3 肝癌の診断・治療

(10) 進行肝癌の治療戦略

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重要なポイント

- 肝動注化学療法の適応は既存の治療，ことに肝動脈塞栓術が明らかに適応外か，無効の症例である．
- 肝動注化学療法を施行するには，化学療法に耐える十分な肝機能，骨髄機能が必要である．
- 肝動注化学療法で Down-staging に成功した場合には，ただちに既存の有効な治療法に変更することが長期生存には必要である．
- 薬剤の有効性を評価する時期は動注継続には重要で，早すぎても遅すぎてもいけない．

Ⅰ．動注化学療法の適応について —腫瘍の進展度からみた適応

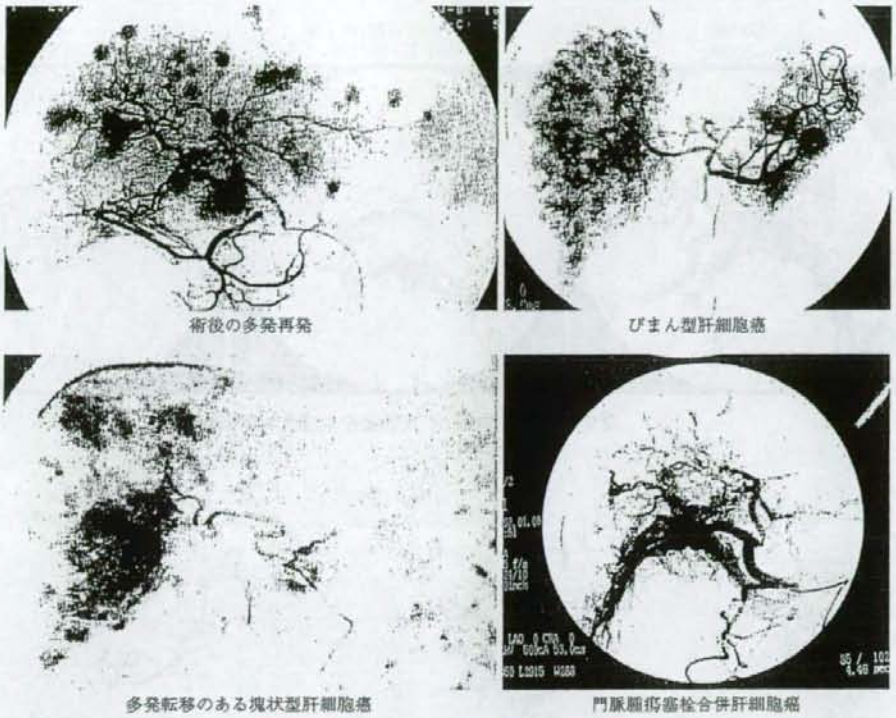
肝癌に対する動注化学療法が患者の予後を延長するという，明らかなエビデンスを実証した報告は，これまでのところ知られていない．いずれも治療例において，これまでの経験により有効な症例があるという報告のみ[1),2)]である．したがって，動注化学療法の適応症例とは，進行肝癌にその治療効果が実証された保存治療である肝動脈塞栓術[3),4)]が明らかに適応外の症例か，肝動脈塞栓術が無効の症例に限って治療対象とすべきである．

腫瘍の進展度からみた肝動注化学療法の適応症例は，① 明らかな脈管内腫瘍塞栓を合併する進行肝細胞癌症例（手術不能例に限る），② びまん型肝細胞癌（とくに肝切除後の再発はよい適応であることが多い），③ 高度の肝内多発転移を伴い，肝動脈塞栓療法（TACE）が無効であったか，明らかに無効であると予測される症例が対象になる．図Ⅱ-3-25 に動注化学療法のよい適応と思われる症例の血管造影像を示す．

図Ⅱ-3-25 に示すように肝切除後に全肝に多発再発した症例，びまん型肝細胞癌，塊状型肝癌で多発転移のある症例は，肝動脈塞栓術が効きにくい発育様式であり，また両葉に病巣がある場合には全肝を治療することが必要になるので，肝動注化学療法を選択する．さらに門脈腫瘍栓があり動脈門脈シャントを伴う場合は，通常の肝動脈塞栓術は肝不全を起こす可能性もあり，これも動注化学療法のよい適応である．

Ⅱ．動注化学療法の適応について —肝予備能と骨髄機能からみた適応

本邦で汎用される肝動注化学療法のプロトコ

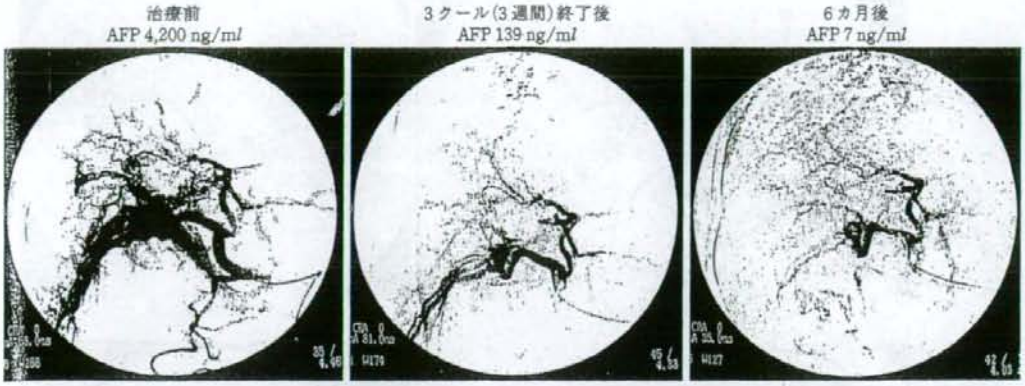


図Ⅱ-3-25　肝動注化学療法の適応症例

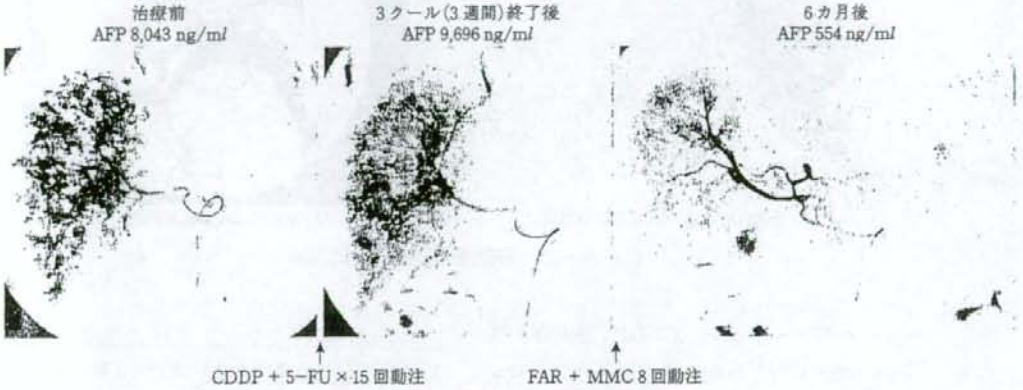
ールは，シスプラチン（CDDP）併用5-FU（Low dose FP）[1] あるいはインターフェロン（IFN）併用5-FU[2] が多い．筆者が多用しているCDDP併用5-FU[1] では，CDDP 10 mgを1時間で動注し，引き続いて5-FUを3時間あるいは4時間で動注する．これを5日間連続で投与し2日間休薬するのを1クールとする．1回のCDDP投与量が少ないので，動注中の確保輸液は500 mlと制吐剤のみである．最初の2週間までは副作用はほとんどないが，3週目以降に吐き気，食欲不振が出現し，これに引き続いて血小板減少，低アルブミン血症，腎機能障害が出現することが多い．したがって，導入化学療法としてCDDP併用5-FU動注化学療法を4週間継続するためには，これに耐える肝予備能と骨髄機能が必要である．

一般には血小板5万以上，白血球3,000以上，Child B 7点以上で，とくにアルブミンは3 g/dl以上が必要である．また10回投与後に採血確認し，以降は毎週確認する．血小板3万以下，白血球2,000以下，アルブミン2.7 g/dl以下では中止，休薬し，血小板2万以下では血小板輸血，好中球500以下では顆粒球コロニー刺激因子（G-CSF），アルブミンの補給を行う．とくに血小板減少は4週間治療を継続した場合には，それ以降にも減少することがあるので注意する．

IFN併用5-FUでは，5-FU 500 mg/日を5日間持続投与する．さらにIFNは週3回筋注し，これを2週間継続，次の2週間はIFNのみ投与，1クールとする投与法がオリジナルである[2]．通常は効果と副作用に応じて2クール（2カ月）以上施行する．IFNによる白血球，血小板減少は認められるが，5-FUのみの投与



図Ⅱ-3-26　症例1（門脈腫瘍塞栓合併肝細胞癌）



図Ⅱ-3-27　症例2（びまん型肝細胞癌）

であるので，肝予備能に与える影響はCDDP併用5-FUよりも軽いことが多く，コンプライアンスは良い傾向にある．また報告されている奏効率はCDDP併用5-FUとほぼ同等で40～50％である．

Ⅲ．CDDP併用5-FU動注化学療法における治療効果予測の実際

進行肝癌に肝動注化学療法を遂行する場合，初回の導入化学療法で有効な割合は45％程度でしかない．ましてや，導入化学療法で著効する割合は10％以下である．このような有効率で無意味に同じプロトコールを継続すると，肝機能の低下や血小板減少のために次の薬剤選択の機会を失うことがある．したがって実際の臨床現場では動注継続中に早期に治療効果を予測して，次の動注薬剤を選択可能にする方法が必要となる．以下にその方法について症例を提示しながら解説する．

CDDP併用5-FU肝動注化学療法では3週間投与までは目立った副作用が出現することが少なく，この時点が同じ薬剤を継続するか，次の薬剤を選択するかを決定するのに重要である．その評価法はリザーバーからの造影による腫瘍濃染の変化と腫瘍マーカーの推移で評価している．

症例1（図Ⅱ-3-26）は門脈腫瘍塞栓合併肝癌で，著明な動脈門脈シャントが血管造影上認められる．動注3週間後のリザーバー造影で，明らかな動脈門脈シャントの減少と腫瘍濃染の減少が認められ，治療前4,200 ng/mlであったAFPも139 ng/mlと低下したので，治療効果は有効と判定し同じ治療を継続し，維持化学療法にも同様の薬剤を用いて完全寛解が得られた症例である．

症例2（図Ⅱ-3-27）はびまん型肝細胞癌で，動注3週間後のリザーバー造影で腫瘍濃染は不変と評価した．治療の継続を迷ったが，治療前8,043 ng/mlであったAFPは9,696 ng/mlと微増していたので，薬剤をファルモルビシン20 mg，マイトマイシンC（MMC）4 mgを隔週動注に変更し，試験動注を施行した．その結果，1回目の動注でAFPは3,383 ng/mlに低下したので同薬剤で維持化学療法を継続し，2カ月後のリザーバー造影で腫瘍濃染の減少が確認できた症例である．

この提示2症例のように，臨床の現場では早期に治療効果を予測して，進行肝癌の肝予備能，骨髄機能をいかに温存しながら有効な薬剤を探していくか（臨床的な試験動注という考え方）が予後延長に重要になる．

現行の肝動注化学療法の奏効率はいまだ不十分で，結果としては，奏効率と予後が有意の相関は示すという成績になるが，実際の症例では抗癌剤に感受性を示さない症例の次の治療選択，治療継続に困惑することのほうが多い．今後はこのような症例に対する治療選択について，具体的な検討がなされていくことが必要である．

Ⅳ．肝細胞癌に対する肝動注化学療法の基本戦略

肝動注化学療法は既存の治療が適応でないか，無効の場合に選択される治療法なので，最後の治療選択と位置づけられることが多い．しかし，肝動注化学療法を適応とするためには，腫瘍の進展度はともかく，肝機能不良例に有効例は非常に少ないことがわかっている．したがって，あらゆる既存の治療をやり尽くした肝機能不良例に対する治療選択とするのは問題である．抗癌効果は感受性に依存しており，十分な投与量を確保できる肝機能を有する症例に施行されるべきであることに留意してほしい．

既存の治療に不応な場合には早めに肝動注化学療法を施行し，Down-stagingに成功した場合には，ただちに既存の有効な治療法に変更して治療を継続する姿勢が，高度に進行した肝細胞癌症例の長期生存を得るためには必要である．

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TGF-ß1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells

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Abstract. Transforming growth factor beta 1 (TGF-ß1) is a proposed regulator of *Ids* (inhibitors of DNA binding/differentiation) gene expression in epithelial cells. We previously reported that Id proteins are variously expressed in human hepatocellular carcinomas (HCC). However, the mechanism of regulation of Ids in HCC remains obscure. Here, we examined the relationship between Id1 and TGF-ß1 in four HCC cell lines, and studied the changes in cell proliferation, cell cycle and differentiation. The four HCC cell lines expressed Id1, TGF-ß1 and their receptors at various levels. TGF-ß1 strongly inhibited the growth of HuH7 cells, while the growth inhibition was moderate in PLC/PRF/5, and was not observed in HLE and HLF cell lines. TGF-ß1-induced growth inhibition in HuH7 cells was associated with cell accumulation in the G1 phase and partial induction of differentiation (with reduction of AFP and AFP-L3). Induction by TGF-ß1 dose-dependently suppressed Id1 expression in HuH7 cells; 1 ng/ml TGF-ß1 inhibited Id1 by 84.0 and 78.6% that of the untreated control at transcriptional and protein levels, respectively. HLE and HLF cells, which did not exhibit a TGF-ß1 growth inhibitory effect, lacked TGF-ß receptors and Id1 expression was not altered. In PLC/PRF/ 5 cells, Id1 augmentation was not observed in response to TGF-ß1, indicating that TGF-ß1-induced growth inhibition was not related to Id1 in this cell line. Our results suggest that, in some HCC cells, the pathway of suppression of Id1 by TGF-ß1 may be important in TGF-ß1-induced growth inhibition and partial differentiation.

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Key words: TGF-ß1, Id1, HCC, proliferation, differentiation, cell cycle

Introduction

Transforming growth factor-ß1 (TGF-ß1) is a multifunctional cytokine that inhibits epithelial cell growth by binding to a heterodimeric receptor consisting of both type 1 (TGFßRI) and type 2 (TGFßRII) serine/threonine kinase receptors. TGF-ß1 binding and activation of the TGF-ß1 receptor complex propagates intracellular signal transduction involving Smad proteins, which regulate numerous developmental and homeostatic processes by regulating gene expression (1,2). Resistance to growth inhibition by TGF-ß1 has been considered an important step in tumorigenesis and contributes to the development of many tumor types (3,4). Recently, *Id1* (inhibitors of DNA binding or differentiation) was revealed to be one of the direct TGF-ß1 target genes (5). Specifically in epithelial cells, Id1 expression can be inhibited through the TGF-ß1-responsive Smad3-activated transcriptional repressor, ATF3, which binds to the ATF/CREB site on the Id promoter, repressing Smad-initiated transcription.

Id proteins are members of the helix-loop-helix (HLH) family of transcription factors and are involved in regulation of various cellular processes, including cellular differentiation and cell cycling, and important functions in oncogenesis (6-9). Generally, Ids inhibit cell differentiation and induce proliferation by inhibiting basic HLH-dependent expression of differentiation-linked genes or suppressing the cyclin-dependent kinase inhibitors. However, these functions vary among cell types. Several studies have revealed a role for Id proteins in primary human tumors. Furthermore, deregulated expression of Ids has been reported in several primary human tumors such as pancreatic, breast, ovarian, prostate and neuronal tumors, and in colorectal adenocarcinoma and squamous cell carcinomas (reviewed in refs. 7 and 10). We have previously reported that Id proteins are down-regulated in human hepatocellular carcinomas (HCC) and their expression is related to tumor differentiation (11). However, the mechanism of regulation of Ids in HCC is not understood.

HCC is the fifth most common malignancy worldwide, and is estimated to cause approximately half a million deaths every year (12). Although curative therapies, such as hepatic resection, liver transplantation and percutaneous ablation, have led to an improvement in the survival of patients with

HCC, the majority of patients are diagnosed in the the inoperable, advanced stages of the disease and/or have recurrence or metastasis after therapy, and their prognosis remains extremely poor (13,14). It is essential to understand the molecular regulation of hepatocarcinogenesis and progression of liver cancer to achieve an improvement in cure rates, and Id proteins are possible candidates for cancer therapy (10). The present study was designed to determine the relationship between Id1 and TGF-ß1 in HCC cell lines, and their effects on hepatoma cell proliferation, cell cycle and differentiation.

Materials and methods

Cell lines, cultures and in vitro assays. Human HCC cell lines, HuH7, PLC/PRF/5, HLE and HLF, were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan). They were from different origins and had distinct characteristics; the first two were established from differentiated hepatomas and expressed hepatic marker proteins, while the latter two originated from undifferentiated HCC and did not express any hepatic marker proteins (15). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2 in air. For experiments with TGF-ß1, the cells were seeded and the next day they were incubated in a medium containing the desired concentrations of human platelet-derived TGF-ß1 (R&D, Minneapolis, MN). At the selected time-points, the cells were harvested and RNA or protein was performed for each experiment as described below. For morphological examination, the cells were studied under light microscopy (Olympus IMT-2; Olympus Corp., Tokyo).

RNA extraction and cDNA generation. Sub-confluent growing cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), and total RNA extraction was performed with a single-step method using Trizol Reagent (Invitrogen, Carlsbad, CA). Purified RNA was quantified and assessed for purity by UV spectrophotometry. Complementary DNA (cDNA) was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) (16).

Quantitative PCR assay with Light Cycler™. Fluorescence PCR was performed using the Light Cycler™ (Roche Diagnostics, Mannheim, Germany) in a 10-μl PCR reaction containing 0.2 μM of each primer, 1x Light Cycler-Fast start DNA Master SYBR-Green I (Roche Diagnostics), 4 mM $MgCl_2$ and 2 μl of cDNA as template. The primer pair sequences were obtained from published sequences of Id1 (17), TGF-ß1 (18), TGFßRI (19), TGFßRII (20) and ß-actin (21), and synthesized from commercial sources. The PCR conditions were as follows: one cycle of denaturing at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 65˚C for 10 sec and 72˚C for 30 sec (or annealing at 58˚C for ß-actin), and a final extension at 72˚C for 10 min. Fluorescence was acquired at the end of each 72˚C extension phase. The melting curves of final PCR products were analyzed after 40 cycles of PCR amplification by cooling the samples to 65˚C, increasing the temperature to 99˚C at a rate of 0.1˚C/sec, and monitoring the fluorescence at each 0.1˚C. Quantification data were analyzed using Light Cycler™ analysis software (Roche Diagnostics) as recommended by the manufacturer. The standard curves for quantification of each mRNA were constructed by using serial dilutions of cDNA from most expressing cell lines. Expression of each mRNA was reported relative to ß-actin.

Growth inhibitory assay. For drawing growth curves with TGF-ß1, the cells were uniformly seeded in 12-well dishes ($4x10^4$ cells/well for HuH7, $3x10^4$ cells/well for PLC/PRF/5, $1.5x10^4$ cells/well for HLE and HLF). The next day, the medium was replaced with fresh medium containing different concentrations of TGF-ß1 (0, 0.1, 1 ng/ml) and this was repeated every 48 h. On days 0, 2, 4 and 6 after starting the treatment, adherent cells were harvested and counted using a Celltac semi-automatic analyzer (Nihon Kohden, Tokyo).

Cell-cycle analysis. Flow cytometric analysis was performed as described previously (22). Briefly, cells were washed twice with PBS and then fixed in 75% cold ethanol overnight and then washed and re-suspended in 1 ml of PBS. After incubation for 30 min at 37˚C in ribonuclease (Wako Pure Chemicals, Osaka, Japan; final concentration of 1 mg/ml), propidium iodide (Sigma Chemical Co., St. Louis, MO; 0.1 mg/ml) was added and incubated at 4˚C for 30 min. Samples were filtered through 44-μm nylon mesh, and data were acquired with a BD FACScan™ cell sorting system (Becton-Dickinson, San Jose, CA). Cell-cycle analysis was carried out using ModFIT software (Becton-Dickinson).

Determination of albumin, AFP and AFP-L3 proteins. Sub-confluent growing cells were washed twice with cold PBS and collected by scraping. Protein lysates were centrifuged at 14000 rpm for 15 min at 4˚C, and the supernatants were collected. Albumin was determined by turbidimetric immunoassay with autometric analyzer (Hitachi-7250, Hitachi, Tokyo) (23). α-fetoprotein (AFP) was measured by chemiluminescence immunosorbent assay (SphereLife180, Wako Pure Chemical Industries, Osaka) (24). Lens culinaris agglutinin-reactive AFP (AFP-L3) was determined by liquid-phase binding assay (LiBASys, Wako) (25).

Western blot analysis. Sub-confluent growing cells were washed twice with ice-cold PBS and collected with a rubber scraper in ice-cold RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% nonidet P-40, 0.2% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride and 500 KIE/ml 'Trasylol®' proteinase-inhibitor (Bayer Leverkusen, Germany)]. The collected lysate was centrifuged at 14000 rpm at 4˚C for 14 min, and the resulting supernatant was collected. The total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) and Western blot analysis was performed as described in our previous study (11). The antibodies were used in dilutions of 1:100 for Id1 (sc-488; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 for actin (A-2066; Sigma) and 1:2000 for secondary donkey anti-rabbit (NA934V; Amersham Biosciences, Buckingham-shire, UK) antibodies. Expression was evaluated by measuring the optical densities of Id1 protein

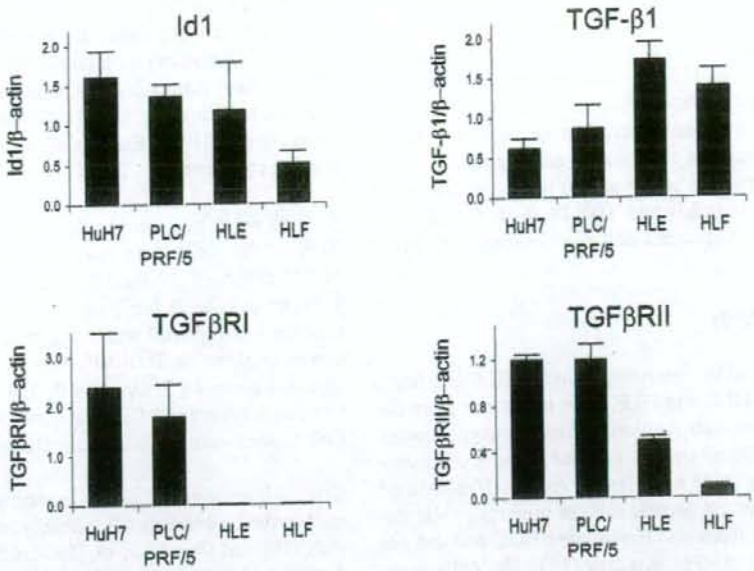

Figure 1. Expression of Id1, TGF-ß1 and TGF-ß1 receptors in HCC cell lines. Quantitative PCR assay using the Light Cycler® revealed that hepatoma cells expressed Id1 and TGF-ß1 at various levels. The expression of TGF-ßRs was higher in HuH7 and PLC/PRF/5 cells; however, HLE and HLF cells lacked TGF-ßRI and expressed TGF-ßRII at a low level. Values are expressed as mean ± SD of four independent experiments (normalized with the copies of ß-actin).

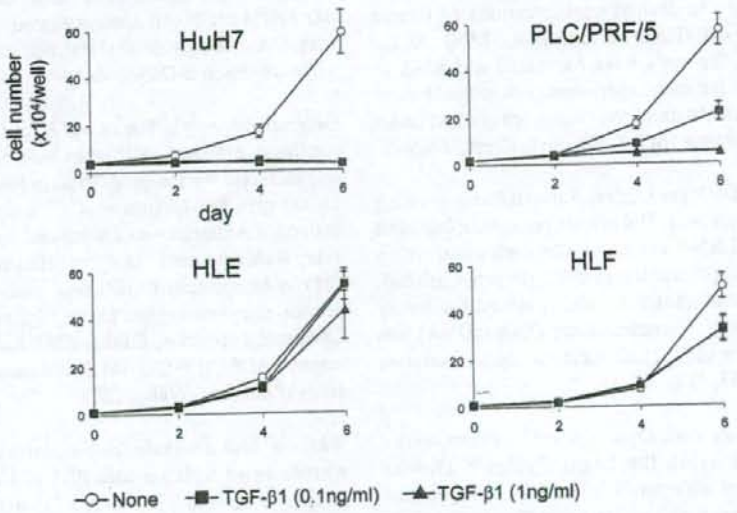

Figure 2. Growth curves of hepatoma cells cultured with or without TGF-ß1 for the initial 6 days. TGF-ß1 induced strong cell growth suppression in HuH7 cells and had a moderate effect on PLC/PRF/5 cells. However, HLE and HLF cells were resistant to TGF-ß1 treatment. Data represent mean ± SD of three independent experiments.

bands, using the ImageJ1.33u software (National Institutes of Health, Bethesda, MD) and the expression value was calculated relative to that of actin.

Statistical analyses. Statistical analyses were performed using the StatView-5.0.1 program (SAS Institute Inc., Cary, NC). The unpaired t-test was used to examine the correlations between two variables. In all analyses, p-values <0.05 were considered statistically significant.

Results

Expression of Id1, TGF-β1 and TGF-β1 receptors in HCC cell lines. We first examined whether the four HCC cell lines expressed Id1, TGF-ß1 and TGFßRI/II. As shown in Fig. 1, quantitative PCR assay using the Light Cycler® revealed that the hepatoma cells expressed various levels of Id1, and the expression was higher in HuH7, PLC/PRF/5 and HLE cells. TGF-ß1 was also expressed at different levels, with lower levels

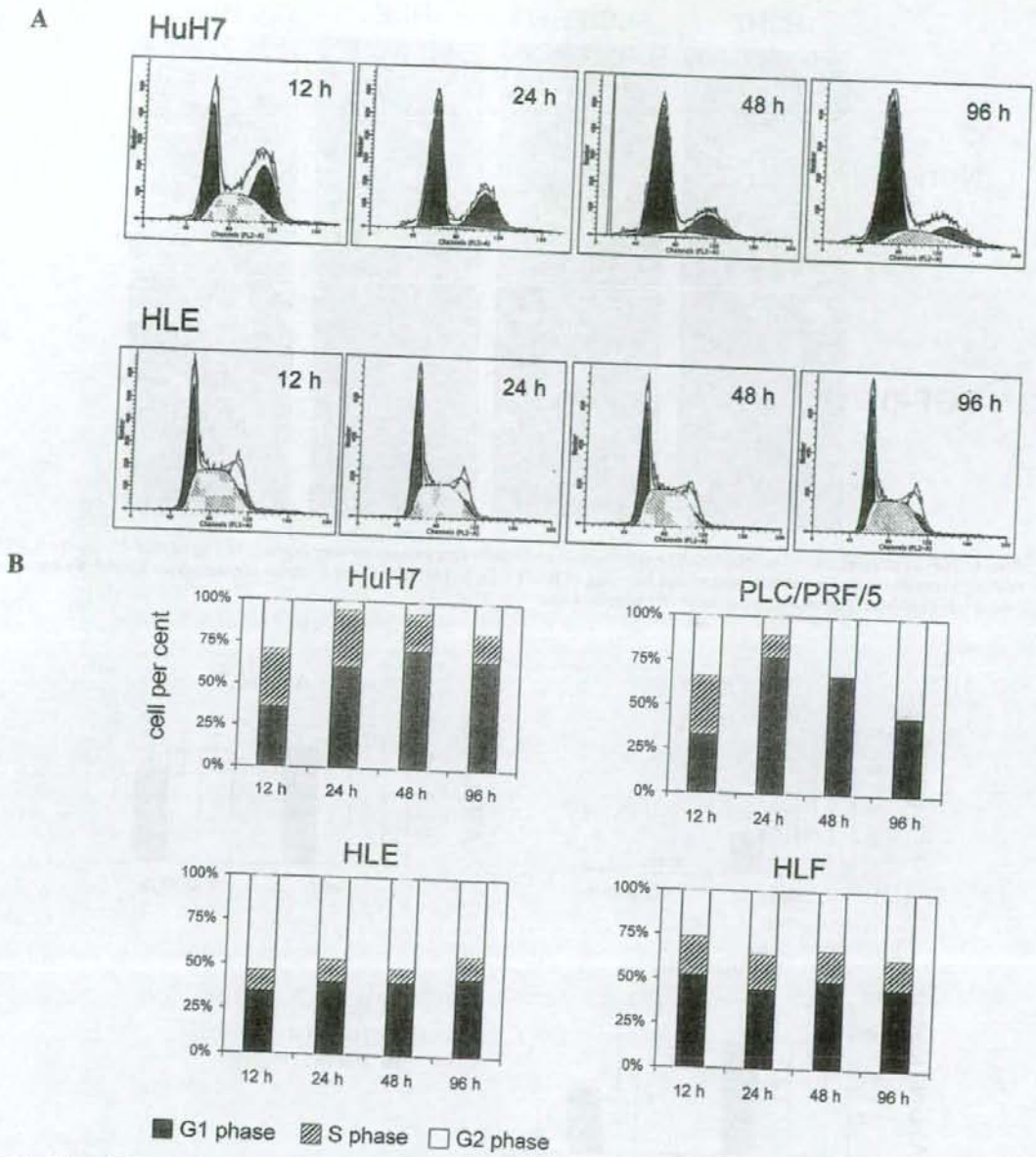

Figure 3. Effect of TGF-ß1 on the cell cycle. Cell-cycle analysis was performed on cells treated with 1 ng/ml TGF-ß1 for up to 96 h; representative cell-cycle graphs of HuH7 and HLE cell lines are shown in A, and data from three independent experiments are summarized in B. The percentage of HuH7 cells in G1 state stably increased from 36.4 to 60.8, 71.2 and 65.6% after exposure to TGF-ß1. However, no changes in cell cycle were observed in HLE and HLF cells during the treatment, and the accumulation of cells in G1 phase returned to the former cell-cycle balance for PLC/PRF/5 cells.

in HuH7 and PLC/PRF/5 cells. HuH7 and PLC/PRF/5 were positive for both TGFßRI and II, while HLE and HLF cells were negative for TGFßRI and only had a low level of TGFßRII.

Effect of exogenous TGF-ß1 on cell proliferation and the cell cycle. For the examination of cell growth, the cell lines were treated with different concentrations of TGF-ß1 and viable cells were counted every 48 h for 6 days. As shown in Fig. 2, TGF-ß1 treatment resulted in a reduced cell growth rate in HuH7 and PLC/PRF/5 cells at the concentrations tested. In HuH7 cells in particular, a low concentration (0.1 ng/ml) of TGF-ß1 stopped cell proliferation. In PLC/PRF/5 cells, the TGF-ß1-induced growth inhibition was less than in HuH7 cells and exhibited dose- and exposure time-dependency. On the other hand, HLE and HLF cells did not respond to TGF-ß1 treatment.

Cell-cycle analysis showed that the number of HuH7 cells in G1 phase was stably increased with exposure to 1 ng/ml TGF-ß1; however, no cell-cycle changes were observed in HLE and HLF cells during the treatment (Fig. 3A and data not shown). The percentage of HuH7 cells in G1 state was

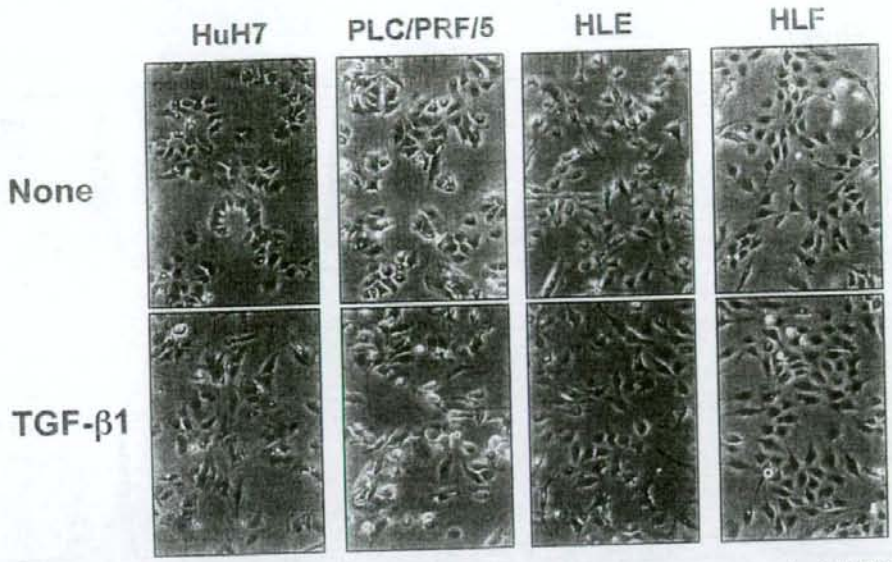

Figure 4. Effect of TGF-ß1 (1 ng/ml) on cell morphology. The cells were observed under light microscopy after exposure to 1 ng/ml TGF-ß1 or without TGF-ß1 for 48 h. TGF-ß1 treatment resulted in the enlargement and flattening of HuH7 cells, and increased their nucleus:cytoplasm ratio. No such visible changes were observed in other cell lines. Photos were taken under x60 magnification.

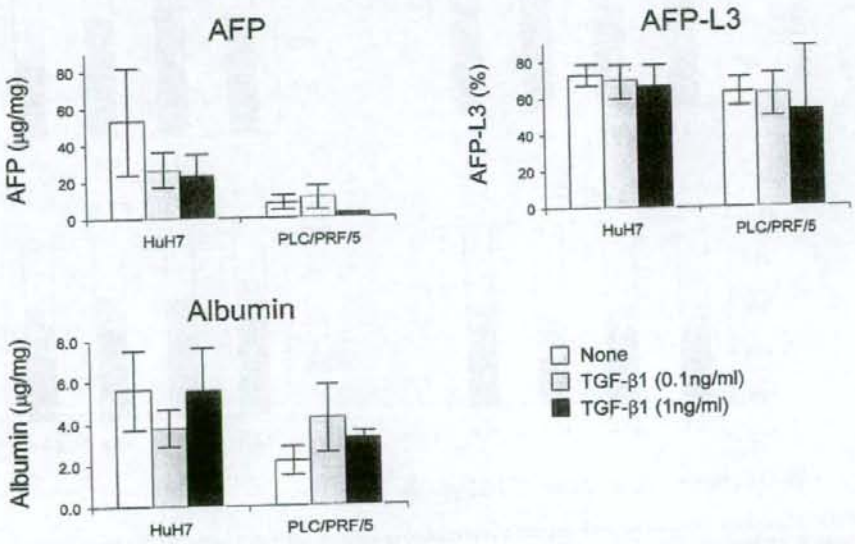

Figure 5. Effects of TGF-ß1 on AFP, AFP-L3 and albumin expression in hepatoma cell lines. The cells were cultured with desired concentrations of TGF-ß1 for 48 h and protein expression was measured in whole cell lysates by protein assays as described in Materials and methods. HLE and HLF cell lines do not express these proteins (data not shown). In the HuH7 cells TGF-ß1 exposure decreased the expression of both AFP and AFP-L3. In PLC/PRF/5 cells, the expression of AFP-L3 decreased. The changes were not statistically significant. Values are expressed as mean ± SD of three independent experiments.

36.4, 60.8, 71.2 and 65.6%, respectively, at each time-point of observation, while for PLC/PRF/5 cells the accumulation of cells in G1 phase returned to the former cell-cycle balance, and the cell cycle of HLE and HLF cell lines was constant during the treatment (Fig. 3B).

Effect of TGF-β1 on cell differentiation. Morphological examination of TGF-ß1-treated cells showed enlargement and flattening of HuH7 cells after exposure to 1 ng/ml TGF-ß1 for 48 h, together with an increase in nucleus:cytoplasm ratio (Fig. 4), which perhaps resembles the morphological characteristics of cells undergoing maturation. These changes correlated with exposure time and concentrations of TGF-ß1 (data not shown). In other cell lines, no visible changes were observed during TGF-ß1 treatment.

To clarify the morphological changes and the effect of TGF-ß1 on differentiation of hepatoma cells, the expression of albumin, and AFP and AFP-L3 proteins was studied because

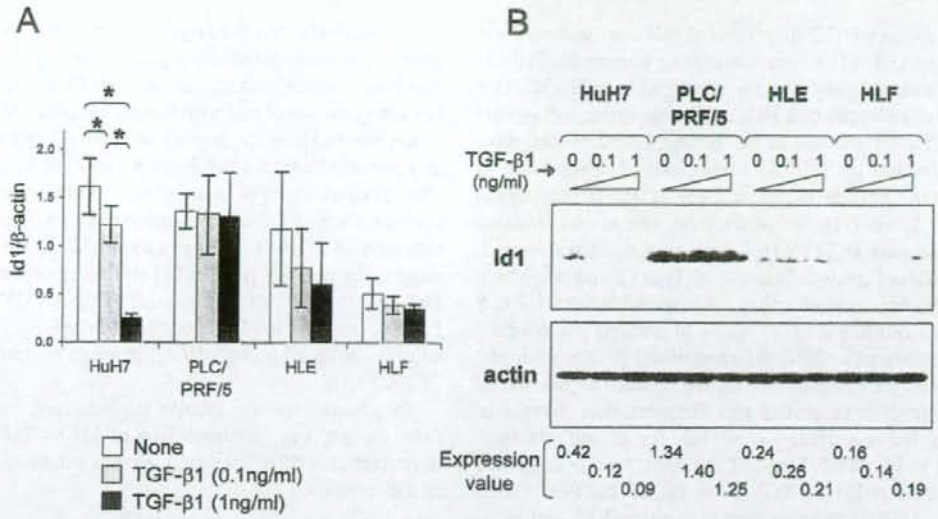

Figure 6. Effect of TGF-ß1 on Id1 expression. Cells were treated with TGF-ß1 (0, 0.1 and 1 ng/ml) and the RNA and protein expression levels of Id1 were examined after 48 h. (A) Quantitative PCR analysis revealed that Id1 mRNA expression in HuH7 cells was suppressed dose-dependently by TGF-ß1 treatment (by 24.6, 84.0% of control). Data represent mean ± SD of three independent experiments. *P<0.05 by the unpaired t-test. (B) Western blot analysis showed that Id1 protein expression was also suppressed in HuH7 cells; however, no significant change of Id1 expression was observed in other cell lines. The expression value was calculated relative to that of actin. Data are representative of similar results of repeated experiments.

AFP and particularly AFP-L3 are known to be specific for transformed hepatocytes with a high grade of malignancy, and albumin secretion is recognized as one of the phenotypes specific for mature hepatocytes (26,27). However, HLE and HLF cell lines did not express those proteins (data not shown). As shown in Fig. 5, at 48 h post exposure to TGF-ß1, a dose-dependent decrease in both AFP and AFP-L3 expression was observed in the treated HuH7 cells. In PLC/PRF/5, a tendency toward increased albumin expression and decreased AFP and AFP-L3 was observed. However, these changes were not statistically different.

Effect of exogenous TGF-ß1 on Id1 expression. To study the effect of TGF-ß1 on the expression of Id1, cells were treated with TGF-ß1 (0, 0.1 and 1 ng/ml) and the RNA and protein expression levels of Id1 were examined after 48 h. In the HuH7 cell line, which was most sensitive to TGF-ß1, Id1 expression was suppressed by TGF-ß1 in a dose-dependent manner at both the transcriptional and protein levels. However, no significant change in Id1 expression was observed in other cell lines (Fig. 6). In HuH7 cells, Id1 mRNA expression was decreased by 24.6 and 84.0%, and protein expression was decreased by 71.2 and 78.6%, respectively, by each dose of TGF-ß1 compared to untreated control. This decrease was dependent on exposure time (data not shown).

Considered together, the above results suggest that TGF-ß1-induced growth inhibition of HuH7 cells may be the result of cell-cycle arrest in G1 phase and partial re-differentiation, which may be associated with down-regulation of Id1. However, in PLC/PRF/5 cells, the growth inhibition did not involve the TGF-ß1-Id1 pathway, and the interruption of TGF-ß1 signaling in HLE and HLF cells resulted in a resistance to TGF-ß1 effects.

Discussion

Although Id proteins have been shown to function as negative regulators of differentiation, and are up-regulated in major cancers (reviewed in refs. 7 and 10), our study revealed that Id proteins were down-regulated in human HCCs, and their expression levels were related to tumor differentiation (11). Our results and data obtained by gene expression profiling analysis are comparable to those of a previous report (28 and our unpublished data). The discrepancy between Ids expression in HCC and other tumors and the regulation of Id proteins in HCC are issues that remain unclarified at present. Therefore, the present study was performed in an effort to investigate the relationship and regulation of Id1 protein with TGF-ß1 in HCC cells, and the effects on cell proliferation, cell cycle and differentiation.

Recent studies have revealed that *Ids* are direct target genes of TGF-ß1 (29,30). TGF-ß1 is overexpressed in HCCs compared with non-tumor liver tissue and is expressed at higher levels in less-differentiated tumors, which is distinct from other malignancies (31,32). This expression pattern of TGF-ß1 was the inverse of our previous findings with Ids, which led us to speculate on the possibility of suppression of Ids by TGF-ß1 in HCC (11).

TGF-ß1 is one of the most well known and potent inhibitors of epithelial cell growth (1). The autocrine and paracrine effects of TGF-ß1 on tumor cells and the tumor micro-environment exert both positive and negative influences on cancer development. Accordingly, the TGF-ß1 signaling pathway has been considered as both a tumor suppressor pathway and a promoter of tumor progression and invasion (2). In the liver, TGF-ß1 is mainly produced by non-parenchymal cells and acts as a paracrine negative regulator of hepatocyte proliferation (33,34).

However, human HCC displayed significant intracellular expression of TGF-ß1 protein, whereas no staining for TGF-ß1 has been detected in hepatocytes of normal liver (31,32). Our present results showed that HCC cell lines expressed various levels of TGF-ß1 as well as Id1 mRNA. Furthermore, exogenous induction by TGF-ß1 suppressed Id1 expression in HuH7 cells in a dose-dependent manner, at both transcriptional and protein levels (Fig. 6). In addition, our results revealed that the decrease in Id1 expression was comparable with TGF-ß1-induced growth inhibition (Figs. 2 and 6), which resulted from the arrest of cell-cycle progression from G1 to S phase in this cell line (Fig. 3). Since Id proteins are involved in the regulation of cellular differentiation, proliferation and the cell cycle, we hypothesize that the reduced expression of Id1 may contribute to growth and differentiation changes in HuH7 cells. However, this was not true for growth inhibition by TGF-ß1 in PLC/PRF/5 cells. The reason for this difference in suppression of Id1 by TGF-ß1 in HuH7 and PLC/PRF/5 cells, when TGF-ß1 induces growth arrest in both cell lines, remains unclear. It may relate to differences in various molecules specific to the TGF-ß1-Id1 pathway; for instance, the expression of ATF3 was 1.74-fold less in PLC/PRF/5 cells than in HuH7 cells (unpublished data). Our results also revealed that HLE and HLF cell lines did not exhibit the TGF-ß1 effect, and Id1 expression was not altered in these lines. These cells lacked TGF-ßRs (Fig. 1), which might explain the interruption of the TGF-ß1-Smad signaling pathway in these cell lines.

The molecular pathway for the role of Ids in cell proliferation might involve cyclin-dependent kinase inhibitors, such as $p16^{INK4a}$, $p21^{WAF1}$, $p27^{KIP1}$ and retinoblastoma proteins that are suppressed by Id1 (7,9). Similarly, this was shown in liver cancer cells where the overexpression of Id1 induced cell proliferation through inactivation of the $p16^{INK4a}$/retinoblastoma pathway (35). Id expression was also enhanced during cellular proliferation and in response to mitogenic stimuli (36,37). Our unpublished data show an increase of Id with serum addition in medium, and it is well known that FBS is a mitogenic stimulator in cell cultures. Similar results with induction of Ids by serum were previously described in studies on breast and pancreatic cancer cell lines (38,39). *In vivo*, in experiments on rats, Id1 was activated from 6 h after partial hepatectomy, which is used as a model for stimulation of hepatocyte proliferation (40).

Id proteins function as inhibitors of cell differentiation. Our results showed a possible link between the inhibition of Id1 by TGF-ß1 and activation of cell differentiation in HuH7 cells; i.e. the morphological changes toward maturation and decrease of AFP and AFP-L3 expression (Figs. 4-6). However, the changes in these markers were not statistically significant, which might reflect the short observation time. To confirm these findings, we also studied the effect of acyclic retinoid (all *trans*-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid; Nikken Chemicals Co., Tokyo) on Id expression. Acyclic retinoid demonstrates the potential to induce the differentiation of human hepatoma cell lines (41). The treatment of HCC cells with acyclic retinoid inhibited the expression of Id1, which is associated with up-regulation of mature hepatocyte-specific genes, such as albumin, and down-regulation of AFP gene expression (unpublished data).

Collectively, our findings suggest that TGF-ß1-induced growth inhibition (involving cell-cycle arrest in G1-S transition and partial re-differentiation) of TGF-ßR-positive HCC cell lines may be associated with down-regulation of Id1, at least in some cells. However, in other cell lines, TGF-ß1 effects were less powerful and did not involve the TGF-ß1-Id1 pathway. Our previous findings in an *in vivo* study, showing that Id1 correlates with HCC differentiation (11), might be an indirect outcome of TGF-ß1 in liver cancer. Moreover, the results suggest the possible roles of Id1 protein in the carcinogenesis and development of HCCs under direct TGF-ß1 control. Finally, our data imply a possible strategy for the treatment or prevention of human HCC, through targeting Ids with TGF-ß1 (10).

In conclusion, our results suggest that, in some HCC cells, the pathway of suppression of Id1 by TGF-ß1 may be important in TGF-ß1-induced growth inhibition and partial re-differentiation.

Acknowledgements

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Angiogenesis in cholangiocellular carcinoma: Expression of vascular endothelial growth factor, angiopoietin-1/2, thrombospondin-1 and clinicopathological significance

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Abstract. Angiogenesis in cholangiocellular carcinoma (CCC) has rarely been investigated. The aim of this study was to determine the angiogenesis status of CCC and assess its relationship with angiogenic factors and clinicopathological characteristics. We examined 33 surgically resected CCC specimens. Tumor angiogenesis was assessed by microvessel density (MVD) using the anti-CD34 antibody, and the expression of VEGF, Ang-1, Ang-2, and TSP-1 was determined by immunohistochemistry. The mean (± SD) MVD was 87.2±52.6/mm² (range, 0-229/mm²). A total of 75.6% cases were positive for VEGF expression, 36% for Ang-1, 57.6% for Ang-2 and 45.5% for TSP-1. VEGF and Ang-2 expression was associated with a significantly higher level of MVD (p=0.004 and 0.015, respectively). TSP-1 expression was associated with a significantly lower level of MVD (p=0.005) and a higher level of intrahepatic metastasis (46.7% vs. 5.6%, p=0.012). There was no significant correlation between VEGF, Ang-1, Ang-2, and TSP-1 expression and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or CCC differentiation. There was no significant correlation between MVD levels, VEGF, Ang-1, Ang-2, and TSP-1 expression and postoperative survival. A considerable degree of angiogenesis, comparable to that of other solid tumors, was observed in CCC. VEGF and Ang-2 might play a proangiogenic role, and TSP-1 may play an inhibitory role in CCC. Although TSP-1 may increase intrahepatic CCC metastases, neither MVD levels nor the expression of VEGF, Ang-1, or Ang-2 was associated with clinicopathological factors and prognosis.

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Abbreviations: Ang, angiopoietin; CCC, cholangiocellular carcinoma; HCC, hepatocellular carcinoma; LN, lymph node; MVD, microvessel density; MCC, metastatic colorectal cancer; PBS, phosphate-buffered saline; SD, standard deviation; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor

Key words: cholangiocellular carcinoma, vascular endothelial growth factor, angiopoietin, thrombospondin, angiogenesis, prognosis

Introduction

Cholangiocellular carcinoma (CCC) is the second most frequent primary liver cancer in adults, and patients with CCC generally have a poorer prognosis than those with hepatocellular carcinoma (HCC) (1). Macroscopically, CCC can be divided into three major types, namely the mass forming, periductal infiltrating and intraductal growth types (2,3). In comparison with the hypervascular HCC, CCC is regarded as hypovascular; in contrast to HCC, there is a much smaller volume of published research pertaining to angiogenesis in CCC. Inconsistent vascularity of this tumor has also been reported, i.e. the reported microvessel density (MVD) of this tumor ranges from as low as 10/mm² to as high as 169/mm² (4,5).

We previously demonstrated that the marginal tumor field harbors more extensive angiogenesis in comparison to the intermediate and central fields during angiogenesis in hepatic metastases of colorectal cancer (6). Like metastatic colorectal cancer (MCC), CCC is regarded as a hypovascular cancer (4,5). However, the vascularity patterns of the different macroscopic types of CCC have yet to be investigated.

Several growth factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and thrombospondin-1 (TSP-1), are associated with tumor angiogenesis. VEGF has demonstrated specificity and is essential for the formation of blood vessels in tumors (7). Ang-1 and Ang-2 are ligands for endothelium-specific tyrosine kinase receptor Tie-2 (8,9). Angiopoietins have also been demonstrated to participate in the angiogenesis of

tumors through their impact on endothelial migration, proliferation and microvascular permeability (10,11). Ang-1 promotes vessel stability by increasing the connections of endothelial cells to the surrounding supporting pericytes (8,11). In contrast, Ang-2 disrupts the stabilizing effect of Ang-1 and induces the loosening of interactions between endothelial and perivascular support cells and the matrix, thus compromising vascular integrity and facilitating access to proangiogenic inducers such as VEGF (9,12). To date, the expression of Ang-1 and Ang-2 in CCC and their relationship with clinicopathological factors remains unclear. Unlike VEGF and angiopoietins, TSP-1 is a multifunctional matrix protein (13). The role of TSP-1 in angiogenesis and tumor progression remains controversial. Both positive and negative effects of TSP-1 on tumor angiogenesis have been reported (5,14,15). In addition, research on the relationship of TSP-1 with tumor clinicopathological factors is limited.

In the present study, we determined the angiogenesis status of CCC in our group of patients, investigated the relationship with angiogenic factors and clinicopathological factors, and assessed the MVD in 33 surgically resected CCC samples. We also evaluated the relationship between angiogenesis and other clinicopathological factors with the expression of VEGF, Ang-1, Ang-2 and TSP-1. In addition, the prognostic significance of MVD, VEGF, Ang-1, Ang-2 and TSP-1 in 23 curative resected CCC cases was investigated.

Materials and methods

Patients and specimens. A total of 33 patients (18 males and 15 females) with surgically resected CCC, who were admitted to the Osaka University Hospital between 1990 and 2003, participated in this study. The median age of the patients was 63 years (range, 33-80 years). There were 7 patients who were positive for hepatitis B surface antigen or hepatitis C virus antibody. Macroscopically, 20 cases were of the mass forming type, 4 cases were the intraductal growth type and 9 cases were the periductal infiltrating type. Some 23 patients underwent curative resection, and 10 cases underwent palliative resection. Resected CCC specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin for assessment of the pathological features of CCC in accordance with the classification proposed by the Liver Cancer Study Group of Japan (3).

Assessment of microvessel density (MVD). MVD was assessed using the method recommended by Weidner *et al* (16). After immunostaining with anti-CD34 antibody (mouse monoclonal IgG2a, diluted 1:50), large and small microvessels, and single brown immunostained endothelial cells separate from adjacent microvessels and stromal structures were included in the microvessel count. An Olympus microscope BX50 with image capture device DP-70 was used for this study. The software DP Controller 2.11 was used for image scaling and capturing. The MVD count was calculated as the number of microvessels found within 1 mm^2 of the microscopic field under a magnification of x100. The MVD of the peripheral, intermediate and central areas of the tumor, as well as the surrounding normal liver, were recorded. For each area, the average MVD of the five highest MVD counts was recorded as the MVD of that area. The MVD of the tumor was thus calculated as the average MVD of five highest MVD counts within the tumor.

Immunohistochemical staining. Formalin-fixed, paraffin-embedded specimens of tumors and associated periphery were selected for analysis. Sections measuring 4 μm in thickness were deparaffinized in xylene, rehydrated, and stained with hematoxylin-eosin solution for histopathological examination. After deparaffinization in xylene and rehydration in a graded series of ethanol, immunohistochemical procedures were performed using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) as described previously (17). Briefly, sections were treated with an antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) for 40 min at 95˚C and incubated in methanol containing 0.3% hydrogen peroxide at room temperature for 20 min) to block endogenous peroxidases. The sections were then incubated with normal protein block serum solution at room temperature for 20 min to block non-specific staining, followed by overnight incubation at 4˚C with anti-VEGF (rabbit polyclonal IgG, diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-angiopoietin-1 (goat polyclonal IgG, diluted 1:50; Santa Cruz Biotechnology), anti-angiopoietin-2 (goat polyclonal IgG, diluted 1:50; Santa Cruz Biotechnology), and anti-thrombospodin-1 (mouse polyclonal IgG2a, diluted 1:50; Lab Vision Corp., Fremont, CA). Sections were washed 3 times for 5 min in phosphate-buffered saline (PBS) before incubation at room temperature for 20 min with a biotin-conjugated secondary antibody (goat anti-rabbit for VEGF and rabbit anti-goat for ANG1 and ANG2), and finally with peroxidase-conjugated streptavidin at room temperature for 20 min. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrachloride (Wako Pure Chemical Industries, Osaka, Japan). The sections were counterstained with Meyer's hematoxylin. Other sections treated with Tris-buffered saline instead of the primary antibody served as the negative control.

Tumor fields with the highest degree of MVD were identified for each tissue section. The intensity of immunohistochemical staining for VEGF, Ang-1, Ang-2, and TSP-1 in these fields was scored on a scale ranging from level 0 to 2. Level 0, 1, and 2 represented negative or faint, moderate and strong staining, respectively. The vascular epithelium in the normal liver field of the same specimen expressed moderate levels of Ang-1, Ang-2 and TSP-1 and the bile duct epithelium in the normal liver field of the same specimen expressed moderate levels of VEGF. Accordingly, these levels of staining were used as an internal control within the sample, which was arbitrarily designated as intensity level 1.

Statistical analysis. Unless otherwise indicated, numerical data are presented as mean ± SD. Differences in the proportions of categorical data were tested using the Chi-square test. Unless otherwise indicated, differences in mean values of numerical data were tested using the two-tailed Student's t-test. Survival (mean, medium survival days and 1-, 3- and 5-year cumulative survival rates) was assessed using the Kaplan-Meier method, and comparisons were made using the

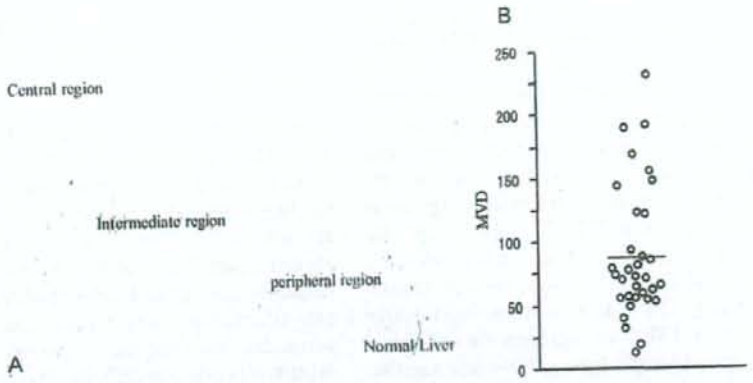

Figure 1. (A) CD34-stained photomicrography of mass forming CCC type. The tumor periphery displayed a higher degree of angiogenesis in comparison to the intermediate and central tumor regions. Original magnification, x20. (B) Scatter plot of MVD in CCC, with a range of 10-229/mm², mean of 87.2/mm² and median of 70.7/mm².

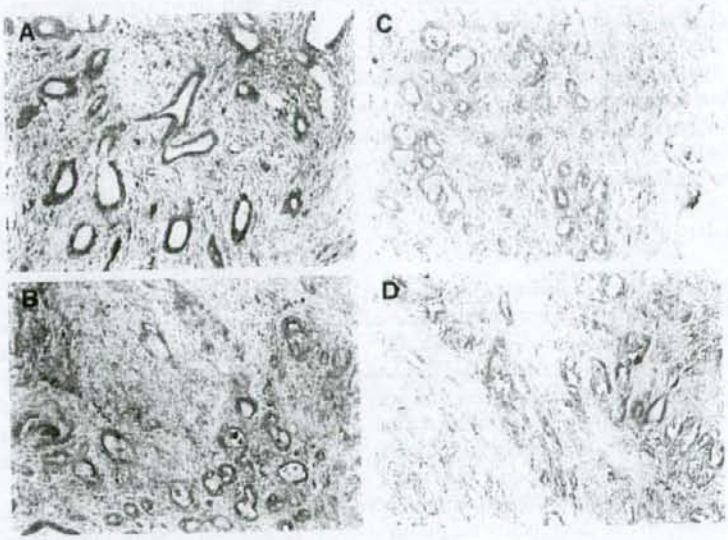

Figure 2. (A) Anti-VEGF-stained photomicrograph of a tissue sample from a CCC patient. Strong positive staining can be observed in the tumor cell cytoplasm. (B) Anti-Ang-1-stained photomicrograph of a tissue sample from the same CCC patient. Strong positive staining can be observed in the tumor cell cytoplasm. (C) Anti-Ang-2-stained photomicrograph of a tissue sample from the same CCC patient. Moderate positive staining can be observed in the tumor cell cytoplasm. (D) Anti-TSP-1-stained photomicrograph of a tissue sample from the same CCC patient. Strong positive staining can be observed in the tumor cell and stromal cell cytoplasm. Original magnification, x100.

log-rank test. A p-value <0.05 denoted the presence of a statistically significant difference. All analyses were performed using SPSS statistical software (version 11.5, Chicago, IL).

Results

Vascular distribution pattern in tumor and surrounding non-tumorous liver. The tumor region and surrounding normal liver tissue were included in the assessment of all CCC cases evaluated in this study. Within CCC, high vascularity with short, tortuous blood vessels was observed. Of the 20 cases of mass forming CCC type, 16 displayed a more condensed CD34 staining in the peripheral region in comparison with the central and intermediate regions (Fig. 1A). There was a significant decrease in MVD from the periphery (85.0±49.1/mm²) to intermediate zone (44.2±32.4/mm²), and also from the intermediate to the central zone (6.3±9.4/mm²) (paired t-test, $p<0.05$ for each comparison). The other 4 cases displayed an almost evenly distributed CD34 staining within the entire tumor region. Uneven and irregular vascular distribution patterns within the tumor were found in 13 cases of intraductal growth type and periductal infiltrating type CCC. It seemed that massive fibrous tissue together with unevenly distributed cancerous tubular structure contributed to the irregular vascularity within the tumor. In the surrounding normal liver tissue, positive CD34 staining was also observed in the Glisson's triangle area and the central vein. These blood vessels were not as condensed as those within the tumor, were smaller in size, and generally associated with a regular round shape. Inflammatory white blood cell

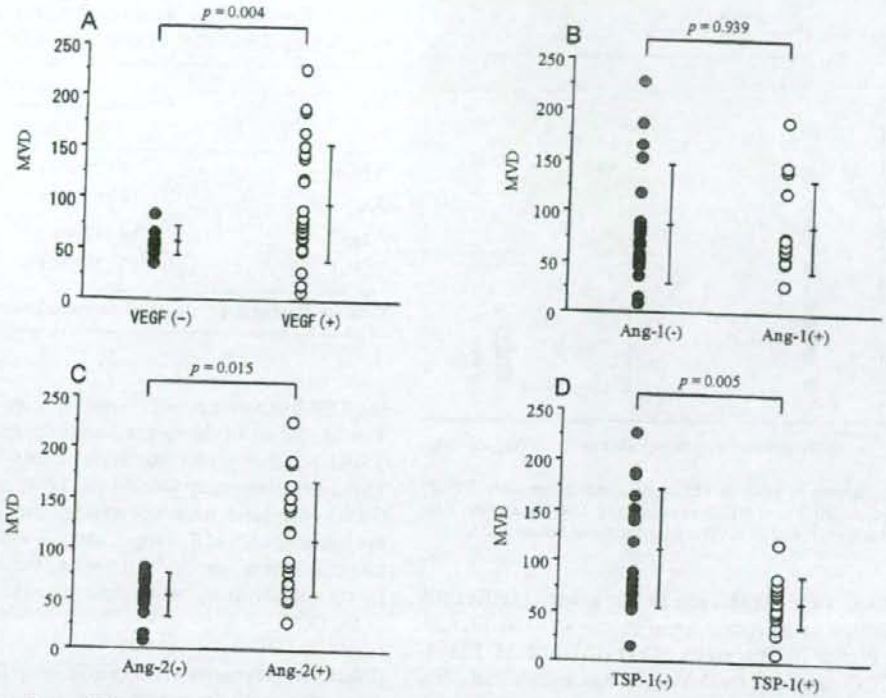

Figure 3. Comparison of mean MVD (± SD) between: (A) VEGF-positive (n=25) and VEGF-negative (n=8) cases; (B) Ang-1 positive (n=14) and Ang-1 negative (n=19) cases; (C) Ang-2-positive (n=17) and Ang-2-negative (n=16) cases; and (D) TSP-1-positive (n=15) and TSP-1-negative (n=18) cases. Data are displayed in scatter plots of MVD, and the bar showed mean ± SD.

infiltration was also noted in the Glisson's triangle, particularly in the intraductal growth and periductal infiltrating CCC types.

MVD of tumor and surrounding normal liver tissue. The MVD of tumor tissue (87.2±52.6/mm²; range, 10-229/mm²) (Fig. 1B) was significantly higher than that of the surrounding normal liver tissue (40.6±22.0/mm²; range, 14-109/mm²). For the 20 cases of mass forming CCC type, the MVD of the marginal area was significantly larger than that of the intermediate and central areas. The MVD of the intermediate area was significantly larger than that of the central area. The difference in MVD between the mass forming CCC type and intrahepatic growth/periductal infiltrating CCC type was not statistically significant (93.0±50.9 vs. 78.3±56.1/mm²; p=0.442). However, the MVD of surrounding normal liver was significantly lower than that of the mass forming CCC type (34.2±17.6 vs. 50.4±25.0/mm²; p=0.036).

Correlation between MVD and clinicopathological factors. We divided the patients into two subgroups based on the median value of MVD (75/mm²), the high MVD and low MVD groups, and examined the correlation between the MVD level and several clinicopathological features. There was no correlation between MVD and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or CCC differentiation.

Immunohistochemical staining of VEGF, Ang-1, Ang-2, and TSP-1. VEGF immunoreactivity was found in both the cytoplasm of cancer cells and the hepatocytes of adjacent liver tissue (Fig. 2A). However, the expression was considerably less intense in hepatocytes than in the CCC tissue. VEGF expression was not detected or faintly stained in 9 (27.2%) cases. However, 13 (39.4%) and 11 (33.3%) cases showed moderate and strong VEGF staining, respectively. Ang-1 and Ang-2 proteins were found in both the cytoplasm of cancer cells and the hepatocytes of adjacent liver tissue (Fig. 2B and C). Ang-1 expression was not detected or faintly stained in 19 (57.6%) cases. However, 9 (27.3%) and 5 (15.2%) cases showed moderate and strong Ang-1 staining, respectively. Ang-2 expression was not detected or faintly stained in 14 (42.4%) CCC cases. However, 14 (42.4%) and 5 (15.2%) CCC cases showed moderate and strong Ang-2 staining, respectively. TSP-1 immunoreactivity was found in the cytoplasm of cancer cells and stromal cells, but not in the hepatocytes or stromal cells from adjacent normal liver tissue (Fig. 2D). TSP-1 expression was not detected or faintly stained in 17 (51.1%) CCC cases. However, 15 (48.5%) and 1 (3.0%) CCC cases showed moderate and strong TSP-1 staining, respectively.

Correlation between MVD and VEGF, Ang-1, Ang-2 and TSP-1 expression. The mean MVD (± SD) of 25 VEGF-positive CCC cases (96.5±57.0/mm²) was significantly higher than that of 8 VEGF-negative cases (58.0±14.4/mm²; p=0.004) (Fig. 3A). However, the mean MVD (± SD) of 14 Ang-1-positive CCC cases (86.4±45.4/mm²) was not different from that of 19 Ang-1-negative cases (87.8.5±58.6/mm²; p=0.939) (Fig. 3B). The mean MVD (± SD) of 17 Ang-2-

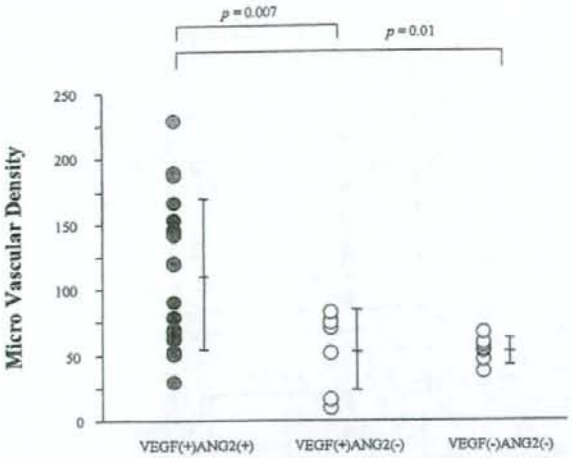

Figure 4. Comparison of MVD in VEGF-positive/Ang-2-positive, VEGF-positive/Ang-2-negative, and VEGF-negative/Ang-2-negative cases. Data are displayed in scatter plots of MVD and the bar showed mean ± SD.

positive CCC cases (108.2±56.0/mm²) was significantly higher than that of 16 Ang-2-negative cases (64.9±39/mm², p=0.015, Fig. 3C). The mean MVD ((SD) of 15 TSP-1-positive CCC cases (61.0±25.8/mm²) was significantly less than that of 18 TSP-1-negative cases (109.0±59.6/mm²; p=0.005) (Fig. 3D). The patients were further grouped into group 1 (18 VEGF- and Ang-2-positive cases), group 2 (8 VEGF-positive and Ang-2-negative cases) and group 3 (7 VEGF- and Ang-2-negative cases). None of the cases was VEGF-negative and Ang-2-positive. The MVD of group 1 was significantly higher than that of groups 2 and 3 (p=0.013 and 0.001, respectively) (Fig. 4).

Relationship between VEGF, Ang-1, Ang-2, and TSP-1 expression and clinicopathological characteristics. Patients were divided into two groups, positive (moderate or strong staining) and negative (faint or no staining) for VEGF, Ang-1, Ang-2, and TSP-1 expression (Table I) in order to examine the correlation between the intensity of VEGF, Ang-1, Ang-2, and TSP-1 expression and several clinicopathological features. The likelihood of developing intrahepatic metastases in the TSP-1-positive group was significantly higher than in the TSP-1 negative group (46.7% vs. 5.6%; p=0.012) (Table II). On the other hand, there was no significant correlation between the intensity of VEGF, Ang-1, Ang-2, and TSP-1 expression and tumor size, capsule formation, infiltration of capsule, portal vein invasion, intrahepatic metastasis or differentiation of the CCC.

Table I. Results of immunohistochemical staining for VEGF, Ang-1, Ang-2 and TSP-1 expression in CCC tumors.

	Expression	
	Negative	Positive
VEGF	8 (24.2%)	25 (75.8%)
Ang-1	19 (57.6%)	14 (42.4%)
Ang-2	14 (42.4%)	19 (57.6%)
TSP-1	17 (51.5%)	16 (48.5%)

Data are number (percentage) of examined tumors.

Relationship between MVD, VEGF, Ang-1, Ang-2 and TSP-1 expression and postoperative overall survival. For statistical analysis of cumulative survival, cases were divided into two groups, positive (moderate or strong staining) and negative (faint or no staining) for VEGF, Ang-1, Ang-2, and TSP-1. We subdivided patients into two further groups of high MVD and low MVD based on the median value of MVD (75/mm²). There was no significant correlation between MVD, VEGF, Ang-1, Ang-2, and TSP-1 expression and postoperative survival (Table III).

Discussion

The vascularity of CCC remains relatively poorly investigated or conflicting results have been reported. In one study, the mean MVD was as low as 10/mm² in CCC, as determined by

Table II. Relationship between TSP-1 expression and various clinicopathological parameters.

Factors	TSP-1 Positive (n=16)	TSP-1 Negative (n=17)	p-value
Age (years)[a]	66.3±9.1	59.8±11.7	0.088
Sex ratio (M/F)	9/7	9/8	1.000
Viral hepatitis B/C (-/+)	14/2	12/5	0.398
Portal vein invasion of cancer (-/+)	13/3	12/6	0.443
Lymphatic permeation of cancer (-/+)	9/7	9/8	1.000
Intrahepatic metastasis (-/+)	8/8	17/0	0.001
Tumor size (cm)[a]	4.7±2.8	5.5±3.9	0.480
Differentiation (H/L+M)	8/8	7/10	0.732

[a]Data are mean ± SD.

Table III. Results of univariate analysis of prognostic factors in 23 radically resected CCC.

Factors	Cumulative survival rates			p-value
	1-year	3-year	5-year	
Age				
≥64 (n=13)	67.7%	58.0%	46.4%	
<64 (n=10)	66.7%	35.6%	0%	0.988
Sex				
Male (n=10)	45.0%	45.0%	45.0%	
Female (n=13)	83.9%	65.3%	46.6%	0.157
Differentiation				
Well (n=12)	91.7%	41.2%	41.2%	
Moderate and poor (n=11)	45.5%	45.5%	45.5%	0.467
Lymphatic permeation of cancer				
(-) (n=15)	70.0%	56.0%	56.0%	
(+) (n=8)	62.5%	25.0%	25.0%	0.245
Intrahepatic metastasis				
(-) (n=22)	65.7%	54.7%	41.0%	
(+) (n=1)	100.0%	0%	0%	0.523
Portal vein invasion of cancer				
(-) (n=17)	67.9%	59.4%	59.4%	
(+) (n=6)	66.7%	16.7%	0%	0.241
MVD				
>70/mm² (n=13)	58.7%	44.1%	44.1%	
≤70/mm² (n=10)	78.8%	42.2%	0%	0.862
VEGF expression				
(-) (n=5)	40.0%	0%	0%	
(+) (n=18)	63.0%	42.0%	42.0%	0.847
Ang-1 expression				
(-) (n=13)	76.9%	46.1%	46.1%	
(+) (n=10)	51.4%	34.3%	34.3%	0.506
Ang-2 expression				
(-) (n=10)	70.0%	37.5%	37.5%	
(+) (n=13)	64.6%	48.5%	48.5%	0.716
TSP-1 expression				
(-) (n=15)	64.6%	29.1%	29.1%	
(+) (n=8)	72.9%	72.9%	72.9%	0.249

FVIII-RAb staining (5). In another study, the MVD was 61/mm² in CCC, as determined by CD31 staining, with mean and median values of 21±7/mm² and 17/mm², respectively (18). In contrast, assessment of 19 surgically resected CCC samples in another study showed a mean MVD of 126.5±45.1/mm² (19). In another study of 102 surgically resected cholangiocarcinoma cases, the mean MVD was 179.4±81.8/mm² for 49 cyclooxygenase-2 negative cholangiocarcinoma cases and 169.6±76.8/mm² for 53 cyclooxygenase-2 positive cholangiocarcinoma cases (4). These discrepancies in MVD may be partially due to the different biological characteristics of CCC in different regions, methodological differences, such as the anti-endothelial antibodies used (FVIII-RAb, anti-CD31, or anti-CD34, etc.) and the method of MVD quantification (highest microvascular density, average microvascular density and microvascular volume) or inter-observer variations. In one study, the mean MVD was calculated as the average number of microvessels observed in five random areas (5). This method counts the microvessel density in the central necrotic area of the tumor

and may explain the low MVD in CCC reported in that study. In addition, the anti-endothelial antibody used for the staining of vascularity may also have an effect. FVIII-RAb is considered to be less accurate due to the absence of its target in some small immature capillaries and single endothelial cells (20). In addition, significant stromal background staining that assists the interpretation of staining data is often associated with the use of this antibody (21). Although anti-CD31 has better sensitivity and superior specificity to FVIII-RAb (20-22), it can also stain lymphatic endothelial and inflammatory cells, and antigens can sometimes be lost due to the use of fixatives that contain acetic acid (21,23). The anti-CD34 antibody stains small and large vessels with equal intensity in normal and tumor tissue; furthermore, it also stains immature capillaries and single endothelial cells with high specificity (24) and a staining performance superior to that of anti-CD31 (25). Therefore, in this study, anti-CD34 antibody was selected for the determination of MVD in formalin-fixed paraffin-embedded CCC specimens.

In the present study, the evaluation of MVD was based on the method proposed by Weidner *et al* (16). We initially identified regions of the highest vascular density under low magnification and marked them as 'hot spots.' For the mass forming CCC type, this was usually located in the peripheral zone of the tumor. The mean MVD of the five highest MVD counts in these 'hot spots' was recorded and regarded as the MVD of the tumor. Our results showed that the MVD of CCC ranged from 10 to 229/mm^2, with a mean value of 87.2±52.6/mm^2. We previously reported that the mean MVD in the peripheral region of metastatic colon carcinoma of the liver was 75.5±32.6/mm^2 (6). In another study (unpublished data), we found the mean MVD to be 93.1±43.8/mm^2, as determined by anti-CD34 staining of 60 HCC cases. The MVD in breast cancer and non-small cell lung cancer, as determined by anti-CD34 staining, was 51.4±23.8/mm^2 and 41.34±11.67/mm^2, respectively (26,27). Our results showed a considerable degree of tumor-associated angiogenesis comparable to that recorded in other well-vascularized tumors.

We found that the 16 cases of mass-forming CCC displayed a more condensed CD34 staining pattern in the peripheral regions. Indeed, there was a statistically significant progressive reduction in staining from the peripheral region to the intermediate zone and then to the central zone, which was often accompanied by complete or incomplete necrosis. The vascularity of the other 4 cases of mass-forming CCC was evenly distributed within the tumor. The robust angiogenesis at the tumor periphery may explain the usually observed 'ring enhancement' phenomenon of the mass forming CCC type in the artery phase of enhanced CT. In our previous study, the same phenomenon was observed in liver metastatic colorectal cancer (6). However, in another study of angiogenesis in HCC, we seldom found this phenomenon (unpublished data). These differences demonstrate the different patterns of angiogenesis in HCC, CCC and MCC. Nevertheless, in the other 13 intraductal growth type and periductal infiltrating type CCC cases, no regular vascular distribution pattern was observed. In some of these cases, the central tumor region showed a higher degree of vascularity in comparison to the peripheral tumor tissue and was often mixed with massive fibrous tissue.

Tumor-associated angiogenesis is a result of the combined action of stimulatory and inhibitory factors (28). VEGF is a specific and critical growth factor for blood vessel formation in various tumors (7). Several studies reported the presence of strong VEGF expression in CCC cell lines and surgically resected CCC tissues (19,29). Our results showed that 75.6% (25/33) of CCC samples displayed strong or moderate VEGF staining. This result is compatible with previously reported high VEGF expression rates in CCC (19,29). Our results also showed that the MVD was significantly higher in the VEGF-positive cases than that in VEGF-negative cases, suggesting that VEGF plays an important role in CCC angiogenesis.

Ang-2 is another family of endothelial cell-specific growth factors (8,9). It can loosen the interactions between endothelial and perivascular support cells and the matrix, compromise vascular integrity and facilitate access to proangiogenic inducers such as VEGF (9,12). To date, the role of Ang-2 in CCC angiogenesis and its relationship to other biological features have not been investigated. Our results showed that Ang-2 expression was observed in 57.6% of cases. The MVD in Ang-2-positive cases was significantly higher than that of Ang-2-negative cases. These results imply that angiopoietin-2 may play an important role in CCC angiogenesis. Our results also showed that in both VEGF- and Ang-2-positive cases, the MVD was significantly higher than that of VEGF-positive and Ang-2-negative cases, or both VEGF- and Ang-2-negative cases. In this group of patients, we did not observe VEGF-negative and Ang-2-positive cases. This result implies that VEGF and Ang-2 may act together to promote CCC angiogenesis. *In vitro* experiments showed that in cultured endothelial cells, Ang-2 mRNA was up-regulated by VEGF (30,31), and Ang-2 expression was associated with VEGF expression in the process of tumor angiogenesis (31). Furthermore, Holash *et al* found that VEGF up-regulation coincided with Ang-2 expression at the tumor periphery and was associated with robust angiogenesis (32). However, further studies that include a combination of immunohistochemistry, laser capture microdissection and real-time quantitative reverse transcription-PCR are necessary to understand the relationship between VEGF and Ang-2 expression in CCC.

In addition to VEGF and angiopoietins, TSP-1 may be another important factor with influence on CCC angiogenesis. Our results showed that TSP-1 immunoreactivity was found in the cytoplasm of cancer cells and stromal cells in 45.5% (15/33) of cases. The MVD of TSP-1-positive cases was significantly lower than that of the TSP-1-negative group. This result suggests that TSP-1 may be inhibitory to CCC angiogenesis and is in agreement with other similar studies (5,15). However, in HCC, a positive correlation between TSP-1 and VEGF expression was found, suggesting that TSP-1 may play a proangiogenic role in HCC (14). In another study (unpublished data), we did not find any correlation between TSP-1 expression and MVD in HCC. Our results showed that patients of the TSP-1-positive group are significantly more likely to develop intrahepatic metastasis than the TSP-1-negative group. This implies that TSP-1 expression may be associated with increased invasiveness of CCC. In this regard, TSP-1 expression is associated with an increased rate of lymphatic permeation in

intrahepatic cholangiocarcinoma (15) and an increased rate of venous invasion in HCC (14). The role of TSP-1 in angiogenesis and invasiveness of tumors deserves further investigation.

The relationship between angiogenesis and the prognosis of patients with CCC is seldom reported. In the present study, we found that neither MVD level nor the expression of VEGF, Ang-1, Ang-2, and TSP-1 was associated with prognosis. Shirabe *et al* reported that tumor angiogenesis was associated with a poorer prognosis in node-negative intrahepatic cholangiocarcinoma (18). They reported MVD in CCC as low as 21±7/mm^2, and only concentrated on the node-negative intrahepatic cholangiocarcinoma. Discrepancies in the relationship between angiogenesis and prognosis have been reported for other tumor types (24) and may be caused by particular tumor biological factors, such as the tumor location and accompanying liver disease, or by small study population, different case selection or heterogeneous therapy patterns. More cases and better standardized grouping are required to understand the clinicopathological and prognostic implications of tumor vascularity and associated factors.

In conclusion, a considerable degree of angiogenesis, comparable to that of other solid tumors, was observed in CCC. VEGF and Ang-2 might play a proangiogenic role, while TSP-1 may play an inhibitory role. Although TSP-1 may increase the intrahepatic metastasis of CCC, neither MVD nor the expression of VEGF, Ang-1, or Ang-2 was associated with tumor invasiveness and prognosis.

Acknowledgements

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