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Expression of survivin during liver regeneration☆

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Abstract

Survivin functions to suppress cell death and regulate cell division, and is observed uniquely in tumor cells and developmental cells. However, the expression and regulation of survivin in non-transformed cells are not well elucidated. Therefore, we investigated the expression of survivin in a murine liver regeneration model after partial hepatectomy and intraperitoneal carbon tetrachloride (CCl_4) injection. We found that the expression of survivin transcript and protein were markedly elevated with the onset of DNA synthesis and remained elevated during G2 and M phases during liver regeneration. In a normal mouse liver cell line, over-expression of survivin resulted in a decrease in the G0/G1 phase and an increase in the S and G2/M phases, resulting in Rb phosphorylation. These findings suggest that survivin is dramatically expressed in a cell cycle-dependent manner during liver regeneration and provide a new insight into the regulation of cell proliferation and differentiation.

Keywords: Survivin; Liver regeneration; Cell cycle; Cell proliferation; Apoptosis

The genomic control of apoptosis plays a crucial role in differentiation, development, and maintenance of normal homeostasis [1]. Among the regulators of apoptosis [2], the inhibitors of apoptosis proteins (IAP) [3] have emerged recently as potential modulators of the terminal effector phase of cell death or survival. Among the IAP members, human survivin is virtually undetectable in normal adult tissue, but abundantly expressed in transformed cells and a variety of human cancers [4]. Survivin is atypical as an IAP in this sense [3,4]. It has also been shown that human survivin inhibits apoptosis in cells exposed to diverse apoptotic stimuli [5–7] by associating with microtubles of the mitotic spindle [6] and inhibiting caspase activity [7]. Additionally, expression of human survivin is highly correlated with prognosis in patients with neuroblastoma [8], gastric cancer [9], colorectal cancer [10], and bladder cancer [11].

Recent studies revealed that non-transformed cells also express survivin protein. In normal proliferating cells, human survivin was found in cellular districts with high mitotic indices, including keratinocytes of the basal layer of the skin [5], non-neoplastic proliferative lesions in normal colonic mucosa [12], and normal bone marrow [13]. However, the role of survivin expression in normal liver tissues is not well understood.

Mouse survivin (TIAP) shows a high homology (84% identity and 92% similarity) to human survivin [14]. The mouse survivin gene has a four-exon/three-intron organization similar to the human gene [14]. Unlike human survivin, mouse survivin is expressed in several growing tissues such as thymus, testis, and intestine of adult mice, and many tissues of the embryo [15]. In adult mouse tissues, survivin is also expressed in proliferating tissues [16]. These observations suggest us that survivin plays an important role in cellular differentiation and proliferation in normal tissues.

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The current study evaluates the expression and regulation of survivin in non-transformed cells. We investigated survivin expression in mouse liver after partial hepatectomy and CCl_4 injury, providing an in vivo model for the study of cell cycle-dependent expression [17–19].

Materials and methods

Molecular cloning. Mouse survivin cDNA was obtained by reverse transcription-coupled PCR using a first-strand cDNA derived from mRNA of a day 14.5 murine embryo as template and primers (forward, 5′-ATCATGGGAGCTCCGGCGCT-3′; reverse, 5′-GCATT AGGCAGCCAGCTGCT-3′). PCR was carried out using Takara Pyrobest (Takara Shuzou, Kusatsu, Japan). After separation of fragments by gel electrophoresis, the desired band was excised, purified from the agarose, and inserted into pcDNA3.1/V5/His-TOPO vector (Invitrogen, Groningen, The Netherlands).

Cell culture and transfection. The mouse hepatocyte cell line, BNL-CL2 (#TIB-73, ATCC, Rockville, MD), was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum. Transfections were performed using FuGENE 6 (Boehringer–Mannheim, Mannheim, Germany) after serum starvation for 24 h and control cells received only pcDNA3.1-LacZ plasmid (Invitrogen). Transfection efficiency was uniformly greater than 50%.

Animal surgery. Adult male C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Animals were kept in a temperature-controlled room with alternating 12-h dark/light cycles and acclimatized for at least 1 week before the experiment. Experimental mice were 12–15 weeks old and of approximately 20 g body weight. All animal procedures were performed according to the guidelines of the NIH Intermural Animal Care and Use Program. A partial (two-thirds) hepatectomy was performed on some mice according to the method of Higgins and Anderson [20] under diethyl ether anesthesia. Liver regeneration was followed for the next 2 weeks. Other mice were injected intraperitoneally with 1 mg/kg CCl_4 dissolved in olive oil and liver regeneration was followed for the same period.

Cell cycle analysis in liver regeneration. Livers were surgically removed and the DNA labeling index of hepatocytes was determined after injection of 50 mg/kg of 5′-bromo-3′deoxyuridine (BrdU; Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England) intraperitoneally 2 h before sacrifice. The staining was performed using a cell proliferation kit (Amersham Pharmacia Biotech). Mitotic figures in the hepatocytes were counted and quantified as a percentage of the total number of hepatocytes. Mice were sacrificed by cervical dislocation and the remnant livers were dissected at the indicated time points after surgery.

Preparation of tissue samples and cell extracts. Tissue samples were crushed with a mortar with pestle while on ice and homogenized three times with a sonicator (Branson Ultrasonics, Danbury, CT). After centrifugation at 15,000 rpm for 15 min at 4 °C, supernatants were collected and protein concentrations were determined with the Bio-Rad Protein Assay System (Bio-Rad Laboratory, Melville, NY).

After transfection, cells were washed, scraped, and suspended in ice-cold PBS, and placed on ice. Cell extracts were homogenized in lysis buffer (50 mmol/L Tris–HCl, pH 8.0, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, and 1 mmol/L phenylmethylsulfonyl fluoride) and a protease inhibitor cocktail (Sigma). Protein concentrations were determined as described above.

Real-time PCR. Survivin mRNA was detected using a real-time PCR method. Total RNA was extracted from 5 mg tissue at several time points by the acid guanidinium thiocyanate–phenol–chloroform extraction method using RNAzolB (Sawady, Tokyo, Japan) and collected from the precipitate in ethanol. cDNA was prepared with a survivin cDNA primer (5′-CCCGTTTCCCCAATGACTTAGA-3′), a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primer (5′-CTTGATGTCATCATATTTGGCAGG-3′). The PCR mixture was prepared using a TaqMan Universal Master Mix (PE Applied Biosystems, CA, USA). The primer set to amplify survivin mRNA was designed according to GenBank NM001168, using primers exon 1 (SVN-F): 5′-AGAACTGGCCCTTCTTGGAGG-3′ and exon 2–3 (SVN-R): 5′-CTTTTTATGTTCCTCTATGGGGTC-3′. The probe (exon 1–2 (SVN-P): 5′-AGCGGATGGCCGAGGCTGGCTTC-3′) was designed to target an internal region between SVN-F and SVN-R primers. This primer set did not detect a survivin-β mRNA (GenBank AB028869). The primer set for amplification of G3PDH mRNA was designed according to GenBank M33197, using primers exon 7: 5′-TGCACCACCAACTGCTTAGCACCC-3′ and exon 8: 5′-CTTGA TGTCATCATATTTGGCAGG-3′. The probe for G3PDH-P was designed from exons 7 to 8: 5′-TGACCACAGTCCATGCCATC ACTGC-3′. Each PCR was 50 cycles using a real-time PCR system (ABIPRISM 7700 Sequence Detection System: PE Applied Biosystems). The PCR products of survivin were directly sequenced using the BigDye terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems). The sequence was finally compared with each target mRNA sequence.

Western blotting. Equivalent amounts of lysate from each extract were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride. After blocking with 5% non-fat dried milk at 4 °C overnight, the blots were incubated with 3 μg/ml anti-survivin antibody (Santa Cruz Biotech, Santa Cruz, CA) or anti-α-tubulin antibody (Oncogene, San Diego, CA). The immunoblots were washed and probed with horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Tokyo, Japan). After washing, the antigen–antibody complexes were visualized using the ECL detection system as recommended by the manufacturer (Amersham). PhosphoPlus Rb Antibody Kit (Cell Signaling Technology, Beverly, MA) was also used.

Cell cycle analysis in culture cells. Cell cycle distribution was determined by measuring the cellular DNA content using flow cytometry. To prepare samples for analysis, 1×10^6 cells were collected 48 h after transfection and fixed with 70% cold ethanol. Rnase A (10 μg/ml) was added and the cells were resuspended in 0.5 ml propidium iodide solution (50 μg/ml in 0.1% sodium citrate with 0.1% NP-40). Propidium iodide-stained cells were analyzed with a FACScan cytometer using Cell Quest software (Becton–Dickinson, Tokyo, Japan).

Results

Cell cycle dynamics in mouse liver regeneration

To define the relevant parameters in this model system, we first examined the time course of DNA synthesis during mouse liver regeneration after partial hepatectomy or CCl_4 intraperitoneal injection. We measured the incorporation of BrdU and the mitotic index in hepatocytes at various time points after the surgery and injection. In the adult mouse liver, most hepatocytes are quiescent. However, after partial hepatectomy and CCl_4 intraperitoneal injection, DNA synthesis increased sharply at day 1.5, declined rapidly at first, and then gradually decreased to baseline after 7 days (Fig. 1). The peak in DNA labeling index occurring at day 1.5 represented the first major wave of DNA synthesis in the hepatocytes, when many cells were in the S phase of the

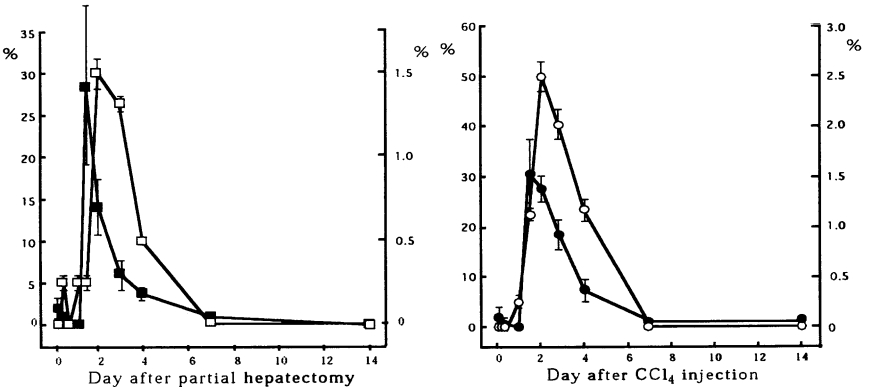

Fig. 1. Time course of labeling index and mitotic index in hepacytes after partial hepatectomy or CCl_4 intraperitoneal injection. Mice were subjected to partial hepatectomy and CCl_4 injection, and livers were harvested at the indicated time points following BrdU injection 2 h before sacrifice. Samples were fixed, sectioned, and then stained with antibodies to BrdU. Positively stained hepatocyte nuclei in each sample were counted and the labeling index was plotted as the percentage of the total number of hepatocytes in the field (hepatectomy, ■; CCl_4, ●). Mitotic figures in hepatocytes were counted and quantified as a percentage of the total number of hepatocytes in the field (hepatectomy, □; CCl_4, ○). Mean values ± SD are indicated.

cell cycle. The mitotic index was maximal at 2 days after partial hepatectomy and CCl_4 injection, consistent with completion of this first round of proliferation. These results indicated that liver regeneration in mice showed highly synchronized cell proliferation.

Survivin expression is markedly induced during S and G_2/M phases in liver regeneration

At first, survivin expression was analyzed by real-time PCR in regenerating mouse liver. Survivin mRNA was detected at a level of 3×10^6 copies per μg total RNA in the pre-treated mouse. There was a transient decrease for 1 day followed by a rapid increase that was observed at day 1.5 reaching a maximum at day 2 in transcript. The transcript signal decreased by day 3 and returned to baseline after 7 days. Typically, the maximal induction of survivin transcript was approximately 30-fold (Fig. 2). Increasing expression occurred during the peak in the BrdU labeling index, corresponding to S phase, whereas maximal expression occurred at or near the peak in the mitotic index, or during G_2/M phase (Figs. 1 and 2). These dynamics of transcriptional expression survivin were observed similarly in both the hepatectomy and the CCl_4 injection model.

To confirm survivin protein expression during liver regeneration, we immunoblotted the same liver homogenates with anti-survivin antibody. In day 15.5 embryonic liver tissue, mouse survivin was detected prominently at the expected molecular weight of 16.2 kDa. Therefore, we used this homogenate as a positive control. Survivin protein was detected in pre-treated adult liver tissues. After treatment, survivin protein expression increased by day 1.5, reached a maximum at day 2, and then gradually decreased but was still detectable 14 days after surgery or CCl_4. Thus, the time courses of survivin protein and mRNA expression were almost in good agreement (Figs. 2 and 3). These results indicated that survivin protein was primarily transcriptionally regulated in regenerating liver tissue after partial hepatectomy or CCl_4 intraperitoneal injection. Thus, the expression pattern of survivin protein in regenerating liver was time-dependent, consistent with the progression of cell proliferation.

Effect of survivin on cell cycle and phosphorylation of Rb

To confirm the involvement of survivin in cell proliferation, we transfected the normal mouse liver cell line, BNL-CL2, with survivin after cell cycle synchro-

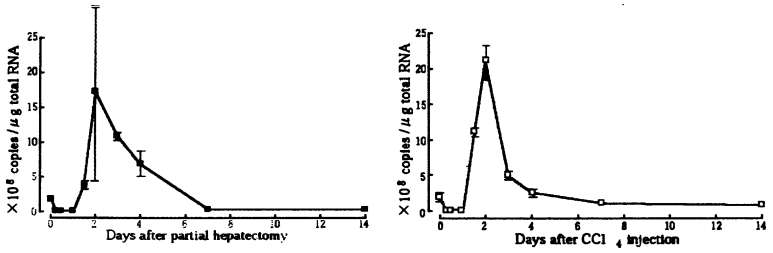

Fig. 2. Survivin mRNA was detected in harvested liver tissue at indicated time points using a real-time PCR method (hepatectomy, ■; CCl_4, □). Mean values ± SD are indicated.

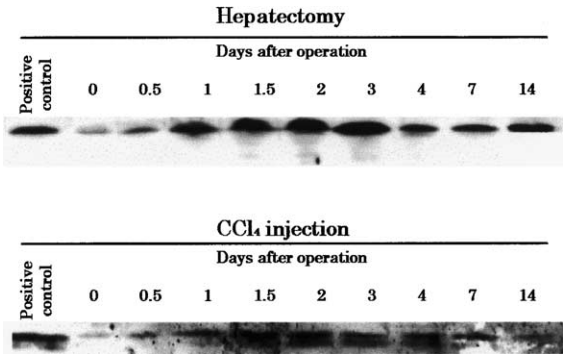

Fig. 3. Expression of survivin protein during liver regeneration using Western blot analysis. An equal amount of total protein in tissue was electrophoresed on SDS–polyacrylamide gels. After transfer to membranes, the blots were probed with antibody to survivin.

nization by serum starvation for 48 h. Cell cycle changes were analyzed by flow cytometry 48 h after transfection with mock vector or survivin. We confirmed over-expression of survivin in the transfectant by Western blotting (Fig. 4A). The cell line transfected with survivin showed a decrease in cells in the G0/G1 phase and an increase in cells in the S phase and G2/M phase (Fig. 4B). Finally, we examined the phosphorylation of Rb (Ser780, Ser785), since it is an event critical to S phase entry. Up-regulation of phosphorylated Rb was observed by Western blotting in cells over-expressing survivin (Fig. 4C).

Discussion

In this study, we examined the expression of mouse survivin during cell cycle progression in regenerating liver after 70% partial hepatectomy or CCl_4 injection. Survivin transcripts induction began in the S phase and increased through the G2/M phases in the cell cycle. Immunoblotting experiments suggested that expression of survivin protein was highest in the cells undergoing mitosis, as manifested by the presence of mitotic figures. Further, we found that over-expression of survivin phosphorylates Rb protein and promotes cell cycle progression from G1 phase to G2/M phase in a normal mouse hepatocyte cell line.

In the adult human, survivin expression is among the most tumor-specific of human gene products. While fetal tissues contain abundant survivin mRNA and protein, most adult tissues were believed not to express survivin [4]. However, recent work revealed that survivin is also expressed in non-neoplastic lesions, hyperplastic polyps, and the crypt epithelium of normal colonic mucosa [12], and in normal endometrium [21] and placental development [22].

In contrast to the human, mouse survivin is expressed in growing tissues, such as the thymus, testis, and intestine of adult mice and in many tissues of the embryo. Most survivin-positive cells in embryos at these stages are proliferating rapidly to form mature organs. In adult mouse tissues, survivin is also expressed in proliferating tissues, most of which have a large population of cells undergoing mitosis, from S phase to G2/M phase [16]. These observations suggest that survivin may be related to cell proliferation and differentiation, even in non-transformed adult tissues. However, how survivin is regulated in these normal cells has not been well elucidated.

The regenerating liver has proven to be an excellent in vivo model system to study the mechanism of growth control within a natural tissue environment. In this study, we confirmed that liver regeneration after partial hepatectomy or CCl_4 injection also exhibits well-synchronized DNA synthesis and cell cycle progression. The peak of DNA synthesis occurred at 1.5 days after treatment and mitosis was maximal after 2 days. Within 7–14 days after chemical damage or 70% surgical resection, these cells undergo one or two rounds of replication until the liver reaches its initial mass, then the cells re-enter the quiescent phase [17–19].

Using this mouse model, we analyzed survivin expression. Surprisingly, the 12–15-week-C57BL/6 mouse used in this study expressed both survivin mRNA and protein in non-treated liver tissues. However, survivin expression was low when compared with that observed

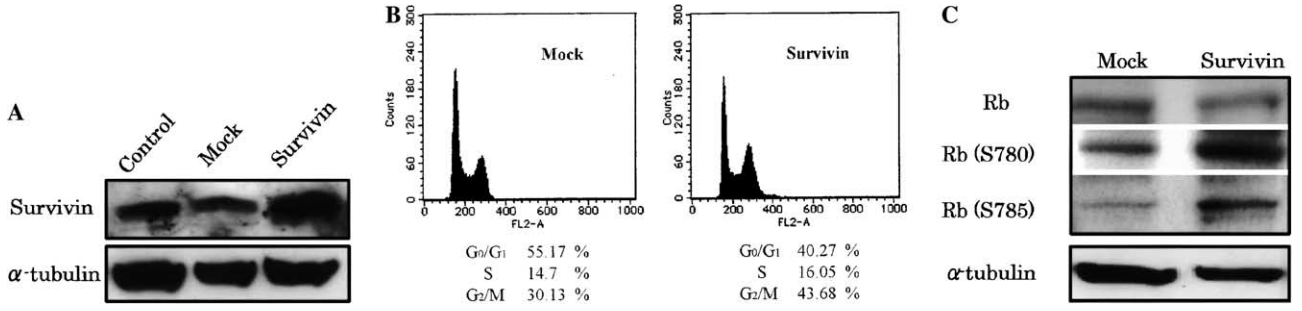

Fig. 4. Cell cycle analysis of BNL-CL2 cells (mouse normal hepatocytes) transfected with survivin after cell cycle synchronization by serum starvation for 48 h. Over-expression of survivin in the transfected cells by Western blotting (A). Cell cycle changes were analyzed by flow cytometry 48 h after transfection (B). Phosphorylation of Rb (Ser780, Ser785) was examined by Western blotting after survivin trasfection (C).

in 15.5-day mouse embryonic liver tissue, which is known to express survivin abundantly. A previous study failed to detect survivin mRNA in adult mouse liver tissues by Northern blot analysis [16]. Such differences may be due to the different analysis methods used. We have also detected survivin mRNA in human non-transformed liver tissue by in situ hybridization (unpublished data, H. Hisatomi, et al.). Our findings suggest that survivin may play a role, even in normal cellular pathways.

The current results demonstrated that survivin expression began to increase in the S phase, with maximal expression occurring in G2/M phase of the cell cycle during liver regeneration. Our immunoblotting results also suggested that the expression level of mouse survivin was highest in cells undergoing mitosis, as manifested by the presence of mitotic figures. During mitosis, survivin localizes to kinetochores until metaphase and then redistributes to the spindle midzone and to the telophase disk during anaphase, ending in the midbody during cell cleavage [23].

A previous study showed that survivin plays an important role in maintaining cell viability at mitosis, potentially coupling apoptosis control to regulation of cell division. Survivin also associates with Cdc2 on the mitotic apparatus and is phosphorylated on Thr^{34} by Cdc2. Moreover, Cdc2 localizes with survivin on mitotic spindle microtubules and midbodies [24]. We have also recently shown that human survivin interacts with cyclin-dependent kinase 4 (Cdk4) and over-expression of human survivin releases p21 from Cdk4 in human hepatocellular carcinoma cell lines [25]. The cyclin-dependent kinases Cdk2 and Cdk4 regulate progression through the G1 phase of cell cycle in a complex with cyclin [26]. These observations support the notion that survivin might function at certain points in the cell cycle transition.

Our finding that the expression of mouse survivin is related to cell proliferation is compatible with the observation that human survivin is expressed prominently in most common human cancers and found frequently in high-grade non-Hodgkin's lymphomas, but not in low-grade lymphoma [4]. The characteristic expression pattern of survivin in proliferating cells suggests that high expression of survivin in tumors may be a causative factor in the generation of neoplasm, or at least a deteriorating factor, but may also be the result of rapid growth after neoplastic transformation. Therefore, we examined whether survivin over-expression could affect the cell cycle phase distribution. In a normal adult mouse hepatocyte cell line, survivin over-expression decreased the G0/G1 phase population and increased the S and G2/M phase population. These results suggested that survivin has an important role directly or indirectly in cell proliferation of in vitro cultured non-transformed cells. There are some reports regarding the effect of survivin antisense expression on cell proliferation. Forced expression of survivin antisense induced morphologic changes characteristic of apoptosis in human cancer cell line, resulting in an increase in the apoptosis of cells transfected with survivin antisense compared with control cells. In addition, cells transfected with survivin antisense revealed an increase in the sub-G0/G1 fraction corresponding to apoptotic cells and a reduction in the G2/M fraction corresponding to dividing cells [5,14]. These observations also support our findings obtained from the liver regeneration study and survivin transfection experiments.

We propose that this novel function of survivin plays an important role in the proliferation and differentiation pathways during development of normal cells. Further evaluation of this and other possible roles of survivin and the regulation of survivin expression are crucially important for the development of new strategies for controlling the normal and neoplastic tissue development.

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