Survivin associates with cell proliferation in renal cancer cells: Regulation of survivin expression by insulin-like growth factor-1, interferon-γ and a novel NF-κB inhibitor

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Abstract. Although survivin has been widely recognized as an attractive target for cancer therapy, the exact mechanism regarding the regulation of survivin and its effect on cell proliferation have yet to be clearly defined in renal cell carcinoma (RCC). We investigated herein the association between survivin expression and cell proliferation in a RCC cell line, KU19-20. In KU19-20 cells, cell proliferation and survivin expression were significantly induced by IGF-1, whereas they were inhibited by a novel NF-κB inhibitor dehydroxymethyl-epoxyquinomicin (DHMEQ) and IFN-γ. The combination of DHMEQ and IFN-γ inhibited cell proliferation synergistically with a pronounced attenuation of survivin expression. Furthermore, treatment with survivin-specific siRNA reduced expression of survivin and significantly inhibited cell proliferation. Survivin expression was thus associated with cell proliferation in KU19-20 cells. The regulation of survivin by IFN-γ and/or an NF-κB inhibitor may therefore be a potential treatment modality for RCC.

Introduction

Renal cell carcinoma (RCC) accounts for 2-3% of all adult cancers and for 2% of cancer related deaths (1). A patient with metastatic RCC usually has an extremely poor prognosis. Treatment modalities for metastasized disease are limited. Systemic treatment with cytotoxic chemotherapy is usually ineffective, and less than 20% of patients will respond to cytokine-based treatments, such as interleukin (IL)-2, interferon (IFN)-α, and IFN-γ (2). Further improvements have to await the development of a more effective systemic therapy.

Apoptosis is the process by which activated cells undergo a suicide program that results in individual cell death. Abnormalities in the control of apoptosis play an important role in tumorigenesis. Defects in apoptotic mechanisms also play an important role in the resistance to chemotherapy and radiation (3). An abundance of apoptosis inhibitors could, therefore, protect cells from dying and contribute to tumorigenicity. Inhibitors of apoptosis (IAP) proteins were first discovered in baculoviruses and are conserved across evolution. These proteins contain variable numbers of a unique 80-amino-acid repeat, the baculovirus inhibitor of apoptosis repeat (BIR) domain, and a single carboxy-terminal RING zinc finger motif (4). The central mechanism of apoptotic suppression by IAP is thought to be through direct caspase and pro-caspase inhibition and by modulation of the transcription factor NF-κB. Five human IAP homologues (XIAP, cIAP1, cIAP2, NAIP, and survivin) have been identified to date (5). Survivin is a novel member of the IAP proteins detected in many tumors including lung, colon, breast, prostate, pancreas cancers, high-grade lymphoma, but is absent in most normal differentiated adult tissues (6). Although survivin has been widely recognized as an attractive target for cancer therapy, the exact mechanism of survivin regulation and its effect on cell proliferation are not clearly defined, especially in RCC.

To learn more about the role of survivin in RCC, we investigated the expression of survivin in the KU19-20 RCC cell line. The effects of insulin-like growth factor-1 (IGF-1), IFN-γ, and dehydroxymethyl-epoxyquinomicin (DHMEQ), a novel NF-κB inhibitor, on the expression of survivin were also explored. In addition, we evaluated the importance of survivin in RCC cell survival by silencing its expression using siRNA in cultured RCC cells.

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Materials and methods

Cell culture. The KU19-20 cell line, derived from human RCC, was established at Keio University. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 0.3% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA).

Cytokines, antibodies and inhibitor. IFN-γ with a specific activity of $2x10^7$ IU/mg (Pierce Biotechnology, Rockford, IL, USA) and IGF-1 (PeproTec, London, UK) were commercially obtained. Optically active DHMEQ ((-)-DHMEQ) was prepared by us according to the previously reported method (7). The anti-survivin rabbit polyclonal antibody (Alpha Diagnostic International, San Antonio, TX, USA) was also commercially obtained.

Preparation of cell lysates. Cells were cultured in 60-mm dishes until they reached about 80% confluence. Cells were then treated under the indicated conditions. To prepare whole cell lysates, cells were harvested and lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1% NP40 and protease inhibitor mixture) for 15 min on ice. The lysates were then centrifuged at 12,000 g for 5 min at 4˚C. Protein concentrations were quantitated using the DC Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA) and stored at -80˚C until required.

Western blot analysis. Samples were mixed with loading buffer, resolved by 12.5-15% SDS-PAGE, and electrotransferred (100 V, 60 min) onto nitrocellulose membranes. After blocking with 5% dry skim milk, the membranes were incubated for 1 h with primary antibodies in TBS-T (2 mM Tris, 50 mM NaCl, 0.05% Tween-20) and were subsequently incubated with a biotinylated secondary goat anti-rabbit antibody (Bio-Rad Laboratories) at room temperature. Membranes were then incubated for 1 h in streptavidin-biotinylated alkaline phosphatase complex (Bio-Rad Laboratories) at room temperature. Immunoreactivity was detected using an alkaline phosphatase substrate kit (Bio-Rad Laboratories).

Cell viability assay. Cells were plated in 96-well culture plates 48 h prior to treatment. Under the indicated conditions, cells were incubated for a further 48 h. Cell viability was determined using an Alamar Blue assay (Alamar Biosciences, Sacramento, CA, USA). Alamar Blue is bioreduced by viable cells. Briefly, 10% of Alamar Blue was added to each well and the cells were incubated for 3 h. The fluorescence of this reaction was read at 560 and 590 nm. Differences in cell proliferation were subjected to the unpaired t-test and probability values at <0.05 were considered statistically significant.

Silencing of survivin using siRNA. The survivin-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and non-sense siRNA (FASMAC, Kanagawa, Japan), manufactured according to the previously reported method (8), were commercially obtained. Briefly, one day before transfection,

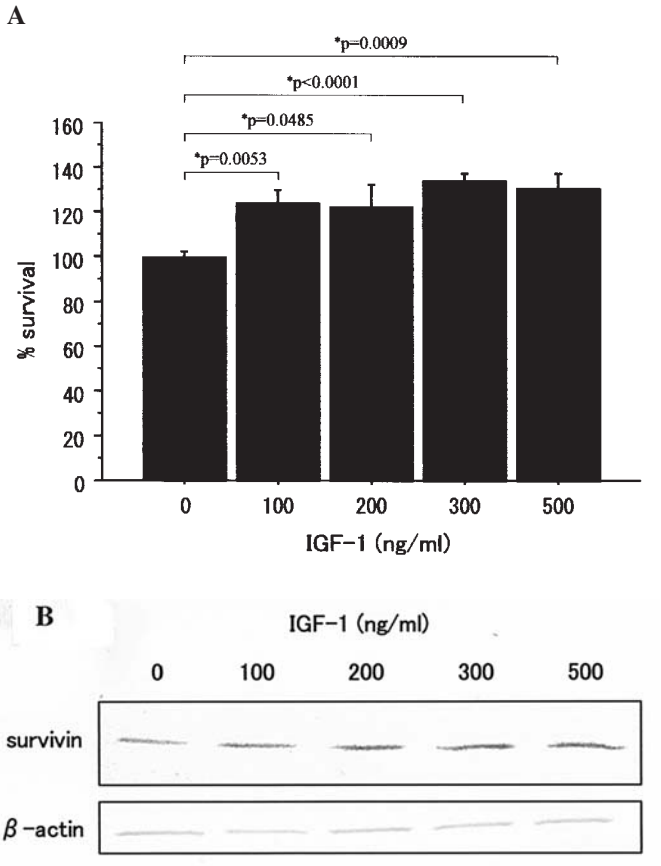

Figure 1. Induction of cell proliferation and survivin expression in KU19-20 cells 48 h after treatment with IGF-1. (A), Cell viability using Alamar Blue assay. (B), Survivin Western blot analysis. *Statistical significance.

KU19-20 cells were plated in 6- and 96-well culture plates so that they would reach 50% confluence at the time of transfection. The survivin-specific siRNA or non-sense siRNA (each 100 nM) was mixed with Lipofectamin 2000 (Invitrogen) following the manufacturer's instruction and administered to the cells in the RPMI-1640 medium without antibiotics. Cells were maintained in this medium for 72 h and then assayed for survivin silencing and cell viability using Western blot analysis and the Alamar Blue assay.

Results

IGF-1 has been reported to increase survivin expression in other cell lines (9). To investigate the effect of IGF-1 on survivin expression and cell proliferation in RCC cells, KU19-20 cells were cultured for 48 h in the absence or presence of IGF-1 (100-500 ng/ml). Cell proliferation was significantly induced by IGF-1 in a dose-dependent manner. The survivin protein level, as determined using Western blot analysis, was also increased by IGF-1 in a dose-dependent manner (Fig. 1).

Several studies have reported the regulation of other IAPs by IFN-γ (10,11). We postulated that survivin also could be regulated by IFN-γ. KU19-20 cells were cultured for 48 h in the absence or presence of IFN-γ (100-1000 U/ml). IFN-γ inhibited cell proliferation in all concentrations tested. Survivin expression was also decreased by IFN-γ as determined using Western blot analysis (Fig. 2).

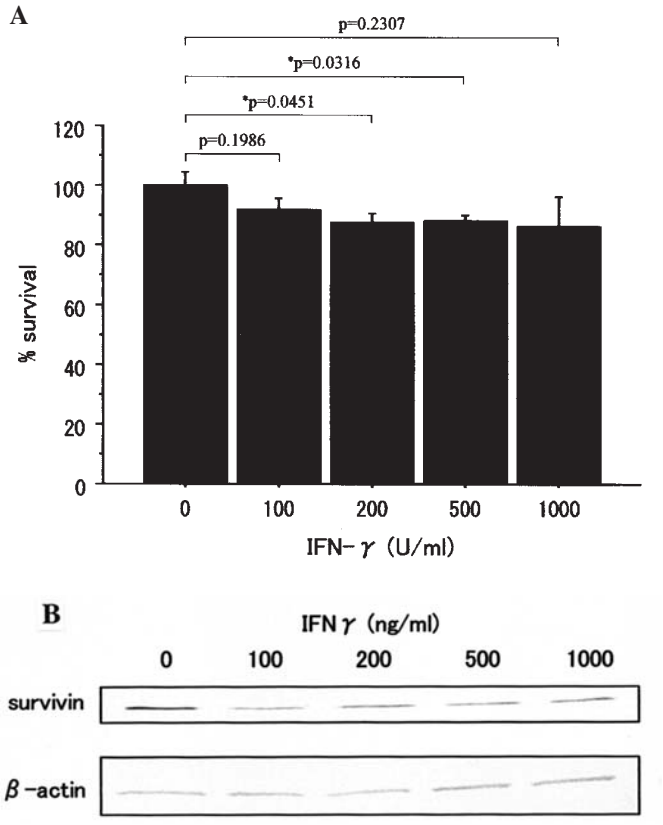

Figure 2. Inhibition of cell proliferation and survivin expression in KU19-20 cells 48 h after treatment with IFN-γ. (A), Cell viability using Alamar Blue assay. (B), Survivin Western blot analysis. *Statistical significance.

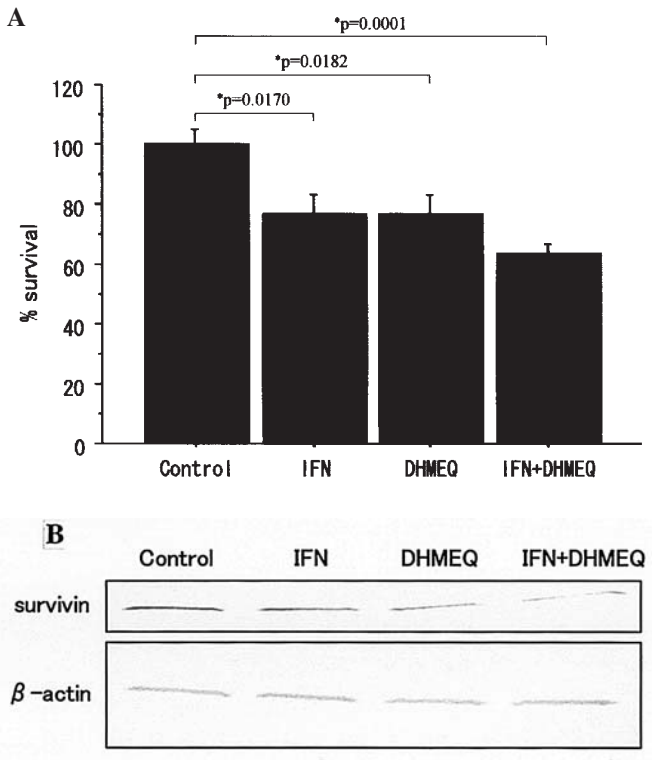

Figure 4. Effect of IFN-γ and DHMEQ combination on cell proliferation and survivin expression in KU19-20 cells. The cells were treated with IFN-γ, DHMEQ or both for 48 h respectively. (A), Cell viability using Alamar Blue assay. (B), Survivin Western blot analysis. IFN, IFN-γ 200 U/ml; DHMEQ, DHMEQ 10 μg/ml. *Statistical significance.

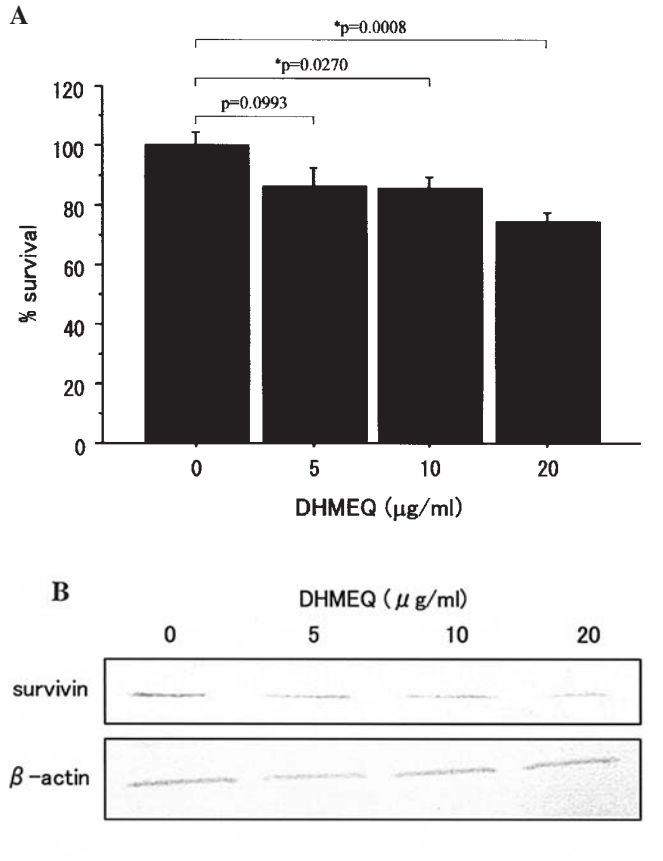

Figure 3. Inhibition of cell proliferation and survivin expression in KU19-20 cells 48 h after treatment with DHMEQ. (A), Cell viability using Alamar Blue assay. (B), Survivin Western blot analysis. *Statistical significance.

To evaluate the influence of NF-κB on survivin expression and cell proliferation, KU19-20 cells were cultured for 48 h in the absence or presence of DHMEQ (5-20 μg/ml). DHMEQ inhibited cell proliferation in a dose-dependent manner. The survivin expression was also significantly decreased by DHMEQ (Fig. 3).

We then combined DHMEQ and IFN-γ to evaluate their synergistic effect on survivin expression and cell proliferation. KU19-20 cells were treated with DHMEQ (10 μg/ml), IFN-γ (200 U/ml) or both for 48 h. The combination of DHMEQ and IFN-γ significantly inhibited cell proliferation. Also, the expression of survivin was decreased synergistically compared with each agent alone (Fig. 4).

To show that suppression of survivin expression directly lead to decreased cell proliferation, we utilized RNA interference to silence survivin. After transfection with survivin-specific siRNA for 72 h in KU19-20 cells, survivin was significantly silenced and the cell proliferation was inhibited. On the contrary, treatment with non-sense siRNA did not affect survivin expression and cell proliferation (Fig. 5A and B). Successful uptake of siRNA was confirmed by an epifluorescence microscope using fluorescein-labeled non-sense siRNA (Fig. 5C). These results suggest the presence of a direct relationship between survivin expression and cell proliferation in KU19-20 cells.

Discussion

Abnormalities in the control of apoptosis play an important role in tumorigenesis (3). First discovered in baculoviruses,

IAP proteins are thought to block apoptosis through direct caspase and pro-caspase inhibition and by modulation of the transcription factor NF-κB (5). However, their exact role in RCC is unknown.

Survivin is a novel member of IAP proteins detected in many tumors, but is absent in most normal differentiated tissues (6). Unlike other IAP proteins, survivin contains only one BIR domain and lacks a carboxyl-terminal RING zinc finger (6). Survivin is the only apoptosis inhibitor that is expressed in a cell cycle-dependent manner in the G2 and M phases and associates with the microtubules in the mitotic spindle. Disruption of the survivin-microtubule interactions results in loss of survivin's anti-apoptosis function and increased caspase-3 activity. When overexpressed, survivin has oncogenic potential because it can overcome the G2/M checkpoint to ensure mitotic progression (12). Regulation of survivin by cytokines and/or an NF-κB inhibitor would be an attractive method to develop a new treatment modality for RCC.

To show that survivin expression associates with cell proliferation in RCC, we investigated the effects of IGF-1, IFN-γ, DHMEQ and their combination in the KU19-20, RCC cell line.

IGF-1 is a growth and survival factor for a diverse range of malignant cells. However, only a few studies have demonstrated IGF-1 receptor expression and function in RCC. An elevated amount of insulin- and IGF-1 receptor expression with increased specific autophosphorylation and tyrosine-kinase activity in surgically resected samples has been reported (13). In contrast, others found no expression of IGF-1 and its receptors in RCC cell lines (14). Therefore, the role of IGF-1 in RCC is controversial. We herein revealed that IGF-1 induced cell proliferation in a dose-dependent manner and increased survivin expression in KU19-20 cells. Similar observations have been reported in human multiple myeloma cells (9). Our results suggest that one of the mechanisms of cell proliferation by IGF-1 in RCC is through increased survivin expression.

IFNs are glycoproteins that exert antiproliferative effects on tumor cell growth, as well as immunomodulatory and antiviral effects (15). IFN-γ is a type II IFN that is structurally unrelated to the type I IFNs and distinctly recognizes cell surface receptors from those recognized by the type I IFNs. Recent studies demonstrated that IFN-γ alone induces apoptosis in different cell types (10). IFN-γ has also been shown to make target cells susceptible to various apoptotic stimuli, such as Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (11,16). However, little is known about the mechanism of the effects of IFN-γ, especially in RCC. We demonstrated that IFN-γ inhibited cell proliferation and survivin expression in KU19-20 cells. Although several studies have reported the regulation of IAPs by IFN-γ, such as downregulation of cIAP-2 and

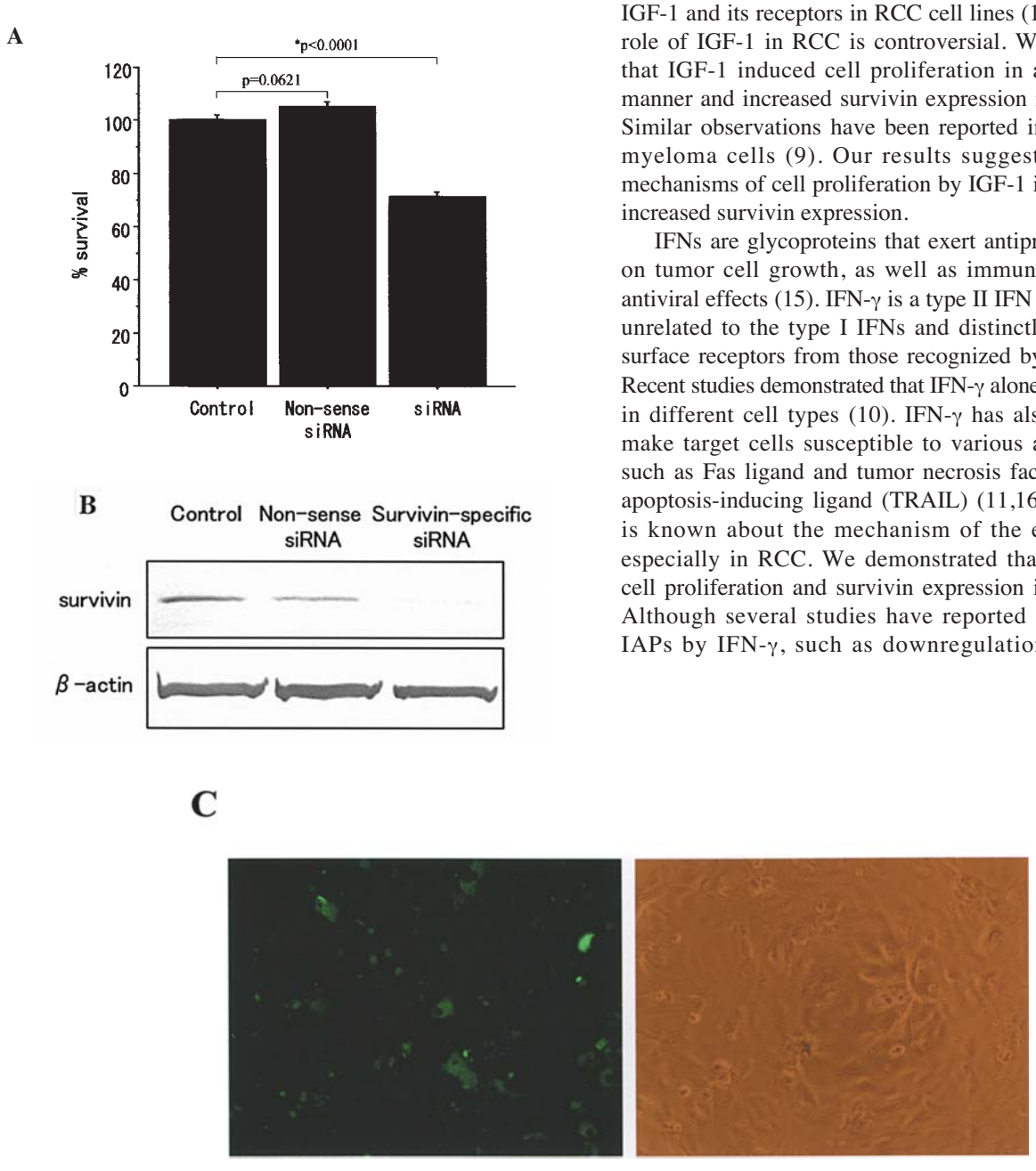

Figure 5. Silencing of survivin expression and reduction in cell proliferation using survivin-specific siRNA. KU19-20 cells were treated with survivin-specific siRNA or non-sense siRNA for 72 h. (A), Cell viability using Alamar Blue assay. (B), Survivin Western blot analysis. (C), Uptake of fluorescein-labeled non-sense siRNA 48 h after transfection. Left, image by an epifluorescence microscope. Right, light image of the same field. *Statistical significance.

inhibition of TRAIL-induced IAP-2 upregulation (10,11), decreased survivin expression by IFN-γ alone has not been reported before. Decreased survivin expression is one of the potential mechanisms of growth suppression by IFN-γ in RCC. However, the clinical response rate to IFN-γ in phase III study was only 4.4% in RCC patients (17). A more effective way of survivin inhibition, such as combination of IFN-γ with other survivin inhibitors, must be explored.

The role of the NF-κB family members in cancer development is becoming increasingly evident. NF-κB has been shown to play an important role in tumor development and is thought to be a possible molecular target in the treatment of RCC (18). NF-κB has been shown to induce cIAP1, cIAP-2 and inhibition of IAP induction by IκB dominant negatives leads to cell death (19). The IAP proteins may be the predominant anti-apoptotic factor induced by NF-κB (5). Therefore, we examined the effect of an NF-κB inhibitor, DHMEQ, on survivin expression and cell proliferation in a RCC cell line. DHMEQ is a newly designed and synthesized NF-κB inhibitor and its mechanism was found to be inhibition of nuclear translocation of NF-κB (7). We have shown that DHMEQ inhibited cell proliferation and survivin expression in KU19-20 cells, suggesting a close relationship between NF-κB and survivin in RCC. A recent study by Matsumoto *et al* also showed the usefulness of DHMEQ in the treatment of pancreas cancer (20). DHMEQ appears to be a potential candidate for a more effective systemic therapy in the treatment of RCC.

Interestingly, the combination of IFN-γ and DHMEQ inhibited cell proliferation and survivin expression more effectively than each compound alone. However, modulation of NF-κB by IFNs is controversial. The limited efficacy of IFN in the treatment of cancers may result from its induction of cell survival signals through an NF-κB-dependent pathway (21). Other investigators demonstrated a reduction in the TNF-α-mediated NF-κB activation by IFN-γ (22). Our results may be explained in part by the further suppression of NF-κB by the synergistic effects of the combined IFN-γ and DHMEQ treatment. Combination of IFN-γ and DHMEQ is an attractive treatment to improve the low clinical response rate to IFN-γ through survivin suppression.

RNA interference (RNAi) is an evolutionarily ancient mechanism of gene regulation in eukaryotes and has evolved into a powerful tool for probing gene function (23). We showed that DHMEQ, IFN-γ and their combination decreased survivin expression and cell proliferation. However, the direct relationship between survivin expression and cell proliferation was unclear from these experiments. Therefore, we utilized RNAi to prove that decreased cell proliferation resulted from survivin suppression. We successfully depleted the survivin expression using siRNA and this lead to a reduction in cell proliferation. However, despite of successful silencing of the survivin expression, cell proliferation was not completely inhibited. Correlation of survivin suppression and cell proliferation seems to vary among cell lines (8,24). It is suggested that, in the KU19-20 cell line, other signaling pathways may also play a role in cell proliferation along with survivin. However, we found survivin to be one of the important regulatory proteins in RCC. Although successful knockdown of survivin expression using siRNA has been reported in several other cancer cell lines (8,24), there have been no reports regarding RCC. To our knowledge, this is the first study showing the role of survivin in RCC using RNAi.

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