, however caspases role in these processes is not fully understood [8-11]. It has previously shown that intrinsic apoptotic pathway, activation of caspase-9 and effector caspases have critical effects on mitochondria. Caspase-9 can prevent accessibility of cytochrome c to complex III in the mitochondria, resulting in increased ROS production, but in the presence of effector caspase activity, ROS production is terminated [12]. These data showed that a possible feedback loop on the mitochondria after release of cytochrome c and activation of caspase, previous studies demonstrated that caspase-8 divides Bid into tBid, that can remodel the mitochondria, but tBid role in intrinsic apoptotic pathway has not been determined [9]. Also, previous studies have shown that caspase-9 is a highly specific protease that cause cleavage of only few proteins, while caspase-3 and caspase-7 are responsible for the majority of protein cleavage that occurs during apoptosis, but the distinct roles of each caspase is not determined [13]. Based upon cleavage- specificity criteria of caspase-3 and caspase-7, it was showed that these caspases were critically redundant compared to substrate cleavage during apoptotic cell death [14,15]. But, current studies shows that caspase-3 and caspase-7 must have critical functions as mice that are deficient in these caspases have effective phenotypes as well as caspase-7 and caspase-3 posse differential activity toward natural, synthetic and purified substrates [16,17].

Morphological Changes in the Cell During Apoptosis

Morphological changes of apoptosis are greatly similar across cell types and species for both nucleus and cytoplasm [18].

The process from the initiation of cell death to the final cellular fragmentation takes several hours. However, the time taken depends on several factors include apoptotic pathway, stimulus and the cell type [19].

Morphological alterations of apoptosis that occur in the nucleus are nuclear fragmentation and, chromatin condensation which are accompanied by pyknosis (reduction in nuclear volume), rounding up of the cell, and retraction of pseudopods [20]. Chromatin condensation begins at the margin of the nuclear membrane, forming a crescent or ring-like structure. Further condensation of the chromatin continues till it divides inside the cell with an intact plasma membrane, a character called karyorrhexis [21]. Throughout the total process, the plasma membrane still intact while at the later stage of apoptosis, morphological alterations of the membrane occur, including ultrastructural alteration of organelles inside cytoplasm , membrane blebbing and a loss of membrane integrity [20].The reason that apoptosis was discovered very late in the history of cell biology in 1972 and *in vitro*, apoptotic bodies are seen only under special conditions is that phagocytic cells engulf apoptotic cells before apoptotic bodies formation. If the remnants of apoptotic cells are not phagocytosed as in artificial cell culture, they will undergo degradation, described as secondary necrosis [19]

Biochemical Changes in the Cell During Apoptosis

Three main types of biochemical alterations occurred in apoptosis include: 1) Caspases activation, 2) breakdown of DNA & protein and 3) membrane alterations and recognition by phagocytic cells [22]. Early in apoptosis, there is phosphatidylserine (PS) expression which has been "flipped out" from the inner layers to outer layers of the cell membrane so early recognition of dead cells by macrophages that result in phagocytosis without the release of proinflammatory cellular components [23], accompanied by DNA breakdown into large 50 to 300 kilobase pieces [24]. Later, endonucleases cleave internucleosomal DNA into multiples of 180 to 200 base pairs of oligonucleosomes. This feature can be seen in necrotic cells as well as in apoptotic cell [25].

Another feature of apoptotic cell death is activation of group of caspases enzymes that one of cysteine protease family. The "c" of "caspase" abbreviated to a cysteine protease, and the "aspase" abbreviated to the enzyme's property to divide aspartic acid residues [22]. Activated caspases cleave vital cellular proteins and degrade the nuclear scaffold and cytoskeleton, as well as activation of DNAase, which further break up nuclear DNA [26]. Moreover, the biochemical changes demonstrate in part the morphological alterations of apoptosis. It is noted that apoptosis can occur without oligonucleosomal DNA fragmentation and can be caspase-independent. Therefore, biochemical analyses of DNA fragmentation or caspase activation should not be used to define apoptosis [27].

Defects in apoptosis mechanism play important roles in tumor pathogenesis, increasing lifespan of neoplastic cells, subverting the need for exogenous survival factors and giving protection from oxidative stress and hypoxia as the tumor mass expands so give chance for accumulation of genetic changes during tumor

progression that interfere with cell proliferation, deregulate differentiation, promote angiogenesis, and increase tumor invasiveness [28].

Defects in the regulation of apoptosis represent one of the molecular mechanisms involved in carcinogenesis that contribute to the pathogenesis and progression of cancer. Dysregulation of oncogenes and tumor suppressor genes involved in apoptosis has been correlated to tumor development and progression [29].

Apoptosis regulatory genes include the Bcl-2 gene family, which codes for both proapoptotic and antiapoptotic proteins such as Bcl-2, Bax, Bcl-x, Bad, Bak, and others. The Bcl-2 oncogene encodes a 26-kDa protein that the expression of which is topographically restricted to cells in proliferating zones and cells with long lifespan, and is downregulated in terminally differentiating cells. In normal stratified squamous epithelia, Bcl-2 expression has been found only in the basal cell layer or in all tissue layers (Fig.1) [29].

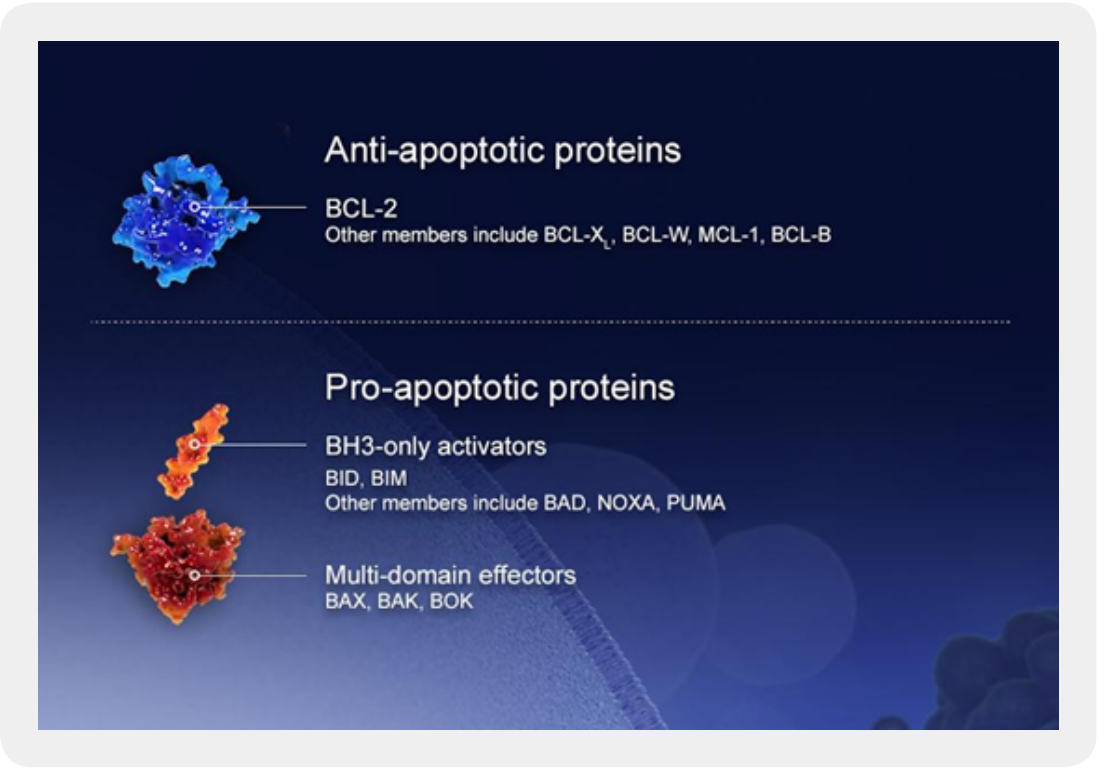

Figure 1: *The BCL-2 family [31,32]*

Tumor cells use different molecular mechanisms to suppress apoptosis that include resistance to apoptosis by the expression of antiapoptotic proteins such as B cell lymphoma 2 (Bcl-2) or by the downregulation or mutation of proapoptotic proteins such as Bcl-2- associated X protein (Bax). Some forms of human B-cell lymphoma have Bcl-2 overexpression as the expression of both Bcl-2 and Bax is regulated by the p53 tumor suppressor gene. So that example represents the strongest line of evidence that failure of cell death contributes to cancer [28].

The Bcl-2 family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members such as Bcl-2 and Bcl-XL, prevent apoptotic cell death through preventing the release of mitochondrial apoptogenic factors as cytochrome c and AIF (apoptosis- inducing factor) into the cytoplasm and through sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome). Cytochrome c and AIF, after entering the cytoplasm, they directly stimulate caspases that degrade group of cellular proteins to cause apoptotic alterations. In contrary, pro-apoptotic members of this family, such as Bax and Bak, activate the release of caspases from death antagonists via heterodimerization and also acting on mitochondrial permeability transition pore lead to stimulating release of mitochondrial apoptogenic factors into the cytoplasm, thereby leading to activation of caspase. Thus, the Bcl-2 family of proteins acts as an important life-death decision point within the common pathway of apoptotic cell death [30].

Bax is a 21-kDa protein that forms homodimers or heterodimers with Bcl-2. Apoptosis depends on the ratio of these two proteins, because it is promoted by Bax and inhibited by the Bax/Bcl-2 heterodimer [29].

Bax and Bcl-2 represent the major members of Bcl-2 family whose potential roles in tumor progression and prognosis of different human malignancies have been of interest during the last decade in various studies. Bax activates apoptosis in response to different cellular stresses; through permeabilization of mitochondrial outer membrane. In contrast, Bcl-2 prevents apoptosis by inhibiting the activity of Bax [33]. Colorectal cancerous cells showed absence of Bax expression that stimulates resistance to apoptosis induced by different chemotherapeutic agents [34].

Bcl-2 and Bax are two typical proteins of the Bcl family that restrain and promote apoptosis through regulating mitochondrial function, mitochondrial membrane permeability and cytochrome-c (cyt-c) release [35,36]. These proteins acting by insertion into membranes of organelles, influence membrane permeability, serve as docking sites for other proteins, and interact with other proteins including other Bcl-2 family members [37].

Location of Bcl-2 is in the nuclear, endoplasmic reticulum and mitochondrial membranes while Bax "the family members that promote apoptotic cell death", are located in the cytoplasm. With apoptosis stimulation, Bax translocate to the mitochondria, this leads to decrease the mitochondrial membrane potential that directly or indirectly increases the mitochondrial membrane permeability. The apoptotic factors found in the mitochondrial intermembrane space are released into the cytoplasm then enter the nucleus where they bind to DNA, resulting in nuclear condensation, fragmentation of DNA and stimulation of the mitochondrial caspase-independent apoptosis pathway (Fig.2) [38]. While Bcl-2 activates the barrier function of the mitochondrial membrane and prevent the transfer of apoptosis-inducing factors into the nucleus [39]. So, Bcl-2 family play important role in promoting or inhibiting intrinsic apoptotic pathway controlled by mitochondrial dysfunction [40,41].

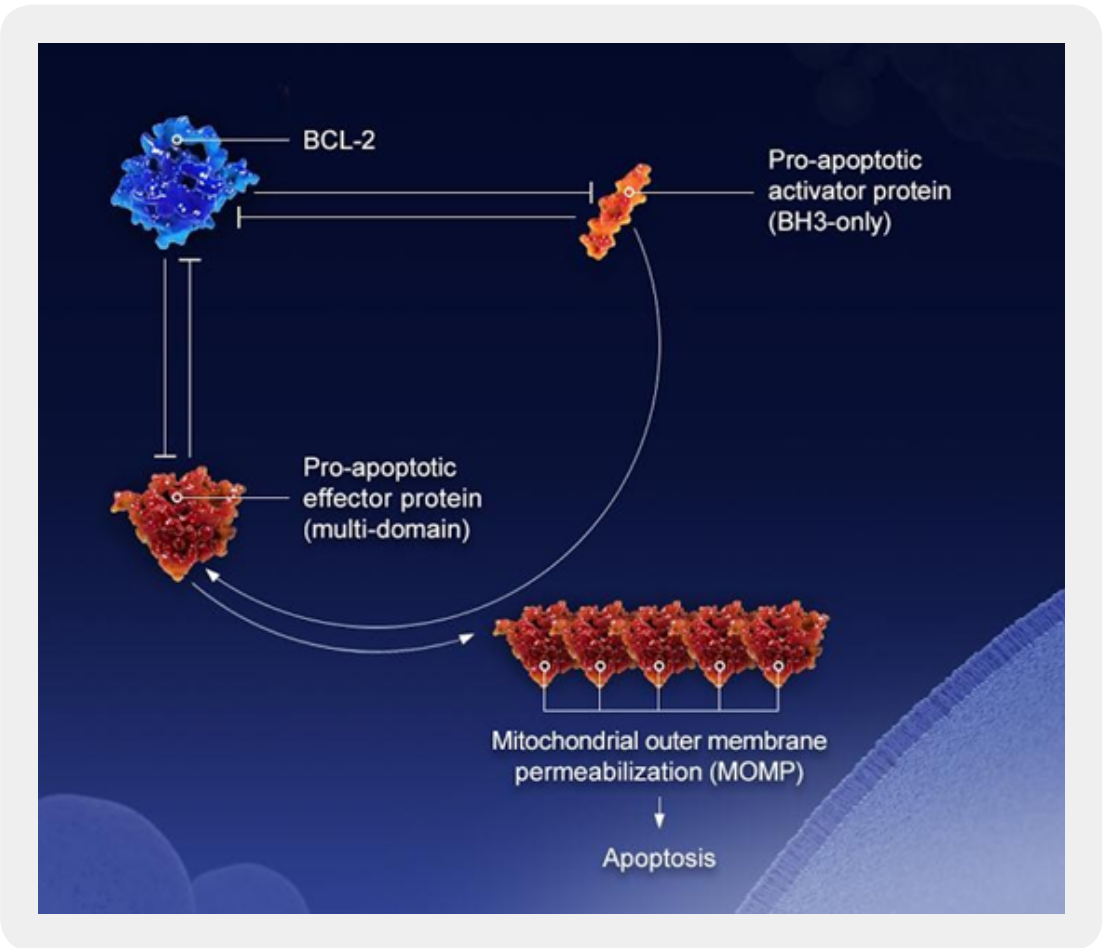

Figure 2: *Functional role of BCL-2 [42]*

Bcl-2 family control apoptosis, with the Bax/Bcl-2 ratio acting as a rheostat to determine the susceptibility of cells to apoptotic cell death, the importance and the correlation of the Bax/Bcl-2 or the Bcl-2/Bax ratio with prognosis of several diseases or malignant tumors have been proven [43].

Cell and mitochondrial membranes damage accompanied with release of cyt-c and Bax gene can enhance the apoptotic process through induction of the ratio of Bax: Bcl-2 protein expression and the cleavage of pro-caspase-3 to active caspase-3, which is a key step of apoptosis. The gene expression profiles of Bax and Bcl-2 have been reported to play a crucial role in apoptotic response mediated by many agents [44]. The expression of the anti-apoptotic protein Bcl-2 is up regulated by the transcription factor NF-kB and down regulated by p53. In contrast, the level of pro-apoptotic Bax is transcriptionally up regulated by p53 [45]. Over expression of Bcl-2 gene has been reported in malignancies and several solid tumors including oral cancer. Bcl-2 has been documented to prolong cell survival and may inhibit apoptosis by inhibiting the release of cyt-c from mitochondria and may promote tumor development [46].

The importance of p53 gene can be drawn from the fact that this gene is reported to be mutated in 80% of all human malignancies, because of its role in regulation of cell cycle; alterations in p53 are critical events in carcinogenesis. Alteration of the p53 gene and high frequency of p53 expression were detected in a number of human solid tumors including oral cancer [47].

Overexpression of Bcl-2 has been reported to protect tumor cells from apoptosis whereas increased Bax expression promotes apoptosis induced by cytotoxic drugs and radiation. Induction of apoptotic process by p53 through up regulation of Bax and down-regulation of Bcl-2 has been suggested to determine death of cells following an apoptotic stimulus. Imbalance in Bax/Bcl-2 ratio leads to malignant transformation and pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins control the fate of damaged cells [48].

Regarding for tumors with altered Bax/Bcl-2 Ratio, previous studies have showed Bax/Bcl-2 ratio as a predictive marker, using western blot analysis, Lee *et al*. showed that Bax/Bcl-2 ratio indicated the cellular radiosensitivity of some cell lines in pancreatic cancerous cells [49].

Similarly, an association was found between Bax/Bcl-2 ratio and clinical response to chemoradiotherapy in bladder cancer patients based on immunohistochemistry [50]. Significant correlation between Bax/Bcl-2 ratio determined before radiation therapy and clinical response in breast cancerous patients acting as biomarker to determine radiosensitive individuals [51]. Bax and Bcl-2 expression was most predictive of outcome when combined as Bax/Bcl-2 expression ratio in colorectal tumors compared to expression levels of Bax and/or Bcl-2 genes alone [52].

How we could direct Bax/Bcl-2 Ratio in cancer therapy; Given numerous reports underlining reduced expression of Bax and increased expression of Bcl-2 in many drug-resistant tumor cells and recent reports showing the ability of 4-aryl-4H-chromenes family to induce procaspase-9 cleavage and chromene compounds induce mitochondrial apoptotic pathway that might be mediated through the Bcl-2 and Bax proteins [53].

Various anticancer agents that induce apoptosis of cancer cells involve the participation of the pro-apoptotic protein Bax. Examples include Hsp90 inhibitor 17-AAG [54] and histone deacetylase (HDAC) inhibitors [55], Hsp90 inhibitors activate p53-dependent apoptosis that mediated by Puma and Bax [56], Cells lacking Bax and Bak prevent apoptosis mediated by HDAC inhibitors [57], Human colorectal cancer cells that lack functional Bax genes are partially resistant to the apoptotic effects of chemotherapeutic agent 5-fluofouracil, and totally remove the apoptotic response to chemopreventive agent sulindac and other nonsteroidal antiinflammatory drugs [58].

Since the molecular cloning of Bcl-2 by Korsmeyer *et al*. [59], there has been tremendous progress in identifying Bcl-2 family members as targets for drug development. In the last 20 years, the anti-apoptotic properties of Bcl-2 were discovered, the overexpression of Bcl-2 conferring chemoresistance was demonstrated and the 3-dimentional protein structure of Bcl-XL was reported, which led to the development of protein inhibitors [60]. The first agent targeting Bcl-2 that entered clinical trials is a Bcl-2 antisense (oblimersen sodium), which has shown chemosensitizing effects when combined with conventional chemotherapy drugs in chronic lymphocytic leukemia patients, leading to improved survival, More recent advances include the

discovery of small molecule inhibitors of the Bcl-2 family proteins. They are designed to bind the hydrophobic groove of anti-apoptotic Bcl-2 proteins in place of BH3-only proteins (i.e., BH3-mimetics). They can oligomerize Bax or Bak that can subsequently depolarize mitochondrial membrane potential leading to release cyto-c., many agents targeting the Bcl-2 proteins family have been developed, and three of these have entered clinical trials [61].

Many agents have been identified or designed to target the Bcl-2 family at the mRNA or protein level. Pharmacologic and cellular aspects of agents targeting the Bcl-2 family should be considered when exploring their potential application as chemotherapy. The binding affinity for inhibiting the anti-apoptotic Bcl-2 family members, a large and redundant family of proteins, should optimally be in the clinically achievable concentrations for each agent. Agents with high specificity give good opportunities for cancer cell drug resistance, while broader acting agents provide unexpected responses to the agents and increase systemic toxicities. There have been six anti-apoptotic Bcl-2 family members identified and several of these seem to contribute to drug resistance in cancer cells, suggesting that inhibition of multiple Bcl-2 family members will be necessary to achieve optimal therapeutic effect. One approach to enhancing the therapeutic effect of the inhibitors of anti-apoptotic Bcl-2 family members would be to use a combination of drugs that target different Bcl-2 family members [62].

To date, one Bcl-2 antisense and three small molecule Bcl-2 protein inhibitors are being tested in clinical trials. Preclinical studies seem promising, especially in combination with additional chemotherapy agents. Ongoing clinical trials to determine the effect of single agents and drug combinations will define the direction of future clinical development of the Bcl-2 inhibitors.

Conclusion

The mitochondrial-mediated pathway of apoptosis is regulated by the Bcl-2 family of antiapoptotic (Bcl-2) and proapoptotic proteins (Bax), The Bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with Bax/bak. High Bax level considered as a good prognostic indicator while Bcl-2 overexpression has been correlated with drug resistance in malignancies. Bax/Bcl-2 ratio has more important role than either promoter alone in controlling apoptosis.

This biologic ratio is feasible and reliable and is a potentially useful prognostic indicator for tailoring the intensity of treatment. This astonishing prognostic effect interprets and confirms the great role of mitochondrial apoptotic proteins, such as Bax and Bcl-2, both in sensitivity to chemotherapy and future strategies for smashing multidrug resistance in malignant tumors.

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