Abstract. Defects in apoptosis signaling in hepatocytes contribute to tumorigenesis in hepatocellular carcinoma (HCC). In addition, treatment with chemotherapeutic drugs is often ineffective in HCC patients due to the apoptosis resistance of cancer cells. Anti-apoptotic members of the Bcl-2 family, including myeloid cell leukemia-1 (Mcl-1), which regulate intrinsic apoptosis induction at the mitochondrial level, are often overexpressed in human cancer, and are implicated with disease grade and prognosis. Yet, little is known about the role of Mcl-1 in HCC. In this study, we analyzed the relevance of Mcl-1 expression for the apoptosis resistance of human HCC. Mcl-1 protein expression was considerably enhanced in human HCC tissue compared to adjacent non-tumor tissue. In addition, Mcl-1 was prominently expressed in various HCC cell lines. Mcl-1 basal expression is dependent on a functional phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway; treatment of the cells with a specific PI3 kinase inhibitor led to both decreased Mcl-1 expression and a sensitization towards chemotherapeutic drug-induced apoptosis. Furthermore, the hepatocyte growth factor and epidermal growth factor induced Mcl-1 expression in an Akt- and ERK-dependent manner. Finally, specific upregulation of Mcl-1 in HCC cells inhibited chemotherapeutic drug-induced apoptosis. Our data suggest that Mcl-1 is an important factor for the apoptosis resistance of human HCC, and constitutes an interesting target for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide, with an incidence of approximately 30/100,000 in developing countries. HCC in Western countries occurs mainly in patients with liver cirrhosis and has an annual incidence of about 3-4 cases per 100,000. Yet, the incidence is dramatically increasing in these countries, mostly due to the large number of HCV-seropositive people. The survival rates of patients depend primarily on the stage of the tumor at diagnosis. Most patients show an advanced tumor stage at the time of diagnosis and, therefore, curative surgical treatment, such as resection and liver transplantation, can only be achieved in a minority of patients (1). Even those patients who undergo resection only have a five-year survival rate of approximately 60% (2), and a recurrence rate of approximately 50% after 2 years (3). In a palliative situation, systemic chemotherapy does not significantly improve survival (4). Therefore, new treatment regimens for patients with advanced HCC are desperately needed.

The suppression of apoptosis is one of the mechanisms contributing to tumorigenesis and the resistance of malignant cells towards chemotherapeutic drugs (5). The decreased sensitivity of mitochondria towards intracellular apoptosis stimuli is an important survival factor for cancer cells. Mitochondrial integrity is regulated by pro- and anti-apoptotic members of the Bcl-2 family, such as Bcl-2, Bcl-xl, and Mcl-1 (anti-apoptotic), or Bid, Bax and Bak (pro-apoptotic).

Mcl-1 (Myeloid cell leukemia-1) was originally identified as an early induction gene during differentiation of myeloid leukemia cells (6). It is both structurally and functionally an anti-apoptotic member of the Bcl-2 protein family (7). Mcl-1 protects cells from apoptosis induction through blockage of cytochrome-c release from mitochondria by interacting with pro-apoptotic members of the Bcl-2 protein family, e.g. Bim (8), Bak (9,10), and NOXA (11). A characteristic feature of Mcl-1 is its rapid regulation in response to specific environmental signals, such as growth factors, and cellular stress (7). Recently, we have shown that the hepatocyte growth factor (HGF) protects primary human hepatocytes against CD95-mediated apoptosis, eventually via induction of Mcl-1 (12). As Mcl-1 promotes the survival of cells, it may contribute to
the development of cancer (13). This effect was first observed in Mcl-1 transgenic mice, which show a high incidence of B-cell lymphoma (14). During the last few years, it has been demonstrated that Mcl-1 plays a role in the survival of various cancer cells. It is overexpressed in human malignancies, e.g. in multiple myeloma (15) and non-small cell lung cancer (16). Moreover, Mcl-1 expression correlates with the relapse and shorter survival of patients with multiple myeloma (17). In B-cell non-Hodgkin’s lymphoma, Mcl-1 expression correlates with disease grade (18). Mcl-1 is also involved in the response to anti-cancer treatment. In patients with chronic lymphocytic leukemia or patients with liver metastases of colorectal cancer, high Mcl-1 expression correlates with a low or absent response towards chemotherapeutic treatment (19,20). Recently, it has been suggested that the modulation of Mcl-1 expression might be effective for the treatment of cancer patients. Mcl-1 downregulation sensitizes cholangiocarcinoma cells to TRAIL-induced apoptosis in vitro (21) and CML cells towards treatment with imatinib (22). However, little is known about the role and regulation of Mcl-1 in HCC.

In this study, we analyzed the expression of Mcl-1 in HCC and found a significant upregulation in human HCC tissue. Furthermore, we analyzed Mcl-1 regulation and its role in the apoptosis resistance of HCC cells. Mcl-1 basal expression is dependent on a functional phosphatidylinositol-3 kinase (PI3K)/Akt pathway, whereas induction of Mcl-1 by HGF and the epidermal growth factor (EGF) depends on the functional PI3K/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway. Finally, modification of Mcl-1 expression triggers apoptosis resistance towards chemotherapeutic drugs.

Materials and methods

Reagents and cell lines. The human hepatoma cell lines, Hep3B and HepG2, were grown in MEM, and Huh7 was grown in DMEM, both supplemented with 10% fetal bovine serum. Reagents were purchased from the following suppliers: LY294002, PD98059, AG490, and 5-Fluorouracil (all solubilized in dimethyl sulfoxide) from Calbiochem (Schwalbach, Germany); HGF and EGF from Sigma (Deisenhofen, Germany), and valproic acid (Orfiril) from Desitin (Hamburg, Germany).

Transfection. The open-reading frame of Mcl-1 was cloned into the pEF4/Myc-His vector (Invitrogen). Huh7 cells were transfected using Transfectin (Biorad, München, Germany) according to the manufacturer’s protocol. As a control for...
transfection efficiency, co-transfection with a GFP-expressing plasmid was regularly performed and exhibited >80% GFP-positive cells on average.

Immunohistochemistry. Formalin-fixed and paraffin-embedded liver specimens from 10 HCC patients [all with liver cirrhosis of different origin (chronic hepatitis B and C, non-alcoholic steatohepatitis, alcoholic steatohepatitis and hemochromatosis)] were sectioned and stained with hematoxylin and eosin. For immunohistochemical staining, epitope retrieval was performed by microwave pretreatment of slides in citrate buffer. Incubation with the Mcl-1 primary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was performed in a 1:50 dilution. For negative controls, the primary antibody was omitted. After washing in PBS-buffer, the slides were incubated with a biotinylated secondary antibody. The slides were mounted. The images were captured using a Zeiss Axiophot microscope (Zeiss, Göttingen, Germany) equipped with an Olympus Camedia camera (Olympus, Hamburg, Germany).

Cell lysis and Western blotting. Cells or tissue were lysed by incubation on ice for 15 min in lysis buffer containing 120 mM NaCl, 50 mM Tris/HCl (pH 8.0), 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 0.1% sodium dodecyl sulfate, 100 μM Na₃VO₄, 1 mM DTT, and a commercial protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell debris was removed by centrifugation (10,000g, 4°C). The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes.

Immunodetection was performed using the following primary antibodies: anti-phospho-Ser⁴⁷³ Akt, anti-Akt1, anti-phospho-ERK1/2 (New England Biolabs, Frankfurt, Germany), anti-ERK-1 (Becton-Dickinson Biosciences, Heidelberg, Germany), anti-Mcl-1 (Santa Cruz Biotechnology), anti-Bcl-2 (Calbiochem), and anti-α-Tubulin (Sigma). Peroxidase-conjugated antibodies (Santa Cruz Biotechnology) were incubated at a concentration of 1:10,000. Bound antibody was visualized using chemiluminescent substrate (Perkin-Elmer, Zaventem, Belgium) and exposure to Fuji Medical X-Ray film.

Detection of apoptosis. The cells were collected, washed with PBS, and resuspended in a hypotonic buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/ml propidium iodide (Sigma). After overnight incubation at 4°C, nuclei from apoptotic cells were quantified by flow cytometry according to the method of Nicoletti et al (23). Specific apoptosis was calculated as follows: 100x (experimental apoptosis - spontaneous apoptosis) / (100 - spontaneous apoptosis).

Data analysis. All results are expressed as means ± SD and represent at least three independent experiments. The data were analyzed by Student's t test (paired, two sided) with p<0.05 considered significant.

Results

Mcl-1 is expressed in human HCC tissue. Hepatocellular carcinoma (HCC) shows a rather resistant phenotype towards apoptosis induction after chemotherapy (24). However, little is known about the alterations in apoptosis pathways responsible for apoptosis resistance in HCC. As Mcl-1 exerts anti-apoptotic functions, it is a potential protooncogen (25) and its contribution to apoptosis resistance in human cancer has been discussed. Thus, we analyzed if Mcl-1 expression is altered in human HCC tissue.

We used the paraffin embedded tissue of HCC patients undergoing transplantation. Mcl-1 expression in HCC tissue was visualized immunohistochemically and compared to adjacent non-malignant liver tissue as control. As shown in Fig. 1A, Mcl-1 was prominently expressed in HCC tissue. Then, we isolated the proteins of cryopreserved HCC and normal liver tissue from the same patients and performed Western blotting. Again, Mcl-1 protein expression was significantly higher in the HCC tissue than in the adjacent non-tumor liver tissue (Fig. 1B).

Mcl-1 is expressed in hepatocellular carcinoma cell lines. As we could detect Mcl-1 expression in HCC tissue, we analyzed the role of Mcl-1 in the apoptosis resistance of HCC cells. First, we examined different HCC cell lines and primary human hepatocytes (PHH) for expression of Mcl-1 and Bcl-2. Bcl-2 was expressed to a low extent in HCC cell lines as well as in PHH. In contrast, all cell lines and PHH showed a profound expression of Mcl-1 (Fig. 1C). Consequently,
HCC cell lines were used to analyze the role of Mcl-1 in the apoptosis resistance of HCC.

Basal Mcl-1 expression in HCC cells is regulated via the PI3K/Akt pathway. Various kinase inhibitors have already entered clinical trial for the treatment of cancer patients. We analyzed which kinase pathways are responsible for Mcl-1 expression in HCC cells. Several pathways have been described as playing a role in Mcl-1 expression in different cellular systems, including the PI3K/Akt, MEK/ERK and signal transducer and activator of transcription 3 (STAT3) pathway (7). Recently, we have shown that growth factor-induced expression of Mcl-1 in PHH is dependent on the PI3K/Akt pathway (12). Here, we analyzed the role of the PI3K/Akt, MEK/ERK and Janus kinase 2 (Jak2)/STAT3 pathways in the regulation of Mcl-1 expression in HCC cells. We treated Hep3B cells with the specific kinase inhibitors: LY294002, a PI3K inhibitor; PD98059, a MEK1 inhibitor; and AG490, a Jak2 inhibitor (26). Since both Mcl-1 mRNA and protein have short half-lives (7), we performed these experiments after short treatment periods of 2 to 6 h.

Treatment of HCC cell lines with LY294002 resulted in a lower phosphorylation status of Akt and a decrease in Mcl-1 expression in Hep3B cells (Fig. 2A). PD98059 and AG490, however, had no effect on Mcl-1 expression, despite their inhibitory effects on ERK1 and STAT3 phosphorylation (data not shown). To determine if downregulation of Mcl-1 by PI3K inhibition is accompanied by a decrease in cell viability or a sensitization towards chemotherapeutic drug-induced apoptosis, we treated HCC cells with LY294002 or PD98059, and, concomitantly, with the chemotherapeutic drug, bleomycin. After 24 h, cells were harvested and analyzed for apoptosis induction by flow cytometry. We found that treatment with LY294002 led to significantly higher apoptosis rates, both when applied alone and, more so, in combination with bleomycin. In contrast, PD98059 had no additive effect on apoptosis induction (Fig. 2B).

Growth factors induce Mcl-1 expression in HCC cells. Recently, it was shown that HGF serum levels in HCC patients were significantly elevated compared to healthy controls, and negatively correlated with survival time (27). Furthermore, elevated mRNA levels of the HGF receptor, c-met, and the EGF receptor were found in HCC patients with poorly differentiated tumors (28). We have shown that HGF induces Mcl-1 expression in PHH (12). In esophageal carcinoma cell lines, EGF has been described to induce Mcl-1 expression (29). Growth factor-induced Mcl-1 expression might contribute to the poor prognosis of HCC patients.

To test whether HGF and EGF influence Mcl-1 expression in HCC cells, cells were treated with HGF or EGF for different time periods. As shown in Fig. 3A, HGF and EGF were capable of inducing Mcl-1 expression. Furthermore, treatment with HGF and EGF activated both the PI3K/Akt and the MEK/ERK pathway, reflected by elevated phosphorylation of Akt and ERK.

We then determined if the blockage of these pathways inhibits growth factor-induced Mcl-1 expression. Cells were preincubated with LY294002 or PD98059 for 1 h. Then, the cells were treated with HGF for an additional 3 h. Kinase
inhibition by specific inhibitors abrogated the effect of HGF on Mcl-1 expression (Fig. 3B). These data demonstrate that growth factor-induced Mcl-1 expression is dependent on the PI3K/Akt and MEK/ERK pathways.

**Specific upregulation of Mcl-1 enhances the chemoresistance of HCC cells.** As we assumed that Mcl-1 might contribute to the apoptosis resistance of HCC, we determined whether enhanced Mcl-1 expression influences the resistance of HCC cells towards chemotherapeutic drugs. Therefore, we transfected either a control plasmid (pEF4) or an Mcl-1 expression plasmid (pEF4/Mcl-1) in Huh7 cells. As shown in Fig. 4A, the transfection of Huh7 cells with pEF4/Mcl-1 resulted in increased Mcl-1 expression after 24 and 48 h compared to cells transfected with the control plasmid. We then analyzed if increased Mcl-1 expression protects cells from chemotherapeutic drug-induced apoptosis. As Huh7 cells show a rather resistant phenotype towards chemotherapeutic drugs, we combined 5-Fluorouracil (5-FU) with the histone deacetylase inhibitor (HDACI), valproic acid (VA), which has been shown to sensitize cells towards chemotherapeutic drugs (30). HDACIs induce histone hyperacetylation in tumor and normal cells but are almost nontoxic to normal cells (31).

As shown in Fig. 4B, the enhanced Mcl-1 expression in Huh7 cells resulted in an increased apoptosis resistance towards treatment with 5-FU and VA compared to control transfected cells. These data demonstrate that enhanced Mcl-1 expression increases the apoptosis resistance of HCC cells towards chemotherapy.

**Discussion**

The aim of the present study was to investigate the role of Mcl-1 in the apoptosis sensitivity of human hepatocellular carcinoma (HCC). The results of this study show that Mcl-1 is highly expressed in human HCC tissue, and the enhanced expression of Mcl-1 inhibits chemotherapeutic drug-induced apoptosis in HCC cells. Our data suggest that Mcl-1 is an important survival factor for HCC and is a promising target for therapeutic approaches in patients with HCC.

Apoptosis is a common property of multicellular organisms, which eliminates unwanted and potentially harmful cells (32). Defects in apoptosis signaling pathways contribute to tumor development. These defects include, among others, stabilization of mitochondria and alterations in survival signaling and death receptor signaling (5). Members of the Bel-2 family of proteins critically regulate the integrity of mitochondria. Anti-apoptotic members, such as Bcl-2, Bcl-xL, and Mcl-1, stabilize mitochondria and inhibit mitochondrial outer membrane permeabilization (MOMP) by interacting with pro-apoptotic BH3-only family members (such as Bid, Bim, PUMA and NOXA), thus preventing the activation of Bax and Bak (33). Furthermore, Bcl-2, Bcl-xL, and Mcl-1 exert anti-apoptotic functions by interacting with Ca²⁺ signaling pathways (34-36). An enhanced expression of anti-apoptotic Bcl-2 proteins is frequently observed in malignancies of diverse origin and confers resistance towards anticancer treatment.

Little is known about the role of Mcl-1 in the apoptosis resistance of HCC cells. In this study, we demonstrated, immunohistochemically and by Western blotting, that Mcl-1 expression is enhanced in human HCC tissue compared to adjacent non-malignant tissue. Mcl-1 is known to have a prominent mitochondrial localization, but also associates with other membranes through its hydrophobic carboxyl tail. Furthermore, it is also detectable in the cytosol (37,38). Immunohistochemical analysis of HCC tissue revealed that Mcl-1 expression is enhanced in virtually all cells. Moreover, Mcl-1 expression is localized to the cytoplasm and shows a granular pattern, suggesting that Mcl-1 is associated with intracellular organelles. This subcellular distribution has also been observed in other human malignancies with enhanced Mcl-1 expression (16,18,20,39). Since Mcl-1 has a short half-life, it is constantly synthesized and degraded. Therefore, synthesis at the ribosomes, degradation at the proteasomes, and transport between different cellular compartments might also contribute to the subcellular distribution pattern observed in immunohistochemical samples.

The enhanced expression of Mcl-1 in human HCC might contribute to apoptosis resistance. Various factors have been described as contributing to tumorigenesis as well as to apoptosis resistance of HCC, such as mutations in the p53 tumor suppressor system (40,41), downregulation of death receptors such as CD95 (APO-1/Fas) (42), and enhanced...
Furthermore, HCC patients with poorly differentiated tumors have elevated levels of HGF and that serum HGF levels negatively correlate with survival time (27). Patients with HCC have elevated levels of HGF and that serum HGF levels negatively correlate with survival time (27). It has recently been demonstrated that EGF protects esophageal carcinoma cells from staurosporine-induced apoptosis (29) and blocks TRAIL-induced apoptosis (30). In addition, the induction of Mcl-1 by HGF inhibits the CD95-mediated apoptosis induction in other malignancies. In UV-irradiated HeLa-cells, degradation of Mcl-1 at the proteasomes is a prerequisite for Bcl-xL/Bax translocation to mitochondria, Bax oligomerization and cytochrome-c release (37). In addition, upon treatment to cytotoxic agents, neutralization of both Mcl-1 and Bcl-xL by BH3-only proteins is necessary for Bak activation and apoptosis induction (10). However, the role of the individual anti-apoptotic Bcl-2 family members and their synergism for survival of HCC cells requires further elucidation. Mcl-1 has also been implicated in p53-mediated apoptosis. Upon cellular stress, p53 antagonizes the interaction of Mcl-1 and Bak, thereby allowing the translocation of Bak to the mitochondria followed by mitochondrial activation (9). Mutations in the TP53 gene, which are frequently observed in HCC patients, might impair the dissociation of Mcl-1 and Bak and thereby contribute to the apoptosis resistance of HCC cells. The role of p53/Mcl-1 interactions in the apoptosis induction of HCC cells was not analyzed in this study and needs further elucidation.

The data presented in this study show that Mcl-1 expression is regulated via the PI3K/Akt signaling pathway in HCC cells. Inhibition of PI3K by the specific inhibitor, LY294002, results in a rapid downregulation of Mcl-1 expression. Recently, we have shown that PI3K is also important in regulating Mcl-1 expression in PHH (12). Mcl-1 expression has been linked to several signal transduction pathways, such as the MEK/ERK, PI3K/Akt, and Jak/STAT pathways in different cellular systems (7). In HCC cells, both inhibition of MEK1 by PD98059 and inhibition of Jak2 by AG490 had no significant effect on Mcl-1 basal expression (data not shown). However, HGF- as well as EGF-induced Mcl-1 expression could be abrogated by concomitant treatment with LY294002 or PD98059. These data suggest a role for the PI3K/Akt pathway in the regulation of both basal and growth factor-induced expression of Mcl-1. In contrast, the MEK/ERK pathway is only involved in growth factor-induced Mcl-1 expression. Recently, it has been shown that BAY 43-9006, a Raf kinase inhibitor, which has entered clinical phase III testing (47), stabilizes disease in patients with cancer of different origin, including HCC, and downregulates Mcl-1 in different cell lines in a dose- and time-dependent manner (48). In addition, it has been shown that BAY 43-9006 downregulates Mcl-1 through inhibition of translation, and thereby facilitates BAY 43-9006-induced apoptosis (49).

This study has shown that Mcl-1 expression is enhanced in HCC cells upon treatment with the growth factors, HGF and EGF. The induction of Mcl-1 expression by growth factors has already been observed in other cellular systems. The induction of Mcl-1 by HGF inhibits the CD95-mediated apoptosis of PHH (12). In addition, the induction of Mcl-1 by EGF protects esophageal carcinoma cells from staurosporine-induced apoptosis (29) and blocks TRAIL-induced apoptosis in NIH3T3-cells (50). It has recently been demonstrated that patients with HCC have elevated levels of HGF and that serum HGF levels negatively correlate with survival time (27). Furthermore, HCC patients with poorly differentiated tumors show elevated c-met and EGFR mRNA levels (28). EGF and HGF might contribute to increased Mcl-1 levels in HCC and thereby contribute to the poor prognosis observed in HCC patients. Further studies are needed to correlate the extent of Mcl-1 expression in HCC tissue with HGF and EGF serum levels and the prognosis of HCC patients.

Chemotherapeutic treatment in patients with HCC is often ineffective (24). To scrutinize if this phenomenon is partly due to enhanced Mcl-1 expression, we analyzed whether the apoptosis resistance of HCC cells is increased if Mcl-1 expression is enhanced. Therefore, HCC cells were transfected with an Mcl-1 expression plasmid and, subsequently, treated with a combined therapy consisting of 5-FU and the histone deacetylase inhibitor (HDACI), valproic acid (VA). It has been shown that HDACIs sensitize malignant cells towards chemotherapeutic drugs (30). The treatment of Huh7 cells with 5-FU alone resulted in minor apoptosis induction. However, upon co-treatment with VA, apoptosis was increased two- to three-fold. VA alone had no effect on cell viability (data not shown). This study has shown that specific upregulation of Mcl-1 significantly inhibits the chemotherapeutic drug-induced apoptosis of HCC cells; in cells with enhanced Mcl-1 expression, apoptosis induction upon treatment with 5-FU and VA was only half of that observed in control transfected cells. These data confirm our hypothesis that enhanced Mcl-1 expression in HCC contributes to apoptosis resistance and a poor response to chemotherapy.

In summary, the present study shows for the first time that Mcl-1 is an important survival factor for human hepatocellular carcinoma. Mcl-1 expression is enhanced in HCC cell lines as well as in human HCC tissue. Mcl-1 expression is dependent on PI3K/Akt signaling and can be induced by growth factors via activation of PI3K and MEK1. Finally, enhanced expression of Mcl-1 confers resistance of HCC cells towards chemotherapy. Thus, Mcl-1 is a promising target in the therapy of patients suffering from HCC.

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References


