Research article

A study into the potential role of Survivin localization in resistance to drug-induced apoptosis

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The aim of this study was to test the hypothesis that diverting the cytoplasmic subcellular localization of the anti-apoptotic form of Survivin to the nucleus would sensitize cancer cells to chemotherapeutics. Apoptosis is a morphologically and biochemically distinctive form of cell death, critical in the maintenance of tissue homeostasis. Caspases are a family of cysteine proteases that have a vital role in the implementation of apoptosis, and their activity is regulated by Inhibitors of Apoptosis Proteins. Recent studies indicate that one such inhibitor, Survivin, may have dual functions that are specific to its cellular location, including suppression of apoptosis (cytosolic) and regulation of cell division (nuclear). Since both apoptosis and proliferation are altered in cancer, identifying whether these roles for Survivin are dependent on its subcellular localization will inform future approaches to treat chemotherapeutically resistant tumour cells. After initially confirming the specificity of several Survivin antibodies, the distribution of Survivin was examined by immunofluorescence microscopy and sub-cellular fractionation in breast cancer cell lines. The threshold of drug-induced apoptosis was compared in cells over-expressing either wildtype Survivin or a form of Survivin unable to exit the nucleus due to a mutation in its nuclear export sequence. Endogenous Survivin localized to both nucleus and cytoplasm of breast cancer cell lines. Over-expressed Survivin had an anti-apoptotic, protective function. In contrast, cells expressing Survivin with the mutated nuclear export sequence had a lower apoptotic threshold to chemotherapeutic drugs. These results demonstrate for the first time that Survivin is localized to both the nucleus and cytoplasm of breast cancer cell lines. Importantly, the sensitivity of cells to chemotherapeutic drugs was increased when Survivin’s localization was restricted to the nucleus, consistent with cytoplasmic Survivin having the anti-apoptotic role. Since clinical studies have shown that nuclear Survivin is a positive prognostic factor in breast cancer patients, the data suggest that strategies to alter Survivin distribution may be useful in the fight against cancer.

Key words: apoptosis, Survivin, subcellular localization, breast cancer.

Introduction

Apoptosis is the complex process of regulated cell death, essential for maintenance of tissue homeostasis and embryonic development. Numerous signalling pathways are involved in triggering apoptosis and determining the fate of a cell. Apoptosis is orchestrated by the caspases, a family of cysteine-dependent death proteases, activated in apoptotic cells.1, 2 The caspase family can be subdivided into initiator caspases and effector caspases. Initiator caspases mediate cellular signalling to activate downstream effector caspases that then execute apoptosis via a ‘caspase cascade’ within the extrinsic and intrinsic pathways.1, 3 Crucial regulation of apoptosis is mediated by the inhibitor of apoptosis protein (IAP) family.4 IAPs function as intrinsic regulators of the caspase cascade, where they inhibit active caspases.5, 6 IAPs are distinguished by containing one or more baculoviral IAP repeat (BIR) domains, essential for inhibitory function.7–11 Mis-regulated apoptosis and promotion of cell survival can lead to cancer.12 IAPs are therefore becoming increasingly targeted within oncology research.

One member of the IAP family, Survivin, binds and inhibits caspase-9 thereby contributing to its anti-apoptotic function.7, 13 In addition, Survivin can inhibit Smac/DIABLO...
function, while conflicting literature debates whether it can bind to other caspases. Survivin also binds and stabilizes other IAPs, e.g. XIAP, promoting their anti-apoptotic effect.

In addition to its anti-apoptotic function, Survivin has a second role within the cell cycle, as a regulator of cell proliferation. Current evidence suggests that Survivin is a chromosomal passenger protein, involved in orchestrating the chromosomal passenger complex (CPC). Since Survivin is found in both the cytoplasm and the nucleus of cells, there has been controversy as to whether Survivin's functions can be separated according to localization, and if this can then be used as a prognostic indicator for cancer patients. The prevailing opinion is that cytosolic localization appears to be a favourable prognostic factor in breast cancer patients, while a cytoplasmic localization is unfavourable. Survivin is transported from the cytosol by nuclear export receptor, Crm1, which is regulated by a leucine-rich nuclear export signal (NES).

Influencing Survivin’s nuclear export as a strategy to antagonize its proliferative activity has been demonstrated using Leptomycin B, a Crm1-specific inhibitor. In addition, mutations in the Survivin NES region result in the accumulation of nuclear Survivin, unable to exit the nucleus. For example, Survivin-L98A causes a lower apoptotic threshold to X-irradiation than wild type Survivin. A predominant nuclear localization could also influence a cell’s susceptibility to apoptosis by increased transcription of pro-apoptotic genes.

Survivin is highly expressed in most cancers, where it may have an anti-apoptotic function. We suggest that tumour cell resistance to chemotherapeutic agents may be mediated by increased nuclear export of Survivin. Survivin localization is of considerable clinical relevance, as nuclear localization is a favourable prognostic factor in breast cancer patients while a cytoplasmic localization is unfavourable.

The current study aimed to investigate further the sub-cellular localization of Survivin, how this localization links with Survivin function, and whether Survivin localization might affect apoptotic susceptibility in response to chemotherapeutic agents. It is hypothesized that cytoplasmic Survivin contributes to IAP function while the nuclear form results in a non-protective role for apoptosis. Sub-cellular fractionation will determine Survivin localization in breast cancer cell lines. Survivin over-expression studies will investigate if increased cytoplasmic Survivin results in a protective anti-apoptotic function, and whether Survivin restricted to the nucleus, using Survivin containing a mutation within the NES (Survivin-L98A-GFP), has a lower threshold to apoptotic stimuli.

**Materials and methods**

**Tissue culture**

MDA-MB-231, MDA-MB-468, BT20, and 293T cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% v/v Foetal Bovine Serum (Invitrogen), 1% v/v Penicillin/Streptomycin, 1% v/v glutamine. MCF10A cells were cultured in DMEM:Hams F-12 1:1 mix supplemented with 5% v/v horse serum (Invitrogen), 10 µg/ml insulin, 500 ng/ml hydrocortisone, and 20 ng/ml EGF, 1% v/v Penicillin/Streptomycin, 1% v/v glutamine.

**Sub-cellular fractionation**

Cells were gently scraped in 1 ml of hypotonic buffer; 10 mM Tris–Cl pH 7.5, 10 mM NaCl, 1.5 mM MgCl2, 1:100 PIC (Protease Inhibitor Cocktail, Calbiochem®), and transferred to ice for 5 min in Dounce homogenizer. Cells were homogenized and NaCl was then added back to regain the correct physiological concentration (i.e. 150 mM). Samples were centrifuged at 100 000g for 30 min at 4°C (TLA 110 rotor, 55 000 rpm). The supernatant was collected, forming the cytosolic fraction. The remaining pellet was re-suspended in 150 µl of CHAPS buffer (1% CHAPS, 0.75 M aminocaproic acid, 1:100 PIC) and stored on ice for 5 min, then spun at 100 000g for 30 min (4°C) and the supernatant collected, forming the CHAPS soluble (enriched membrane fraction). The pellet was then finally re-suspended, forming the CHAPS insoluble (enriched nuclear fraction), in 2% SDS in 5 mM Tris–Cl pH 6.8 and boiled for 10 min until fully re-suspended. The protein concentration for each sample of cell fraction was calculated using the BCA assay (Pierce).

**Immunoblotting**

Twenty micrograms of protein was run on a 15% resolving gel, transferred onto nitrocellulose membrane (Whatman) before being treated with block buffer (5% v/v BSA, 0.1% v/v NaN3, 0.1% v/v Tween 20). Primary antibodies for protein detection were: 1/1000 rabbit anti-Apaf-1 (Stressgen Bioreagents); 1/1000 rabbit anti-Histone H3 and 1/1000 mouse anti-Survivin (Cell Signalling Technology); 1/1000 mouse anti-HSP 70 (Affinity Bioreagents); 1/150 rabbit anti-X-IAP (Cell Signalling Technology). IR-dye-conjugated secondary antibodies (1/10 000 diluted in 50% Odyssey Block Buffer, 50% 1× PBS) were used for detection. Membranes were read on an Odyssey LI-COR™, and band staining intensities were obtained from the gel using Odyssey V1.1 software.

**Over-expression of Survivin constructs**

293T cells were plated at 0.8×10⁵ cells per well of a 12-well plate, and the following day transfection was carried out with QIAGEN QIAprep®-purified pcDNA3.1 vectors (Invitrogen) encoding Survivin-GFP, Survivin-L98A-GFP or GFP alone, using Lipofectamine™ (4 h). Forty-eight hours following transfection, microscopic examination on a Zeiss Axiosvert 40CFL showed a 30% transfection efficiency. A sample of cells were lysed (in sample buffer containing...
5% v/v Na$_2$VO$_4$, 1% v/v NaF, 1× PIC) and separated on a 15% gel to confirm transfection levels. Localization of Survivin constructs was also confirmed by confocal microscopy. Transfected cells were stained with the nuclear marker, DAPI, and visualized on a Leica SP2 inverted microscope. Single sections through the mid-point of the cell are presented.

**Drug treatment**

Twenty-four hours post-transfection, cells were drug treated overnight with 100 μM Etoposide (Calbiochem), 1 μM Melphalan (Sigma, St Louis) or 1 μM Staurosporine (Calbiochem). Etoposide and Melphalan are both DNA damaging agents, currently in clinical use. Staurosporine is a kinase inhibitor, which acts to induce cell stress and apoptosis. Drug concentrations were chosen to induce maximal apoptosis in the cell lines used, over 24 h, without resulting in complete cellular loss, based on previous concentration-dependence studies performed in the lab (unpublished) and elsewhere.32 Cells were detached and cytospun onto slides, fixed in 4% formaldehyde (10 min) and permeabilized in 0.2% Triton-X100 (5 min). Cells were treated with 10% calf serum in PBS before staining with 1/500 mouse anti-Ki67 primary antibody (BD Transduction Laboratories), Alexa 594 goat anti-mouse secondary antibody (1:500) and DAPI. Slides were visualized on an Axioplan2 Imaging microscope and images analysed using OpenLab software.

**Statistical analysis**

A Student’s two-tailed t-test was used.

**Results**

**Endogenous Survivin localization in breast cancer cell lines**

In order to investigate the sub-cellular localization of endogenous Survivin in breast cancer cells, a panel of three cancer lines (MDA-MB-231; MDA-MB-468; BT20) was compared with a normal line (MCF10A). Cells were separated into enriched fractions of cytoplasm (soluble), membranes (CHAPS soluble) and nuclei (CHAPS insoluble) (Fig. 1). To confirm accurate fractionation of samples, immunoblots were probed with antibodies to (i) anti-Apaf-1 antibody, a cytoplasmic marker which migrates at ~130 kDa;33-35 (ii) Heat Shock Protein 70 (HSP70), a 70 kDa protein that localizes to the mitochondria and was used as a membrane and mitochondrial marker16 and (iii) anti-Histone H3 antibody, a 17 kDa nuclear marker.35

In addition, anti-Survivin and anti-XIAP antibodies were used to probe for IAP levels and location (Fig. 1). Notably, both Survivin, which migrates at ~16.5 kDa, and XIAP, migrating at ~50 kDa, were expressed at considerably higher level in the three breast cancer cell lines than in MCF10A cells. While XIAP was largely cytoplasmic, the distribution of Survivin was more diverse, being present in both cytosolic and nuclear fractions. These results indicate that IAPs may be upregulated in cancer cells, and second that Survivin, but not XIAP, is not only present within the cytoplasm but is also nuclear.

**Survivin protects against Etoposide-induced apoptosis in 293T cells**

In order to determine whether the sub-cellular localization of Survivin influences the sensitivity of cells to chemotherapeutics, the ability of a mutant form of Survivin, unable to exit the nucleus, was compared with the ability of wildtype Survivin to protect cells from drugs known to induce apoptosis, and which are currently in clinical use.

Initially 293T cells were transfected with plasmids expressing a GFP control, Survivin-GFP and Survivin-L98A-GFP. To confirm equivalent levels of transfection, cell lysates were extracted and probed for the presence of Survivin and GFP (Fig. 2A). In the Survivin-GFP (both WT and mutant) expressing cells, the Survivin (red) and GFP bands (green) overlap to give a yellow merged image. Confocal microscopy confirmed that wildtype Survivin-GFP was present within the cytosol, whereas Survivin-L98A-GFP was limited to the nucleus (Fig. 2B). Previous studies using tagged survivin have shown that it behaves exactly the same as over-expressed survivin constructs.28, 37, 38

GFP, Survivin-GFP and Survivin-L98A-GFP transfected cells were then treated with Etoposide, Melphalan or Staurosporine for 24 h, and the GFP-expressing cells were examined for either apoptosis or proliferation. Nuclear DAPI stain was used to identify the distinct morphological features associated with apoptotic cells including nuclear fragmentation and the presence of apoptotic bodies.39 Cells undergoing proliferation were analysed by Ki67 staining.40 The percentage of apoptotic or proliferating cells was calculated and expressed as a percentage of the total number of transfected cells (Fig. 2C and D).

Transfection with the GFP-construct did not result in an increase in basal apoptosis rates, and transfection with Survivin-GFP and Survivin-L98A-GFP caused 1.5% and 6.5%, respectively, of the cells to apoptose spontaneously. However, in each case, the percentage of cells undergoing apoptosis increased significantly (P < 0.05) in the drug-treated cells in comparison to the untreated controls (Fig. 2C, white bars). Moreover, and importantly, the cells expressing Survivin-GFP showed a significant protection against Etoposide-induced apoptosis compared with the GFP control cells. This resistance to apoptosis was not afforded by over-expression of the nuclear-restricted Survivin-L98A-GFP. Interestingly, neither Survivin nor Survivin-L98A conferred significant resistance to cells treated with Melphalan or Staurosporine.
In contrast to the apoptosis results, all the drugs inhibited proliferation, though there was no significant additional effect with either WT or L98A Survivin (Fig. 2D). Interestingly, there was a significant inhibition of basal proliferation (i.e. no drug treatment) in the cells transfected with either Survivin-GFP or Survivin-L98A-GFP.

Together these experiments demonstrate a protective role for certain forms of Survivin on Etoposide-induced apoptosis in breast cancer cells.

Discussion

The main discovery arising from this study is that over-expressed Survivin can protect breast cancer cells against apoptosis induced by some chemotherapeutic drugs, but only if Survivin is present within the cytosol. Since Survivin was found to be upregulated in three independent cancer lines, we speculate that it may have an apoptosis-suppressing function that contributes to disease progression.

Endogenous Survivin localization by sub-cellular fractionation

Both XIAP and Survivin are up-regulated in the three cancer cell lines, MDA-MB-231, MDA-MB-468 and BT20, compared with the normal breast cell line, MCF10a. This is consistent with the general observation that IAPs are up-regulated in cancer and are part of the mechanism of apoptosis resistance displayed by cancer cells.41 In each of the cell lines tested, XIAP localized to the cytoplasm, correlating with the central function of XIAP, which is to buffer the caspase cascade.42, 43 In contrast, Survivin localized to both the nucleus and the cytoplasm in each of the cancer lines. These data correlate with a study of tissue from breast cancer patients including atypical hyperplasias and malignant lymph tissue, where Survivin was most frequently present either only in the cytoplasm (31.3%) or in the cytoplasm and nucleus (22.5%), but was restricted to the nucleus in 11.3% of cases.44 A separate statistical analysis showed that cytoplasmic Survivin expression correlates with tumour stage and histological grade, and with a lower recurrence-free patient survival rate.45

Over-expression of exogenous Survivin

The decrease in the percentage of cells undergoing Etoposide-induced apoptosis in cells over-expressing Survivin-GFP is consistent with the protective role of Survivin within the apoptotic pathway. This supports previous literature showing a protective IAP function of over-expressed Survivin in cells treated with various apoptotic stimuli.46, 47 However, we found that over-expressed Survivin did not prevent Melphalan or STS-induced apoptosis. The differential response to Etoposide and the other two agents might reflect differences in the caspase activation. For example, in HeLa cells, Etoposide induced greater activation of caspase 9 than STS, while STS induced greater activation of caspases 8 and 3.32 Survivin has previously been shown to
Figure 2. Survivin protects against Etoposide-induced apoptosis. (A) 293T cells were transfected with Survivin-GFP, Survivin-L98A-GFP and GFP alone. Whole cell lysates were separated on a 15% gel, with (a) Rainbow marker, (b) un-transfected control, (c) GFP control, (d) transfected Survivin-GFP and (e) transfected Survivin-L98A-GFP. The gel was probed with anti-Survivin (chemifluorescence at 700 nm) and anti-GFP antibody (chemifluorescence at 800 nm). Data are representative of n = 2. (B) 293T cells transfected with Survivin-GFP (top panel), Survivin-L98A-GFP (middle panel) or GFP (bottom panel) were stained with DAPI (left column), and examined for Survivin localization (middle column and merged picture in right column) by confocal microscopy. (C and D) 293T cells were transfected with GFP alone, Survivin-GFP (SUR-GFP) or Survivin-L98A-GFP (SUR-NES-GFP). Cells were treated for 24 h with drugs as indicated, detached and cytospun onto slides, then fixed, permeabilized and stained with antibodies to Ki67. DAPI was used as a nuclear marker and transfected cells located by their GFP-tag. (C) Cells with condensed, apoptotic nuclei were scored in the GFP-positive population of the transfected cells. (D) Similarly, GFP-positive cells were scored for Ki67 staining. Results are expressed as percentages, taken from an average of 60 samples from three independent experiments. Error bars depict standard error of the mean. Asterisks represent significant difference (P < 0.05) compared with GFP-only transfected cells for each drug treatment.
inhibit caspase 9, whereas its effects on caspase 3 are indirect and mediated through interactions with other IAPs. It is therefore possible that at least in breast epithelia, an over-expressed IAP only suppresses specific drug-induced caspase pathways.

In comparison to the results with Survivin-GFP, no protective effect to therapeutic agents was seen in samples transfected with Survivin-L98A-GFP, where the over-expressed protein is restricted to the nucleus. This is consistent with the hypothesis that only cytoplasmic IAPs are able to protect apoptosis responses. It also correlates with a previous study, where mutating the Survivin-NES region removes its cytoprotective function while still maintaining its role in mitosis.28 Nuclear Survivin has also reduced stability compared with cytoplasmic Survivin, which prevents it having a cyto-protective effect.48 Our data therefore support the suggestion that Survivin exists in two separate pools, in which it has distinct apoptosis and cell cycle roles.

In summary, this study demonstrates that over-expressed Survivin in breast cancer cells can provide resistance to some types of therapeutic agents that are in current use to treat breast cancer. Moreover, it is tempting to hypothesize that a post-translational alteration of the location of Survivin from nucleus to cytosol might provide a further mechanism of drug resistance. For example, Survivin phosphorylation by protein kinase A or cdc2 can affects its localization and caspase-binding activity.49 Pharmaceutical strategies aimed at changing the distribution of endogenous IAPs such as Survivin might therefore be an attractive approach to sensitize tumor cells to chemotherapy.

Acknowledgement

The authors are grateful to Dr Sally Wheatley for providing the Survivin constructs.

Funding

Funding for this project was provided by the Faculty of Life Sciences, University of Manchester. Fiona Foster is supported by Breast Cancer Campaign.

References