Cytotoxic Effect of Hep88 mAb: A Novel Monoclonal Antibody Against Hepatocellular Carcinoma

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Abstract

Fifteen novel monoclonal antibodies (mAbs) against S102, an established hepatocellular carcinoma cell line from a Thai patient, were tested at 50-500 μg/ml concentration for cell proliferation inhibitory activity against various types of commercial human cancer cell lines i.e., HepG2 (liver), Chago (lung), A375 (melanoma), Kato-III (gastric) and SW 620 (lymph node). To compare these inhibitory activity effects, we also verified them against a normal liver cell line, Chang liver, as well as against two of the established hepatocellular carcinoma cell line from Thai patients, S102 and R12 at the same mAbs concentration. The viability of cells after exposure to these 15 novel mAbs was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric methods. The result showed that, at their IC_{50} concentration against S102, only 4 of them (Hep20: IC_{50} = 100 μg/ml, Hep44: IC_{50} = 300 μg/ml, Hep88: IC_{50} = 100 μg/ml and Hep94: IC_{50} = 200 μg/ml mAbs) illustrated cell proliferation inhibitory activity against the hepatocellular carcinoma cell lines including HepG2, S102 and R12. Interestingly, Hep88 mAb inhibited S102 and R12 cell growth by approximately 70 and 95%, respectively, at 100 μg/ml, whereas it gave a tumoricidal effect to the HepG2 cell line at the same concentration. It had no effect on the normal liver cell line, Chang liver. Moreover, when compared with the effect from an anti-oncogenic cytokine, an interferon alfa-n1 (IFN) at its IC_{50} concentration (IC_{50} = 1,000 U/ml), Hep88 mAb showed lesser cytotoxicity on the normal liver cell line. These results suggest that Hep88 mAb might play an important role not only in inhibitory effects but also in lethal effects on hepatocellular carcinoma cells without disturbing normal cells. Accordingly, this antibody may be a promising tool for the therapeutic era of hepatocellular carcinoma in the next decade.

Keywords: Hepatocellular carcinoma, Monoclonal antibody, Cell cytotoxicity, MTT colorimetric assay, Growth inhibition

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. In Thailand it ranks first as the leading cancer in males and third in females [1]. The major etiologic risk factors for HCC development include hepatitis B virus (HBV), hepatitis C virus (HCV) infection, and aflatoxin ingestion [2-5]. Although technologies in treatment and diagnosis of the disease have been continuously

developed, incidence and the mortal rates of HCC patients still increase annually. This is due to a lack of a sensitive and specific early detection, and an effective treatment to cure any remaining cancer cells. The main HCC treatment modalities are: surgical (hepatic resection), non-surgical management including Percutaneous Ethanol Injection, PEI [4, 6], Transcatheter Oily Chemoembolization, TOCE [7-9]and Radiofrequency Ablation (RFA) [10]. The survival rates depend on many factors, but especially on tumor size and staging. [11-14]. Alternatively, a targeted therapy such as the immunotherapy via monoclonal antibodies (mAbs) specific to tumor-associated antigen is nowadays a fascinating tool to target specific HCC cells that are critical to tumor progression while reducing toxicity to normal cells [15-19]. The possible anti-tumor effect of mAbs starts after it binds a portion of the extracellular domain of the receptors which may block growth factor receptors resulting in cell death or induce apoptosis of the tumor cells [20-23]. 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 [24, 25].

With the advantages of mAb by the development of hybridoma technology [26], it has been reported of the identify of several novel mAbs against HCC [27-30]. In this regard, Laohathai K, *et al*. [31] have produced anti-hepatoma mAbs against S102, an established hepatocellular carcinoma cell line from a Thai patient by The murine system. 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 been further investigated in detail. Sandee D, *et al*. [32-33] demonstrated that the Hep27 mAb alone can inhibit both tumor cell growth *in vitro* and human solid tumor growth in an animal experiment, *in vivo*. 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, the greater its specificity, the lower its sensitivity, therefore the combination of anti-HCC mAbs in a cocktail formula will increase the sensitivity and therapeutic accuracy. Hence, to search for a new mAb with superior characteristics is an urgent need and this will lead to the identification of pharmacological interventions for HCC in the near future.

In this present study, we report an alternative novel mAbs produced from S102, an established hepatocellular carcinoma cell line from a Thai patient. We have used these mAbs to determine the HCC cell proliferation inhibitory activities by MTT (3- [4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide) colorimetric methods. Interestingly, one of those clones i.e., Hep88 mAb is able to either kill an HepG2 cell line (hepatoblastoma, ATCC strain HB 8065) or inhibit the proliferation of hepatoma cell lines: S102, R12 (established Thai patient's hepatoma cell lines) but had no effect on the normal liver cell line: Chang liver (human, hela marker, ATCC strain CCL 13). Therefore, Hep88 mAb might be a promising tool for the development of an effective treatment of HCC in the next decade.

2. Material and Methods

Cell lines

Seven cancer cell lines including; HCC cell lines HepG2, S102, R12; lung cancer cell line Chago; gastric cancer cell line Kato-III; lymph node metastasis cell line SW620; melanoma cell line A375; and one normal liver cell line, Chang liver, were cultured in RPMI 1640 with 10% fetal

bovine serum (Biochrom AG, Berlin). All cell lines except HCC cell lines S102 and R12 were obtained from the American Type Culture Collection (ATCC, Rockville, MD), with ATCC No. as shown in Table 1. All of them were maintained at 37°C in a CO_2 incubator and subcultured every 3-4 days until used.

Table 1 Cell lines and their origins, type of carcinoma, sources and ATCC No.

Cell lines		Origin	Type of carcinoma	Source	ATCC No
Cancer cell lines	HepG2	Liver	Hepatocellular carcinoma	Human	HB-8065
	S102	Liver	Hepatocellular carcinoma	Thai patient	-
	R12	Liver	Hepatocellular carcinoma	Thai patient	-
	Chago	Lung	Undifferentiated carcinoma	Human	-
	Kato-III	Gastric	Carcinoma	Human	HTB-103
	SW 620	Lymph node metastasis	Colon adenocarcinoma	Human	CCL-227
	A-375	Skin	Melanoma, Malignant	Human	CRL-1619
Normal cell lines	Chang liver	liver	Normal liver	Human	CCL-13

Production of anti-HCC mouse mAbs

The anti-HCC mouse mAbs were produced as previously described [31]. In brief, Balb/c mice were immunized by intraperitoneal injection with 0.3 ml (3 x 10^6cells/ml) of the suspension of S102 HCC cell line. Booster injections were given at day 20 with the same dose. Two weeks after boosting, the mice were bled and antisera were collected to test for the presence of antibody against HCC antigen, by an indirect ELISA using the pre-immunized serum from the same mice as negative control. Three days prior to fusion, the final booster was performed intravenously with 0.1 ml (1 x 10^6 cells/ml) of the suspension of S102. The mouse titer was selected for fusion. Splenocytes from immunized mice that represented the highest antibody wear then fused with an NS-1 myeloma cell line according to standard protocols. After 10–14 days, the culture supernatants were screened with an indirect ELISA test in which the solid phase was coated with the S102 HCC cell line, the cell line used for the immunization. By 2 or 3 times limiting dilution, antibody-producing hybridomas were subcloned, and then frozen in liquid nitrogen. Monoclonal antibodies were produced *in vitro* by collecting high concentrated supernatants. The mAbs from positive clones were further purified from the supernatant using a protein A-Sepharose (GE Healthcare) column according to the manufacturer's instructions.

Cytotoxicity assay

The fifteen supernatants from different hybridoma clones were assessed for their cytotoxic activity via the MTT colorimetric assay. Approximately 5 x 10^3 cells of HCC S102 cell line were seeded in 96-well plates in RPMI-1640 supplemented with 10% fetal bovine serum and incubated in a CO_2 incubator at 37°C for 24 hours prior to treatment. The MTT assay was performed as described by Mosmann [34] with the modifications suggested by Denizot and Lang [35]. To start the coloring reaction, 100 μl of fifteen

supernatants from antibody-producing hybridoma clones against S102 were added. In addition, negative control with the culture media alone and positive control with the interferon alfa-n1, an anti-oncogenic cytokine, instead of the test sample, were also performed. After incubation for 1, 3, 6 days with renewal of the medium plus mAb at day 4, 10 μL of 5 mg/mL MTT solution was supplementary added into each well, and incubated for 4 hours at 37°C. After that, the solution containing media, MTT and dead cells were removed, 150 μl of DMSO was subsequently added to each well and the plates were shaken for 5 min to dissolve the purple formazan crystals formed. In addition, 25 μl of 0.1M glycine pH10.5 was then added. The plates were next shaken yet again and the tests were read using an ELISA plate reader at a wavelength of 540 nm (OD data not shown). The amount of color produced is directly proportional to the number of viable cells. The percentage of MTT conversion to its formazan derivative for each well (percent cell growth) was calculated by dividing the OD at 540 nm of the wells with that of the control based on the following equation: (percent cell growth = $[A_{540}$ test $- A_{540}$ zero$] \times 100/[A_{540}$ control $- A_{540}$ zero$]$,A_{540} zero = A_{540} of solution after cell was incubated for 24 hours before mAb addition , A_{540} test = A_{540} of solution after mAb addition , A_{540} control = A_{540} of solution without mAb addition). The assay was conducted in triplicate for each sample concentration, negative and positive control. The cytotoxic hybridoma clones against HCC S102 have been selected and further purified. The concentrations of purified mAb giving 50% inhibition concentration (IC_{50}) were determined from three separate experiments. The IC_{50} of each mAbs were then used as the desired concentration against various cell lines to compare those effects with HCC S102 by MTT colorimetric assay.

3. Results

After incubation of fifteen supernatants from different hybridoma clones with MTT for different time periods, the results showed that a 50% growth inhibition effect could be detected after 3 days (as shown by an example of this effect on those mAbs in Figure 1). Therefore, this was chosen as the incubation period for all further experiments. When the HCC S102 cell line was treated with mAbs in doses ranging from 50 to 500 μg/ml for three days, the cytotoxic effect was observed in a dose dependent manner in all cases. From those, only four of them i.e. Hep20, Hep44, Hep88 and Hep94 mAb exhibited strong inhibitory effect on HCC S102. Figure 1 represents 50% growth inhibition effect of these four mAb. After purification by protein A Sepharose column, it was found that all of them were IgG_{2a} isotype.

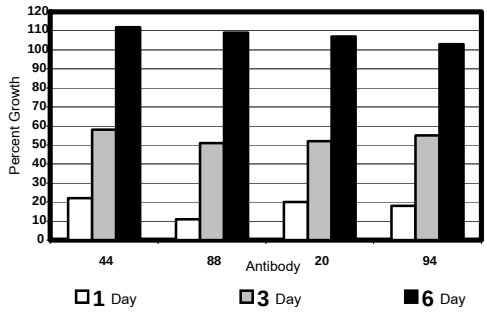

Figure 1 The 50% growth inhibition effect of Hep20, Hep44, Hep88 and Hep94 mAb on HCC S102 cell viability. This indicated that HCC S102 was half-inhibited at day 3.

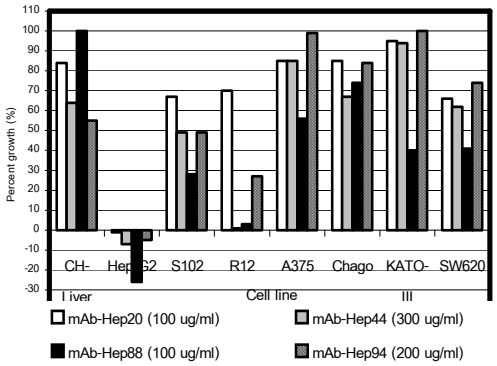

Figure 2 The cytotoxic effect of Hep 20, Hep44, Hep88 and Hep94 mAb. The results showed the cydal effect of Hep88 mAb on HepG2.

The cytotoxic effect of these four mAbs are represented in Figure 2. At its IC_{50} concentration as shown in Figure 3, Hep20 mAb (IC_{50} = 100 μg/ml) inhibited the growth of the hepatocelluar carcinoma cell line HepG2, whereas it slightly inhibited (from range 15% to 35% inhibition) the growth of other cancer cell lines (Figure 2). Moreover, it exhibited cytotoxic effect by 16% inhibition on the normal liver cell line, Chang liver. Similarly, at their IC_{50} concentrations of Hep44 and Hep94 mAb as shown in Figure 3 (IC_{50} = 300 and 200 μg/ml, respectively), they showed not only cidal effect on HepG2 but also exhibited the cross reactivity effect (from range 10% to 80% inhibition) on other cancer cell lines and the normal liver cell line (Figure 2). By contrast, at its IC_{50} concentration of Hep88 mAb as shown in Figure 3 (IC_{50} = 100 μg/ml), it was extremely effective on cell proliferation inhibition of all cancer cell lines (from range 30% to 95% inhibition), but had no effect on the normal liver cell line used in the experiment. Moreover, when considering the hepato-celluar carcinoma cell lines HepG2, S102 and R12, it was found that Hep88 mAb showed the highest cytotoxicity for all of them. Interestingly, it killed the HCC HepG2 cell line, whereas it showed approximately 70%-95% inhibition on the growth of HCC S102 and R12 cell lines (Figure 2). Surprisingly, it does not interfere with the growth of the normal liver cell line, Chang liver. In addition, when compared with the effect of an anti-oncogenic cytokine, the IFN, at its IC_{50} concentration (IC_{50} = 1,000 unit/ml), Hep88 mAb exhibited lesser cytotoxicity on the normal liver cell line.

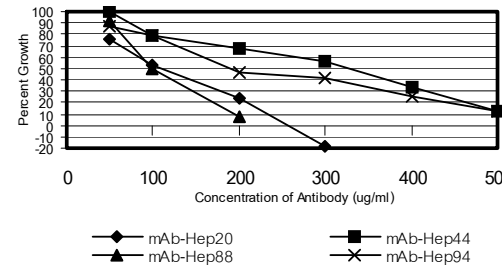

Figure 3 The IC_{50} of Hep20, Hep44, Hep88 and Hep94 mAb. Their IC_{50} are 100, 300, 100 and 200 μg/ml, respectively against S102 HCC cell line.

4. Discussion

For most patients with hepato-cellular carcinoma, malignant cancer has a poor prognosis. The therapeutic methods nowadays involve an operation and non-operation approach. However, survival rates depend on tumor size and staging [13-14]. Alternatively, novel strategies such as immunotherapy by using mAbs targeted to specific tumor antigens have been continuously developed for the past ten years [15-25]. Currently, many mAbs have been approved by the FDA for clinical use as reviewed in references 37-38. Mostl of them are targeted to CD markers, which are surface components found on leukocytes and lymphocytes resulting in therapeutic efficacy on cancer derived from haematopoietic stem cells, such as non-Hodgkin's lymphoma,

leukemia, etc. [38-41]. Moreover, many mAbs also have important contributions to the therapeutic treatment of breast cancer, and now colorectal cancer [42-44]. Nevertheless, little has been report about the mAb therapeutic effect on HCC [20, 32-33, 45-47]. Thus, in this study, we report the cytotoxic effect of a novel mAb, Hep88 mAb, against HCC cell lines HepG2, S102 and R12, but not on the normal liver cell line, Chang liver. We furthermore compare the effect of Hep88 mAb with those having cell inhibitory proliferation effect, i.e., Hep20, Hep44 and Hep94 mAb, on other cancer cell lines as well.

The cytotoxic effects of Hep20, Hep44, Hep88 and Hep94 mAb on HCC S102 were evident at 3 days of continuous exposure. This result was in agreement with the effect reported for Hep27 mAb on its original HCC S102 cancer cell line *in vitro* [33]. This indicated that all of them could cause injury to cells but not acutely. Therefore, the proposed mechanisms include the blocking of tumor growth receptors or inducing an apoptosis pathway after its binding at their specific receptor on the cell surface [48-49].

When considering IC_{50} concentrations under the same condition, Hep20 and Hep88 mAbs (IC_{50} = 100 μg/ml) showed 2-3 times lower effects than that of Hep44 and Hep94 mAb (IC_{50} = 300 and 200 μg/ml, respectively). This suggests that Hep20 and Hep88 mAbs are more cytotoxic than Hep44 and Hep94 mAb. In other words, these results also imply that Hep44 and Hep94 mAb could inhibit cell growth in a more delayed manner.

In addition, regarding their inhibitory effects on liver, lung, gastric, colon and melanoma cancer cell lines as well as normal liver cell line at each IC_{50} concentration, it was found that Hep20 mAb strongly inhibits growth of HCC HepG2 but slightly inhibits not only other cancer cell lines but also the normal liver cell line. These supported our proposed mAb-mediated immunotherapy mechanism *in vitro* by growth factor signaling a blocking effect, or apoptosis induction action soon after they bind to their specific antigen. These resulted in signal transduction alteration within the tumor cell or cell-surface antigen elimination and cancer cell growth inhibition.

The level of inhibition obtained from this result might rely on the fact that it was different in degree of protein expression either on each cancer or normal cells [50]. Similar rationales would be applied to explain those of Hep44 and Hep94 mAbs, which showed a cidal effect on HCC HepG2, and various degrees of inhibition on other cancer cell lines along with the normal liver cell line. In contrast, the effect of Hep88 mAb demonstrated its harmful activity only on HCC cell lines though various degrees, i.e. *in vitro* tumoricidal, inhibition or no effect (Figure 2). This, in turn, directly associates with the effect only on the up-regulating protein normally observed in cancer [24, 36, 50]. Another possibility to explain this phenomenon is that Hep88 mAb affects the mutated protein which exists on mutated cells such as cancer. Therefore, when Hep88 mAb bind to its specific tumor associated Ag, it would rather induce an apoptotic activation pathway or inhibit a signal transduction pathway, p53 activation pathway, growth factor receptor related signalling pathways. In support of this view, not only in the implementation of a cancer treatment with monoclonal antibodies therapy a number of strategies include “small molecule inhibitors” such as tyrosine kinase inhibitors and antisense oligonucleotides, which have already been evaluated for their potency to inhibit the activity and downstream signalling cascades of these receptors in HCC [52]. These finally lead to the death of only the cancer cells. In addition, binding of Hep88 mAb and its tumor Ag might also impede

any cell growth signal which over stimulates a cell to become cancerous [49-53].

In summary, all of this evidence indicates that Hep88 mAb might be a promising tool in targeting therapy to HCC, however, the precise functions have yet to be determined. This is a preliminary report in the therapeutic efficacy of Hep88 mAb, which will have to be further investigated in detail.

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6. References

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