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Exosomal Survivin-T34A: A Novel, Potential Cancer Therapeutic

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Exosomal Survivin-T34A: A Novel, Potential Cancer Therapeutic

by

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ABBREVIATIONS

ΔΨm	Mitochondrial membrane potential
4C7	YUSAC 2 that overexpresses Survivin-WT (tet-free)
5-FU	5-fluorouracil
Ad-	Adenovirus-vector expressing -
AIF	Apoptosis inducing factor
Bcl-2	B-cell lymphoma 2
BIR	Baculoviral inhibitor of apopotosis protein repeat
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5 (Survivin)
Caspase	Cysteine-ASPartic proteASES
Cdc2	Cell division protein 2 (cyclin-dependent kinase 1, CDK1)
cIAP1 or 2	Cellular inhibitor of apopotosis 1or 2 protein (BIRC2 or 3)
CM	Conditioned media
DAPI	4',6-diamidino-2-phenylindole stain
ELISA	Enzyme-linked immunosorbent assay
F5C4	YUSAC 2 that overexpresses Survivin-T34A (tet-free)
G1	Gap 1/Growth 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
Gem	Gemcitabine
HA-tag	Hemagglutinin epitope tag
IAP	Inhibitor of APoptosis
kDa	Kilodalton
M	Mitosis of cell cycle

mAb	Monoclonal antibody
Mdm2	Mouse double minute 2 homolog
PARP	Poly (ADP-ribose) polymerase
S-phase	Synthesis-phase of cell cycle
Smac/DIABLO	Second mitochondrial activator of caspases
T34A	Threonine mutated to Alaline – 34 amino acid of protein
T34E	Threonine mutated to Glutamate – 34 amino acid of protein
Tet-	Tetracycline
Thr34 (T^{34})	Threonine – 34 amino acid of protein
WCL	Whole cell lysate
WT	Wild-type protein
XIAP	X-linked inhibitor of apoptosis

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, June 2014
Dr. Nathan R. Wall, Chairperson

Pancreatic cancer is the fourth most common cause of cancer fatality in American men and women with a less than 5% survival rate. Currently, if diagnosed early, surgical resection remains the only viable cure. However, only 20% of pancreatic cancer patients meet these criteria. It is therefore necessary to discover new therapies or therapeutic combinations in order to impact significantly this deadly disease. The anti-metabolite agent Gemcitabine is currently being employed to treat pancreatic cancer. While Gemcitabine has shown significant benefit in clinical applications, its ability to more than modestly impact pancreatic cancer is limited. It has been speculated that combinatory treatments using Gemcitabine could improve survival rates. Survivin, a member of the inhibitor of apoptosis (IAP) protein family, is expressed in virtually all cancer cells, but not detectable in most normal cells outside of development. Mutation of Survivin's Threonine 34 to Alanine (Survivin-T34A) abolishes a phosphorylation site for p34cdc2-cyclin B1 resulting in the initiation of the mitochondrial apoptotic pathway in cancer cells with little to no direct effects on normal cells. The possibility that targeting Survivin in this manner may provide a novel approach for selective cancer gene therapy has yet to be fully evaluated. We have recently described that cells generated to express a stable form of the mutant protein, released this Survivin-T34A to the conditioned medium. When this

conditioned medium was collected and deposited on naive tumor cells, conditioned medium containing Survivin-T34A was as effective as chemotherapy in induction of tumor cell apoptosis. When combined with other forms of genotoxic stress, Survivin-T34A potentiated their killing effects. We further determined that Survivin-T34A is trafficked by microvesicles called exosomes, which are released into the conditioned media. We showed strong evidence that exosomes containing Survivin-T34A elicited cellular death and synergistically enhanced cellular death during combination with low doses of Gemcitabine and we propose that these findings may lead to novel modalities for cancer therapies. This dissertation provides the rational for Survivin-T34A's potential as a therapeutic. Next, we provide evidence that extracellular Survivin-T34A can elicit its anti-tumor effects on treated cells. Finally, I show that extracellular Survivin-T34A is found in exosomes.

CHAPTER ONE

SURVIVIN-T34A: MOLECULAR MECHANISM AND THERAPEUTIC POTENTIAL

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Abstract

The inhibitor of apoptosis (IAP) protein Survivin's Threonine 34 to Alanine mutation abolishes a phosphorylation site for p34cdc2-cyclin B1 resulting in the initiation of the mitochondrial apoptotic pathway in cancer cells yet has little known direct effects on normal cells. The possibility that targeting Survivin in this way may provide a novel approach for selective cancer gene therapy has yet to be fully evaluated. Though a flurry of work was undertaken in the late 90s and early 2000s, only minor advances on this mutant have recently taken place. We recently described that cells generated to express a stable form of the mutant protein, released this Survivin-T34A to the conditioned medium. When this conditioned medium was collected and deposited on naive tumor cells, conditioned medium T34A was as effective as some chemotherapeutics in induction of tumor cell apoptosis and when combined with other forms of genotoxic stressors, potentiated their killing effects. In addition to determining the therapeutic dose and the duration of drug therapy required at the disease site, a better understanding of other key factors is also important. These include knowledge of target cell populations, cell-surface receptors, changes that occur in the target tissue at the molecular and cellular level with progression of the disease, and the mechanism and site of therapeutic action.

Introduction

Survivin is Important in Cancer

Apoptosis is a physiological cell suicide program that is critical for the development and maintenance of healthy tissues (Deveraux and Reed 1999). The evasion of apoptosis, as identified by Hanahan and Weinberg, is a primary characteristic of human cancer (Hanahan and Weinberg 2000) with proteins involved in its control of significant biological interest as they could prove to be important targets for therapy development. The inhibitor of apoptosis (IAP) family consists of proteins with proven ability to inhibit apoptosis, although much of how this works mechanistically is still to be revealed. Survivin is an important IAP because its expression is among the most tumor specific of all human gene products (Velculescu, Madden et al. 1999, Reed 2001). It has been classified as a bifunctional protein as it can inhibit apoptosis and cell cycle (Li, Ambrosini et al. 1998). More specifically, Survivin's promoter exhibits M phase inducible transactivation and in HeLa cells, both Survivin protein and mRNA are upregulated at G_2/M phase of the cell cycle (Li, Ambrosini et al. 1998). Survivin expression is enhanced during embryonic and fetal development, but not in terminally differentiated normal tissue (Li 2003). Its expression has also been seen in virtually every type of human cancer (Andersen, Svane et al. 2007), making Survivin an attractive protein in the study of cancer pathophysiology, drug discovery and medical diagnosis. Our laboratory has recently shown that cancer cells release Survivin into the extracellular space (Khan, Aspe et al. 2009) packaged in small 50-150 nm vesicles called exosomes (Khan, Jutzy et al. 2010). This is significant as it may reveal new mechanisms into how cancer cells communicate with one another and how they affect the tumor microenvironment and we hope that this discovery may pro-

vide other options for targeted therapy. However, this discovery begs for studies about why and how Survivin is packaged within exosomes as well as how Survivin from exosomes is utilized by the recipient cells as well as what are the effects of exosomal Survivin on normal human cells?

Survivin physically associates with and is phosphorylated on its 34 Threonine (Thr^{34}) by the cyclin-dependent kinase p34(cdc2)–cyclin B1 protein complex (O'Connor, Grossman et al. 2000). Loss of Thr^{34} phosphorylation results in dissociation of the caspase-9-Survivin protein complex and caspase-dependent apoptosis (O'Connor, Grossman et al. 2000, Mesri, Wall et al. 2001, Wall, O'Connor et al. 2003). Initially, Thr^{34} through site-directed mutagenesis was changed to Ala (T34A) as a non-phosphorylated mimic of Survivin (O'Connor, Grossman et al. 2000). When replication-deficient adenoviruses encoding this Survivin-T34A mutant were produced and used to infect cancer cell lines, spontaneous apoptosis resulted. In contrast, the viability of normal human cells including fibroblasts, endothelium and smooth muscle cells were unaffected (Mesri, Wall et al. 2001). In studies analyzing normal human oral mucosa, oral submucosa fibrosis and oral squamous cell carcinomas (Zhou, Li et al. 2008), a gradation from no Survivin Thr^{34} phosphorylation in normal to significantly enhanced amounts of phosphorylated Survivin Thr^{34} in the cancer tissues was recorded. Furthermore, phosphorylated Thr^{34} and p34(cdc2) kinase expression was not detected in normal tissue samples but increased with advancing stages in the cancer tissue samples (Zhou, Li et al. 2008). Agents that elevate p34(cdc2) kinase activity during spindle checkpoint activation drive increased Survivin expression and cancer cell viability (O'Connor, Wall et al. 2002) resulting in limited anti-cancer effects. Pharmacologic, genetic, or molecular ablation of p34(cdc2) kinase after

microtubule stabilization resulted in apoptosis independent of p53, suppression of tumor growth, and indefinite survival without toxicity in mice (O'Connor, Wall et al. 2002). Taken together, the ablation of Survivin-dependent apoptosis inhibition could improve the efficacy of many agents used to treat cancer.

One of the most daunting questions in any gene-based approach for cancer treatment is in the method of delivery. We have recently shown that Survivin exists in a novel extracellular pool in tumor cells. Furthermore, we have constructed stable cell lines that provide the extracellular pool with either wild-type Survivin or dominant-negative mutant Survivin (T34A) (Khan, Aspe et al. 2009). Cancer cells grown in the conditioned medium taken from wild-type Survivin expressing cells absorbed Survivin and experienced enhanced protection against genotoxic stress as well as exhibited an increased replicative and invasive potential (Khan, Aspe et al. 2009). Alternatively, cancer cells grown in conditioned medium from T34A cells began to apoptose through a caspase-2- and caspase-9-dependent pathway that was further enhanced by the addition of chemo- and radiotherapeutic modalities (Khan, Aspe et al. 2009), We believe that this extracellular Survivin-T34A could be used as a possible "White Knight" to Survivin's "dark family". The following sections detail T34A's journey as a novel potential cancer therapeutic. The studies above have already provided impetus for further studies on T34A, but there is still a lot unknown. The advancement of future T34A studies may provide a more comprehensive understanding of cancer and the potential of using T34A to battle it.

T34A Induces Apoptosis

What makes this Survivin-T34A mutant important as a potential therapeutic is that little or no toxicity has resulted from its treatment *in vitro* or *in vivo* (Wall, O'Connor et al. 2003, Yan, Thomas et al. 2006, Peng, Yang et al. 2008). T34A treated mice showed significantly longer survival rates compared to control mice as well as had no pathologic changes in major organs (Li, Zhao et al. 2009). More specifically, tumor cells treated *in vitro* with T34A exhibited reduced cell survival that was associated with morphological features of apoptosis (Mesri, Wall et al. 2001, Blanc-Brude, Mesri et al. 2003, McKay, Bell et al. 2003, Fukuda and Pelus 2004, Liu, Brouha et al. 2004, Wang, Fukuda et al. 2004, Zhang, Mukherjee et al. 2005, Ma, Zheng et al. 2006, Yan, Thomas et al. 2006, Peng, Yang et al. 2008, Zhang, Wang et al. 2008, Khan, Aspe et al. 2009, Shen, Liu et al. 2009, Xiao, Chen et al. 2010, Yu, Wang et al. 2010). Studies of mouse-xenographs treated with Survivin-T34A concluded with significant tumor size reduction and increased animal survival rates (Blanc-Brude, Mesri et al. 2003, Yan, Thomas et al. 2006, Peng, Yang et al. 2008, Li, Zhao et al. 2009, Yu, Wang et al. 2010) except in mice subcutaneously injected with the IGROV1 ovarian carcinoma cell line, where there were no significant differences between T34A infected tumors and controls (McKay, Bell et al. 2003). These results may however be explained as IGROV1 cells do not express significant levels of endogenous Survivin and T34A, as shown in normal, Survivin non-expressing cells, does not induce apoptosis (Mesri, Wall et al. 2001). It was not until IGROV1 cells were infected with adenovirus-vector expressing caspase-3 (Ad-caspase-3) or co-infected with Ad-caspase-3 & Ad-T34A that tumor size was reduced and survival rates extended (McKay, Bell et al. 2003). It is of much interest that the adenoviruses used

in these studies upregulated survivin mRNA, as shown by RT-PCR, and concomitantly resulted in a half-fold reduction in another IAP, XIAP (McKay, Bell et al. 2003). Furthermore, in melanoma cells that have been engineered to express an inducible Survivin-T34A (Grossman, Kim et al. 2001), XIAP and the IAP Livin were cleaved upon T34A induction (Liu, Brouha et al. 2004). These reductions may in part be responsible for the more favorable outcomes observed in these studies. In contrast, it has been postulated that the transfection of cells may affect endogenous levels of XIAP, but there was no effect on XIAP protein levels following transfections using virus vector controls, Ad-Survivin or Ad-T34A treatment (Mesri, Wall et al. 2001, McKay, Bell et al. 2003, Liu, Brouha et al. 2004). There were also no changes in other IAPs or members of the Bcl-2 family (Liu, Brouha et al. 2004). While Survivin-T34A may not affect endogenous levels of other IAPs, a Survivin-IAP (XIAP, cIAP1, and cIAP2) complex has been confirmed as well as suggestion for direct interaction between Survivin and XIAP through their BIR domains (Dohi, Okada et al. 2004). Furthermore, evidence that T34A can only kill cells expressing Survivin has been shown in its inability to kill normal human cells treated *in vitro* (Mesri, Wall et al. 2001, McNeish, Lopes et al. 2005) and *in vivo* (Mesri, Wall et al. 2001, Yan, Thomas et al. 2006). However, T34A has shown significant killing affects in normal endothelial cells that express high levels of endogenous Survivin (Blanc-Brude, Mesri et al. 2003). Whether this is the result of some interaction between endogenous Survivin, other IAPs, and the T34A mutant or a competition between them for protein interactors is currently unknown.

Mechanistic examination of T34A-induced apoptosis revealed mitochondrial events that preceded caspase activation, including depolarization and the release of cyto-

chrome c, AIF and Smac/DIABLO (Mesri, Wall et al. 2001, Blanc-Brude, Mesri et al. 2003, Liu, Brouha et al. 2004, Ogura, Watanabe et al. 2008). Also, T34A treatment of melanoma cells has also been shown to involve Apaf-1, Mcl-1 and PARP cleavage (Blanc-Brude, Mesri et al. 2003, Liu, Brouha et al. 2004, Zhang, Wang et al. 2008). Stress-induced cytochrome c release results in apoptosome formation and caspase activation (Boya and Kroemer 2008), thus several groups have reported that caspase-3 may be important to T34A-induced apoptosis (Mesri, Wall et al. 2001, Blanc-Brude, Mesri et al. 2003, McKay, Bell et al. 2003, Fukuda and Pelus 2004, Liu, Brouha et al. 2004, Wang, Fukuda et al. 2004, Barrett, Osborne et al. 2009). A significant increase in apoptosis was reported in ovarian carcinoma cells treated with T34A and caspase-3 *in vivo* and *in vitro* (McKay, Bell et al. 2003). We, however, working in cervical carcinoma HeLa cells, were unable to show cleavage of caspase-3 or caspase-8, though cleavage of caspase-9 and caspase-2 was recorded (Khan, Aspe et al. 2009). Further experiments revealed T34A-induced a reduction in the proforms of caspase-2, caspase-7 and caspase-9, a cleavage of PARP and an induction of mitochondrial depolarization. Treatment with caspase inhibitors, Z-DEVD-FMK and Z-VAD-FMK, has been shown to counter the effect of T34A treatment suggesting T34A-induced apoptosis may act through the caspase-dependent pathway (Liu, Brouha et al. 2004). Additionally, the earliest proapoptotic event observed in T34A treated cells was nuclear translocation of mitochondrial apoptosis-inducing factor AIF, which triggers both mitochondrial associated apoptosis and caspase-independent DNA fragmentation (Liu, Brouha et al. 2004). Taken together, Survivin-T34A targets the intrinsic (Kroemer and Pouyssegur 2008) or mitochondria-associated apoptotic pathway to induce most probably caspase-dependent and independent apoptosis (Figure 1).

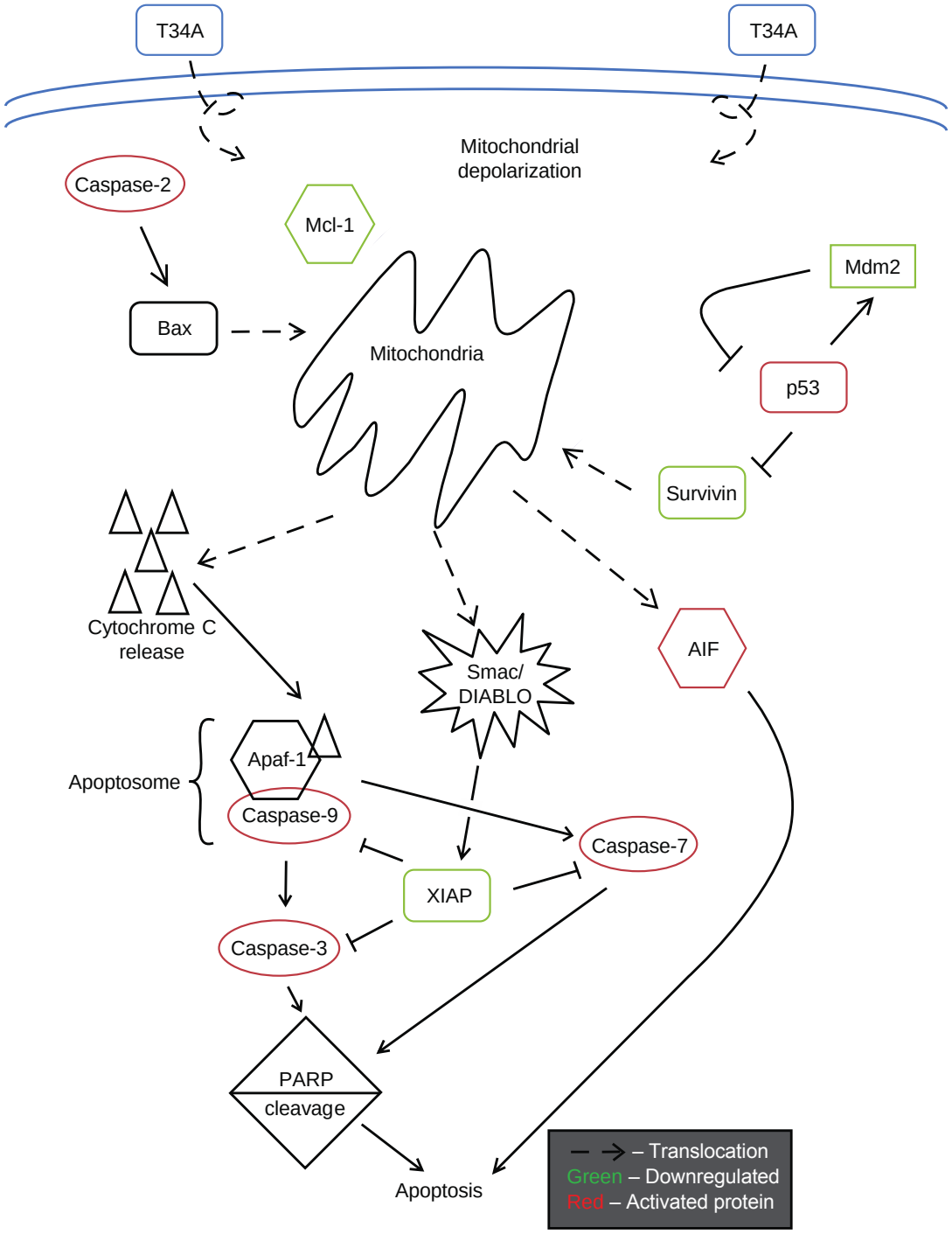

Figure 1. Survivin-T34A induces apoptosis by modulating the intrinsic (mitochondrial) pathway of apoptosis. After the mitochondrial depolarization, mitochondrial proteins such as Cytochrome c and SMAC/Diablo which activate caspases as well as those that are caspase-independent such as AIF are released by way of transmembrane channels across the mitochondrial outer membrane.

However, with the exception of one study (Shin, Sung et al. 2001), Survivin has not been shown to directly inhibit caspases and whether Survivin-T34A disrupts caspase activity has yet to be fully elucidated. We postulate that Survivin-T34A-induced apoptosis begins farther upstream in the intrinsic apoptotic pathway initiating mitochondrial depolarization as well as inhibition of IAPs known to directly inhibit caspases (e.g. XIAP-caspase-3).

The tumor suppressor gene, p53, has been widely studied and is known to induce apoptosis (Haldar, Negrini et al. 1994, Strobel, Swanson et al. 1996). As a transcription factor, p53 is activated by a variety of genotoxic and cytotoxic stresses. Upon activation, p53 prevents the proliferation of genetically compromised cells by regulating the expression of a battery of genes that initiate cell cycle arrest, apoptosis and DNA repair (Figure 2) (Ashcroft and Vousden 1999). Transcriptionally, p53 activates Mdm2, which in turn targets p53 for ubiquitin proteasome-dependent degradation. In response to stress, a decrease in Mdm2 protein levels and/or its activity and the interaction between Mdm2 and p53 lead to p53 stabilization (Marine and Lozano). The relationship between Survivin and p53 may be important, as Survivin regulates cell division and inhibits apoptosis whereas p53 inhibits cell cycle progression and induces apoptosis (Wang, Fukuda et al. 2004). Survivin was recently shown to be transcriptionally repressed by wild type p53 and when overexpressed in cells sensitive to p53-dependent cell death, markedly inhibited apoptosis induced by ultraviolet light (Hoffman, Biade et al. 2002).

When cancer cells that had been stably transfected with Survivin-T34A were treated with the DNA damage-inducing Adriamycin, p53 protein levels were significantly

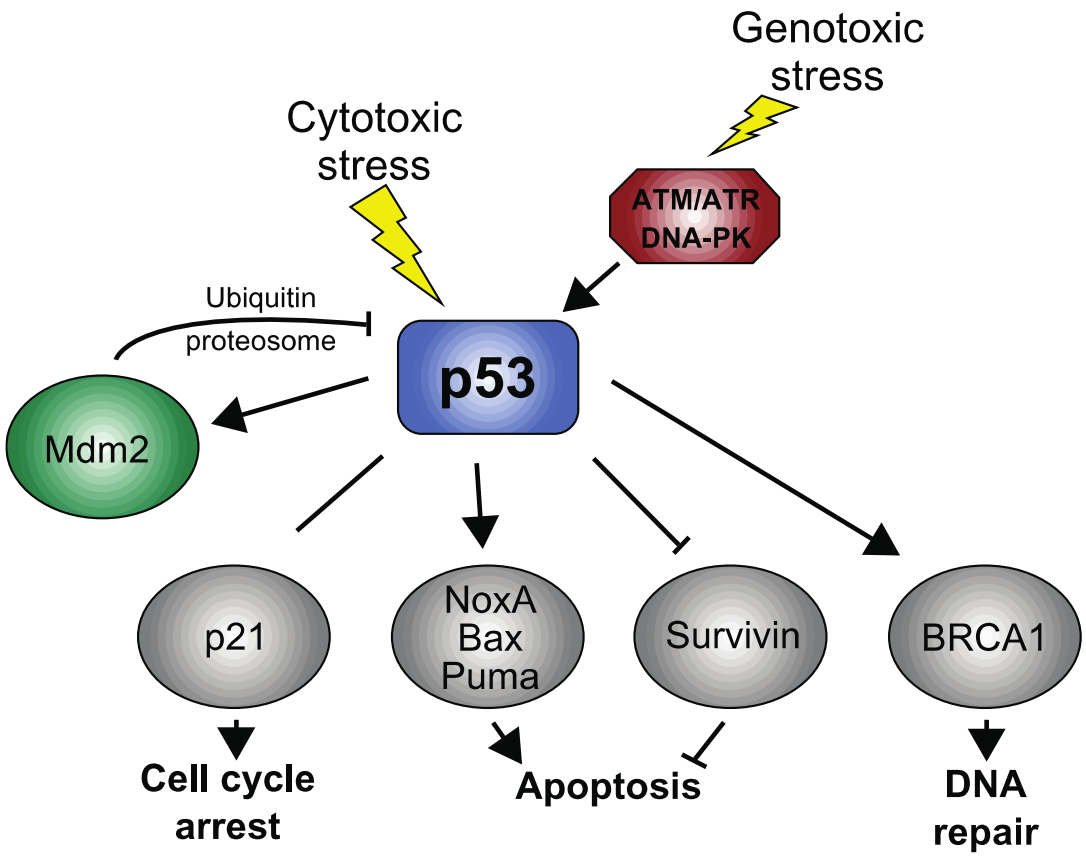

Figure 2. Survivin-T34A promotes p53-associated apoptosis. Upon activation, p53 prevents the proliferation of genetically compromised cells by regulating the expression of a battery of genes that initiate cell cycle arrest, apoptosis, and DNA repair. Survivin disruption by T34A treatment of cancer cells resulted in increased p53 protein levels and proteasomal degradation of Mdm2.

elevated compared to DNA damage alone or when combined with stably transfected Survivin (Wang, Fukuda et al. 2004). Increased levels of p53 mRNA preceded the elevation in p53 protein levels seen with T34A-treated cells, suggesting that p53 may be directly upregulated by Survivin-T34A. Survivin disruption by Survivin-T34A also resulted in the degradation of Mdm2. Proteasome inhibition and caspase-3 inhibition blocked this Survivin-T34A-associated decrease in Mdm2 resulting in the stabilization of p53. Survivin-T34A was not shown to affect mRNA levels of Mdm2 (Wang, Fukuda et al. 2004). Furthermore, the p53 homologues TAp63, ΔNp63, TAp73 and ΔNp73 were analyzed for changes following Survivin-T34A treatment. The response of these p53 homologues is important because TAp63 and TAp73 both transactivate similar genes as p53, whereas the ΔNp63 and ΔNp73 function as antagonists to p53 and the TA-homologues (Yang, Kaghad et al. 1998, Irwin and Kaelin 2001, Yang, Kaghad et al. 2002). ΔNp63, TAp73 and ΔNp73 were all decreased in response to Survivin-T34A, while TAp63 was not detectable. These findings suggest that p53 transcription may be differentially regulated by Survivin-T34A (Wang, Fukuda et al. 2004). Taken together, the ability of Survivin-T34A to perturb the p53/Mdm2 feedback loop resulting in increasing levels of p53 and p53's ability to then transcriptionally repress Survivin may prove to be just the therapeutic strategy needed for targeting p53-associated stress-induced repair pathways in cancer.

Survivin Threonine 34 Plays a Role in Cell Cycle Regulation

Survivin is a structurally unique IAP family protein that is expressed in mitosis in a cell cycle-dependent fashion and localized to components of the mitotic apparatus (Li, Ambrosini et al. 1998). Survivin has been described as bifunctional, in that it is involved

in both inhibition of apoptosis and control of cell division (Altieri 2003, Altieri 2006). In order to study the effect Survivin phosphorylation at Thr^{34} had on cell division, the non-phosphorylatable T34A form of Survivin was compared to T34E, which acts as a phosphomimetic (Barrett, Osborne et al. 2009). As a phosphomimetic, T34E assumes the role of a phosphorylated Thr^{34} and therefore mimics an activated Survivin. T34A-treated cells grew 2-fold faster than control cells while T34E cells grew 2.5-fold slower. Depletion studies showed that only T34A was normally distributed and able to support cell proliferation while T34E could not.

Survivin has been known to be upregulated in G2/M of the cell cycle, but T34A's ability to induce apoptosis is G2/M-independent (McKay, Bell et al. 2003). The cell cycle profile of T34A treated cells was normal while T34E treated cells had a significantly increased G2/M population suggesting that cell division failed (Barrett, Osborne et al. 2009). Furthermore, cancer cells which exhibited endogenous levels of functional Survivin, when treated with T34A showed significant mitotic index inhibition (Mesri, Wall et al. 2001), as well as reduction in colony formation (Khan, Aspe et al. 2009). Ultimately, when endogenous Survivin was depleted, T34A restored Survivin's mitotic function while T34E was mitotically incompetent. We therefore conclude that phosphorylated Thr^{34} inhibits mitosis (Barrett, Osborne et al. 2009), but non-phosphorylated Survivin is important for mitosis.

T34A has been shown to decrease the proportion of cells in S-phase following S-phase arrest (Fukuda and Pelus 2004). Evidence for Survivin-T34A regulation of chromosomal passenger complexes and central spindle checkpoint during mitosis has been generated (Lens, Rodriguez et al. 2006). In this work, Survivin-T34A was also shown to

be able to restore the spindle checkpoint defect following mitosis arrest unlike the other non-wildtype forms as well as rescue the cells from cytokinesis failure (Lens, Rodriguez et al. 2006). These findings strongly suggest Survivin-T34A is a functional complement for endogenous Survivin. Because Survivin-T34A functionally behaves as Survivin, it can localize appropriately during mitosis and thus correctly colocalize Aurora B at the centromeres and midzone (Lens, Rodriguez et al. 2006, Barrett, Osborne et al. 2009), allowing proper mitosis regulation. Further experiments show that Survivin-T34A correctly localizes BubR1 to the kinetochores and can restore localization of endogenous Borealin/Dasra B to both centromeres and central spindles (Ruchaud, Carmena et al. 2007). Taken together, Survivin-T34A allows for the enhanced proliferative effects of Survivin while Survivin-T34E does not. This suggests that the non-phosphorylated Survivin Thr^{34} (T34A) accounts for cellular proliferation as opposed to the phosphorylated Survivin Thr^{34} (T34E). Also, contrasting Survivin Thr^{34} for its cytoprotective activity revealed that T34E is responsible for the antiapoptotic characteristics whereas T34A allows for caspase activation and induced apoptosis (Barrett, Osborne et al. 2009).

T34A Opposes Classical Cancer Characteristics

Angiogenesis is crucial for cell survival and function and without it tumor cells are not able to obtain oxygen and nutrients. Targeting tumor angiogenesis is a relatively new therapeutic strategy and it is believed that if the tumor's oxygen and nutrient supply is limited, tumor growth and development will be inhibited and may result in apoptosis. Survivin-T34A treatment of tumor-bearing mice resulted in inhibited tumor-induced angiogenesis and increased apoptosis (Peng, Yang et al. 2008, Li, Zhao et al. 2009, Shan,

Wang et al. 2010, Yu, Wang et al. 2010). Tumor sections taken from human breast cancer transplants in mice were stained with anti-CD31 antibody to determine microvessel density (Pan, Peng et al. 2010). Survivin-T34A treated groups showed significantly reduced microvessel density compared to controls (Peng, Yang et al. 2008). *In vivo* results showed T34A-induced apoptotic characteristics in murine endothelial cells as well as decreased new vessel formation when compared to control mice (Blanc-Brude, Mesri et al. 2003, Peng, Yang et al. 2008). Furthermore, experiments using CT26 colorectal cancer cells showed similar results (Li, Zhao et al. 2009). It is still unknown how Survivin-T34A treatment reduces tissue staining for the angiogenesis marker CD31. The elucidation of this mechanism may provide insight into this tumor-acquired capability. Survivin has been shown to be upregulated by vascular endothelial growth factor (VEGF) (Blanc-Brude, Mesri et al. 2003), an important promoter of angiogenesis. Perhaps, Survivin regulation by VEGF promotes angiogenesis by protecting developing vessels from apoptosis. Therefore, Survivin-T34A inhibits VEGF's promotion of angiogenesis, which may be mediated through loss of p53, activation of phosphatidylinositol 3'-kinase, or phosphorylation of signal transducers and activators of transcription 3 (Tran, Rak et al. 1999, O'Connor, Schechner et al. 2000, Papapetropoulos, Fulton et al. 2000, Mahboubi, Li et al. 2001, Harfouche, Hassessian et al. 2002).

Once a tumor mass becomes constricted, cells acquire the ability to invade surrounding tissue and metastasize to distant sites, which accounts for 90% of human cancer deaths (Hanahan and Weinberg 2000). The inhibition of Survivin using adenoviruses (Ad-T34A) or short hairpin RNA dramatically inhibited invasiveness of prostate cancer cells in the in vitro invasion assay, and spontaneous metastasis in the Dunning prostate

cancer in vivo model (Zhang, Coen et al. 2010). Treatment of subcutaneous breast cancer tumors in mice using T34A plasmids (Peng, Yang et al. 2008) or Ad-T34A (Mesri, Wall et al. 2001) inhibited metastasis of breast tumor cells from the primary tumor site to the lungs and peritoneum, respectively (Peng, Yang et al. 2008). Specifically, control treatment groups were observed to have a greater number of metastatic nodules and structural destruction of pulmonary alveoli compared to the T34A treatment group in transplanted mice (Mesri, Wall et al. 2001, Peng, Yang et al. 2008). In light of the role metastasis plays in cancer death, T34A's metastasis-inhibitory role is important as current therapeutics offer limited capabilities.

Cancerous lesions promote tumor growth, motility, invasion and angiogenesis via oncogene-driven immunosuppressive leukocyte infiltrates (Melief 2008). T34A has been shown to enhance T lymphocyte activity against autologous tumor cells (Peng, Yang et al. 2008). In addition, T cells derived from mouse xenographs exhibited higher cytotoxicity against target tumor cells in T34A treated mice (Li, Zhao et al. 2009). Further studies revealed that adoptive transfer of $CD8^+$ T lymphocytes, and not their $CD4^+$ T lymphocyte counterparts, isolated from the spleen of T34A treated mice exhibited a decrease in tumor volume. Perhaps the treatment of tumor cells with T34A or with immune cells that have been primed by Survivin-T34A association will redress the immunosuppression associated with cancer.

Translational Approaches and Obstacles

Given Survivin's role in chemotherapy and radiation resistance (Mesri, Wall et al. 2001, Wall, O'Connor et al. 2003, Chakravarti, Zhai et al. 2004, Fujie, Yamamoto et al.

2005, Zhang, Latham et al. 2005, Zhang, Mukherjee et al. 2005, Ferrario, Rucker et al. 2007, Ogura, Watanabe et al. 2008) results using T34A would suggest its role is to promote sensitivity to chemotherapy and radiotherapy. In the first study to investigate T34A's ability to enhance cancer cell chemosensitivity, transfected Survivin-T34A-induced apoptosis was as effective as Taxol alone, whereas transfected Survivin-T34A cells enhanced Taxol-induced apoptosis (Mesri, Wall et al. 2001). Subsequent studies involving other chemotherapeutics have also revealed enhanced apoptosis in T34A-expressing cancer cells and xenograph models (Flutamide (Zhang, Latham et al. 2005), Paclitaxel (Zhang, Mukherjee et al. 2005)). Survivin-T34A combined with Adriamycin exhibited no added effect over Adriamycin alone (Mesri, Wall et al. 2001). This study was followed by studies designed to characterize phosphorylated Survivin Thr^{34}'s protective role. Following Adriamycin treatment of tumor cells, p34(cdc2) was coimmunoprecipitated with Survivin. These findings suggest that p34(cdc2) phosphorylates Survivin on Thr^{34} following Adriamycin treatment thus protecting the cells against the chemotherapeutic. These results were correlated with elevated MPM-2 mitotic phosphoepitope expression (Wall, O'Connor et al. 2003). While Survivin-T34A combined with Adriamycin exhibited no added effect over Adriamycin alone, it may still be possible to synergistically enhance Adriamycin's effect with pretreatment of Survivin-T34A. The same study further showed that increased doses of Flavopiridol treatment were associated with suppression of Survivin Thr^{34} phosphorylation.

Human glioblastoma multiforme (GBM) cells were treated with adenoviral vectors containing T34A or Survivin, *in vivo* and *in vitro*. No change was seen in normal cells treated with either Survivin form, but radiosensitivity was exhibited in T34A treated

cells compared to Survivin-treated or control cells. Several other studies have confirmed similar results suggesting that Survivin only affects radiosensitivity of tumors cells and radioresistance can be reduced by T34A treatment (Ferrario, Rucker et al. 2007, Ogura, Watanabe et al. 2008, Barrett, Osborne et al. 2009, Khan, Aspe et al. 2009, Yuan, Wang et al. 2010). Interestingly, when cancer cells were irradiated and then treated with T34A, radiosensitivity was enhanced even more (Chakravarti, Zhai et al. 2004). Enhanced radiation sensitivity may be explained by cleavage and activation of caspase-3 in T34A treated cells. Caspase inhibitors, except those for caspase-9, have been able to reduce apoptotic phenotypes in X-irradiation of T34A-treated cells (Ogura, Watanabe et al. 2008). Radiosensitivity may also be induced via a caspase-independent mechanism (Chakravarti, Zhai et al. 2004). DNA double strand breakage was greater in T34A treated cells suggesting that T34A may interfere with the cell's ability for DNA repair post irradiation (Chakravarti, Zhai et al. 2004).

Conclusion

Survivin-T34A has a rich history, having been first utilized as a mimic of non-phosphorylated Survivin, and now as a possible cancer therapeutic. It is full of potential as a molecular therapy because of its minimal toxicity and its ability to induce apoptosis and immune modulation while reducing angiogenesis, metastasis and cell cycle progression in Survivin expressing cells **(**Figure 3). With the discovery that T34A conditioned medium, taken from cells that overexpress and release Survivin-T34A to the extracellular space, is able to kill cancer cells *in vitro* (Khan, Aspe et al. 2009), we hope to further enhance the scientific data banks and pave new paths for clinical modalities and regimens.

For this to occur however, additional testing of this agent on normal tissues – specifically testing its myelosuppressive effects, will have to be completed. Also, additional studies to synergistically enhance chemo-and radiotherapies, whether in combination with or through Survivin-T34A pretreatment, must be accomplished. The knowledge that will make this possible includes: the elucidation of Survivin-T34A target cell populations, cell-surface receptors, changes that occur in the target tissue at the molecular and cellular level, the presence or development of resistance which could limit its use, the mechanism of therapeutic action, and its site of action. As Survivin and p53 are important opposing regulators of cancer, further research on their interactions and signaling pathways will likely provide specific clues for understanding this complicated relationship.

Acknowledgments

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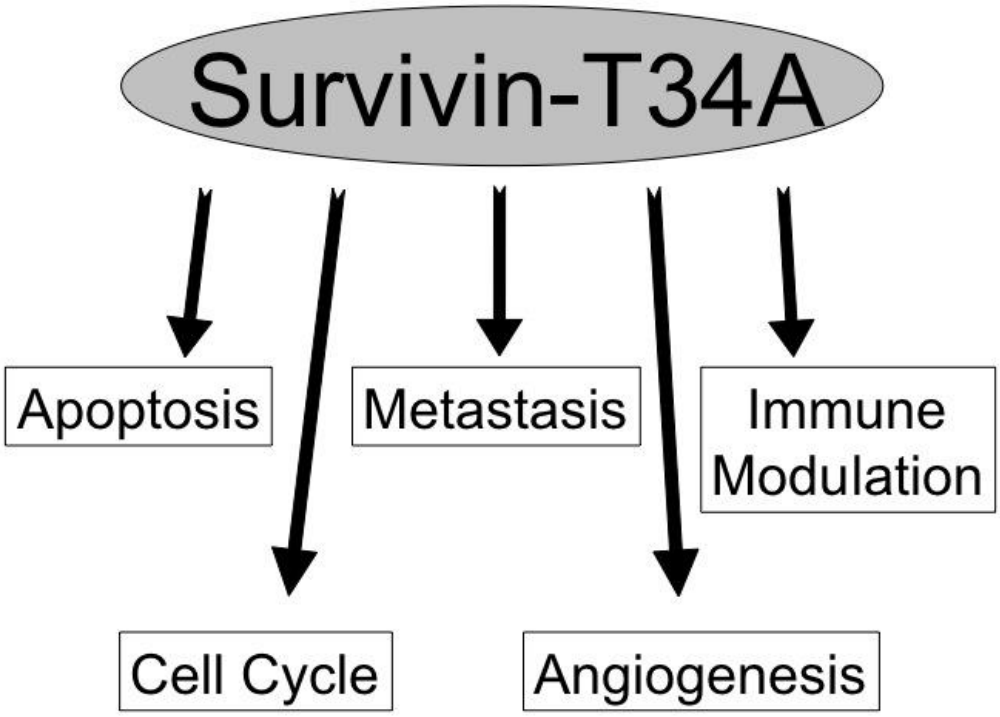

Figure 3. Survivin-T34A has therapeutic potential, as it has been shown to induce apoptosis and immune modulation while reducing angiogenesis, metastasis, and cell cycle progression in Survivin-expressing cells.

CHAPTER TWO

EXTRACELLULAR, CELL-PERMEABLE SURVIVIN INHIBITS APOPTOSIS WHILE PROMOTING PROLIFERATIVE AND METASTATIC POTENTIAL

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Abstract

The tumor microenvironment is believed to be involved in development, growth, metastasis, and therapy resistance of many cancers. Here we show Survivin, a member of the inhibitor of apoptosis protein (IAP) family, implicated in apoptosis inhibition and the regulation of mitosis in cancer cells, exists in a novel extracellular pool in tumor cells. Furthermore, we have constructed stable cell lines that provide the extracellular pool with either wild-type Survivin (Surv-WT) or the previously described dominant-negative mutant Survivin (Surv-T34A), which has proven pro-apoptotic effects in cancer cells but not in normal proliferating cells. Cancer cells grown in conditioned medium (CM) taken from Surv-WT cells absorbed Survivin and experienced enhanced protection against genotoxic stresses. These cells also exhibited an increased replicative and metastatic potential, suggesting that Survivin in the tumor microenvironment may be directly associated with malignant progression, further supporting Survivin's function in tumorigenesis. Alternatively, cancer cells grown in CM taken from the Surv-T34A cells began to apoptose through a caspase-2- and caspase-9-dependent pathway that was further enhanced by the addition of other chemo- and radiotherapeutic modalities. Together our findings suggest a novel microenvironmental function for Survivin in the control of cancer aggressiveness and spread, and should result in the genesis of additional cancer treatment modalities.

Keywords: tumor microenvironment, survivin, apoptosis, metastasis

Introduction

The complexity and heterogeneity of the tumor microenvironment (Tredan, Galmarini et al. 2007) is believed to have an important function in the development, growth, metastasis, and therapy resistance of many cancers (Ohtani 1998). The tumor is not merely a single genotype-containing clonal mass but a community of heterogeneous cancer cells and stromal cells embedded in an extracellular matrix and nourished by vascular and fibroblast cell networks (Aznavoorian, Stracke et al. 1990).

The multifunctional protein Survivin controls diverse cellular functions, including surveillance checkpoints, suppression of cell death, the regulation of mitosis, and the adaptation to unfavorable environments (Li, Ambrosini et al. 1998, Altieri 2003, Altieri 2006). The suppression of cell death activities and baculovirus inhibitor of apoptosis protein (IAP) repeat (BIR) domain characterizes Survivin as a member of the IAP family (Ambrosini, Adida et al. 1997). However, Survivin's lack of a C-terminal RING finger domain and a caspase recruitment domain (Deveraux and Reed 1999) makes it structurally unique among the mammalian IAPs. The multifaceted functionality of Survivin is still being intensely scrutinized, although it appears that protein compartmentalization may be important. Survivin was recently shown to colocalize in the mitochondria, where it abolishes tumor cell apoptosis and promotes tumorigenesis in immunocompromized animals (Dohi, Beltrami et al. 2004). Survivin may therefore possess a function in apoptosis similar to the pro-apoptotic Bcl-2 family proteins. Furthermore, Survivin has been also found in the nucleus and cytosol where the implication is that it has functions in mitosis regulation and apoptosis inhibition, respectively (Fortugno, Wall et al. 2002). Survivin is expressed in most common human cancers and although present during embryonic and

fetal development, it is undetectable in a variety of adult tissues (Adida, Crotty et al. 1998) and for this reason it is currently recognized as an important anticancer target (Fukuda and Pelus 2006). For this purpose, multiple strategies have been successfully investigated, including the molecular antagonists such as antisense oligos, RNA inhibition, dominant-negative mutants, Survivin-specific cytolytic T cells, a non-phosphorylatable Survivin mutant Thr34 → Ala (T34A), and most recently, binding interface mimetics (Olie, Simoes-Wust et al. 2000, Andersen, Pedersen et al. 2001, Grossman, Kim et al. 2001, Kanwar, Shen et al. 2001, Mesri, Wall et al. 2001, Wall, O'Connor et al. 2003, Plescia, Salz et al. 2005).

In the present study, we investigate whether extracellular compartmentalization of Survivin participates in the cytoprotection, tumorigenesis, and enhanced metastasis encountered in the tumor microenvironment. We have identified a novel pool of Survivin, localized extracellularly that is readily taken up by cancer cells but not by normal stromal cells. Furthermore, cancer cells that absorb Survivin show enhanced growth patterns and are more resistant to genotoxic stress than controls containing only endogenous levels of Survivin. Further investigations to define uptake-induced phenotypes of the apoptosis inducing non-phosphorylatable Survivin-mutant (T34A) have shown a caspase-dependent enhancement of chemotherapy-induced cell death, *in vitro*.

Materials and Methods

Cell Lines and Cultures

Cervical carcinoma (HeLa and HeLa-S), osteosarcoma (U2OS and SaOS2), pancreatic carcinoma (Panc1, Capan1, and Capan2), prostate carcinoma (PC3, DU-145, and

RWPE-2), and breast carcinoma (MCF-7, HCC 1806) cell lines were obtained from the American Type Culture Collection (ATCC) as were the human normal prostate stromal cells (PrSC). The acute monocytic leukaemia cell lines (MOLM-14 and MV4-11) were a kind gift from CS Chen (Loma Linda University). Normal prostate epithelial cells (PrEC) were obtained from Clonetics-BioWhittaker (Walkersville, MD, USA) and cultured in PrEMB medium (Clonetics-BioWhittaker). The normal human bone marrow stromal (HBMS) cells were a generous gift of Kimberly Payne (Loma Linda University). Stromal cells were isolated and propagated as previously described (Hao, Shah et al. 1995) from adult human bone marrow that was purchased from Poietics Cell Systems (Lonza Walkersville Inc., Gaithersburg, MD, USA). Human peripheral blood mononuclear cells were isolated from a peripheral blood draw using Ficoll-Hypaque (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) as has been previously described (Wang, Azzo et al. 1984). The use of all human tissues was reviewed and approved by the institutional review board at Loma Linda University with informed written consent obtained from each subject. Cells were maintained as defined in DMEM, McCoy's, RPMI, or IMEM (ATCC) supplemented with 100 U of penicillin, 100 μg ml−1 streptomycin, 300 μg of l-glutamine, and 10–20% heat inactivated fetal bovine serum (FBS; ATCC). Cells were grown at 37°C in a humidified atmosphere of 95% air, 5% CO2 until 60% confluent and supernatants were removed. New medium was given and cells were grown to 90% confluent, after which supernatants were collected, centrifuged for 10 min at 2500 r.p.m., and stored at −20°C until use.

Expression Plasmid and Generation of Stable Cell Lines

The detailed procedure for cloning and propagation has been described previously (Ogawa, Ishiguro et al. 2002, Shi, Sawada et al. 2003). In brief, recombinant retroviruses expressing a bicistronic messenger RNA containing open-reading frames of Flag-HA (hemagglutinin)-tagged human Survivin or human T34A-Survivin and interleukin-2 receptor (IL-2R)-α were constructed and transduced into HeLa cells. The infected HeLa cells were sorted by anti-IL-2R monoclonal antibody (mAb) conjugated with magnetic beads, and the resulting Flag-HA-Survivin or Flag-HA-T34A-Survivin stable cell lines propagated as suspension cultures. The expression level of both the wild-type (WT) and mutant (T34A) Survivin was evaluated by western analysis and immunohistochemistry with anti-Flag and HA antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

Survivin Depletion

Conditioned medium (CM) from stable Survivin-expressing HeLa cells contains Survivin that has a Flag-HA tag as well as normal endogenous Survivin. To deplete the medium of Survivin, we added anti-Flag beads (20 μl/ml) to the medium and rotated overnight at 4°C. The medium was centrifuged at 2000 r.p.m. for 5 min to pellet the beads. The supernatant was collected and 100 μl set aside for analysis by ELISA. The pelleted beads were collected and put in at −20°C. The remaining medium was treated with anti-HA beads (20 μl/ml) in a similar manner to remove the remaining tagged Survivin. To deplete the endogenous Survivin, we added anti-Survivin polyclonal antibodies (10 μl/ml) and anti-rabbit beads (20 μl/ml) to the medium and rotated overnight at 4°C.

The 100 μl aliquots at each stage were used in an ELISA to confirm the depletion. The beads collected at each step were incubated in 40 μl sample loading buffer at 100°C for 4 min, then centrifuged at 2000 r.p.m. for 5 min. A western blot of the supernatant was used to further confirm depletion.

Quantitation of Survivin from CM (Cell Culture Supernatants)

Conditioned medium was collected from 90% confluent cell cultures with cell viability assessed by Trypan blue exclusion (Sigma-Aldrich, St Louis, MO, USA). One-half of the medium was concentrated 4 × using a speed-vac (Savant OligoPrep 120) after which unconcentrated and concentrated samples were assayed for the presence of Survivin using the Quantikine Human Survivin immunoassay kit (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions. To quantify representative Survivin protein from cultures experiencing cell death, we grew HeLa cells to 90% confluence as previously described after which 0.1, 1.0, and 10% of these cells were lysed and their protein used to ‘spike' control medium for ELISA.

Immunofluorescence Localization of Protein

Cells were plated in six-well plates on 22 × 22 mm coverslips and cultured with 2 ml of control or CM (Surv-WT-CM, Surv-T34A-CM). Wells were washed with PBS and fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized with 0.5% NP-40 in PBS for 15 min, blocked with 5% FBS in 0.01% NP-40/PBS for 30 min, incubated for 1 h with 1 : 500 dilution of anti-Flag/HA mAbs (Sigma-Aldrich). Cells were incubated with Alexa Fluor-555-conjugated rabbit anti-mouse IgG secondary

antibody (Invitrogen/Molecular Probes, Eugene, OR, USA), mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA, USA) and observed (× 1000) under an Olympus BX50 fluorescent microscope (Olympus America Inc., Center Valley, PA, USA).

Image Acquisition Using Laser-scanning Confocal Microscopy

Sampled sections were imaged and analyzed with an Olympus FV 1000 laser-scanning confocal imaging system mounted onto an Olympus IX81 microscope (Olympus America Inc.). Microscopic data were acquired with a 60× oil immersion objective lens. In each section, confocal stack images from representative fields of view per section were captured. The distance between each focal plane was 1 μm.

Protein Expression and Purification

Recombinant WT or mutated (T34A) proteins were expressed as glutathione-*S*-transferase (GST) fusion proteins in the *Escherichia coli* BL21-CodonPlus-RIL (Stratagene, La Jolla, CA, USA) strain with induction in 0.2 mm isopropyl-β-d-thiogalactopyranoside (IPTG) for 5 hrs. at 30°C. BIRC5 (Survivin) was purchased from Abnova (Walnut, CA, USA). The cells were harvested by centrifugation at 6000 **g** for 20 min, suspended in 50 mm Tris (pH 7.4), 150 mm NaCl, 5 mm MgCl2, and 1 mm dithiothreitol, and lysed by sonication. After centrifugation at 15 000 **g** for 30 min, we mixed the soluble fractions with glutathione-agarose beads (Sigma-Aldrich) and incubated for 1 h at 4°C. After centrifugation at 1000 **g** for 1 min, we washed beads containing proteins three times in 50 mm Tris (pH 7.4), 500 mm NaCl, 5 mm MgCl2, and 1 mm dithio-

threitol and further purified by chromatography over Econo-Column (Bio-Rad, Hercules, CA, USA) after overnight thrombin (Sigma-Aldrich) treatment to release the GST frame. The protein concentrations were measured using a protein assay reagent (Pierce, Rockford, IL, USA) with BSA as standard. Recombinant transferrin protein was purchased from Sigma-Aldrich. The expression vector PRSET-CTB contained a gene encoding the entire cholera toxin B subunit (CTB) protein (11.6 kDa), driven by the T7 bacteriophage promoter region and containing an oligonucleotide encoding 6 histidines immediately upstream of the CTB gene for nickel column isolation of the recombinant protein was built as described (Carter, Yu et al. 2006). Briefly, CTB protein synthesis was stimulated by addition of 90 mg IPTG to the bacterial culture for 6 h at 37°C. Cells were harvested by centrifugation with the pellet resuspended in 10 mm HEPES buffer (pH 7.5), containing 100 mm imidazole. The cells were disrupted by sonication at 3 × 10 s bursts at 10 W, with a Sonic 60 Dismembrator. Cholera toxin B subunit protein was isolated from the homogenate by a Maxwell Model 16 robotic protein purification system (Promega Inc., Madison, WI, USA), according to the protein isolation protocol provided by the manufacturer. Purity of the isolated CTB protein was determined by electrophoretic mobility measurement in a 12% polyacrylamide gel. Imidazole was removed from the protein mixture by dialysis of the preparation against 10 mm HEPES buffer (pH 7.5), for 4 hrs. at 4°C.

IR-Dye Labeling of Recombinant Proteins and In-cell Western Blotting

IR-Dye labeling was performed according to the manufacturer's instructions (LI-COR Biosciences, Lincoln, NE, USA). Briefly, 100 μg of transferrin, CTB, and 10 μg of

BIRC5 was labeled at 20°C for 2 hrs.. Labeled protein was column purified as described by the manufacturer. HeLa cells (5 × 103 per well) were plated in 96-well plates. After overnight culture, we added labeled BIRC5, transferrin, and CTB proteins at doses of 1 μg, 0.01 μg, and 0.001 μg in duplicate wells. After overnight culture, we washed cells vigorously 3–4 times with PBS to remove all remaining unattached recombinant protein. Cells were fixed with 4% paraformaldehyde and an in-cell western blot was performed using Survivin polyclonal antibody (Novus, Littleton, CO, USA) according to the manufacturer's instructions. The 96-well plate was then scanned immediately in both 700 and 800 nm channels. Relative fluorescent intensity was determined using our Odyssey Infrared Imaging system and software.

Colony Formation Assay

The cells were plated in 10 cm dishes at a density of 100 000 cells per well, incubated at 37°C overnight, and then were treated with the indicated control, Surv-WT-CM, and Surv-T34A-CM for up to 48 hrs.. The cells were fixed with methanol and stained with crystal violet.

Cell Cycle Synchronization

HeLa-S cells were G1-arrested for 16 hrs. using mimosine as previously described (Li, Ackermann et al. 1999). Cells were then released and treated with either Surv-WT-CM or control medium and then harvested after 0, 3, 6, 9, and 12 hrs.. Cells were analyzed for DNA content by propidium iodide staining and flow cytometry (Becton Dickinson, San Jose, CA, USA).

Apoptosis and Cell Cycle Analysis

Subconfluent cultures of the various cell lines were incubated with vehicle (DMSO), taxol (4 μm; Sigma-Aldrich), 5-flurouracil (5-FU, 200 nm; Sigma-Aldrich), adriamycin (Ad, 100 nm, Sigma-Aldrich), cisplatin (CDPD, 2 μm; Sigma-Aldrich) or exposed to ultraviolet B (UVB) irradiation at 50 J m−2 with or without the presence of WT-CM, T34A-CM, or control for 24 and 48 hrs. at 37°C. Cells were harvested, prepared, and analyzed for DNA content as described previously (Li, Ackermann et al. 1999). DNA content was analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson). The distribution of cells in the different phases of the cell cycle was analyzed from DNA histograms using BD CellQuest software (Becton Dickinson).

AlamarBlue (AB) Assay for Cell Proliferation

Initial experiments were carried out to follow per cent AB (%AB) reduction over time. The aim was to determine the optimal seeding density and culture period. HeLa cells, trypsinized from subconfluent cultures as described earlier, were suspended in culture medium containing 1% FBS, T34A-CM, and WT-CM and then seeded into duplicate wells of a 96-well plate (200 ml well) at concentrations of 1.5×10^4–1×10^6 cells per ml at standard culture conditions of 5% CO2 in air at 37°C. After an initial 4 hrs. period to allow for cell attachment, we added AB (BioSource, Camarillo, CA, USA) directly into culture medium at a final concentration of 10% and the plate was returned to the incubator. Optical density of the plate was measured at 540 and 630 nm with a standard spectrophotometer at 1, 3, 6, 12, 24, 48, and 72 hrs. after adding AB. Because the culture medium was not changed during this period, the calculated %AB reduction is a cumula-

tive value. As a negative control, AB was added to medium without cells.

Caspase Activity Assay

Caspase activity was quantitated using the ApoAlert Caspase Assay Plates (Clontech, Mountain View, CA, USA). Fluorogenic substrates specific for caspase-3, caspase-8, caspase-9, and caspase-2 were immobilized in the wells of a 96-well plate. Fluorescence was detected using a standard fluorescence plate reader in the presence or absence of the specific caspase inhibitor.

Western Blot Analysis

Cells were solubilized, proteins (20–40 μg) separated using 12% Bis-Tris polyacrylamide gels, proteins transferred onto polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA) and probed using the following antibodies: mouse monoclonal anti-Flag and anti-HA (Sigma-Aldrich), rabbit polyclonal anti-Survivin (Novus), rabbit polyclonal antibodies to caspase-7, caspase-9, PARP and GAPDH (Cell Signaling Technologies, Beverly, MA, USA), mouse mAbs to β-actin (Abcam, Cambridge, MA, USA), and mouse mAb to caspase-2 (Cell Signaling Technologies). Secondary antibodies (IR-Dye-conjugated) were goat anti-rabbit and goat anti-mouse immunoglobulin (LI-COR Biosciences). Immunoreactive bands were detected using the Odyssey imaging system (LI-COR Biosciences) and quantified using ImageQuant software (Amersham Biosciences, Piscataway, NJ, USA). Protein quantifications presented in this report were normalized with respect to β-actin or GAPDH.

Mitochondrial Permeability Detection

Mitochondrial depolarization is detected by a unique fluorescent cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, also known as JC-1 (Invitrogen/Molecular Probes). Cells were collected and washed with PBS and prepared for the analysis according to the manufacturer's instructions.

Collagen Cell Invasion Assay

Cellular invasion was studied using the QCM 96-well Fluorometric Collagen-based Cell Invasion Assay from Chemicon International (Millipore) according to the manufacturer's instructions. Briefly, single-cell suspensions were obtained by trypsinization of monolayer cultures of HeLa cells. Cell counts and viability were performed using Trypan blue staining after overnight serum starvation. Cells (25 000 cells per well) were plated on 8 μm pore size collagen-coated inserts in 96-well plates. Cells were grown for 24 h with control media, Surv-WT-CM, Surv-T34A-CM, medium that was depleted of Survivin introduced into the lower chamber or medium taken from cells treated with cytochalasin D, a known inhibitor of exocytosis that works by specifically disrupting microfilaments. Survivin was depleted using one round of anti-Flag antibody, one round of anti-HA antibody, and either 1 (depleted 1 ×) or 3 (depleted 3 ×) rounds of anti-Survivin antibody. A control, termed blank, was performed using only cell detachment buffer, lysis buffer, and CyQuant dye without cells. Invasive cells, migrating through the polymerized collagen layer, were dissociated using cell detachment buffer as described by the manufacturer and lysed. Fluorescence emission (CyQuant GR dye) was assessed by a fluorescent plate reader (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Statistical analysis was performed using a two-way analysis of variance with the aid of JMP statistical software (Cary, NC, USA). A paired *t*-test was used for group analysis. Caspase-2, caspase-7, and caspase-9 densitometric analysis was conducted using our AlphaImager EC (Alpha Innotech, San Leandro, CA, USA). Density of individual bands was divided by GAPDH as the internal control with each timed sample then divided by the particular time period control. As 6 hrs. staurosporine did not have a 6 hrs. control, these were also controlled against the 24 hrs. control samples.

Results

Stable Expression of Wild-Type and Mutant T34A Survivin leads to Survivin Secretion

We and others (Bokarewa, Lindblad et al. 2005, Mera, Magnusson et al. 2008) recently found and reported a form of Survivin that is found in the extracellular space. Using a commercially available Survivin ELISA, we were able to quantitate picogram amounts of Survivin from CM taken from tumor cell lines representative of the most common cancer types, including pancreatic, breast, prostate, cervical, sarcoma, and acute monocytic leukaemia (Table 1). To determine whether this extracellular form of Survivin was merely released as a result of an excretory process, the result of cellular necrosis, or whether its function was secretory and would be important in the context of the tumor microenvironment, that is absorbed into cells, we needed to construct a form of Survivin different from the endogenous form. We therefore generated stable HeLa cells expressing human Survivin (Surv-WT) tagged with both the Flag and HA epitopes at their N terminus. Immunohistochemistry (Figure 4A) as well as western blotting (Figure 5) using

antibodies to either HA or Flag revealed the Survivin fusion protein. The Surv-T34A mutant has shown promise in initiating the mitochondrial apoptotic pathway (Mesri, Wall et al. 2001) and if also detected extracellularly could provide a form of Survivin suitable for therapeutic manipulation. We therefore generated stable HeLa cells expressing the non-phosphorylatable Surv-T34A mutant. Immunohistochemistry (Figure 4B) and western blotting (Figure 5) revealed the HA-Flag-Survivin fusion protein. The generation of this cell line took many attempts, and many months, as the Surv-T34A mutant was very toxic to the HeLa cells. Further analysis of allowing mutations has not yet taken place.

Conditioned medium taken from these stable Surv-WT or Surv-T34A HeLa cells showed picogram amounts of Survivin by ELISA (Table 1). Interestingly, it was quite apparent that accumulation of the T34A mutant Survivin and that from the parent HeLa-S cells was limited in comparison to that which accumulated in the medium from the Surv-WT cells (Table 1). To evaluate this disparity, we evaluated Survivin protein concentration, determined using ELISA, on a per cell basis. Dividing the measured amount of protein by the number of cells that were present provides evidence that the Surv-WT cells truly do release more protein into the CM than does the control cells or the Surv-T34A-producing cells (Figure 6A). AB staining was next used to determine if this phenomenon was the result of cellular division. As recorded in Figure 6B, AB reduction in the Surv-WT HeLa-S cells represented a state of enhanced cellular growth when compared to the controls or the Surv-T34A cells. Surv-T34A HeLa-S cell proliferation is significantly reduced when compared to the Surv-WT and control HeLa-S cells. Also, there was no significant difference in the level of cell proliferation recorded in the Surv-T34A-expressing cells when compared to medium where no cells were being grown (Figure 6B,

blank), indicating that Surv-T34A may reduce the level of released Survivin as a result of its stifling cell growth. We next evaluated the functional importance of this extracellular Survivin protein. HeLa cells were incubated for 24 h in CM taken from control, Surv-WT, or Surv-T34A stable cells. Immunohistochemical staining (Figure 7A) and western blotting (Figure 7B), using antibodies to HA, showed that the Survivin fusion proteins are readily taken up. A retarded band of approximately 18 kDa appeared in the HeLa-S lysates of cells incubated in WT- and T34A-CM representing the Flag-HA-Survivin fusion protein (Figure 7B), which was not observed in control conditioned medium grown HeLa cells. Interestingly, the cells that were incubated in Surv-T34A-CM took on a shrunken appearance and were fewer in number compared to the control medium-incubated cells (Figure 7A) whereas the Surv-WT, CM-incubated cells appeared to be healthier and in greater numbers. Phase-contrast microscopy allowed enhanced visualization of this shrunken phenotype and the initiation of apoptotic bodies in these T34A-CM-treated cells. To ensure that the ability of cells to uptake Survivin from the CM was a general occurrence and not specific for the HeLa cells, we also incubated Capan1, Capan2, MCF-7, Panc1, PC3, SaOS2, U2OS, HCC, and HBMS cells in HA-Flag-survivin-containing CM. All cancer cells were able to take up the Survivin from the medium whereas the normal HBMS cells did not (Figure 8A). Western blots (Figure 8B) from these cells confirmed the uptake results as every cell type except HBMS, when incubated in Surv-WT-CM, expressed 18 kDa band representing the Flag-HA-Survivin fusion

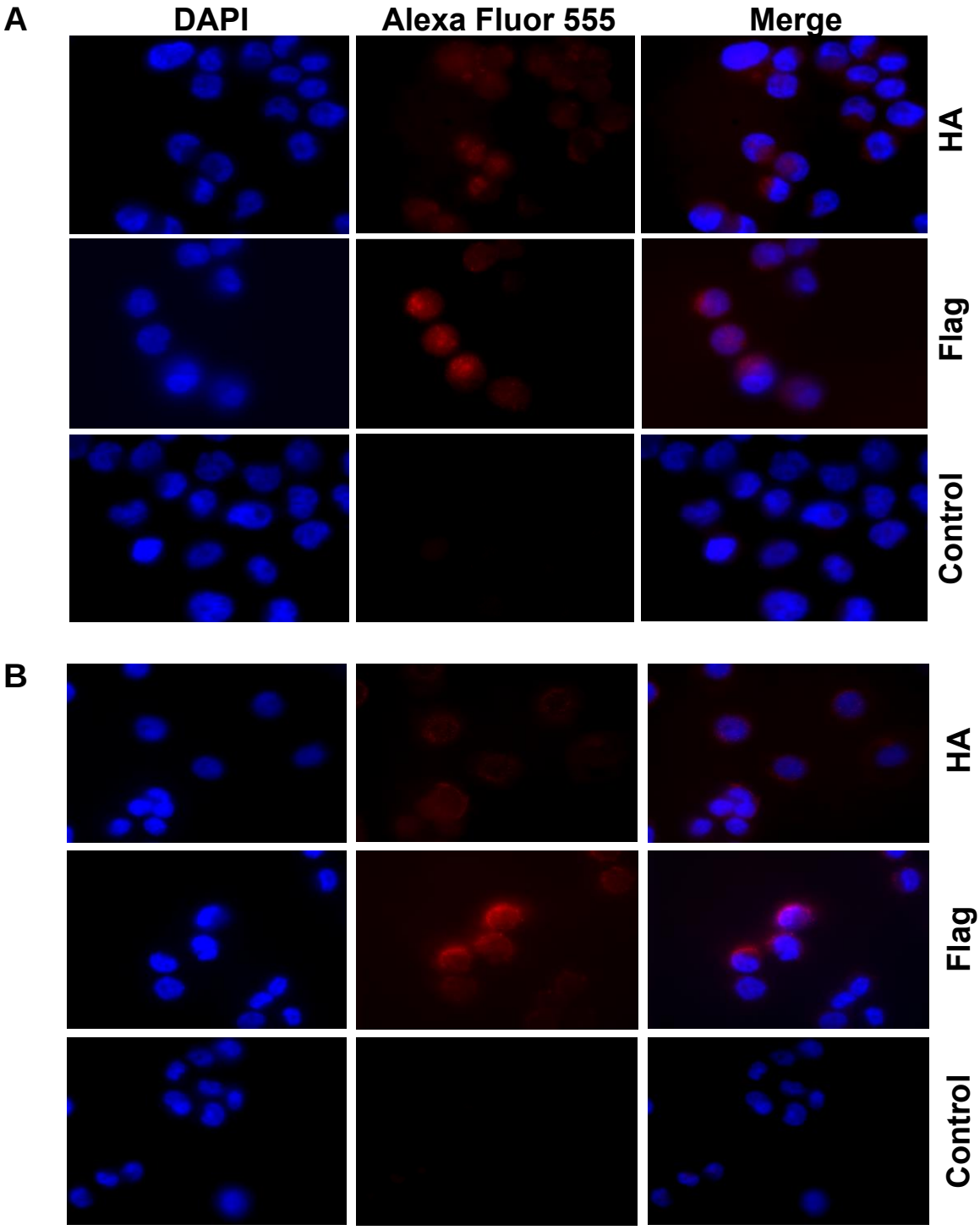

Figure 4. HeLa cells express the Flag-HA-Survivin fusion protein. Wild type Survivin **(A)** and T34A mutant Survivin **(B)** transformed HeLa cells were selected four times using magnetic beads conjugated to the IL-2 Receptor antibody. Cells were fixed and stained using HA and Flag antibodies as described in the methods and materials. Magnification x1000.

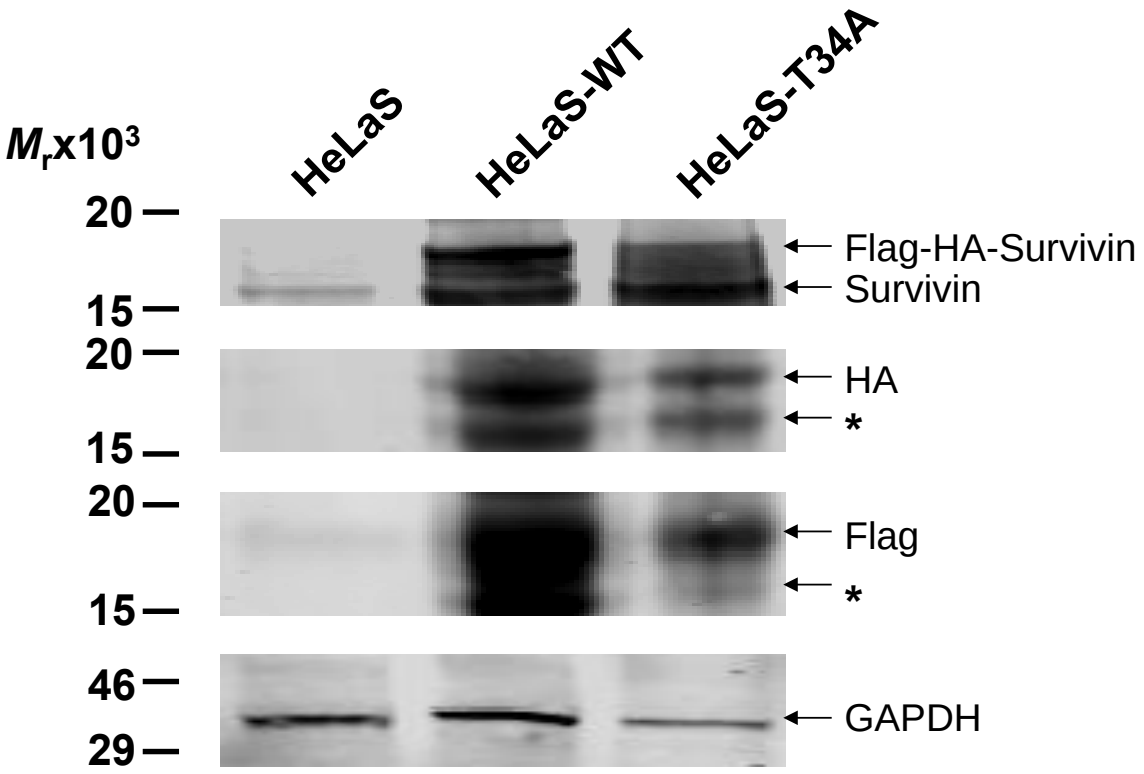

Figure 5. HeLa cells that expressed the stable Flag-HA-Survivin fusion protein were harvested and analyzed by Western blotting with antibodies against Survivin, HA, and Flag in order to support the immunohistochemical findings. As blots were often first probed with antibodies to Survivin and then with antibodies to Flag or HA, a doublet band (indicated by arrows) occurs with Survivin being 16.5 KDa and the Flag/HA retarding the band to ~18 KDa. A third band, labeled * often appears after probing with the antibodies to Flag and HA whose identity is yet unknown to us. GAPDH blotting was used as loading control. Molecular-weight markers in kilodaltons (KDa) are shown on the left.

Table 1. Quantitative analysis of Survivin in conditioned medium of cancer cell lines by ELISA

Cell type	Cell lines	pg ml^{-1}	s.e.m.	Concentrated (pg ml^{-1})	s.e.m.
Cervical cancer	HeLa	ND	—	330.4	11.4
	HeLa-S	94.0	42.1	746.1	1.4
	HeLa-S-Surv-WT	619.0	24.3	1158.3	32.1
	HeLa-S-Surv-T34A	35.2	0.01	81.9	0.01
	HeLa-S-survivin Antibody depletion	ND	—	ND	—
Pancreatic cancer	Capan1	246.9	13.6	1204.7	14.3
	Capan2	ND	—	26.1	8.6
	Panc1	20.4	42.9	174.7	47.1
Prostate cancer	PC3	73.3	32.9	888.3	5.0
	Du-145	136.9	37.4	771.9	24.0
	RWPE-2	29.0	16.0	337.6	7.0
Osteosarcoma	U2OS	ND	—	210.4	14.3
	SaOS2	405.4	50.0	858.3	50.7
Breast cancer	MCF-7	63.0	2.0	175.4	1.7
	HCC TN	ND	—	19.0	1.4
Acute monocytic leukaemia	MOLM-14	ND	—	273.3	23.3
	MV4-11	ND	—	347.6	15.9
Human bone marrow stroma	HBMS (normal)	ND	—	ND	—
Prostate epithelial cells	PrECs (normal)	ND	—	ND	—
Prostate stromal cells	PrSCs (normal)	ND	—	ND	—
Peripheral blood mononuclear cells	PBMCs (normal)	ND	—	ND	—
Media control	Media control	ND	—	ND	—
Spiked media control	0.1% lysed cells	ND	—	3.2	0.7
	1.0% lysed cells	77.6	1.1	121.9	2.0
	10% lysed cells	259.7	8.3	596.1	37.0

ND ¼ none detected.

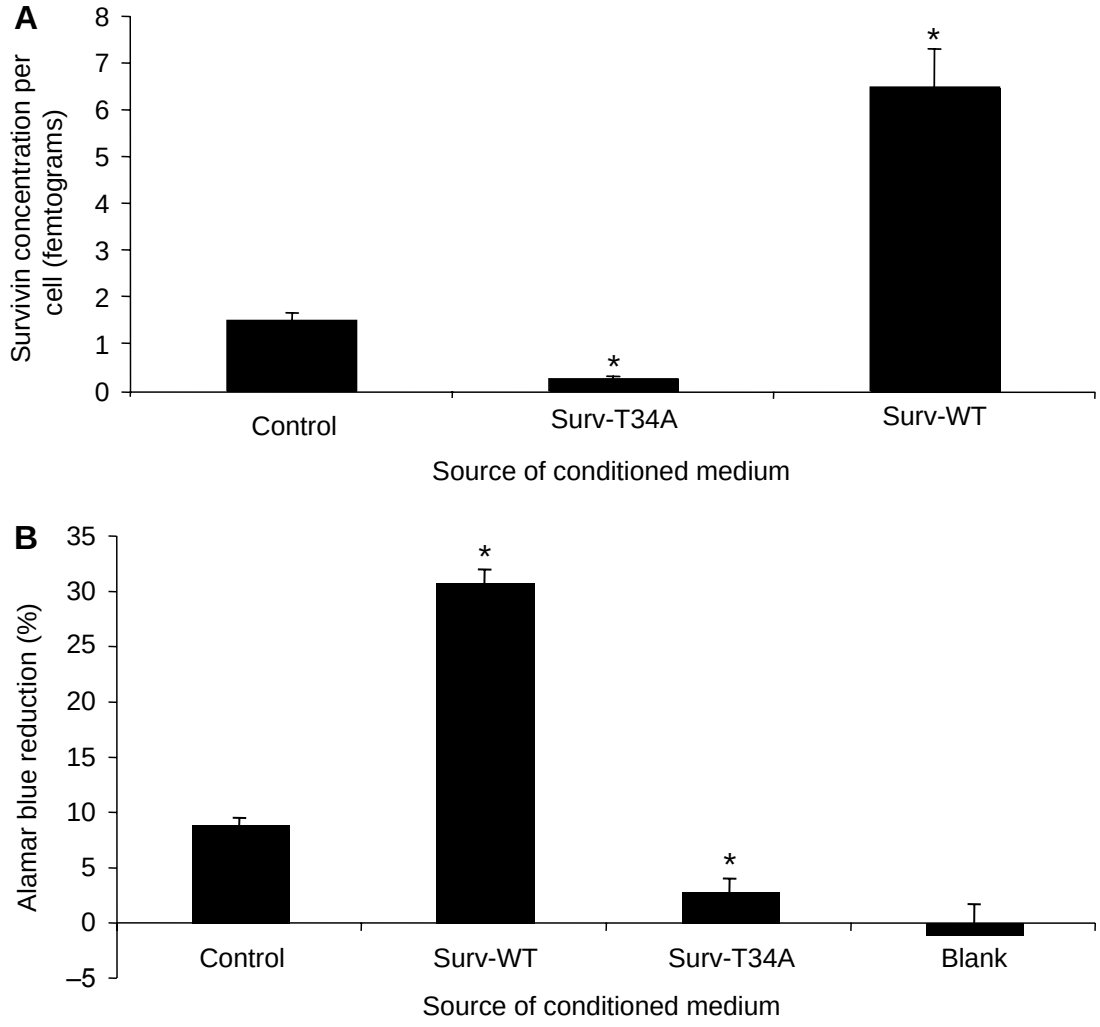

Figure 6. Comparison of extracellular Survivin protein concentrations and cellular metabolism of the cervical carcinoma HeLa cells that were stable transfectants of the Survivin-WT (Surv-WT) or mutant survivin T34A (Surv-T34A). (**A**) ELISA-defined Survivin concentrations were measured in medium taken from the different stable HeLa cells lines as described in Materials and methods section. For each cell line, the Survivin protein concentration was divided by the number of cells defined by Trypan blue exclusion at the time of medium collection. (**B**) Alamar Blue fluorimetry was conducted in the same three cell lines to determine if the stable expression of Survivin or its mutant would affect the cell growth and metabolism. Measurements were also conducted in medium containing no cells (blank), which would represent no growth or metabolism. Data are the mean±s.e. of three independent experiments (*P<0.001) as compared to the control.

protein. Previous work performed on neutrophils showed Survivin not entering the cells but binding to the surface (Mera, Magnusson et al. 2008). It was therefore necessary to perform confocal microscopy to ensure Survivin's relocalization within the cells. Taking photographs at 1 μm increments throughout the cell (shown schematically in Figure 10K) indicated that in these HeLa cells, Survivin was not only inside the cells but also relocalized within the cytoplasm and to the nucleus (Figure 10).

Relationship Between Survivin Absorption, Surv-T34A-induced Apoptosis, and Surv-WT-induced Cell Cycle Progression

Consistent with the data presented in Figure 7A, HeLa cells grown in CM taken from stable Surv-T34A cells experienced enhanced numbers of hypodiploid cells, that is, sub-G1 DNA content or apoptotic cells (Figure 11A, inset) by DNA content analysis and flow cytometry. Furthermore, Trypan blue exclusion and colony formation assay evaluation of these same cells showed a time-dependent, marked reduction in live cell numbers and live cell colonies in the Surv-T34A-CM-grown HeLa cells compared with cells grown in control medium or the CM from the Surv-WT cells (Figures 11A and B and Figure 9). This cell number reduced even further after 48 h as shown by the live cell colonies in the Surv-T34A-CM-grown HeLa cells, a finding that is consistent with what has been recorded by our own group and others (Mesri, Wall et al. 2001, O'Connor, Wall et al. 2002, Wall, O'Connor et al. 2003, Ogura, Watanabe et al. 2008) that the dominant-negative Survivin mutant T34A induces as caspase-dependent apoptosis. In contrast, Trypan blue exclusion and colony formation assay evaluation of Surv-WT-CM-grown cells showed a time-dependent, marked increase in live cell numbers (four times) and live cell colonies (approximately two times), in comparison with cells

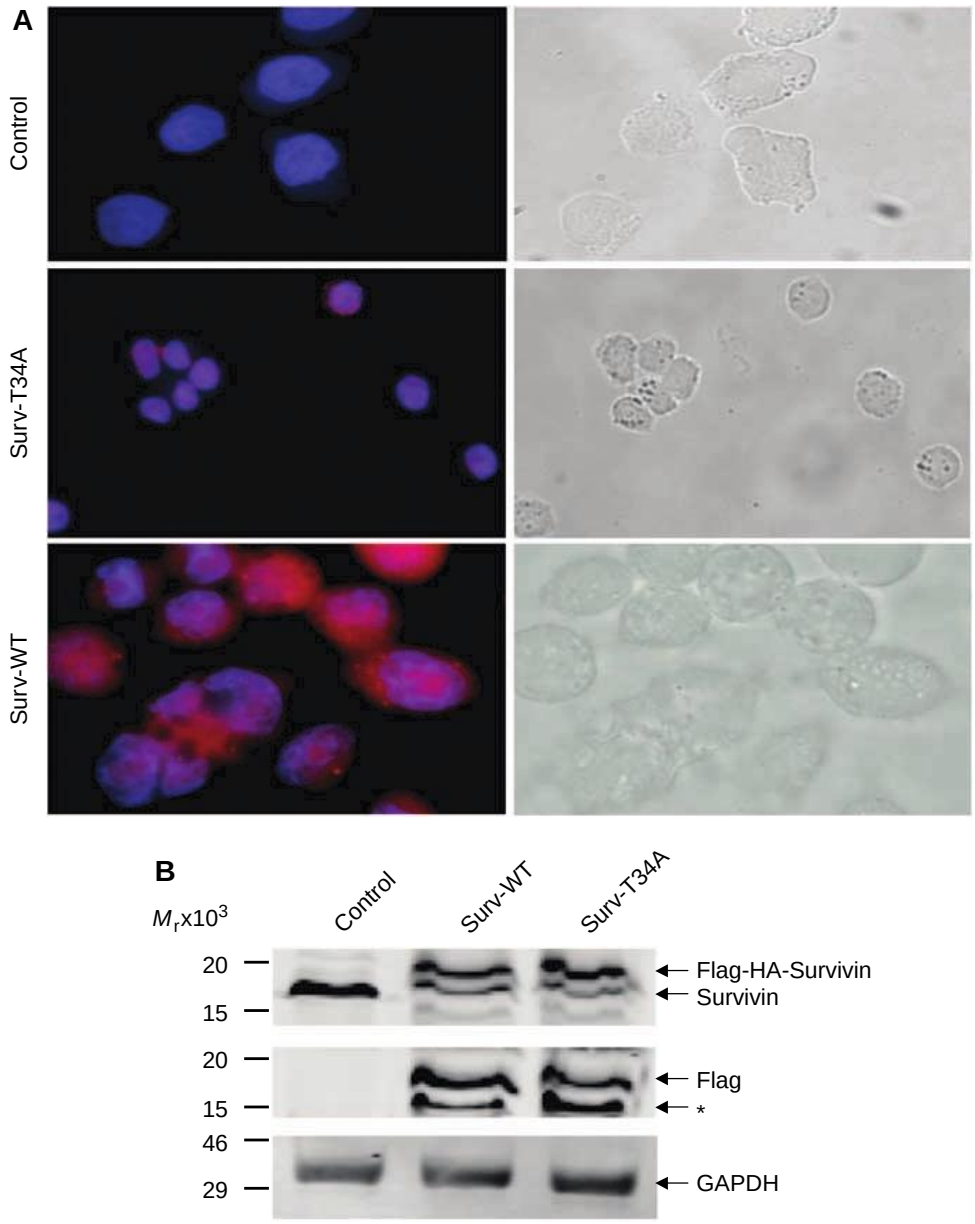

Figure 7. HeLa cells incubated in Surv-WT- or Surv-T34A-conditioned medium take up the Flag-HA-Survivin fusion protein. (**A**) HeLa cells were cultured with control-, Surv-WT-, or Surv-T34A-conditioned medium for 24 hrs. Cells were fixed and stained with antibodies to HA, magnification × 1000. Insets are western blots taken from cell lysates of these cells probed with antibodies to HA. Phase-contrast panels show the T34A-associated shrunken phenotype and early apoptotic bodies in comparison with the control or WT-treated cells. (**B**) Western blot analysis using antibodies to both Survivin and Flag shows the retarded ~18 kDa band of the Flag-HA-Survivin fusion protein. Control lysates only show the presence of the 16.5 kDa endogenous Survivin. Molecular weight markers in kilodaltons (kDa) are shown on the left.

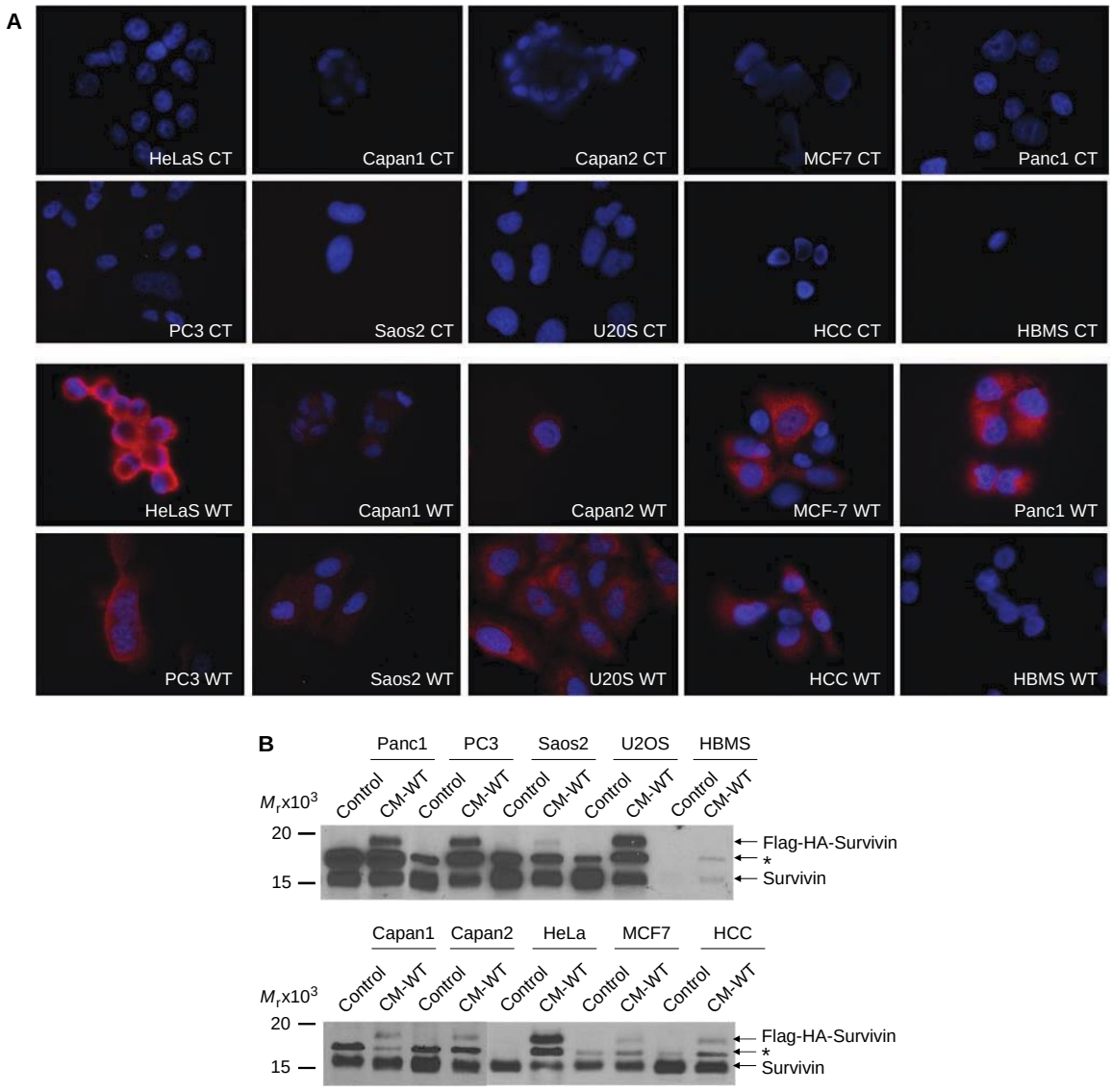

Figure 8. Cancer cells but not normal cells incubated in Surv-WT-conditioned medium take up the Flag-HA-Survivin fusion protein. (**A**) HeLa, Capan1, Capan2, MCF-7, Panc1, PC3, SaOS2, U2OS, HCC, and HBMS cells were cultured with control- or Surv-WT-conditioned medium for 24 h. Cells were fixed and stained with antibodies to HA, magnification × 1000. (**B**) Western blot analysis using antibodies to Survivin shows the retarded ~18 kDa band of the Flag-HA-Survivin fusion protein only in those lysates from the cells grown in conditioned medium. A third band, labeled*, often appears after probing with the antibodies Survivin whose identity is yet unknown to us. Molecular weight markers in kilodaltons (kDa) are shown on the left.

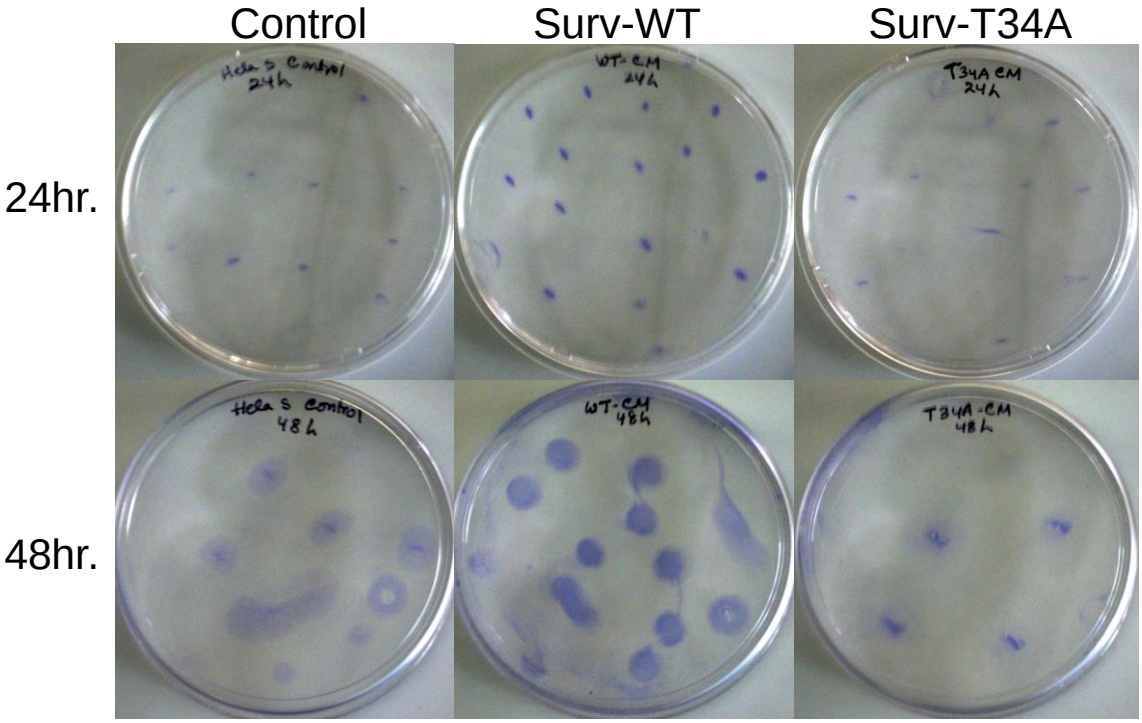

Figure 9. Surv-T34A conditioned medium induces tumor cell apoptosis while Surv-WT conditioned medium promotes cell growth. Cervical Carcinoma HeLa cells grown in the presence of either Surv-T34A or Surv-WT conditioned medium for 24 and 48 hours, were fixed with methanol and stained with crystal violet allowing visualization of the enhanced cellular proliferative effects of Surv-WT or the killing/growth repressive effects of Surv-T34A. Plates shown are representative of one of two independent experiments with comparable results.

grown in control medium or the CM from the Surv-T34A cells (Figures 11A and B and Figure 9).

To further evaluate the cell-cycle progression effects observed in the Surv-WT-CM-grown cells, we mimosine synchronized HeLa cells for 16 hrs., which effectively arrested cells in the G_0/G_1 phase of the cell cycle. Mimosine was then removed and the cells were incubated in the absence or presence of Surv-WT-CM. Cells were then harvested at 0, 3, 6, 9, and 12 hrs. time points following CM addition and cells were analyzed by DNA content analysis and flow cytometry. In comparison with cells grown in control medium, Surv-WT-CM-grown cells had a 10 – 12% increase in S-phase growth in cells harvested after 6 hrs. and a 10% increase in the number of cells in G_2/M by 12 hrs. (Figure 11C). These findings are consistent with those published indicating that Survivin displays both anti- apoptosis and promotion of mitosis in cancer cells (Li, Ambrosini et al. 1998, O'Connor, Grossman et al. 2000, Chao, Su et al. 2007). We are further encouraged as our CM containing fusion proteins may prove useful for the future study of Survivin trafficking and T34A therapy in vivo.

Recombinant Survivin Protein Lacks the Function of Survivin-containing CM

Recombinant Surv-WT and Surv-T34A proteins were incapable of affecting cell growth or apoptosis in our hands (Figure 12). It is uncertain whether bacterial-derived Survivin proteins are folded or modified properly for recognition by cellular mechanisms of uptake. To test this hypothesis further, we evaluated recombinant Survivin, transferrin, and CTB proteins for cellular uptake using in-cell western assay

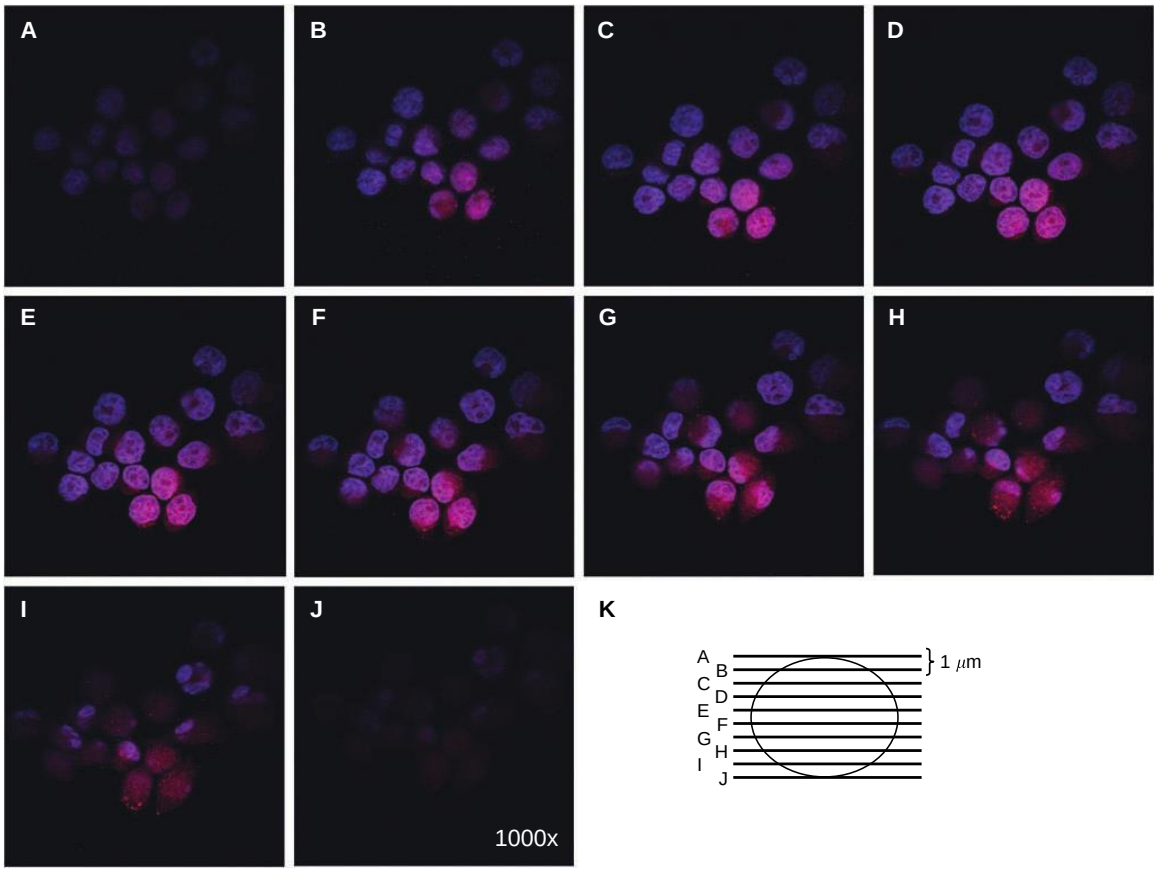

Figure 10. Confocal microscopy indicates that Survivin protein is taken up by cancer cells and is not merely binding to the cell surface. Cells were fixed and stained with antibodies to HA, with pictures taken on a *z*-scale of 1 μm, magnification × 1000. (A–J) are *z*-scale pictures taken at 1μm intervals as represented in (K).

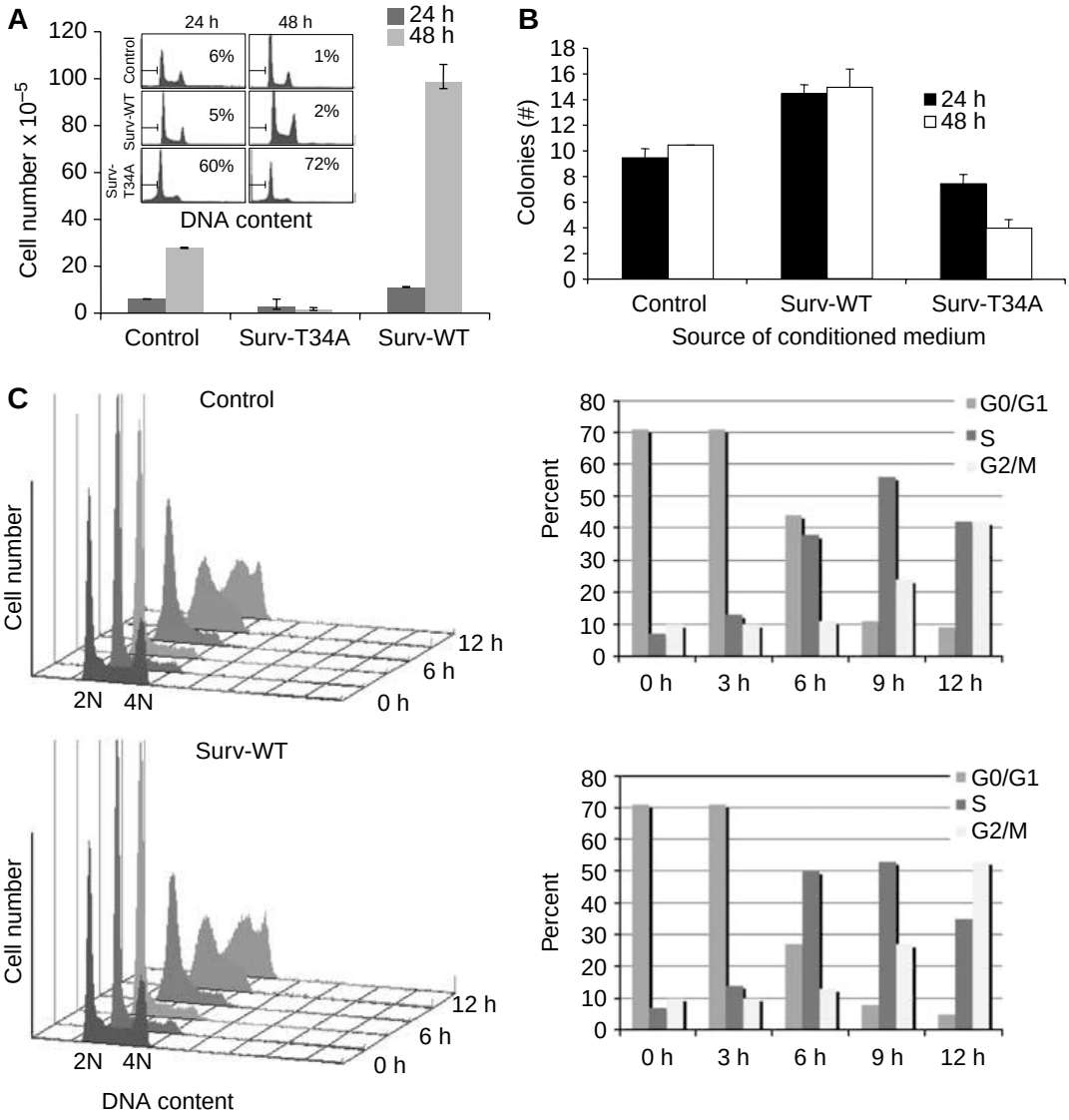

Figure 11. Surv-T34A-conditioned medium induces tumor cell apoptosis whereas Surv-WT-conditioned medium promotes cell growth. (A) Cervical carcinoma HeLa cells were grown in the presence of either Surv-T34A- or Surv-WT-conditioned medium for 24 and 48 hrs., harvested and analyzed for DNA content by propidium iodide staining and flow cytometry (inset) or Trypan blue exclusion. Percentages of apoptotic cells with hypodiploid (sub-G1) DNA content are indicated per each condition tested. Data are representative of one of two or three independent experiments with comparable results. (B) HeLa cells, grown as described, were fixed with methanol and stained with crystal violet allowing visualization of the enhanced cellular proliferative effects of Surv-WT or the killing/growth-repressive effects of Surv-T34A. The data presented are the mean colony formation efficiency ± S.D. in three experiments. (C) Mimosine-synchronized HeLa cells were harvested at the indicated increasing time intervals after release and analyzed for DNA content by propidium iodide staining and flow cytometry. A full color version of this figure is available at the *British Journal of Cancer* online.

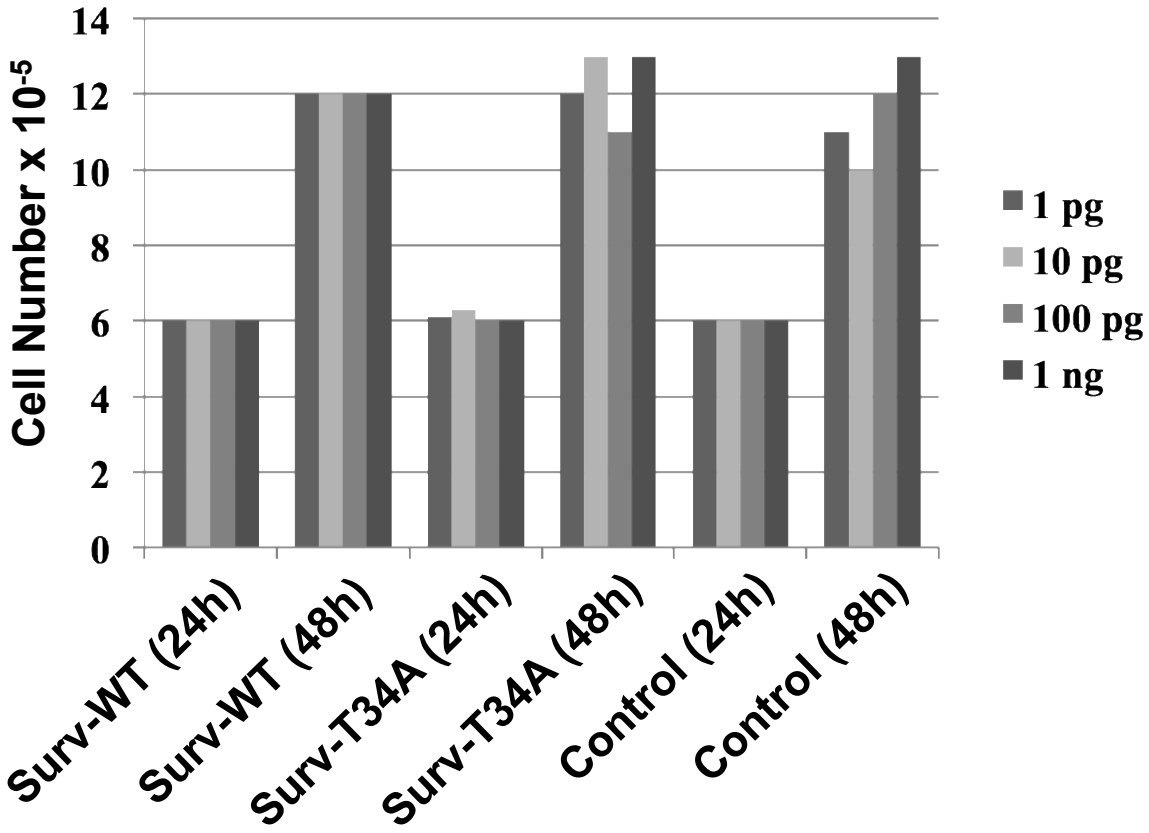

Figure 12. Recombinant Survivin does not affect cell growth (Surv-WT) or life (Surv-T34A). HeLa cells were grown in increasing quantities of recombinant Surv-WT or Surv-T34A protein (1pg, 10pg, 100pg and 1ng) for 24 and 48 hours. Cells were harvested, stained with Trypan blue and counted.

methods (Egorina, Sovershaev et al. 2005). HeLa cells were incubated with increasing concentrations of labeled recombinant protein, and the amount of protein endocytosed after a 12 hrs. incubation was measured as shown in Figure 13. The amount of transferrin and CTB proteins endocytosed increased in a dose-dependent manner. However, Survivin recombinant protein (BIRC5) at the same concentrations was not endocytosed (Figure 13). Transferrin (Sandvig and van Deurs 1990) and CTB (Gizurarson, Tamura et al. 1992, Frey, Giannasca et al. 1996, Hansen, Dalskov et al. 2005) recombinant proteins were previously described as substrates for endocytic uptake and were used as positive controls.

Characterization of Tumor Cell Apoptosis Induced by Surv-T34A *in vitro*

We have shown in Figures 7A and 11A that incubating HeLa cells with Surv-T34A-CM resulted in apoptosis. Cytofluorometric quantification results showed that in comparison with control media or Surv-WT-CM treatment, Surv-T34A treatments induced robust results within 24 to 48 hrs.. Previous studies performed, using an adenovirus-encoding T34A mutant, resulted in apoptosis that was associated with the mitochondrial release of cytochrome *c*, cleavage of caspase-9, and the processing of caspase-3 (Mesri, Wall et al. 2001). Further analysis of Surv-T34A-CM-induced apoptosis, using a commercially available caspase activity assay, revealed that Surv-T34A induced cleavage of caspase-9 and caspase-2 without cleaving the substrates specific for caspase-3 or caspase-8 (Figure 14). The antibiotic and protein kinase inhibitor, staurosporine, was used as a positive control for caspase-3 activity. Surv-WT-CM had no effect on the caspase activity under evaluation. In addition, western blotting provided biochemical data that supported

studies recorded above for caspase-2, caspase-3, and caspase-9. Using antibodies that were specific for the full-length, non-cleaved forms of caspase-2, caspase-7, and caspase-9 and the cleaved form of caspase-3 protein and staurosporine again as a positive control, we found that Surv-T34A induced the parallel time-dependent reduction in caspase-2, caspase-7, caspase-9, and cleavage of the DNA repair protein PARP (Figure 15). In all cases the pan-caspase inhibitor Z-VAD-FMK was able to inhibit the activity of Surv-T34A. In contrast, Surv-WT again had no observable effects on caspase cleavage when cells were treated with it alone for up to 48 hrs.. Caspase-3 was robustly cleaved after 6 hrs. of staurosporine treatment. However, this cleavage product was absent after 24 hrs. indicating a complete cleavage and degradation of caspase-3. Concomitant with this cleavage was the significant level of PARP cleavage recorded at this 24 hrs. time point.

Loss of Mitochondrial Membrane Potential After Treatment with Surv-T34A CM

Mitochondrial membrane potential ($\Delta\psi$m), a phenomenon readily measured using the mitochondrial dye JC-1 and fluorescence-activated cell sorting analysis (Salvioli, Ardizzoni et al. 1997), was evaluated for the possibility that Surv-T34A was stimulating apoptosis by inducing depolarization within the mitochondria. Under normal circumstances, JC-1 accumulates in the inner mitochondrial membrane in which it oligomerizes and fluoresces red. A reduction in $\Delta\psi$m results in diffusion of the dye from the mitochondria and a subsequent reduction in the mean red fluorescent intensity. As expected, control HeLa cells showed a high mean red fluorescence (set to ~96%) after staining with JC-1 (Figure 16). After treatment with staurosporine, we found that the mean red fluorescence of the mitochondria dropped rapidly within 6 h, indicating that the $\Delta\psi$m had

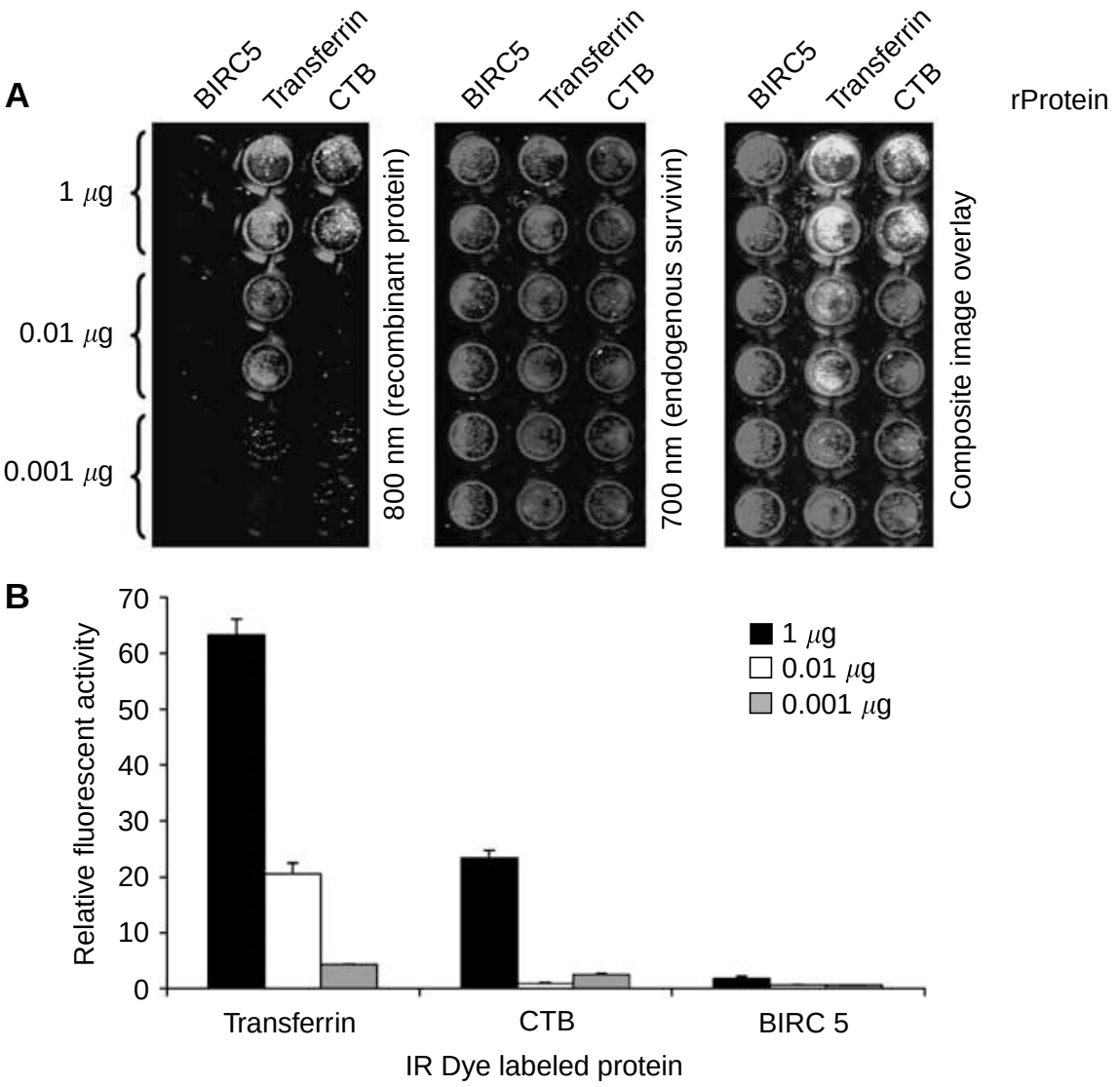

Figure 13. Recombinant Survivin protein is unable to cross the HeLa cell membrane. (A) Detection of cellular recombinant proteins. These images show a portion of a 96-well plate. The right panel is a composite image showing the fluorescence in both the 700- and 800-nm detection channels. Duplicate rows of microplate wells loaded with 0.001, 0.01, or 1.0 μg of protein are shown. The middle panel shows detection of endogenous Survivin protein, which was used as a loading control. The left image shows detection of increasing amounts of recombinant Survivin (BIRC5), transferrin, and CTB. (B) Quantification of fluorescence. Recombinant protein signal has been normalised using the endogenous Survivin protein signal from each well to correct for well-to-well variation in cell number. Recombinant protein (rProtein).

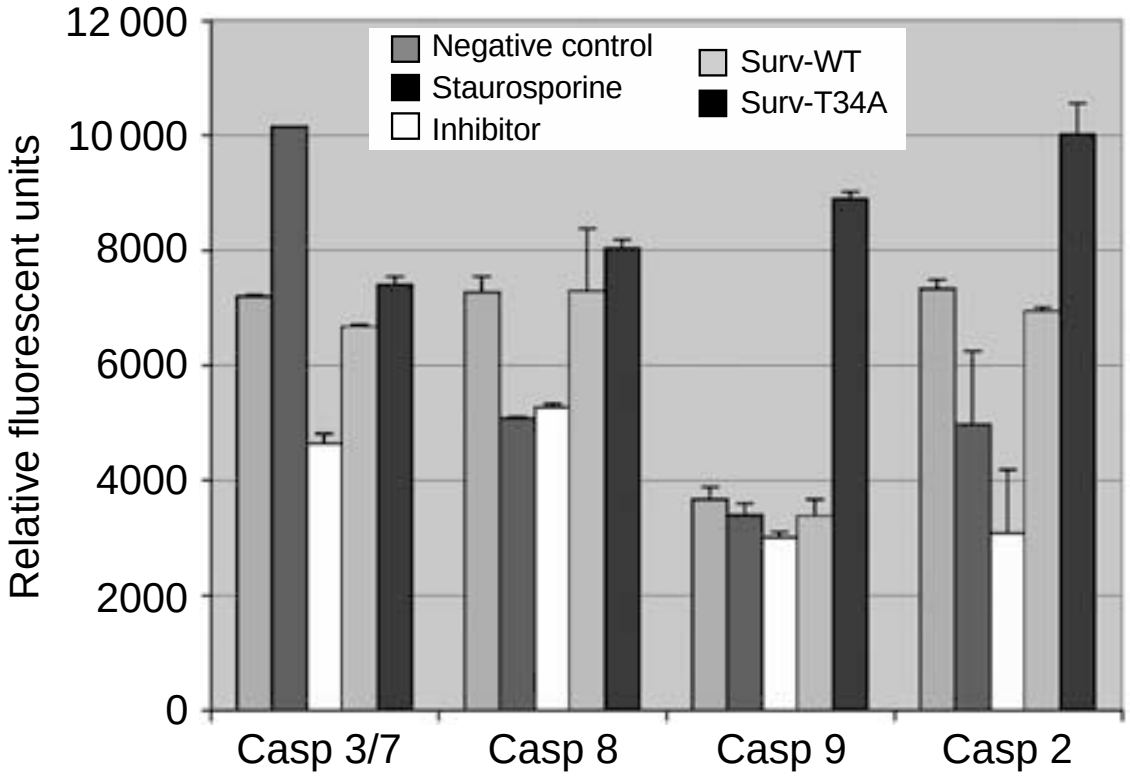

Figure 14. Conditioned medium containing Survivin affects the intrinsic apoptotic pathway. Caspase catalytic activity. Aliquots of HeLa cells grown in the presence of either Surv-T34A- or Surv-WT-conditioned medium were assayed for caspase-3/7, caspase-8, caspase-9, and caspase-2 activity by Ac-DEVD-AMC, IETD-AMC, LEHD-AMC, and VDVAD-AMC hydrolysis, respectively, in the presence or absence of the caspase-3 inhibitor, DEVD-CHO; the caspase-8 inhibitor, IETD-fmk; the caspase-9 inhibitor, LEHD-fmk; or the caspase-2 inhibitor, VDVAD-fmk. Inhibitor (INH), staurosporine (STR). Data are the mean±s.e. of two independent experiments.

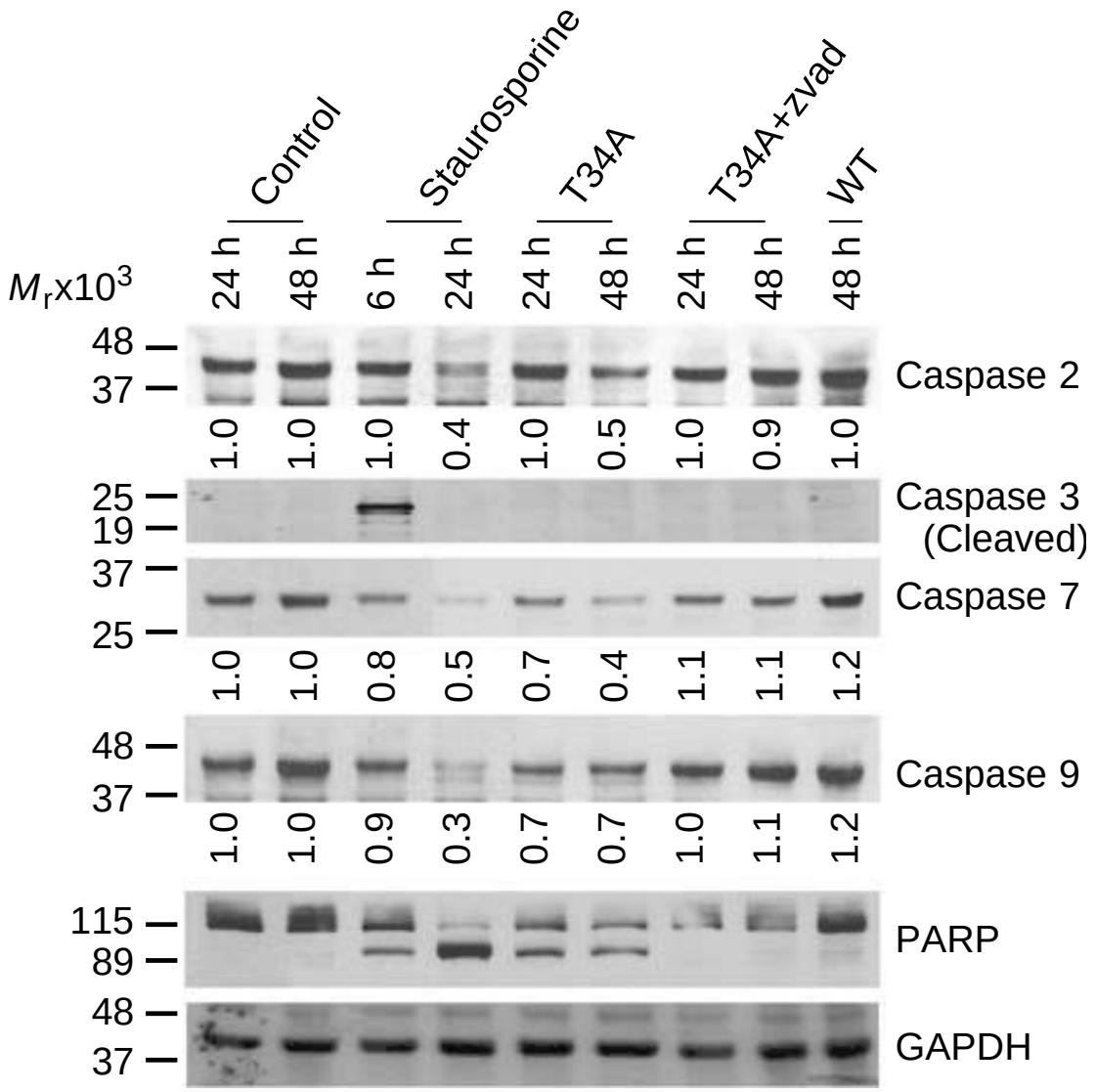

Figure 15. Caspase activation. Detergent-solubilized extracts of HeLa cells grown in Surv-WT- or Surv-T34A-conditioned medium were analyzed at the indicated time intervals for reactivity with antibodies for caspase-2, caspase-3, caspase-7, caspase-9, PARP, and GAPDH (loading control) by western blotting. Molecular weight (*M*r) markers in kilodaltons are shown on the left. Densitometric analysis was conducted on caspase-2, caspase-7, and caspase-9 with their densities as compared to GAPDH located below each blot.

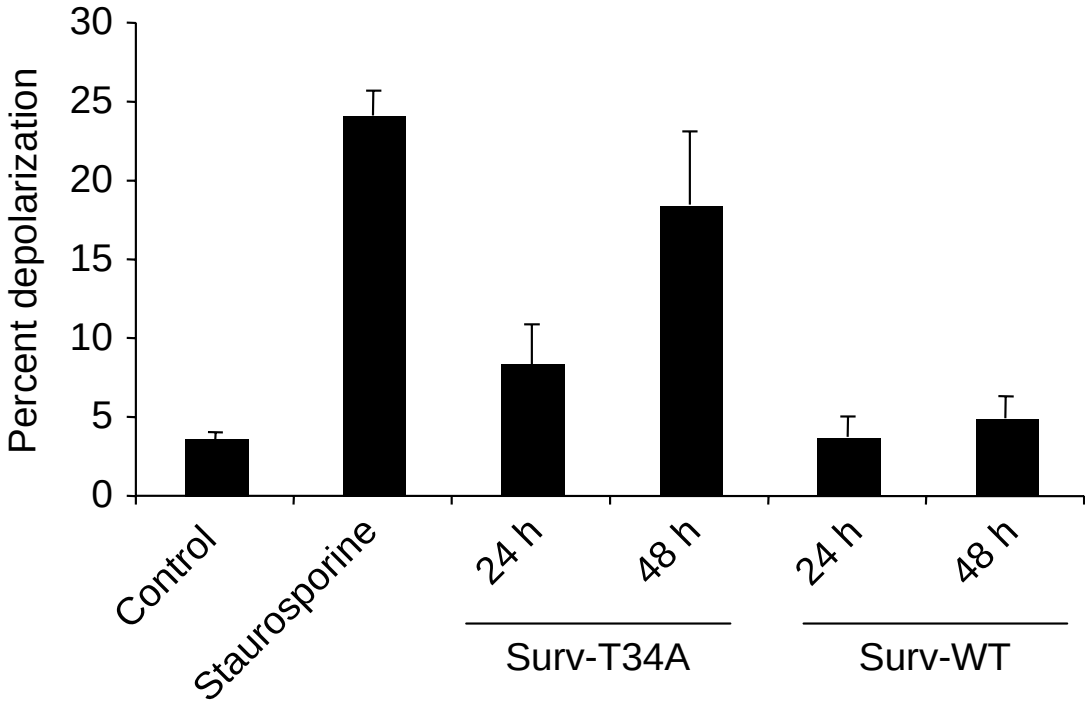

Figure 16. Mitochondrial depolarization. The experimental conditions are the same as in B, except that HeLa cells grown in Surv-WT- or Surv-T34A-conditioned medium were analyzed at the indicated time intervals using the mitochondrial membrane potential monitoring agent JC-1. A reduction in $\Delta\psi$m results in diffusion of the dye from the mitochondria and a subsequent reduction in the mean red fluorescent intensity, which is quantifiable using flow cytometry. Staurosporine treatment for 6 h is used as a positive control. Data are the mean±s.e. of two independent experiments.

collapsed (Figure 16). HeLa cells treated with Surv-T34A, but not Surv-WT, showed a time-dependent reduction in the mean red fluorescence intensity. However, in repeated experiments this was always slower than that seen for staurosporine (Figure 16). This may indicate that mitochondrial depolarisation due to Surv-T34A is a secondary event and not the primary effect of Surv-T34A-induced killing or that mitochondrial depolarization is one of many factors involved in Surv-T34A-induced killing.

Effect of Surv-T34A and Surv-WT and UV Radiation on Tumor Cell Apoptosis

Incubation of HeLa cells with UV radiation resulted in enhanced numbers of hypodiploid cells, which are sub-G1 DNA content or apoptotic cells (Figure 17A) by DNA content analysis and flow cytometry. In comparison to control cells that were set at ~5%, UV radiation treatment resulted in 35% of the cells exhibiting a sub-G1 DNA content. However, cells grown for 24 hrs. in Surv-WT-CM before UV treatment were fully protected from the effects of the UV energy. In turn, Surv-T34A enhanced the killing effects of the UV and resulted in 75% of the cells exhibiting a sub-G1 content (Figure 17A).

Effect of Surv-T34A and Surv-WT and Chemotherapeutic Drugs on Tumor Cell Apoptosis

To establish that the effects recorded in HeLa cells were not specific for this cell line alone or for UV radiation, we selected human pancreatic adenocarcinoma (Capan1 and Capan2) cell lines to study drug sensitivity and responsiveness in the presence or absence of Surv-WT- and Surv-T34A-CM. Effects of taxol, 5-FU, Ad, and CDPD were studied. Capan1 cells exhibited an enhanced susceptibility to taxol, 5-FU, Ad, CDPD-induced cell

death after the addition of Surv-T34A-CM, whereas Surv-WT-CM-incubated cells were protected from taxol, 5-FU, and CDPD-induced cytotoxicity (Figure 17B). Only a modest protection was recorded against Ad. Interestingly, Surv-WT-CM treatment protected Capan2 cells from the cytotoxic effects of 5-FU and again only a modest protection was recorded against taxol, Ad, and CDPD. Treatment of Capan2 cells with Surv-T34A-CM was as effective as taxol, more effective than CDPD and 5-FU and less effective as Ad alone. Surv-T34A when combined with taxol showed an additional cytotoxicity that may be quantitatively additive at best. When combined with CDPD, Surv-T34A did not provide further enhancement to the killing effects over that of Surv-T34A alone, which was substantial, and when paired with 5-FU or Ad, the individual cytotoxic effects of Surv-T34A were reduced (Figure 17C).

Survivin Containing CM Induces Cancer Cell Invasion *in vitro*

To determine the function of secreted Survivin in regulating cancer cell invasion through collagen, we plated HeLa cells on collagen-coated inserts in the presence of control, Surv-WT-, or Surv-T34A-CM. Cells were grown for 24 h, dissociated, lysed, and then evaluated for invasion by measuring the fluorescence emission (CyQuant GR dye). HeLa cells exhibited an average fourfold increase in cell invasion when grown with Surv-WT-CM in the lower chamber as compared to control medium (Figure 18). Surv-T34A-CM invasion levels were little changed from that of the control as were those cells that were treated with medium that had been depleted of Survivin.

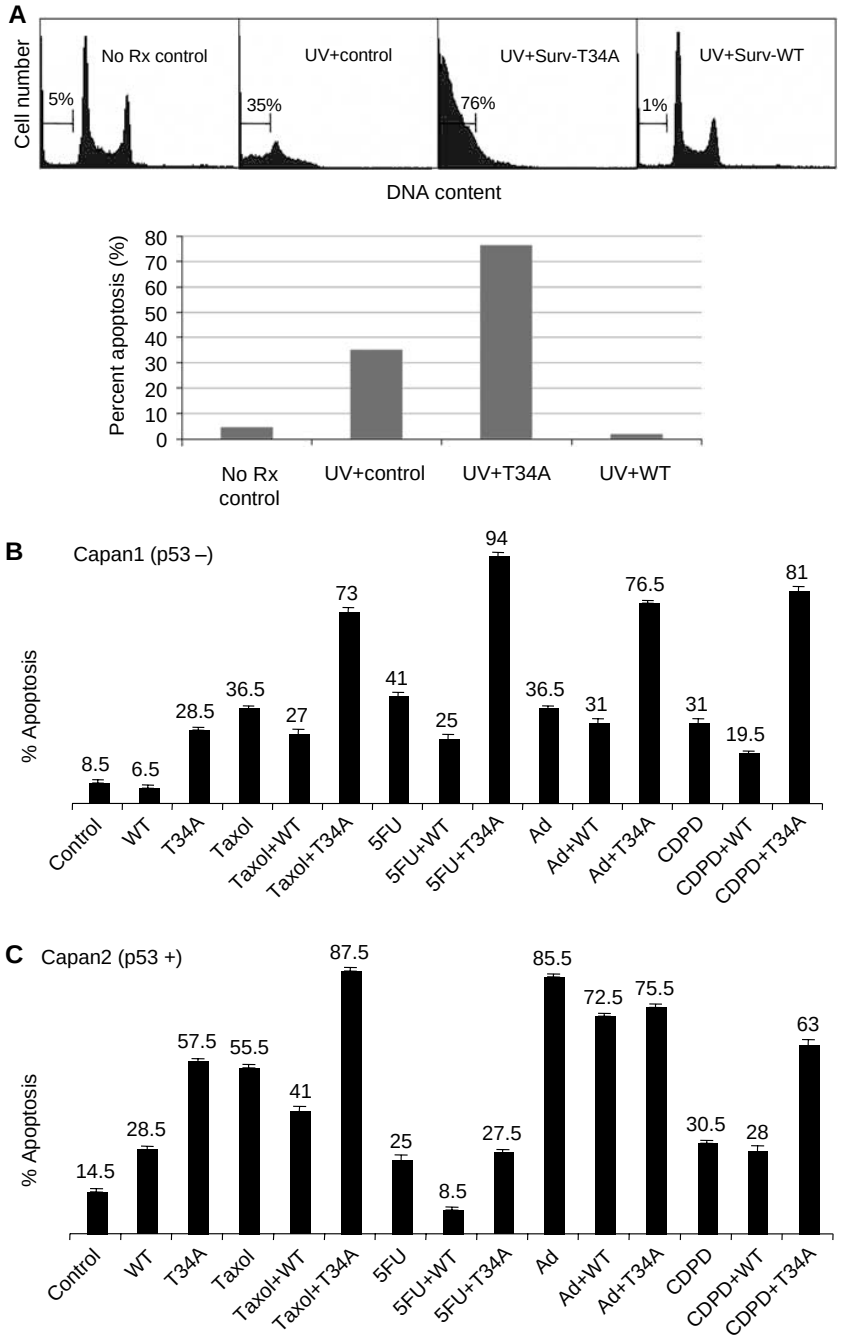

Figure 17. Effect of Surv-WT and Surv-T34A and UV (A) or chemotherapeutic drugs (B and C) on tumor cell apoptosis. Aliquots of HeLa cells were treated with UV (50 J/m^2) (A) or Capan1 (B) and Capan2 (C) cells were treated with Adriamycin (Ad; 100 nm), 5-fluorouracil (5-FU; 200 nm), taxol (4 μm), or cisplatin (CDPD; 2 μm) in the presence or absence of Surv-WT- or Surv-T34A-conditioned medium. Cells were harvested at 48 hrs. and apoptosis was determined by DNA content analysis and flow cytometry. The time point of combination treatments is 48 hrs. after the addition of the drug following a 24 hrs. pretreatment in the conditioned medium. Data are representative of one of two independent experiments with comparable results.

Discussion

The growth and spread of cancer depends as much on the host response to the tumor as on the biological characteristics of the tumor itself. The IAP Survivin has been shown aberrantly expressed in cancer but undetectable in normal differentiated adult tissue. It has been implicated in both control of apoptosis (Ambrosini, Adida et al. 1997, Adida, Crotty et al. 1998)and regulation of cell division (Deveraux and Reed 1999, Li, Ackermann et al. 1999, Gianani, Jarboe et al. 2001). . Indeed, Survivin expression has been shown to be cell cycle regulated with its highest expression in G2/M phase, and it has been shown that much of its function comes from its subcellular localization with residences in the cytosol, nucleus, and mitochondria (Li, Ackermann et al. 1999, Li 2003). Recent reports on patients with rheumatoid arthritis have described a new Survivin localization and the possibility that it may also function in the extracellular space (Bokarewa, Lindblad et al. 2005, Mera, Magnusson et al. 2008). Moreover, in these patients, extracellular Survivin was directly linked to an erosive course of joint disease and its origin was prescribed to peripheral blood leukocytes where its expression is constitutive. Furthermore, Survivin was shown to bind extracellularly to neutrophils inducing the p38-MAPK-dependent expression of α- and β-integrins (Mera, Magnusson et al. 2008). Taken together, the finding of a new and physiologically functional pool of Survivin provides new insight into the function of IAPs as well as other relevant tumor-associated proteins in the tumor microenvironment.

In this study we have shown that an extracellular form of Survivin is detectable in CM taken from cancer cells. We also demonstrate that unlike Survivin from the rheumatoid arthritis study, Survivin from cancer cells is readily taken up by cancer cells rather

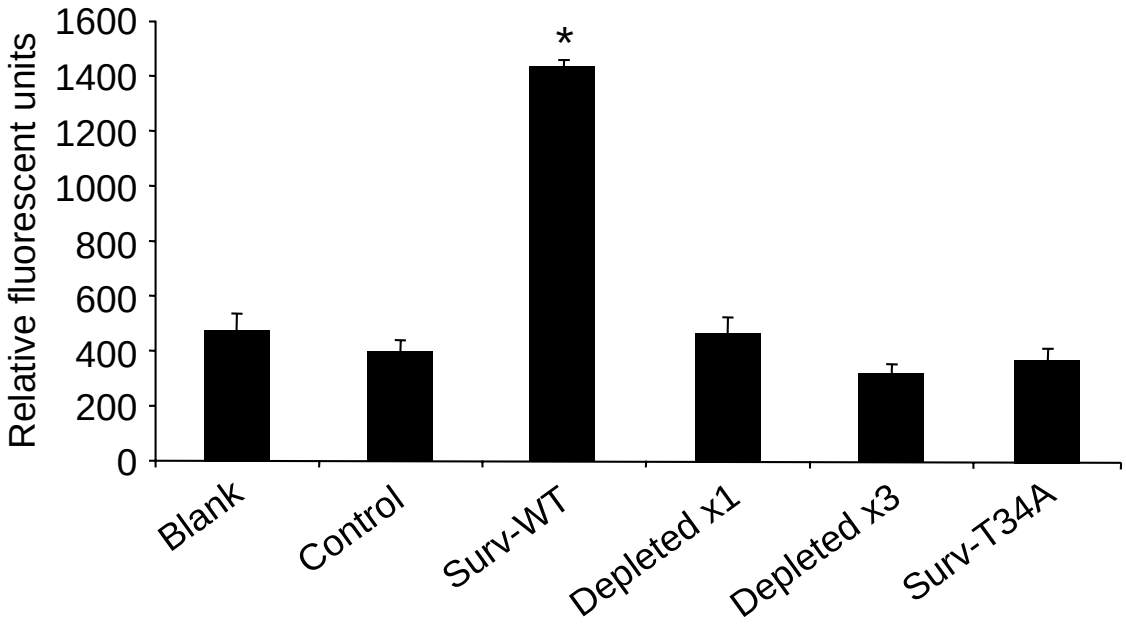

Figure 18. Effect of Surv-WT and Surv-T34A on tumor cell invasion. HeLa cells (1×10^5 cells) were seeded into the upper well of the FIA chamber in 100 μl culture medium. Cells were treated with the presence of Surv-WT- or Surv-T34A-conditioned medium, medium depleted of Survivin using two rounds of fusion protein antibodies (anti-Flag and anti-HA) and one or three rounds of Survivin antibodies in the lower chamber and incubated at 37°C in an atmosphere of 5% CO2. After 24 hrs. of incubation, we lysed and quantitatively analyzed fluorescence intensity of invasive cells that passed through the collagen layer onto the surface of the fluorescence-blocking membrane, giving relative fluorescent units (RFU). Data are the mean±s.d. of four independent experiments (*P<0.01).

than binding only to their surface (Mera, Magnusson et al. 2008). Finally, upon incubation in medium containing this extracellular pool of Survivin, cancer cells responded physiologically, becoming more resistant to therapy, proliferating more rapidly, and having an increased metastatic potential. These findings lead us to believe that extracellular Survivin may modulate, *in vivo*, the tumor microenvironment for the purpose of tumor evolution and these findings in part may be responsible for the observations that patients with a high expression of Survivin protein display advanced disease, high-grade disease, abbreviated survival, resistance to therapy, and accelerated tumor recurrences (Andersen, Svane et al. 2007).

We have also shown that stable cells expressing the previously described (Mesri, Wall et al. 2001, O'Connor, Wall et al. 2002) pro-apoptotic Survivin mutant Thr34 → Ala (Surv-T34A) release a form of Survivin that is able to disrupt the cell cycle and cause a caspase-dependent, mitochondrial depolarization-associated apoptosis. When combined with UV radiation or with the chemotherapeutic agents taxol, Ad, CDPD, or 5-FU, Surv-T34As pro-apoptotic ability was amplified. It was not surprising that these modalities were differentially enhanced or inhibited by Surv-T34A or Surv-WT because they have varied mechanisms of action. Surv-T34A was found to be as effective in enhancing therapy-induced cell killing as Surv-WT was in protecting against therapy-induced cell killing. These differential effects seemed not to follow the p53 status of the cell lines tested as Surv-WT was as effective at protecting those cells lacking the tumor suppressor and transcription factor as it was for those cells positive for p53. It has been shown that Survivin gene transcription is repressed by WT p53 (Li 2003)but little is known of p53's ability to modify the protein's function. A more careful evaluation of this finding is re-

quired.

The precise mechanism by which this Survivin mutant causes apoptosis is still incompletely understood. The mutations that allowed for the generation of stable cells as well as their ability to induce apoptosis through the caspase-2- and caspase-9-dependent pathways are currently being evaluated in our laboratory. Importantly, a quantifiable highly stable extracellular form of Survivin has been identified. This novel T34A Survivin molecule is readily taken up by cancer cells after which characteristics of apoptotic cell death are measurable. Further, it potentiates the killing effects of other cancer therapeutics. It is our belief that this extracellular Surv-T34A molecule has significant potential as a novel therapeutic or as part of a novel therapeutic and possible immunotherapy regimen.

Tumor invasion and metastasis are very complex processes that are yet very poorly understood but account for 90% of all human cancer deaths (Sporn 1996, Hanahan and Weinberg 2000). The mechanism involves coupling and uncoupling of cells to their microenvironment, activation of extracellular proteases, and modulation in tethering proteins such as cadherins, β-catenin, and integrins (Hanahan and Weinberg 2000). As Survivin has already been shown to modulate the integrin proteins CD49d, CD11b, and CD11c on leukocytes (Mera, Magnusson et al. 2008), we hypothesized that it may also modulate the invasive capabilities of cancer cells. In our hands Surv-WT-CM stimulated a 3- to 4-fold increase in collagen invasion in comparison with the control medium alone or medium containing Surv-T34A. Medium depleted of Survivin using antibodies first that recognized the fusion proteins Flag and HA and then one and three rounds of anti-Survivin antibody depletion, completely inhibited Survivin-induced collagen invasion

thus placing Survivin as a central player in this increased metastatic potential.

Stress-induced cancer cell death by means of apoptosis, necrosis, and autophagy occurs continually during tumor development and progression (Fonseca and Dranoff 2008). What the ramifications of this to the tumor microenvironment is only beginning to be evaluated. It is believed that the capture of apoptotic cells serves as an immunoregulatory event that helps maintain tolerance whereas necrotic cells evoke an inflammatory cascade that mobilizes effector responses. On the basis of our findings, we postulate that many of these released cancer antigens could in addition to modulating immune function prime the cells within the tumor microenvironment for the phenotypes associated with tumor invasion and metastasis that are listed above.

Survivin expression in CM was measured extracellularly using ELISA. It is not known whether extracellular Survivin originates from dead cells or is the subject of active secretion. Comparing Survivin quantity from 1 to 10% lysates to that of CM argues for active secretion rather than necrosis as SaOS2, Capan1, and HeLa-S-Surv-WT cells all have equal or more Survivin protein but yet have no dead or dying cells as determined visually and by Trypan blue exclusion. Importantly, Survivin found extracellularly creates a more erosive disease in patients with rheumatoid arthritis (Bokarewa, Lindblad et al. 2005) and in our cancer cell culture models, parameters that are considered clinically unfavorable in cancer patients: proliferative advantage, enhanced resistance to therapy, and enhanced metastatic potential. Investigating recombinant Survivin's ability to recapitulate the effects recorded using CM-containing Survivin proved unprofitable. From these experiments, we conclude that the Survivin recombinant protein, which has been severed from its GST handle, is unable to induce similar phenotypes as the CM-

containing Survivin as it is unable to enter the cancer cells. Further studies using circular dichroism mass spectrometry will be required to compare these two forms of Survivin to define differences in structure and or posttranslational modifications that may aid Survivin in binding with its yet to be defined receptor or trafficking proteins.

A deeper understanding of the mechanisms underlying this extracellular pool of Survivin, the possible ramifications for tumorigenesis, and the possibility of exploiting it for therapy are only a few of the many properties of this most fascinating of proteins that we are continuing to evaluate.

Acknowledgements

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CHAPTER THREE

ENHANCEMENT OF GEMCITABINE SENSITIVITY IN PANCREATIC ADENOCARCINOMA BY NOVEL EXOSOME-MEDIATED DELIVERY OF THE SURVIVIN-T34A MUTANT.

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Running title: Exosome therapy and pancreatic cancer.

Adapted from Aspe, J.R., Diaz Osterman, C.J., Jutzy, J.M.S., DeShields, S., and Wall, N.R. Journal of Extracellular Vesicles (2014)

Abstract

Background

Current therapeutic options for advanced pancreatic cancer have been largely disappointing with modest results at best, and though adjuvant therapy remains controversial, most remain in agreement that Gemcitabine should stand as part of any combination study. The inhibitor of apoptosis (IAP) protein Survivin is a key factor in maintaining apoptosis resistance, and its dominant-negative mutant (Survivin-T34A) has been shown to block Survivin, inducing caspase activation and apoptosis.

Methods

In this study, exosomes, collected from a melanoma cell line built to harbor a tetracycline-regulated Survivin-T34A, were plated on the pancreatic adenocarcinoma (MIA PaCa-2) cell line. Evaluation of the presence of Survivin-T34A in these exosomes followed by their ability to induce Gemcitabine-potentiative cell killing was the objective of this work.

Results

Here we show that exosomes collected in the absence of tetracycline (tet-off) from the engineered melanoma cell do contain Survivin-T34A and when used alone or in combination with Gemcitabine, induced a significant increase in apoptotic cell death when compared to Gemcitabine alone on a variety of pancreatic cancer cell lines.

Conclusion

This exosomes/Survivin-T34A study shows that a new delivery method for anti-cancer proteins within the cancer microenvironment may prove useful in targeting cancers of the pancreas.

Keywords: Survivin, Survivin-T34A, exosomes, pancreatic cancer, Gemcitabine

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States, with an average 5-year survival rate of 5% for all stages of the disease (Kleeff, Michalski et al. 2006). Pancreatic cancer has an annual mortality rate of approximately 95% with over 250,000 patients dying worldwide (Jemal, Bray et al. 2011). Pancreatic cancer exhibits no clear early warning signs or symptoms and it is often detected after it is too late for pancreatic resection. Currently, if diagnosed at early stages, surgical resection remains the most efficacious treatment and offers the best patient outcome. However, only 20% of pancreatic cancer patients meet these criteria (Muller, Friess et al. 2008). There is a need to discover and implement new therapies or therapeutic combinations that increase the survival rate of those afflicted with this pancreatic cancer. Gemcitabine remains the gold-standard for chemotherapy (Ueno, Kiyosawa et al. 2007). However, while Gemcitabine has shown significant benefit in clinical applications, its ability to effectively impact pancreatic cancer is limited. Currently, combinatory treatments using Gemcitabine and other therapeutics have shown no significant improvements in survival rates (Oettle and Neuhaus 2007, Reni, Cereda et al. 2007, Galloway, Aspe et al. 2009). However, the cancer research field is moving rapidly towards combinatorial therapies including combined multiple chemotherapy drugs (Vishnu, Colon-Otero et al. 2012), radiation with chemotherapy (Galloway, Aspe et al. 2009), and virotherapy with chemotherapy (Karapanagiotou, Roulstone et al. 2012). The IAP Survivin seems to be one of the key players in resistance to many of these cancer therapies (Asanuma, Kobayashi et al. 2002, Li 2003, Kami, Doi et al. 2005) and therefore a strategy to inhibit its action, when combined with standard treatment options may prove beneficial.

Survivin is a possible prognostic marker for pancreatic cancer patients (Satoh, Kaneko et al. 2001, Sarela, Verbeke et al. 2002, Kami, Doi et al. 2004). Though expressed in most human cancers and present during embryonic and fetal development (Adida, Crotty et al. 1998), its aberrantly high protein expression in cancer cells and low level of expression in most normal tissues makes Survivin an important anticancer target (Fukuda and Pelus 2006). Survivin overexpression in cancer has been described as a predictive factor in determining response to chemotherapy and radiotherapy (Galloway, Aspe et al. 2009, Zhu, Wang et al. 2011, Shen, Zheng et al. 2012). Survivin reduces cell death induced by several anticancer agents including paclitaxel, etoposide and tumor necrosis factor alpha. Conversely, inhibition of Survivin reduces tumor growth potential and sensitizes tumor cells to many of the same chemotherapeutic agents (Mita, Mita et al. 2008).

We have reported a marked enhancement of Survivin's role in therapeutic resistance to both chemo- and radiotherapy in pancreatic cancer (Galloway, Aspe et al. 2009). Reduction of Survivin levels and/or inhibition of the protein's anti-apoptotic properties may assist in making cancer cells more susceptible to existing (and future) therapeutic regimens. Successful strategies against intracellular Survivin include molecular antagonists such as antisense oligos, RNA inhibition, Survivin-specific cytolytic T cells, the nonphosphorylatable dominant negative Survivin mutant $Thr^{34}\rightarrow$ Ala (T34A), and most recently, binding interface mimetics (Olie, Simoes-Wust et al. 2000, Andersen, Pedersen et al. 2001, Grossman, Kim et al. 2001, Kanwar, Shen et al. 2001, Mesri, Wall et al. 2001, Wall, O'Connor et al. 2003, Plescia, Salz et al. 2005, Andersen, Svane et al. 2007).

The loss of phosphorylation at the Survivin Thr^{34} site is significant as it results in the dissociation of the caspase-9/Survivin protein complex, leading to antitumor effects (Grossman, Kim et al. 2001, Peng, Yang et al. 2008, Li, Zhao et al. 2009, Shen, Liu et al. 2009, Aspe and Wall 2010). Mesri, *et al.* employed Survivin-T34A treatment *in vivo* via adenoviral vectors, with their results yielding only modest levels of success (Mesri, Wall et al. 2001). A better delivery method will need to be utilized if this Survivin inhibitor is to prove efficacious *in vivo*.

Conditioned media (CM) collected from cervical, pancreatic, prostate, breast cancer, osteosarcomas, leukemia cell lines (Khan, Aspe et al. 2009) and CM collected from a Survivin-T34A overexpressing HeLa cell line provided evidence that functional Survivin can be found extracellularly in the CM. We have recently shown extracellular Survivin to reside in small 50-100 nm vesicles called exosomes (Khan, Jutzy et al. 2011). Exosomes have been described as a pivotal mechanism in the multicellular organism for cell to cell communication as they allow for cells to exchange information through transferal of soluable factors such as proteins, RNAs and miRNA (Bobrie and Thery 2013). More recently, exosomes have been exploited for cancer immunotherapy as there may be opportunity to adapt them as drug delivery vehicles for therapeutic intervention(S, Mager et al. 2013). The typical exosome is 50-100 nm in size and originates intracellularly, displaying MHC class I and class II, heat shock proteins, tetraspanin proteins and in our hands inhibitor of apoptosis (IAP) proteins (Tan, De La Pena et al. 2010, Khan, Jutzy et al. 2011).

We showed in this undertaking Survivin-T34A CM eliciting anticancer effects such as induced apoptosis with loss of mitochondrial potential. The goal of the present

study was to determine if the cells engineered to overexpress the Survivin-T34A dominant-negative mutant would produce a functional, exosomally packaged, Survivin-T34A which when used in combination with Gemcitabine might significantly enhance the death of pancreatic cancer cells, *in vitro*.

Materials and Methods

Cell Cultures

Pancreatic adenocarcinoma (MIA PaCa-2) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM, supplemented with 100 units of penicillin, 100 μg/mL streptomycin, 300 μg of L-glutamine, 10% heat inactivated FBS (ATCC), and 2.5% horse serum. Exosomes were isolated from YUSAC 2, a melanoma cell line obtained from Dr. Doug Grossman at the Huntsman Cancer Institute in Salt Lake City, Utah. The two cell line derivatives from YUSAC 2 were designed to overexpress either Survivin-WT (4C7 cells) or Survivin-T34A (F5C4 cells) in the absence of tetracycline (tet), otherwise only normal endogenous levels of Survivin are produced. YUSAC 2 cells were maintained in DMEM (CellGro, Manassas, VA) supplemented with 100 units of penicillin, 100 μg/mL streptomycin, 300 μg of L-glutamine, 5% newborn calf serum (Thermo Scientific HyClone, Rockford, IL), 0.5 μg/mL tetracycline (tet-off system), 1.5 mg/mL Geneticin G418 (Teknova, Holister, CA), and 2 mM NaOH. YUSAC 2 cells were grown to 60% confluency in the presence of tetracycline in order to establish a healthy monolayer culture. Subsequently, cells were washed carefully twice in PBS followed by the addition of media in the absence of tetracycline. All cells were grown at 37 °C in a humidified

atmosphere containing 5% CO_2.

Gemcitabine Treatment

Gemcitabine (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO and added to cells simultaneously with exosome treatment. Final DMSO concentration was 0.03%. Final Gemcitabine concentration used was 10 μM. The cells were returned to the incubator and harvested after 24, 48, or 72 hrs. for apoptosis analysis by flow cytometry.

Exosome Isolation and Quantification

Exosomes were isolated from conditioned media (CM) as we have previously reported (Khan, Jutzy et al. 2011). In brief, the culture media was collected from cells in culture after 24 hrs.. The CM was centrifuged at 10,000 × *g* for 10 min at 4 °C to pellet the minor amounts of cells and cell debris. CM was filtered through 0.22 µm syringe followed by centrifugation at 100,000 × *g* for 18 hrs. at 4 °C on a 30% sucrose cushion (Thery, Amigorena et al. 2006). The sucrose cushion containing exosomes was collected and washed with PBS followed by additional centrifugation at 100,000 × *g* for 18 hrs. at 4 °C. Exosome pellets were collected and stored at -80 °C. Exosome quantification was accomplished using acetylcholinesterase assay, according to the protocol of Lancaster and Febbraio (Lancaster and Febbraio 2005). Briefly, acetylcholinesterase activity was employed to determine the presence of exosome vesicles. 40 µL of the exosome fraction was suspended in 110 µL of PBS. 37.5 µL of this PBS-diluted exosome fraction was then added to individual wells on a 96-well flat bottom plate. 112.5 µL of 1.25 mM acetylthiocholine and 150 µL of 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) were added to PBS-

diluted exosomes. The change in absorbance at 412 nm was monitored every 5 min for 30 min. The data presented represents acetylcholinesterase enzymatic activity after a 30 min incubation. Exosome and whole cell lysate (WCL) protein was quantified using the BCA assay on a μQuant microplate spectrophotometer (Bio-Tek, Winooski, VT) and analyzed using KC Junior Software (Bio-Tek).

Apoptosis and Cell Cycle Analysis

Sub-confluent cultures of the pancreatic cancer cells were incubated with vehicle (DMSO), Gemcitabine (0 to 500 μM) and/or exposed to exosomes treatment (0-1500 μg/mL total protein, exosomes were sterilized using 0.22μm syringe filter) for 0, 24, 48, and 72 hrs. at 37°C. Cells were harvested, prepared, and analyzed for DNA content using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Li, Ackermann et al. 1999, Khan, Jutzy et al. 2011). The distribution of cells in the different phases of the cell cycle was analyzed from DNA histograms using BD CellQuest software (Becton Dickinson and Company, San Jose, CA).

Western Blot Analysis

Whole cell lysates (WCLs) were prepared as previously described (Galloway, Aspe et al. 2009) and quantified as described above. Proteins (30 μg) were separated using 12 or 15% Bis-Tris polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad) and probed using 1-5 μg/ml of the following antibodies: rabbit polyclonal anti-Survivin (Novus, Littleton, CO), rabbit anti-ppSurvivin-Thr^{34} (Novus), rabbit polyclonal anti-β-actin (BioLegend, San Diego, CA), and mouse polyclonal anti-

LAMP-1 (Cell Signaling Technologies, Beverly, MA). Secondary antibodies (IR-Dye-conjugated) were goat anti-rabbit and goat anti-mouse immunoglobulin (LI-COR, Lincoln, Nebraska) used at a 1:5000 dilution. Membranes were blocked for 1 hr. using blocking reagent purchased from LI-COR. Membranes were incubated overnight in primary antibody followed by three 15 minute PBS-Tween wash steps and a final 1 hour secondary antibody incubation followed again by three 15 minute PBS-Tween washings. Immunoreactive bands were detected using the Odyssey imaging system (LI-COR). β-actin or LAMP-1 was used as Western blot loading controls for either cell lysates or exosomal protein, respectively.

Statistical Analysis

Multiple comparisons among different groups were calculated by using Multiple Analysis of Variance (MANOVA) as we have done in the past (Khan, Jutzy et al. 2012). Student t-test (two-tailed) was used to evaluate the significance of changes between control groups and experimental groups. Probability values $P<0.05$ were considered statistically significant.

Results

The Presence of Survivin-T34A Induces Time-dependent Apoptosis

The melanoma cell line, YUSAC 2, was engineered to overexpress WT Survivin (4C7) or mutant T34A (F5C4), as previously reported (Grossman, Kim et al. 2001). The YUSAC 2 cells were continuously treated with 0.5 μg/mL of tetracycline (tet) to prevent overexpression of Survivin-WT (4C7 cells) or Survivin-T34A (F5C4 cells) in this tet-off

system. Cells were harvested 6, 12, 18 and 24 hrs. after removal of tet for analysis by Western blot and flow cytometry (Figure 19). Following tet removal, Survivin protein was concomitantly increased in both cell lines in a time-dependent manner (Figure 19A) as it has been previously reported (Grossman, Kim et al. 2001). Removal of tet from F5C4 cells resulted in increased apoptosis in a time-dependent manner, maximizing at 72 hrs., while no increase in apoptosis was recorded in the 4C7 cells treated under the same conditions (Figure 19B).

Survivin-T34A Overexpressing Cells Showed Decreased Phosphorylated Survivin in a Time-dependent Manner

4C7 and F5C4 cells were incubated in either the presence or absence of tet for 24 hrs., and levels of Survivin, phospho-Survivin and β-actin were analyzed by Western blotting. 4C7 and F5C4 cells expressed increased Survivin levels in the absence of tet compared to tet-treated control cells (Figure 20). Survivin phosphorylation at Thr^{34} was confirmed by Western blotting using a phospho-specific Survivin Thr^{34} antibody as has been previously shown (Grossman, Kim et al. 2001). Tet-free F5C4 cells showed a decreased phospho-Survivin band compared to tet-free 4C7 cells.

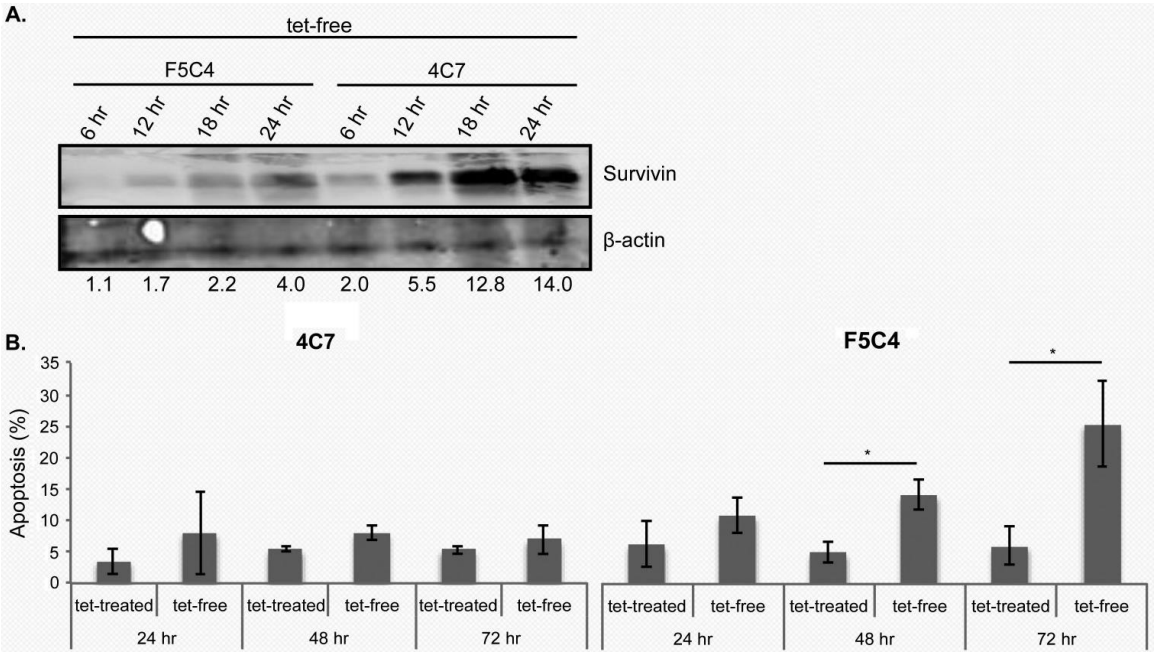

Figure 19. (A) Western blots of whole cell lysates of YUSAC 2 cell line derivatives. 4C7 or F5C4 cells were incubated in the presence or absence of tetracycline for 6, 12, 18, and 24 hrs., which in the absence of tetracycline will overexpress Survivin-WT or Survivin-T34A, respectively. Survivin is increased in a time-dependent manner when incubated in the absence of tetracycline. Beta-actin was used as the loading control and molecular weights (kDa) are shown on the left. Densitometry was undertaken to show the degree of Survivin increase. (B) Histogram representing the percentage of apoptosis using propidium iodide (PI) analysis by flow cytometry, 4C7 show similar apoptosis levels for both tet-treated and tet-free conditions. F5C4 cells have increasing apoptosis in a time-dependent manner when incubated in tet-free media. Data are the mean ± SD of three independent experiments (*$p<0.05$).

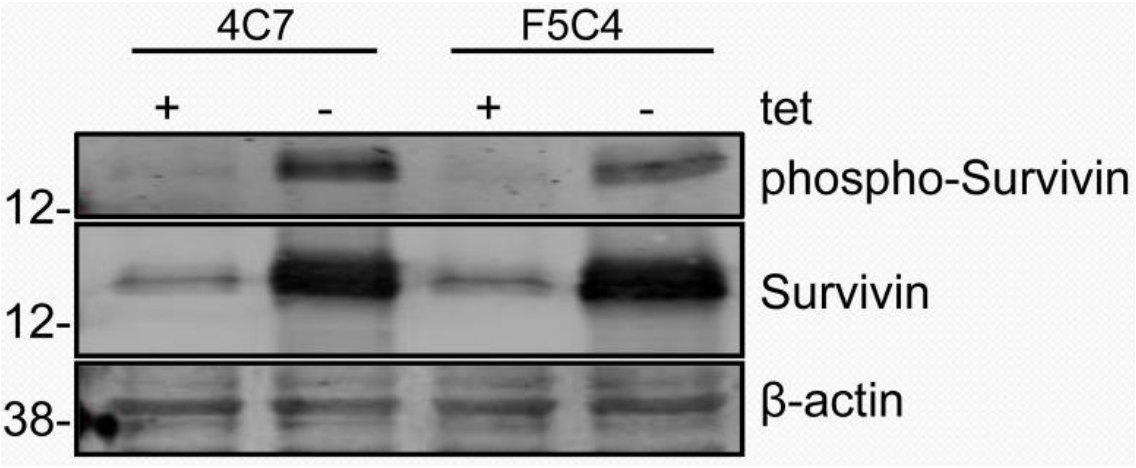

Figure 20. Western blot of whole cell lysates of YUSAC 2 cell line derivatives. 4C7 and F5C4 cells were incubated in the presence or absence of tetracycline for 24 hrs., which in the absence of tetracycline will overexpress wild-type or Survivin-T34A, respectively. 4C7 has increased Survivin in absence of tetracycline, which is concurrent with phosphorylated Survivin-Thr34. However, in the absence of tetracycline F5C4 also overexpresses Survivin, but it is not phosphorylatable at the Thr34 site.

YUSAC 2 Cells Release Survivin-containing Exosomes

In order to confirm that F5C4 cells release exosomes, we isolated exosomes by ultracentrifugation using a sucrose cushion as has been previously described (Thery, Amigorena et al. 2006, Khan, Jutzy et al. 2011). Following ultracentrifugation, exosome levels were analyzed using the acetylcholinesterase enzyme (AChE) activity assay (Figure 21A). No significant difference in exosome levels between tet-treated and tet-free cells was measured at 24 hrs., signifying that the number of exosomes released is independent of the tet-system.

Exosomal Survivin content was evaluated by Western blotting. Survivin expression was elevated after tet removal in exosomes from both 4C7 and F5C4 cells (Figure 21B). This was similar to observed levels of Survivin expression in whole cell lysates from YUSAC 2 cells. In order to evaluate Survivin-T34A presence in the exosomes released from F5C4 cells, we employed the phospho-specific Survivin Thr^{34} antibody for Western blots of these exosomes. Survivin was increased in the exosomes from tet-free F5C4 as was recorded in the immunoblots from the parent cells. However, there were no phosphorylated-Survivin bands from either exosomes of tet-treated or tet-free F5C4 cells (Figure 21C). The presence of tet-removal-enhanced Survivin, coupled with the absence of phosphorylated-Survivin, provides strong evidence that these exosomes contain the dominant-negative Survivin-T34A.

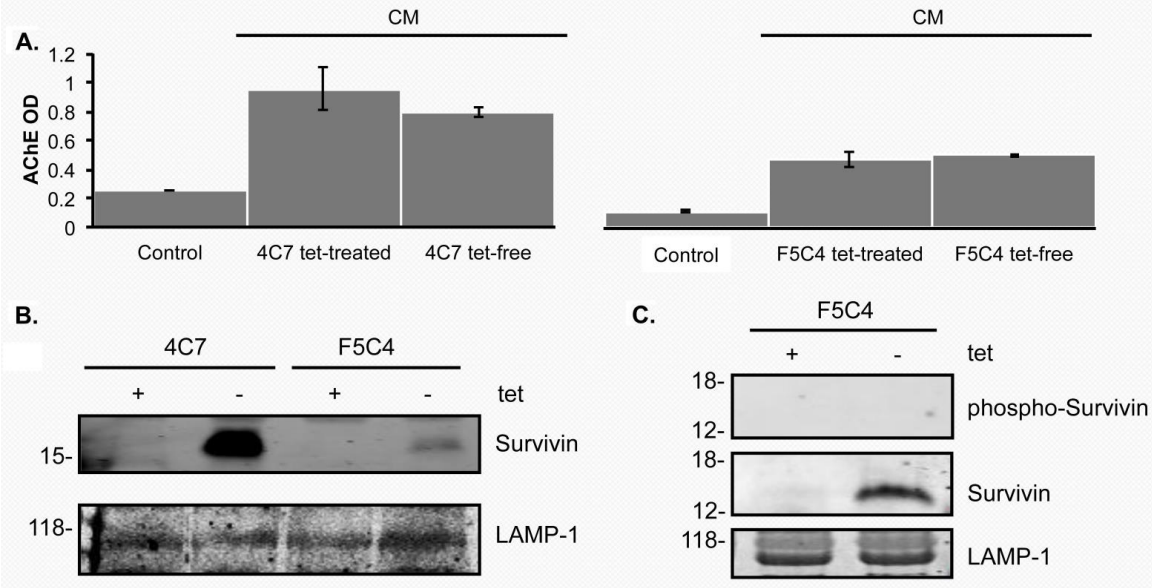

Figure 21. (A) Histogram representing an acetylcholinesterase enzyme activity assay to quantify exosome numbers relative to fresh complete media. Exosomes are present as represented by the graph when compared to control. There is no difference within the same cells when treated with tetracycline or without. Data are the mean ± SD of 3 independent experiments. (B) Both YUSAC 2-derived cell lines overexpress Survivin in the absence of tetracycline. (C) Western blots of proteins isolated from exosomes collected from F5C4 cells after incubation in the presence or absence of tetracycline for 24 hrs.. LAMP-1 was used as the loading control with molecular weights (kDa) shown on the left.

Exosomes Containing Survivin-T34A Induce Apoptosis in MIA PaCa-2 Pancreatic Cancer Cells

Exosomes isolated from tet-treated F5C4 (endogenous WT Survivin) cells were employed as the control for exosomes isolated from tet-free F5C4 cells (Survivin-T34A). There was no significant apoptotic induction (≤10%) after MIA PaCa 2 cells were treated with 200, 1000 or 2000 μg/mL of exosomes purified from tet-treated F5C4 cells after 24 and 48 hrs. (Figure 22). MIA PaCa 2 cells were then treated using exosomes containing Survivin-T34A (150 and 750 μg/mL based on total exosomal protein). Results showed little apoptosis (~5%) versus treatment by control exosomes (from tet-treated F5C4 cells) at 24 hrs. (Figure 22). However, using Survivin-T34A exosomes (1500 μg/mL), a marked 48 hrs. increase in apoptosis (30.5%) was measured.

Survivin-T34A Exosomes Enhance Gemcitabine Treatment on Pancreatic Cancer Cells

In order to investigate whether exosomes containing Survivin-T34A could enhance Gemcitabine-cytotoxicity, MIA PaCa 2 cells were treated with either exosomes containing Survivin-WT (200 μg/mL) or Survivin-T34A (150 μg/mL) or 10 μM Gemcitabine. Gemcitabine dosage levels conformed to the protocols previously established in our laboratory (Galloway, Aspe et al. 2009). Exosome concentrations were chosen that did not induce a measurable level of apoptosis (Figure 22) at 24 and 48 hrs. as an induced additive or potentiative response from the combination was the goal. We also measured apoptotic cell death after pancreatic cancer cells were treated with a combination of exosomes and Gemcitabine. At 24 hrs., no marked differences

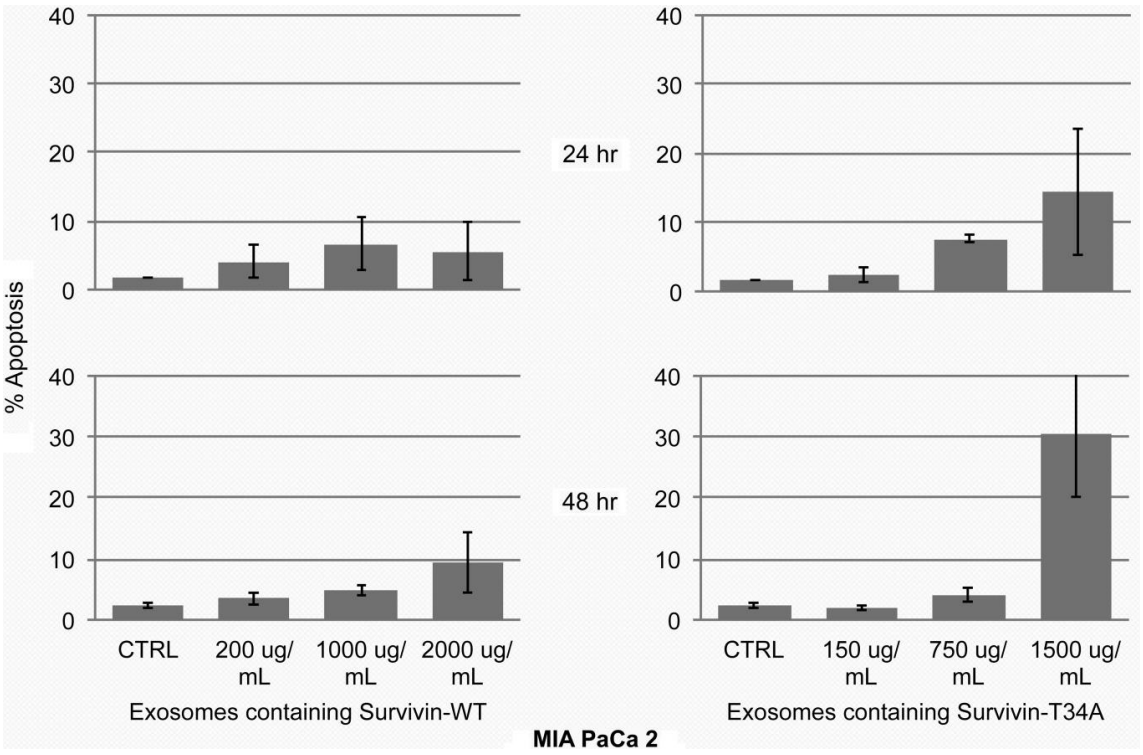

Figure 22. Histograms representing the percent apoptosis in MIA PaCa2 cells following exosome treatment for 24 and 48 hrs.. MIA PaCa 2 cells treated for 24 hrs. showed little to no increase in apoptosis after exosomes treatment. After 48 hrs. treatment, only 1500 μg/mL of exosomes containing Survivin-T34A induced apoptosis (30.5%). Percent apoptosis was determined from sub-G1 DNA content analyzing propidium iodide staining by flow cytometry. Data are the mean ± SD of 2 independent experiments.

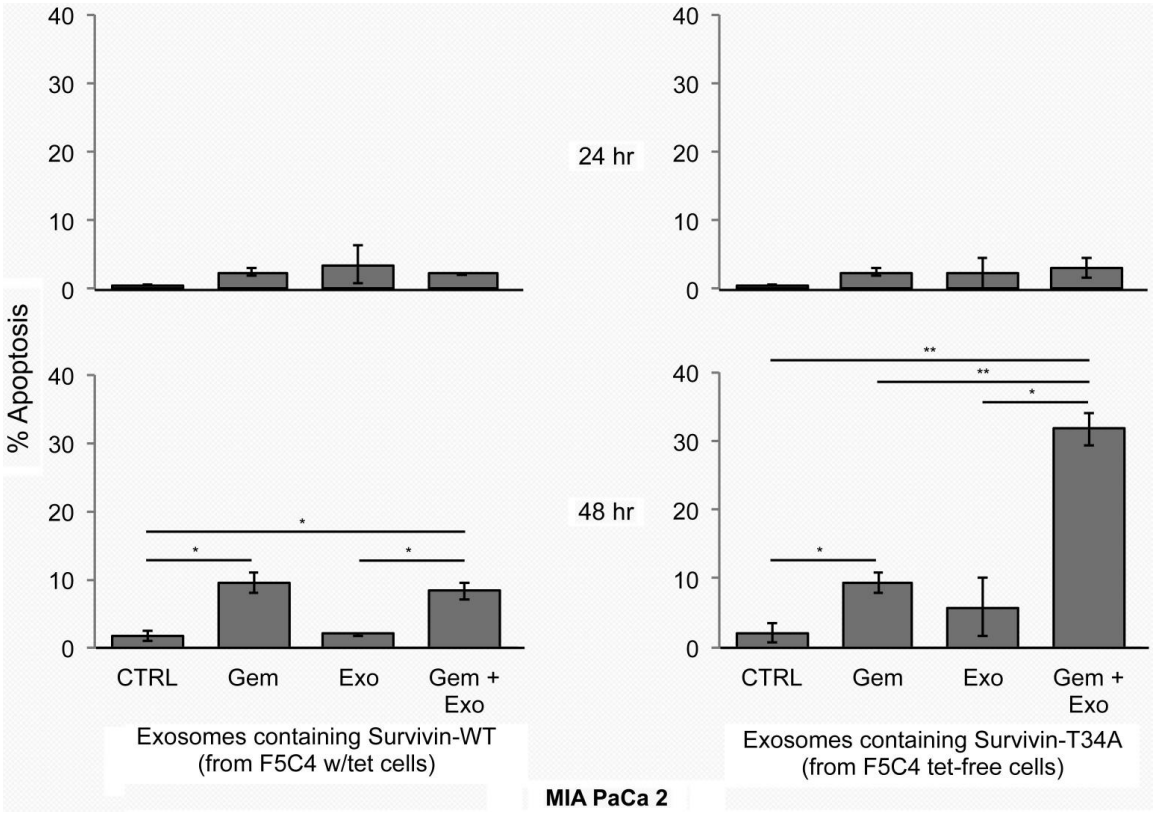

Figure 23. MIA PaCa 2 cells were treated with either 10 μM Gemcitabine, 150 or 200 μg/mL (total exosomal protein from F5C4), or the combination of both. MIA PaCa 2 cells treated for 24 hrs. showed little to no marked increase in apoptosis (<5%) after exosomes treatment. 10 μM Gemcitabine had modest apoptosis induction over control (~11% vs. 2%, respectively). The combination of 10 μM Gemcitabine with 150 μg/mL exosomes containing Survivin-T34A yielded 32% apoptosis (~30% greater than control) after 48 hrs. Percent apoptosis was determined from sub-G1 DNA content analyzing propidium iodide staining by flow cytometry. Data are the mean ± SD of 2 independent experiments (*$p<0.05$, **$p<0.01$).

between the various treatments (24 hrs., <4%) were recorded (Figure 23). As expected Gemcitabine induced modest apoptosis vs. control (10% and 2%, respectively) after 48 hrs.. However, the combination of exosomes containing Survivin-T34A with 10 μM Gemcitabine treatment displayed the greatest enhanced apoptosis compared to control and the single modality treatments (32% apoptosis vs. less than 10% apoptosis) after 48 hrs.. The combination treatment utilizing exosomes containing Survivin-T34A yielded the same levels of apoptosis as did treatment using 1500 μg/mL Survivin-T34A exosomes.

Discussion

We have previously described Survivin's (WT and T34A) presence in the extracellular conditioned media (Bokarewa, Lindblad et al. 2005, Mera, Magnusson et al. 2008, Khan, Aspe et al. 2009, Khan, Jutzy et al. 2011), and that extracellular Survivin can be taken up by cancer cells resulting in functional effects such as proliferation, invasion and resistance (Khan, Aspe et al. 2009). We have also shown that Survivin is released via small membrane-bound vesicles called exosomes (Khan, Jutzy et al. 2011). Building upon these findings, we have sought to test whether or not exosomes containing the apoptosis-inducing Survivin mutant, T34A, could be produced and if so, used either alone or in combination with other treatment methods as a cancer therapeutic.

To this end, the YUSAC 2 tet-off system (Grossman, Kim et al. 2001) was employed. Exosomes must be collected early so that the vesicles being harvested are intact and functional with the production of apoptotic bodies kept at a minimum. The F5C4 cell lines provide a good system in which the tet-off regulatory mechanisms allow for Sur-

vivin-T34A to be controlled systematically in all cells. The released quantity of exosomes was independent of the tet-system, which is important because any changes in protein levels within the exosomes are the result of the tet-system and not the quantity of exosomes. Our results demonstrate for the first time that Survivin-T34A is released from the cells via exosomes.

Utilizing exosomes for anticancer therapy an emerging and rapidly growing field (Khan, Jutzy et al. 2011). Dendritic exosomes (DEX) are the most commonly utilized exosomes for such therapy (Zitvogel, Regnault et al. 1998, Wolfers, Lozier et al. 2001, Couzin 2005, Chaput, Flament et al. 2006, Tan, De La Pena et al. 2010). However, DEXs are typically used as a form of immunotherapy – a way to prime the immune system against tumor cells (Khan, Jutzy et al. 2011). Our aim in this study was to directly attack tumor cells with exosomes containing Survivin-T34A.

MIA PaCa 2 cells treated in culture with exosomes containing Survivin-T34A undergo enhanced apoptosis. When we compared Survivin-T34A exosome-induced apoptosis levels to that induced by Gemcitabine alone, 100 μM Gemcitabine yielded 50% less apoptosis in the pancreatic cancer cell lines PANC1 and MIA PaCa-2 (Galloway, Aspe et al. 2009) then did 1500 μg/mL of Survivin-T34A-containing exosomes on the same cell lines. We could not obtain similar levels of apoptosis as exosomal Survivin-T34A using our highest concentration of Gemcitabine (500 μM, data not shown). We therefore combined low doses of exosomal Survivin-T34A (150 μg/mL total protein) with Gemcitabine (10 μM) to investigate whether Survivin-T34A could enhance Gemcitabine's cytotoxic effects. Alone, these doses did not yield an increase in apoptosis over control. However, there was a marked enhancement of apoptosis when Survivin-T34A

exosomes and Gemcitabine were combined. Using 150 μg/mL of Survivin-T34A exosomes and 10 μM Gemcitabine, apoptosis levels reached greater than 30% after 48 hrs. of combined treatment. We believe the enhancement from the combination treatments occurred because of the mechanism of Gemcitabine. Gemcitabine has been shown to reduce G_2/M cell cycle arrest which reduces cellular Survivin levels (Aspe and Wall 2010). We also have found that a modest amount of stress induces wild-type Survivin in this model system (data not shown). This combined with Survivin-T34A exosomes, which target Survivin, enhances apoptosis with much lower dosages because it is believed that Survivin-T34A targets and disrupts Survivin-associated protection of the cancer cell (Blanc-Brude, Mesri et al. 2003, Liu, Brouha et al. 2004, Aspe and Wall 2010).

In summary, delivery has been the confounding factor for using the novel Survivin-T34A as an effective therapeutic. This study not only adds to the importance of using Survivin-T34A as a cancer therapeutic for the treatment of pancreatic cancer, but that exosome delivery may provide a potential mechanism for effective tumor delivery. Cancer research and therapy have been rapidly moving towards combinatorial therapies but clinically, Gemcitabine remains the most prominent player in effective pancreatic cancer therapy. In closing we would like to emphasize the importance of continued exploration of the potential of combining exosome delivery of Survivin-T34A with Gemcitabine and other anticancer therapeutic regimens. These studies may prove relevance for the discovery and implementation of novel pancreatic cancer therapeutic strategies to improve the efficacy of chemotherapy-induced apoptosis in patients (Nicholson 2000).

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CHAPTER FOUR

CONCLUSION

Our finding that Survivin, a unique human inhibitor of apoptosis (IAP), has intercellular transport and signaling capabilities via exosomes is a significant and potentially seminal discovery. This led to another significant, potential discovery of Survivin-T34A being packaged into exosomes from genetically engineered YUSAC-2 cells which overexpress Survivin-T34A via a tet-off system. Consistent with Survivin's association with unfavorable clinicopathological parameters, trafficking Survivin throughout the tumor microenvironment can drive the aggressive status of the tumor prohibiting or minimizing therapeutic results. Thus, treatment of a tumor using Survivin-T34A induces apoptosis and sensitizes Gemcitabine induced cell death in MIA PaCA 2 cells. With the new discovery that exosomes containing Survivin-T34A, taken from cells that overexpress Survivin-T34A, is able to kill cancer cells *in vitro*, we hope to further enhance the scientific data banks and pave new paths for clinical modalities and regimens. Thus, the outcomes of this research project is significant to both cancer biology and clinical cancer research as a potentially novel cancer treatment. The knowledge that will make this possible includes: the elucidation of T34A target cell populations, cell-surface receptors, changes that occur in the target tissue at the molecular and cellular level, the mechanism of therapeutic action, and its site of action. Other potential future studies have also been described in Appendix A of this dissertation.

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APPENDIX A

FUTURE STUDIES

Part 1: ***To characterize the combinatorial therapy of exosomes with Gemcitabine in vivo to induce apoptosis and increase animal survival rates.***

Rationale

Based on the project in this document, proof of concept *in vitro* has shown the potential for enhancing the "Gold Standard" (Gemcitabine) for pancreatic cancer. Proof of concept *in vivo* is the next step. Because of the current poor prognosis for all pancreatic cancer patients, great strides to advance treatment of pancreatic cancer is essential. The novelty of this study is the delivery of exosomal based Survivin-T34A which may enhance Gemecitabine treatment while reducing toxicity. **The objective of this study is to show proof of concept that exosomal Survivin-T34A enhances Gemcitabine treatment in mice.** To accomplish this project, SCID mice will be injected orthotopically into the pancreas with MIA PaCa 2 cells. Once the tumor has been established, treatment with exosomes and Gemcitabine will follow. Then, apoptosis, tumor size, and animal survival rates will be characterized.

Part 2: ***To characterize the mechanism by which Survivin-T34A causes release of Cytochrome c from the mitochondria inducing the intrinsic pathway of apoptosis as well as the possibility that Survivin through physical as-***

sociation with players within the intrinsic apoptotic pathway, inhibits or mediates this phenomenon.

Rationale

Proof of concept *in vitro* has revealed promising results, a logical next step is proof of mechanism. Currently, the intrinsic pathway of apoptosis involves Cytochrome c, which is known to be released from the mitochondria as well as other proapoptotic proteins into the cytosol. Cytochrome c and cleaved Caspase-9 then bind to Apaf-1 to form the apoptosome therefore promoting caspase activation, PARP cleavage, and cell death. Phosphorylated Survivin-Thr^{34} is believed to the mechanism by which Cytochrome c is released into the cytosol is still unknown. Initially, Cytochrome c was thought to be released through mitochondrial membrane depolarization and mitochondrial permeability transition (MPT) - the result of the permeability transition pore (PTP). The PTP in the inner membrane opens and causes swelling of the matrix space, thus causing the outer membrane to rupture and spill Cytochrome c and other proapoptotic proteins into the cytosol. Recent studies using cyclophilin D knockouts have shown that PTP is primarily involved in necrosis and ischemia-reperfusion injury and less likely in intrinsic apoptosis and that Cytochrome c can be released in the absence of mitochondrial membrane depolarization. Instead, the mitochondrial apoptosis induced channel (MAC) has been proposed as the alternative mechanism by which Cytochrome c is released into the cytosol. Following Cytochrome c release, Survivin has been speculated to directly inhibit caspases. Although several groups have reported that Survivin does not bind directly to Caspase-3, one group has shown a Survivin direct inhibition of both Caspase-3 and Caspase-7 through physical chemistry techniques. Only one study has reported that phos-

phorylated Survivin-Thr^{34} can form a complex with Caspase-9 at midbodies and concomitantly inhibits apoptosis at cell division. From this we believe that Survivin physically associates with the apoptosome and directly inhibits cleaved Caspase-3 within the complex. **The objective of this study is to define the mechanism by which the phosphorylation status of Survivin-Thr^{34} inhibits or induces Cytochrome c release and its sequentially affected caspases.** To accomplish this project, the use both 4C7 and F5C4 cell lines will be utilized to compare and contrast the subcellular localization of WT-Survivin and Survivin-T34A and their association with key mitochondrial factors.

APPENDIX B

BIOGRAPHICAL SKETCH

Jonathan R. Aspe Ph.D. Candidate

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Education/Training

Loma Linda University, Loma Linda, CA	Ph.D.	2009-current	Biochemistry
Loma Linda University, Loma Linda, CA	Master's Track	2006-Ph.D.	Biochemistry
Pacific Union College, Angwin, CA	BS	2006	Chemistry

Research Experience

Graduate Dissertation: Loma Linda University School of Medicine, Loma Linda, CA
Advisor: Dr. Nathan R. Wall, Department of Biochemistry
Dissertation Title: Survivin-T34A, a potential exosomal-based cancer therapy.

Publications

Manuscripts

Aspe JR, Diaz Osterman CJ, Jutzy JMS, Deshields S, Whang S, Wall NR. Enhancement of Gemcitabine sensitivity in pancreatic adenocarcinoma by novel exosome-mediated delivery of the Survivin-T34A mutant. *J Extracellular Vesicles*, Accepted for Publication, January 21, 2014.

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Book Chapters

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Abstracts and Poster Presentations

Aspe, J.R., DeShields, S., and Wall, N.R. (2011). Survivin-T34A Overexpressing Cells Release Exosomes that can Induce Apoptosis and Enhance Gemecitabine-Induced Apoptosis; American Association for Cancer Research Pancreatic Cancer: Progress and Challenges Special Conference, Abstrac (#21).

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Manuscripts in Preparation

Khan S, Asumen MG, Jutzy JMS, **Aspe JR**, Galloway NR, Neidigh JW, Wall NR. Cellular import of the inhibitor of apoptosis protein survivin.

Khan S, Jutzy JMS, **Aspe JR**, Asumen MG, Wall NR. Tumor-released exosomes and their implications in cancer health disparities.

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Mentorships Experiences

Research Mentor at Loma Linda University
Simone DeShields
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2010 Summer, Apprenticeship Bridge to College Program

Professional Memberships

2007-Present American Association for Cancer Research (AACR)

Work Experiences

2005-2006 Pacific Union College Pre-Med/Dent Club Officer: Webmaster
Job Description: Designed and maintained the club's webpage

2005-2006 Chemistry Department Stockroom Student Manager
Job Description: Supervised the Chemistry Department's Stockroom employees and was in charge of managing the orders and supplies of the chemistry stockroom

2004-2006 Computer Science/Mathematics/Physics Teaching Assistant
Job Description: Tutored algebra and calculus students; graded math student's homeworks and quizzes

2003-2005 Chemistry Department Stockroom
Job Description: Organized the chemical stockroom and assisted lab T.A.'s with chemical supplies

Honors

2005 Pacific Union College Chemistry Department Commendation Award