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NSAIDs enhance proteasomic degradation of survivin, a mechanism of gastric epithelial cell injury and apoptosis

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ABSTRACT

NSAIDs cause severe gastrointestinal injury, in part by suppressing survivin, an inhibitor of apoptosis protein, both in cultured gastric epithelial cells and in human and rat gastric mucosa. The mechanism(s) of survivin down-regulation by NSAIDs is unclear. In this study, we examined whether NSAID treatment decreases survivin mRNA expression and/or enhances degradation of survivin protein via ubiquitin proteasome system in rat gastric mucosal, RGM-1 cells, and whether survivin overexpression prevents indomethacin-induced cell injury and apoptosis. Effects of indomethacin on survivin mRNA expression, survivin protein half-life and ubiquitination were examined in RGM-1 cells. Proteasome inhibitors were utilized to prevent indomethacin-induced survivin protein degradation in RGM-1 cells. The effects of stable overexpression of survivin on indomethacin-induced RGM-1 cell injury and apoptosis were examined. Results showed: (1) Indomethacin treatment did not alter survivin mRNA expression, but significantly reduced survivin protein half-life from 1.5 h to approximately 1 h and increased survivin ubiquitination. (2) Inhibition of ubiquitin proteasome prolonged survivin protein half-life to over 2 h and inhibited indomethacin-induced survivin degradation. (3) Overexpression of survivin significantly reduced indomethacin-induced cell injury and apoptosis. In conclusion, indomethacin treatment enhances degradation of survivin via the ubiquitin proteasome machinery in RGM-1 cells, and maintenance of survivin levels is important for prevention of gastric epithelial cell injury and apoptosis.

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1. Introduction

Survivin is a member of the inhibitors of apoptosis protein family (IAP). IAPs were first identified as baculovirus proteins that prevent apoptosis in infected host cells, and are characterized by presence of at least one baculovirus IAP repeat (BIR) domain [1,2]. Several cellular IAP homologues were found. A subset of IAPs, such as XIAP, cIAP-1 and cIAP-2, mainly function to suppress apoptosis by inactivating effector caspases via their BIR domains [3,4]. A distinct group of IAPs, including survivin, mainly function to regulate cell division, and may or may not be directly involved in inhibiting apoptosis [5–8].

Survivin expression is essential during development and carcinogenesis. Survivin knockout mice exhibit defects in microtubule assembly, absence of mitotic spindles and formation of multinucleated cells, culminating in embryonic lethality [8]. Survivin expression is widespread during fetal development [5,9], but is undetectable in most adult tissues, with the exception of a few, such as colon, placenta and

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stomach [10–12]. Survivin is prominently reexpressed during preneoplastic and malignant transformation, and is a prognostic indicator of tumor progression in breast, lung, colorectal, gastric, liver, neuroblastoma, lymphoma and several other common human cancers [5,13]. Anticancer agents and regimen, including radiation, NSAIDs and chemotherapy, induce cell death via apoptosis, and survivin overexpression in cancer plays a role in resistance to these agents, suggesting that survivin participates in inhibiting apoptosis induced by these agents [14].

Survivin expression is also important during tissue regeneration, and suppression of survivin may contribute to injury. Survivin expression is elevated in endothelial cells involved in angiogenesis in granulation tissue of inflamed skin biopsies, and by mitogenic stimulation of quiescent endothelial cells in culture, indicating that survivin function is essential for angiogenesis, a fundamental process in tissue regeneration [15]. Survivin expression is also elevated in regenerating liver cells after partial hepatectomy [16]. NSAIDs are known to induce severe gastrointestinal injuries. Previously, we showed that indomethacin significantly decreases survivin protein levels in the gastric mucosal epithelium and in cultured gastric epithelial cells, and suppression of survivin expression promotes indomethacin-induced gastric epithelial cell injury [17]. The mechanism(s) of survivin down-regulation in gastric epithelial cells by NSAIDs remains unclear, and is the focus of this study.

The ubiquitin-proteasome plays a central role in regulating essential cellular processes such as cell cycle progression, transcription, signal transduction, and apoptosis. Free ubiquitin (Ub) is recruited by the E1 Ub-activating enzyme and subsequently transferred to the E2 Ub-conjugating enzyme (UBC). E3 Ub-ligases, which are responsible for substrate recognition, binds to E2 UBC, and Ub is then transferred from E2 to specific substrates. Polyubiquitinated proteins are then recognized by the 26S proteasome complex, and degraded quickly into short peptides. Several key regulator proteins such as cyclins, cyclin kinase inhibitors [18], E2F transcription factors [19], beta-catenin [20] and p53 tumor suppressor [21] are degraded via the ubiquitin proteasome system. Survivin is also degraded via the ubiquitin proteasome pathway in human embryonic kidney cells [22].

Our hypothesis is that indomethacin treatment enhances degradation of survivin via the ubiquitin proteasome machinery, and this in turn facilitates indomethacin-induced cell injury and apoptosis. We will examine, in RGM-1 cells, whether: (1) indomethacin treatment decreases survivin mRNA expression and/or increases survivin protein ubiquitination, (2) proteasome inhibitors could prevent survivin degradation by indomethacin and (3) overexpression of survivin could inhibit indomethacin-induced cell injury and apoptosis.

2. Materials and methods

2.1. Cell lines and treatments

Normal rat gastric mucosal, RGM-1, epithelial cells were cultured in DMEM:F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. Cells were grown in 100 mm tissue culture dishes until they were about 60% confluent, incubated for 24 h in serum-free DMEM:F12 medium, then underwent various treatments: (1) cells were treated with 0.05, 0.1, and 0.5 mM of indomethacin for 6 h to examine concentration effects on survivin protein reduction and induction of apoptosis. 0.1 mM indomethacin was selected for subsequent experiments involving quantitaion of survivin protein levels, since it is necessary to have intact cells in order to accurately measure survivin protein levels in the cells, and 0.1 mM was the highest concentration that markedly reduced survivin protein levels but induced minimal toxicity and apoptosis within the time frame of these experiments. (2) To examine whether indomethacin treatment affects survivin expression at the transcription level, cells were treated with 0.1 mM indomethacin for 0, 1, 3 and 6 h, then harvested for total RNA isolation. (3) To determine survivin protein half-life in RGM-1 cells, cells were first treated with 10, 50, 100 and 200 μg/ml cycloheximide (CHX) to determine the lowest effective dose with minimal cytotoxicity. One hundred micrograms per milliliter CHX, which is commonly utilized in numerous publications to inhibit protein synthesis in a wide variety of cultured cells, was also chosen for this study. Cells were treated with 100 μg/ml CHX for 0, 0.5, 1, 1.5, and 2 h. (4) To examine whether indomethacin treatment could decrease the half-life of survivin, cells were pretreated with 0.1 mM indomethacin for 2 h, before significant decreases in survivin protein level is observed, then 100 μg/ml CHX was added for 0, 0.5, 1, 1.5, and 2 h. (5) Cells were treated with 0.5, 5, 10 and 25 μM of MG-132 or lactacystin for 6 h to establish the minimum concentration of proteasome inhibitors required for inhibition of survivin protein degradation in RGM-1 cells. Ten micromoles was selected as the minimum effective concentration for studies that followed. (6) To examine the effect of indomethacin on survivin protein ubiquitination, cells were treated with 0.1 mM indomethacin in the presence or absence of 10 μM MG-132 (pretreated for 20 min) for 0, 3 and 6 h. Then survivin protein was immunoprecipitated and ubiquitination was examined by Western blot analysis. The MG-132 inhibitor does not prevent protein ubiquitination, and was added to prevent rapid survivin protein degradation following ubiquitination induced by indomethacin. (7) To examine whether inhibition of ubiquitin proteasome could increase survivin protein half-life, RGM-1 cells were pretreated with 10 μM of MG-132 or lactacystin for 20 min, then with 100 μg/ml CHX for 1, 1.5 and 2 h. (8) To examine whether survivin is also degraded via other protease pathways, RGM-1 cells were pretreated with 0.2 mM PMSF and 1mM leupeptin for 20 min, then with 100 μg/ml CHX for the above durations. (9) To determine whether inhibition of proteasome and other protease pathways would prevent degradation of survivin by indomethacin, cells were pretreated with inhibitors for 20 min and then with or without 0.1 mM indomethacin for 2 h, followed by 100 μg/ml CHX for 2 h. Vehicle-treated RGM-1 cells were included in all the above experiments as control. Protein extracts were then isolated for immunoprecipitation and/or Western blot analyses, and total RNA was isolated for realtime quantitative PCR (qPCR) analyses. All treatments and time-points were performed in triplicate in order to calculate statistical significance.

2.2. Total RNA isolation and quantitative realtime PCR analysis

Total RNA was isolated from RGM-1 cells using the RNeasy mini kit (Qiagen Biosciences, Valencia, CA), following the manufacturer's instructions. 0.3 μg of RNA per sample was reverse transcribed using 25 units of MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Five microliters of the reverse transcription (RT) mixture was utilized in each subsequent qPCR reaction. QPCR was performed using a method described by Pfaffl [23] for realtime RT-PCR. This method accounts for effects of amplification efficiency in the following formula:

$$\text{ratio} = \frac{(E_{\text{target}})^{\delta \text{Ct target(control-treated)}}}{(E_{\text{ref}})^{\delta \text{Ct ref(control-treated)}}}$$

Target refers to survivin PCR products, and beta-actin is used as reference to which all survivin PCR products were normalized. *E* refers the amplification efficiency based on the slope of the standard dilution curve. realtime PCR analysis was performed on the iCycler (BioRad, Hercules, CA) using IQ SYBR Green Supermix (BioRad, Hercules, CA), and survivin PCR primers that were described previously [10]. PCR reaction conditions were: pre-incubation at 95 °C for 10 min (1×); denaturation at 95 °C for 1 min, reannealing at 51 °C for 30 s and extension at 72 °C for 30 s (40×). Fluorescence emission data were collected at the annealing step. All treatments and conditions were performed in triplicate in order to calculate statistical significance.

2.3. Immunoprecipitation, Western blot analysis and quantitation

RGM-1 cells were lysed in buffer containing 20 mM Tris–HCl (pH 7.9), 1.5 mM $MgCl_2$, 550 mM NaCl, 0.2 mM EDTA, 2 mM DTT and 20% (v/v) glycerol. To examine the effect of indomethacin on ubiquitination of survivin protein, 800 μg total protein from each sample was incubated at 4 °C for overnight with 5 μg rabbit polyclonal anti-survivin antibody (Novus Biologicals, Littleton, CO) adhered to protein A sepharose beads. Then protein was eluted from beads with 50 μl fresh lysis buffer. Equal volumes per sample of immunoprecipitated lysates were analyzed for protein ubiquitination by Western blot analysis. Western blot analysis was performed as follows: immunoprecipitated lysates or 100 μg per sample of total protein was extracted from the supernatant after centrifugation, and separated on a 15% SDS-PAGE, then transferred onto nitrocellulose membranes. The membranes were blocked in skim milk, incubated with rabbit-polyclonal anti-survivin antibody (Novus Biologicals, Littleton, CO) or with rabbit-polyclonal anti-ubiquitin antibody (Novus Biologicals, Littleton, CO) for 1 h, and then with peroxidase conjugated goat-anti-rabbit antibodies (Sigma, St. Louis, MO) for 1 h. Protein signals were visualized using ECL chemiluminescence reagent (Amersham Life Science, Piscataway, NJ) and by exposure to Kodak X-omat film (Eastman Kodak, Pitsburgh, PA). The membranes were stripped and re-incubated with monoclonal anti-β-actin antibody (Sigma, St. Louis, MO), then with peroxidase conjugated anti-mouse antibody (BD Transduction Laboratories, Lexington, KY).

Protein signal densities were quantified using a Metamorph Imaging System, version 3.0 (Universal Imaging Corp., Downingtown, PA). Protein signal densities were subtracted from background densities and normalized to the corresponding β-actin signal densities.

2.4. Stable overexpression of survivin in RGM-1 cells

Total RNA was isolated from rat gastric mucosa using Rneasy (Qiagen Biosciences, Valencia, CA), and reverse transcribed to cDNA by random priming, using 25 units of MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). The full-length survivin cDNAs (corresponding to the complete mRNA coding sequence) was amplified by PCR using gene specific primers that include an EcoRI restriction site 5′ and in frame with the survivin start codon, and a BamHI site 3′ of the stop codon. The PCR product was ligated into TA cloning vector (Invitrogen, Carlsbad, CA), and amplified in bacterial culture. Those containing an insert were isolated by blue/white colony selection. Constructs containing the expected 440 bp insert were confirmed by restriction analysis and agarose gel electrophoresis. The appropriate inserts were purified and sequenced. Correct sequence was confirmed by comparison with that of the rat survivin gene (accession no. NM022274). The survivin insert was then subcloned into a pcDNA 3.4 mammalian expression vector (Invitrogen, Carlsbad, CA). The constructs were resequenced to confirm the correct insert, and that the survivin insert is in frame with the promoter. Stable survivin expression was achieved by Lipofectamine (Invitrogen, Carlsbad, CA) mediated transfection of the pcDNA3.4-survivin plasmid construct into RGM-1 cells according to the manufacturer's instructions, and selection of stable transfectants in medium containing the antibiotic Zeocin (Invitrogen, Carlsbad, CA). The control cell line, which does not overexpress survivin, was established by stable transfection with empty vector. Western blot analysis was used to examine survivin protein levels in control and survivin transfectants.

Cell growth data for the control and survivin transfectants were obtained by seeding 5×10^6 cells from each cell line in each well of six-well plates, and culturing them in medium containing zeocin for 24 and 48 h, at which time the number of cells in each well was counted using a hemacytometer. Six wells were counted for each cell line at each time point.

2.5. Cell injury and apoptosis assays

Cell injury data was obtained using the Cytoscan LDH assay kit (Bioworld, Dublin, OH), following the manufacturer's instructions, to measure the amount of LDH released into the culture supernatant after treatment of cells with indomethacin or vehicle only. Results were expressed in a graph as percent cell death relative to the positive control (100% cell death by complete disintegration of cells by sonication).

Annexin V binding assay was used to determine the amount of apoptosis induction by indomethacin. Cells were plated on glass coverslips and grown to 50% confluence in a 12-well dish (Corning Inc., New York). They were serum starved for 24 h and then treated with vehicle only or indomethacin.

Cells were then washed and incubated with FITC-conjugated Annexin V and propidium iodide according to the manufacturer's instructions (Abcam Inc., Cambridge, MA). Cells were analyzed under fluorescent microscopy at 10× and 20× magnification using a dual filter set for FITC and rhodamine. Percentage of FITC-labeled cells in each sample, indicating cells at early stages of apoptosis, was determined per 5000 total cells counted.

2.6. Statistical analyses

Student's two-tailed *t*-test was used to compare data between two groups. One-way analysis of variance and Bonferroni's correction were used to compare data between three or more groups. *P*-value < 0.05 was considered statistically significant.

3. 3. Results

3.1. Indomethacin treatment reduced survivin protein but not mRNA expression in RGM-1 cells, and reduction of survivin coincided with induction of apoptosis

The relationship between survivin levels and apoptosis in indomethacin-treated RGM-1 cells was examined in initial experiments. Cells were treated with vehicle only or 0.5, 0.1 or 0.5 mM indomethacin for 0, 1, 3 and 6 h. At each time point, survivin protein levels and percentages of apoptotic cells were determined. Indomethacin treatment at concentrations up to 0.1 mM did not significantly reduce survivin protein levels or induce apoptosis at 0, 1 and 3 h, compared to vehicle treatment. At 3 h, 0.5 mM indomethacin treatment significantly reduced survivin protein levels (to 74.2 + 3.8%, $p < 0.05$) and induced apoptosis (to 8.6 + 2.1%, $p < 0.05$) compared to vehicle treatment (survivin, 100.0 + 4.9%; apoptosis, 0.0 + 2.2%). All indomethacin treatments significantly reduced survivin protein levels and induced apoptosis by 6 h, compared to vehicle treatment, and the effects were concentration-dependent (Fig. 1A).

To examine whether indomethacin treatment also down-regulates survivin expression at the transcription level, survivin mRNA expression in indomethacin-treated cells were compared to the basal expression levels at 0 h of treatment by realtime qPCR analysis. Survivin mRNA expression was not significantly altered by indomethacin treatment at all concentrations and time points examined (Fig. 1B).

Based on these results, 0.1 mM indomethacin treatment was chosen to perform subsequent protein half-life experiments, since it caused a marked reduction in survivin protein levels with minimal cytotoxic effects.

3.2. Indomethacin treatment decreased survivin protein half-life

Since indomethacin treatment reduced survivin protein but not mRNA levels, down-regulation of survivin by indomethacin likely occurs at the post-translational level. Therefore the effect of indomethacin on survivin protein half-life was examined. First, survivin protein half-life in RGM-1 cells was determined. RGM-1 cells were treated with vehicle, or

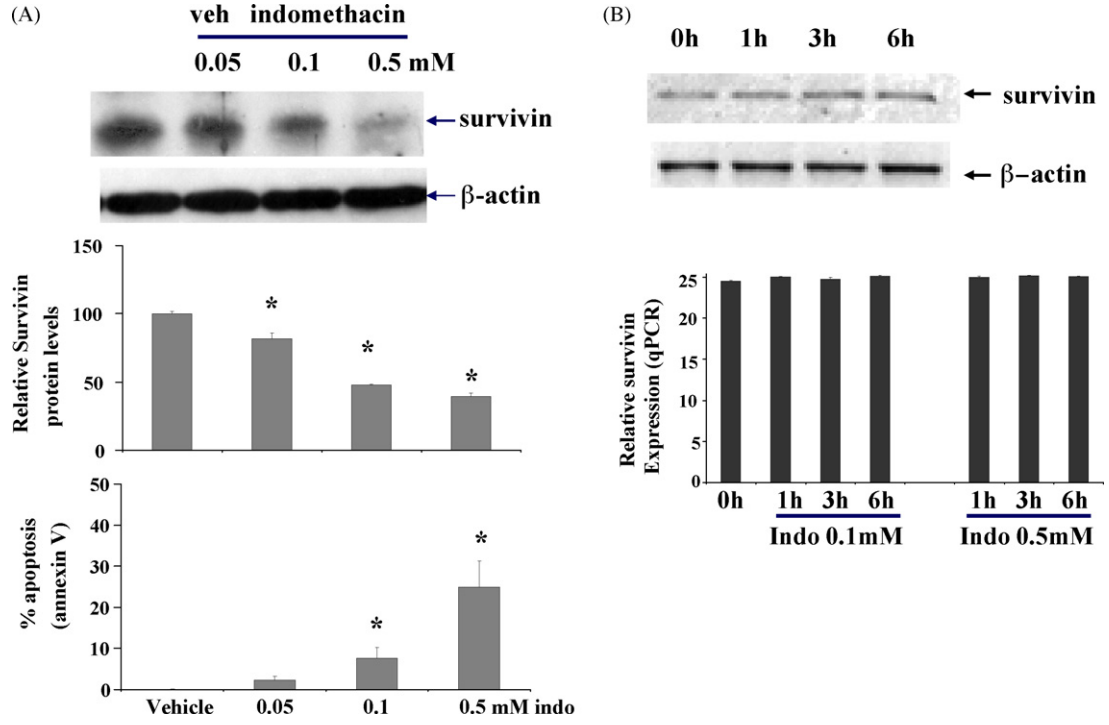

Fig. 1 – Indomethacin reduces survivin protein but not mRNA, and induces apoptosis in cultured RGM-1 cells. (A) Cells were treated with increasing doses of indomethacin as indicated for 6 h. Indomethacin significantly decreases survivin protein and induces apoptosis in cultured RGM-1 cells in a concentration-dependent manner. (B) Realtime quantitative PCR showed that indometacin treatment does not affect survivin mRNA expression. Beta-actin serves as control for equal protein and mRNA amount in each sample. *Statistically significant reduction of survivin or induction of apoptosis, $p < 0.05$.

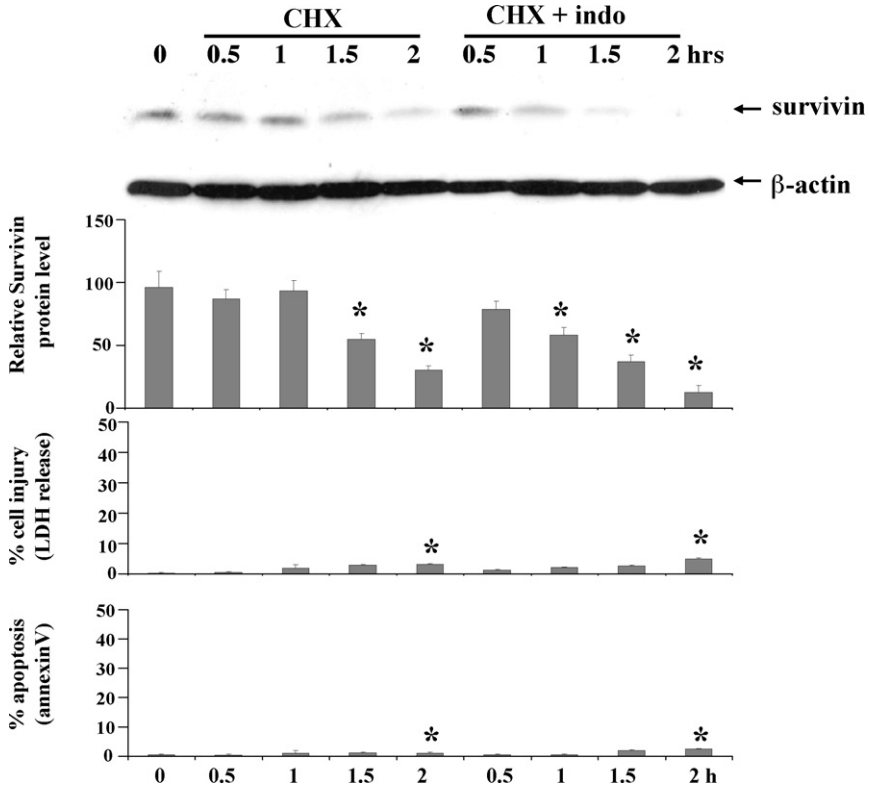

Fig. 2 – Indomethacin treatment reduces survivin protein half-life in RGM-1 cells. Cells were cultured in the presence of 100 μg/ml CHX for the indicated times, and survivin protein levels were monitored by Western blot analysis. Survivin half-life in RGM-1 cells is approximately 1.5 h. When treated with 0.1 mM indomethacin, survivin half-life is reduced to between 1 and 1.5 h, in the presence of CHX. Beta-actin serves as control for equal protein amount in each sample. Cell injury and apoptosis were not significant factors in reducing survivin protein levels, since all treatments up to 1.5 h did not induce significant cell injury or apoptosis, and induced minimal amounts of cell injury and apoptosis at 2 h. *Statistically significant reduction of survivin protein levels, or induction of cell injury and apoptosis, $p < 0.05$.

100 μg/ml CHX to stop new protein synthesis, and survivin protein levels were examined. Vehicle treatment did not alter survivin protein levels at all time points examined. At 1.5 h after addition of CHX, survivin protein levels decreased to 54.68 + 4.9% of baseline (0 h) (Fig. 2). Thus survivin protein half-life in RGM-1 cells is approximately 1.5 h. The percentages of cell injury and apoptosis induced by CHX treatment were insignificant up to 1.5 h, and minimal (2.91 + 0.3% and 1.2 + 0.18%, respectively) after 2 h (Fig. 2). Therefore, cell injury and apoptosis were unlikely to be significant factors in decreasing survivin protein levels within the duration of these experiments.

To determine the effect of indomethacin on survivin protein half-life, RGM-1 cells were pretreated with vehicle only or indomethacin then treated with vehicle only or CHX, and total survivin protein levels were examined at various timepoints. Again, treatment with vehicles only did not alter survivin protein levels at all time points examined. Indomethacin pretreatment reduced survivin protein levels to 58.31 + 5.08% of baseline at 1 h, and 35.09 + 4.98% of baseline at 1.5 h after addition of CHX, indicating that survivin protein degradation is enhanced by indomethacin treatment (Fig. 2). Again, cell injury and apoptosis were unlikely to be significant factors in decreasing survivin half-life, since percentages of cell injury and apoptosis induced by cycloheximide plus indomethacin treatments were insignificant up to 1.5 h, and minimal (4.45 + 0.36 and 2.03 + 0.19%, respectively) after 2 h (Fig. 2).

3.3. *Indomethacin treatment increased survivin ubiquitination*

The ubiquitin proteasome machinery is a major system in the cell that regulates protein degradation. Survivin is shown to be degraded via the ubiquitin proteasome pathway in human embryonic kidney cells [22]. It is likely that survivin down-regulation by indomethacin also occurs via proteasomic degradation in RGM-1 cells. We determined whether indomethacin treatment targets survivin for proteasomic degradation by examining the effect of indomethacin on survivin protein ubiquitination. RGM-1 cells were first treated with 0.1 mM indomethacin up to 6 h, and survivin ubiquitination was examined by immunoprecipitation with anti-survivin antibodies, then Western blotting using anti-ubiquitin antibodies. Indomethacin treatment alone did not significantly increase survivin ubiquitination up to 3 h, and significantly decreased both total and ubiquitinated survivin protein by 6 h (Fig. 3A). This was expected since indomethacin treatment

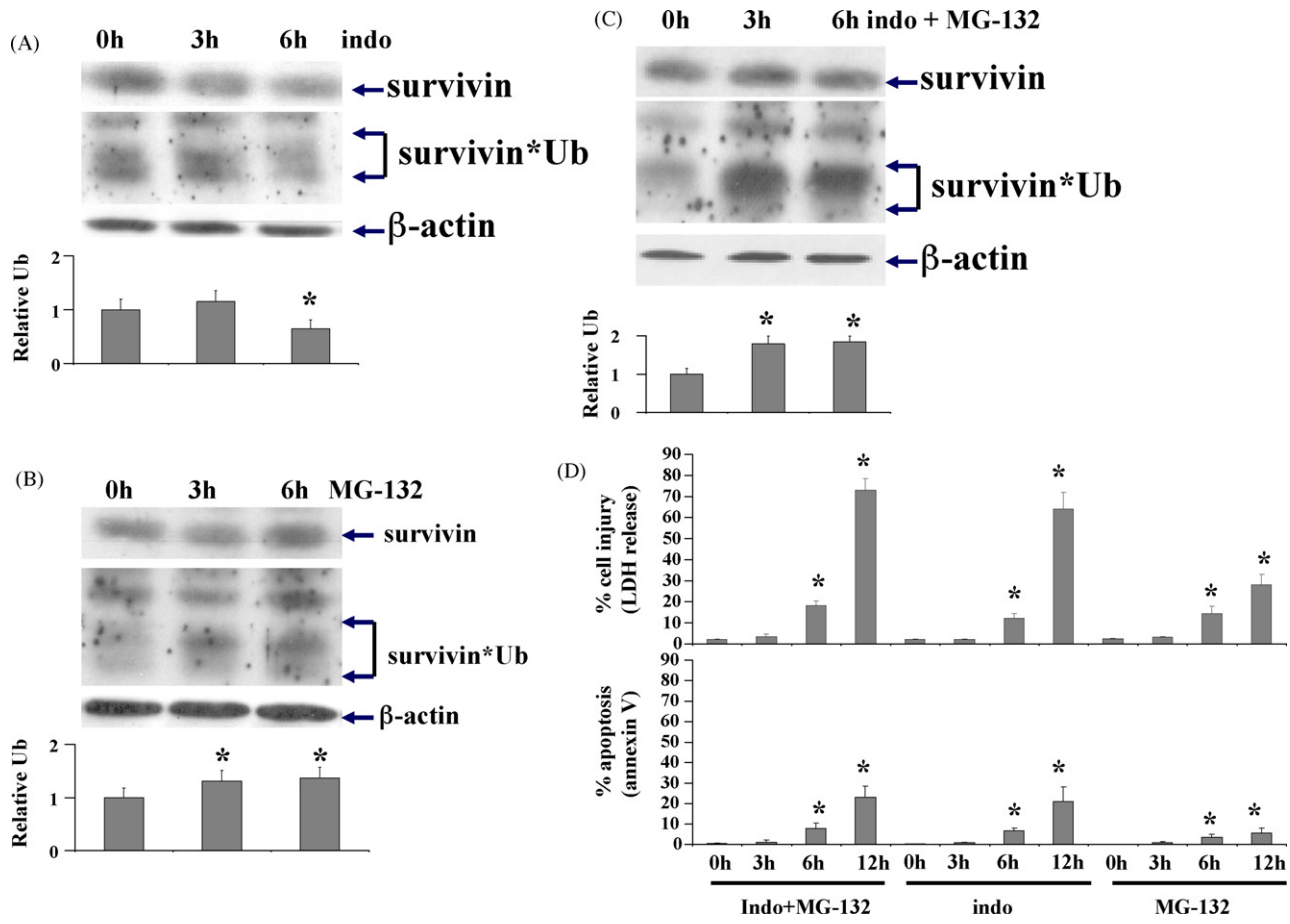

Fig. 3 – (A) Indomethacin treatment increases ubiquitination of survivin protein in RGM-1 cells. Cells were treated with indomethacin alone, MG-132 alone, or indomethacin plus MG-132 for the indicated durations. Then survivin protein was immunoprecipitated using polyclonal anti-survivin antibodies, and ubiquitination was examined by Western blot analysis using polyclonal anti-ubiquitin antibodies. (A) Treatment with 0.1 mM indomethacin reduced cellular survivin protein levels, and thus ubiquitinated survivin. Reduction was significant by 6 h. (B) Ten micromoles MG-132 caused accumulation of survivin protein and ubiquitinated survivin as early as 3 h of treatment. (C) Treatment with 0.1 mM indomethacin in the presence of 10 μM MG-132 significantly increased survivin protein ubiquitination by 3 h, and proteasome inhibitor prevented the reduction of survivin protein levels at 6 h by indomethacin. (D) Treatments with indomethacin induced significant cell injury and apoptosis by 12 h. Treatment with MG-132 alone markedly induced cell injury, but not apoptosis. Beta-actin serves as control for equal protein amount in each sample. *Statistically significant increase of total ubiquitin content or apoptosis induction, $p < 0.05$.

ultimately significantly reduced survivin levels and induced cell injury and apoptosis by 12 h (Fig. 3D). To circumvent this problem, cells were pretreated with MG-132, which does not interfere with ubiquitination but inhibits proteasomic protein degradation, for 20 min before addition of indomethacin. Treatment with MG-132 alone resulted in significant accumulation of ubiquitinated survivin protein (1.31 + 0.31-fold by 3 h, and 1.37 + 0.32-fold by 6 h, compared to 0 h) (Fig. 3B). Treatment with indomethacin in the presence of MG-132 significantly increased survivin ubiquitination above the level of accumulation achieved by MG-132 alone (1.89 + 0.34-fold by 3 h, and 1.95 + 0.19-fold at 6 h, compared to 0 h) (Fig. 3C). The increase in survivin ubiquitination at 6 h was insignificant compared to 3 h, possibly due to significant cell injury by MG-132 alone and indomethacin plus MG-132 treatments at 6 h (Fig. 3D). By 12 h, indomethacin alone and indomethacin plus MG-132 dramatically induced both cell injury and apoptosis, but MG-132 alone dramatically induced cell injury but not apoptosis, indicating that toxicity associated with MG-132 alone occurs via different mechanisms (Fig. 3D).

3.4. *Inhibitors of ubiquitin proteasome prevented survivin degradation by indomethacin in RGM-1 cells*

To confirm that survivin protein degradation normally occurs via the ubiquitin proteasome pathway in RGM-1 cells in the absence of indomethacin, the ability of proteasome inhibitors to prolong survivin protein half-life was examined. Vehicle treatment alone did not alter survivin protein stability at all timepoints examined. Treatment with CHX alone reduced survivin protein levels to half by 1.5 h (Fig. 2). Treatment of

RGM-1 cells with proteasome inhibitors MG-132 and lactacystin extended survivin protein half-life to at least 2 h in the presence of CHX (Fig. 4). In contrast, treatments with other protease inhibitors such as leupeptin and PMSF did not prolong survivin protein half-life (Fig. 4), indicating that survivin protein degrdation normally occurs mainly via the ubiquitin proteasome machinery in RGM-1 cells.

To examine whether the ubiquitin proteasome is involved in down-regulation of survivin protein levels by indomethacin, the ability of proteasome inhibitors to prevent survivin down-regulation by indomethacin was examined. Cells were pretreated with inhibitors, followed by addition of indomethacin in the presence of CHX. Treatment with indomethacin alone significantly reduced survivin protein levels (lane 2, Fig. 5) when compared to vehicles only treatment (lane 1, Fig. 5). Pretreatment of RGM-1 cells with proteasome inhibitors MG-132 and lactacystin prevented survivin down-regulation by indomethacin (lanes 4 and 5, Fig. 5), but pretreatment with leupeptin and PMSF did not (lanes 6 and 7, Fig. 5). MG-132 and lactacystin treatments alone, in the absence of indomethacin, did not alter survivin protein levels (Fig. 5). All treatments induced minimal cell injury and apoptosis within the duration of these experiments (Fig. 5). These results indicate that indomethacin treatment enhances survivin protein degradation by the ubiquitin–proteasome pathway in RGM-1 cells.

3.5. *Overexpression of survivin confered resistance to indomethacin-induced RGM-1 cell injury and apoptosis*

To determine whether survivin expression plays a role in preventing RGM-1 cell injury and apoptosis by indomethacin, we examined whether overexpression of survivin could prevent indomethacin-induced cell injury and apoptosis. A stable cell line that ectopically overexpresses survivin was established, and compared to a control cell line (empty vector transfectant) that expresses basal survivin levels (Fig. 6A) for resistance to indomethacin-induced cell injury and apoptosis. The survivin overexpressing cell line was characterized by approximately 4-fold higher survivin expression (Fig. 6A), and significantly more rapid growth than the control cell line (Fig. 6B). The survivin overexpressing cell line is significantly more resistant to injury (Fig. 6C, left) and apoptosis (Fig. 6C, right) than the control cell line when treated with a high concentration (0.5 mM) of indomethacin. These results indicate that survivin expression plays a role in resistance to indomethacin-induced cell injury and apoptosis.

4. Discussion

In this study we showed that indomethacin down-regulates survivin protein levels in normal gastric epithelial cells via

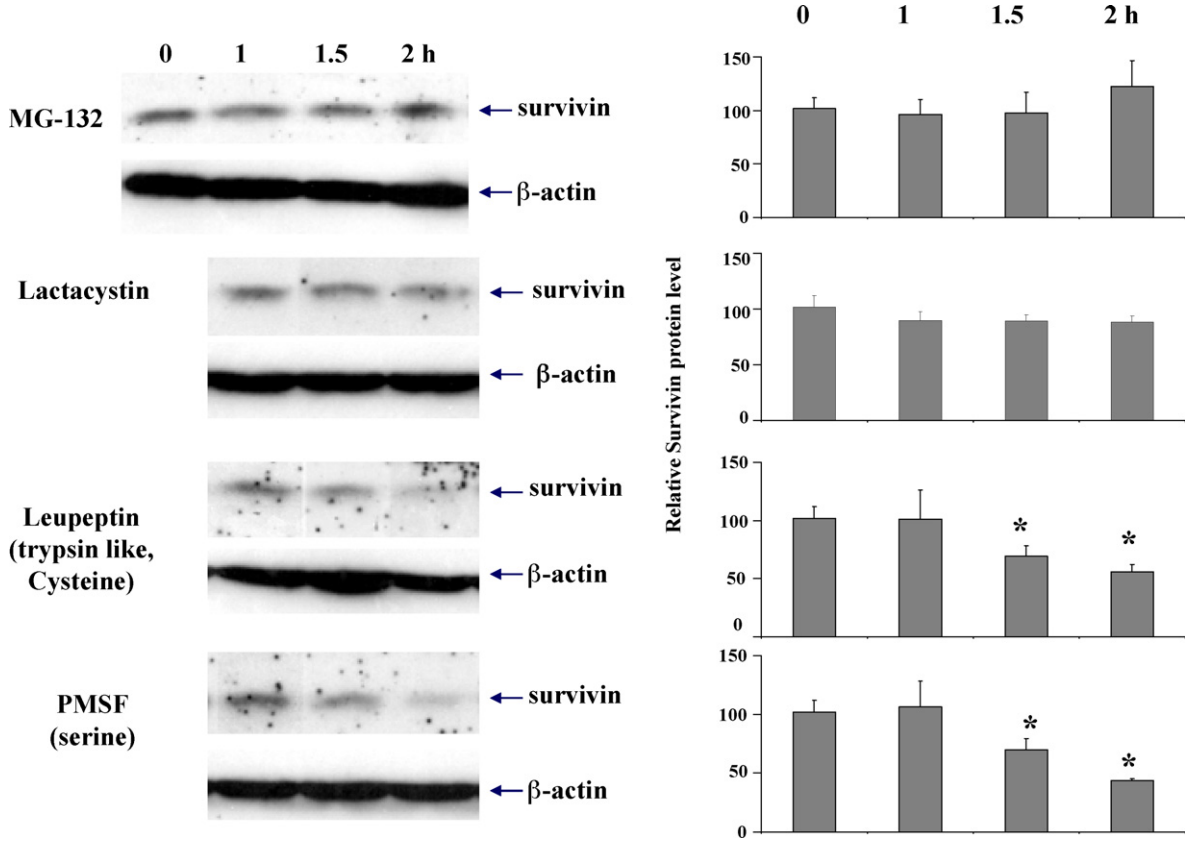

Fig. 4 – Proteasome inhibitors prolong survivin protein half-life in RGM-1 cells. Treatment with ubiquitin proteasome inhibitors MG-132 and lactacystin increased survivin protein half-life to at least 2 h in the presence of CHX, and in the absence of indomethacin. Treatment with other protease inhibitors, leupeptin and PMSF, did not affect survivin protein half-life in the presence of cycloheximide. Beta-actin serves as control for equal protein amount in each sample. *Statistically significant reduction of survivin protein levels, $p < 0.05$.

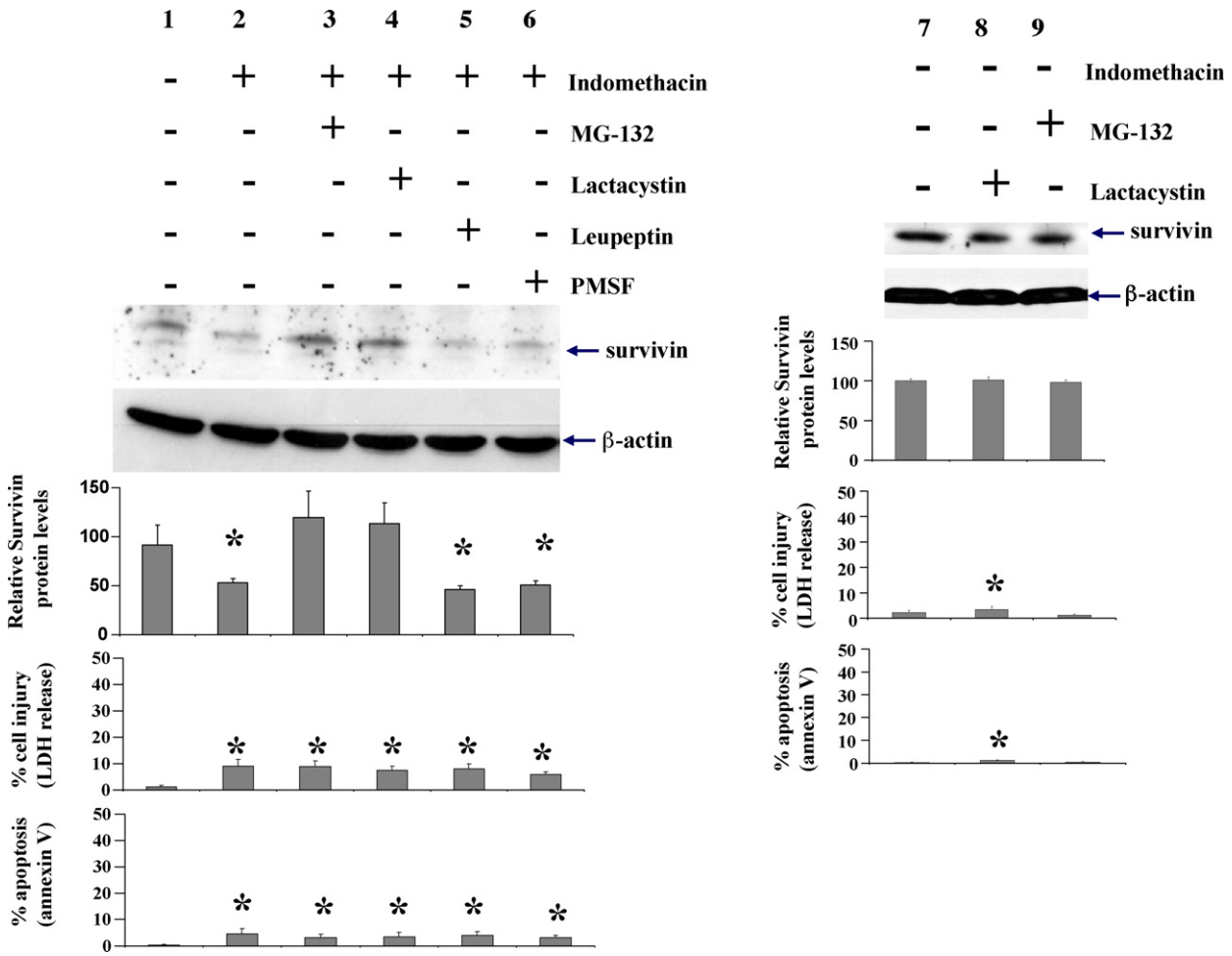

Fig. 5 – Proteasome inhibitors prevent reduction of survivin by indomethacin in RGM-1 cells. Cells were cultured in 0.1 mM indomethacin for 6 h in the presence or absence of the indicated inhibitors. Indomethacin treatment alone significantly reduced survivin protein levels (lane 2) compared to vehicle only treatment (lane 1). Treatments with proteasome inhibitors MG-132 and lactacystin prevented reduction of survivin by indomethacin (lanes 3 and 4). Treatments with other protease inhibitors, leupeptin and PMSF, had no effect on survivin protein reduction by indomethacin (lanes 5 and 6). Treatments with MG-132 and lactacystin alone (lanes 8 and 9) did not affect survivin protein levels. All treatments induce minimal cell injury and apoptosis. Beta-actin serves as control for equal protein amount in each sample. *Statistically significant reduction of survivin protein levels, $p < 0.05$.

enhanced proteasomic degradation of survivin protein: indomethacin treatment decreased survivin protein half-life but not mRNA expression, targeted survivin for ubiquitin proteasomic degradation by increasing survivin protein ubiquitination, and proteasome inhibitors but not other protease inhibitors prevented indomethacin-induced survivin protein degradation. These data are in agreement with an earlier observation that the ubiquitin proteasome plays a role in NSAIDs-induced gastric injury, since pre-treatment of rats with proteasome inhibitors reduces indomethacin-induced gastric injury [24]. Proteasomic degradation has also been shown to play a role in regulation of apoptosis and cell proliferation by other NSAIDs treatments. For example, celecoxib induces apoptosis in lung cancer cells by down-regulation of cellular FLICE-inhibitory protein (c-FLIP), a major negative regulator of the death receptor-mediated extrinsic apoptotic pathway, through a ubiquitin/proteasome-dependent mechanism [25], and Curcumin inhibits proliferation of a variety of human cancer cells by increasing proteasomic degradation of cyclin E, a proto-oncogene that is over-expressed in many human cancers and mediates the G(1) to S transition [26].

Suppression of survivin at the post-translational level by NSAIDs has not been shown previously. Thus far, NSAIDs have been reported to exert transcriptional repression of survivin expression. Numerous factors that influence survivin expression at the transcriptional level have been described. Survivin expression is transcriptionally repressed by the p53 and Rb tumor suppressors and via APC/beta-catenin/TCF-4 signaling, and induced by the E2F transcription factors and by growth factors such as VEGF and bFGF in endothelial cells [27–30]. Sulindac treatment results in transcriptional inhibition of survivin expression in colon cancer cells, although the molecular mechanism(s) involved in this process remain unexamined [31]. Celecoxib treatment also down-regulates survivin expression in a variety of human cancer cell lines via

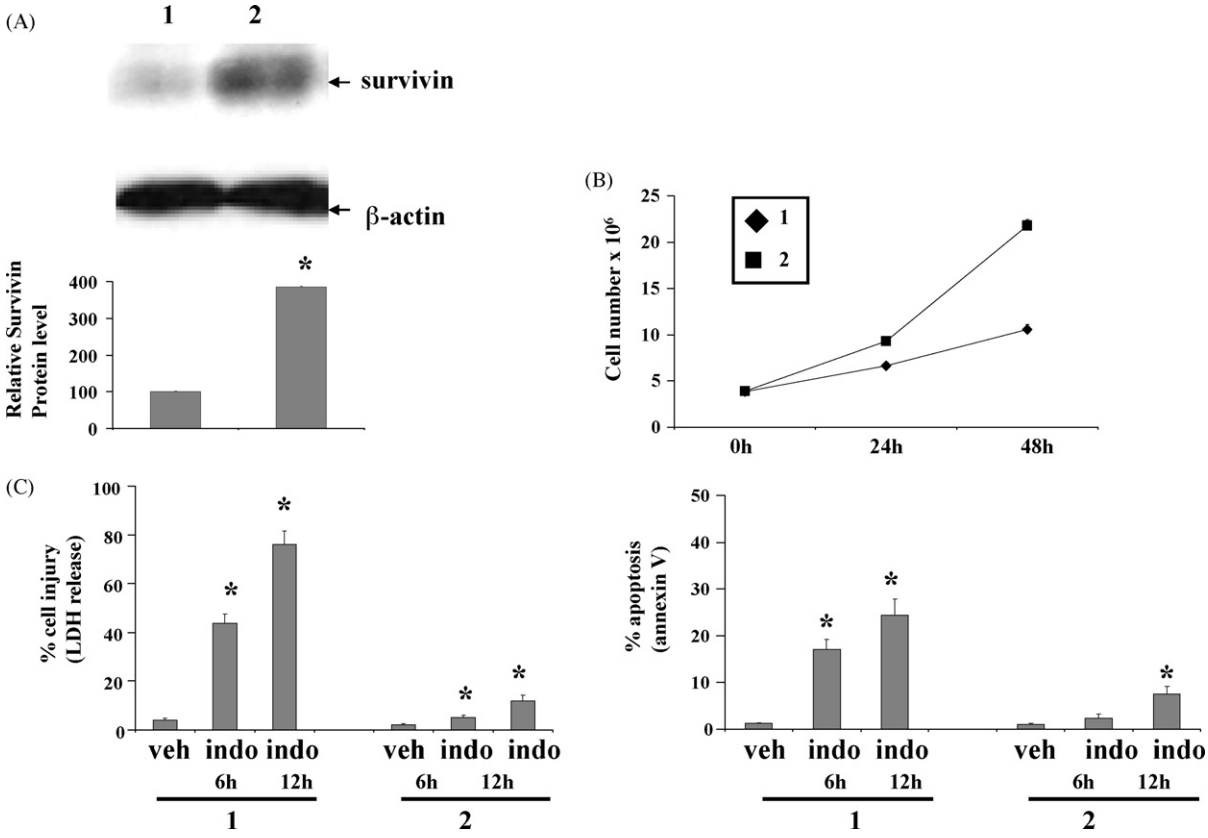

Fig. 6 – (A) Western blot (top panel) and quantitation of survivin protein by densitometry (bottom graph) in the empty pcDNA3 vector transfectant control (1) and the survivin overexpressing (2) RGM-1 cell lines. (B) Cell growth data showing that survivin overexpressing cell line (2) increases in cell number faster than control cell line (1). (C) Left, 0.5 mM indomethacin treatment significantly induced injury in the control cell line, but not survivin overexpressing cell line. Right, the survivin overexpressing cell line was also more resistant to indomethacin-induced apoptosis than the control cell line. Magnification 20×. *Statistically significant overexpression of survivin protein or increase in cellular injury and apoptosis, $p < 0.05$.

a p53-independent mechanism of transcriptional repression, and this culminates in induction of apoptosis [32]. Survivin is also a short-lived protein that is degraded via the ubiquitin proteasome pathway in a cell cycle-dependent manner in embryonic kidney cells [22]. Our results showed that survivin protein is also degraded by the ubiquitin proteasome in RGM-1 cells, since proteasome inhibitors prolonged survivin protein half-life in the presence of CHX and in the absence of indomethacin (Fig. 4). Our results also showed that pretreatment of cells with indomethacin for 2 h prior to addition of CHX decreased survivin protein half-life (Fig. 2), and proteasome inhibitors prevented survivin protein reduction by indomethacin treatment (Fig. 5), suggesting that indomethacin treatment triggers molecular events that enhance survivin protein degradation. Thus far, molecular factors that mediate proteasomic degradation of survivin are unknown. Physical interaction of XIAP with recombinant survivin has been described in MCF-7 and HEK293T cells [33], and XIAP is a potent regulator of apoptosis, which is attributed in part to its E3 Ub-ligase activity in ubiquitylating itself and pro-apoptosis factors such as capase 3 and Smac/Diablo for proteasomic degradation [34–36]. However, XIAP has not been demonstrated to target other IAPs for proteasomic degradation. Further studies are required to determine whether XIAP and/or other factors are involved in survivin protein degradation. This study is the first to demonstrate that indomethacin treatment did not inhibit survivin mRNA expression, but rather reduced survivin protein levels by increasing proteasomic degradation of survivin in gastric epithelial cells.

Our results showed that while indomethacin treatment induced significant levels of cell injury and apoptosis, it did not abolish survivin protein expression in RGM-1 cells, and did not decrease survivin protein half-life dramatically (from 1.5 to 1 h), although both were statistically significant. These results suggest that, in addition to down-regulation of survivin protein levels, more complicated mechanisms of cytotoxicity underlie indomethacin-induced cell injury and apoptosis. Indeed, NSAIDs such as indomethacin are known to illicit a wide variety of molecular effects in the cell, including inhibition of cyclooxygenase 1 and 2, activation of pro-apoptotic caspase cascade, and induction of tumor necrosis factor alpha pathway [37], all of which may contribute to NSAID-induced cytotoxicity. The question arises as to whether survivin down-regulation is one of the significant mechanisms involved in indomethacin-induced cell injury and apoptosis. Numerous studies showed the function of

survivin as a chromosomal passenger protein that participates in stabilizing the mitotic spindle during cell division, but there is ongoing debate about the involvement of survivin in inhibiting apoptosis [5–8]. Our studies consistently showed that survivin levels are significantly down-regulated by indomethacin treatment prior to onset of cell injury and apoptosis both *in vitro* and *in vivo* (Fig. 2, [17]). This suggests that reduction of survivin protein levels is an early molecular event triggered by indomethacin treatment that eventually leads to the demise of the cell, and that maintaining survivin levels is necessary for gastric cell viability. In support of this, CHX treatment alone also resulted in a significant reduction of survivin protein levels (1.5 h) prior to onset of cell death (2 h), indicating a role for survivin reduction in cell death (Fig. 2). Indeed, results of this study showed that survivin expression is important for resistance to indomethacin, since survivin overexpressing RGM-1 cells were more resistant than control cells that express basal survivin levels to indomethacin-induced injury and apoptosis (Fig. 6). Previously, we showed that siRNA suppression of survivin expression increases indomethacin-induced RGM-1 cell injury, confirming that indomethacin induces cellular injury in part by down-regulation of survivin [17]. Taken together, our current data and available literature strongly indicate that one significant mechanism by which indomethacin may cause gastric cell injury and apoptosis is by facilitating degradation of survivin via the ubiquitin proteasome system. Further studies are required to determine whether NSAIDs also down-regulate other anti-apoptosis proteins, and the molecular factors downstream of survivin reduction that lead to gastric cell injury and apoptosis.

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