Basic fibroblast growth factor upregulates survivin expression in hepatocellular carcinoma cells via a protein kinase B-dependent pathway

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Abstract. Basic fibroblast growth factor (bFGF) plays an important role in tumor angiogenesis. Several studies have reported that bFGF may influence cell apoptosis through different signaling pathways. The aim of the present investigation was to study the effect of bFGF on the activities of protein kinase B (PKB)/survivin and cell apoptosis in hepatocellular carcinoma cells (Bel-7402). We treated Bel-7402 cells with bFGF and wortmannin [phosphatidylinositol 3-kinase (PI3K)-specific inhibitor] separately to observe the expression of PKB and survivin detected with RT-PCR and western blotting. The cell cycle and apoptosis were assayed with flow cytometry. We found a significant increase in PKB expression in the group treated with 25 ng/ml bFGF for 10 min ($P<0.05$), and this effect was significantly inhibited by pretreatment with wortmannin (200 nM) for 1 h. After treatment with 10 ng/ml bFGF, the expression of survivin mRNA in Bel-7402 cells increased significantly, and reached the peak at 16 h ($P<0.05$); however, this effect could be significantly inhibited by pretreatment with wortmannin (200 mM) in a time-dependent manner. Following incubation with 25 ng/ml bFGF for 10 min, the apoptosis rate and M phase were significantly decreased and S phase cells increased compared with the wortmannin (200 nM)-treated group. When this group was pretreated with wortmannin (200 nM) for 1 h, the apoptosis rate and S phase were significantly increased, M phase cells decreased. The results revealed that wortmannin could induce high apoptosis rates in hepatocellular carcinoma cells, and bFGF could inhibit the cell apoptosis induced by wortmannin. These findings indicate that bFGF could rapidly activate the PKB activities, enhance the expression of survivin and the proliferation of hepatocellular carcinoma cells via the PI3K pathway, thus it may serve as a novel molecule for early targeting therapy of hepatocellular carcinoma.

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Introduction

Liver cancer is one of the most malignant diseases. The early diagnosis and treatment will greatly improve patient prognosis. In recent years, there has been a growing awareness that the cellular microenvironment during tumorigenesis plays a critical role in cancer cell proliferation, survival, migration and invasion (1-3). Substantial evidence shows that cancer is the result of imbalance of endogenous angiogenesis inhibitor and accelerator local concentrations in the tumor tissue. The most important angiogenesis promoting agent is basic fibroblast growth factor (bFGF) and vascular growth factor (VEGF) (4,5). Several recent reports have also implicated bFGF as a major survival factor, which has been shown to mediate this additional function through the induction of Bcl-2 and the activation of the PI3 kinase-Akt/protein kinase B (PKB) signaling pathway. bFGF can also mediate the induction/upregulation of members of a newly discovered family of antiapoptotic proteins, i.e., the inhibitors of apoptosis (IAP) in vascular endothelial cells (6,7). PKB is an enzyme that covalently attaches ATP-phosphate groups to the serine/threonine on protein substrates to alter the activity of the targeted protein (8,9). The activation of Akt is initiated with the plasma membrane recruitment of phosphatidylinositol 3-kinase (PI3K), an upstream enzyme stimulated by a variety of activated growth factor receptors such as epidermal growth factor receptor (EGFR), vascular growth factor receptor (VEGFR), bFGF, and insulin-like growth factor receptor (IGFR) (10). The activation of Akt is completed via the phosphorylation of Thr-308 and Ser-473, leading to increased Akt activity toward a variety of downstream substrates, such as the family of forkhead transcription factors for inhibiting tumor proliferation, the mammalian target of rapamycin (mTOR) for modulating protein synthesis, and the Bcl-xL/Bcl-2-associated death promoter (BAD) for uncontrolled proliferation (9,11). Since Akt acts as a signal hub in the regulation of cell survival,

proliferation and growth, the elevation in Akt activity is found to be correlated with increased tumorigenicity (8). Thus, a variety of inhibitors targeting Akt or its up- or downstream events are currently under clinical trials (12). Wortmannin is a potent and selective inhibitor of Akt, and molecular imaging of Akt activity has become an important approach in monitoring Akt activity *in vivo*. Numerous studies have confirmed that PI3K/PKB plays a crucial role in promoting proliferation and inhibiting apoptosis (13-16).

Survivin is the smallest protein of the known IAP family (17). It is a unique bifunctional protein that inhibits apoptosis by suppressing caspase-3 and caspase-7 and modulates the G2/M phase of the cell cycle through association with mitotic spindle microtubules (18). Survivin contains a single BIR domain and can bind caspases, preventing caspase-induced apoptosis (19); it is the least-expressed IAP family candidate in adult tissues (almost absent in normal tissue), but can be recognized in developing fetal tissues, with the exception of placenta and thymus (20-23). Survivin is able to inhibit apoptosis and promote proliferation of tumor cells. Recently, it was found that survivin protein degradation is associated with the disorder of pantothenic acid - proteasome pathway in a cell cycle-dependent manner (24). The potential value of survivin in tumor diagnosis and treatment has gained considerable attention. It is possible to inhibit growth and recurrence of liver cancer through inhibiting the expression of survivin. Survivin is the strongest inhibitor of apoptosis and its regulation and signaling pathway has yet to be fully clarified. It was reported that bFGF and its receptor FGFR1 highly expressed in hepatocarcinoma cells, play an important role in the occurrence and development of liver cancer and promote the growth of liver cancer cells by autocrine or paracrine mechanisms (25). Investigations into the FGF/FGFR signal pathway may reveal the pathogenesis of numerous diseases, and it may become a new target for the treatment of liver cancer and other diseases. In the present study, we examined whether bFGF could regulate the expression of survivin and affect the apoptosis of liver cancer cells, and we elucidated its signal pathway so as to provide a basis for bFGF in regulating apoptosis via the PI3K/PKB pathway in the treatment of liver cancer.

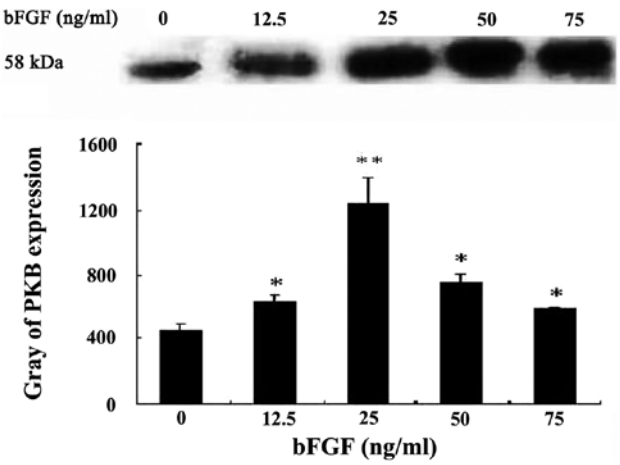

Figure 1. PKB activity of Bel-7402 cells at different concentrations of bFGF (n=3, mean ± SD). *Compared with the control group, P<0.05. **Compared with the control group, P<0.01.

Materials and methods

Cells lines and culture. Human hepatocarcinoma cell line Bel-7402 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) was maintained in DMEM medium supplemented with 10% fetal calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) in 5% CO_2 at 37˚C. Bel-7402 cells were exposed to bFGF (Beijing Shuanglu Pharmaceutical Co., Ltd., China) at different concentrations (0, 12.5, 25, 50 and 75 ng/ml) for 10 min, or to 25 ng/ml bFGF at different times (0, 5, 10, 30 and 60 min) respectively. Each group had 3 parallel wells. Wortmannin (Sigma) was dissolved in dimethyl sulfoxide (DMSO) in serum-free medium with a final concentration of 100, 200 and 400 nM.

Western blot analysis. Cultured cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 1% SDS (sodium dodecyl sulfate), 50 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethylsulfonyl fluoride), 10 mM sodium fluoride, and 1 mM sodium orthovanadate. Cell samples were then agitated at 4˚C for 1 h followed by centrifugation for 15 min. Equivalent amounts of protein (40 μg) from each sample were separated on 10% SDS-PAGE (polyacrylamide gel electrophoresis) and the separated proteins were transferred to nitrocellulose membranes. Membranes were routinely blocked in 5% nonfat milk in PBS with 0.1% Tween-20 for an hour with agitation and washed, and primary antibodies PKB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or survivin were added (1:500-1,000 dilution in 5% bovine serum albumin in PBS buffer). Membranes were incubated overnight at 4˚C with agitation, washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilutions in 5% bovine serum albumin in PBS with 0.1% Tween-20) for 3 h at room temperature. Proteins were detected using the Enhanced Chemiluminescence Western Blotting Detection Reagent (Pierce, Rockford, IL, USA). The results were described as a ratio of relative absorbance value of the protein band of interest to β-actin.

RNA extraction and RT-PCR. Total RNA was extracted, reverse transcribed, amplified and analyzed as previously described (26). Briefly, total RNA was extracted from Bel-7402 cells using a total RNA isolation kit (TransGen) according to the manufacturer's protocol. An ultraviolet spectrophotometer was used to determine the quantity and quality of total RNA. β-actin was used to normalize cDNA quantities and was amplified with the following primers: forward, 5'-ATCATGTTT GAGACCTTCAACA-3' and reverse, 5'-CATCTCTTGCTC GAAGTCCA-3'. For survivin, the following primers were used: forward, 5'-GCACTTTCTTCGCAGTTTCC-3' and reverse, 5'-GGACCACCGCATCTCTACAT-3' (Biosune). The PCR reaction was carried out under the following conditions: 35 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 1 min, and an extension at 72˚C for 2 min. A final extension was performed at 72˚C for 1 min. PCR products were separated by 1% agarose gel electrophoresis.

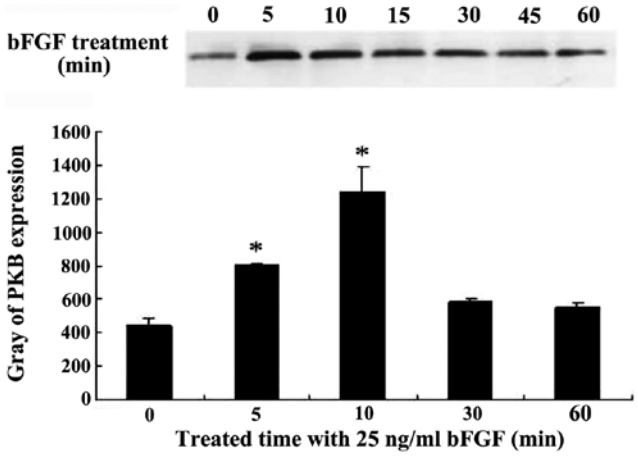

Figure 2. PKB activity of Bel-7402 cells after treatment with bFGF at different times. *Compared with the control group, P<0.05 (n=3, mean ± SD).

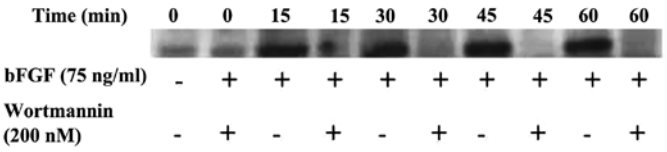

Figure 3. PKB activity in hepatoma cells after bFGF stimulation and pretreatment with wortmannin.

Cell-cycle and apoptosis assay. Bel-7402 cells were treated with different concentrations of bFGF for different times in serum-free medium. Following incubation, cells were harvested, washed in PBS, fixed in cold 70% ethanol for 45 min, stained with propidium iodide solution that contains RNase A for 30 min, and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). CellQuest analysis software was used for DNA content analysis of the cell cycle.

Statistical analysis. All values were expressed as means ± SD. Statistical analysis was processed using one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

PKB activity increases in hepatocarcinoma cells following bFGF treatment. The cytosolic PKB activity gradually increased with bFGF 0-25 ng/ml following different concentrations of bFGF co-incubated with Bel-7402 cells for 10 min, and reached the highest value at 25 ng/ml, 2.81-fold of control (P<0.01); then, cytosol PKB activity declined. There was a significant difference of PKB expression between the bFGF-treated and -untreated groups (P<0.05) (Fig. 1). Bel-7402 cells were co-incubated with 25 ng/ml bFGF for 5, 10, 30 and 60 min. The PKB cytosol activity began to increase at 5 min, 1.82-fold for the control group, and it increased the most at 10 min, which was 2.81-fold that of the control group, and subsequently PKB activity began to decline at 60 min. There was a statistically significant difference (P<0.05) (Fig. 2). Western blot analysis showed that PKB expression was increased at 15, 30, 45 and 60 min with the stimulation of 25 ng/ml bFGF, but PKB expression was clearly inhibited at each time-point when wortmannin (200 nM) was added. The inhibition occurred in a time-dependent manner (Fig. 3). When pretreated with different concentrations of wortmannin under 25 ng/ml bFGF, the expression of PKB was inhibited significantly, in a dose-dependent manner, compared with the wortmannin control group P<0.05, compared with the bFGF control group P<0.01 (Fig. 4).

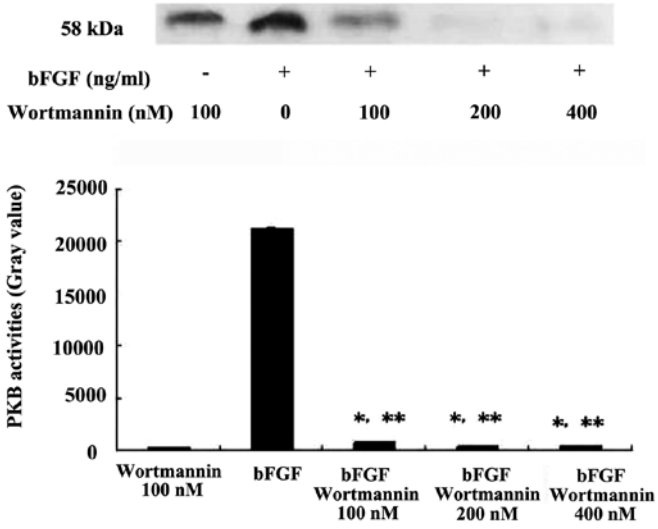

Figure 4. PKB activity after treatment with bFGF and wortmannin (n=3, mean ± SD). *Compared with the wortmannin control group, P<0.05. **Compared with the bFGF control group, P<0.01.

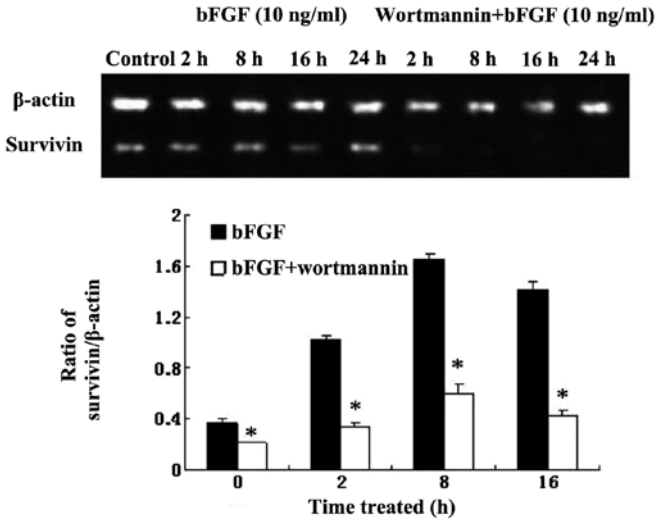

Figure 5. Expression of survivin mRNA in Bel-7402 cells treated with 10 ng/ml bFGF and wortmannin (n=3, mean ± SD). *Compared with the bFGF group, P<0.05.

Survivin mRNA is upregulated with bFGF and downregulated with wortmannin. RT-PCR analysis showed that survivin mRNA expression of Bel-7402 cells was increased, and reached a peak at 16 h, 7.86-fold upregulated compared with the control group after treatment with 10 ng/ml bFGF (Fig. 5) (P<0.05), which suggested that bFGF could induce the expression of survivin mRNA in liver cancer cells. While survivin

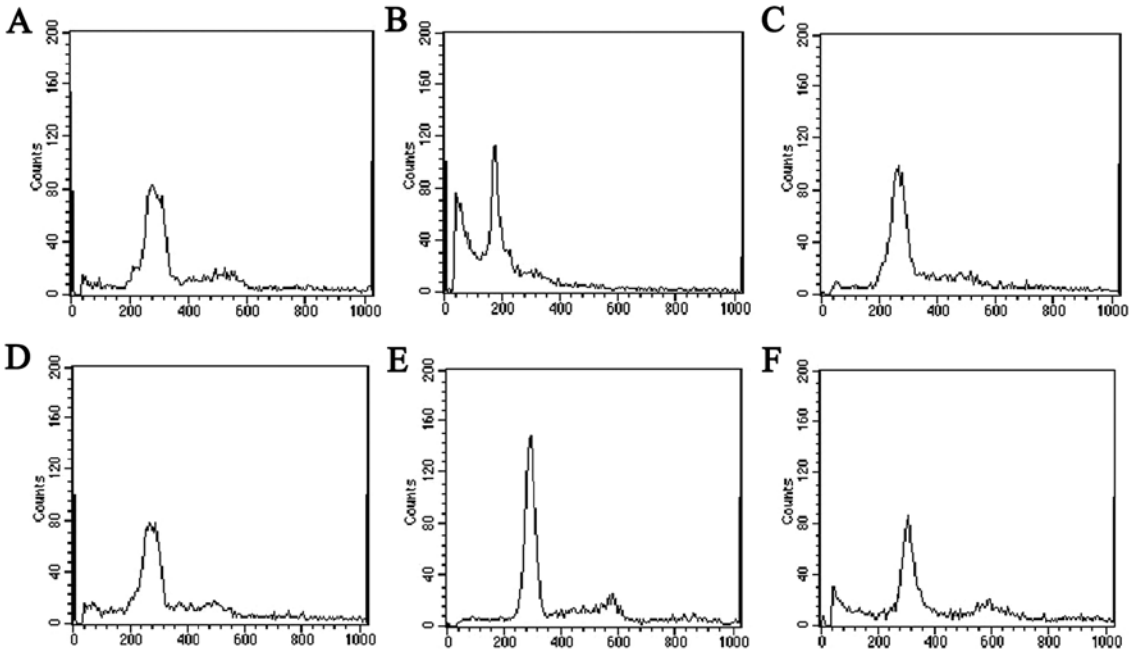

Figure 6. Quantitative detection of cell cycle and apoptotic Bel-7402 cells by flow cytometry.

mRNA expression was significantly suppressed by pretreatment with wortmannin (200 nM) for 1 h, P<0.05, the highest inhibition occurred at 8 h (Fig. 5). The results suggested that increasing survivin mRNA expression induced by bFGF can be blocked by wortmannin in Bel-7402 cells.

bFGF and wortmannin are closely related to cell proliferation and apoptosis. The results of flow cytometry showed lower diploid peak and a small sub-G1 peak (apoptosis cells) in the Bel-7402 cell control group (Fig. 6A). Bel-7402 cells pretreated with wortmannin (200 nM, 1 h) only showed significant hypodiploid peak, accompanied by a significant decline of S phase peak and apoptosis rate up to 50% compared with the cell control group, P<0.05 (Fig. 6B). Following incubation with 25 ng/ml bFGF for 10 min, the apoptosis rate and M phase cells were apparently decreased, and S phase cells increased compared with the wortmannin-treated group (Fig. 6C); however, no marked difference was detected compared with control cells. When this group was pretreated with wortmannin (200 nM) for 1 h, the apoptosis rate and S phase increased significantly, M phase cells decreased (Fig. 6D). Following treatment with 10 ng/ml bFGF for a longer time (16 h), Bel-7402 cells showed typical diploid peak, G1 phase cells increased and the apoptosis rate decreased significantly (Fig. 6E) compared with the control cell group (P<0.05 vs. cell control and wortmannin-treated group), S phase cells increased clearly compared with the wortmannin-treated group. However, when this group was pretreated with wortmannin (200 nM) for 1 h, G1 phase cells decreased apparently, significant hypodiploid peak (sub-G1) appeared and the apoptosis rate increased significantly (Fig. 6F). The effects of bFGF and wortmannin on the apoptosis of Bel-7402 cells are also shown in Fig. 7, demonstrating that wortmannin induced high apoptosis rates and bFGF inhibited apoptosis induced by wortmannin.

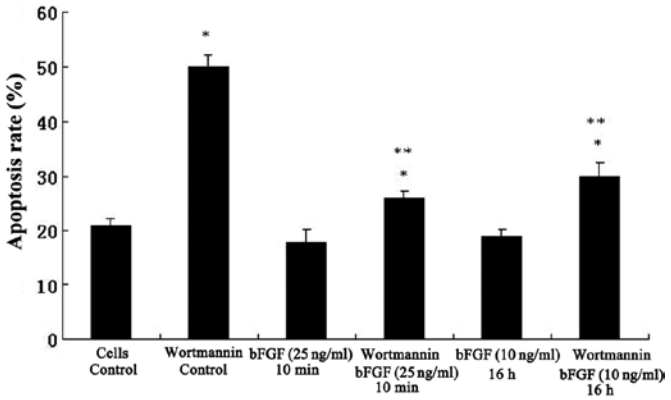

Figure 7. The effects of bFGF and wortmannin on the apoptosis of Bel-7402 cells (n=3, mean ± SD). *Compared with the wortmannin control group, P<0.05. **Compared with the bFGF control group, P<0.05.

Discussion

The complex process of tumorigenesis in humans has been revealed to be a series of stochastic events which occur in almost all types of human cancer and involve the sequential acquisition of a number of genetic, epigenetic or somatic-alterations as a result of increasing genomic instability (26,27). Cancer cells launch various signaling pathways to respond to the altered external microenvironment and to coordinate cell growth with stress responses (28). High levels of TGF-β and bFGF were found in a high percentage of human lung cancer tissues and have been associated with poor patient prognosis (29,30). Although activation of the PBK signaling pathway by TGF-β and bFGF is known to confer resistance to apoptosis in cancer cells, the exact mechanism by which this pathway regulates cell survival has yet to be elucidated.

bFGF in the intracellular transduction pathway has previously been reported, but, to date, it is not fully understood. On the one hand, it can play a regulatory role by different intracellular transduction pathways, such as PLC/PKC, PI3K/PKB, JAK/STAT (31-33). On the other hand, the role in regulating is not consistent in different cells; there is a negative regulatory role in certain cells (particularly tumor cells), while in other cells is the proliferation of the positive regulatory factors (34-36). PKB is the major downstream signal transduction pathway of PI3K. The effect of FGF on PKB activity has yet to be clarified. Although the point that PKB activation is required for cell cycle progression remains to be confirmed, studies show that PKB in some downstream target substances can directly influence cell cycle progression.

It has been reported that survivin inhibits apoptosis by suppressing caspase-3 and caspase-7 and modulates the G2/M phase of the cell cycle through association with mitotic spindle microtubules (18). Cytokines and intracellular growth signal transduction can regulate the expression of survivin. To examine whether bFGF can activate PKB and survivin via the PI3K pathway in the hepatoma cell line Bel-7402, we observed the changes of cytoplasmic PKB and survivin mRNA expression in the Bel-7402 cells with bFGF stimulation and PI3K pathway inhibitor wortmannin. The results showed that the expression of PKB and survivin significantly increased with bFGF (10-25 ng/ml) treatment. These effects were clearly inhibited by pretreatment with wortmannin (200 nM). bFGF treatment inhibited the apoptosis rate of Bel-7402 cells and decreased G2/M phase of the cell cycle, while this effect was blocked by pretreatment with wortmannin (200 nM) for 1 h. The results showed that wortmannin could induce high apoptosis and bFGF could inhibit apoptosis induced by wortmannin. The results revealed that bFGF could rapidly activate the PKB activities, enhance the expression of survivin and the proliferation of hepatocarcinoma cells via the PI3K pathway, thus it may be a novel molecule for early targeting therapy of hepatocarcinoma.

The ability of bFGF to inhibit apoptosis and promote survival of cells could be explained by the following pathways: first, upregulation of Bcl-2 (37-39); secondly, P13 kinase-Akt/PKB signaling pathway which is further confirmed by our results (37,40); the third, upregulation of at least two members of the IAP gene family, XIAP and survivin. For the third pathway, our results showed approximately 8-fold upregulation of survivin induced by bFGF, while PKB was upregulated approximately 3-fold under the same conditions. Previous studies suggested that human survivin, while absent in most adult differentiated tissues, could be detected in almost all transformed cell lines and types of cancer, as well as in fetal tissue (23). Thus, survivin behaves as an 'oncofetal' protein. Our results therefore suggest that therapeutically targeting survivin at the gene expression or protein expression/function levels may result in suppression of angiogenesis. It is also notable that the cell cycle dependency of bFGF induced upregulation of survivin expression. Li *et al* suggested that survivin may counteract a default induction of apoptosis in the G2/M phase by associating with the mitotic spindle at the beginning of mitosis (41). It could be hypothesized that the bFGF functions in survival and mitogenesis of endothelial cells may not be mutually exclusive. Indeed, survivin may be upregulated at the G2/M interface in order to counteract an apoptotic signal, thereby allowing liver cells to survive cell cycle progression and to finally proceed to mitosis. This could therefore represent a tighter association between the mitotic and survival functions. Based on these results, it is also of interest to determine whether agents which block bFGF, or bFGF receptor function, inhibit angiogenesis, in part, by the simultaneous downregulation of multiple effectors of cell survival such as survivin and XIAP. Furthermore, certain other antiangiogenic agents or regulators which are known to induce cell apoptosis, e.g., tubulin-binding agents such as combretastatin-A4, or endogenous inhibitors of angiogenesis, such as angiopoietin-2, may do so, at least in part, by interfering with the survival functions of IAP proteins such as survivin and XIAP expressed by such cells.

Promoting proliferation is one of the main functions of bFGF. Survivin is currently the strongest known inhibitor of apoptosis inhibitory proteins. Our results revealed the correlation of both signaling pathways. The result is consistent with the functionality of bFGF and survivin. Following pretreatment with PI3K inhibitor wortmannin, and then bFGF treatment, cell proliferation was inhibited, survivin mRNA expression levels correspondingly decreased, suggesting that bFGF affects the expression of survivin PKB signaling pathway, which regulates cell proliferation and apoptosis.

Emerging data indicate a pivotal role for IAP family members in maintaining cancer cell survival and inhibiting apoptosis induced by anticancer drugs. Our data provide further validation of IAP family members as potential drug discovery targets for the improved treatment of liver cancer. The association between bFGF, survivin and hepatoma cancer warrants further investigation.

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