RESEARCH ARTICLE

Survivin S81A Enhanced TRAIL's Activity in Inducing Apoptosis

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Abstract

BACKGROUND: Survivin is rarely expressed in normal healthy adult tissues, however, it is up-regulated in the majority of cancers. Survivin, which belongs to IAPs family, has been widely reported to protect cells from apoptosis by inhibiting caspases pathway. Survivin's mitotic activity is modulated by many kinases, and its phosphor status can also influence its ability to inhibit apoptosis. There are several important survivin's phosphorylation sites, such as S20 and T34. We have continued our investigation on other potential survivin's phosphorylation sites that could be important site for regulating survivin's cyto-protection.

METHODS: By assuming that S81 could be a potential target to modify activity of survivin, wild-type survivin (Survivin), antisense survivin (Survivin-AS), mutated-survivin Thr34Ala (Survivin-T34A) and mutated-survivin Ser81Ala (Survivin-S81A) were constructed and inserted into pMSCV-IRES-GFP vector with cytomegalovirus (CMV) promoter. Each retroviral product was produced in BOSC23 cells. LY294002 pretreatment and TRAIL treatment along with infection of retroviral products were performed in murine fibrosarcoma L929 cells. For analysis, flow cytometric apoptosis assay and western blot were performed.

RESULTS: In our present study, survivin for providing cytoprotection was regulated by PI3K. The results showed that LY294002, an inhibitor of PI3K, effectively suppressed survivin-modulated cytoprotection in a TRAIL-induced apoptotic model. In addition, mutated survivin S81A showed marked suppression on survivin's cytoprotection. Along with that, TRAIL's apoptotic activity was enhanced for inducing apoptosis.

CONCLUSION: We suggested that survivin could inhibit apoptosis through PI3K and S81A could be another potential target in order to inhibit Survivin-modulated cytoprotection as well as to sensitize efficacy of TRAIL or other related apoptotic inducers.

KEYWORDS: apoptosis, survivin, TRAIL, S81A, L929, LY294002.

Introduction

Survivin acts as a suppressor of apoptosis and promotes cell proliferation (1,2). Survivin is rarely expressed in normal healthy adult tissues, however, it is up-regulated in the majority of cancers (1,2). By molecular profiling, survivin has been consistently identified as a risk-associated gene in various malignancies, carrying unfavorable implications for cancer prognosis, disease recurrence and abbreviated survival (1).

Survivin is the smallest member of the Inhibitor of Apoptosis (IAP) gene family, containing 142 amino acid residues (2-4). Unlike other IAPs, survivin contains a single baculovirus IAP repeat (BIR) domain that stretches

from amino acid residue 15 to 87 (2,4). Similar to all other IAPs, except X-linked inhibitor of apoptosis (XIAP), survivin does not directly bind caspases (3). The expression of survivin is up-regulated at a transcriptional level by the nuclear factor-κB (NFκB) (3,4). Survivin can be activated indirectly by growth factors via phosphatidylinositol 3-kinase (PI3K)/Akt pathway (4). On the other hand, survivin is one of the genes repressed at the transcriptional level by wild-type p53 and p75 (4).

Alternative splicing of survivin pre-mRNA from chromosome 17q25 produces five different mRNAs, which potentially encode five distinct proteins: survivin, survivin 2B, survivin ΔEx3, survivin 3B and survivin 2α (2). It has been proposed that the splice variants function to modulate the function of full-length survivin (4). While this may be true for apoptosis inhibition, where survivin and survivin ΔEx3 interact within the mitochondria to inhibit mitochondrial-dependent apoptosis, recent evidence suggests that the splice variants cannot modulate survivin's function during cell division (4).

When phosphorylated, survivin will gain opposite and balancing effects (1). Phosphorylation of survivin on Thr34 by the mitotic kinase cyclin dependent kinase 1 (Cdk1) has a positive effect, stabilizes survivin at prometaphase and metaphase against proteasomal degradation and heightens an anti-apoptotic threshold in cells traversing mitosis (1). On the other hand, phosphorylation of survivin on Thr117 by Aurora B kinase has a negative effect, lowering its affinity for the interaction with centromeric chromatin (1). Besides these two phosphorylation sides, more other phosphorylation sides of survivin have been reported such as Ser20 (3) and Thr48 (5).

Many studies on regulating survivin have been conducted. For instance, quercetin was reported to promote degradation of survivin in glioma cells (6). Studies on sensitizing TRAIL to induce apoptosis were largely performed in its combination with celecoxib (7), troglitazone (8), celastrol (9), tunicamycin (10), beclin1 (11), capsaicin (12), sodium butyrate (13) and aspirin (14). In addition, there are current approaches to target survivin by anti-sense oligonucleotides, small interfering RNAs, dominant-negative mutants and small molecule antagonists (2).

Ablation of survivin-dependent apoptosis inhibition could improve the efficacy of many agents used to treat cancer (15). In the attempt to target survivin, modulation of its phosphorylation status could be one of the solutions. Since we are interested in knowing survivin's phosphorylation properties, we conducted a study on its potential phosphorylation sites, previously screened by using bioinformatic tools. Here we report our investigation in targeting a phosphorylation site of survivin on Ser81 in order to enhance TRAIL to induce apoptosis.

Methods

Bioinformatics and Preparation of Survivin Constructs

An investigation on potential phosphorylation sites was conducted by using Scansite tools (scansite.mit.edu). Sequence of *Mus musculus* baculoviral IAP repeat-containing 5 (Birc5)/survivin was in concordance to NCBI reference with gene ID: 11799. Antisense survivin (Survivin-AS) was constructed with 5' HpaI and 3' HpaI sites, while Survivin Thr34Ala (Survivin-T34A) and Ser81Ala mutants (Survivin-S81A) with 5' BglII and 3' HpaI sites. Each cDNA of Survivin, Survivin-AS, Survivin-T34A and Survivin-S81A were inserted into pMSCV-IRES-GFP vector with cytomegalovirus (CMV) promoter. Each vector was transformed in DH5α *Escherichia coli*. Colonies were selected in ampicillin-contained media. cDNA constructs were later purified and confirmed.

Viral Production and Confirmation

Each cDNA construct (20 μg) was transfected in $4x10^6$ BOSC23 cells by calcium phosphate method with addition of 10 μg of pCl3-EcotR in 100 mm plate. Transfection was done for 10 hrs at 37°C. After transfection, the medium was replaced with fresh DMEM containing 10% fetal bovine serum (FBS). Cultures were monitored for expression of green fluorescent protein (GFP). Viruses were harvested 72 hrs after transfection. For retroviral tittering, $2x10^5$ NIH3T3 cells in 60 mm plate were infected with 100 μl of each virus and addition of 4 μg/ml polybrene for 6 hrs at 37°C. Then the medium was replaced with fresh DMEM containing 10% fetal calf serum (FCS). Forty-eight hrs after infection, NIH3T3 cells were collected and measured with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Survivin protein expression of infected cells was determined by immunoblotting, using a rabbit polyclonal to mouse Survivin antibody (Novus Biologicals, Littleton, CO, USA).

Cell Culture and Infection of L929 Cells

L929 cells were cultured in α-MEM containing 10% horse serum. Infection was carried out using viral product of BOSC23 cells (Survivin, Survivin-AS, Survivin-T34A, Survivin-S81A or vector only) for 48 hrs.

Apoptosis Assay

Flow cytometric detection of apoptotic cells was performed as described by Sandra *et al*. (16). Briefly, pretreated cells were harvested and suspended in 1 ml of hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1 % sodium citrate plus 0.1 % Triton X-100). Cell suspensions were placed at 4°C in the dark for 2 hrs before the flow cytometric analysis. The propidium iodide fluorescence of individual nuclei was measured with an FACSCalibur (Becton Dickinson).

Immunoblotting

Treated cells were harvested and incubated with lysis buffer. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk in Tris-buffered saline (TBS, 150 mM NaCl and 50 mM Tris-HCl, pH 7.4), the sheets were incubated with first antibody. The second antibody was horseradish peroxidase-conjugated donkey anti-rabbit (Amersham) or sheep anti-mouse (Amersham) IgG antibody, diluted 1:2000. The bound antibodies were visualized using the ECL system (Amersham).

Results

Survivin expression of L929 cells

Based on the Scansite tools, S81 was suggested as a potential phosphorylation site of survivin. Therefore, beside T34, S81 mutant was also constructed. Figure 1 shows low basal survivin expression of L929 cells. Marked increase of survivin expression was observed in viral product of Survivin-infected L929 (L929 Survivin) cells. Increased survivin expression was observed in viral product of Survivin-T34A or Survivin-S81A-infected L929 (L929 Survivin-T34A or L929 Survivin-S81A) cells as well. These results implied that viral products of Survivin, Survivin-T34A and Survivin-S81A could perform well. Viral product of Survivin-AS also performed well by eliminating basal survivin expression in viral product of Survivin-AS-infected L929 (L929 Survivin-AS) cells. As a control, viral product of vector did not affect the basal survivin expression in viral product of vector-infected L929 (L929 Vector) cells.

Survivin cytoprotected TRAIL-induced apoptosis in L929 cells through PI3K

As shown in Figure 2, TRAIL induced apoptosis in L929 cells for 26.70%, and with pretreatment of LY294002, percentage of apoptotic cells was increased to 37.35%. Infection with survivin decreased percentage of TRAIL-treated apoptotic cells in viral particle concentration dependent manner. The highest survivin viral concentration, $7.5x10^7$ viral particle/ml, cytoprotected TRAIL-induced L929 cells markedly, decrease of apoptotic cells from 26.70% to 15.04%. However, pretreatment of LY294002 suppressed cytoprotective activity of survivin, a marked increase of the percentage of apoptotic cells was observed in pretreatment of LY294002 on $7.5x10^7$ viral particle/ml-infected L929 cells, marked increase from 15.04% to 33.03%.

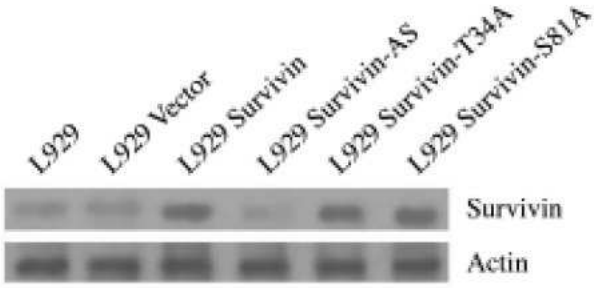

Figure 1. Survivin expression of L929 cells. Each 3x105 L929 cells in 60 mm plate were/not infected with 75x107 viral particle/ml retrovirus of Survivin, Survivin-AS, Survivin-T34A, Survivin-S81A or vector for 48 hrs. Infected cells were cell sorted, then lysed and immunoblotted using rabbit polyclonal to mouse Survivin antibody (Novus Biologicals, Littleton, CO, USA).

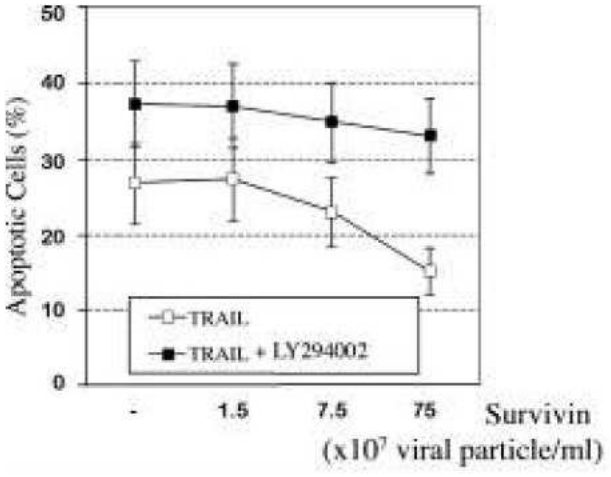

Figure 2. Survivin cytoprotected TRAIL-induced apoptosis in L929 cells through PI3K. Each 3x105 L929 cells in 100 mm plate were infected with different viral particle concentration of Survivin retrovirus for 48 hrs as indicated in the panel. Pretreatment with/without 25 µM LY294002 for 12 hrs was performed prior to treatment of 100 ng/ml TRAIL for 30 hrs. All treated cells were collected and subjected to apoptosis assay. Detailed procedures were described in "Material and Methods". These experiments were repeated 3 times.

Ser81 site of survivin is important in providing cytoprotection

As shown in Figure 3, TRAIL induced marked percentage of apoptotic L929 Survivin-AS cells. A result showed that survivin-moderated cytoprotection of L929 Survivin-AS cells was markedly suppressed. Suppressed-cytoprotection of L929 Survivin-T34A and Survivin-S81A cells were also exhibited. Addition of LY294002 did not accelerate percentage of apoptotic L929 Survivin-AS, L929 Survivin-T34A and Survivin-S81A cells.

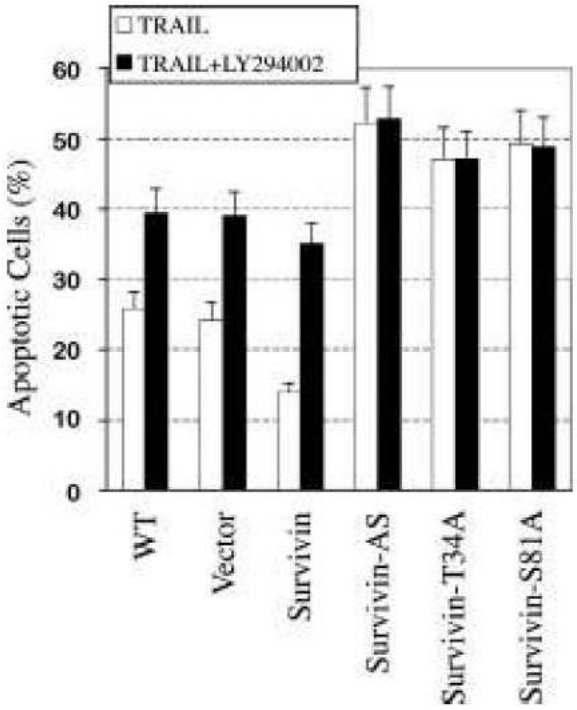

Figure 3. Ser81 site of survivin is important in providing cytoprotection. Each 3x105 L929 cells in 60 mm plate were infected with 75x107 viral particle/ml retrovirus of Survivin, Survivin-AS, Survivin-T34A, Survivin-S81A or vector for 48 hrs and cell sorted. Each 7x105 sorted L929 cell were re-plated and pretreated with/ without 25 μM LY294002 for 12 hrs was performed prior to treatment of 100 ng/ml TRAIL for 30 hrs. All treated cells were collected and subjected to apoptosis assay. Detailed procedures were described in "Material and Methods". These experiments were repeated 3 times. WT: wild type.

Discussions

Survivin's cytoprotective activity has been reported widely (1-4). Survivin can bind to XIAP. Functionally, a survivin–XIAP complex enhances XIAP stability against ubiquitin-dependent degradation, synergistically increases the activity of XIAP for caspase inhibition, promotes tumor growth *in vivo* and directly participates in XIAP-mediated intracellular signaling, in particular NF-κB activation (3). Survivin blocks apoptosis by mechanisms other than direct effector caspase inhibition (2,17,18). Thus, survivin is now thought to antagonize cell death upstream of effector caspases (2). In one situation, this was accomplished by inhibition of caspase 9 (2,19). In another, a survivin-hepatitis B X-interacting protein (HBXIP) complex bound to pro-caspase-9 and prevented the recruitment of apoptosis protease activating factor-1 (Apaf-1) to the apoptosome (2,20) A further mechanism by which survivin may inhibit apoptosis is by interacting with second mitochondria-derived activator of caspase (SMAC) / direct IAP-binding protein with low PI (DIABLO), thus displacing bound IAPs (2,21).

Growth factors can activate survivin via PI3K/Akt pathway (4). Additionally, insulin like growth factor I/mTOR signaling has been reported to up-regulate survivin (4,22) In our present study, survivin in providing cytoprotection was regulated by PI3K. Results showed that LY294002, an inhibitor of PI3K, effectively suppressed survivin-modulated cytoprotection in a TRAIL-induced apoptotic model. From these results, we assumed that survivin could inhibit apoptosis through PI3K.

Post-translation, survivin's mitotic activity is modulated by many kinases, including Cdk1, aurora-B and polo-like kinase 1 (plk1) on T34, T117 and S20 respectively (5,23-27) and its phosphostatus can also influence its ability to inhibit cell death (23) Mutation of T34 to a nonphosphorylatable alanine (T34A) abolishes survivin's cytoprotective activity and decreases its stability (23,28,29) Consistent with this, suppression of T34 phosphorylation by cdk1 inhibition also enhances apoptosis (28,29) Based on a bioinformatic study, we found another potential survivin's phosphorylation site, S81. A mutated form of survivin was created to replace potential phosphorylation site on S81 to a nonphosphorylatable alanine (S81A). Our results showed that mutated survivin S81A showed marked suppression on survivin's cytoprotection as well. In addition, TRAIL's apoptotic activity in inducing apoptosis was enhanced. These results suggested that S81 could be another potential target to inhibit survivin-modulated cytoprotection as well as to sensitize efficacy of TRAIL or other related apoptotic inducers.

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