Abstract. Heparan sulfate proteoglycans (HSPGs) were isolated from normal human liver and a monoclonal antibody (MAb) was raised against them. Preliminary studies showed that MAb clone 1E4-1C2 was able to react with many cell lines tested, including hematopoietic cells and solid tumors. MAb1E4-1C2 was used to study whether HSPG was involved in growth and proliferation of human liver cancer using hepatocellular carcinoma (HCC) cell line (HepG2) as a model. Inhibition by MAb1E4-1C2 of HepG2 cell proliferation was studied in vitro by MTT assay. For in vivo assay, xenograft induction in athymic mice was performed. The results showed that MAb1E4-1C2 inhibited proliferation of HepG2 cells significantly, compared to isotype and medium control. MAb1E4-1C2 also suppressed the growth of tumor, resulting in smaller tumor size and weight. The investigation also showed that MAb1E4-1C2 inhibited proliferation and restricted tumor growth through the induction of apoptosis. The results suggest that HSPG might be involved in liver cancer cell proliferation. Therefore, a specific MAb that was raised against liver HSPG might be an alternative therapeutic agent for the treatment of human liver cancer.

Interactions of cells with extracellular materials are mediated by cell surface matrix receptors (1). Many molecular processes associated with tumor growth, angiogenesis and metastasis are influenced by specific interactions between cells and the extracellular matrix (ECM). Heparan sulfate proteoglycans (HSPGs), with one or more HS glycosaminoglycan (GAG) chains linked to a protein core are found at or near the surface of all adherent cells (2) and are among those molecules (3, 4) found to be key components of such interactions.

HSPGs bind to and regulate the activities of numerous signaling molecules such as growth factors and cytokines (5, 6). There are two forms of cell surface HSPGs, cell surface bound through glycosylphosphatidyl inositol (GPI) anchor (7) or through an integral membrane protein (4, 7, 8). HSPGs are also found in the ECM in both parenchymal cell basement membrane (9) and pericellular matrix of fibroblasts (10). HSPGs appear to have multiple functions such as regulation of cell adhesion (11-13), cell migration and differentiation (14, 15), and basement membrane permeability (16). HS GAG chains are extracellular, suggesting that they act as receptors or co-receptors for various kinds of heparin-binding proteins (17). Liver is known to be a rich source of GAGs. HSPG in liver serves as a receptor for many molecules (18, 19), is involved in certain diseases (20), and is a target of pathogens (21-25). A recent study, focused on the structural characterization of HS isolated from human liver, demonstrated that the isolated GAG was HS, not heparin (26).

Monoclonal antibodies (MAb) were raised against human liver HSPGs and characterized. One of the clones obtained is 1E4-1C2, a MAb that specifically reacts with membrane molecules of various malignant cells lines, including hematopoietic cells in erythromyeloid series, various kinds of solid tumor cell lines (unpublished data), and peripheral blood leukocytes isolated from patients with acute myeloid leukemia (27). These data suggest that cell surface HS is involved and influences tumor growth and metastasis (28-32). Based on the results showing that MAb1E4-1C2 specifically reacted with HepG2, human hepatocellular carcinoma (HCC) cell line (HepG2), we decided to study the
effect of MAb1E4-1C2 on the growth of this cell type both in vitro and in vivo to address whether MAb1E4-1C2 might have a role in the progression of liver cancer cells.

In this study, we examined the potential of this novel MAb specific to carcinoma cells for use as an anticancer agent and potentially for the high specificity delivery of cytotoxic drugs to solid tumors.

Materials and Methods

Monoclonal anti-human liver HSpg (MAb1E4-1C2) production and purification. BALB/c mice (8 weeks old, weighing 26-28 g) were intraperitoneal primed with pristane (500 μl). Hybrid clone 1E4-1C2 (28), which produced monoclonal anti-human liver HSpg (1×10^5 cells/500 μl/dose), was injected 1 week later. The monoclonal antibody from ascites was purified using Protein G affinity agarose beads (PIERCE, Rockford, IL, USA) according to the manufacturer’s directions. Briefly, ascites was centrifuged at 14,000 xg for 5 min to eliminate cell debris. Supernatant was diluted 1:1 with binding buffer. The diluted sample was applied and allowed to flow completely into the resin. The column was washed with binding buffer before eluting with 5 ml of elution buffer. Fractions of 1.0 ml were collected and adjusted to physiologic pH with 100 μl of neutralization buffer. The fractions were then measured for absorbance at 280 nm. The purified monoclonal antibody was dialyzed against phosphate buffered saline (PBS) (pH 7.2) and concentrated. Aliquots were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and specificity by indirect immunofluorescence staining with HepG2 cell lines.

Expression of molecules on HepG2 cells specifically binding MAb1E4-1C2. Indirect immunofluorescence staining was performed to determine whether HepG2 cells expressed membrane molecules specific to MAb1E4-1C2. Fifty microliters of HepG2 cells (1×10^5 cells/ml) (CLS Cell Lines Service, Eppelheim, Germany) were incubated with heat-inactivated normal human AB serum (Blood Bank Unit, Maharaj Hospital, Chiang Mai University, Chiang Mai, Thailand) on ice at a final concentration of 10% for 30 min to block the Fc receptor. MAb1E4-1C2 of 50 μl was added to a final concentration of 20 μg/ml and cells were incubated on ice for another 30 min. MAb anti-HIV p24 (IgG1, in-house production, Department of Associated Medical Science, Chiang Mai University, Chiang Mai, Thailand) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) in PBS (pH 7.2), with 0.02% sodium azide, were used as IgG1 isotype matched control and negative control, respectively. After three washes with 1% BSA-PBS (pH 7.2) and 0.02% sodium azide, cells were re-suspended in 50 μl with 1% BSA-phosphate buffered saline and 50 μl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgGs, 1:25 (DakoCytomation, Glostrup, Denmark) was added. The reactions were extended by incubation on ice for 30 min and washed out with four changes of 1% BSA-PBS (pH 7.2), with 0.02% sodium azide. Finally, stained cells were suspended with 500 μl of sheath fluid (Becton Dickinson, CA, USA) and analyzed by flow cytometry (Coulter, Danvers, MA, USA).

Isotype matched control using MAb anti-HIV p24. Isotype matched control experiments were performed to demonstrate that target molecule interaction was an antigen-antibody specific reaction and not the result of a MAAb isotype. An isotype matched MAb should not stain target cells. MAb 1E4-1C2 was isotyped as IgG1, so MAb anti-HIV p24, which had been produced and characterized as isotype IgG1 in our laboratory, was selected as the isotype control.

Inhibition of HepG2 cell growth and proliferation by MAb1E4-1C2. Cytotoxicity assay of HepG2 cells was performed to determine the half maximal inhibitory concentration (IC50) of MAb1E4-1C2. HepG2 cells were trypsinized and washed with Ca2+/Mg2+-free PBS (pH 7.2). Cells were adjusted to 4×10^4 cells/ml with DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA) and plated (50 μl/well) in 96-well cell culture plate (Corning, Corning, NY, USA) overnight at 37℃ with 5% CO2 and 95% humidity. Fifty microliters of serial 2-fold diluted sterile MAb1E4-1C2 or MAb anti-HIV p24 (isotype control) were added to final concentrations of 0-0.50 μg/ml. Culture medium was used as negative control. Cultures were incubated for another 2 days. Supernatants were discarded, added to 20 μl/well of methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent (Promega, Madison, WI, USA) and incubated for 4 h at 37℃ with 5% CO2. Sterile SDS (10% v/v in PBS) was added at 25 μl/well and the plate was kept at room temperature for 18 h before measuring optical density at 490 nm. Each assay was performed in triplicate, and DMEM was used as a blank control. The percentage of viable cells was calculated as [(O.D. of cell control–O.D. of initiated cells)/O.D. of cell control] ×100.

Induction of apoptosis by monoclonal antibody 1E4-1C2. Cell surface changes and intracellular caspase-8 activity were analyzed in parallel to explore whether MAb1E4-1C2 induces apoptosis of HepG2 cells. Briefly, HepG2 cells (1×10^5 cells/ml) were plated in a 24-well culture plate (Corning) for 24 h at 37℃ with 5% CO2 and 95% humidity. Different concentrations (0-31.2 μg/ml) of MAb1E4-1C2 or MAb anti-HIV p24 (IgG1, isotype matched control) were added. Cells were incubated for another 24 h in the absence or presence of antibodies of interest. Cells were trypsinized with 0.25% trypsin in Ca2+/Mg2+ free 2% BSA-PBS (Sigma, St. Louis, MO, USA) and washed twice with Ca2+/Mg2+-free PBS. Cells were added to 1×10^6 cells/ml with annexin V incubation reagent (TACS™ Annexin V-FITC; R&D Systems, Minneapolis, MN, USA). The reaction was allowed to proceed in the dark for 30 min at room temperature. Finally, 400 μl of 1x binding buffer was added and flow cytometry carried out within 1 h. Intracellular caspase-8 activity was
also quantified (ApoStat; R&D Systems). ApoStat (10 µl) was added to the culture wells for the last 30 min of the culture period. Cells were harvested, washed and analyzed by flow cytometry.

**Western blot analysis.** Cells treated under all conditions mentioned above were subjected to western blot analysis to confirm the expression of caspase-8. Briefly, treated cells were washed and extracted in extraction buffer (1% NP-40, 50 mM Tris HCl pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.02% sodium azide) with protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM iodoacetamide, 10 µg/ml aprotinin). Cell extract was run under non-reducing conditions in 10% SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. Non-specific sites on membrane were blocked with 5% skimmed milk in Tris-buffered saline (TBS) (0.1 M Tris HCl, 0.15 M NaCl, pH 6.5) for 1 h at room temperature before adding anti-caspase-8 antibody (R&D Systems) overnight at room temperature. The membrane was washed four-times with washing buffer (0.5% Tween-20, TBS) and horseradish peroxide (HRP) conjugated rabbit anti-mouse IgGs, 1:1000 (DAKO) was applied. The reaction was performed for another 1 h at room temperature and washed out. Finally, the reaction was observed by chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) followed by autoradiography.

**Cell cycle analysis.** Cells were seeded into 6-well plates and treated with MAb1E4-1C2 or MAAb anti-HSPG p24, IgG1, isotype matched control (0 µg/ml, 3.9 μg/ml and 7.8 μg/ml) for 24 h and 48 h. Cells were then trypsinized, washed twice with ice-cold PBS and fixed with 70% ethanol overnight at 4°C. After a further PBS wash, cells were treated with Triton X-100 (1%), stained with 0.1 mg/ml propidium iodide and 2 mg/ml RNase at 37°C for 30 min. DNA content was measured by flow cytometry. Cellular DNA histograms were analyzed by FCS Express V3 software (De Novo Software, Los Angeles, CA, USA).

**Animal experiments.** The animal study was approved by the Experimental Animal Committee of Faculty of Medicine, Chiang Mai University. All animal experiments met the Animal Welfare guidelines. Male BALB/c nude mice (6 weeks old) were purchased from The Institute of Experimental Animal, Mahidol University, Bangkok, Thailand. Mice were housed in laminar-flow cabinets under specific pathogen-free conditions at room temperature with a 24-h night-day cycle and fed with pellets and water ad libitum. Log growth-phase of HepG2 cells (1×10^5 cells in 0.1 ml PBS) were injected subcutaneously into the right flank of athymic nude mice (n=4) to establish a model of tumor-bearing mice. On day 4 after implanting, MAb1E4-1C2 was injected subcutaneously (10 µg/50 µl) daily for 4 doses. Monoclonal anti-platelet antibody (PY-13) and sterile PBS were used as isotype and negative controls, respectively. Tumor growth was observed every 3 days by measuring its diameter with Vernier calipers. Tumor weight (TW) was calculated by TW = a x b^2 x 0.526, where a is the longest diameter, and b is the diameter at right angle. Mice were sacrificed when the tumor size reached 2.0 cm in diameter, and samples were collected.

**Immunohistochemistry.** Tumor specimens collected from the mice, were embedded in paraffin and stained with hematoxylin-eosin. Paraffin-embedded, 5 µm sections were deparaffinized, rehydrated, and subjected to hematoxylin and eosin staining to visualize vessels, arteries and endothelial cells.

### Results

**Expression of MAb1E4-1C2 reactive molecules on HepG2 cells.** Prior to studying the effect of 1E4-1C2, monoclonal antibody specific to HSPG isolated from human liver on the inhibition of HepG2 cells growth, indirect immunofluorescence was performed to verify the expression of 1E4-1C2 on specific cells. The results showed that 1E4-1C2 specifically reacted with HepG2 cells (Figure 1).

**Inhibition effect of 1E4-1C2 on HepG2 cell growth and proliferation.** Prior to studying whether MAb1E4-1C2 inhibited HepG2 cell growth and proliferation, a growth curve was prepared and analyzed. Incubation of 7 days showed a 3-day doubling time of HepG2 cells. MAb1E4-1C2 at a concentration of 25 µg/ml strongly inhibited HepG2 cell growth and proliferation (Figure 2).

**Induction of apoptosis by MAb1E4-1C2.** HepG2 cells were treated with 0, 3.9, 7.8, 15.6 and 31.2 µg/ml of MAb1E4-1C2 for 24 h, and then stained with annexin V-FITC/PI to examine whether MAb1E4-1C2 induced apoptosis in treated HepG2 cells. Stained cells were detected using flow cytometry. We found that MAb1E4-1C2 induced early apoptosis by 20-25% (Figure 3a). Intracellular caspase-8 was also investigated as a function of concentration of MAb1E4-1C2. Typical results showed MAb1E4-1C2 induced caspase-8 activity change as a function of concentration, as indicated in Figure 3a. In the presence of MAb1E4-1C2 (3.9 µg/ml), caspase-8 activity rapidly increased to a maximal value and progressively decreased to 60% of this maximal value. We proposed that MAb1E4-1C2 induced apoptosis through a caspase pathway in a dose-dependent manner. Moreover, results from western blot analysis indicated that caspase-8 processing in cells tested, was about 1.5-fold enhanced compared to its amount in non-treated cells (Figure 3b).

**Cell cycle analysis.** The histogram of the DNA content of control cells is shown in Figure 4a. After MAb1E4-1C2 (3.91 µg/ml) treatment, the histogram pattern was still similar to that of the control group (Figure 4b). A sub-GT1 population, corresponding to cells that underwent apoptosis, was not observed. The cell phase distribution at different concentrations of MAb1E4-1C2 and isotype control is shown in Figure 4c. These results show that neither MAb1E4-1C2 nor the isotype control had any effect on the cell cycle compared to control at 24 h. However, at 48 h, the sub-GT1 peak was undetectable in the histogram for the control group (Figure 4d) but was detected in the MAb1E4-1C2 (3.91 µg/ml)-treated group (Figure 4e). Figure 4f shows the cell phase distribution at different concentrations of MAb1E4-1C2 and isotype control at 48 h. The distribution of each phase in the isotype-treated group did not differ from that of...
the control group and a sub-GT$_1$ population was not detected. In contrast, a sub-GT$_1$ phase was detected in the MAb1E4-1C2 treated-group at 39% (n=3) for 3.91 μg/ml and 54% (n=3) for 7.8 μg/ml.

**Animal experiments.** Four days after tumor cell inoculation, each tumor-bearing mouse was daily subcutaneously injected with MAb1E4-1C2, or MAb anti-HIV p24 (IgG1, isotype matched), or sterile PBS (as controls) for 4 days. The tumor growth slightly increased during the first 18 days and then rapidly increased with specific growth rates of 0.37, 0.61, 0.56 for MAb1E4-1C2, MAb anti-HIV p24 and sterile PBS, respectively, as shown in Figure 5. MAb1E4-1C2 appeared to inhibit tumor growth. The tumor sizes in the MAb1E4-1C2-treated group were smaller than those of the other two control groups. The tumor weight is indicated in Figure 6. It was determined that the tumor weight was 9.88±2.43 g for PBS (n=3), 9.09±0.79 g for isotype control (n=4) and 5.39±1.28 g for the MAb1E4-1C2 group (n=4). The tumor weight of MAb1E4-1C2 group was significantly different from control as indicated by Wilcoxon Signed Ranks Test analysis (p<0.05).

**Immunohistochemistry.** Angiogenesis is crucial for tumor growth and metastasis. The results of hematoxylin-eosin staining showed that the number of blood vessels was
Figure 4. Flow cytometric analysis of the cell cycle distribution in MAb1E4-1C2 treated HepG2. a-e: HepG2 cells were incubated for 24 h and d-f: for 48 h. In b and e, MAb1E4-1C2 (3.9 μg/ml) was present for 24 h and 48 h, respectively. The percentages of cells residing in sub-G1, G0/G1, S- and G2/M phases were counted. In c and f, the cell phase distribution was determined at various concentrations of MAb1E4-1C2 and isotype control (MAb anti-HIV p24) for 24 h and 48 h, respectively. Results are expressed as mean±SE (n=3).

Figure 5. Inhibition of tumor growth by MAb1E4-1C2 in nude mice xenografted with human HCC HepG2. On day 4 after implanting tumor cells, MAb1E4-1C2, MAb anti-HIV p24 (isotype control group) or sterile PBS (vehicle control group) were injected subcutaneously (10 μg/50 μl), daily for 4 doses. Tumor growth was recorded every 3 days by measuring its diameter with Vernier caliper. Tumor weight (TW) was calculated by TW (g)=tumor volume (cm³)=d² × D/2, where d is the shortest and D is the longest diameter, respectively.
Figure 6. MAb1E4-1C2 inhibited xenograft growth in nude mice. Tumor volumes (a) and tumor weights (b) at sacrifice on day 28 were compared between mice treated with MAb1E4-1C2 (n=4), MAb anti-HIV p24 (n=4) and sterile PBS (n=3) (*p<0.05).

Figure 7. Immunohistochemistry of tumor specimens. Tumor specimens were collected from the mice, embedded in paraffin and stained with hematoxylin-eosin. A greater number of blood vessels were observed in tumor from mice treated with MAb anti-HIV1, isotype matched control or PBS control group (d-i) than in tumors from mice treated with MAb1E4-1C2 (a-c). V: Vessel, A: artery, arrows: endothelial cells.
increased in tumors of mice treated with isotype or PBS (Figure 7d-i). Endothelial cells were observed. In contrast, there was a smaller number of blood vessels in the MAb1E4-1C2 treated group (Figure 7a-c).

Discussion

HSPGs interact with a multitude of proteins and have been implicated in the regulation of a broad range of biological processes including cell division, migration, adhesion, tissue organization and viral entry (33). MAb1E4-1C2 had been prepared in our laboratory against a human liver HSPG (26) and showed some activity against acute myeloid leukemia (27). Since HepG2 human HCCs were shown to react with MAb1E4-1C2, we hypothesized that the specific recognized molecule may be involved in tumor cell growth and proliferation. Both in vitro and in vivo experiments in an animal model, clearly demonstrate the activity of this MAb against human HCC.

Flow cytometry with PI staining was used to evaluate the apoptosis of HepG2 cells after treatment with MAb1E4-1C2 for 48 h. The resulting apoptotic cells showed a reduction of DNA content and, therefore, could be recognized, following staining of cellular DNA, as cells with low DNA stainability (sub-G1) that were lower than that of G1 cells (34). The MAb1E4-1C2 treated groups showed a sub-G1 apoptosis peak. The apoptotic rate progressively increased from 39% to 54% in a dose dependent manner. The results correspond to an enhancement of intracellular caspase-8 on exposure to MAb1E4-1C2. Caspase-8 is the typical apoptosis initiator caspase downstream of TNF super-family of death receptors. Indeed, caspase-8 deficient cells are resistant to death receptor-mediated cell death both in vitro and in vivo (35).

Angiogenesis plays an important role in cancer, since vessels are needed to supply nutrients and oxygen to sustain tumor growth, and to excrete metabolic waste. The neovessels provide access for tumor cells to penetrate into the circulation. Tumor cells stimulate the formation of new blood vessels by means of enhanced production of the major angiogenic growth factors. Angiogenesis is controlled by various receptor tyrosine kinases and their ligands. Vascular endothelial growth factor (VEGF) receptors play an important role in angiogenesis. VEGF, secreted by hepatoma cells and hepatic stellate cells, is up-regulated during tumor differentiation and vascular development of HCC (36, 37). VEGF is expressed at high levels in tumor blood vessels (38). Hypervascularity is one of the main characteristics of large and moderately or poorly differentiated HCC. Tissue factor (TF), a plasma membrane glycoprotein expressed by endothelial cells and smooth muscle cells, and known to initiate blood coagulation, can also play a role in tumor progression and angiogenesis (39, 40). An improved knowledge of molecular mechanisms regulating angiogenesis in cancer provides insight into molecular targets for anti-angiogenic treatment. Anti-angiogenic treatment can prevent neovascularization by inhibiting proliferation, migration and differentiation of endothelial cells. The endothelial cell staining by H&E showed both decreased vasculature and distorted vascular morphology in MAb1E4-1C2-treated xenografts and the resulting endothelial vessels were undersupplied. These data suggest that the tumor size in the control group, which was higher than the one for MAb1E4-1C2, might result from tumor vascularization (41).

In conclusion, our results reveal that the human HCC cell line HepG2 express HSPG. HSPGs are reported to be involved in cell growth and proliferation, thus, we hypothesized that HSPG isolated from human liver may take part in cell proliferation both in vitro and in vivo. Using MAb raised against human liver HSPGs as a tool, we demonstrated that this specific MAb inhibited proliferation of HepG2 cells and suppressed tumor growth in an animal model. This is the first report of using this specific MAb to inhibit proliferation and growth of HCC.

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References


