Midkine Protects Hepatocellular Carcinoma Cells against TRAIL-Mediated Apoptosis through Down-Regulation of Caspase-3 Activity

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BACKGROUND. It is believed that midkine (MK), a heparin-binding growth factor, plays an important role in carcinogenesis. However, the biologic mechanism of MK in hepatocellular carcinoma has not been clarified to date. The objective of the current study was to investigate the antiapoptotic role of MK in a human hepatoma cell line.

METHODS. The human hepatoma cell line HepG2 was used to study the antiapoptotic effect of MK. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/actinomycin D (ActD)–induced apoptosis was detected using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) assay, a caspase-3 activity assay, a caspase-8 activity assay, and flow cytometric analysis.

RESULTS. TRAIL had a potent, dose-dependent inductive effect on cell death in HepG2 cells, for which viable cell counts decreased to 6.3% of the control count at a TRAIL concentration of 100 ng/mL in the presence of 500 ng/mL ActD. Flow cytometry was used to demonstrate that apoptosis induced by TRAIL/ActD was in fact the cause of cell death. According to the WST-8 assay, MK pretreatment resulted in the suppression of TRAIL/ActD-mediated apoptosis in HepG2 cells, although cell viability did not increase when HepG2 cells were treated with MK alone. Caspase-3 activity was down-regulated when MK was added, but caspase-8 activity was high in both the absence and presence of MK.

CONCLUSIONS. The results of the current study indicate that MK acts as an antiapoptotic factor in HepG2 cells through the down-regulation of caspase-3 activity. *Cancer* **2004;100:2430–6.** *© 2004 American Cancer Society.*

KEYWORDS: midkine, antiapoptotic factor, carcinoma, liver, caspase-3.

Midkine (MK) is a member of the heparin-binding growth factor family and has been identified as the product of a retinoic acid–responsive gene.[1] It has been reported that MK promotes the survival, growth, and migration of many cell types.[2] Moreover, it can act as an angiogenic,[3] fibrinolytic,[4] and antiapoptotic factor in carcinoma cell lines.[5,6] Thus, it has been suggested that MK plays an important role in carcinogenesis and tumor progression. MK is overexpressed in various malignant tumors, such as lung, breast, esophageal, gastric, colorectal, liver, pancreatic, ovarian, urinary bladder, prostatic, cerebral, and renal malignancies.[7–15] The high expression of MK is correlated with an unfavorable outcome for patients with urinary bladder carcinomas, glioblastomas, and neuroblastomas.[11,13,14] Recently, we found that MK expression was an independent prognostic factor for overall and disease-free survival in patients with hepatocellular carcinoma (HCC) (unpublished data).

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HCC is one of the most common malignancies worldwide, and several mechanisms of carcinogenesis have been proposed for HCC.[16–18] Deregulation of apoptosis in hepatic cells and accelerated proliferative activity in hepatic cells have been reported as significant factors with respect to hepatocarcinogenesis or tumor progression in HCC.[19] In fact, it is known that HCC is resistant to Fas-mediated apoptosis, and a common defect in the expression of Fas, up-regulation of Fas-associated phosphatases, and down-regulation of downstream molecules, such as Fas-associated death domain and caspase-8, have been noted in HCC.[20]

Apoptosis is essential for normal development and tissue homeostasis. Because apoptosis is under genetic control, mutations that disrupt apoptotic programs can produce disease. For example, inappropriate or excessive apoptosis can lead to neurodegenerative disorders, whereas reductions in apoptosis contribute to lymphoproliferative disorders or neoplasia.[21] Apoptosis is controlled via two major pathways: one that originates in the mitochondria and another that originates at the cell membrane. Multiple stimuli, such as Bax, oxidants, chemothrapeutic agents, and active caspases, can trigger mitochondria to release cytochrome C, and the result is activation of caspase-9, which then processes and activates other caspases.[22] All mitochondrial activities in apoptosis can be blocked by overexpression of antiapoptotic protein, such as Bcl-2 or Bcl-XL.[23] The membrane pathway involves death receptors, such as tumor necrosis factor (TNF) receptor 1, death receptor 3 (DR3), DR4, and DR5, which are activated by their respective ligands and engage the intracellular apoptotic machinery. TNF-related apoptosis-inducing ligand (TRAIL) is a newly identified member of the TNF ligand family that can activate DR4 and DR5, and recruitment to caspase-8 drives its autoactivation through oligomerization and subsequently activates the downstream effector caspases (such as caspase-3), which induce apoptosis in various cells.[24] In a previous report,[5] it was shown that MK possessed activity, through the up-regulation of Bcl-2, against cisplatin-induced apoptosis (i.e., apoptosis via the mitochondrial pathway) in vivo and in vitro in a renal carcinoma cell line. However, the effect of MK on the other apoptotic pathway (i.e., the death receptor pathway) has not yet been elucidated, and the biologic mechanism of MK in HCC has not been investigated. Therefore, we investigated whether MK could act as an antiapoptotic factor in the human hepatoma cell line HepG2, in which apoptosis was induced by TRAIL and actinomycin D (ActD).

MATERIALS AND METHODS

Cell Line and Reagents

The human hepatoma cell line HepG2 was obtained from RIKEN Cell Bank (Osaka, Japan) and was cultured in Earle minimal essential medium (MEM; NI-PRO Company, Osaka, Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS; ICN Pharmaceuticals, Aurora, OH), 0.1 mM MEM nonessential amino acids solution (Gibco BRL, Grand Island, NY), and 1% penicillin/kanamycin (Meiji Confectionery Company, Tokyo, Japan). This medium was used to induce apoptosis of HepG2 cells. ActD was purchased from Sigma (St. Louis, MO), and recombinant human TRAIL was purchased from R&D Systems (Minneapolis, MN). The cell counting kit was purchased from Dojin (Cell Counting Kit-8; Dojin, Kumamoto, Japan). The MEBCYTO (annexin V–fluorescein isothiocyanate [FITC]) apoptosis kit and the caspase-3 and caspase-8 colorimetric protease assay kits were purchased from the Medical and Biological Laboratories Company (Nagoya, Japan).

2-(2-Methoxy-4-Nitrophenyl)-3-(4-Nitrophenyl)-5-(2,4-Disulphophenyl)-2H-Tetrazolium Monosodium Salt Assay

To assess the viability of HepG2 cells, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt assay was performed. Cells were plated in Earle MEM plus 5% FBS in 96-well microtiter plates at a density of 5×10^3 cells per well, and each plate was incubated for 24 hours at 37 °C in 5% CO_2. The live cell count was assayed using Cell Counting Kit-8 according to the instructions provided by the manufacturer, and the absorbance of each well was measured at 450 nm with a microtiter plate reader. Cell viability was calculated as the ratio of treated cells to untreated cells.

Flow Cytometric Analysis

Apoptosis was assayed by staining cells with annexin V–FITC and by propidium iodide (PI) labeling, as annexin V can be used to detect the externalization of phosphatidylserine during the apoptotic progression and thus to identify early apoptotic cells.[25] HepG2 cells (2×10^5) were cultured in 60 mm dishes in MEM Earle plus 5% FBS for 20 hours, after which 0.5 ng/mL TRAIL and 500 ng/mL ActD were added. Cells were harvested and labeled with annexin V–FITC and PI using the MEBCYTO apoptosis kit according to the method described by the manufacturer. In all cases, 10,000 cells were analyzed by flow cytometry.

Caspase Activity Assay

TRAIL-induced apoptosis activates several caspases, including initiator caspase-8, initiator caspase-10, ex-

ecutioner caspase-3, and executioner caspase-7. Once activated, the caspases proteolytically cleave a multitude of cellular substrates, leading to apoptotic death. Thus, caspase activation is a key regulatory point in the commitment to apoptosis. Caspase-3 and caspase-8 activity levels were measured using the colorimetric protease assay kit according to the protocol recommended by the manufacturer. HepG2 cells (1×10^7) were cultured in 100 mm dishes in MEM Earle plus 5% FBS for 16 hours, after which 0.5 ng/mL TRAIL and 500 ng/mL ActD were added. Cytosol extracts from the cell culture were diluted to 100 μg protein per 50 μL cell lysis buffer. Protease activity was measured using tetrapeptide p-nitroanilide substrates. Asp-Glu-Val-Asp-pNA was used in the caspase-3 assay, and Ile-Glu-Thr-Asp-pNA was used in the caspase-8 assay. The absorbance of each well was measured at 405 nm with a microtiter plate reader.

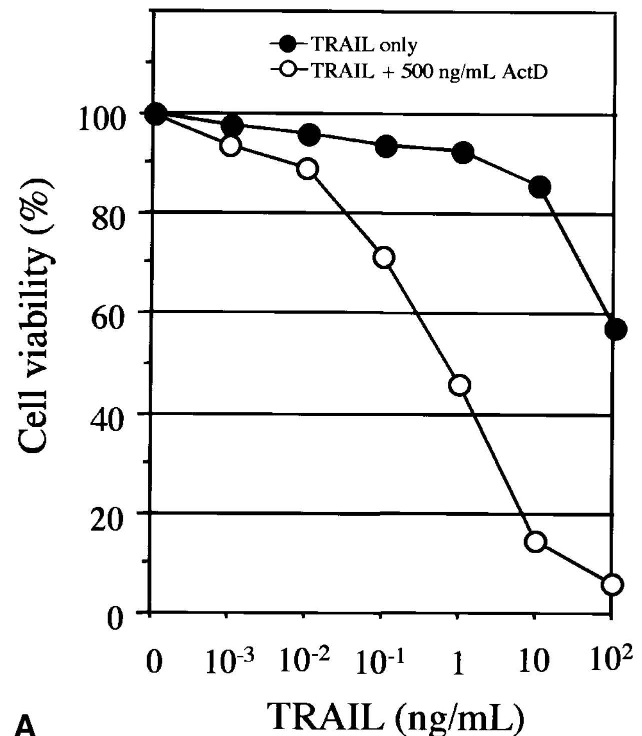
Statistical Analysis

All data are expressed as mean values ± standard errors of the mean. Differences between groups were assessed for statistical significance using the Student *t* test. *P* values < 0.05 were taken to indicate statistically significant differences.

RESULTS

TRAIL-Induced Cell Death

TRAIL is required for pretreatment along with transcriptional inhibitors such as ActD to induce apoptosis in cells.[26] Therefore, the apoptotic effect of TRAIL in the presence of ActD was examined. TRAIL alone, even at a dose of up to 10 ng/mL, failed to induce significant cytotoxicity or apoptosis. A concentration of 100 ng/mL of TRAIL did induce 42.5% cell death in HepG2 cells. However, in the presence of a subtoxic level (500 ng/mL) of ActD, a drug that inhibits RNA synthesis, cell viability decreased in a dose-dependent manner. The viability of cells treated with 100 ng/mL TRAIL in the presence of ActD at 24 hours was 6.3% (Fig. 1A). HepG2 cells were treated with 0.5 ng/mL TRAIL alone and with 0.5 ng/mL TRAIL plus 500 ng/mL ActD. Figure 1B shows the viability of HepG2 cells at 24 hours and at 48 hours. In the untreated group, cell viability had increased in a time-dependent manner. Twenty-four hours after TRAIL treatment, the cell viability was 92.1%; however, when ActD was added, cell viability decreased to 52.1%. At 48 hours,



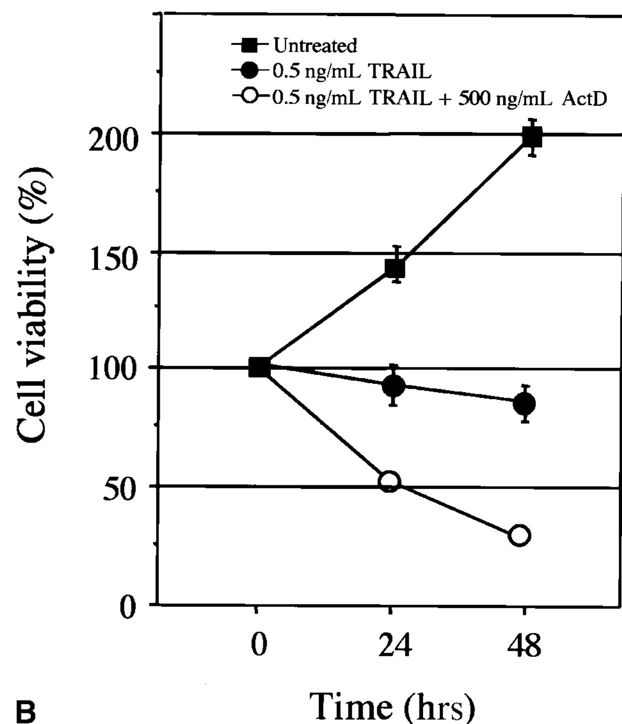


FIGURE 1. (A) HepG2 cells were treated with the indicated concentrations of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in the presence or absence of 500 ng/mL actinomycin D (ActD) for 24 hours. (B) HepG2 cells were treated with 0.5 ng/mL TRAIL only and with 0.5 ng/mL TRAIL plus 500 ng/mL ActD for the indicated periods of time. Cell viability was measured as described in the text (see Materials and Methods). Data points and error bars represent mean values and standard errors of the mean, respectively, from five independent experiments.

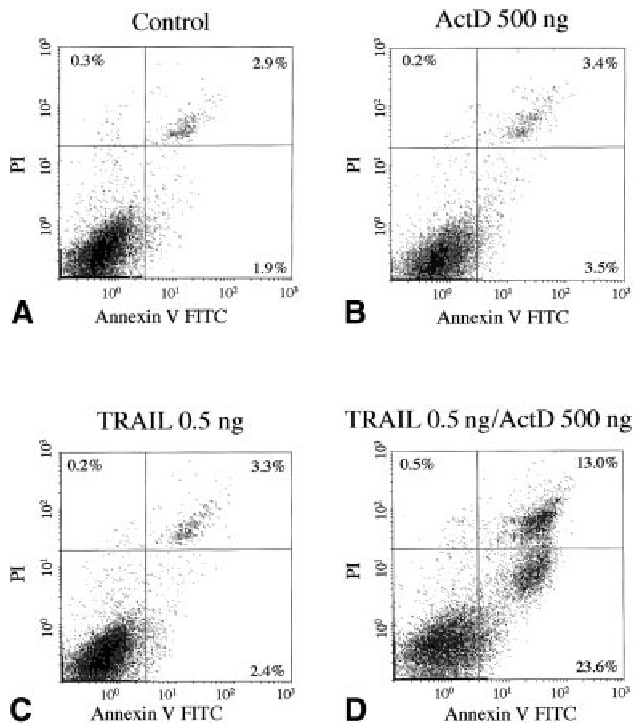


FIGURE 2. Populations of dead cells and apoptotic cells were measured by staining with annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI). HepG2 cells were (A) untreated, (B) treated with 500 ng/mL actinomycin D (ActD), (C) treated with 0.5 ng/mL tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), or (D) treated with TRAIL/ActD for 20 hours. Data were confirmed in three independent experiments.

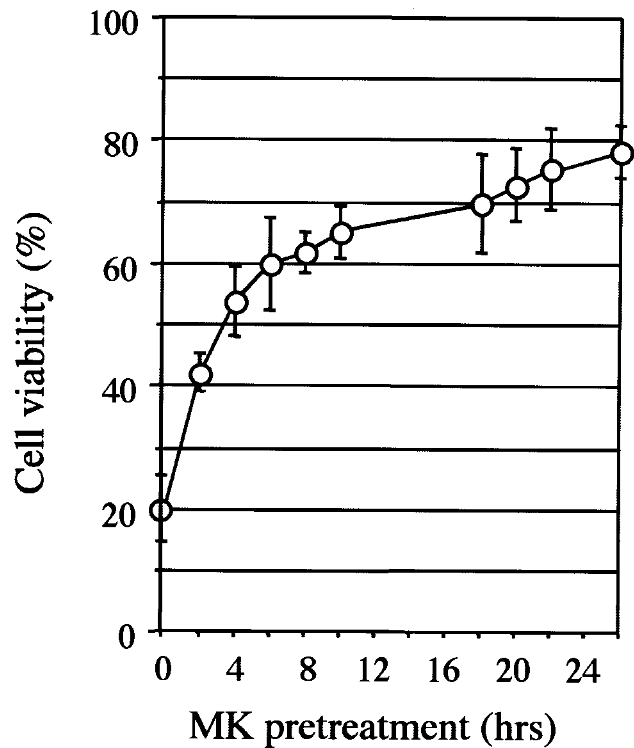


FIGURE 3. HepG2 cells were pretreated with 10 ng/mL midkine (MK) for the indicated lengths of time and then treated with 0.5 ng/mL tumor necrosis factor–related apoptosis-inducing ligand in the presence of 500 ng/mL actinomycin D for 24 hours. Data points and error bars represent mean values and standard errors of the mean, respectively, from five independent experiments.

the cell viability was 84.4% for cells treated with TRAIL alone, whereas the cell viability had decreased to 28.6% for TRAIL/ActD-treated cells (Fig. 1B).

To investigate the type of cell death induced by TRAIL/ActD, flow cytometric analysis was performed using annexin V–FITC and PI. Figure 2 shows that there was no difference in the annexin V and PI staining patterns among HepG2 cells treated with 0.5 ng/mL TRAIL alone, cells treated with 500 ng/mL ActD alone, and untreated cells. In contrast, after 20 hours of exposure to 0.5 ng/mL TRAIL in the presence of 500 ng/mL ActD, a relatively large proportion of HepG2 cells (23.6%, compared with 1.9% of untreated cells) exhibited positivity for annexin V and negativity for PI (Fig. 2D), an expression profile that is considered to be a marker for early apoptosis.[25] Positive staining for both annexin V and PI (Fig. 2A), which represents a marker for late apoptosis, also was more common among cells treated with TRAIL/ActD compared with untreated cells (13.0% vs. 2.9%). These findings indicate that under the current experimental conditions, TRAIL/ActD induced apoptosis in HepG2 cells.

Prevention of TRAIL/ActD-Induced Apoptosis by MK Pretreatment in HepG2 Cells

To investigate whether MK had an antiapoptotic effect on HepG2 cells, we examined the pretreatment effect of MK on TRAIL/ActD-induced apoptosis. After pretreatment with 10 ng/mL MK for the indicated times, HepG2 cells were treated with 0.5 ng/mL TRAIL in the presence of 500 ng/mL ActD for 24 hours. Figure 3 shows that the prevention of TRAIL/ActD-induced apoptosis was observed in a time-dependent manner. MK pretreatment for > 18 hours was sufficient to prevent ~70% of HepG2 cells from undergoing TRAIL/ActD-induced apoptosis; therefore, in subsequent experiments, MK pretreatment was performed for 18 hours.

Next, we examined the number of doses of MK required to inhibit TRAIL/ActD-induced apoptosis. After pretreatment with 10 ng/mL or 100 ng/mL MK for 18 hours, HepG2 cells were treated with 0.5 ng/mL, 1.0 ng/mL, or 2.0 ng/mL TRAIL in the presence of 500 ng/mL ActD for 24 hours. Cell viability was 27.6% at 0 ng/mL MK, 56.3% at 10 ng/mL MK, and 73.9% at 100 ng/mL MK when cells were treated with 0.5 ng/mL

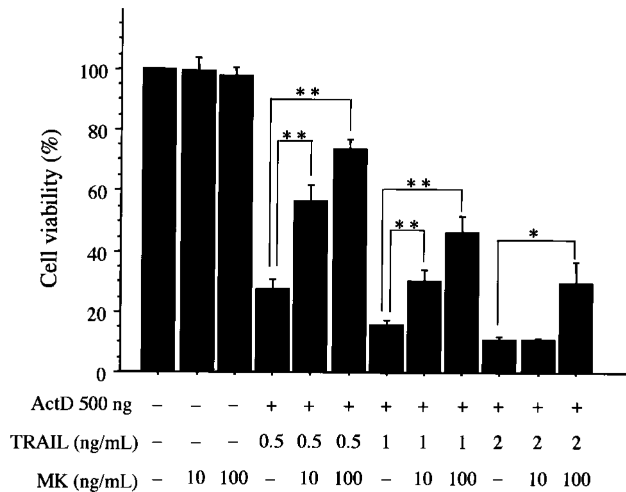


FIGURE 4. HepG2 cells were pretreated with 0 ng/mL, 10 ng/mL, or 100 ng/mL midkine (MK) for 18 hours and then treated with 0.5 ng/mL, 1.0 ng/mL, or 2.0 ng/mL tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in the presence of 500 ng/mL actinomycin D (ActD) for 24 hours. Cell viability was measured as described in the text (see Materials and Methods.) Rectangular bars and error bars represent mean values and standard errors of the mean, respectively, from five independent experiments. −: not administered; +: specified dose administered; *: $P < 0.05$; **: $P < 0.01$.

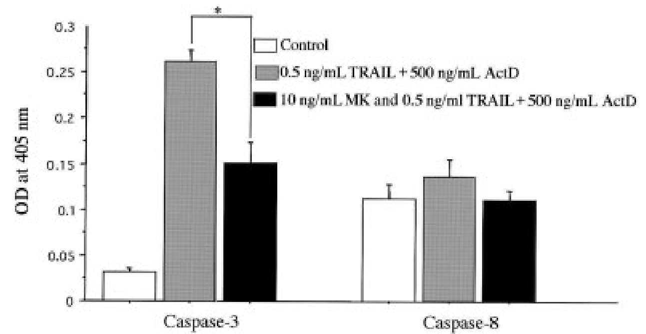


FIGURE 5. HepG2 cells were pretreated with or without 10 ng/mL midkine (MK) for 18 hours and then treated with 0.5 ng/mL tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) in the presence of 500 ng/mL actinomycin D (ActD) for 16 hours (caspase-3) or for 8 hours (caspase-8). Caspase activity was measured as described in the text (see Materials and Methods). Data points and error bars represent mean values and standard errors of the mean, respectively, from five independent experiments. *: $P < 0.05$. OD: optical density.

TRAIL and 500 ng/mL ActD ($P < 0.01$). When cells were treated with 1.0 ng/mL TRAIL and 500 ng/mL ActD, cell viability was 15.7% at 0 ng/mL MK, 30.4% at 10 ng/mL MK, and 46.4% at 100 ng/mL MK ($P < 0.01$). Finally, cell viability was 10.8% at 0 ng/mL MK and 29.9% at 100 ng/mL MK when cells were treated with 0.2 ng/mL TRAIL and 500 ng/mL ActD ($P < 0.05$). Thus, cell viability was significantly increased when HepG2 cells were pretreated with MK (Fig. 4). When HepG2 cells were treated with MK alone, however, cell viability did not increase, indicating that MK contributes to cell survival through up-regulation of the antiapoptotic effect, rather than through the enhancement of cell growth.

Inhibition of Caspase-3 Activation by MK in TRAIL/ActD-Treated HepG2 Cells

Sixteen hours after the induction of apoptotic cell death with 0.5 ng/mL TRAIL in the presence of 500 ng/mL ActD, a 9-fold increase in caspase-3 activity was observed in treated cells compared with untreated cells. The increase in caspase-3 activity was inhibited when cells were pretreated with 10 ng/mL MK for 18 hours ($P < 0.05$) (Fig. 5). In contrast, 8 hours after the induction of apoptosis with TRAIL/ActD, the activity of caspase-8 was high in both the absence and the presence of MK.

DISCUSSION

Several reports have demonstrated the antiapoptotic activity of MK[5,6,27,28]; however, little is known about this activity in tumor cells. In the current study, we have demonstrated that MK acts as an antiapoptotic factor in HepG2 cells; furthermore, we found that MK suppressed the activity of caspase-3, which plays a significant role in the apoptotic pathway. These findings are consistent with previous studies in which it was shown that strong MK expression was correlated with poor prognosis in patients with urinary bladder carcinoma, glioblastoma, neuroblastoma,[11,13,14] or HCC (unpublished data). One possible explanation for the significantly shortened survival of patients with tumors that strongly express MK is that caspase activity is suppressed and malignant cells are more likely to develop in tumor tissue that expresses MK at high levels. In the current study, we have confirmed that MK can act as an antiapoptotic factor and that it may contribute to carcinogenesis.

It has been reported that activation of caspase-8 by DR can lead to two signals: 1) a mitochondria-independent signal that activates caspases downstream of caspase-8[24] and 2) a mitochondria-dependent caspase activation signal following caspase-8 activation and Brd cleavage, which are required for the release of cytochrome C from the mitochondria.[29,30] Bcl-2 overexpression inhibits apoptosis triggered by the latter signal[23] but not apoptosis triggered by the former signal. In the current study, we have demonstrated that MK is capable of inhibiting the two-signal DR pathway through the down-regulation of caspase-3 activity.

Apoptosis is an efficient method of preventing malignant transformation, because it removes cells that carry genetic lesions. Inhibition of apoptosis can promote carcinogenesis. We examined caspase-8, the protease that lies farthest upstream, and caspase-3, a critical downstream protease in the protease cascade. Our results showed that caspase-3 activity was suppressed when the cells were treated with MK. Many previous studies have demonstrated the importance of caspases.[30–32] High levels of interleukin-1β-converting enzyme and caspase-3 expression have been found to be correlated significantly with lower disease stage and better prognosis in patients with neuroblastoma,[33] and it was discovered that the expression levels of these proteins were downregulated significantly in human HCC compared with the surrounding nontumor tissues.[34] Recently, it has been shown that members of the inhibitor of apoptosis protein (IAP) family, such as survivin and X-linked IAP, contribute to resistance to apoptosis by inhibiting caspase activation.[35,36] Therefore, survivin represents a possible new target for the treatment of malignant disease.[37] These findings suggest that the MK inhibitor may play a role in preventing, via upregulation of the apoptotic pathway, the development of HCC. However, in a previous report,[38] an antisense oligodeoxynucleotide targeted against MK did not enhance apoptosis in murine rectal carcinoma cells. In tumor cells, the death program often is compromised and/or regulated abnormally by a process of random mutation and selection, with cells becoming progressively more malignant as they accumulate mutations that improve their ability to survive and proliferate. Thus, further studies will be necessary to evaluate the role of MK in tumor cells.

In conclusion, we have demonstrated the potent antiapoptotic effect of MK in a hepatoma cell line. Because molecules with antiapoptotic activity are frequent targets of cancer therapy, MK inhibitors may be good candidates for use in the treatment of malignant disease.

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