RESEARCH ARTICLE

mRNA Expression of Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9 in the HepG2 Cell Line Following Induction by a Novel Monoclonal Ab Hep88 mAb: Cross-Talk for Paraptosis and Apoptosis

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Abstract

Monoclonal antibodies with specific antigens have been widely used as targeted therapy for cancer. Hep88 mAb is a monoclonal antibody which shows specific binding with anti-cancer effects against the HepG2 cell line. However, its mechanisms of action are still not completely understood. We examined cell cycling and apoptosis by flow cytometry and mRNA expression of factors involved in apoptosis and paraptosis in Hep88 mAb-treated HepG2 cells by real-time PCR. The cell-cycle analysis demonstrated that growth-inhibitory activity was associated with G2/M cell cycle arrest. Hep88 mAb induced a significant increase in apoptotic cell populations in a dose- and time-dependent manner. The mRNA expression results also suggested that the process triggered by Hep88 mAb involved up-regulation of tumor suppressor p53, pro-apoptotic Bax, Cathepsin B, Caspase-3 and Caspase-9, with a decrease of anti-apoptotic Bcl-2 - thus confirming paraptosis and apoptosis programmed cell death. These findings represent new insights into the molecular mechanisms underlying the anti-cancer properties of Hep88 mAb in liver cancer cells.

Keywords: Apoptosis - cell cycle arrest - monoclonal antibody - mRNA expression - paraptosis

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Introduction

Generally, the cancer cells often evade apoptosis by expressing anti-apoptotic proteins, down-regulation of pro-apoptotic genes and alteration in the signaling pathways that let them survive. Hence, many researchers have been concentrated the development and discovery of the route for cancer cell apoptosis cell death induction. There are two main signaling pathways to trigger apoptosis: the intrinsic apoptotic pathway and the extrinsic apoptotic pathway. The intrinsic apoptotic pathway is activated in response to a number of stress conditions including DNA damage, oxidative stress and many others. The extrinsic apoptotic pathway indicates a form of death that is induced by extracellular signals and through their binding to death receptors. Presently, many evidences indicating the existence of caspase constitute the important mediators of apoptosis program cell death.

On the other hand, researchers have recently witnessed a novel concept for program cell death (PCD) that includes caspase-independent PCD. It is induced by a large number of death-inducing factors to form lysosomal membrane permeabilization (LMP) and/or mitochondrial outer membrane permeabilization (MOMP). Several proteins among the mitochondrial proteins are released to stimulate caspase-independent PCD. Meanwhile proteolytic enzymes in lysosome-like cathepsins are also released to degrade intracellular proteins or activate caspases, as well as other enzymes, to induce cell death. It is now recognized this type of PCD, as apoptosis-like PCD or paraptosis by morphological characterization, differs from apoptosis, i.e. numerous vacuolization without apoptotic body or membrane blebbing. The mechanism involving paraptosis has also demonstrated scientifically that it is involved in cathepsins activation but has not responded to caspase inhibitors and caspases activation (Sperandio et al., 2004).

However, the cell can also lead to apoptosis program cell death via the dysfunction of tumor suppressor proteins. One of those that are outstanding in its being recognized as involved in apoptosis is the p53 protein. p53 plays a crucial role in cell cycle and apoptosis as a tumor suppressor in the arresting of the cell cycle or the elimination of DNA-
damaged cells. In the sense of apoptosis, p53 can mediate apoptosis through transcriptional activation of various pro-apoptotic target genes, such as Bax, or through the repression of those of anti-apoptotic genes (Chiang et al., 2014). Hence, most cases of cancer cells, deregulation of p53 by mutation, post-translation modification or even interaction with other proteins will induce the cells to become cancerous. For example: mortalin–p53 interaction was shown to cause inactivation of p53 activities that are scientifically proven to be involved in tumorogenesis. As a result, targeted therapy to recover p53 function is currently being studied extensively and in detail. One such effective tool includes the production of monoclonal antibody that is recognized with a p53-deregulated protein such as Hep88 mAb.

Hep88 mAb is the monoclonal antibody specific to mortalin HSP70 in hepatocellular carcinoma (Laohathai and Bhamarapravati, 1985; Rojpibulstit et al., 2014). It has been shown to have anti-cancer effects against HepG2 cell line, but harmless otherwise to the normal liver cell line (Putthong et al., 2009). The death of HCC induced by Hep88 mAb was formerly demonstrated by way of a paraptosis-like program cell death (paraptois-like PCD) character (Manochantr et al., 2011). However, the focus of many reports has been that various types of cell death could be triggered by multiple mechanisms, whereas the morphological changes might be detected by one which is predominant. We then also reported that Hep88 mAb induced paraptosis-like PCD by up-regulation of Caspase-3, -8 and -9, as well as its activity after 24-hr of incubation (Mitupatum et al., 2015). These findings prompted us to conclude that Hep88 mAb did in fact induce HCC from a paraptosis-like state to apoptosis by the downstream induction of caspases. However, we were still left with the question of vacuolization morphology—namely, whether or not Hep88 mAb was involved in lysosomal breakdown. In this study, we have conducted our tests by increasing the cytotoxic doses of Hep88 mAb treatment to explore the apoptosis induction and the involvement of mitochondria and lysosome in HCC. The HCC cell lines were treated with Hep88 mAb and we then evaluated the induction of apoptosis, cell cycle arrest, and mRNA expression of Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9.

Materials and Methods

Materials

Fetal bovine serum, Trypsin/EDTA Solution and PBS solution were purchased from Biochrom (AG, Berlin). TRI REAGENT® was obtained from Molecular research center, Inc. The oligo nucleotide primers (oligoDT primer), 2X KAPA SYBR® FAST qPCR Master Mix were obtained from KapaBiosystems, Inc.(Wilmington, MA, USA), SuprimeScript RT premix (2X) was obtained from GENET BIO (Daejeon, Korea). The QIAprep® Spin Miniprep Kit was obtained from QIAGEN (Hilden, Germany). PCR Master Mix (2X) and InsTAcione PCR Cloning Kit were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). The FITC Annexin V Apoptosis Detection Kit I, PI/RNase staining buffer, BD FACS Rinse solution, BD FACS Clean solution and BD FACS Flow Sheath Fluid were purchased from BD Biosciences (CA, USA).

Cell Lines and mAb

The human HCC cell lines, specifically the HepG2 cells (American Type Culture Collection [ATCC] HB8065), were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Biochrom AG, Germany). The cell lines were maintained at 37°C in a CO₂ incubator and subculture every 3–4 days until use. Hep88 mAb, the anti-HCC mouse mAb, was produced as previously described (Laohathai and Bhamarapravati, 1985).

Treatment of HepG2 Cells with Hep88 mAb

HepG2 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in 5% CO₂ for 24-hr prior to treatment. From our previous study, it was found that a cytotoxicity dose of Hep88 mAb contained a concentration of 5 times that of the IC₅₀ (12.5 µg/ml). Hence, it was necessary to monitor its apoptotic response while varying time and dose for apoptosis detection and cell-cycle progression by flow cytometry analysis. For this purpose, it was decided to treat the HepG2 cells with 4-6 times the IC₅₀ concentration of Hep88 mAb (i.e. 50, 62.5 and 75 µg/ml), paralleling untreated HepG2 cells as the negative control. After 24, 48 and 72-hr of incubation, the cells were trypsinized and were then washed twice with iced-cold PBS. The cell was collected for the next step in flow cytometry analysis.

Additionally, mRNA expression analysis was performed by HepG2 cells treated with a cytotoxic concentration of 62.5 µg/ml Hep88 mAb. To investigate its time-dependent manner, the cells were incubated in two sets: i.e. for 12-, 18- and 24-hr in the case of one set for Bax, Bcl-2, p53, Cathepsin B, Caspase-3, Caspase-9 and EF-2 gene. The other set was designed to investigate a nearly response which was formerly notified as paraptosis morphology. The incubation thus performed in an earlier incubation period (at 3-, 6-, 9-hr of treatment) was then carried out as an mRNA expression of Bax, Bcl-2, p53, Cathepsin B, Caspase-3, Caspase-9 and EF-2 gene. The untreated HepG2 cells at each time and dose were used as controls. After incubation, the cells were extracted for total RNA by TRI REAGENT® (Molecular research center, Inc).

Apoptosis detection

Treated- and untreated-HepG2 cell pellets were stained with the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™, CA, USA) according to the manufacturing protocol. The suspension was analyzed with a fluorescence-activated cell sorter (FACS, BD FACSCalibur™, BD Biosciences, CA, USA). The percentages of cells were calculated by CellQuest™ software (BD Biosciences, CA, USA). The cells in the early stages of apoptosis were annexin V positive and PI negative, while the cells in the late stages of apoptosis were both annexin V and PI positive.

Cell cycle progression
After the cells had been cultivated, operated on and collected as described above, they were then washed and fixed in 90% ethanol at -20°C overnight. The cell were stained with PI/RNase staining buffer (BD Biosciences, CA, USA) at room temperature away from sunlight for 15 minutes and analyzed with a fluorescence-activated cell sorter (FACS, BD FACSCalibur™, BD Biosciences, CA, USA). Data were analyzed using ModFit LT 3.2.

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis was performed as describe previously (Mitupatum et al., 2015). In brief, the steps would be as followed: (1) extraction of total RNA by TRI REAGENT® referring to the manufacturer’s instruction (Molecular research center, Inc), (2) quantization of RNA's concentration and purity by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE, USA), (3) complementary DNA (cDNA) synthesis by reverse transcription reaction, (4) quantization of cDNA concentration by NanoDrop 1000 Spectrophotometer.

Plasmids construction and copy number determination

Plasmids Construction of Bax, Bcl-2, p53, Cathepsin B, Caspase-3, Caspase-9 and EF-2 gene were performed as previously described (Mitupatum et al., 2015). In brief, the PCR product of those amplified genes with the primer sets as shown in Table 1 was cloned into the TA cloning vector pTZ57R/T using the Instaclone PCR Cloning Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA). After transformation into competent cells (E. coli DH5α), the transformed cells were cultured and isolated for plasmid DNA by the QIAprep® Spin Miniprep Kit (QIAGEN, Hilden, Germany) before sequencing by Macrogen Inc. (Korea) with the 96-capillary 3730xl DNA Analyzer (Applied Biosystems®). Serial dilutions of plasmid DNA were performed before being added into the real-time PCR Master Mix. The gene expression analysis was carried out using the Exicycler™ 96 Bioneer (Bioneer Corporation, Daejoen, Korea). Cx value of each dilution was determined using the Exicycler™96 Bioneer machine (Bioneer Corporation, Daejoen, Korea). Each PCR reaction took place in triplicate. The forward and reverse primers used for PCR amplification of Bax, Bcl-2, p53, Cathepsin B, Caspase-3, Caspase-9 and EF-2 gene are all listed in Table 1. The PCR reactions were made by mixing 2X KAPA SYBR® FAST qPCR Master Mix (KapaBiosystems, Inc. of Wilmington, MA, USA) with each primer and cDNA template. The real-time PCR program was carried out with a following 1 cycle of initial denaturation at 94°C for 10 min through 40 cycles of PCR reaction and then held for 30 seconds at each temperature level. Finally, melting curve analysis was performed over a gradient extending from an annealing to a denaturation temperature. The expression was calculated by using the relative standard curve method of quantification and reported as a fold change of gene expression.

Statistical analysis

The data were expressed as mean values ± standard deviation of the mean (SD). Difference between the two groups was analyzed by One-way ANOVA and student’s t test. A value of p<0.05 was considered statistically significant. Cell-cycle distribution was analyzed by Modfit software.

Results

HepG2 apoptotic cell death induced by different concentrations of Hep88 mAb

The mode of cell death induced by Hep88 mAb in HepG2 cells was confirmed by annexin V/PI double staining. Hep88 mAb-treated cells induced a decrease in the percentage of viable cells (annexin V-/PI-) as compared to control cells with a concurrent increase in the percentage of early apoptotic cells (annexin V+/PI-) and late apoptotic cells (annexin V+/PI+). This indicated that Hep88 mAb causes HepG2 cells to undergo apoptosis.

Table 1. Forward and Reverse Primer PCR Sequences for Real-time PCR

| Primers | Forward | Reverse | Sequence of Nucleotides (nt) | Size (nt) | PCR Product length |
|---------|---------|---------|----------------------------|----------|-------------------|
| Bax (55°C) | Forward | 5'-ATGTTTTTCTGACGAGCAACTTC-3' | 21 | 133 bp |
| | Reverse | 5'-AGTCCAATGTCCAGCCCAT-3' | 19 | |
| Bcl-2 (65°C) | Forward | 5'-ATGTTGTTGAGAGACCCGCAAA-3' | 20 | 141 bp |
| | Reverse | 5'-GCCGTACAGTTCCAACAAAGG-3' | 20 | |
| p53 (60°C) | Forward | 5'-ATGTGTGTGGAGACCGTCAA-3' | 20 | |
| | Reverse | 5'-TGTTTGTGTGCTTCTGAGCC-3' | 20 | |
| Cathepsin B (55°C) | Forward | 5'-TGACCGAGCGCTACATGGTG-3' | 18 | 153 bp |
| | Reverse | 5'-TGCAGGCTCCTCCAATAGCAG-3' | 18 | |
| EF-2 (60°C) | Forward | 5'-CTGAGTGGCGCTGCTAATGCTG-3' | 23 | 155 bp |
| | Reverse | 5'-GGGTCAGATTCTTGTAGGAGAT-3' | 24 | |
| Caspase-3 (60°C) | Forward | 5'-TGTGTTTGTTGTCCTTGACGGC-3' | 20 | 210 bp |
| | Reverse | 5'-CAGCGCATGCTCATCAAC-3' | 20 | |
| Caspase-9 (65°C) | Forward | 5'-CATTTCTGTGGGGAGGCTGGAAG-3' | 21 | 149 bp |
| | Reverse | 5'-GGGACCACCTGCAGCGTGCT-3' | 18 | |
after treated with Hep88 mAb (Figure 1C). The increase in the apoptotic population is associated with the increase in the sub-G1 population observed in the cell cycle analysis (Figure 2). These results suggest that Hep88 mAb induces cell death through an apoptotic mechanism.

**HepG2 cell cycle progression induced by different concentrations of Hep88 mAb**

Apoptotic cells show staining below the G1 population of normal diploid cells. The DNA-specific fluorochrome PI identified a distinct hypo-diploid cell (sub-G1) population as apoptotic cells. Figure 2A shows a representative DNA-histogram of HepG2 cells as observed at 24-, 48- and 72-hr after treatment with 50, 62.5 and 75 µg/ml of Hep88 mAb, respectively. The percentages of each phase population were measured by FCM. Populations of apoptotic cells (sub-G1) and G2/M cells were higher in the experimental group than in the control group (Figure 2A and 2B). Furthermore, at the same concentration of Hep88 mAb, apoptotic cells increased over time.

![Figure 1. The Apoptotic Cell Death in Hep88 mAb-treated HepG2 Cells.](image1)

(A) Flow cytometry analysis of apoptotic cell death. (B) The percentage of early apoptotic cells increased in a dose- and time-dependent manner (mean±SD, n=3), *p<0.05 vs. control, p<0.05 48- and 72-hr incubation vs 24-hr incubation of their corresponding doses. (C) The light microscope photomicrograph.

![Figure 2. Effects on Varying Doses and Times of Hep88 mAb Incubation on Cell Cycle Progression in HepG2 Cells.](image2)

(A) The histograms of cell cycle at 24-, 48- and 72-hr in varying doses. (B) The G2/M cell cycle arrest and apoptosis (sub-G1) were significant increase in doses and incubation times of Hep88 mAb treatment, *p<0.05 vs. control.
Plasmids construction and copy-number determination

After plasmid quantification, the copy numbers of each gene were then calculated. The standard curves of genes were then formed. A linear plot was shown in the range tested for R² = 0.995 (Bax), R² = 0.997 (Bcl-2), R² = 0.991 (p53) R² = 0.999 (cathepsin-B), R² = 0.992 (Caspase-3), R² = 0.996 (Caspase-9), and R² = 0.997 (EF-2).

Real-time PCR

Statistically significant differences in fold change of the mRNA expression levels of Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9 in the HepG2 Cell Line with Hep88 mAb at 12-, 18- and 24-hr.

![Figure 1: Down-regulation of Bax mRNA expression](image)

![Figure 2: Bcl-2/Bax expression ratio](image)

Figure 3. Real-time PCR Analysis for Bcl-2 mRNA expression, Bax mRNA Expression and the Ratio of Bcl-2/Bax after Cytotoxic Treatment with Hep88 mAb at 12, 18 and 24-hr. (A) Graph represents the up-regulation of Bax mRNA expression. (B) The chart shows the Bcl-2/Bax expression ratio with a value lower than 1. These results strongly supported the apoptosis induction of Hep88 mAb through an up-regulation of Bax together with a down-regulation of Bcl-2. 

![Figure 4: Fold Change of mRNA Expression](image)

Figure 4. Real-time PCR Analysis for Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9 mRNA Expression after Cytotoxic Treatment of Hep88 mAb at 12-, 18- and 24-hr. (A) The Bax and Caspase-9 were consistent with the p53 mRNA expression throughout the period of treatment (Figure 4A). It shows up- and down- and up- significant regulation response from the start to the completion of treatment (with 12-, 18- and 24-hr of incubation times). On the other hand, a flipped response was found at the Bcl-2 mRNA expression (Figure 4B). To monitor the response of p53, Bax and Caspase-9 mRNA expression at the earlier time of treatment, we also conducted the real-time PCR at 3-, 6- and 9-hr of treatment. The results of p53 mRNA expression show a gradual increase in fold change (Figure 4B). It was related to Bax mRNA expression (Figure 4B). Meanwhile, the Caspase-9 mRNA expression at the earlier time of treatment wasn’t correlated to Bax and p53 mRNA expression.
Additionally, as regards the protein involved in paraptosis morphology - i.e. Cathepsin B (CTSB) - it shows a sharply contoured expression (Figure 4C). The Cathepsin B mRNA expression displays an up-regulation after 18-hr of treatment while a drop to the lowest expression level is detected both at an early (12-hr) and late treatment (24-hr). This pattern prompted us to reveal the response that would be detected at the earlier point. Figure 5D shows the sharpest up-regulation of the CTSB mRNA expression at the earliest point in time (at 3-hr) of treatment.

However, when focusing on the Caspase-3 mRNA expression (Figure 4D), it is likely to peak with its strongest increase after a prolonged incubation time of treatment, corresponding to flow cytometry results as reported in our former report (Mitupatum et al., 2015). The earlier response of Caspase-3 mRNA expression at 3-, 6- and 9-hr of treatment was also found (Figure 5A).

Discussion

There is a sizable amount of evidence that suggests the utility of various anti-cancer agents against HCC (Taghiyev et al., 2012), but there are also many restrictions especially the specification to HCC. The present study is approach to manifest novel strategies that more specific for HCC treatment using Hep88 monoclonal antibody. We have investigated Hep88 mAb-treated HCC to explore whether it dominantly induces apoptosis and whether mitochondria, lysosome and caspases are involved in stimulated cell death in HCC or not. Our research evaluated the induction of apoptosis, cell cycle arrest, and mRNA expression of Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9. The challenge of this study is the differences in anti-cancer effect of Hep88 mAb that was induced through multi-mechanisms of cell death. Our results shown that the induction of apoptosis by cytotoxic dose of Hep88 mAb is mediated G2/M phase arrest and undergoing apoptosis via cascade reaction.

Previously gathered evidence has shown that Hep88 was targeted to the HCC cell line (Puthong et al., 2009). The alteration in morphological structure following Hep88...
mAb treatment of HepG2 cells illustrated the intracellular vacuolization, endoplasmic reticulum and mitochondria dilation, which is the morphology of parapostosis-like PCD (Manochantr et al., 2011). The parapostosis-like PCD was explained by Sperandio et al. (Sperandio et al., 2000) and Wyllie and Golstein (Wyllie and Golstein, 2001), who described the cascade reaction of caspase-independent PCD. As a result, this pathway might be lacking some of the morphology of caspase-dependent PCD. However, many reports have now demonstrated that cell death might be accompanied by a multi-mechanism. The morphological nature of cell deaths depends on the most effective and fastest pathway (Leist and Jaattela, 2001).

From this point, the results of this study demonstrate that the effects of Hep88 mAb on apoptosis induction, cell cycle progression in HCC cell line were significantly increased in a dose- and time-dependent manner. As cells are entering the apoptosis state, the earliest feature of this activity is the phosphatidylserine translocation from the inner cytoplasmic membrane to the outer membrane, a movement which can be detected by annexin V staining. Our annexin V-PI staining data found a significant increase of an early apoptotic cell population with elevated concentration and prolonged exposure time (Figure 1). This increase correlates well with many pieces of evidence indicating that apoptosis induction not only involves an extrinsic (caspase-8 activation) apoptosis pathway and a mitochondrial/intrinsic (Caspase-9 and Caspase-3 activation) apoptosis pathway (Mitupatum et al., 2015), but also induces cell cycle arrest as well (Lin et al., 2013; Wang et al., 2014b).

From within the scope of these patterns, the cell cycle can be determined by the quantization of DNA content (PI staining) within the cell. The cycle is based on differences in DNA content in each phase by following the cells in a pre-replicative phase (G1), replicate DNA- (S phase) and post-replicative plus mitotic (G2/M)-phase cells. Moreover, DNA content can be used to estimate apoptotic cells by sub-G1 (Darzynkiewicz et al., 2010). During the cell cycle, the potential target for cancer therapy is a G2/M checkpoint (Wang et al., 2009), because G2/M is a checkpoint that prevents DNA-damaged cells from entering mitosis and allows for the DNA repair. If cells cannot repair the damage during cell cycle arrest, the cell cycle progression with the DNA damage causes cell death or apoptosis (Wang et al., 2014a). As based on this point, it has been found that many publications on the novel cytotoxic molecule with regard to its role in cancer therapy are concerned with G2/M phase arrest (Lin et al., 2013; Wang et al., 2014b). Remarkably, our results revealed that Hep88 mAb induced HepG2 cells to arrest at the G2/M phase and to increase in the sub-G1 peak (Figure 2) in a concentration- and-time-dependent manner. This cell cycle data was correlated with significantly increased cell death, as determined by annexin V staining. As shown in the previous report, Hep88 mAb-specific protein is heat shock protein 70 (mortalin) (Rojibulstit et al., 2014). Cell cycle data from our study might be explained by the result of mortalin depletion after recognition by Hep88 mAb. It induces the restoration of p53-activating apoptosis induction and finally activates cell cycle arrest (Starenki et al., 2014).

Interestingly, the heat shock protein 70 (Hsp70) family is found at the lysosomes of many cancer cells and has been shown to prevent LMP (Nylandsted et al., 2004). Hsp70 has been shown to interact with Bax and prevent its translocation (Stankiewicz et al., 2005). Based on our recent report, its effect might be elucidated by the fact that, once Hep88 mAb interacted with Hsp70 mortalin, it may have subsequently resulted in recovering the cascade mechanism which conducted the apoptosis pathway. The postulated mechanism of Hep88 mAb may also be explained by the regaining of p53-mediated apoptosis by withdrawal of Hsp70 mortalin action on p53.

The existence of this mechanism is confirmed by the continual increase in fold change of p53 mRNA expression at 6- and 9-hr following treatment (Figure 5B). Subsequently, mortalin-free-p53 thus induces transcription-dependent apoptosis through transcriptional activation of the pro-apoptotic gene Bax by the translocation of itself into a nucleus and induces apoptotic signals, as reported by Green and Kroemer (Green and Kroemer, 2009). The result of this study has also confirmed that it did show the correlation between the up-regulation of Bax and p53 mRNA expression (Figure 5B). On the other hand, p53 can also induce these effects through the transcription-independent apoptosis, which is mainly achieved by the interaction between p53 and anti-apoptotic Bcl-2 proteins. This response is actually the reaction to apoptotic stresses, which has this consequence: Cytoplasmic p53 consequently moves rapidly to the mitochondria, binds to anti-apoptotic Bcl-2 proteins and then releases the pro-apoptotic Bax from the complex with the anti-apoptotic proteins. Subsequently, the released Bax mediates the mitochondrial outer membrane permeabilization (MOMP) and lysosomal membrane permeabilization (LMP) formation, which elicits cytochrome c (Green and Kroemer, 2009; Chiang et al., 2014) and lysosomal cathepsins release to the cytosol (Kagedal et al., 2005). From this study, it is suggested that up-regulation of Bax mRNA expression and down-regulation of Bcl-2 mRNA expression at each time point in Hep88 mAb-treated HepG2 cells was induced by p53.

On the other consequence, the overexpression of cathepsins has been demonstrated in numerous cancers (Gondi and Rao, 2013). The cathepsins are involved in either directed or indirected cell death. Cathepsin B is an important cysteine protease enzyme, and is involved in either the progression of cancer cells or in cell proliferation; likewise, it is involved in apoptosis induction, especially in HCC. Previously, Foghsgaard et al. reported evidence for the role of cathepsins in the execution of caspase-independent cell death, resulting in a parapostosis-like morphology (Foghsgaard et al., 2001). This involvement correlated well with our results that, following the earliest Hep88 mAb treatment, the rapid up-regulated Cathepsin B (CTSB) mRNA expression is strongly expressed at 3 hr of incubation (Figure 5D). Additionally, our data has shown that mRNA expression of Cathepsin B is up-regulated again at 9- and 18-hr of treatment (Figure 4C and 5D). However, when monitoring the Cathepsin B mRNA expression at the end of a 24-
hr period following treatment, down-regulation was observed. This result corresponds well with the report shown by Malla et al. that down-regulation of Cathepsin B could initiate caspase-dependent cell death by decreases in the Bcl-2/Bax ratio and also induce the activation of Caspase-9 and Caspase-3 in the brain-cancer cell line (Malla et al., 2010).

From all of these data, it can be concluded that Hep88 mAb treatment induces paraptosis until the development of apoptosis with this consequence: At the early stage of Hep88 mAb treatment (3-hr), HepG2 cells suddenly up-regulated the Cathepsin B mRNA expression. Overexpressed Cathepsin B mRNA was transported from the nucleus, packed in the lysosomes and then released to cytosol from the external induction. It contributed to loss of mitochondria membrane potential and the releasing of cytochrome c (Guicciardi et al., 2000). The intrinsic apoptotic pathway was subsequently initiated by the up-regulation of Capase-9 and Caspase-3 mRNA, which correlated well with the previous reports (Joy et al., 2010). The death induction was further stimulated after the binding of Hep88 mAb to its specific antigen (Ag), which might correspond with the death receptor at the cell membrane. The extrinsic apoptotic pathway was subsequently induced (Guicciardi et al., 2000). However, at this time point, Cathepsin B wasn’t completely released because LMP was suppressed by Hsp70 mortalin-BAX and Hsp70 mortalin-p53 binding complexes (Taurin et al., 2002; Stankiewicz et al., 2005; Wadhwa et al., 2006). Taurin and co-workers found that the p53 expression in mortalin-transfected cells was delayed in its response to death induction (Taurin et al., 2002). Moreover, Stankiewicz et al. also found that Hsp70 blocks heat-induced apoptosis by inhibiting Bax activation (Stankiewicz et al., 2005). For this reason, Hep88 mAb-treated HepG2 cells did not show the p53 and Bax mRNA up-regulation for three hours.

At 6-hr of treatment, the cancer cell attempts to combat and escape from the death induction of Hep88 mAb-treatment by the up-regulated Bcl-2 mRNA expression. As a result, MOMP and LMP were suppressed in their release of cytochrome c and Cathepsin B. This suppression probably means that the cancer cells have adapted themselves to avoid harm when exposed to the treatment. As related in many reports, it was demonstrated that an over-expression of Bcl-2 has been found to be associated with apoptotic resistance to anti-cancer treatment (Sartorius and Krammer, 2002; Douarre et al., 2005). Sartorius and Krammer found that the strongly up-regulated Bcl-2 paralleled the apoptotic resistance in lung-cancer cell lines after treatment with anti-cancer drugs (Sartorius and Krammer, 2002). In addition, Douarre et al. demonstrated that transfected Bcl-2 in lung-cancer cell lines resisted the apoptosis cell-death induction (Douarre et al., 2005). Moreover, Hep88 mAb treatment at this time point showed that the balance between anti-apoptosis and apoptosis occurred like a seesaw board; we explored the up-regulated Bcl-2 mRNA that caused the remarkable reduction in the regulation of Cathepsin B, Caspase-9 and Caspase-3 mRNA in the HepG2 cell. Meanwhile, the death induction in this study was still further stimulated through extrinsic apoptotic PCD. Bid was truncated and directly allowed the release of cytochrome c to induce Caspase-9 (Chipuk and Green, 2008). So, we were able to find the Caspase-9 mRNA expression in the Hep88 mAb-treated cell. At the same time, Hep88 mAb might be fully internalized into the HepG2 cell, as it was widely reported (Walter et al., 2005). It was then binding itself to Hsp70 mortalin to restore p53-function. In that way, p53 mRNA was beginning to be up-regulated. Similarly, as Walker and colleagues have reported, the molecule specific to mortal also induced p53 by stealing it from the p53-mortal complex (Walker et al., 2006).

A still larger amount of Hep88 mAb was then internalized into the HepG2 cells. At 9-hr of incubation, the Hsp70 mortalin-p53 complex in cytoplasm was reduced. p53 was thus freely capable of translocation into the nucleus. Those cells of the up-regulated p53 then induced transcription-dependent apoptosis through transcriptional activation of the pro-apoptotic gene Bax. As shown in the results, Cathepsin B, Caspase-3, p53 and Bax mRNA expression were drastically increased. An overt up-regulation of Bcl-2 mRNA also came into sight. This state initiated the signaling of a pathway of gradual inhibition of LMP and MOMP. They were modified into a seesaw board balance between anti-apoptosis and apoptosis, which still occurred. Nevertheless, the strongest expression can show its power to affect the cells. Cathepsin B was gradually increased and released from lysosome such that the dead-escape phenomenon could no longer withstand it. For this reason, when mitochondrial-mediated caspase route did not work, most of the cell death at this time point was induced through Cathepsin B activity that caused caspase-independent PCD or paraptosis (Broker et al., 2004). On the other hand, apoptosis was still stimulated through the extrinsic apoptotic pathway so that we could see the up-regulated levels of Caspase-3 mRNA expression in our results.

With a prolonged incubation time of the Hep88 mAb-treated HepG2 cell extending to 12-hr, the death induction was altered, and was mainly induced through mitochondrial-mediated caspase that led to apoptosis. It was caused by the damage that had accumulated in the cell from the previously released Cathepsin B and other lysosomal proteases activities. Further, it may precede the mitochondria dysfunction by exhibiting the cytochrome c release (Nylandsted et al., 2004) and may also represent a common mechanism for p53-dependent apoptosis (Joy et al., 2010). From our results, we disclosed the fact that the up-regulation of Bax and Caspase-9 correlated well with p53 mRNA expression. Meanwhile, Cathepsin B and Bcl-2 mRNA expression were down-regulated to allow cell death through apoptosis. It is possible that the DNA damage response led to p53 accumulation in the nucleus. Nuclear p53 transactivated the pro-apoptotic gene Bax to stimulate entering into the intrinsic apoptotic pathway.

At 18-hr of our treatment program, the data showed the Cathepsin B mRNA expression to be outstandingly up-regulated once again. It is possible that the Hsp70 mortalin increased by adaptive response to high intracellular stress and was sequestered to cytoplasmic p53 (Taurin et al., 2002). As a result, p53 cannot translocate back into the
mRNA Expression of Bax, Bcl-2, p53, Cathepsin B, Caspase-3 and Caspase-9 in the HepG2 Cell Line with Hep88 mAb

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