Hep 88 mAb Induced Ultrastructural Alteration Through Apoptosis Like Program Cell Death in Hepatocellular Carcinoma

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Hep88 mAbs, a novel monoclonal antibodies against hepatocellular carcinoma cell line from Thai patient, has been proved earlier for its tumoricidal effect on HepG2 cell line. In the present study, we investigated not only Hep88 mAb's targeted proteins from HepG2 cell line by western blot analysis but also its inhibitory activity on those cells by MTT assay. Moreover, the ultrastructural alteration induced by Hep88 mAb of HepG2 cell line compare with Chang liver cell line was also examined. The results demonstrated that Hep88 mAb had cytotoxic effect on HepG2 cell line but not Chang liver cell line. Additionally, recognizing proteins against Hep88 mAb have been found on both cell lines. The ultrastructural alteration detected from transmission electron microscopy included the appearing of intracellular vacuolization as well as the dilatation of endoplasmic reticulum and mitochondria have been observed. These findings are suggested that the death of HepG2 cell line after treatment with Hep88 mAb might be involved by an apoptosis-like program cell death (PCD) pathway. From all of these remarks, it is possible that Hep88 mAb can injure HCC cells by binding with its membrane-bound antigen and activated downstream intracellular signals which is finally leading cell to be death via apoptosis-like PCD.

Keywords: *Hepatocellular carcinoma, Monoclonal antibody, Cell cytotoxicity, Apoptosis, Transmission electron microscopy*

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Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. In Thailand, it ranks the first leading cancer in males and the third in females[1]. The major etiologic risk factors for HCC development include hepatitis B virus (HBV), hepatitis C virus (HCV) infections and aflatoxin ingestion[2]. Although technologies in treatment and diagnosis of the disease have been continuously developed, an incidence and the mortal rates of the HCC patients are still increased annually. This is due to a lack of a sensitive and specific early detection and an effective treatment to cure any remaining cancer cells. Moreover, the survival rates from traditional HCC treatments depend on many factors but especially on tumor size and staging[2,3]. Therefore, a targeted therapy such as the immunotherapy via monoclonal antibodies (mAbs) specific to tumor-associated antigen is nowadays a fascinating tool. It's not only critical on tumor regression by targeting specifically on HCC cells but also reduce toxicity to normal cells[4,5]. The possible anti-tumor effect of mAb started after it binds a portion of the extracellular domain of the receptors which may block growth factor receptor resulting in cell death or induce apoptosis of the tumor cells[6]. Moreover, the recognition of the Fc portion of a mAb bound to its specific tumor-associated antigen *in vivo* may activate not only the complement cascade, resulting in tumor cell lysis, but also result in the phagocytosis by macrophage[7,8].

With the advantages of the mAb by the development of the hybridoma technology, it has been

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reported in identify several novel mAbs against HCC[9]. In this regard, Laohathai et al has produced anti-hepatoma mAbs against S102, an established hepatocellular carcinoma cell line from Thai patient by murine system[10]. Since then, several clones of these mAbs were tested to seek out not only the highest specificity and sensitivity but also the tumoricidal activity against HCC. Among these, an anti-tumor associated antigen, Hep27 mAb has already been further investigated in detail. Sandee et al demonstrated that the Hep27 mAb alone can inhibit both tumor cell growth *in vitro* and human solid tumor in animal experimental model[11]. A single-chain variable fragment (scFv) molecule corresponding to a variable region of both the heavy-chain and light-chain of the Hep27 mAb has been constructed and its DNA sequence has previously been determined. However, the limitation of the mAb tools is that, the more its specificity, the less its sensitivity. Therefore, the combination of anti-HCC mAbs in cocktail formula will increase the sensitivity and therapeutic accuracy. Hence, to search for the new mAb with superior characteristic is an urgently need which will be in front to the identification of new pharmacological interventions for HCC in a near future.

From previous study, the authors reported the tumoricidal effect on HepG2 cell line of Hep88 mAb, a novel mAb produced from S102 cell line[12]. The authors then further verified its specific proteins from HepG2 cell line both from cytoplasmic and membranous parts by western blot analysis. In addition, the authors also examined the ultrastructural alterations of HepG2 cell line after treating with Hep88 mAb at 0 and 3 days using a transmission electron microscopy. The fascinating results from the present study imply that Hep88 mAb might be a promising tool to a development of an effective treatment of HCC in the next decade.

Material and Method

Cell lines

Human HCC cell line, HepG2 cell line (American Type Culture Collection [ATCC] HB8065), and normal liver cell line, Chang liver cell line (American Type Culture Collection [ATCC] CCL-13) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Biochrom AG, Germany). Both cell lines were maintained at 37°C in a CO_2 incubator and subculture every 3-4 days until use.

Production of anti-HCC mouse mAbs

The anti-HCC mouse mAbs were produced as previously described[10]. In brief, Balb/c mice were immunized by intraperitoneal injection of the suspension of S102 HCC cell line. Booster injection was given at day 20 using the same dose. After positive antibody tested was verified, the spleen was then fused with NS-1 myeloma cell line according to standard protocol. After 10-14 days, the positive culture clones were screened with an indirect ELISA and subsequently sub-cloned by limiting dilution technique until isolating out of 1 clone/well. The mAbs from the supernatants of each clone were further purified using a protein A-Sepharose column (GE Healthcare, Sweden) according to the manufacturer's instruction.

Cytotoxicity assay

Hep88 mAb (varying dose from 0 to 200 µg/mL, 3 days incubation) was assessed its cytotoxic activity against HepG2 and Chang liver cell lines (5 x 10^3 cells) via the MTT colorimetric assay. The IC50 concentration of Hep88 mAb against HepG2 cell line was determined from three separated experiments. The IC50 of Hep88 mAb was then used as the treated concentration at 0 and 3 days against HepG2 cell line which were finally detected the result using a transmission electron microscopy.

Western blotting analysis

Total proteins from cytoplasmic and membranous parts of 4.5 x 10^8 cells of HepG2 and Chang liver cell lines were extracted by Qproteome cell compartment kit (Quigen, Singapore) and subsequently separated by gel electrophoresis using 7.5% resolving gel and 4% stacking gel with 4 well-lane. The proteins were then transferred to a nitrocellulose membrane using a semi-dry transblot technique at 6 V, 70 min. After blocking the transferred membrane in blocking solution: TBST (containing 20 mM TBS pH 7.5, 5% skim milk and 0.1% Tween-20), the membrane was then washed in TBST and incubated with Hep88 mAb (1:100) as the primary antibody at room temperature for 2 h. The membrane was subsequently washed in TBST and incubated with 1:3,000 rabbit anti-mouse IgG (H + L) alkaline phosphatase conjugated (Invitrogen, USA) at room temperature for 1 h. The bounded proteins were color developed with BCIP/NBT substrate solution (Invitrogen, USA) according to the manufacturer's protocol.

Ultrastructure examination by a transmission electron microscopy

HepG2 cell line (1 x 10^6 cells) was cultured in RPMI 1640 supplemented with 10% fetal bowine serum

with or without Hep88 mAb at IC50 concentration for 0 and 3 days. The cells were then harvested and fixed in a solution of 4% glutaraldehyde plus 2% paraformaldehyde in PBS, pH 7.4, at 4°C for 1 h, followed by post fixation in 1% osmium tetroxide in the same buffer for 30 min. After fixation, the cells were dehydrated through increasing concentrations of ethyl alcohol at 50%, 70%, 90%, 95%, 100% for 10 min, twice at each concentration, at room temperature, cleared in two changes of propylene oxide, for 15 min each, infiltrated in the mixtures of propylene oxide and Araldite 502 resin at the ratios of 2:1 for 1 h and 1:2 overnight, then in pure Araldite 502 resin for at least 6 h which was finally polymerized at 30°C, 45°C and 60°C for 24, 48 and 48 h, respectively.

Thin sections with interference colour of silver to silver-gold (about 60-90 nm thick) were cut with diamond knife on a Porter-Blum MT-2 ultramicrotome. The sections were picked up on uncoated 300-mesh copper grids, air dried and stained by floatation on saturated aqueous uranyl acetate in the dark for 30 min, then rinsed with several changes of distilled water, and the excess water was blotted off with Whatmann filter paper. The sections were further stained by floatation on saturated aqueous lead citrated for 30 min, rinsed with several changes of CO_2-free distilled water, the excess water was blotted off with Whatmann filter paper and air dried. The sections were then observed in a Philips CM 100 TEM at 80 kV.

Statistical analysis

Data was presented as means ± SD from three separated experiment. The comparison results were proved their significant different at $p < 0.05$ by ANOVA.

Results

Cytotoxicity effect of Hep88 mAb on HepG2 and Chang liver cell lines

After determining its cytotoxic activity by MTT assay in dose ranging from 0 to 200 µg/mL for 3 days, the cytotoxic effect against HepG2 and Chang liver cell lines were observed in a rectangular hyperbola response. These meant the effects were proportional to Hep88 mAb's concentrations from a concentration of 0-50 µg/mL and 0-100 µg/mL for HepG2 and Chang liver cell lines, respectively (Fig. 1). After that the effect is independent on the concentration of antibody and having got the saturated effect until 200 µg/mL. The 50% growth-inhibitory effect or IC50 concentration had been detected at 12.5 ± 9 µg/mL for HepG2 cell line and at 72.2 ± 7 µg/mL for Chang liver cell line. Moreover, the cytotoxicity at its IC50 concentration (12.5 ± 9 µg/mL) against HepG2 cell line showed its tumoricidal effect only on HepG2 cell line but harmless to Chang liver cell line (% cell survival = 93 ± 6) (Fig. 2). Additionally, if the authors consider the cancer selective power of Hep88 mAb by a selective index (IC50 concentration for normal liver/IC50 concentration for HepG2 cell line = 5.776 fold), it also presented the 5 fold strengthening lethal ability of Hep88 mAb over HCC than normal liver.

Targeted proteins of Hep88 mAb

After extracting and blotting total proteins isolated from HepG2 and Chang liver cell lines with Hep88 mAb, the proteins from cytoplasmic fraction was demonstrated that the recognizing proteins of molecular weights about 53-60 kDa and 70-90 kDa have been found not only on HepG2 cell line but also on Chang liver cell line (Fig. 3). Note that some bands on cancer

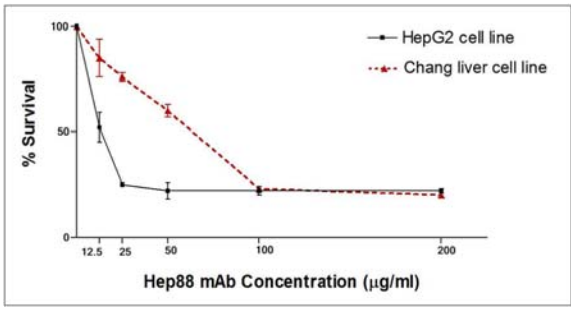

Fig. 1 The cytotoxic effect of Hep88 mAb against HepG2 and Chang liver cell lines. The results showed a rectangular hyperbola response *i.e.* a dose dependent manner from 0-50 µg/mL for HepG2 cell line and 0-100 µg/mL for Chang liver cell line. After that the effect is independent on the concentration of antibody and having got the saturated effect until 200 µg/mL

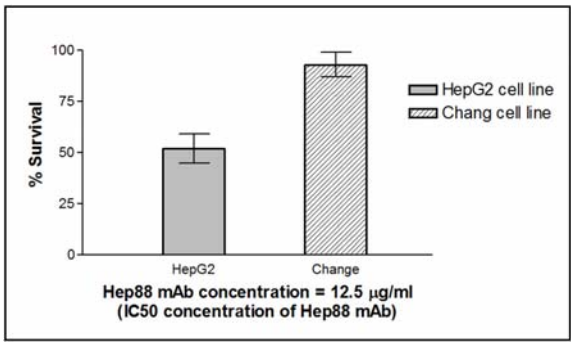

Fig. 2 At IC50 concentration of Hep88 mAb on HepG2 cell line (12.5 µg/mL), it showed harmless effect on Chang liver cell line (% cell survival = 93 ± 6)

cell line when compared with normal liver cell line were more intense whereas some were lighter. Additionally, the proteins from membranous fraction were found that much darker band around 60-76 and 80-90 kDa has been noticed on cancer cell line (Fig. 3).

Ultrastructural alterations of HepG2 cell line induced by Hep88 mAb

After treated HepG2 cell line with IC 50 concentration (12.5 µg/mL) of Hep88 mAb, ultrastructural change was revealed under transmission electron microscope. HepG2 cell line treated with Hep88 mAb at day 0, showed no DNA fragmentation, chromatin condensation, membrane blebing or apoptotic bodies (Fig. 4A). After 3 days of incubation with Hep88 mAb, the morphological changes which implicated to the cytocidal appearance including extensive features of intracellular vacuolization with mitochondria and endoplasmic reticulum dilatations were noticed (Fig. 4B). These findings correlated well with the results from cytotoxicity effect by MTT assay.

Discussion

Nowadays, monoclonal antibodies have widely used as cancer-specific targeting therapy because of their lower toxicity to normal cells. Many of them have been approved by the FDA for clinical use[13]. Mostly of them are targeted to CD markers which are surface components found on leukocytes and lymphocytes resulting in the therapeutic efficacy on cancer derived from haematopoietic stem cells such as non-Hodgkin's lymphoma and leukemia[14,15]. Moreover, many mAbs are also important contribution to the therapeutic treatment of breast cancer and now colorectal cancer[16,17]. Nevertheless, a little has been reported about the mAb therapeutic effect on HCC[6,11]. The previous study illustrated tumoricidal effect of Hep88 mAb on HepG2 cell line at IC50 concentration (100 µg/mL) against HCC cell line from Thai patient (S102 cell line)[12]. Thus, in the present study, the authors not only determined the cytotoxic effect of Hep88 mAb, a novel monoclonal antibody against HCC, but also verified its IC50 concentration against HCC cell line, HepG2, *in vitro*. The authors also tried to specify its recognition tumor-specific protein by western blotting analysis. In addition, the authors described ultracellular alterations of the HepG2 cell lines induced by IC50 concentration of Hep88 mAb. These fascinating outcomes confirm that Hep88 mAb is not only more specific but also more harmful to HCC than normal liver cells *in vitro*.

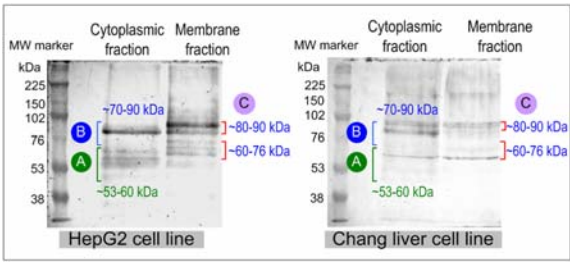

Fig. 3 Western blot analysis of proteins isolated from HepG2 (Right) and Chang liver cell lines (Left). The more intense protein bands recognized by Hep88 mAb between both cell lines were around 53-60 kDa (A), 70-90 kDa (B) from cytoplasmic part and 60-76 kDa, 80-90 kDa (C) from membranous part

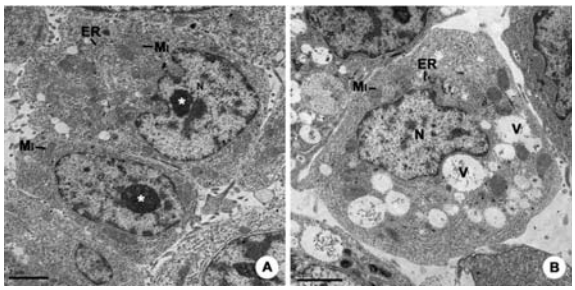

Fig. 4 TEM micrograph of ultrastructural alterations of the HepG2 cell line incubated with Hep88 mAb. At 0 day of incubation, no any morphological changes which correlated with cellular damages have been observed (A). The appearances of cytotoxic changes including intracellular vacuolization with mitochondria and ER dilatation have been remarkably noticed after 3 days of incubation (B). N = Nucleus, ER = Endoplasmic reticulum, Mi = Mitochondria, V = Vacuole, * = Nucleolus

When consider with the cytotoxic effect of Hep88 mAb against HepG2 and Chang liver cell lines, the result showed rectangular hyperbola response. It exhibited dose-dependent killer effect only at the range from 0-50 µg/mL for HepG2 cell line and 0-100 µg/mL for Chang liver cell line. Above that range, its effect was no longer decreased with an increase of Hep88 mAb concentration. That means the maximum cytotoxic effect can be obtained without increasing the amount of antibody. In other words, this relationship might be explained by the saturation with Hep88 mAb of all available binding sites on its specific proteins present. Moreover, when consider with the cancer selective index of Hep88 mAb to HepG2 cells, it shows approximately 5 fold over normal liver cells. This is

almost similar to those of adriamycin and 5-FU, a cancer therapeutic drug, on HCC cell line, Hep3B reported by Wang et al (SI was 5)[18] and on HepG2 reported by Tan et al (SI was 7)[19], respectively. This supports the potential effect of Hep88 mAb if the authors would like to use as cancer therapeutic drug *in vivo* in the near future, however, necessary primarily determine an optimum concentration to get the maximum cytotoxic effect at first.

In addition, the result from this research also demonstrated that the interesting proteins against Hep88 mAb have been found not only on cell membrane but also on cytoplasmic compartment. Their molecular weights are about 53-60 kDa and 70-90 kDa from cytoplasmic part and 60-76 kDa and 80-90 kDa from membranous part. In this regard, those recognizing proteins might be explained by up-regulated proteins which would be expressed once cells turned to be cancer for example, the group of chaperonic protein *i.e.* high molecular weight (HMW) heat shock proteins (HSP 70, HSP 90). These findings are correlated well with those of previous study which present up- and down-regulated protein expression profile of HCC cell line[20,21]. Those reports classified the identified proteins into several groups under different function categories including those of HMW chaperone and stress response proteins. All of them supposed that their results might pave the way for other researchers to find out a new biomarker for HCC. From these expectations, it might be the great possibility that such a definite specify Hep88 mAb's recognizing protein might be the one of these promising biomarkers if further investigation much more closer look in dept in the near future. However, though the monoclonal antibody is produced from identical immune clone of a unique parent cell which should bind only its own specific antigen. But in fact, it can cross recognize to other proteins if those of them hold the same antigenic determinant or having a homologous amino acid sequence leading to the almost similar of conformation at the antigenic site[22]. This is the same reason for an explanation of Hep88 mAb recognition. It can recognize many kinds of proteins which might be because of its cross reactivity against each antigenic sites. It is anticipated that this is not the limitation of Hep88 mAb's efficacy, instead it increase sensitivity of the usage both in therapeutic and diagnosis era.

Furthermore, ultrastructural alteration after incubated HepG2 and Chang liver cell lines with Hep88 mAb demonstrated the gradually appearance of intracellular vacuolization with mitochondria and endoplasmic reticulum dilatation corresponded with time of incubation. These typical morphological characteristics are firstly reported by Cornillon et al[23] and later described as apoptosis-like program cell death (PCD), an alternative form of program cell death by Sperandio et al[24] and Wyllie and Golstein[25]. This pattern of cell death lacks of both chromatin condensation and DNA fragmentation or any sign of caspase activation. The major difference is an existing of enclosing large vacuoles, markly filled with fluid. These findings have been sooner proved the pathway involved and simultaneously classified as caspase-independent pathway. Moreover, the formation of close contact of endoplasmic reticulum with the mitochondria demonstrate that apoptosis is controlled through signaling pathways that engage mitochondria and possibly endoplasmic reticulum. Indeed, mitochondria are now thought to act as key coordinators of cell death. Several the mediators of apoptosis, especially for various proteins from the Bcl-2 family are constitute on mitochondria[26,27]. These proteins translocate to mitochondria and facilitate the release of pro-apoptotic polypeptide factors including cytochrome c, smac (second mitochondria derived activator of caspase), AIF (apoptosis-inducing factor), Endo G (endonuclease G) and Hsp 70 (heat shock protein) into cytosol, where they contribute to the formation of the 'apoptosome' protein complex[28,29]. Emerging evidence suggests that translocation of mitochondrial AIF into the cytosol and then into the nucleus is a hallmark of caspase independent apoptosis[30]. Thus, the mechanism of relocation of pro-apoptotic proteins from cytosol to mitochondria has been considered to be central for their action.

From all of these remarks, there are 2 possibilities to hypothesize how Hep88 mAb can destroy HCC cells *in vitro* after binding with its membrane-bound tumor antigen which up-regulated once cell turn to be cancer (Fig. 5). Firstly, it then triggers downstream cell death signaling pathway without activating caspase activity. Another one is, Hep88 mAb might then be internalized into the HCC cells and binding with its cytoplasmic specific proteins which are initiated signalled cell to be death via caspase independent pathway or inhibit that of apoptosis-inhibitor proteins. Both of them are finally leading Hep88 mAb-treated cancer cell die through apoptosis-like PCD.

Conclusion

In summary, the data obtained from the

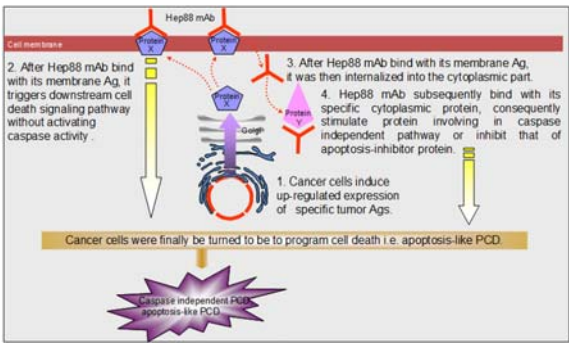

Fig. 5 Proposed mechanism of Hep88 mAb activate apoptosis like PCD

present study significantly identify not only those specific proteins recognized by Hep88 mAb which moreover provided us to greater understanding of the role of Hep88 mAb on liver carcinoma cells but also elucidated the propose mechanism of the mAb in destroying the cancer cells. However, the precise specific antigen and definite pathway has yet to be determined. Importantly, the findings from this research will help us considering for a further investigation on Hep88 mAb which requires huge budget and consumes time. In addition, the knowledge and technology obtained from this study can be applied for studies on other cancers in a future.

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Potential conflicts of interest

None.

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ผลของ Hep 88 mAb ต่อการเปลี่ยนแปลงโครงสร้างของเซลล์มะเร็งตับผ่านกระบวนการตายแบบ apoptosis

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จากการศึกษาก่อนหน้านี้พบว่า Hep88 mAbs ซึ่งเป็นแอนติบอดีต่อเซลล์มะเร็งตับสายพันธุ์ที่สร้างจากผู้ป่วยไทยมีฤทธิ์ในการฆ่าเซลล์มะเร็งสายพันธุ์ HepG2 การศึกษาในครั้งนี้นอกจากจะมีวัตถุประสงค์ เพื่อตรวจสอบหาโปรตีนเป้าหมาย ของ Hep88 mAbs โดยวิธี Western blot แล้วยังได้ทำการทดสอบฤทธิ์ยับยั้งการเจริญเติบโต โดยวิธี MTT นอกจากนี้ยังได้ทำการศึกษาผลของ Hep88 mAb ในการเหนี่ยวนำให้เกิดการเปลี่ยนแปลงทางโครงสร้างของเซลล์ โดยการเปรียบเทียบระหว่างเซลล์มะเร็งตับสายพันธุ์ HepG2 และเซลล์ตับปกติสายพันธุ์ Chang โดยใช้กล้องจุลทรรศน์ อิเล็กตรอนแบบส่องผ่าน ผลการทดลองที่ได้แสดงให้เห็นว่า Hep88 mAb ออกฤทธิ์ทำลายเซลล์มะเร็งตับสายพันธุ์ HepG2 แต่ไม่มีผลต่อเซลล์ตับปกติสายพันธุ์ Chang การตรวจสอบโปรตีนเป้าหมายของ Hep88 mAb พบทั้งในเซลล์มะเร็งตับสายพันธุ์ HepG2 และเซลล์ตับปกติสายพันธุ์ Chang การเปลี่ยนแปลงที่ตรวจพบจากกล้อง จุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบช่องว่างภายในเซลล์จำนวนมากรวมทั้งมีการขยายตัวของร่างแหเอนโด พลาสมิกเรติคูรัม และไมโทคอนเดรีย ผลการศึกษาแสดงให้เห็นว่าการที่ Hep88 mAb ออกฤทธิ์ทำลายเซลล์มะเร็ง สายพันธุ์ HepG2 อาจเกี่ยวข้องกับการตายของเซลล์แบบ apoptosis like program cell death อาจจะเป็นไปได้ว่า Hep88 mAb สามารถทำลายเซลล์มะเร็งตับโดยการจับแอนติเจนบนผิวเซลล์มะเร็งตับและกระตุ้นให้เกิดสัญญาณ ภายในเซลล์ ซึ่งนำไปสู่การตายของเซลล์แบบ apoptosis ในที่สุด