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Induction of Autophagic Cell Death by Targeting Bcl-2 as a Novel Therapeutic Strategy in Breast Cancer

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1. Introduction

Breast cancer is the second leading cause of tumor related death in women in Western countries. It has been estimated in recent reports that this year about 207.000 women will be diagnosed with breast cancer and about 40.000 will die of it in the US. (Jemal et al., 2010). While 5 year survival in early stages is about 90 %, it is as low as 15 % in metastatic stage. Despite the fact that there are many agents to treat the breast cancer, most of the tumors ultimately become unresponsive to these systemic therapeutics (Alvarez et al., 2010). Therefore new therapeutic strategies either alone or in combination with conventional therapies are required to improve the survival rates of breast cancer patients.

2. Autophagy

Autophagy (Greek, meaning self eating) is an evolutionary conserved process whereby damaged or excess organelles, long-lived or unfolded proteins, protein aggregates and invasive microbes are subjected to lysosomal degradation. There are three autophagy types, macroautophagy (autophagy from now on in this chapter) takes cytoplasmic components to the lysosome in a double-membrane vesicle (autophagosome); micro-autophagy, lysosome itself takes the cytoplasmic material inside by invagination of lysosome membrane, and in chaperone mediated autophagy the proteins are targeted to the lysosome by a chaperone protein such as Hsp-70 which is recognized by lysosome membrane proteins (Glick et al., 2010). Autophagy includes some other selective autophagy types such as mitophagy, autophagy for mitochondria, reticulophagy for endoplasmic reticulum), pexophagy for peroxisome, ribophagy for ribosome, aggrephagy for protein aggregates (Beau et al., 2008). Autophagy starts from endoplasmic reticulum as phagophore and expands towards the cytoplasmic content and forms autophagosome, then autophagosome fuses with lysosome and forms autophagolysosome (Klionsky & Emr, 2000). Autophagy takes place in most cells at a basal level to eliminate protein aggregates and damaged organelles, therefore, to maintain the cellular homeostasis (Scarlatti et al., 2009).

2.1 Autophagy as a survival pathway

Autophagy is induced by nutrient or growth factor deprivation, hypoxia, oxidative stress, radiation, hormonal and chemotherapeutic agents (He & Klionsky, 2009). During nutrient

deprivation, autophagy produces energy from the recycling of the organelles or the proteins and prevents the cells to undergo apoptosis by sequestering mitochondria and preventing cytochrome c release - this protective role of autophagy is not limited to apoptosis but also extends to necrosis (Scarlatti et al., 2009). In the tumor hypoxic and acidic microenvironment, autophagy helps the cells to survive. Numerous anticancer agents such as doxorubicin, temozolomide, etoposide, arsenic trioxide, histone deacetylase inhibitors as well as TNF alpha, IFN gamma, imatinib, rapamycin, anti-estrogen tamoxifen and radiation therapy have also been shown to induce autophagy in various human cancer cells as a protective mechanism (Dalby et al.). But, if the stress leads to continuous or excessively induced autophagy, autophagic cell death may occur. Autophagic cell death (programmed cell death type II) is independent of caspase activation and DNA laddering presenting in apoptotic cell death. (Shen & Codogno). But it occurs depending on the nature of stimuli, cell type, presence or activation other autophagy related factors (Shen & Codogno). It has been shown that bax/bak double knockout mice embryonic fibroblasts which are resistant to apoptosis, could undergo autophagic cell death after starvation, etoposide and radiotherapy (Moretti et al., 2007). These studies suggests that autophagic cell death has been induced by growth-factor deprivation and cytosine arabinoside in sympathetic neurons (Xue et al., 1999), etoposide and staurosporine in mouse embryonic fibroblasts (Shimizu et al., 2004), rottlerin in pancreatic cancer cells (Akar et al., 2007), zoledronic acid in prostate cancer cell lines (Lin et al.). Although there is no marker for autophagic cell death, it can be shown, indirectly, by reduced cell death after inhibition of autophagy in two different ways such as pharmacological inhibition (for example 3-methyladenine) or siRNA based silencing of the autophagy genes such as Beclin-1, ATG5, ATG8.

2.2 Autophagy as tumor suppressor mechanism

Recently, autophagy has been shown to function as a tumor suppressor mechanism. Liang et al. reported that one of the autophagy promoting genes, Beclin-1, could inhibit tumorigenesis and is expressed at lower levels in human breast cancers and they suggested that low expression of autophagy proteins may play a role in the development or progression of breast and other malignancies (Liang et al., 1999). Yue et al. reported that Beclin-1 haploinsufficient mice had higher incidence and different spectrum of tumor formation including B cell lymphomas, hepatocellular carcinomas, lung adenocarcinoma (Yue et al., 2003). They showed that tumors were also larger in Beclin-1^{+/-} mice than wild type ones, suggesting that the tumors developed at an earlier age. It has also been shown that heterozygous disruption of beclin-1 resulted in cell proliferation in vivo suggesting beclin-1 as a tumor suppressor (Qu et al., 2003). It has been suggested that autophagy cleans up unwanted proteins to keep genomic stability (Mathew et al., 2007) and prevent the cells to transform into malignant cells. Therefore, the induction of autophagy may help to reverse the malignant phenotype.

2.3 Autophagy as a pro-death and type II programmed cell death mechanism

Apoptosis (programmed cell death-type I, PCD-type I) and necrosis are well known mechanism of cell death induced by anticancer therapies (Dalby et al, 2010). Until recently, apoptosis was a synonym for programmed cell death and thought to be the major mechanism of cell death in response to chemo- and radiotherapy. However, emerging studies have demonstrated the existence of a non-apoptotic form of programmed death called autophagic cell death, which is now considered as a PCD-type II. In contrast to apoptosis, autophagic cell death, in general, is caspase-independent and does not involve

classic DNA laddering and believed to be a result of an extensive autophagic degradation of intracellular content (Lockshin RA, Zakeri Z, 2007). Studies showed that cytotoxic signals can induce autophagy in cells that are resistant to apoptosis (apoptosis defective), such as those expressing high Bcl-2 or Bcl-XL, those lacking Bax and Bak or those being exposed to pan-caspase inhibitors, such as zVAD-fmk (Shimizu et al, 2004). Proapoptotic Bcl-2 family member proteins, *Bak* and *Bax*, regulate intrinsic apoptotic pathway by causing mitochondrial outer membrane permeabilization and cytochrome c release. Bax and Bak (-/-) knockout fibroblast cells have been shown to be resistant to apoptosis and undergo an autophagic cell death after the induction of death, following starvation, growth factor withdrawal, chemotherapy (etoposide) or radiation (Moretti et al, 2007). The evidence suggests that autophagy leads to cell death in response to several compounds, including rottlerin (Akar et al, 2007) cytosine arabinoside (Xue et al, 1999), etoposide and staurosporine as well as growth factor deprivation (Xue et al, 1999). A link between autophagy and related autophagic cell death has been demonstrated using pharmacological (e.g. 3-MA) and genetic (silencing of *ATG5*, *ATG7* and *Beclin-1*) approaches for suppression of autophagy. For example, the knockdown of *ATG5* or *Beclin-1* in cancer cells containing defects in apoptosis lead to a marked reduction in autophagic cell death (and autophagic response) in response to cell death stimuli with no sign of apoptosis (Akar et al, 2007). Studies also suggest that apoptosis and autophagy are linked by effector proteins (e.g., Bcl-2, Bcl-XL, Mcl-1, *ATG5*, p53) and common pathways (e.g., PI3K/Akt/mTOR, NF- κ B, ERK) (Akar et al, 2007; Yousefi et al, 2006, Shimizu S et al, 2004; Akar U et al 2008). Overall, there is evidence that autophagy may function as a type II PCD in cancer cells in which apoptosis is defective or hard to induce. Therefore it is reasonable to propose the notion that the induction of autophagic cell death may be used as a therapeutic strategy to treat cancer (Dalby et al, 2010, Ozpolat et al 2007).

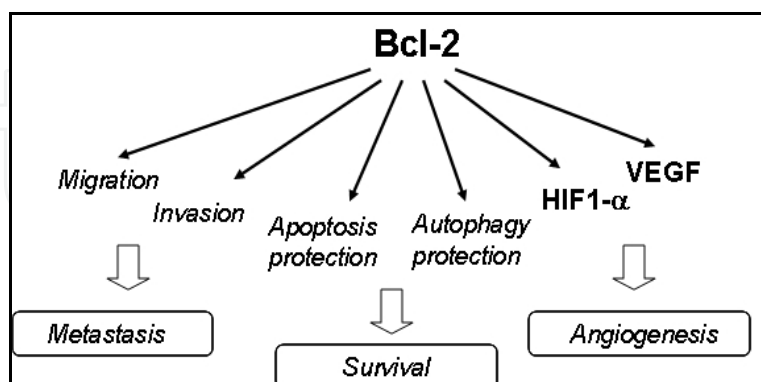
2.4 Targeting autophagy as a novel cancer therapy

Autophagy can be used as a new therapeutic strategy either by inducing the autophagic cell death or inhibiting protective autophagy depending on the context (Dalby et al, 2010). Apoptosis defects such as a lack of caspase 3 or apoptosis resistance such as having overexpression of anti apoptotic proteins lead to resistance to chemotherapy, radiotherapy or some other anticancer agents. Up regulation of the expression of several antiapoptotic Bcl-2 family protein members, including Bcl-2, Bcl-XL, prevents cell to undergo apoptosis induced by death ligands or chemotherapeutic drugs (Bardeesy & DePinho, 2002; Simoes-Wust et al., 2002) Either the induction or the inhibition of autophagy can provide therapeutic benefits to patients and that the design and synthesis of modulators of autophagy may provide novel therapeutic tools and may ultimately lead to new therapeutic strategies in cancer. Defects in apoptosis leads to increased resistance to chemotherapy, radiotherapy, some anticancer agents and targeted therapies. Therefore, induction of autophagic cell death may be an ideal approach in those cancers that are resistant to apoptosis by anticancer therapies (e.g., chemotherapy, radiation). As explained in the previous section cancer cells can undergo autophagic cell death when their apoptosis is inhibited, or they are resistant to therapy-induced apoptosis (e.g. in response to DNA-damaging agents such as etoposide), suggesting that autophagic cell death can be induced as an alternative cell death mechanism when cells fail to undergo apoptosis. Therefore, induction of autophagic cell death may serve as a novel therapeutic tool to eliminate cancer cells with defective apoptosis, which is the case in many advanced, drug resistant and metastatic cancers (Dalby et al, 2010). We have recently

demonstrated that the inhibition of some protein kinases (e.g., PKC δ in pancreatic cancer) or the targeting of key proteins that are involved in the suppression of autophagy (e.g. Bcl-2, TG2) can trigger autophagic cell death without any other treatment (Akar et al., 2008; Akar et al., 2007; Ozpolat et al., 2007). On the other hand, because a number of cancer therapies, such as radiation therapy, chemotherapy and targeted therapies (e.g. imatinib) induce autophagy as a protective resistance mechanism against anticancer therapies for cancer cell survival, the inhibition of autophagy can be used to enhance the efficacy of anticancer therapies.

3. Bcl-2

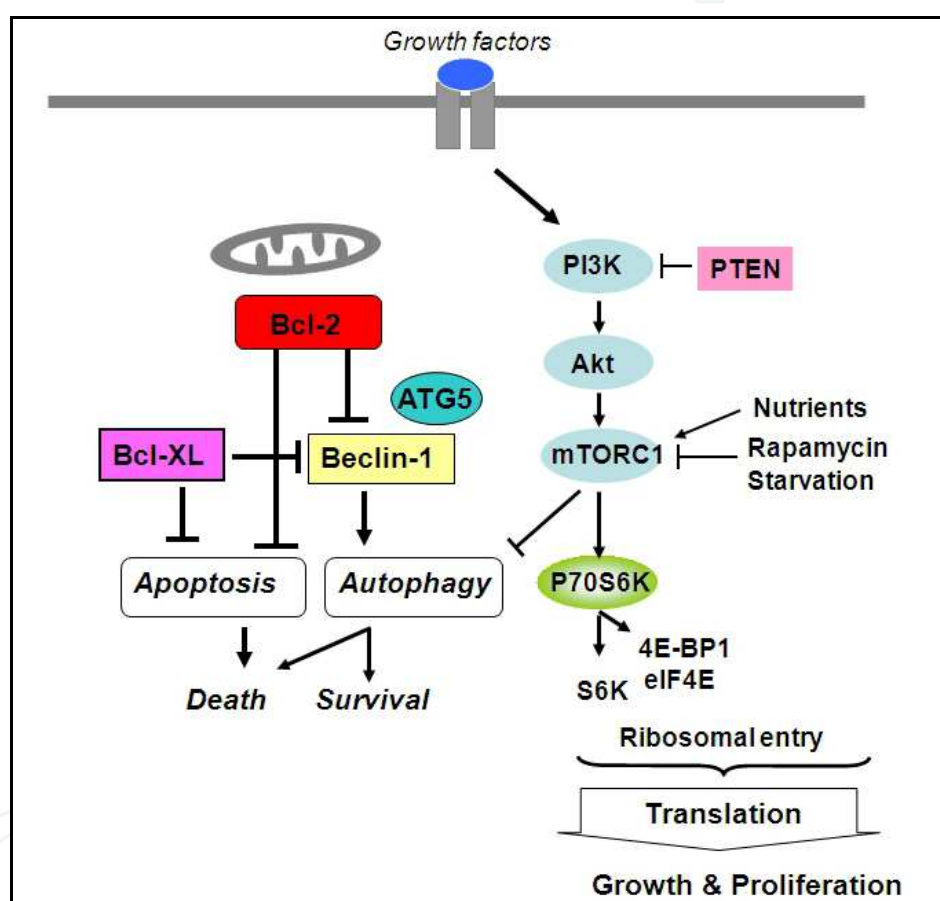
The Bcl-2 gene encodes a 26-kDa Bcl-2 proto-oncogene is overexpressed in 40-80 % of breast cancer patients and more than half of all human cancers (Hellemans et al., 1995; Oh et al.2011). Bcl-2 is a gene family consisting of several anti-apoptotic (such as Bcl-2, Bcl-XL, Mcl-1) and pro-apoptotic members (such as Bax, Bak, puma). The balance between pro- and anti-apoptotic proteins determines the cell's fate, to survive or die. Although some studies suggest that enhanced Bcl-2 expression is associated with improved survival in human colon cancer (Buglioni et al., 1999; Meterissian et al., 2001) and breast cancer studies (Cheng et al., 2004). The role of Bcl-2 in cancer cells was shown to be related to its ability to promote the tumorigenesis through interfering with apoptosis and autophagy (Reed et al, 1995, Oh et al.2011). It has been demonstrated that Bcl-2 overexpression leads to an aggressive tumor phenotype in patients with a variety of cancers as well as to the resistance of cancer cells against chemotherapy, radiation, and hormone therapy (Bishop, 1991; Reed, 1995). Recently, Buchholz et al, found 61% of the breast cancer patients treated at MD Anderson Cancer Center were Bcl-2 positive and they had a poor response to chemotherapy compared to those had less Bcl-2 expression (Buchholz et al., 2005). Figure below summarizes the novel functions of Bcl-2 in cancer cells, including metastasis, survival and tumor progression, these functions will be explained in the following sections. Overall, Bcl-2 over expression confers drug resistance, an aggressive clinical course, and poor survival in patients (Patel et al., 2009; Pusztai et al., 2004).



3.1 Bcl-2 as an inhibitor of apoptosis and autophagy

Bcl-2 family proteins work in pairs with their proapoptotic counterparts for example Bcl-2 heterodimerize with BAX, Bcl-XL with BAK. Proapoptotic members of this family mostly localized to cytosol. Following a death signal the proapoptotic members undergo a conformational change that enables them to target and integrate into membranes,

particularly mitochondrial outer membrane (Gross et al., 1999). But anti-apoptotic Bcl-2 is predominantly a mitochondrial protein, and it can prevent mitochondrial changes which take place with apoptosis, including loss of mitochondria membrane potential, release of mitochondria proteins cytochrome c and apoptosis-inducing factor (AIF), and opening of the mitochondria permeability transition pore which is a large conductance pore that evolves in mitochondria after necrotic and apoptotic signals then cytochrome c is released and caspase 9 and 3 are activated (Gross et al., 1999). Therefore, downregulation of Bcl-2 leads to induction of apoptosis, reduction of the apoptotic threshold. Tormo et al. have shown the induction of apoptosis by lipid incorporated Bcl-2 antisense in transformed follicular lymphoma cells (Tormo et al., 1998). An siRNA based inhibition of Bcl-2 is also increased apoptosis in MCF7 breast cancer cells (Lima et al., 2004).



Regulation of autophagy and apoptosis through the crosstalk between Bcl-2 and Bcl-XL may determine the predominant response to anticancer therapies.

Recently, Pattingre et al., have reported that stable transfection of Bcl-2 in HT29 colon carcinoma cells inhibited starvation induced autophagy, decreased the association of beclin-1 and Vps34 and magnitude of beclin-1 associated class III phosphoinositol-3- kinase activity (Pattingre & Levine, 2006). The proposed mechanism is that Beclin-1 has a BH3 domain that is required to bind to Bcl-2 and bcl-XL for Bcl-2 mediated inhibition of autophagy (Boya & Kroemer, 2009) (Please see the Figure)(Dalby et al, 2010). It has been shown that the pharmacological BH3 mimetic ABT737 competitively inhibited the interaction between Beclin-1 and Bcl-2/Bcl-XL, antagonized autophagy inhibition by Bcl-2/Bcl-XL and hence

stimulated autophagy (Maiuri et al., 2007). A recent study demonstrated that anti-autophagic property of Bcl-2 is a key feature of Bcl-2-mediated tumorigenesis (Oh et al., 2011). MCF7 cells expressing Bcl-2 mutant defective in apoptosis inhibition but competent for autophagy suppression grew *in vitro* and *in vivo* as efficiently as wild-type Bcl-2. The growth-promoting activity of this Bcl-2 mutant is strongly correlated with its suppression of autophagy in xenograft tumors, suggesting that oncogenic effect of Bcl-2 arises from its ability to inhibit autophagy but not apoptosis.

Recent studies also suggested that silencing of Bcl-2 by siRNA induced autophagic cell death (up to 55%) in estrogen receptor (+) MCF7 breast cancer cell line, but not apoptotic cell death (Akar et al., 2008). An increase in autophagy with increased number of punctates in GFP-LC3 transfected cells, increased LC3-II formation and acridine orange accumulation in autophagosomes as well as induction of autophagy genes (e.g., *ATG5* and *BECN1*) were observed in response to Bcl-2 silencing. We further blocked autophagy with ATG5 siRNA - autophagy related gene 5- and inhibition of ATG5 significantly blocked Bcl-2-siRNA-induced cell killing, suggesting the autophagic cell death (Akar et al., 2008). Bcl-2 mediated-autophagic cell death pathway induction is most likely related to MCF-7 cells caspase 3-deficiency thus, presenting a higher threshold for the induction of apoptosis, additionally; we reported that doxorubicin induces autophagy and apoptosis. These findings led to the hypothesis that apoptosis resistant cancer cells can be killed by autophagic cell death as an alternative death mechanism and this strategy may be used as a therapeutic intervention for targeted silencing of genes for induction of autophagic cell death. It is important to recognize the conditions and genetic make up of the cells in order for the induction of autophagic cell death. Furthermore, doxorubicin at a high dose (IC₉₅) induced apoptosis but at a low dose (IC₅₀) induced only autophagy and Beclin-1 expression. In addition, when combined with chemotherapy (doxorubicin), therapeutic targeting of Bcl-2 by siRNA induced significant growth inhibition (83%) and autophagy in about 80% of the MCF-7 breast cancer cells (Akar et al., 2008). We also found that *in vivo* targeted silencing of Bcl-2 by systemically administrated nanoliposomal Bcl-2 siRNA induced autophagy and tumor growth inhibition in mice bearing MDA-MB231 tumors (Tekedereli et al, in press). These results provided the first evidence that targeted silencing of Bcl-2 induces autophagic cell death in breast cancer cells and that Bcl-2 siRNA may be used as a therapeutic strategy alone or in combination with chemotherapy in breast cancer cells that overexpress Bcl-2.

3.2 Bcl-2 induces cell proliferation and cell cycle progression

We have shown that silencing Bcl-2 decreased the clonogenicity and induced cell proliferation inhibition either alone or in combination with doxorubicin, which is a widely used anti-cancer agent, in estrogen receptor (+) MCF7 breast cancer cell line (Akar et al., 2008). We did not observe growth inhibition in Bcl-2-negative MDA-MB-453 cells after treatment with the siRNA, suggesting that Bcl-2 siRNA specifically inhibits growth of Bcl-2-overexpressing breast cancer cells (Akar et al., 2008). We also showed that Bcl-2 knockdown inhibited clonogenicity and cell proliferation in estrogen receptor (-) MDA-MB-231 cells (unpublished data). Emi et al. reported 50-70 % proliferation inhibition by Bcl-2 antisense oligonucleotides (ASO) in BT-474 and ZR-75-1 breast cancer cells. They also showed that pretreatment with bcl-2 antisense led to 2.5 to 10 fold increase in sensitivity to chemotherapy with either doxorubicin, mitomycin C, docetaxel or paclitaxel in MDA-MB-231, BT-474 and ZR-75-1 breast cancer cell lines *in vitro* (Emi et al., 2005). Inhibition of bcl-2 expression by

ASO has been shown to inhibit colony formation in AML progenitor cells (Konopleva et al., 2000). Inhibition of bcl-2 by ASO led the cells to arrest in G1 phase of cell cycle in PC3 prostate cancer cell line (Anai et al., 2007). Some other recent studies in breast cancer experimental models have also demonstrated that in vitro and in vivo downregulation of Bcl-2 by ASO enhanced the sensitivity to chemotherapeutic drugs, such as doxorubicin, paclitaxel, mitomycin C and cyclophosphamide suggesting the downregulation of Bcl-2 may be a useful strategy to prevent drug resistance and enhance chemosensitivity (Emi et al., 2005; Tanabe et al., 2003). In melanoma, lymphoma and breast cancer xenografts pretreatment with Bcl-2 antisense enhanced antitumor activity of various chemotherapeutic agents such as cyclophosphamide, dacarbazine and docetaxel (Nahta & Esteva, 2003). George et al., reported that bcl-2 siRNA combine with taxol (100 nM) increased the apoptotic cells in Tunel assay up to 70 % when compared to 30 % in taxol alone (100 nM) group in human glioma cells (George et al., 2009).

There are several conflicting studies on the effect of Bcl-2 on cell proliferation. Huang et al. (Huang et al., 1997) have shown that mutated Bcl-2 on BH4 domain which didn't interfere the ability of Bcl-2 to inhibit apoptosis led starved quiescent cells to enter the cell cycle much faster than wild type protein expressing cells on stimulation with cytokine or serum. It has also been suggested that whereas Bcl-2 deficiency caused the accelerated cell cycle progression, increased levels of Bcl-2 led to retarded G0 to S transition in T cells (Linette et al., 1996). It has also been reported that Bcl-2 delayed cell cycle progression by regulating S phase in ovarian carcinoma cells (Belanger et al., 2005). On the other hand, it has been reported that downregulation of Bcl-2 expression by anti-sense oligonucleotide did not change prostate cancer cells proliferation (Anai et al., 2007). Lima et al. have reported that inhibition of Bcl-2 by siRNA led to a decrease in viable cells when compared to control group. However, they further analyzed the cells with BrdU proliferation assay and there was no significant difference between the groups, concluding that decreased cell number was due to the spontaneous induction of apoptosis in MCF7 breast cancer cell line (Lima et al., 2004). Holle et al., used A T7 promoter driven siRNA expression vector system that targets Bcl-2 mRNA in MCF-7 human cancer cells, and inhibition of Bcl-2 expression inhibited cell proliferation and induced apoptosis (Holle et al., 2004).

3.3 Bcl-2 induces angiogenesis and metastasis

Recent studies suggested that Bcl-2 plays roles in metastasis, angiogenesis and autophagy. It is now established that angiogenesis plays an important role in the growth of solid and hematological tumors. Bcl-2 has been shown to induce VEGF expression, which plays a main role of in angiogenesis by regulating differentiation, migration and proliferation of endothelial cells by interacting with its receptors. Moreover, VEGF has also been recently shown to be a survival factor for both endothelial and tumor cells, preventing apoptosis through the induction of Bcl-2 expression (Biroccio et al., 2000; Fernandez et al., 2001; Iervolino et al., 2002; Nor et al., 2001). Anai et al (Anai et al., 2007) recently showed for the first time that knock-down of Bcl-2 by ASO leads to inhibition of angiogenesis in human prostate tumor xenografts. Bcl-2 ASO decreases rates of angiogenesis and proliferation by inducing G₁ cell cycle arrest and apoptosis. This was the first study which shows that therapy directed at Bcl-2 affects tumor vasculature. An increase in angiogenic potential of tumor cells after Bcl-2 transfection was also observed using different in vivo assays. In addition, Bcl-2 overexpression increases VEGF promoter activity through the HIF-1 α transcription factor and transcription factors (Iervolino et al., 2002). Indeed, Bcl-2 increases

nuclear factor κ B (NF- κ B) transcriptional activity in the MCF7 ADR line (Ricca et al., 2000). Since NF- κ B signaling blockade has been demonstrated to inhibit in vitro and in vivo expression of VEGF, it is possible that Bcl-2 affects VEGF expression through modulation of the activity of NF- κ B or other transcription factors.

Bcl-2 overexpression increases the metastatic potential of MCF7 ADR breast cancer cell line by inducing cellular invasion, and migration, in vitro and in vivo (Del Bufalo et al., 1997; Ricca et al., 2000). It has also been shown that bcl-2 involves in tumorigenicity, invasion, migration, and metastasis of different tumors (Takaoka et al., 1997; Wick et al., 1998). In glioma cell lines, bcl-2 expression has been shown to correlate with matrix metalloproteinase-2 (MMP-2) therefore the invasiveness (Wick et al., 1998). On the other hand, the in vivo aggressiveness of tumors derived from cells overexpressing Bcl-2 is much more than cells which do not. It has been attributed to the anti-apoptotic properties of Bcl-2 (Fernandez et al., 2001). Zuo et al. (Zuo et al.) demonstrated a decrease in epithelial markers such as desmoglein-3, zonula occluding-1, cytokeratin and E-Cadherin and a increase in mesenchymal markers such as N-Cadherin, vimentin, fibronectin and also a transition from a cobblestone to a scattered appearance with increased bcl-2 expression. Therefore, they suggested that bcl-2 overexpression induced epithelial to mesenchymal transition and enhanced mobility and invasive character of HSC-3 human squamous carcinoma cells by promoting persistent ERK signaling and elevating MMP-9 production. Wang et al. have shown that a bcl-2 small inhibitor, TW-37, led to increased apoptosis, decreased MMP-9 and VEGF gene transcriptions and their activities consequently inhibited tumor growth in a pancreatic cancer model (Wang et al., 2008). It has been shown that bcl-2 upregulation in tumor associated endothelial cells was sufficient to enhance tumor progression in vivo (Nor et al., 2001). The same group also showed that bcl-2 expression was significantly elevated in tumor blood vessels from head and neck cancer patients as compared to control samples and when they compared bcl-2 expression in tumor blood vessels from lymph node-positive cancer patients with lymph-node negative patients, they found that lymph node-positive cancer patients had significantly higher number of bcl-2 positive blood vessels (Kumar et al., 2008). They showed in human head and neck cancer specimens that bcl-2 expression in tumor associated endothelial cells was directly linked to metastasis, they further found in an in vivo SCID mouse model that tumors with bcl-2 expressing endothelial cells showed significant increase in lung metastasis suggesting bcl-2 expression mediated metastasis through increase in angiogenesis, tumor cell invasion and blood vessel leakiness (Kumar et al., 2008).

4. Bcl-2 as a candidate for targeted therapy in breast cancers

Bcl-2 anti-apoptotic and anti-autophagic protein has been proposed as an excellent therapeutic target in various cancers to overcome resistance to conventional therapies and enhance the effects of these therapies. Previous studies suggested that downregulation of bcl-2 by ASO enhances their sensitivity to chemotherapeutic drugs, such as doxorubicin, paclitaxel, mitomycin C and cyclophosphamide in breast cancer experimental models, suggesting a downregulation of Bcl-2 may be a useful strategy to prevent drug resistance and enhance-chemosensitivity (Emi et al., 2005; Tanabe et al., 2003). Bcl-2 specific ASO (Oblimersen) in clinical studies have ended up somewhat disappointing results and toxicity (Tanabe et al, 2003). SiRNA has been shown to be 10 to 100-fold more potent than ASO and causes its degradation, leading to shut down protein expression (Bertrand et al, 2002). *In*

in vivo efficient delivery of the siRNA-based therapeutics into tumors, remains a great challenge. Traditionally cationic (positively charged) liposomes have been used as nonviral delivery systems for oligonucleotides (e.g., plasmid DNA, ASO, and siRNA). However, their effectiveness as potential carriers for siRNA has been limited due to the toxicity. We recently developed non-toxic, neutrally charged 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC)-based nanoliposomes (mean size 65nm) leading to significant and robust target gene knock down in human tumors animal models (Landen et al, 2005). We found that liposomal siRNA targeting Bcl-2 led to 73 % and 61 % inhibition in Bcl-2 target protein expression on 4 day and day 6, respectively in MDA-MB-231 tumors in mice (Tekedereli et al, in press), indicating that Bcl-2 siRNA therapeutics can be used successfully inhibit overexpressed proteins in *in vivo* therapeutic modality in cancer.

4.1 Bcl-2 expression in prognosis of breast cancer patients

Overexpression of Bcl-2 occurs in about 40 to 80% of human breast tumors (Doglioni et al., 1994; Hellemans et al., 1995; Joensuu et al., 1994) and confers drug resistance, an aggressive clinical course, and poor survival in patients (Reed, 1995). Recently, Buchholz et al, found that 61% of breast cancer patients are Bcl-2 positive and patients with positive Bcl-2 expression had a poor response to chemotherapy compared to those had less Bcl-2 expression (Buchholz et al., 2005). Because most Bcl-2 positive breast cancers express estrogen and/or progesterone receptors and respond hormonal therapy, Bcl-2 does not seem to be an independent prognostic marker in short-term (5 year) follow up (Joensuu et al., 1994). However, Bcl-2 fails to maintain its prognostic relationship in breast cancer when considered in multivariate analysis and long-term follow up studies (Daidone et al., 1999; Joensuu et al., 1994). Lack of Bcl-2 expression was associated with a higher probability of complete pathological response to doxorubicin-based chemotherapy (Pusztai et al., 2004). Antiestrogens such as tamoxifen and ICI 164384 promote apoptosis by downregulating Bcl-2 without affecting Bax, Bcl-X_L or p53 (Kumar et al., 2000) and Bcl-2 upregulation plays a role in resistance to estrogens (Teixeira et al., 1995). Overall, these data suggest that tumors with a decreased level of Bcl-2 had better response to chemotherapy and hormonal therapy, and targeting Bcl-2 is a viable strategy.

5. Concluding remarks

Apoptosis (type I) and Autophagic (type II) programmed cell death play crucial roles in such physiological processes as the development, homeostasis and elimination of unwanted or cancer cells. Autophagy is characterized by the sequestration of cytoplasmic contents through the formation of double-membrane vesicles (autophagosomes). Subsequently, the autophagosomes merge with lysosomes and digest the organelles, leading to cell death if its induced excessively. In contrast to apoptosis, autophagic cell death is caspase-independent and does not involve classic DNA laddering (Ng & Huang, 2005). Targeting autophagy is can be used as a therapeutic strategy where autophagy is induced as a protective mechanism or induction of autophagic cell death can also be used as a therapeutic strategy where apoptosis is defective or anti-apoptotic proteins is overexpressed.

6. References

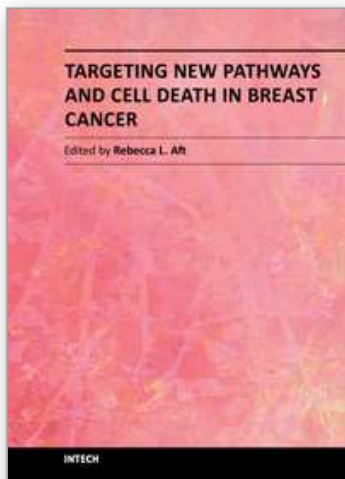
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