薬物トランスポーターを高発現していることから，現在でも*in vitro*での標準細胞として薬物毒性試験等で用いられている[1]。しかしながら，ヒト初代培養肝細胞は，ドナーが制限され日本人由来のヒト初代培養肝細胞は入手が困難であり100％輸入に頼っていること，ロット差が大きいこと，高価であること，増殖しないために安定供給が難しいこと，培養後急速にシトクロムP450薬物代謝酵素等の活性低下がみとめられること，等の問題点が指摘されている[2~4]。したがって，無限増殖能を有するヒトiPS細胞から効率良く肝細胞が分化誘導できればこれらの問題点が解決できると期待されている。

3　ヒトiPS細胞から肝細胞への分化誘導

ヒトiPS細胞はヒトES（embryonic stem）細胞と同様に分化多能性を有し，神経や皮膚，肝臓，血液，心筋等の三胚葉由来の組織へ分化することができる[5, 6]。ヒトiPS細胞の分化誘導はヒトES細胞の分化誘導と基本的に同等であり，いずれも共通の手法を用いて分化誘導できる。したがって，以下に解説するヒトiPS細胞から肝細胞への分化誘導法は，ヒトES細胞から肝細胞への分化誘導法に関する報告も混在していることに留意されたい。

3.1　ヒトiPS細胞から内胚葉への分化誘導

ヒトiPS細胞の分化誘導研究において，肝細胞等の内胚葉分化に関する研究は，神経細胞等の外胚葉分化に関する研究や心筋細胞・血液細胞等の中胚葉分化に関する研究よりも遅れていた（図1）。ヒトES細胞から肝細胞への最初の分化誘導の報告では，胚様体（embryoid body：EB）を形成させた後，各種液性因子を作用させることで肝分化が試みられた[7]。しかしながら，EB形成法では細胞集団が不均一であり分化がランダムに進行し，肝細胞への選択的な分化が制御できない。そこで，効率よく肝細胞へ分化させるために，均一な分化誘導ができる平面培養で，生体内での肝発生・分化の環境を模倣してサイトカインや増殖因子などの各種液性因子を作用させることによって，中内胚葉，内胚葉，肝幹前駆細胞，肝細胞へと段階的に分化させる肝分化誘導

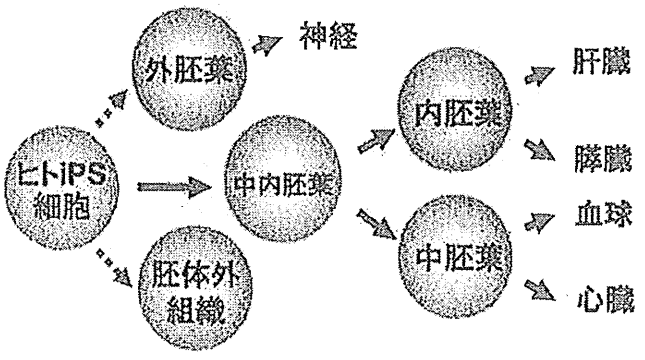

図1　ヒトiPS細胞から三胚葉への分化誘導
ヒトiPS細胞はヒトES細胞とおなじく三胚葉に分化することができる。

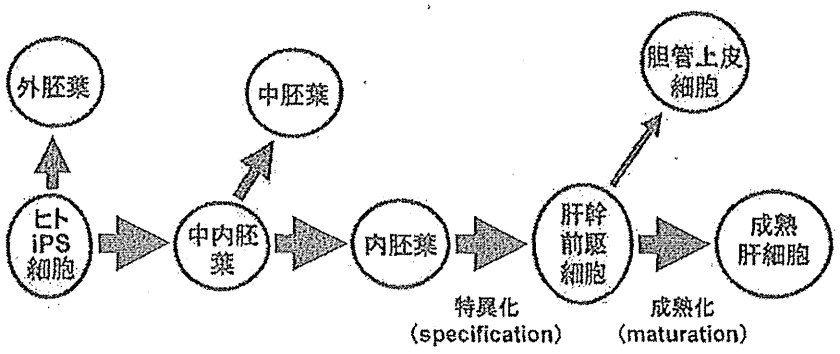

図2　ヒトiPS細胞から肝細胞への分化
ヒトiPS細胞から成熟肝細胞への分化は複数の過程に分けることができる。

法が開発された（図2）[8]。

ヒトiPS細胞から内胚葉への分化誘導ステップでは，アクチビンAがほぼ全てのプロトコールで使われている[9, 10]。アクチビンAはTGF（transforming growth factor）-βファミリーに属する増殖因子であり，受容体に結合した後，細胞内でSmadとよばれるアダプター分子群を活性化する[11]。アクチビンAと同時作用させる形で，FGF（fibroblast growth factor）2やWnt3aも内胚葉分化誘導に用いられることがある[12, 13]。

3.2　内胚葉から肝幹前駆細胞への分化

内胚葉が肝幹前駆細胞へ分化（特異化；specification）すると，細胞はα-フェトプロテインやトランスサイレチンを発現するようになる（図2）[14, 15]。この過程ではFGFシグナルとBMP（bone morphogenetic protein）シグナルが重要であることが知られており，FGF4とBMP2を作用させることにより肝特異化が著明に亢進することが報告されている[16]。またその他にも，FGF1/2/4とBMP2/4の組み合わせによって，内胚葉から肝細胞が分化誘導できるという報告や[12]，ヒストン脱アセチル化酵素であるDMSO（dimethyl sulfoxide）やSodium butyrateが肝細胞への方向付けにおいて有効であることが報告されている[17]。

3.3　肝幹前駆細胞から肝細胞への分化・成熟化

肝幹前駆細胞は肝実質細胞と胆管上皮細胞という2種類の系列細胞に分化することができる（図2）。肝幹前駆細胞から肝実質細胞へ分化するにつれてα-フェトプロテインの発現量が低下し，代わってアルブミンの発現量が上昇してくる。この過程において重要な液性因子はHGF（hepatocyte growth factor）とオンコスタチンMである[18, 19]。HGFは肝前駆細胞の増殖を促進させるとともに胆管への分化を阻害し，オンコスタチンMは肝前駆細胞の成熟化を促進することが知られている。

さらに各分化ステップで，基礎培地や細胞外マトリックス（I型コラーゲンやマトリゲルが汎用される）の種類，血清やフィーダー細胞の有無等が各プロトコールで工夫されている。ヒト

iPS細胞由来分化誘導肝細胞を再生医療に利用する場合には，血清やフィーダー細胞等の異種動物由来成分を排除し，かつ組成の明らかな培地（chemically defined medium）で分化誘導する必要がある。一方，iPS細胞由来分化誘導肝細胞を創薬研究に応用する場合にはそのような制限は必要ではなく，むしろ創薬応用においては可能な限り成熟度が高い肝細胞を分化誘導する必要がある。しかしながら，これらの増殖因子やサイトカインの添加だけからなる分化誘導法は，肝細胞への分化効率もまだまだ不十分なのが現状であり，更なる分化効率の向上が必要となっている。

3.4　遺伝子導入による肝細胞分化誘導

先述したように，iPS細胞から肝細胞への分化誘導効率は未だ十分ではなく，毒性評価系に応用するにはさらなる技術改良が必要である。著者らや他のグループは，内胚葉分化に重要な転写因子であるSOX17遺伝子をヒトiPS細胞からにアクチビンAで分化誘導した中内胚葉に導入することにより，内胚葉への分化誘導効率が著明に向上することを明らかにした[20, 21]。また，内胚葉で強く発現している転写因子のFOXA2遺伝子を中内胚葉に導入することでも内胚葉分化は促進される[22]。肝特異化のステップでは，肝発生に重要な転写因子であるHEX遺伝子をiPS細胞由来内胚葉に導入することにより，肝細胞分化が強く促進されることが著者らと他のグループにより報告されている[23, 24]。

著者らはさらに，複数の遺伝子を分化の適切な時期に順次導入することにより，ヒトiPS細胞から成熟肝細胞までの一連の分化を飛躍的に向上させることに成功した。即ち，未分化iPS細胞からアクチビンA処理で分化させた中内胚葉にSOX17遺伝子を，内胚葉から肝幹前駆細胞への分化ステップではHEX遺伝子を，さらに肝幹前駆細胞から肝細胞への分化ステップではHNF4α遺伝子を導入することで，高いアルブミン産生能や薬物代謝機能を有した肝細胞を効率よく分化誘導することに成功した[25]。さらに最近では，ヒトiPS細胞から肝細胞への各分化ステップにおいて7種類の肝関連転写因子（FOXA2, SOX17, HEX, HNF1α, HNF1β, HNF4α, HNF6）による分化促進効果をスクリーニングした結果，FOXA2およびHNF1α遺伝子を組み合わせて発現させることにより，さらに効率良く成熟肝細胞を分化誘導することに成功した（図3）[26]。

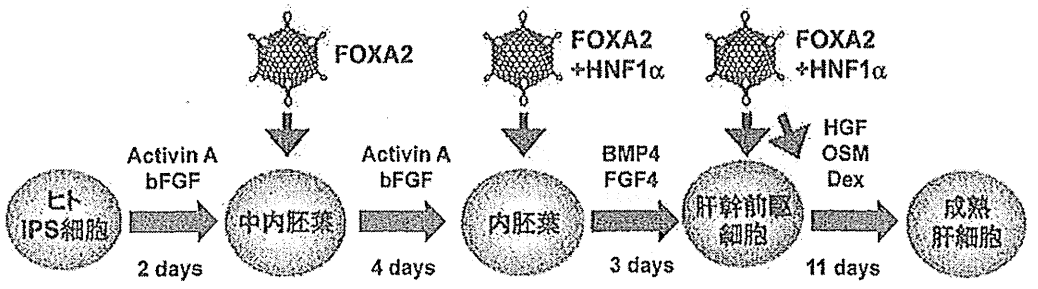

図3　遺伝子導入を用いたヒトiPS細胞から成熟肝細胞への分化誘導
分化の適切な時期に適切な遺伝子を一過性に発現させることにより，効率良く肝細胞を分化誘導できる。

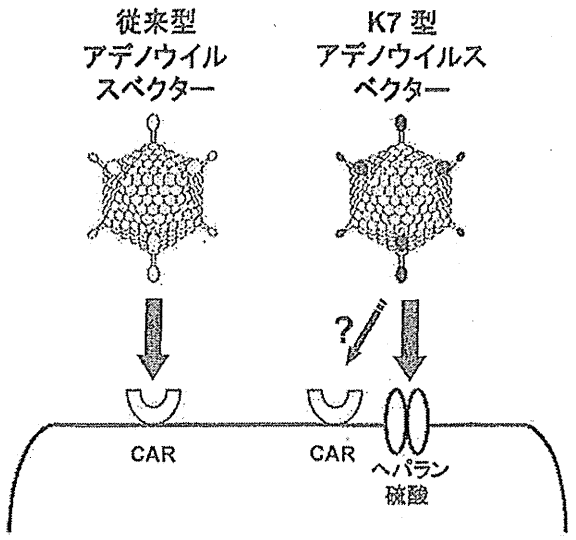

図4　改良型アデノウイルスベクター
改良型（K7型）アデノウイルスベクターはアデノウイルス受容体（CAR）だけでなく，
ヘパラン硫酸も認識することにより，多くの細胞種に効率よく遺伝子導入が可能となる。

なお，本分化誘導における遺伝子導入には，機能性に優れ，独自開発した改良型アデノウイルスベクターを用いた。iPS細胞から肝細胞への分化のように，分化の各ステップが段階的に起こる場合には，各分化ステップでだけ導入遺伝子が機能するように（後の細胞分化に影響を与えないように）遺伝子発現期間は一過性であること，そして効率よく細胞集団を分化させるためには，100％の遺伝子発現効率で遺伝子発現させることが必須となるが，改良型アデノウイルスベクターはこのような目的に唯一叶うベクターである。本研究で用いた改良型アデノウイルスベクターは，細胞への感染に関与するウイルス表面タンパク質のファイバータンパク質のC末端領域にポリリジン配列（KKKKKKK；リジン（K）が7つ続くのでK7と略称）を遺伝子工学的に付与しており，細胞表面のヘパラン硫酸を認識して多くの細胞種に効率よく遺伝子導入が可能となる（図4）[27]。K7型アデノウイルスベクターは，未分化ヒトiPS細胞や，ヒトiPS細胞から分化した細胞に対しても100％の効率で遺伝子導入が可能であった[24]。

3.5　三次元培養技術による肝細胞の成熟化

ヒト初代培養肝細胞は，培養すると急速に肝細胞特異的な性質が失われていくことが知られている。例えば，シトクロムP450酵素やアルブミンの遺伝子発現は，最適化された培養条件で培養しても，48時間も培養すると，解凍（凍結肝細胞の場合）直後の遺伝子発現と比較すると10～100分の1程度にまで低下する。一方で，スフェロイド培養等の三次元培養や，繊維芽細胞や血管内皮細胞との共培養系でヒト初代培養肝細胞を培養すると，シトクロムP450酵素やアルブミン等の肝特異的な機能の減弱は，ある程度抑制されることが知られている。そこで，細胞シート工学技術を用いることで，シート状に回収したSwiss3T3細胞とヒトiPS細胞から分化誘導した肝細胞とを積層三次元共培養し，肝機能の向上が可能か検討した[25]。その結果，単層のヒト

iPS細胞由来分化誘導肝細胞と比較し，肝細胞特異的な遺伝子発現量やアルブミン分泌量が有意に増加することが明らかとなった。また，ヒトiPS細胞由来分化誘導肝細胞の成熟化には肝細胞とSwiss3T3細胞との物理的な接触が重要であることを見出した。さらに，ヒトiPS細胞由来分化誘導肝細胞へ，1型コラーゲンゲルを重層することにより肝細胞成熟化が促進される一方で，コラーゲン合成阻害剤存在下においてはSwiss3T3細胞との積層三次元共培養時の成熟化が抑制されたことから，Swiss3T3細胞が産生する1型コラーゲンが肝細胞成熟化を担う主要な因子のひとつであることが明らかとなった。

最近では，簡便に三次元培養が可能な基材が各社から販売されており，これらの基材を用いてもヒトiPS細胞由来分化誘導肝細胞の成熟化亢進が期待できる。著者らはナノピラープレート（日立ハイテクノロジーズ社）に，ヒトiPS細胞から分化誘導した肝幹前駆細胞を播種し，スフェロイドを形成させ長期間培養，肝成熟化を施したところ，シトクロムP450酵素やアルブミン等の遺伝子発現量が上昇し，シトクロムP450酵素による薬剤代謝能も上昇することを確認し[29]，培養法の工夫によっても肝細胞への分化効率が向上することを明らかにした。このような基材は，特殊な技術を必要とせず，スループット性にも優れるため，毒性評価等の創薬研究には極めて有用と考えられる。

4　iPS細胞由来肝細胞を用いた薬物毒性評価系の開発

このようにしてヒトiPS細胞から分化誘導した肝細胞は，形態学的には二核を有した成熟肝細胞様の形状をしており，80～90％以上の細胞がアルブミン，アシアロ糖タンパク質受容体，LDL（low density lipoprotein）取り込み能，インドシアニングリーン取り込み能，薬物代謝酵素（シトクロムP450 3A4，7A1，2D6等）陽性であり，ヒト初代培養肝細胞に匹敵する薬物代謝酵素の遺伝子発現レベルを示した。また，シトクロムP450酵素などで代謝される9種類の薬物の代謝プロファイルを調べたところ，分化誘導肝細胞の薬物代謝能はヒト初代培養肝細胞より低いものの（シトクロムP450酵素の種類により異なるが，分化誘導肝細胞はヒト初代培養肝細胞の1～40％程度の活性），いずれの薬物に対しても代謝能を有していることが確認された[23]。各シトクロムP450酵素の遺伝子発現と代謝能との間に，iPS細胞由来分化誘導肝細胞とヒト初代培養肝細胞で乖離が認められたが，この原因としては，そもそもシトクロムP450酵素の活性は個人差が大きいことが知られており（数十倍～千倍程度の個人差），低いシトクロムP450酵素活性の個人からiPS細胞が樹立されていた可能性や，シトクロムP450酵素の活性発現に必要な補酵素群の発現が未だ分化誘導肝細胞では十分でないこと等が考えられた。今後，異なった個人から樹立した様々なヒトiPS細胞由来分化誘導肝細胞を用いて同様の検討を行う予定である。また，Rashidらはα1-アンチトリプシン欠損症・家族性コレステロール血漿症・グリコーゲン貯蔵疾患症1αの患者の皮膚細胞からiPS細胞を作製し，肝細胞へ分化誘導させ，それぞれの病態を反映した肝細胞を作製できることを示した[30]。したがって，将来的には病態，あるいは個人差を反映したヒ

トiPS細胞由来分化誘導肝細胞を用いた毒性評価も可能になるであろう。

著者らは，ヒトiPS細胞由来分化誘導肝細胞を用いて，薬剤に対する毒性評価についても検討した。肝毒性を示す化合物について，ナノピラープレート上で分化誘導した肝細胞を用いて細胞毒性を生じるか評価したところ，通常の2次元培養したヒトiPS細胞由来分化誘導肝細胞やHepG2細胞（ナノピラープレート上で培養）を用いた場合に比べ，より感度良く細胞毒性を示し，かつその毒性はシトクロムP450酵素の阻害剤を加えると部分的に消失した[29]。したがって，ヒトiPS細胞由来分化誘導肝細胞を用いることによって，シトクロムP450酵素で代謝された代謝物（反応性代謝物）によって生じた細胞傷害性を再現性良く検出できることが明らかとなった。反応性代謝物は薬物性肝障害の主な原因と考えられており，ヒトiPS細胞由来分化誘導肝細胞で反応性代謝物による細胞傷害性を検出できたことは，極めて大きな意義をもつと考えられる。以上のことから，著者らが作製したヒトiPS細胞由来分化誘導肝細胞は，薬物の毒性スクリーニングに使用できる可能性が示唆された。

5　おわりに

従来のヒトiPS細胞から分化誘導した肝細胞は，機能面において初代培養肝細胞に比べて大きく劣っており，創薬研究への応用は困難であった。しかしながら，著者らが開発した遺伝子導入を駆使した分化誘導法により，創薬応用に向けてようやく最低限の解析が可能なレベルにまで分化した肝細胞を得ることが可能になった。一方で，ヒトiPS細胞由来分化誘導肝細胞を幅広く創薬研究に応用するためには，実験毎に3週間に及ぶ分化誘導を行う必要があり，細胞供給の観点から効率が悪いと考えられる。そこで現在著者らは，分化途中の肝幹前駆細胞の段階で，分化細胞を大量に増幅できないかという課題にも取り組んでいる。今度，より一層高機能な（成熟度が高い）ヒトiPS細胞由来分化誘導肝細胞の作製法の開発を進めるとともに，本分化誘導肝細胞が創薬研究で広く活用されることを期待している。なお，本稿で紹介した分化誘導法で作製されたヒトiPS細胞由来分化誘導肝細胞は，株式会社リプロセルよりReproHepatoとして市販されている（用いているヒトiPS細胞の由来が著者らとは異なっているため性質は同一ではない）。

文　献

1) N. J. Hewitt *et al.*, *Drug Metab. Rev.*, **39**, 159 (2007)
2) C. Terry, R. D. Hughes, *Methods Mol. Biol.*, **481**, 25 (2009)
3) M. A. Baxter *et al.*, *Stem Cell Res.*, **5**, 4 (2010)
4) N. Safinia, S. L. Minger, *Methos Mol. Biol.*, **481**, 169 (2009)
5) J. A. Thomson *et al.*, *Science*, **282**, 1145 (1998)

6) K. Takahashi *et al.*, *Cell*, 131, 861 (2007)
7) T. Hamazaki *et al.*, *FEBS Lett.*, 497, 15-19 (2001)
8) Y. Duan *et al.*, *Stem Cells*, 25, 3058-3068 (2007)
9) K. A. D'Amour *et al.*, *Nat. Biotechnol.*, 23, 1534 (2005)
10) S. Sulzbacher *et al.*, *Stem Cell Rev.*, 5, 159 (2009)
11) Y. G. Chen *et al.*, *Exp. Biol. Med.*, 231, 534 (2006)
12) G. Brolen *et al.*, *J. Biotechnol.*, 145, 284 (2010)
13) D. C. Hay *et al.*, *Proc. Natl. Acad. Sci. U S A*, 105, 12301-12306 (2008)
14) R. Gualdi *et al.*, *Genes Dev.*, 10, 1670 (1996)
15) S. Asgari *et al.*, *Stem Cell Rev.* (in press)
16) J. Cai *et al.*, *Hepatology*, 45, 1229 (2007)
17) D. C. Hay *et al.*, *Stem Cells*, 26, 894-902 (2008)
18) K. Si-Tayeb *et al.*, *Dev. Cell*, 18, 175 (2010)
19) S. Snykers *et al.*, *Stem Cells*, 27, 577 (2009)
20) C. A. Seguin *et al.*, *Cell Stem Cell*, 3, 182 (2008)
21) K. Takayama *et al.*, *PLoS One*, 6, e21780 (2011)
22) S. Kanda *et al.*, *Hepatol. Res.*, 26, 225 (2003)
23) A. Kubo *et al.*, *Hepatology*, 51, 633 (2010)
24) M. Inamura *et al.*, *Mol. Ther.*, 19, 400 (2011)
25) K. Takayama *et al.*, *Mol. Ther.*, 20, 127 (2012)
26) K. Takayama *et al.*, *J. Hepatol.*, 57, 628 (2012)
27) N. Koizumi *et al.*, *J. Gene Med.*, 5, 267-276 (2003)
28) Y. Nagamoto *et al.*, *Biomaterials*, 33, 4526 (2012)
29) K. Takayama *et al.*, *Biomaterials*, 34, 1781-1789 (2013)
30) S. T. Rashid *et al.*, *J. Clin. Invest.*, 120, 3127 (2010)

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The combination therapy of α-galactosylceramide and 5-fluorouracil showed antitumor effect synergistically against liver tumor in mice

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α-Galactosylceramide (α-GalCer) has been reported to be therapeutic against metastatic liver tumors in mice. However, little is known regarding the efficacy of combined chemo-immunotherapy using α-GalCer and anticancer drugs. In this study, we evaluated the antitumor effect of the combination therapy of α-GalCer and 5-fluorouracil (5-FU) against liver tumors of MC38 colon cancer cells. The liver weights of tumor-bearing mice treated with the combination were significantly lower than those of nontreated mice and of mice treated with 5-FU or α-GalCer alone. No toxic effects on the liver and renal functions were observed in any of the treatment groups. α-GalCer treatment induced significant activation of liver NK cells *in vivo*, but 5-FU treatment did not. 5-FU treatment resulted in a significant upregulation of NKG2D activating molecules (Rae-1 and H60) and DNAM-1 ligands (CD112 and CD155) on MC38 cells, but α-GalCer did not. The cytolytic activity of α-GalCer-activated liver mononuclear cells against 5-FU-treated MC38 cells was significantly higher than that against nontreated cells. The increase of the cytolytic activity induced by 5-FU partially depended on NKG2D-Rae-1 or H60 signals. Depletion of NK cells significantly inhibited the antitumor efficacy of 5-FU against MC38 liver tumors, which suggested that the antitumor effect of 5-FU partially depended on the cytolytic activity of NK cells. These results demonstrated that the combination therapy of α-GalCer and 5-FU produced synergistic antitumor effects against liver tumors by increasing the expression of NK activating molecules on cancer cells. This study suggests a promising new chemo-immunotherapy against metastatic liver cancer.

Colon cancer is one of the most common cancers in the world. Despite recent progress in the development of treatment, the overall 5-year survival rate is only 50–60% due to local recurrence or distant metastasis.[1] In particular, patients with metastatic colon cancer have a median survival rate of only six months. 5-Fluorouracil (5-FU) remains key-drug in chemotherapy against colon cancer. However, colon cancer cells are becoming increasingly resistant to existing chemotherapies including 5-FU.[2] Therefore, novel strategies are needed especially for the treatment of advanced colon cancers including metastatic liver cancer.

A normal liver contains abundant lymphocytes that are usually enriched with NK and NKT cells in contrast to peripheral blood.[3,4] Thus, the effective activation of innate immune cells might be beneficial in the treatment of metastatic liver cancer. To date, however, immunotherapy has not yet been established against metastatic liver cancer. α-Galactosylceramide (α-GalCer) induces the activation of NKT cells in a CD1d-dependent manner.[5,6] Recently, α-GalCer has been attracting attention as a novel antitumor therapy. Systemic administration of α-GalCer has demonstrated antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma, and lymphoma) *in vivo* in animal models of hepatic and lung metastasis.[7,8] We and others have demonstrated that sequential activation of both NKT and NK cells could be observed in the liver after α-GalCer administration.[8–10] Although most NKT cells had disappeared from the liver within 12 hr of α-GalCer administration, strong activation and proliferation of liver NK cells could be

Key words: α-GalCer, 5-FU, NK cells, liver tumor

Abbreviations: 5-FU: 5-fluorourcil; Alb: albumin; ALT: alanine aminotransferase; Cr: creatinine; IFN-α: interferon-α; MICA: major histocompatibility complex class I-related chain A; MNCs: mononuclear cells; PBS: phosphate buffered saline; T-Bil: total bilirubin; α-GalCer: α-galactosylceramide

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What's new?
α-Galactosylceramide (α-GalCer) is effective against metastatic liver tumors in mice. In this study, the authors evaluated the antitumor effect of a combination therapy of α-GalCer plus 5-FU. They found that the combination therapy produced synergistic antitumor effects against liver tumors of colon cancer cells in mice, by both increasing the activation of natural killer (NK) cells and enhancing the sensitivity of the cancer cells to those NK cells. This combination may therefore represent a promising new chemo-immunotherapy against metastatic liver cancer.

observed, and the antitumor effect of the α-GalCer treatment against liver tumors depended primarily on NK cells. Based on the promising results of preclinical studies, several Phase 1 clinical studies using intravenous administration of α-GalCer have been conducted, but clinical responses of α-GalCer have been limited.[11] In view of future α-GalCer treatment of metastatic liver cancer, new strategies should be explored. We have previously reported that anticancer drugs enhance the expression of the human NKG2D ligand, membrane-bound major histocompatibility complex class I-related chain A (MICA), and the NK sensitivity of human hepatocellular carcinoma cells *in vitro*.[12,13] These findings suggest that the efficient activation of liver innate immunity after chemotherapy might represent a promising approach to the suppression of liver tumor growth.

In this study, we investigated the therapeutic potential of the combination of α-GalCer and 5-FU in the treatment of liver tumor of colon cancer cells. We found that 5-FU can enhance the NK sensitivity of colon cancer cells by increasing the expression of NK activating molecules. In addition, the combination therapy of α-GalCer and 5-FU showed synergistic antitumor effects against liver tumor of colon cancer cells. This study demonstrates a promising new therapeutic strategy for the treatment of metastatic liver cancer.

Material and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from Charles River Laboratories Japan, INC (Yokohama, Japan) and were used at 6–10 weeks of age. The mice were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guidelines.

Cell lines

MC38, a mouse colon cancer cell line derived from C57BL/6 mice, was generously provided by Dr. Michio Imawari (Showa University School of Medicine, Tokyo, Japan). Colon26, a mouse colon cancer cell line derived from BALB/c mice, was kindly provided by Dr. Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). This cell line was maintained in complete medium (CM, RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C.

Reagents

α-GalCer was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*[5] 5-FU was purchased from Kyowa Hakko Kirin (Tokyo, Japan) and dissolved in phosphate buffered saline (PBS). MC38 cell viability was determined 24 hr after the addition of 5-FU (used at 10 nmol/l to 2 μmol/l) or PBS by the WST assay using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan) as previously described (10).

Flow cytometry

MC38 cells were cultured with or without α-GalCer (100 ng/ml) or 5-FU (500 nmol/l) for 24 hr and evaluated for the expression of NK activating molecules. Treated and non-treated MC38 cells were incubated with PE-conjugated antibodies (Abs) against anti-Rae-1 (R&D Systems, Minneapolis, MN), H60 (R&D Systems), CD112 (Nectin-2) (Abcam, Cambridge, UK), and CD155 (BioLegend, San Diego, CA). Flow cytometric analysis was performed using a Canto II flow cytometer (Becton Dickinson, San Jose, CA).

Preparation of hepatic mononuclear cells from 5-FU- or α-GalCer-treated mice

C57BL/6 mice were administered 5-FU (20 mg/kg body weight)or PBS intraperitoneally (i.p.) for 3 consecutive days. Liver mononuclear cells (MNCs) were prepared as previously described.[8] In some experiments, C57BL/6 mice were administered α-GalCer (0.4 μg/mouse) or PBS i.p. on Day 0. On Day 3, hepatic MNCs were prepared. NK cells were identified as DX5+/TCRβ- by flow cytometry as previously described.[8] The expression levels of NKG2D and DNAM1 were evaluated with anti-NKG2D (R&D Systems) and anti-DNAM1 (BioLegend) Abs by flow cytometry.

Cytolytic assays

C57BL/6 mice were injected i.p. with α-GalCer (2 μg/mouse) for the preparation of activated NK cells as previously described.[8] Liver MNCs were prepared on Day 3 after α-GalCer injection. MC38 cells were cultured with or without 5-FU (500 nmol/l) for 1 day. α-GalCer-activated liver MNCs

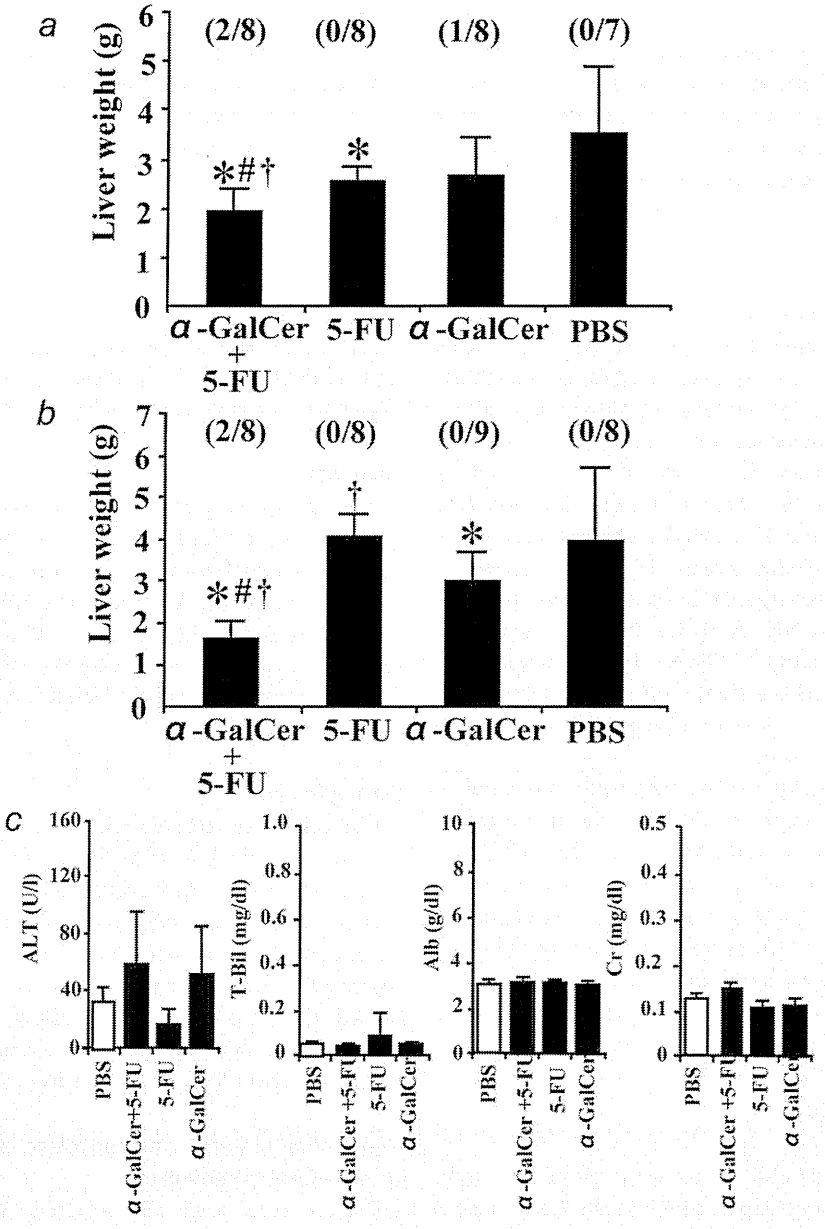

Figure 1. The antitumor effect of the α-GalCer and 5-FU combination therapy against MC38 liver tumors. (*a*, *b*) C57BL/6 mice or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0. To evaluate the efficacy of the α-GalCer and 5-FU combination therapy, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine intrahepatic tumor growth. $N = 7–9$ mice/group. Each data point represents the mean liver weight ± SD. The fraction of mice achieving tumor rejection in each treatment group is shown in parentheses. *$p < 0.05$ *versus* PBS group, #$p < 0.05$ *versus* 5-FU group, †$p < 0.05$ *versus* α-GalCer group. (*c*) Blood samples from treated C57BL/6 mice were obtained 1 day after the final injection of each treatment. The serum levels of ALT, T-Bil, Alb, and Cr were examined. $N = 3$ /group. No significant differences were observed between any of the groups.

were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated or nontreated MC38 cells as previously described.[12] The assays were performed in triplicate, and the spontaneous release of all assays did not exceed 25% of the maximum release. In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 (R&D Systems) or H60 (R&D Systems).

Animal experiments

C57BL/6 or BALB/c mice were injected in the liver with 3×10^5 MC38 cells or 5×10^5 Colon26 cells on Day 0.

To evaluate the efficacy of the combination therapy of α-GalCer and 5-FU, the mice were treated with α-GalCer (0.4 μg/mouse) on Day 0 and/or 5-FU (C57BL/6, 10 mg/kg body weight; BALB/c, 20 mg/kg body weight respectively) for 5 consecutive days after tumor inoculation. Two weeks after the tumor injection, the liver weight was measured to examine the intrahepatic tumor growth. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-asialo GM-1 (ASGM1) Ab (WAKO, Osaka, Japan) on Days −1, 4, and 9 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometric analysis of splenocytes using PE-conjugated anti-DX5 mAbs (BD-Pharmingen) as previously described.[8] NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of treated mice were removed, and the liver weight was measured to examine the intrahepatic tumor growth.

NKG2D lignads and DNAM1 ligands expression in MC38 tumor tissues and nontumor tissues in 5-FU-treated mice

C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry.

Blood biochemistry test

Blood samples were obtained 24 hr after treatment. The levels of serum alanine aminotransferase (ALT), total bilirubin (T-Bil), albumin (Alb), and creatinine (Cr) were measured with a standard UV method using a Hitachi type 7170 automatic analyzer (Tokyo, Japan).

Statistics

All values are expressed as the mean and SD. Statistical analyses were performed by the unpaired Mann–Whitney *U* test or one-way ANOVA unless otherwise indicated. When ANOVA analyses were applied, differences in the mean values among groups were examined by the Scheffe post hoc correction. We defined statistical significance as $p < 0.05$.

Results

The combination therapy of α-GalCer and 5-FU showed a synergistic antitumor effect against MC38 liver tumors

We examined the antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 liver tumors. C57BL/6 mice were injected intrahepatically with MC38 cells. The mice were treated with α-GalCer on Day 0 and/or 5-FU for 5 consecutive days after tumor inoculation. As shown in Figure 1*a*, the liver weights of the mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with 5-FU were significantly lower than those of nontreated mice, but treatment with α-GalCer did not produce this effect. We also examined the antitumor effect of α-GalCer plus 5-FU in a Colon26 liver tumor model. The liver weights of mice treated with α-GalCer plus 5-FU were significantly lower than those of nontreated mice and mice treated with either 5-FU or α-GalCer alone. The liver weights of mice treated with α-GalCer were significantly lower than those of nontreated and 5-FU-treated mice (Fig. 1*b*). Tumor rejection in the MC38 liver tumor model was observed in 2/8 of the α-GalCer plus 5-FU-treated

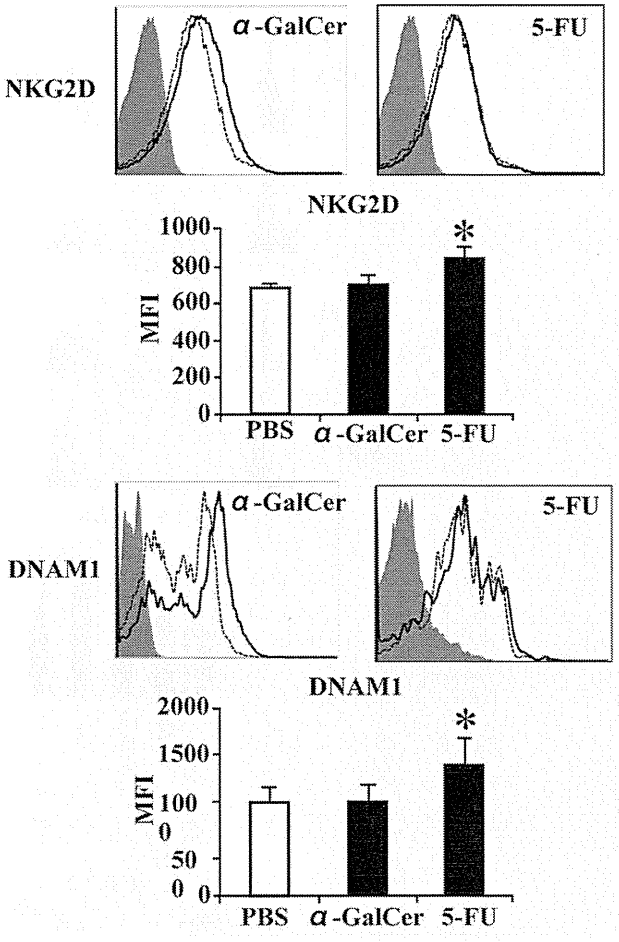

Figure 2. Expression of NKG2D and DNAM1 on liver NK cells isolated from α-GalCer- or 5-FU-treated mice. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or with 5-FU (10 mg/kg body weight) for 3 consecutive days. Liver NK cells were isolated from α-GalCer or 5-FU-treated mice, and the expression levels of NKG2D and DNAM1 were evaluated by flow cytometry. Black bold line histograms: NKG2D or DNAM1 staining of NK cells from α-GalCer or 5-FU-treated mice; dotted line histograms: NKG2D or DNAM1 staining of NK cells from PBS-treated mice; shaded/gray histograms: control staining. The data are represented as the average of the MFI obtained from 3 separate experiments. $^*p < 0.05$ *versus* PBS-treated group.

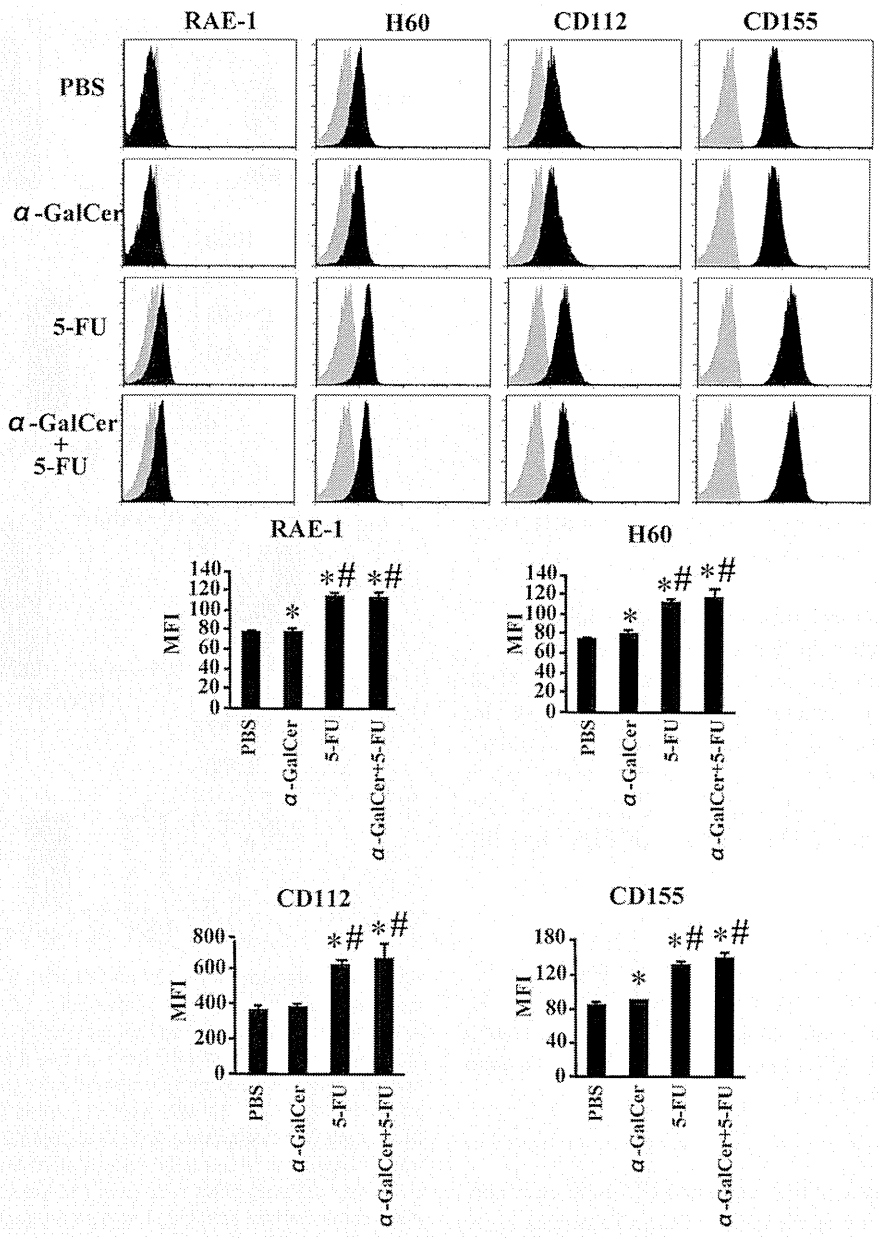

Figure 3. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells treated with α-GalCer and/or 5-FU. MC38 cells were cultured with or without α-GalCer (100 ng/ml) or 5-FU (500 nmol/l) for 24 hr. The treated cells were harvested and evaluated for the expression levels of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells by flow cytometry. Upper panel: representative data. Shaded/black histograms: NKG2D or DNAM1 ligand staining of α-GalCer or 5-FU-treated MC38 cells; shaded/gray histograms, control staining. Lower panel: data are represented as the average of MFI obtained from 3 separate experiments. *$p < 0.05$ *versus* PBS group, #$p < 0.05$ *versus* α-GalCer group.

mice, 0/8 of the 5-FU-treated mice, 1/8 of the α-GalCer-treated mice, and 0/7 of the PBS-treated mice (Fig. 1*a*). These results were consistent with those of another Colon26 liver tumor model in BALB/c mice, where tumor rejection was observed in 2/8 of the α-GalCer plus 5-FU-treated mice, 0/8 of the 5-FU-treated mice, 0/9 of the α-GalCer-treated mice, and 0/8 of the PBS-treated mice (Fig. 1*b*). These results demonstrated that the combination therapy of α-GalCer and 5-FU produced a synergistic antitumor effect against liver tumors in both the MC38 and Colon26 models. To evaluate the safety of this combination therapy, serum levels of ALT, T-Bil, Alb, and Cr were evaluated in C57BL/6 mice immunized with α-GalCer plus 5-FU, 5-FU, α-GalCer, or PBS. There was no toxic effect upon the ALT, T-Bil, Alb, or Cr levels for any of the treatment groups (Fig. 1*c*). These results demonstrated that the combination therapy of α-GalCer and

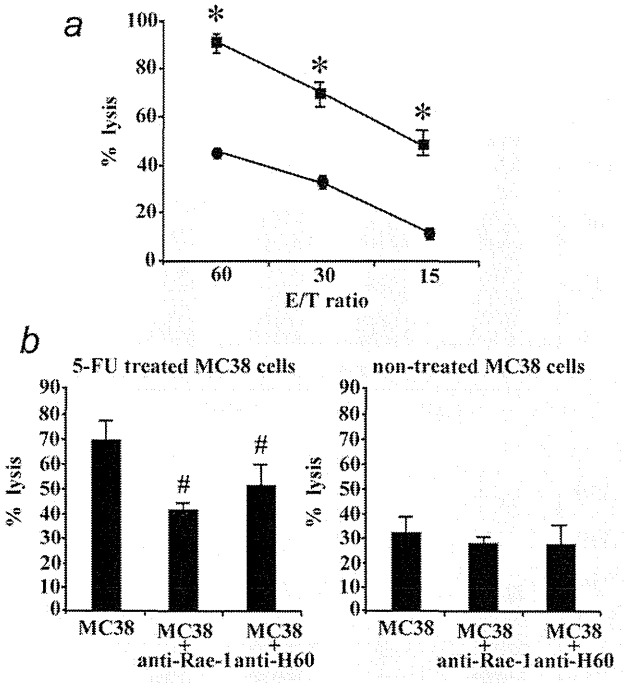

Figure 4. The cytolytic activity of α-GalCer-activated MNCs against 5-FU-treated MC38 cells. C57BL/6 mice were injected i.p. with α-GalCer (2 μg/mice) to activate NK cells. Liver MNCs were prepared on Day 3 after α-GalCer injection. (*a*) MC38 cells were cultured with or without 5-FU (500 nmol/l) for 24 hr. α-GalCer-activated liver MNCs were subjected to a 4-hr ^{51}Cr release assay against 5-FU-treated (■) or nontreated (●) MC38 cells. (*b*) In some experiments, the cytolytic ability of activated NK cells was assessed by a 4-hr ^{51}Cr-release assay with or without blocking Abs against Rae-1 or H60 at an E/T ratio of 30:1. Similar results were obtained from 3 independent experiments. $*p < 0.05$ *versus* the cytolytic activity of activated NK cells against nontreated cells, $\#p < 0.05$ *versus* the cytolytic activity of activated NK cells against 5-FU-treated cells.

5-FU is not toxic to hepatocytes and does not harm the liver or kidney.

α-GalCer, but not 5-FU, treatment induced NK activating receptors on NK cells

We examined the expression levels of activating (NKG2D and DNAM1) receptors on liver NK cells. C57BL/6 mice were treated with α-GalCer (0.4 μg/mouse) i.p. on Day 0 or 5-FU (10 mg/kg body weight) for 3 consecutive days and liver NK cells were isolated from 5-FU- and α-GalCer-treated mice. As shown in Figure 2, the expression levels of NKG2D and DNAM1 on liver NK cells from α-GalCer-treated mice were significantly higher than those from PBS-treated mice. In contrast, the expression of NKG2D and DNAM1 on liver NK cells from 5-FU-treated mice was similar to that of PBS-treated mice. These results demonstrated that α-GalCer, but not 5-FU, could activate liver NK cells.

5-FU, but not α-GalCer, treatment induced NK activating molecules on colon cancer cells

We next examined the expression of the NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 colon cancer cells treated with α-GalCer and/or 5-FU. We first examined the cytotoxicity of 5-FU on MC38 cells by the WST-8 assay. The addition of more than 1 μmol/l of 5-FU resulted in a significant decrease in the growth of MC38 cells (data not shown). On the basis of these findings, we used 500 nmol/l of 5-FU to evaluate the biological effect on MC38 cells. MC38 cells were incubated with α-GalCer (100 ng/ml) and/or 5-FU (500 nmol/l) for 24 hr and the expression levels of NK activating molecules on MC38 cells were evaluated by flow cytometry. 5-FU induced the expression of Rae-1, H60, CD112, and CD155 on MC38 cells (Fig. 3). The expression of these molecules on 5-FU-treated MC 38 cells was significantly higher than that of nontreated MC38 cells. The induction of these NK activating molecules was dose-dependent (data not shown). In contrast, α-GalCer could not induce the expression of Rae-1, H60, CD112, or CD155 on MC38 cells. Even in the MC38 cells treated with this combination of α-GalCer and 5-FU, α-GalCer failed to induce additional expression of NK activating molecules. These results demonstrated that 5-FU, but not α-GalCer, could enhance the expression of NK activating molecules on colon cancer cells.

The cytolytic activity of α-GalCer activated liver MNCs against 5-FU-treated MC38 cells

We next examined the cytolytic activity of α-GalCer-activated liver MNCs against 5-FU-treated MC38 cells. We isolated liver MNCs from normal α-GalCer injected mice and the cytolytic activity of these α-GalCer-activated liver MNCs was measured. The cytolytic activity of liver MNCs against 5-FU-treated MC38 cells was significantly higher than that against nontreated cells (Fig. 4*a*). The cytolytic activity against 5-FU-treated MC38 cells decreased significantly following the addition of blocking Abs against Rae-1 or H60 (Fig. 4*b*).

5-FU treatment induced the expression of NK activating molecules in MC38 liver tumor tissues but not in MC38 nontumor tissues

We examined the induction of NKG2D ligand (Rae-1 and H60) and DNAM1 ligand (CD112 and CD155) expression in MC38 liver tumor or nontumor tissues of 5-FU-treated mice. As shown in Figure 5, the expression of Rae-1 in liver tumor tissues of 5-FU-treated mice was significantly higher than that of liver tumor and nontumor tissues of PBS-treated mice and that of nontumor tissues of 5-FU-treated mice. The expression of H60 and CD112 was similar to that of Rae-1. The expression of CD155 in liver tumor tissues of 5-FU-treated mice tended to be higher than that of PBS-treated mice, although the difference was not statistically significant. These results demonstrated that 5-FU treatment induced the expression of NK activating molecules in liver tumor tissues but not in nontumor tissues consistent with the *in vitro* results.

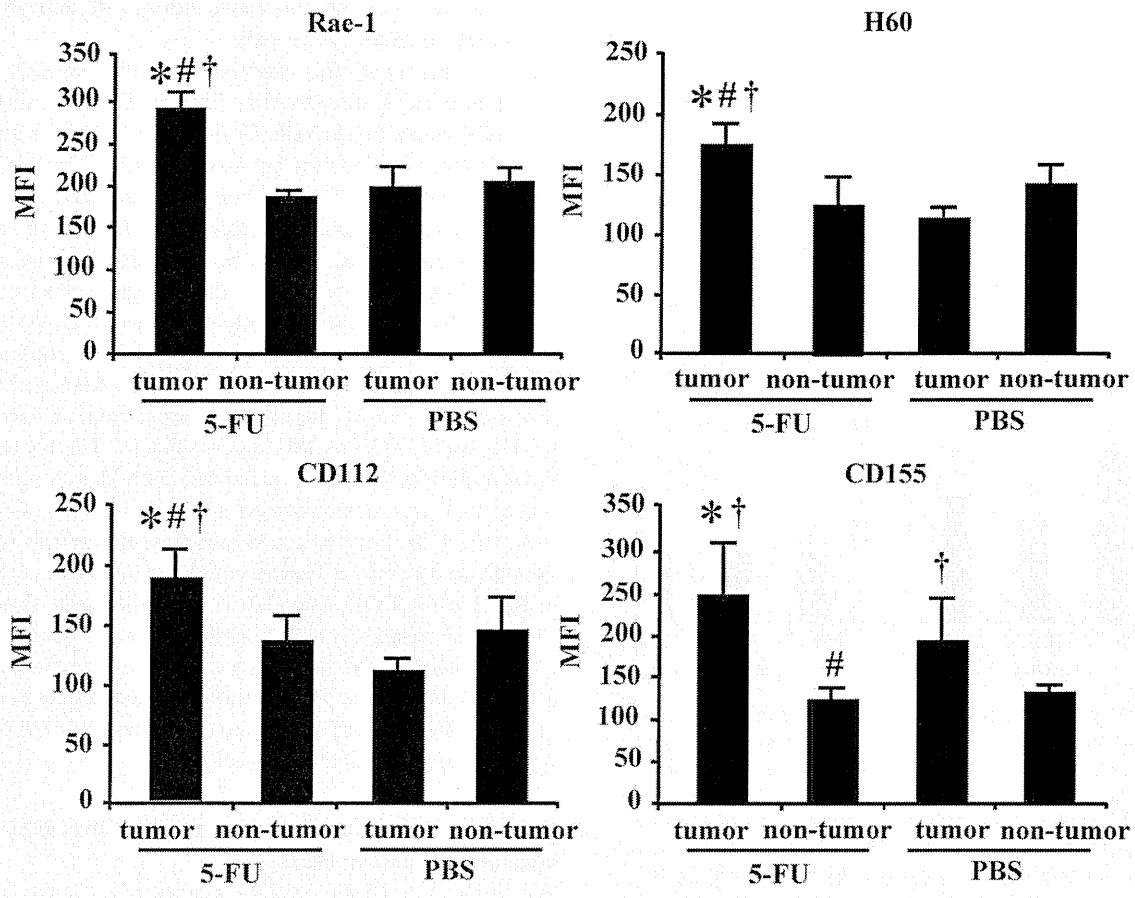

Figure 5. Expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 liver tumor tissues of mice treated with 5-FU. C57BL/6 mice were injected in the liver with 3×10^5 MC38 cells on Day 0 and were treated with 5-FU on Day 4–8 after tumor inoculation. On Day 8, MC38 liver tumor or nontumor tissues were harvested and divided into single cells to evaluate the expression of NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) by flow cytometry. $N = 3$/group. *$p < 0.05$ *versus* nontumor tissues of PBS group, #$p < 0.05$ *versus* tumor tissues of PBS group, †$p < 0.05$ *versus* nontumor tissues of 5-FU group.

The antitumor effect of 5-FU depended on both direct cytotoxicity and the cytolytic activity of NK cells in mouse colon cancer

The above results suggested that 5-FU could enhance the NK sensitivity of MC38 cells. To confirm that NK activity played a role in the antitumor effect of 5-FU, we examined the antitumor effect of 5-FU against MC38 liver tumors in NK depleted mice. As shown in Figure 6, the liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. Depletion of NK cells significantly inhibited the antitumor efficacy of 5-FU against MC38 liver tumors. These results suggested that the antitumor effect of 5-FU depended on not only on the direct cytotoxic effect of 5-FU but also on the cytolytic activity of NK cells. Therefore, NK activity plays a role in the antitumor effect of 5-FU in the liver which contains abundant NK cells.

Discussion

The lymphocytes in the liver are typically enriched with a higher number of NK cells than that found in the peripheral blood in a normal mouse.[3,4] Efficient activation of the abundant NK cells in the liver might be important in antitumor defense against liver tumors. Interferon-α (IFN-α) could activate liver NK cells efficiently.[14] Bui *et al.*[15] reported that IFN-α reduced the expression of H60 on MCA sarcoma cells, suggesting that IFN-α treatment may reduce the NK sensitivity of cancer cells. We and others have previously demonstrated that the systemic administration of α-GalCer can lead to antitumor effects against metastatic liver tumors through the efficient activation of liver NK cells.[8,16] Although α-GalCer has not yet been officially accepted for clinical application in cancer treatment, these previous results encouraged us to evaluate the antitumor effect of the combination of α-GalCer and 5-FU against MC38 liver tumors. In most reports, high dose (2 μg/mouse) α-GalCer was applied for the treatment of liver tumors. However, administration of these high dose resulted in liver injury.[9,17,18] In the present study, we used low dose (0.4 μg/mouse) α-GalCer in the combination therapy. The administration of low dose α-GalCer is enough to activate liver NK cells and did not affect

the expression of NK activating molecules on MC38 cells. Importantly, the administration of this low dose α-GalCer did not cause liver injury. The antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 and Colon26 liver tumors was stronger than that of 5-FU alone or α-GalCer alone. The antitumor effect of the combination therapy of low dose (0.4 μg/mouse) α-GalCer and 5-FU was equal to that of the combination therapy of high dose (2 μg/mouse) α-GalCer and 5-FU (Aketa *et al.*, unpublished data). Our results might offer new chemo-immunotherapy strategies, especially for those patients with advanced stages of cancer.

In this study, we demonstrated that 5-FU treatment enhanced the expression of both NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells. In contrast, 5-FU treatment did not affect the activating molecules on NK cells. Both pathways involving NKG2D and DNAM1 play critical roles in the activation of NK cells and have been implicated in tumor surveillance.[19] The expression of NKG2D ligands has been associated with a good prognosis in patients with colon cancer.[20] Thus, these results suggest that the upregulation of NKG2D ligand expression might improve the prognosis of patients with colon cancer. Gasser *et al.*[21] previously reported that DNA-damaging agents and DNA-synthesis inhibitors including 5-FU could induce the expression of NKG2D ligands on tumor cells. We also demonstrated that 5-FU treatment could induce the expression of NK activating molecules in MC38 liver tumor tissues but not in nontumor tissues, which was consistent with the *in vitro* results. Our present results suggest that 5-FU treatment might have strong immune-editing potential to enhance the NK sensitivity of colon cancer cells by regulating DNAM1 and NKG2D ligands.

In this study, we demonstrated that 5-FU treatment enhanced the susceptibility of MC38 cells to the cytolytic activity of liver MNCs *via* the NKG2D-NKG2D ligand pathway. Because the blocking antibody of the DNAM1-DNAM1 ligand is not commercially available, we could not evaluate the involvement of this pathway. We have previously demonstrated that membrane-bound MICA, an activating molecule of NK cells, on HCC cells is essential in the NK sensitivity of HCC cells.[12,13] The addition of both epirubicin and sorafenib enhanced the NK sensitivity of HCC cells by increasing the membrane-bound MICA.[12,13] This finding is consistent with this study of a colon cancer model. Interestingly, the expression of death receptors, such as FAS and TRAIL receptors, on MC38 cells was significantly increased by 5-FU treatment (Aketa *et al.*, unpublished data). This result may also explain the enhancement of the susceptibility of MC38 cells to the cytolytic activity of liver MNCs. We previously demonstrated that α-GalCer administration resulted in rapid and strong activation of liver NK cells and that the cytolytic activity of liver MNCs early after α-GalCer administration mainly depended primarily on liver NK cells and not on NKT or T cells.[8,10] Taken together, these results suggest that the addition of 5-FU enhanced the NK sensitivity of MC38 cells by increasing the

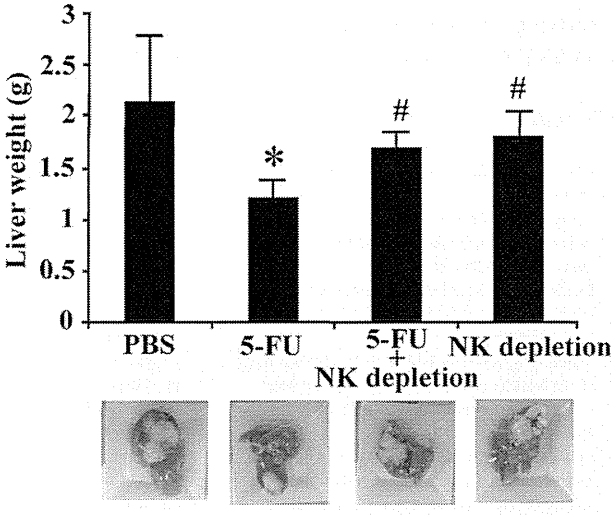

Figure 6. The antitumor effect of 5-FU against MC38 liver tumors in NK-depleted mice. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-ASGM1 Ab. NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine the intrahepatic tumor growth. *$p < 0.05$ *versus* PBS group, #$p < 0.05$ *versus* 5-FU group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of Rae-1 or H60 on MC38 cells. Therefore, 5-FU treatment might be expected to enhance the susceptibility of MC38 cells to the cytolytic activity of NK cells by modifying the expression of NKG2D and DNAM1 ligands.

NK depletion decreased the antitumor effect of 5-FU against MC38 liver tumors, demonstrating that the antitumor effect of 5-FU depends on NK activity in addition to direct cytotoxicity. We also examined the antitumor effect of 5-FU against the Colon26 liver tumor model, derived from BALB/c colon cancer. The liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. The depletion of NK cells also significantly inhibited the antitumor efficacy of 5-FU against Colon26 liver tumors in BALB/c mice. A significant upregulation of Rae-1, H60, CD112, and CD155 could also be observed in 5-FU-treated Colon26 cells derived from BALB/c mice (Aketa *et al.*, unpublished data). These results were consistent with the results of C57BL/6 mice and suggest that the antitumor effect of 5-FU may always depend on NK activity in the liver. The liver contains abundant NK cells. In cancer tissues that are rich in NK cells, the combination therapy of α-GalCer and 5-FU might have a potential as a new chemo-immunotherapeutic strategy.

The liver is the most common site of metastasis of gastrointestinal cancers (*i.e.*, colorectal, gastric, and pancreatic cancers). Thus, new therapeutic approaches of cancer immunotherapy for metastatic liver cancer need to be developed. We have shown here that 5-FU can enhance the NK sensitivity of cancer cells by inducing the expression of NK activating molecules in

addition to the direct cytotoxicity of 5-FU to the cancer cells. In addition, the combination therapy of α-GalCer and 5-FU showed sufficient antitumor effects against MC38 liver tumors. These findings indicate that this new combination chemo-immunotherapy might represent a particularly promising approach for patients with metastatic liver cancer.

References

1. Andre N, Schmiegel W. Chemoradiotherapy for colorectal cancer. *Gut* 2005;54:1194–202.
2. Gallagher DJ, Kemeny N. Metastatic colorectal cancer: from improved survival to potential cure. *Oncology* 2010;78:237–48.
3. Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev* 2000;174:5–20.
4. Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology* 2001;120: 250–60.
5. Kawano T, Cui J, Koezuka Y, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997;278:1626–9.
6. Fujii S, Shimizu K, Kronenberg M, et al. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide -loaded DCs. *Nat Immunol* 2002;3:867–74.
7. Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M, et al. alpha -galactosylceramide-activated Valpha 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci USA* 2000;97:8461–6.
8. Miyagi T, Takehara T, Tatsumi T, et al. CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer cells to eliminate experimentally disseminated hepatoma cells in murine liver. *Int J Cancer* 2003;106:81–9.
9. Osman Y, Kawamura T, Naito T, et al. Activation of hepatic NKT cells and subsequent liver injury following administration of alpha-galactosylceramide. *Eur J Immunol* 2000;30: 1919–28.
10. Tatsumi T, Takehara T, Yamaguchi S, et al. Decreased expressions of CD1d molecules on liver dendritic cells in subcutaneous tumor bearing mice. *J Hepatol* 2008;49:779–86.
11. Giaccone G, Punt CJ, Ando Y, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002;8: 3702–9.
12. Kohga K, Takehara T, Tatsumi T, et al. Anticancer chemotherapy inhibits MHC class I-related chain a ectodomain shedding by downregulating ADAM10 expression in hepatocellular carcinoma. *Cancer Res* 2009;69:8050–7.
13. Kohga K, Takehara T, Tatsumi T, et al. Sorafenib inhibits the shedding of major histocompatibility complex class I-related chain A on hepatocellular carcinoma cells by down-regulating a disintegrin and metalloproteinase 9. *Hepatology* 2010;51:1264–73.
14. Takehara T, Uemura A, Tatsumi T, et al. Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. *Int J Cancer* 2007;120: 1252–60.
15. Bui JD, Carayannopoulos LN, Lanier LL, et al. IFN-dependent down-regulation of the NKG2D ligand H60 on tumors. *J Immunol* 2006;176: 905–13.
16. Nakagawa R, Motoki K, Ueno H, et al. Treatment of hepatic metastasis of the colon26 adenocarcinoma with an alpha-galactosylceramide, KRN7000. *Cancer Res* 1998;58:1202–7.
17. Trobonjaca Z, Kröger A, Stober D, et al. Activating immunity in the liver. II. IFN-beta attenuates NK cell-dependent liver injury triggered by liver NKT cell activation. *J Immunol* 2002;168:3763–70.
18. Biburger M, Tiegs G. Alpha-galactosylceramide-induced liver injury in mice is mediated by TNF-alpha but independent of Kupffer cells. *J Immunol* 2005;175:1540–50.
19. Guerra N, Tan YX, Joncker NT, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* 2008;28:571–80.
20. McGilvray RW, Eagle RA, Watson NF, et al. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. *Clin Cancer Res* 2009;15:6993–7002.
21. Gasser S, Orsulic S, Brown EJ, et al. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 2005;436:1186–90.

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The Bcl-2 Homology Domain 3 (BH3)-only Proteins Bim and Bid Are Functionally Active and Restrained by Anti-apoptotic Bcl-2 Family Proteins in Healthy Liver*[S]

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Background: A fine balance between the anti- and pro-apoptotic multidomain Bcl-2 family proteins controls hepatocyte apoptosis in the healthy liver.
Results: Disruption of the BH3-only proteins Bim and Bid prevents spontaneous hepatocyte apoptosis in the absence of anti-apoptotic Bcl-2 family proteins.
Conclusion: Hepatocyte integrity is maintained by the well orchestrated Bcl-2 network.
Significance: We demonstrated the novel involvement of BH3-only proteins in the healthy Bcl-2 network of the liver.

An intrinsic pathway of apoptosis is regulated by the B-cell lymphoma-2 (Bcl-2) family proteins. We previously reported that a fine rheostatic balance between the anti- and pro-apoptotic multidomain Bcl-2 family proteins controls hepatocyte apoptosis in the healthy liver. The Bcl-2 homology domain 3 (BH3)-only proteins set this rheostatic balance toward apoptosis upon activation in the diseased liver. However, their involvement in healthy Bcl-2 rheostasis remains unknown. In the present study, we focused on two BH3-only proteins, Bim and Bid, and we clarified the Bcl-2 network that governs hepatocyte life and death in the healthy liver. We generated hepatocyte-specific Bcl-xL- or Mcl-1-knock-out mice, with or without disrupting Bim and/or Bid, and we examined hepatocyte apoptosis under physiological conditions. We also examined the effect of both Bid and Bim disruption on the hepatocyte apoptosis caused by the inhibition of Bcl-xL and Mcl-1. Spontaneous hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice was significantly ameliorated by Bim deletion. The disruption of both Bim and Bid completely prevented hepatocyte apoptosis in Bcl-xL-knock-out mice and weakened massive hepatocyte apoptosis via the additional *in vivo* knockdown of *mcl-1* in these mice. Finally, the hepatocyte apoptosis caused by ABT-737, which is a Bcl-xL/Bcl-2/Bcl-w inhibitor, was completely prevented in Bim/Bid double knock-out mice. The BH3-only proteins Bim and Bid are functionally active but are restrained by the anti-apoptotic Bcl-2 family proteins under physiological conditions. Hepatocyte integrity is maintained by the dynamic and well orchestrated Bcl-2 network in the healthy liver.

Apoptosis via the intrinsic pathway, which is known as the mitochondrial pathway, is regulated by Bcl-2 family members. These members are divided into two groups as follows: core Bcl-2 family proteins, which possess three or four Bcl-2 homology domains (BH1–BH4)[2] and the Bcl-2 homology domain 3 (BH3)-only proteins (1). The former, which are multidomain proteins, are subdivided into pro- and anti-apoptotic proteins. Pro-apoptotic core Bcl-2 family members, such as Bax and Bak, serve as effector molecules of this apoptotic machinery. Upon activation, these members can form pores to permeabilize the mitochondrial outer membrane. Apoptogenic factors, such as cytochrome *c*, can then be released through this membrane into the cytosol, leading to the activation of the caspase cascade and to cellular demise (2). Anti-apoptotic core Bcl-2 family members, including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1/A1, inhibit the intrinsic pathway of apoptosis by either directly or indirectly antagonizing Bak/Bax activity (3–5). In the original rheostasis model, cellular life and death are regulated by a balance between these anti- and pro-apoptotic core Bcl-2 family proteins (6). We previously reported that the hepatocyte-specific deletion of the *bcl-x* gene resulted in spontaneous hepatocyte apoptosis, and this effect could be completely prevented by the additional deletion of the *bak* and *bax* genes (7). These findings elucidated the importance of the rheostatic balance of the core Bcl-2 family proteins in controlling hepatocyte apoptosis in the healthy liver.

The BH3-only proteins, which include at least eight members, are considered to function as pro-apoptotic sensors, and these proteins set this rheostatic balance toward apoptosis upon activation by a variety of apoptotic stimuli (8, 9). It has been reported that hepatocyte apoptosis through the activation of these BH3-only proteins is involved in the pathophysiology of various liver diseases (10–12). Alternatively, we previously reported that the slight activation of Bid, which can trigger hepatocyte apoptosis, occurs even in the healthy liver and that the inactivation of Bid partially ameliorated spontaneous hepato-

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[2] The abbreviations used are: BH1–BH4, Bcl-2 homology domains 1–4; SCID, severe combined immune deficiency; ALT, alanine aminotransferase.

cyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice (7, 13). In the present study, we focused on another BH3-only protein, Bim, which promotes hepatocyte apoptosis upon activation by free fatty acids or by reactive oxygen species in pathological settings, and we further clarified the orchestration of the Bcl-2 network, which governs hepatocyte life and death in the physiological state (10, 11, 14, 15). We found that the disruption of Bim ameliorated hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice, indicating the involvement of Bim in this hepatocyte apoptosis machinery in the healthy liver as well as that of Bid. Additionally, the deletion of both Bim and Bid prevented the massive hepatocyte apoptosis caused by the inhibition of both Bcl-xL and Mcl-1, suggesting that Bim and Bid are functionally active in the healthy liver and are essential regulators for promoting the intrinsic pathway of apoptosis in hepatocytes in the absence of anti-apoptotic Bcl-2 family proteins. Our present study unveiled the fine and dynamic Bcl-2 networks, the orchestration of which determines hepatocyte life and death in the healthy liver.

EXPERIMENTAL PROCEDURES

Mice—Mice carrying a *bcl-x* gene with two *loxP* sequences at the promoter region and a second intron (*bcl-x*$^{flox/flox}$), mice carrying an *mcl-1* gene encoding amino acids 1–179 flanked by two *lox*P sequences, and heterozygous *alb-cre* transgenic mice expressing the Cre recombinase gene under regulation of the *albumin* gene promoter have been described previously (16–18). Hepatocyte-specific Bcl-xL-knock-out mice (*bcl-x*$^{flox/flox}$*alb-cre*) (17), hepatocyte-specific Mcl-1-knock-out mice (*bcl-x*$^{flox/flox}$*alb-cre*) (13), systemic Bid-knock-out mice (*bid*$^{-/-}$) (12), and Bcl-xL/Bid double knock-out mice (*bid*$^{-/-}$*bcl-x*$^{flox/flox}$*alb-cre*) (7) have also been described previously. We purchased C57BL/6J mice from Charles River (Osaka, Japan), systemic Bim-knock-out mice (*bim*$^{-/-}$) from the Jackson Laboratory (Bar Harbor, ME), and NOD/ShiJic-*scid* Jcl mice from Clea Japan Inc. (Osaka, Japan). We generated Bcl-xL/Bim double knock-out mice (*bim*$^{-/-}$*bcl-x*$^{flox/flox}$*alb-cre*), Mcl-1/Bim double knock-out mice (*bim*$^{-/-}$*mcl-1*$^{flox/flox}$*alb-cre*), Bcl-xL/Bim/Bid triple knock-out mice (*bim*$^{-/-}$*bid*$^{-/-}$*bcl-x*$^{flox/flox}$*alb-cre*), and Bim/Bid double knock-out mice (*bim*$^{-/-}$*bid*$^{-/-}$) by mating the strains. We generated mice with a hepatocyte-specific deletion of Mcl-1 and homozygote severe combined immune deficiency (SCID) mutations (*mcl-1*$^{flox/flox}$*prkdc*$^{scid/scid}$*alb-cre*) by mating hepatocyte-specific Mcl-1-knock-out mice (*bcl-x*$^{flox/flox}$*alb-cre*) and NOD/ShiJic-*scid* Jcl mice. Genotyping of *prkdc*^{scid} gene mutation was performed by the PCR-confronting two-pair primer (PCR-CTPP) method reported previously (19). The mice were maintained in a specific pathogen-free facility and were afforded humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Histological Analyses—Liver sections were stained with hematoxylin and eosin (H&E). To detect apoptotic cells, the liver sections were also subjected to staining by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) according to a procedure reported previously (20). For immunohistochemical detection of cleaved caspase-3, the liver sections were incubated with the polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA) according to a procedure reported previously (20).

Caspase-3/7 Activity—Serum caspase-3/7 activity was measured by a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega) according to the manufacturer's protocol.

Western Blot Analysis—Liver tissue was lysed in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× protein inhibitor mixture (Nacalai tesque, Kyoto, Japan), 1× phosphatase inhibitor mixture (Nacalai tesque), and phosphate-buffered saline, pH 7.4). The liver lysates were cleared by centrifugation at 10,000 × *g* for 15 min at 4 °C. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). The protein lysates were electrophoretically separated with SDS-polyacrylamide gels and were transferred onto a polyvinylidene fluoride membrane. For immunodetection, the following antibodies were used: a rabbit polyclonal antibody to Bcl-xL (Santa Cruz Biotechnology, Inc.), a rabbit polyclonal antibody to Bid, a rabbit polyclonal antibody to Bax, a rabbit polyclonal antibody to cleaved caspase-3, a rabbit polyclonal antibody to cleaved caspase-7, a rabbit polyclonal antibody to Puma (Cell Signaling Technology, Beverly, MA), a rabbit monoclonal antibody to Bad, a rabbit polyclonal antibody to Noxa (Abcam, Cambridge, MA), a rabbit polyclonal antibody to Bak (Millipore, Billerica, MA), a rabbit polyclonal antibody to Bim (Enzo Life Sciences Inc., Farmingdale, NY), a rabbit polyclonal antibody to Mcl-1 (Rockland, Gilbertsville, PA), and a mouse monoclonal antibody to β-actin (Sigma-Aldrich).

Real-time Reverse Transcription Polymerase Chain Reaction (Real-time RT-PCR) for mRNA—Total RNA was extracted from liver tissues using an RNeasy minikit (Qiagen, Valencia, CA), was reverse-transcribed, and was subjected to real-time RT-PCR as described previously (21). The mRNA expression of specific genes was quantified using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) as follows: murine *bcl2l11* (assay ID: Mm00437796_m1), murine *fas* (assay ID: Mm01204974_m1), murine *bik* (assay ID: Mm00476123_m1), murine *hrk* (assay ID: Mm01208086_m1), murine *bmf* (assay ID: Mm00506773_m1), and murine *actb* (assay ID: Mm02619580_g1 or Mm00607939_s). The transcript levels are presented as -fold inductions.

siRNA-mediated in Vivo Knockdown—The hepatocyte-specific Bcl-xL-knock-out mice (*bcl-x*$^{flox/flox}$*alb-cre*) and the Bcl-xL/Bim/Bid triple knock-out mice (*bim*$^{-/-}$*bid*$^{-/-}$*bcl-x*$^{flox/flox}$*alb-cre*) were injected with 5 mg/kg *in vivo* grade siRNA against *mcl-1* (MSS275671_e0N), which was mixed with Invivofectamine (Invitrogen), via the tail vein according to the manufacturer's protocol. The mice were sacrificed and examined as indicated by the time courses. The Stealth RNAi negative control with low GC content (Invitrogen) was used as the control.

In Vivo ABT-737 Experiment—ABT-737 was dissolved in a mixture of 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water) with pH 4–5. ABT-737 (100 mg/kg) was intraperitoneally administered to the Bim/Bid double knock-

out mice ($bim^{-/-}bid^{-/-}$) or to the Bid-knock-out mice ($bid^{-/-}$). The mice were sacrificed and examined 6 h later.

Statistical Analysis—All of the data are expressed as means ± S.D. unless otherwise indicated. Statistical analyses were performed using an unpaired Student's *t* test or a one-way analysis of variance unless otherwise indicated. When the analyses of variance were applied, the differences in the mean values among the groups were examined by Scheffe's post hoc correction unless otherwise indicated. $p < 0.05$ was considered statistically significant.

RESULTS

The Disruption of Bim Alleviated Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Bcl-xL-knock-out Mice—To investigate the involvement of the BH3-only protein Bim in the hepatocyte apoptosis caused by Bcl-xL deficiency, hepatocyte-specific Bcl-xL-knock-out mice ($bcl\text{-}x^{fl/fl}alb\text{-}cre$) were mated with systemic Bim-knock-out mice ($bim^{-/-}$). Offspring from the mating of $bim^{+/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$ mice and $bim^{+/-}bcl\text{-}x^{fl/fl}$ mice were examined at 6 weeks of age. A Western blot study confirmed the disappearance of both Bcl-xL and Bim protein expression in the liver tissue of the double knock-out mice ($bim^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$) (Fig. 1*A*). In agreement with our previous report (7, 17), H&E staining of the liver sections showed an increase in the number of hepatocytes, with chromatin condensation and cytosolic shrinkage in the liver lobules of the Bcl-xL-knock-out mice (Fig. 1*B*). The staining also showed a significant increase in TUNEL-positive cells and cleaved caspase-3-positive cells in the liver (Fig. 1, *B–D*). Consistent with these histological observations, the levels of serum caspase-3/7 activity and serum alanine aminotransferase (ALT), which can be used as indicators of hepatocyte apoptosis (22, 23), were significantly higher in the Bcl-xL-knock-out mice than in their wild-type littermates (Fig. 1, *E* and *F*). Additionally, cleaved caspse-3 and -7 were detected in the livers of the Bcl-xL-knock-out mice by Western blotting (Fig. 1*A*). All of these findings indicated spontaneous hepatocyte apoptosis in these mice. Bim-knock-out mice did not show any phenotypes in the liver under physiological conditions (Fig. 1, *B–F*). Alternatively, the disruption of Bim significantly improved all of the parameters that are indicative of hepatocyte apoptosis in Bcl-xL-knock-out mice, including the TUNEL-positive cell counts, cleaved caspase-3-positive cell counts, serum ALT levels, and serum caspase-3/7 activity (Fig. 1, *B–F*). These findings clearly demonstrated that Bim was involved in the hepatocyte apoptosis caused by Bcl-xL disruption. It should be noted that the gene and protein expression levels of Bim were not different between the Bcl-xL-knock-out mice and their wild-type littermates (Fig. 1, *A* and *G*), indicating that the Bim expression levels observed in the healthy liver could induce hepatocyte apoptosis in the absence of the Bcl-2 family proteins.

The Disruption of Bim Alleviated Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Mcl-1-knock-out Mice—Of the five members of the anti-apoptotic Bcl-2 family proteins, we previously reported that Mcl-1 and Bcl-xL played a pivotal anti-apoptotic role in maintaining hepatocyte integrity in the healthy liver (13). We thus examined the role of Bim in the hepatocyte apoptosis caused by Mcl-1 deficiency. We generated Mcl-1/Bim double knock-out mice ($bim^{-/-}mcl\text{-}1^{fl/fl}alb\text{-}cre$) by mating the hepatocyte-specific Mcl-1-knock-out mice ($mcl\text{-}1^{fl/fl}alb\text{-}cre$) with the systemic Bim-knock-out mice ($bim^{-/-}$). A Western blot study confirmed the disappearance of both Mcl-1 and Bim protein expression in the liver tissue of the double knock-out mice ($bim^{-/-}mcl\text{-}1^{fl/fl}alb\text{-}cre$) (Fig. 2*A*). Consistent with our previous report (13), hepatocyte-specific Mcl-1-knock-out mice showed apoptosis phenotypes very similar to those of the Bcl-xL-knock-out mice, as assessed by TUNEL staining (Fig. 2, *B* and *C*), cleaved caspase-3 staining (Fig. 2, *B* and *D*), serum caspase-3/7 activity (Fig. 2*E*), and serum ALT levels (Fig. 2*F*). In contrast, Mcl-1/Bim double knock-out mice showed significant improvement in these parameters (Fig. 2, *B–F*), indicating that Bim is also involved in the hepatocyte apoptosis induced by the disruption of Mcl-1.

The Disruption of Bim and Bid Prevented Spontaneous Hepatocyte Apoptosis in Hepatocyte-specific Bcl-xL-knock-out Mice—We previously reported that a small amount of Bid, which is another BH3-only protein, was constitutively active and was involved in the spontaneous hepatocyte apoptosis in Bcl-xL- or Mcl-1-knock-out mice (7, 13). We thus examined whether these BH3-only proteins redundantly or cooperatively promoted hepatocyte apoptosis in the absence of Bcl-xL. To this end, Bim/Bid/Bcl-xL triple knock-out mice ($bim^{-/-}bid^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$) were generated by mating the Bim/Bcl-xL double knock-out mice ($bim^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$) with the Bid/Bcl-xL double knock-out mice ($bid^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$). The offspring from the mating of $bim^{+/-}bid{-}/{-}bcl\text{-}x^{fl/fl}alb\text{-}cre$ mice with $bim^{+/-}bid^{-/-}bcl\text{-}x^{fl/fl}$ mice were examined at 6 weeks of age. A Western blot study confirmed that Bcl-xL, Bid, and Bim protein expression disappeared from the liver tissue of the triple knock-out mice ($bim^{-/-}bid^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$) (Fig. 3*A*). Liver sections of the Bim/Bid/Bcl-xL triple knock-out mice were histologically normal compared with those of the Bid/Bcl-xL double knock-out mice ($bim^{+/+}bid^{-/-}bcl\text{-}x^{fl/fl}alb\text{-}cre$), which still contained some hepatocytes with apoptotic morphologies (Fig. 3*B*). Both the number of TUNEL-positive cells and the serum caspase-3/7 activity in the triple knock-out mice were significantly lower than those in the Bid/Bcl-xL double knock-out mice and did not differ from their control Bid-knock-out or Bim/Bid double knock-out littermates (Fig. 3, *B–D*). Moreover, in contrast to the mild elevation of serum ALT levels in the Bid/Bcl-xL double knock-out mice, the levels in the triple knock-out mice were completely normal (Fig. 3*E*). These findings demonstrated that hepatocyte apoptosis in the absence of Bcl-xL was completely dependent on these two BH3-only proteins.

Bim and Bid Are Essential Regulators for the Promotion of the Intrinsic Pathway of Apoptosis in Hepatocytes in the Absence of Anti-apoptotic Bcl-2 Family Proteins—We then attempted to further examine the involvement of Bim and Bid in hepatocyte apoptosis in the absence of both Bcl-xL and Mcl-1, which are two major anti-apoptotic proteins in the liver. Because, as we reported (13), the hepatocyte-specific Bcl-xL and Mcl-1 double knock-out mice died within 1 day after birth due to impaired liver development, we performed an siRNA-mediated *in vivo* knockdown of *mcl-1* in the Bcl-xL-knock-out mice and in the Bim/Bid/Bcl-xL triple knock-out mice. *mcl-1* siRNA administration efficiently reduced Mcl-1 protein expression in the liver

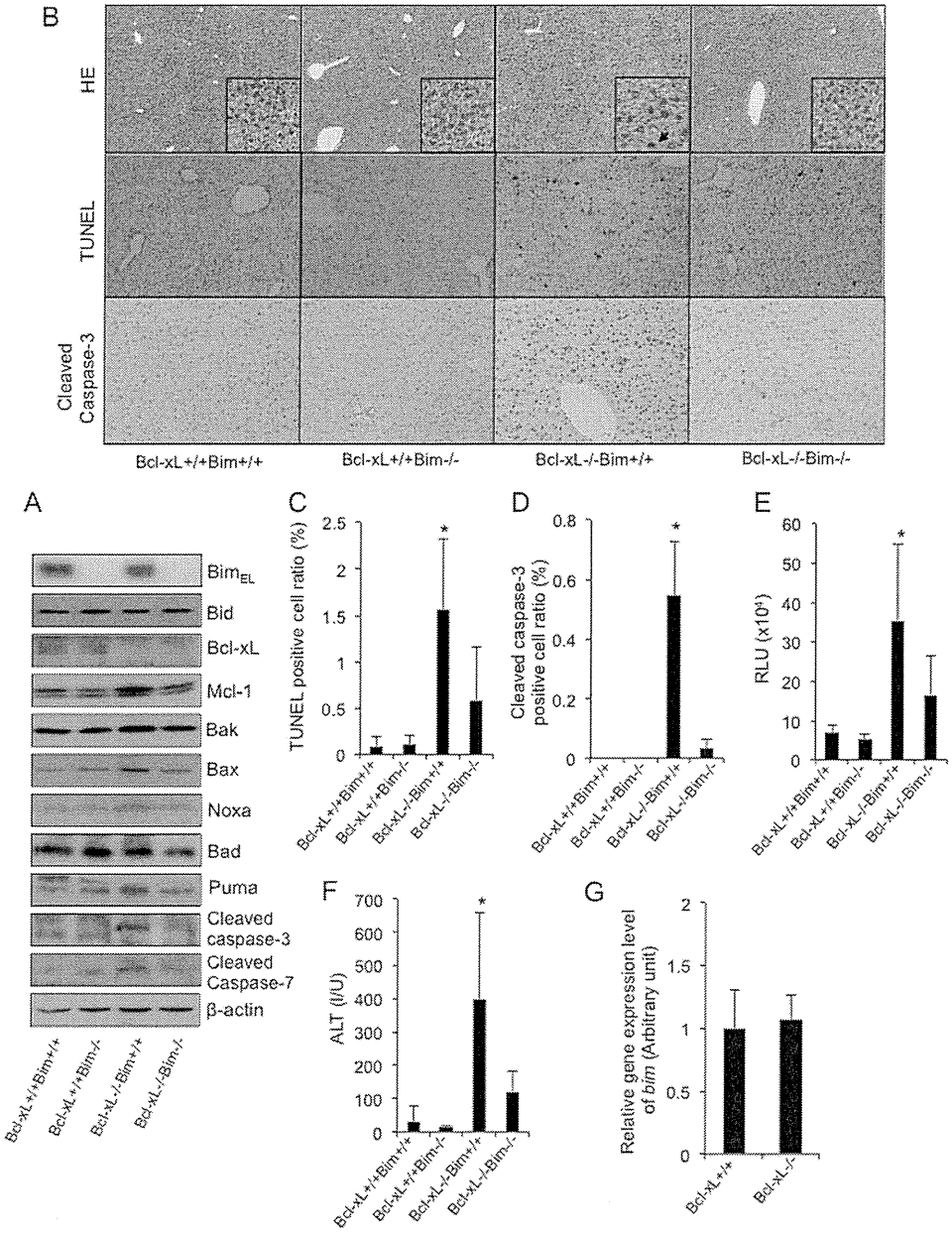

FIGURE 1. **The disruption of Bim alleviated spontaneous hepatocyte apoptosis in the absence of Bcl-xL.** *A–F*, the offspring from the mating of *bim*±*bcl-x$^{flox/flox}$alb-cre* mice with *bim*±*bcl-x$^{flox/flox}$* mice were examined at 6 weeks of age. *Bcl-xL$^{+/+}$* and *Bcl-xL$^{-/-}$*, *bcl-x$^{flox/flox}$* and *bcl-x$^{flox/flox}$alb-cre*, respectively. *A*, Western blot analysis of whole liver lysates for the expression of Bim$_{EL}$, Bid, Bcl-xL, Mcl-1, Bak, Bax, Noxa, Bad, Puma, cleaved caspase-3, cleaved caspase-7, and β-actin. *B*, representative images for liver histology stained with hematoxylin-eosin (*HE*), TUNEL, and cleaved caspase-3 (original magnifications, ×100 (*large panels*) and ×400 (*insets*)); *black arrows* indicate apoptotic bodies. *C*, TUNEL-positive cell ratio; $n = 8$ mice/group; *, $p < 0.05$ *versus* all. *D*, cleaved caspase-3-positive cell ratio; $n = 3$ mice/group; *, $p < 0.05$ *versus* all. *E*, serum caspase-3/7 activity; $n = 11$ mice/group; *, $p < 0.05$ *versus* all. *F*, serum ALT levels; $n = 13$ mice/group; *, $p < 0.05$ *versus* all. *G*, offspring from the mating of *bcl-x$^{flox/flox}$alb-cre* mice with *bcl-x$^{flox/flox}$* mice were examined at 6 weeks of age. *Bcl-xL$^{+/+}$* and *Bcl-xL$^{-/-}$*, *bcl-x$^{flox/flox}$* and *bcl-x$^{flox/flox}$alb-cre*, respectively. *bim* mRNA levels in the whole liver tissue were determined by real-time RT-PCR; $n = 6$ mice/group. *Error bars*, S.D. *RLU*, relative light units; *I/U*, international units.

tissue of both mice (Fig. 4*A*), but it caused severe liver injury only in the Bcl-xL-knock-out mice (Fig. 4*B*) when assessed by the H&E staining of liver sections. Notably, *mcl-1* siRNA administration caused massive hepatocyte apoptosis in the Bcl-xL-knock-out mice, but this apoptosis was weakened in the Bim/Bid/Bcl-xL triple knock-out mice, as evidenced by the TUNEL staining of the liver sections, serum caspase-3/7 activity, and serum ALT levels (Fig. 4, *C–E*). In agreement with these findings, *mcl-1* siRNA treatment impaired the liver function of the Bcl-xL-knock-out mice, as evidenced by an increase in the serum bilirubin levels, but not the liver function of the triple knock-out mice (Fig. 4*F*). These findings demonstrated that the massive hepatocyte apoptosis and liver failure caused by decreases in these anti-apoptotic Bcl-2 family proteins were dependent on Bid and Bim.

The Presence of Bim- and Bid-induced Constant BH3 Stress in the Healthy Liver Causes Hepatotoxicity with the Use of Anti-cancer Agents That Target the Anti-apoptotic Bcl-2 Family Proteins—Recent advances in cancer therapy have enabled the selective targeting of some anti-apoptotic Bcl-2 family proteins,