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Targeted Apoptosis in Breast Cancer Immunotherapy

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1. Introduction

Apoptosis is programmed and precisely regulated cell death characterized by morphological and biochemical alterations distinct from necrosis (Edinger & Thompson, 2004). The development of breast cancers, like other processes of carcinogenesis, involves uncontrolled cell proliferation and insufficient apoptosis due to either the lack of pro-apoptotic stimuli in the in vivo environment or the disturbance of cellular apoptotic pathways (Brown & Attardi, 2005). Whereas both chemotherapy and radiation caused massive apoptotic cell death in the tumor tissues, we are far from conquering the breast malignancies until the establishment of targeted pro-apoptotic therapeutic protocols or the development of apoptosis-inducing drugs that target the tumor without causing severe impairment of the normal organism (Alvarez et al, 2010; Fulda & Debatin, 2006; Motyl et al, 2006; Muschel et al, 1998). However, thanks to the elucidation of mechanisms underlying physiological and pathological apoptosis, studies have been addressed in the development of pro-apoptotic strategies targeting the cancer cells, which has provided novel approaches to the successful immunotherapy of breast cancers (Schlotter et al, 2008).

2. Unbalanced proliferation and apoptosis in breast cancers

Cells undergo consistent proliferation and apoptosis during ontogenesis and in maintenance of normal morphology and function of multiple organs. These vital behaviors of cells are regulated by requisite molecular mechanism so that they are balanced to avoid uncontrolled expanding or degeneration of certain tissues (Domingos & Steller, 2007). During carcinogenesis, however, these mechanisms were disturbed by or compromised to genetic alterations either occurring spontaneously or caused by environmental stress, resulting in over-proliferation and resistance to apoptosis (Brown & Attardi, 2005; de Bruin & Medema, 2008).

2.1 Apoptotic signaling pathways

As a process of cell death with hallmarks of morphological abnormalities, e.g. shrunken and bubbled cytoplasm, condensed nucleus, fragmented chromatin but intact membrane or organelle at the early stage, apoptosis is triggered by extracellular or intracellular stimuli,

and results from intracellular signaling thereafter, which ultimately leads to the degradation of functional proteins, destroy of cytoskeletons and fragmentation of DNA (Hengartner, 2000; He et al, 2009). The major initiators, mediators and executioners involved in apoptotic signaling have been unraveled, which contribute to a better understanding of carcinogenesis in diverse tissues, e.g. the mammary gland (Johnstone et al, 2002).

Both extrinsic and intrinsic stimuli can initiate apoptotic signaling, which involves the activation of downstream mediators in diverse pathways, and converge on the processing of cysteine-dependent aspartate-directed proteases, caspases (Hengartner, 2000). Caspases exist as proenzymes in cells, and once activated they can cleave various cellular protein substrates at consensus amino acid sites, leading the cells to apoptosis (Hengartner, 2000). So far two types of caspases have been identified: initiator (apical) caspases, e.g. caspases-2, -8, -9 and -10, and effector caspases, e.g. caspases-3, -6 and -7 (Thornberry & Lazebnik, 1998). Initiator caspases processed and activated by upstream stimuli could cleave inactive pro-forms of effector caspases, and effector caspases in turn cleave other protein substrates including divergent proteins maintaining normal cell structure, metabolism and physiological function, e.g. poly ADP-ribose polymerase (PARP) involved in DNA repair, lamin A protein of the cytoskeleton, and the DNA-fragmentation factor DFF45 (Riedl & Shi, 2004; Timmer & Salvesen, 2007).

2.1.1 Extrinsic pathway: Death receptor-mediated signaling

Death receptors are a class of transmembrane receptors that, once engaged by their ligands, initiate intracellular signaling resulting in cell death. These receptors belong to a tumor-necrosis factor receptor (TNFR) superfamily binding to a homotrimeric TNF protein family, among which Fas ligand (FasL or CD95L)/Fas have been well-documented (Lavrik, 2005). As a type II transmembrane protein, FasL binds and induces the trimerization of Fas, which in turn recruits the adaptor molecule Fas-associated death domain (FADD) via interaction between their death domains (DD). FADD also contains a death effector domain (DED), which aggregates and activates another DED-containing protein, FADD-like interleukin-1 β -converting enzyme (FLICE)/caspase-8. This is followed by a cascade of caspase activation and ultimately the cleavage of various protein substrates and apoptosis of the cell (Houston & O'Connell, 2004; Wajant, 2002).

In addition to FasL/Fas, other death receptors and ligands have also been found to play vital roles in mediating apoptotic signaling (Houston & O'Connell, 2004; Wajant, 2002). Of note are TNF/TNFR and TRAIL/TRAIL-R1, each of which triggers specific signaling pathways, thus resulting in apoptosis in a variety of cell types or physiological or pathological processes (Baud & Karin, 2001; Gonzalez & Ashkenazi, 2010). Upon activated by TNF, TNFR1 interacts with various death domain-containing proteins, forming a complex comprising TRADD, TNF Receptor Associated Factor-2 (TRAF2), cellular inhibitor of apoptosis-1 (CIAP1), and the receptor-interacting protein-1 (RIP1). The complex then recruits I-kappaB-kinase (IKK) and releases and activates Nuclear Factor-KappaB (NF- κ B), which actually promotes cell survival (Baud & Karin, 2001; Shen & Pervaiz, 2006). However, in a following step, the TRADD-based complex can also dissociate from the receptor and bind to FADD, which consequently causes the activation of Caspase-8 and end up with apoptosis (Baud & Karin, 2001; Shen & Pervaiz, 2006). The caspase-8 inhibitor FLIP, which is a target gene of NF- κ B, dictates the outcome of TNF signaling, i.e. whether cells continue to survive or undergo apoptosis (Hyer et al, 2006).

Growth factors (GFs) represent pro-survival stimuli counteracting the apoptotic signaling. After associating with their receptors, GFs activate PI3K (Phosphatidylinositide-3 Kinase) and subsequently Akt. Akt suppresses apoptosis via disrupting Bad inhibition of Bcl-2/Bcl-X_L (Duronio, 2008). The protein kinase C (PKC) also inhibits Bad via activation of ribosomal S6 kinases (p90RSKs) (Thimmaiah et al, 2010).

2.1.2 Intrinsic pathway: Role of mitochondrion and endoplasmic reticulum-related signaling

The intrinsic pathway of apoptosis begins when an injury, such as oncogene activation and DNA damage, occurs within the cell, or alternatively, cells are in stress, e.g. hypoxia or survival factor deprivation (Fulda & Debatin, 2006). The mitochondrion plays a crucial role in sensing and regulating intrinsic signaling pathway, in particular, by providing a platform for normal functioning of the Bcl-2 family proteins (Chipuk et al, 2010; Yip & Reed, 2008).

As a family of proteins containing one or more Bcl-2 homology (BH) domains, which share sequence homology and mediate heterodimeric interactions among different members, the Bcl-2 family proteins differentially affect mitochondrial outer membrane permeabilization, and thus can be divided as anti-apoptotic and pro-apoptotic protein subfamilies (Chipuk et al, 2010; Yip & Reed, 2008). The anti-apoptotic proteins, e.g., Bcl-2 and Bcl-X_L, are usually located on the surface of the mitochondrion and block cell death by preventing the activation and homo-oligomerization of the pro-apoptotic Bcl-2 family members. The pro-apoptotic family members, such as Bax, Bad, Bid and Bak, are often found in the cytosol and relocate to the surface of the mitochondria in response to cellular damage or stress (Chipuk et al, 2010; Yip & Reed, 2008). Consequently, an interaction between anti-apoptotic proteins and excessive pro-apoptotic proteins leads to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. The released cytochrome C interacts with Apaf-1 to recruit pro-caspase 9 into a multi-protein complex called the apoptosome, where caspase-9 is activated. The activated caspase-9 thus induces the processing of effector caspases, the degradation of diverse substrates of caspases and ultimately the morphological and biochemical changes by which apoptosis is featured (Scorrano & Korsmeyer, 2003; Inoue et al, 2009).

Other proteins released from the mitochondria include the apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (SMAC)/ Diablo, Arts and Omi/high temperature requirement protein-A2 (HTRA2). As a ubiquitous mitochondrial oxidoreductase, AIF could migrate into the nucleus, bind and cause the destruction of genomic DNA, and induce apoptosis in a caspase-independent manner (Modjtahedi, 2006), while SMAC/Diablo and HTRA2, once released from the damaged mitochondria, counteract the effect of inhibitor of Apoptosis Proteins (IAPs), which normally bind and prevent activation of Caspase-3 (Wang & Youle, 2009). The interaction between Bcl-2 family members, IAPs, SMAC and Omi/HTRA2 is central to the intrinsic apoptosis pathway (Wang & Youle, 2009).

The tumor suppressor p53 is also a sensor of cellular stress and is a critical activator of the intrinsic pathway. As a transcription factor, p53 is phosphorylated and stabilized by DNA checkpoint proteins in response to DNA damage, and transcriptionally activates pro-apoptotic proteins of Bcl-2 family, e.g. Bax and Bid, and other tumor suppressor such as PTEN, the outcome of which is cell cycle arrest to allow DNA repair, and apoptosis in cases of severe DNA damage (Manfredi, 2010; Robles & Harris, 2001). The mouse double minute-2

homolog (MDM2) protein negatively regulates p53 function by mediating the nuclear export and ubiquitination of p53 (Manfredi, 2010).

As an organelle mainly involved in correct protein folding and intracellular trafficking, the endoplasmic reticulum (ER) is highly sensitive to stresses that perturb cellular energy levels, the redox state or Ca²⁺ concentration. These ER stresses initiate unfolded protein responses (UPR), which promote cell survival and switch to pro-apoptotic signaling when the ER stress is prolonged (Rasheva & Domingos, 2009; Szegezdi et al, 2006). ER stress-induced apoptosis is a complicated process mediated by a series of specific proteins, in particular, the pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) in the initiation phase, the transcription factor C/EBP homologous protein (CHOP), growth arrest and DNA damage-inducible gene 34 (GADD34), Tribbles-related protein 3 (TRB3) and Bcl-2 family members in the commitment phase, and ultimately caspases during the execution of apoptosis (Rasheva & Domingos, 2009; Szegezdi et al, 2006).

2.2 Alterations of apoptotic signaling in breast cancer cells

Breast cancer is a malignancy with a wide spectrum of genetic alterations, phenotypic heterogeneity, and a variety of contributing etiological factors like age, family history, parity, and age of menarche or menopause (McCready et al, 2010). While breast cancers share the characteristics, e.g. deregulated proliferation and apoptosis with carcinomas of other origins, the molecular mechanisms underlying these characteristics are quite different, or even unique for certain processes of breast cancer development or metastasis. To date, several molecular markers and related signaling pathways have been revealed to play key roles in breast carcinogenesis by causing persistent proliferation and blocked apoptosis of breast epithelial cells (McCready et al, 2010).

2.2.1 Attenuated or blocked signaling in classical apoptotic pathways

The neoplastic breast epithelial cells have evolved diverse mechanisms to resist apoptosis via the extrinsic or intrinsic pathway. The downregulation of Fas or Fas ligand is found in numerous breast cancers, and is implicated in prognosis evaluation of patients with breast malignancies (Mottollese et al, 2000). Meanwhile, the expression of FasL may also be upregulated in breast cancers, which contributes to excessive apoptosis of T cells and thus serves as a mechanism of immune escape (Muschen et al, 2000). Signaling by death receptors can also be negatively regulated by overexpression of their inhibitors, e.g. the FLICE-like inhibitory proteins (FLIP) which dampens caspase-8 activation after recruited to the death-inducing signaling complex (DISC) (Rogers et al, 2007). Another inhibitor of death receptors, phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15 kDa (PED/PEA-15), has also been implicated in mediating AKT-dependent chemoresistance in human breast cancer cells (Eramo et al, 2005). As crucial regulators of mitochondrial apoptotic pathway, several Bcl-2 family members have been found aberrantly expressed or frequently mutated in breast cancers. For example, overexpression of Bcl-2 or Bcl-X_L is associated with the development or metastasis of breast carcinomas (Alireza et al, 2008; Martin et al, 2004). In contrast, the absence or inactivation of the pro-apoptotic Bcl-2 family members, such as Bax, Bid and Bim is involved in breast carcinogenesis (Sivaprasad et al, 2007; Sjöström-Mattson et al, 2009; Whelan et al, 2010). Among the caspase-recruiting adaptors, the downregulation of Apaf-1 was found to correlate with the progression of some

clinical breast adenocarcinomas (Vinothini et al, 2011). Finally, the polymorphisms and loss of function mutations within caspase genes have also been detected in breast cancers (Ghavami et al, 2009).

2.2.2 Reinforced estrogen signaling in breast carcinogenesis

As a sex hormone, estrogens exert their actions by binding to the intracellular receptors-estrogen receptors (ER)- α or ER- β . While estrogen/ER regulates growth, differentiation and homeostasis of the normal mammary gland, sustained engagement of ER with endogenous or exogenous estrogen (E2) is well established to cause breast cancer (Hayashi et al, 2003). In fact, ER-positive breast cancers account for 70% of the nearly 200,000 new cases diagnosed annually in the USA. Activated ER promotes breast cancer development via three major mechanisms: stimulation of cellular proliferation through the receptor-mediated hormonal activity, direct genotoxic effects by increasing mutation rates through a cytochrome P450-mediated metabolic activation, and induction of aneuploidy (J. Russo & I.H. Russo, 2006). Estrogen-bound ERs become activated transcription factors via induced dimerization and translocation to the nucleus. This is followed by the recognition of the estrogen-responsive element (ERE) in the 5' regulatory sequences of the target genes with the assistance of a "pioneer factor", FoxA1, and consequently the altered expression of the gene via recruitment of related transcriptional factors (Yamaguchi, 2007). A growing list of genes have proved to be the target of estrogen signaling, among which are cell cycle genes like E2F1 and cyclin D1, and those involved in cell survival and oriented differentiation. A systemic analysis suggested that estrogen/ER signaling is crucial for the regulation of genes involved in an evolutionarily conserved apoptosis pathway (Liu & Chen, 2010). It is also hypothesized that estrogen promotes the survival of ER-positive breast cancer cells mainly by suppressing the apoptotic machinery or upregulation of the anti-apoptotic molecules, e.g. Bcl-2 and Bcl-X_L (Gompel et al, 2000; Rana et al, 2010).

2.2.3 Elevated HER2 expression and signaling in breast carcinogenesis

Human epidermal growth factor receptor 2 (HER2) is a member of the avian erythroblastosis oncogene B (ErbB) protein family with alternative names ErbB2, neu, CD340 (cluster of differentiation 340) and p185. As a receptor tyrosine kinase encoded by the *ERBB2* proto-oncogene, HER2 over-expression has been found in a wide variety of cancers (Moasser, 2007). Approximately 30% of breast cancers exhibit an overexpression of HER2 due to aneuploidy or the amplification of the *ERBB2* gene. Transcriptional deregulation involving cis-acting element mutation or abnormal activation of transcription factors due to dysfunction of tumor suppressors like p53 also contribute to HER2 overexpression (Freudenberg et al, 2009; Moasser, 2007). HER2 gene amplification and over-expression are frequently detected in high-grade ductal carcinoma in situ (DCIS) and high-grade inflammatory breast cancer (IBC), but not in benign breast biopsies such as the terminal duct lobular units (TDLUs), suggesting that over-expression of HER2 usually occurs at the transition from hyperplasia to DCIS (Freudenberg et al, 2009; Moasser, 2007). HER2 overexpression in breast cancers correlates with high metastasis capacity, increased disease recurrence and worse prognosis, and are therefore routinely examined in breast cancer patients for a determination of therapeutic protocol and prediction of the treatment outcome (Eccles, 2001).

Despite its well-documented association with transformation of normal breast epithelial cells and metastasis and poor outcome of breast cancers, the detailed mechanisms underlying HER2-mediated signal events and cell behavior are far from being fully understood. Nevertheless, it is established that HER2 functions through homodimerization and more frequently forming heterodimers with other human epidermal growth factor receptors (HERs) (Moasser, 2007; Park et al, 2008). These HERs are commonly activated upon binding of a ligand in their extracellular domain, resulting in dimerization of HERs and triggering the intrinsic tyrosine kinase activity of the receptors responsible for a mutual or monodirectional phosphorylation between the dimerized HERs. The phosphorylated tyrosine-containing motif provides a docking station for intracellular signaling molecules (Moasser, 2007; Park et al, 2008). Given the existence of several tyrosine phosphorylation sites in the intracellular sites, the phosphorylation patterns are unique for a certain HER2 dimer, and thus trigger downstream signaling different from other dimers. Although none of the known HER ligands bind directly to HER2 with high affinity, heregulin, a cytokine secreted by the breast stromal cells, can activate HER2 by inducing or stabilizing heterodimers with other HER receptors. More importantly, HER2 is the preferred heterodimerization partner of other HER receptors like HER3, and strengthens their binding to a cognate ligand (Park et al, 2008).

The HER dimers containing HER2 modulate diverse signaling pathways involved in cell proliferation, apoptosis and migration. Adaptor proteins in Ras-MAPK pathway, e.g. Grb2 and Shc, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) can bind directly to the dimers, leading to prolonged signaling of both pathways (Moasser, 2007; Park et al, 2008). In addition to inducing cell over-proliferation via well-defined mechanisms like NF- κ B activation downstream of PI3K, these signaling events also efficiently inhibit apoptosis via negatively regulating tumor suppressors p53 and PTEN, and cell cycle inhibitors p21 Cip1/WAF1 and p27Kip1 (Park et al, 2008). Whereas the molecular machinery utilized by HER2 to promote cell migration and invasion remains unclear, the upregulation of the chemokine CXCR4 and thus the stromal cell-derived factor-1 (SDF-1)/CXCR4 axis are believed to play a central role in mediating metastasis of HER2-positive cancers (Li et al, 2004).

2.2.4 Other genetic alterations

It is now widely accepted that all cancers are attributed to alterations of the genomic information or gene expression (Teixeira et al, 2002; Jovanovic et al, 2010). In this regard, both germline mutations that increase the risk of carcinogenesis and somatic chromatin alterations in specific gene locus have been implicated in the development of breast cancers (Teixeira et al, 2002; Jovanovic et al, 2010). Like malignancies in other tissues, breast cancer occurs as a result of the activation of oncogenes or dysfunction of a tumor suppressor gene. In addition to the well-documented cancer-related genes, e.g. c-Myc, Ras, ATM, p53 and PTEN, accumulated data have unraveled a class of genes whose functional abnormalities are specifically associated with the development of breast carcinoma (Geyer et al, 2009; Prokopcova et al, 2007; Teixeira et al, 2002). This is exemplified by the breast cancer-susceptibility genes BRCA1 and BRCA2, the mutation of which leads to a lifetime risk of as high as 80% of developing breast cancer and accounts for 15% of total breast cancer cases. Germline mutations in the BRCA1 and BRCA2 genes result in chromosome instability and deficient repair of DNA double-strand breaks by homologous recombination. BRCA-mediated homologous recombination and DNA repair require their interaction with ataxia telangiectasia mutated gene (ATM), RAD51C, BRIP1, Checkpoint kinase 2 (CHEK2) and the

partner and localizer of BRCA2 (PALB2), the mutations of which have also been found in breast cancer development (Byrnes, 2008; Fulda & Debatin, 2006; Venkitaraman, 2009). PIK3CA, an oncogene encoding the PI3K catalytic subunit, exhibits a high frequency of gain-of-function mutations in breast cancers, leading to constitutive PI3K/AKT pathway activation in breast cancer. PIK3CA mutations have been observed in more than 30% of ER α -positive breast cancers (Cizkova et al, 2010).

3. Strategies of targeted apoptosis in breast cancer cells

Given that resistance to apoptosis is a major causative factor of breast carcinogenesis, correction of the deregulated apoptotic process or enforced induction of apoptosis will be beneficial in the treatment of breast cancers. However, an ideal apoptosis-based therapeutic protocol must be cancer cell-specific in order to avoid impairment of adjacent normal tissues or a systemic cytotoxicity of the therapeutics. This could be achieved either by targeted delivery of pro-apoptotic molecules in the cancer cells, or by strategies that confer the candidate therapeutics apoptosis-inducing activity specifically in the cancer cells (Alvarez et al, 2010).

3.1 Therapeutics that trigger apoptosis in breast cancers

3.1.1 Apoptosis-inducing chemicals

Despite a relatively late elucidation of molecular mechanisms of apoptosis, chemical drugs or radiation traditionally used for cancer therapy have proved efficient in apoptosis induction. DNA-damaging agents like doxorubicin, etoposide, cisplatin or bleomycin may induce apoptosis via both extrinsic and intrinsic apoptotic pathways (Fulda & Debatin, 2006). Treatment of patients with some of these anticancer drugs causes an increase in the expression of CD95L/FasL, which stimulates the receptor pathway in an autocrine or paracrine manner; conventional chemotherapeutic agents also trigger intrinsic apoptotic pathway by eliciting mitochondrial permeabilization (Fulda & Debatin, 2006). In addition, detailed mechanism underlying the apoptosis-inducing effect of chemicals may include the perturbations of intermediate metabolism, increased expression or activity of p53 or an apoptotic mediator, or changes in the ratios of the anti-apoptotic and pro-apoptotic Bcl-2 family members. For example, paclitaxel treatment causes the accumulation of BH3-only Bcl-2 family protein Bim and induces Bim-dependent apoptosis in epithelial tumors (Tan et al., 2005); paclitaxel also causes hyperphosphorylation and inactivation of Bcl-2, and facilitates the opening of the permeability transition (PT) pore (Ruvolo et al., 2001).

3.1.2 Apoptosis initiators, mediators and executioners

The past two decades has witnessed an increasingly clear depiction of the molecular machinery of apoptosis, which facilitated the development of strategies aiming at apoptosis induction of breast cancer cells (Brown & Attardi, 2005). Theoretically, introduction of any active molecule in the irreversible apoptotic pathway is sufficient to trigger apoptosis of cancer cells (Waxman & Schwartz, 2003). These active molecules involve extracellular cytokines or ligands representing death stimuli, e.g. FasL, tumor necrosis factor- α (TNF- α) or the TNF-related apoptosis-inducing ligand (TRAIL), and cellular mediators in the apoptotic pathway such as the pro-apoptotic members of Bcl-2 family and adaptors that link death signal sensors and caspases. Finally, introduction of apoptotic executioners, in

particular, effector caspases in cancer cells, will directly trigger apoptosis independently of upstream apoptotic signaling machinery (Ashkenazi, 2008; Fulda & Debatin, 2006).

While a simple overexpression or accumulation of the apoptotic proteins could commit killing of cancer cells, it is also common that a structural modification is needed before delivery or ectopic expression in cancer cells due to the following reasons. First, a tumoricidal dose of the pro-apoptotic protein, e.g. TNF- α , may also be very closer to a dose that causes systemic toxicity. In this case, screening from the mutated or modulated counterparts to obtain a lowerly toxic mutant is necessary (Meany et al, 2008). Second, the mediators or executioners of apoptosis, such as the Bcl-2 family members and caspases, exist as inactive zymogen or precursors in the cells, and will not trigger apoptosis unless activated (Yip & Reed, 2008). Constitutively active caspases-3 and -6 have been generated by removal of the prodomain and rearrangement of the large and small subunits (Srinivasula et al, 1998a). Active forms of Bax or Bid can be acquired by deletion of an amino-terminal domain, whereas an amino-terminal moiety of AIF is sufficient to trigger the caspase-independent apoptosis (Yu et al, 2006). Finally, strategies to generate cancer-targeted molecules are beneficial to improving the tumoricidal efficacy while alleviating the side effect, and therefore add weight to the applicability of the antitumor studies from bench to bedside (Alvarez et al, 2010).

3.1.3 Therapeutics targeting apoptosis inhibitors and growth signals

During carcinogenesis or acquiring resistance to chemotherapy, many breast epithelial cells have developed apoptosis-escaping mechanisms by upregulating a class of apoptosis inhibitors (Hyer et al, 2006; Liston et al, 2003). These involve the anti-apoptotic members of Bcl-2 family, e.g. Bcl-2 and Bcl-X_L, as well as endogenous inhibitors of caspases, e.g. the IAP family and c-FLIP proteins (Hyer et al, 2006; Liston et al, 2003). Among the IAPs, both survivin and X-linked inhibitor of apoptosis (XIAP) have been targeted in breast cancer treatment (Liston et al, 2003). Therefore, antisense oligonucleotides or small interfering RNAs (siRNAs) targeted to these inhibitors holds out great promise to counteract these inhibitors and possibly restore the apoptotic signaling in these cells (Crnkovic-Mertens, 2003; Li et al, 2006). The targeting of growth signals that counteract the cellular apoptotic machinery was also widely exploited. Of note are the monoclonal antibodies or chemical agents which target HER2, vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) (Alvarez et al, 2010; Ludwig et al, 2003). Also targeted are the heat shock proteins (HSPs), the molecular chaperones required for the stability and function of the growth factor signaling and anti-apoptotic proteins (Sánchez-Muñoz et al, 2009).

3.2 Targeted introduction of apoptosis-inducing proteins

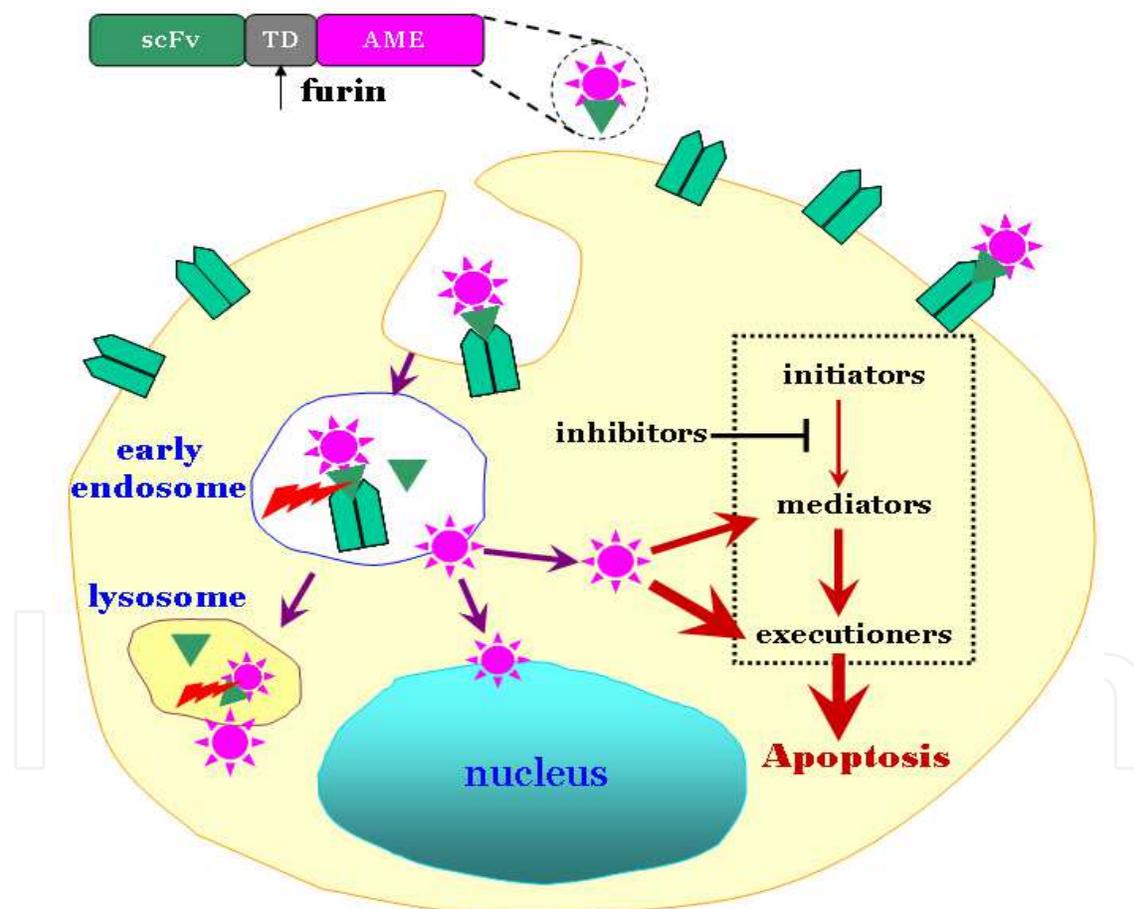
It has been an inherent challenge to selectively introduce the therapeutics or cytotoxic mechanism into the malignant cells in cancer therapy (Alvarez et al, 2010). Theoretically, targeted apoptosis induction in breast cancer cells could be achieved via two basic approaches. First, pro-apoptotic molecules could be delivered specifically into breast cancer cells. Thanks to the characteristic expression of a class of cell surface markers by breast cancer cells, antibodies that recognize these markers have been utilized to construct apoptosis-eliciting recombinant proteins, or alternatively, to generate targeted delivery system for pro-apoptotic genes (Alvarez et al, 2010). Second, the regulatory element, e.g. promoter of a gene specifically expressed in breast cancer cells could be used to control the

expression of an apoptosis-inducing gene, which results in the tumor-specific expression of the gene (Lee, 2009). Despite an overall limited clinical application of these targeted strategies, both approaches have proved effective *in vitro* or in xenograft breast cancer models (Alvarez et al, 2010; Lee, 2009).

In our study, a series of cancer-targeted pro-apoptotic fusion proteins were generated based on the active apoptosis mediators or executioners and a single chain antibody, e23sFv, which binds HER2 with high affinity (Jia et al, 2003; Xu et al, 2004; Zhao et al, 2004). These fusion proteins, designated “immunoapoptotins” after the well documented immunotoxins, consist of e23sFv in the amino terminal, a translocation domain and a constitutively active apoptotic protein (Pastan et al, 2006). The constitutively active apoptotic proteins were obtained by reversing the large and small subunits of caspases-3 or -6, or through generating a truncated form of granzyme B, AIF or the pro-apoptotic Bcl-2 family member, Bid (Figure 1; Jia et al, 2003; Qiu et al, 2008; Xu et al, 2004; Zhao et al, 2004). A translocation domain originating from a protein toxin, *Pseudomonas* Exotoxin A (PEA), was embedded in the fusion protein to mediate a translocation of the apoptotic proteins from an intracellular vesicle to the cytosol, which is required for apoptosis execution by a majority of apoptotic proteins (Jia et al, 2003; Qiu et al, 2008; Wang et al, 2010; Wang et al, 2007; Xu et al, 2004; Zhao et al, 2004). In principle, the single chain antibody mediates the recognition of HER2-overexpressing breast cancer cells and internalization of the fusion protein via endocytosis. In the endosome, the fusion protein undergoes proteolytic processing by a proprotein convertase, furin, which is enriched in the intracellular vesicles like lysosome, endosome or Golgi apparatus (Wang et al, 2010; Wang et al, 2007). This results in the generation of a carboxyl-terminal fragment encompassing the active apoptotic protein, which is released into the cytosol and triggers the apoptosis of the cells (Figure 1). Unless processed inside the cells, these recombinant proteins are nontoxic due to a steric hindrance of pro-apoptotic moiety by the N-terminal antibody and translocation domain (Wang et al, 2010; Wang et al, 2007). Unlike previously reported tumor killer proteins, such as immunotoxins, the cytotoxic activities of the immunoapoptotins are based on human endogenous proteins that kill tumor cells in an intrinsic physiologic manner, resulting in relatively weak immunogenicity and minor systemic toxicity over repeated treatments (Chen et al, 1997; Jia et al, 2003; Pastan et al, 2006).

These immunoapoptotins could be prepared by engineered bacteria or mammalian cells. Alternatively, the construct expressing the immunoapoptotins could be delivered into *in vivo* cells via a retroviral vector or non-viral systems (Jia et al, 2003; Wang, 2010; Xu et al, 2004). In addition, we also generated lymphocytes that could secrete immunoapoptotins after genetic modifications (Jia et al, 2003; Zhao et al, 2004). The apoptosis-inducing capacities of the immunoapoptotins were examined *in vitro* by incubation of the fusion proteins with the target cancer cells, coculture of the immunoapoptotin-secreting lymphoma Jurkat cells with target cells, or direct transfection of target cells with the construct of an amino-terminal signal sequence-containing immunoapoptotins, which is anticipated to exert their tumoricidal effect via an autocrine pattern (Jia et al, 2003; Qiu et al, 2008; Wang et al, 2010). As a result, the immunoapoptotins selectively induce apoptosis of HER2-positive breast cancer SKBr-3 and ovary cancer SKOv-3 cell lines, but not of those that barely express HER2. Xenograft tumor models were next generated via subcutaneous injection of the nude mice with HER2-positive or -negative breast cancer cells (Jia et al, 2003; Qiu et al, 2008; Wang et al, 2010). Intratumoral injection of the immunoapoptotin-producing adenovirus resulted in dramatic suppression of tumor growth and significantly prolonged mice

survival. In addition, intravenous administration of either the immunoapoptotin-producing adenovirus or the lymphocytes that secrete these recombinant proteins also accounted for marked tumor suppression *in vivo* (Jia et al, 2003; Qiu et al, 2008; Wang et al, 2010; Xu et al, 2004; Zhao et al, 2004). A distribution of immunoapoptotin in the tumor tissue but not other major organs was observed, consistent with the detection of redundant apoptosis specifically in the xenograft tumors (Jia et al, 2003; Wang et al, 2010; Xu et al, 2004; Zhao et al, 2004). These results indicate that the immunoapoptotins, which combines the properties of a tumor-specific antibody with the potent tumoricidal activity of apoptotic mediators or executioners, may provide novel approaches to immunotherapy or gene therapy of HER2-positive breast cancers. The genetically modified somatic cells, especially lymphocytes, are expected to suppress primary tumors and micrometastases, owing to continuous secretion of the fusion proteins and their diffusion through blood and lymph fluid. These modified cells remain viable because they are HER2-negative and the immunoapoptotins are directed to the lumen of the endoplasmic reticulum and secreted co-translationally (Jia et al, 2003; Xu et al, 2004; Zhao et al, 2004).



The “immunoapoptotin” fusion proteins consist of a single-chain antibody (scFv), a translocation domain (TD) and a constitutively active apoptosis mediator/executioner (AME). The antibody recognizes a distinguished surface marker (e.g. HER2) of breast cancer cells, and internalizes via endocytosis; the translocation domain mediates the processing of the fusion protein in the endosome or lysosome by furin to release into the cytosol a C-terminal moiety; the released moiety containing the AME triggers apoptosis of the cell independently of the endogenous upstream apoptotic machinery.

Fig. 1. Immunoapoptotins specifically induce apoptosis of breast cancer cells.

Another constituent in the immunoapoptotins that need to be addressed is the translocation domain, which is exogenous to human and thus has potentials to elicit immunological responses, resulting in either systemic toxicity or neutralization of the killer protein (Wang et al, 2010; Wang et al, 2007). To ameliorate the primary structure of the killer protein, we generated truncated variants of PEA or diphtheria toxin (DT) translocation domains to identify the minimal sequence required for furin processing and translocation (Wang et al, 2010; Wang et al, 2007). Consequently, we found that peptides encompassing the 273rd to 282nd amino acids of PEA (TRHRQPRGWE), or 187th to 196th amino acids of DT (AGNRVRRSVG) are furin-sensitive sequences, and immunoapoptotins based on these translocation sequences exhibited relatively low systemic toxicity while maintaining potent pro-apoptotic activities when applied to HER2-positive cancers (Wang et al, 2010; Wang et al, 2007). Considering that cancer specific antigens other than HER2 can also be targeted by an antibody, our immunoapoptotin strategy would have a versatile applicability in the treatment of breast cancers with distinguished cell surface biomarkers.

Estrogen receptor overexpression and estrogen-dependence are found in a majority of breast cancer cases (Ghazoui et al, 2011). A class of selective estrogen receptor modulators (SERMs), as represented by tamoxifen (TAM), have been used for targeted therapy of these breast cancers with encouraging outcomes at least partially via reversing the anti-apoptotic effect of estrogen receptor signaling (Jordan & O'Malley, 2007). Unfortunately, use of SERMs is associated with de novo and acquired resistance and some undesirable side effects (Jordan & O'Malley, 2007). In contrast, a targeted introduction or activation of a cellular apoptosis executioner will elicit irreversible cell death without inducing drug resistance (Waxman & Schwartz, 2003). According to the well-documented "induced proximity model", the activation of the initiators occurs only when they are recruited by upstream oligomerization inducers, which bring the procaspases into close proximity and thus allow intramolecular processing (Shi, 2004). This model has been applied in developing pro-apoptotic strategies in cancer treatment based on the oligomerization capacity of either mouse IgG Fc portion or the tandem FK506-binding domain (FKBP) plus its dimeric ligand FK1012 (Muzio et al, 1998; Shi, 2004; Srinivasula et al, 1998b). In a study focusing on ER-positive breast cancer treatment, we generated a fusion gene encoding the chimeric protein of caspase-8 and the ligand-binding domain (LBD) of ER α and introduced the gene into breast cancer cell lines (Zhao et al, 2011). Upon administration of estrogen, the fusion protein will be induced to form a dimer, which triggers the activation of caspase-8 and apoptosis (Tamrazi et al, 2002; Shi, 2004). Indeed, incubation of estradiol with the chimeric gene-modified breast cancer cells induced a rapid dimerization of the chimeric protein, which in turn caused the activation of caspase-8 within the chimeric protein and leads to apoptosis of the modified cells (Zhao et al, 2011). Estradiol also significantly impaired the development of the xenograft breast cancers derived from the chimeric gene-modified SKBr-3 cells, and inhibited the growth of in vivo tumors originating from MCF-7 cells when administered in combination with the chimeric gene recombinant adenovirus (Zhao et al, 2011).

Unlike previously reported artificial caspase recruitment models, our study employed the receptor of endogenous estrogenic hormones, which otherwise promote the growth of breast cancer cells, to trigger the apoptotic signaling in breast cancers, thus switching the estrogenic hormones from potential mitogens to apoptosis inducers in breast cancers (Shi, 2004; Zhao et al, 2011). Therefore, the caspase-8 and estrogen receptor-based chimeric protein has implications in developing novel therapeutics of estrogen-dependent and -independent breast cancers. Despite the effectiveness of the chimeric protein, optimization

of this suicidal system using an accurate dimerization-related domain of ER α is necessary for preventing inappropriate autoactivation of chimeric caspase-8; given that the estrogen antagonist, tamoxifen, is also found to induce the formation of ER dimers in a much lower efficiency, it remains elusive whether tamoxifen in place of estradiol could trigger dimerization and activation of caspase-8 conjugated to the ER ligand-binding domain (Tamrazi et al, 2002; Zhao et al, 2011).

In addition to the generation of fusion/chimeric proteins, the pro-apoptotic gene regulatory elements, as well as the gene construct carriers, either non-viral or viral particles, have also been modified to target breast cancers. The antibody-drug conjugate, Trastuzumab-DM1 is currently in a late clinical trial for treatment of HER2-positive breast cancers (Alley et al, 2010; Senter, 2009). Breast cancer-targeted delivery systems are also constructed by modifying the envelop of viral particles, liposomes or other nanomaterials with tumor cell-binding phage peptides, or with ligands or antibodies that recognize HER2, E-selectin, transferrin or erythropoietin-producing hepatocellular receptor tyrosine kinase receptor class A2 (EphA2) (Alvarez et al, 2010; Mann et al, 2010; Normanno et al, 2009; Sarkar et al, 2005; Tandon et al, 2011). Tumor-specific regulatory elements, such as promoters of human telomerase reverse transcriptase (hTERT), survivin, Muc1 and the homologous recombination-related protein Rad51, as well as the hypoxia-responsive elements (HRE) have been employed in the regulation of pro-apoptotic genes like Bax and truncated Bid in the development of breast cancer-targeted therapeutic strategies (Lee, 2009; Kazhdan et al, 2006; Hine et al, 2008).

3.3 Selective blockade of anti-apoptotic signaling

It is widely accepted that intracellular apoptosis-inhibitory mechanisms account for a rapid progression and chemotherapy resistance of a variety of breast cancers. Therefore, targeted restoration of the apoptotic signaling by suppression of the anti-apoptotic factors helps reverse the malignant phenotype of breast cancers (Fulda & Debatin, 2006; Waxman & Schwartz, 2003). This could be achieved in the post-transcriptional level by targeted siRNA or in the post-translational level via targeted degradation of the anti-apoptotic proteins (Fulda & Debatin, 2006; Waxman & Schwartz, 2003). Considering that the short hairpin RNA (shRNA) expression is commonly driven by an RNA polymerase III promoter, which somehow excludes a modification of the promoter using a tumor-specific elements, specific delivery of siRNAs instead of selective expression of the shRNAs in neoplastic cells would be appropriate to targeting breast cancer cells (Couto and High, 2010; Wen et al, 2008). Again, the antibodies or ligands that recognize breast cancer cells are utilized in these targeted delivery systems (Couto and High, 2010; Wen et al, 2008). In one of our studies, a subset of siRNA delivery systems were generated by fusing a tumor-targeting antibody with the nucleic acid-binding peptide derived from the arginine-rich protein, protamine. The targeted delivery of the synthesized siRNA or shRNA-expressing cassette in cells expressing the corresponding antigen was corroborated in *in vivo* models (Wen et al, 2008). The protein ubiquitination process governs the degradation of unfavored endogenous proteins in the cells. In an attempt to trigger targeted degradation of the breast cancer-related proteins, Li et al generated a series of chimeric molecules in which the Src homology 2 (SH2) domain of the ubiquitin ligase Cbl was replaced with that from growth factor receptor binding protein 2 (Grb2), Grb7, p85 or Src (Li et al, 2007). These chimeric proteins could interact with HER2 and accelerate the degradation of this oncoprotein, suggesting a novel approach to the targeted therapy of HER2-overexpressing cancers (Li et al, 2007).

4. Targeted apoptosis in clinical treatment of breast cancers

The development of targeted pro-apoptotic strategies in laboratory studies on breast cancer treatment is in parallel with the emerging of novel targeted agents for clinical breast cancer therapy (Fulda & Debatin, 2006; Waxman & Schwartz, 2003). As aforementioned, monoclonal antibodies or small molecule inhibitors targeting HER2 (Herceptin/trastuzumab), EGFR (Gefitinib/Iressa) and VEGF (Avastin/Bevacizumab) have found their clinical applications in the war against breast cancers (Schlotter et al, 2008). In particular, as a well-documented adjuvant drug for metastatic breast cancer therapy, Herceptin has provided startling benefits to patients by reducing suffering and mortality of breast cancers, although frequent resistance development and cardiovascular toxicity have limited its repeated administration (Paik et al, 2008). In clinical trials of breast cancers, the death receptor ligands recombinant TNF- α and TRAIL have exhibited notable apoptosis-inducing potentials (Gonzalvez & Ashkenazi, 2010; Li et al, 2010). Intriguingly, TRAIL has displayed minimal adverse effects on normal tissues and the most striking therapeutic benefits in patients with HER2, ER and progesterone receptor (PR) triple-negative breast cancers (Oakman et al, 2010; 19: 312). Targeted expression of TRAIL under the control of a radiation-inducible RecA promoter delivered by *Salmonella typhimurium* significantly improved the survival of mice bearing breast cancers (Ganai et al, 2009).

Small RNAs or chemicals targeting the anti-apoptotic genes are also on their way towards the prescription for breast cancer patients. However, as a pioneering anticancer nucleic acid drug, G3139- the antisense oligonucleotide targeting Bcl-2 showed limited therapeutic efficacy when combined with doxorubicin and docetaxel in phase I/II studies on breast cancers (Moulder et al, 2008). Inhibitors to poly(ADP-ribose) polymerase-1 (PARP-1), a well-defined substrate of caspase-3, have showed single agent activity in treatment of breast cancers in a phase I trial, and conferred therapeutic benefits in combination with chemotherapy in triple-negative breast cancers without an increase in normal tissue toxicity in a phase II clinical trial. (Drew & Plummer, 2010).

5. Conclusion and perspective

Accumulating evidence on breast malignancies has supported that insufficient apoptosis contributes crucially to the occurrence and progression of breast cancers, and apoptosis resistance accounts for failure of traditional anticancer therapy on a majority of clinical breast cancers (Brown & Attardi, 2005; Evan & Vousden, 2001). In recent years, an extensive understanding of the canonical apoptosis signaling mechanisms has allowed the development of novel approaches to reversing or compensating for the apoptosis deficiency of cancer cells, either by introduction of a pro-apoptotic molecule or via blockade of the anti-apoptotic signals in breast cancers (Fulda & Debatin, 2006; Waxman & Schwartz, 2003). Given the distinguished expression of the definitive tumor-specific antigens (TSA) in breast carcinomas, strategies targeting these cells could be developed based on specific recognition mediated by antigen/antibody or ligand/receptor binding or on a selective expression of the therapeutic genes under the control of the TSA regulatory elements (Rakha et al, 2010; Schlotter et al, 2009). Despite a successful clinical application of the targeted drugs, e.g. antibodies or chemical reagents to trigger apoptosis in numerous breast cancer cases, modifications to these drugs are probably necessary for two purposes (Alley et al, 2010; Fulda & Debatin, 2006). First, combination of the therapeutic antibodies or chemicals with

other potent cytotoxic mechanisms will improve their apoptosis-inducing efficacy in breast cancers. Second, additional approaches to achieving a “genuine” breast cancer targeting in drug delivery or gene expression facilitate reducing a systemic cytotoxicity or other adverse effects on patients (Alley et al, 2010; Lee, 2009). Nevertheless, the ongoing studies aiming at targeted apoptosis induction in breast cancers have opened new avenues to successful breast cancer immunotherapy.

6. References

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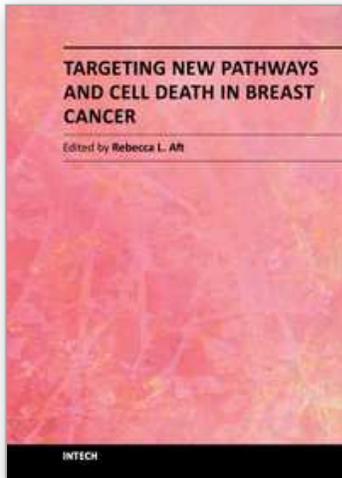
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