shown to promote metastases or tumor growth after re-challenge with primary tumor cells [46, 65, 68]. Therefore, augmentation of NK-mediated innate immune responses may be an attractive strategy for preventing HCC recurrence.

Recently, not only in nude mice but in immunocompetent mice, we have found that adenovirally-delivered monocyte chemoattractant protein (MCP)-1 highly augmented the antitumor effects of the HSV-tk/GCV system [69]. In an immunocompetent mouse model of colon cancer, subcutaneous tumor foci were directly transduced with both rAds expressing an HSV-tk gene and one of the MCP-1, CD80 and LacZ genes, followed by GCV administration. The growth of tumors was markedly suppressed by codelivery of HSV-tk and MCP-1 genes, which was exclusively associated with the recruitment of monocytes/macrophages, T helper 1 (Th1) cytokine gene expression and cytotoxic activity of the splenocytes. Furthermore, the antitumor effects were more efficient than that obtained by the combination of HSV-tk and CD80 genes. These results suggest an immunomodulatory effect of MCP-1 in the context of suicide gene therapy via orchestration of innate and acquired immune responses.

Perspective

Although the findings presented here are promising, a number of problems remain to be solved before this approach can be used clinically. In general, the presence of rAd vector immunity has impeded clinical use of these vectors [70]. Innate immune responses to the vector particles drastically limit the vector transduction efficiency and the duration of transgene expression. However, in cancer gene therapy, transgene expression for a short duration may be enough for the desired effects. Induction of proinflammatory cytokines and chemokines by rAd vectors might not be a limitation in every situation since rAd vectors can efficiently transduce a broad range of cell types. In terms of the safety of replication-defective adenovirus vectors [71], although the clinical trial designed to correct partial ornithine transcarbamylase deficiency was overshadowed by the death of a patient at the highest dose [72, 73], the first gene therapy treatment for cancer was approved in 2004 [74]. A replication-defective Ad5 vector expressing p53 from a Rous sarcoma virus (RSV) promoter, Gendicine (SiBiono, Shenzen, China), is now approved for use in head and neck squamous cell carcinoma, glioma [75], non-small cell lung cancer [76], bladder carcinoma [77], and ovarian cancer [78]. The use of an E1-deleted adenovirus expressing the HSV-tk shows promise for glioma [79, 80] and prostate cancer [81]. Finally, we look forward toward how these new insights into the development of gene therapy will impact on therapeutic options for cancer in the near future.

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最新医学・別冊　新しい診断と治療のABC 50 （別刷）

消化器 7　肝　癌

血液検査

江口潤一　　広石和正　　井廻道夫

最新医学社

第3章 診 断

血液検査

要旨

肝細胞癌のほとんどが肝硬変あるいは線維化の進行した慢性肝炎を合併している．したがって，肝癌患者においては比較的早期より，血液生化学検査による背景肝で，肝硬変や慢性肝炎のパターンを示しているケースが多い．肝機能の悪化，肝炎や肝硬変の進行が発癌のリスクを高める要因になることから，慢性肝炎や肝硬変の患者では血液検査を含めた厳重な経過観察が必要である．

はじめに

我が国の原発性肝癌において95％は肝細胞癌であり，慢性肝炎や肝硬変などの慢性肝疾患を背景に発症する．そして，肝細胞癌の80％以上が肝硬変あるいは線維化の進行した慢性肝炎を合併している．したがって，肝細胞癌患者は腫瘍が小さい比較的早期より，血液生化学検査において肝硬変や慢性肝炎のパターンを示していることがほとんどである．その後腫瘍が増大して進展すると，それに従い血液生化学検査も増悪し，黄疸や血清酵素，腫瘍マーカーの上昇などがみられる．

本稿では前癌病変と言える慢性肝疾患，特に肝硬変における血液生化学検査の特徴と肝細胞癌の腫瘍マーカーについて述べる．

● キーワード

肝細胞癌
血液検査
慢性肝疾患
肝硬変
腫瘍マーカー

血液検査

肝硬変の症例においては門脈圧亢進症に伴い脾腫を認めることが多く，脾機能亢進による汎血球現象，特に血小板減少と白血球減少を高頻度に認める．しかし，肝癌合併例では白血球増多も少なからずみられる．また，腫瘍随伴症候群としてまれに赤血球増多症を認めることがある．

血小板数については慢性肝炎において肝線維化ステージとよく相関することが知られており，12万/μl以下になると肝硬変の割合が高くなる．また，C型慢性肝炎においては肝線維化ステージの進展に伴い発癌率が上昇することが知られており，F1が0.5％，F2が1.5％，F3が3～4％，そして肝硬変であるF4は7～8％である．すなわち，これらの結果を組み合わせることにより血小板数から肝線維化の進展度と年間発癌率の推定が可能で，肝癌の高危険群の推定が可能である．C型肝細胞癌の発見時において約60％以上の症例で血小板数は10万/μl以下である．

生化学検査

1．逸脱酵素

アスパラギン酸アミノトランスフェラーゼ（AST，またはGOT），アラニンアミノトランスフェラーゼ（ALT，またはGPT），乳酸脱水素酵素（LDH）は肝細胞内に存在する酵素で，肝細胞の障害がみられると血液中に漏れ出てくる．これらの酵素を逸脱酵素と呼び，血液中の濃度を測定することによって肝細胞障害の程度を推測することができる．

1）AST，ALT，AST/ALT比

AST，ALTはビタミンB_6を補酵素とするアミノ基転移酵素であり，この2つの酵素を合わせてトランスアミナーゼと呼ぶ．ASTは肝以外でも筋肉や赤血球にも含まれるため，ASTの上昇だけで肝障害があるとは言えない．また，ASTが肝細胞全体に均一分布するのに対し，ALTは門脈域周囲の肝細胞に存在するとされている．また，ASTとALTでは血中の半減期が異なる．これらの理由で種々の肝疾患によりAST，ALTの比率が異なるため，肝疾患診断の大きな助けとなる．正常肝ではAST＞ALTでいずれの酵素も正常値を示すが，慢性肝炎，脂肪肝，急性肝炎の回復期などではAST＜ALTの比率でトランスアミナーゼ上昇を認める．一方で，肝硬変，肝癌，アルコール性肝障害，急性肝炎極期，劇症肝炎などではAST＞ALTの比率でトランスアミナーゼの異常値を示す．

一般的に肝癌においてはトランスアミナーゼの絶対値の上昇は肝硬変と同様に軽度である場合が多い．また，肝硬変と比較して，より

AST 値が優位となることが特徴的であり，AST / ALT の比が 3 以上を呈することもしばしば認める．すなわち，肝硬変の経過観察中に AST / ALT 比の上昇がみられたら肝癌合併の可能性があることを考慮しなければならない．

2）LDH

LDH は肝疾患以外でも心疾患（特に心筋梗塞の際のマーカーになる），骨格筋疾患（筋炎），血液疾患（白血病，悪性貧血，溶血など），感染症，悪性腫瘍などの場合にも上昇する．LDH はアイソザイムを測定することにより，LDH_1，LDH_2 が多い場合には心筋，血液疾患，LDH_4，LDH_5 が多い場合には肝細胞，骨格筋障害を考える．

2．胆道系酵素

肝癌により腫瘍占拠部位の肝内胆管が閉塞して胆汁排泄が障害されると，胆汁うっ滞の血清マーカーであるγ－グルタミルトランスフェラーゼ（γ－GT），アルカリホスファターゼ（ALP），ロイシン・アミノペプチターゼ（LAP）などが上昇する．したがって，前述の AST / ALT 比の上昇時と同様に，肝硬変の経過観察中にこれら胆道系酵素の上昇がみられたら肝癌合併の可能性を考えなくてはならない．

1）γ－GT

γ－GT は肝細胞の小胞体で合成され，一部は胆汁中に分泌されるため肝細胞の毛細血管膜や胆管上皮に分布する．肝細胞の機能異常，特に胆汁分泌異常と小胞体機能異常を認めるときに異常値を示す．γ－GT は肝癌において上昇を示すが通常著増することはまれである．肝細胞癌で産生される血清γ－GT は正常の 4 倍ほどの糖鎖を含んでおり，糖鎖の約半分が N－アセチルグルコサミン残基を持っているとされる．この分画はポリアクリルアミドゲル電気泳動で検出される．

2）ALP

ALP は肝胆道疾患のほか，骨疾患，内分泌疾患でも上昇することがあり，電気泳動法で分析すると ALP1 ～ 6 および ALPI の 7 つのアイソザイムに分けることができる．肝癌では主に ALP1，ALP2 または ALP5 の上昇がみられ，特に ALP2 の増加が著明である．ALP2 は肝性 ALP と呼ばれ，肝細胞毛細胆管膜，胆管細胞膜に局在している．成人健常者の血清 ALP の主体をなし，胆管に何らかの障害が及べば

肝での合成が亢進して血中で増加していく．ALPIはポリアクリルアミドゲル電気泳動で検出されるアイソザイムであり，肝癌 ALP (variant ALP) とも呼ばれる．肝細胞癌により産出され肝細胞癌の腫瘍マーカーとなりうる．ALPIの肝細胞癌での陽性率は14～31％と高くはないが，特異度は極めて高いとの報告がある．

3．血清ビリルビン

赤血球中のヘモグロビンはヘムに代謝された後にビリルビンとなって胆汁中に排泄される．ビリルビンには肝でグルクロン酸抱合を受ける前の間接（非抱合型）ビリルビンと，抱合を受けた後の直接（抱合型）ビリルビンとがある．血清総ビリルビンが3 mg / dl 以上に上昇すると（基準値は1 mg / dl 以下）眼球結膜や皮膚に黄疸がみられるようになる．黄疸が認められる疾患としては肝疾患や胆道系疾患のほか，溶血性貧血などの血液疾患や不適合輸血，感染症，または体質性黄疸が挙げられる．肝炎などの肝細胞障害による黄疸では直接と間接の両方のビリルビンが上昇するが，一般には直接ビリルビンが優位で，末期の肝硬変や劇症肝炎では間接ビリルビンが優位となる．肝癌により胆管に閉塞を来すと，直接ビリルビンの上昇がみられる．

4．タンパク合成能

免疫グロブリンを除いてほとんどすべての血漿タンパクは肝細胞で合成される．肝硬変によるタンパク合成能低下の結果，血清アルブミン，コリンエステラーゼ，各種血液凝固タンパクなどの成分の減少が認められる．

1）アルブミン

アルブミンは肝で合成され血液中に分泌されるタンパク質で，血漿浸透圧の維持や血液中で多くの物質の運搬に関与する．進行した慢性肝炎や肝硬変，劇症肝炎ではアルブミン合成能の低下から血清アルブミン値が減少する．血清アルブミンはほかに，ネフローゼ症候群，栄養障害，タンパク漏出性胃腸症，悪性腫瘍などの疾患でも低下する．

2）コリンエステラーゼ（CHE）

CHE も肝で合成されるタンパク質で，肝タンパク合成能の指標になる．アルブミンと同様に肝硬変や劇症肝炎で低下する．一方，肥満による脂肪肝では上昇する．

3）プロトロンビン時間（PT），ヘパプラスチンテスト（HPT）

第Ⅷ因子以外の凝固因子は肝臓で合成される．PT にはフィブリノーゲン，プロトロンビン，第Ⅴ因子，第Ⅶ因子，第Ⅹ因子が関与するが，これらの凝固因子は血中の半減期が5時間～3日間と短いため，PT を測定することにより検査時の肝タンパク合成能を鋭敏に反映する．肝硬変や劇症肝炎ではタンパク合成能の低下から，また閉塞性黄疸時にはビタミンKの吸収障害から凝固因子（第Ⅱ，Ⅶ，Ⅸ，Ⅹ因子）の合成が阻害されるため，PT の延長（パーセントは低下）がみられる．

HPT は PT と同じく凝固因子（第Ⅱ，Ⅶ，Ⅹ因子）を反映するが，ビタミンK欠乏時に誘導されるタンパクの影響を取り除き，外因系凝固機序異常，特に肝凝固因子合成能の低下を検出できるよう改良された検査である．

5．タンパク分画，A/G 比

肝硬変ではγ-グロブリンの多クローン性の増殖を認める．さらに，アルブミンの低下などが加わり電気泳動による分画の特徴的変化がみられる．硫酸亜鉛混濁反応（ZTT）やチモール混濁反応（TTT）などの膠質反応の上昇もこれらの変化により間接的にみられる現象である．A/G比とは，血清中に存在するアルブミン値とグロブリン値の比率のことで，慢性肝疾患ではアルブミンが低下しグロブリンは上昇するため，A/G比は低下する．

6．血清アミノ酸分析

肝硬変ではバリン，ロイシン，イソロイシンのような分枝鎖アミノ酸（BCAA）の低下とフェニルアラニン，チロシンのような芳香族アミノ酸（AAA）の増加をみる．両者のモル比（BCAA / AAA）であるフィッシャー比は肝障害の重症度に相関して低下する．

7．線維化マーカー

Ⅲ型プロコラーゲンN末端ペプチド（PⅢP），Ⅳ型コラーゲン，ヒアルロン酸などの線維化マーカーの上昇が肝硬変においてみられる．肝細胞癌や転移性肝癌におけるⅣ型コラーゲンの高値は癌の進展に伴う脈管への浸潤や破壊，癌内部のコラーゲン代謝の亢進を反映していると考えられている．

8．腫瘍随伴症候群

肝細胞癌患者においては，しばしば腫瘍随伴症候群を示すことがある．頻度の順に高コレステロール血症，低血糖，多血症，高カルシウム血症がみられる．

腫瘍マーカー

1．AFP

αフェトプロテイン（AFP）は代表的な癌胎児抗原の1つであり肝細胞癌に最も古くから使用されている重要な腫瘍マーカーである．成人において1,000 ng / ml を超える症例や月単位で連続上昇をみる症例では肝細胞癌の疑い濃厚であると考えられる．肝細胞癌症例中AFP 陽性（15 ng / ml 以上）を示した症例は約65％であり，報告されている腫瘍マーカーの中では最も陽性率は高い．しかし，定期的な画像スクリーニングによって発見されるような小肝癌においてAFP値が高値であることはまれであり，画像診断の進歩によりAFP の肝癌診断における地位は近年低下している．また，AFP は肝癌以外の慢性肝炎などの良性疾患においてもしばしば上昇を認めることがある．しかし，画像検査にて肝細胞癌が認められなくても，AFP 陽性例は発癌の危険因子とされている．肝硬変や慢性肝炎での陽性例，特にB型肝炎では，軽度の慢性肝炎例やトランスアミナーゼ正常例からの発癌もみられることから，厳重な経過観察が必要であると考えられる．

2．AFP－L3

AFP はレンズ豆レクチンを用いたレクチン親和電気泳動により3本のバンドに分画される．肝硬変などの良性肝疾患ではレクチン非結合型のAFP－L1 バンドが主であり，肝細胞癌になるとレクチン結合型のAFP－L3 バンドが増加してくる．肝癌におけるAFP－L3 の陽性率は40％程度とあまり高くはない．また，AFP 中の濃度を測定するため，AFP がカットオフ以下の症例での陽性率は数％とされ，感度は低くAFP 陽性例での測定が望ましい．しかし，その反面特異度は90％以上と非常に高く薬剤などの影響も受けない．また，AFP－L3 陽性例は陰性例に比べ進展度が速いため予後が悪く，また低分化型の肝細胞癌である特徴を持つとされる．さらに，AFP－L3 陽性例

は初発であってもすでに肝内転移を示すことが多く，AFP－L3は悪性度の指標として優れた腫瘍マーカーであると言える．

3．PIVKA－Ⅱ

ビタミンK依存性凝固因子前駆物質（PIVKA－Ⅱ）は肝臓で合成される凝固活性を持たない異常プロトロンビンで，des γ carboxy prothrombin（DCP）とも呼ばれる．肝細胞癌症例での PIVKA－Ⅱ（40 mAU／m*l* 以上）の陽性率は60％弱と報告されている．また，AFP と同様に小肝癌においての陽性率は高くないが，慢性肝炎や肝硬変などの良性肝疾患での陽性例はほとんど認められず，肝細胞癌において90％以上の特異性があるとされている．注意点としては閉塞性黄疸や肝内胆汁うっ滞などでビタミンK欠乏を来した場合，あるいはビタミンKサイクルを阻害するようなワルファリンや N－methyltetrazolethiol（MTZ）基を有するセフェム系抗生物質の投与例で陽性となることが挙げられる．PIVKA－Ⅱは肝細胞癌の予後を左右する門脈浸潤の最も有用な予測因子であるとの報告があり，PIVKA－Ⅱ陽性群は陰性群に比べて生存率が低い傾向にあり，予後予測の有用な指標になると考えられる．

肝細胞癌での AFP（AFP－L3）値と PIVKA－Ⅱ値との相関はなく，両者は互いに相補的な関係にある．両者を同時に測定することにより，AFP（AFP－L3）低値ないし陰性例は PIVKA－Ⅱで，逆に PIVKA－Ⅱ陰性例は AFP（AFP－L3）でカバーすることで肝細胞癌の診断を高めることができる．肝癌診療ガイドラインでも，小肝癌において2種類以上の腫瘍マーカーを測定することが，特異度の低下を最小限に抑えつつ感度を上昇させるとして，グレードAに推奨されている．

おわりに

以上，肝癌における血液検査の特徴を特に肝硬変における変化を中心に述べてきた．前述のように肝癌のほとんどが慢性肝疾患を合併しており，肝機能の悪化，肝線維化などにより肝癌発癌のリスクが高まることが明らかにされている．ウイルス性肝炎患者などの高危険群の患者では定期的な血液検査を施行し，肝癌の存在を示唆する小さな変化も見逃さずに経過観察をすることが必要である．また，グリピカン

3などの新たな肝癌のマーカーも研究が進められており，今後の臨床応用が期待される．

江口潤一・広石和正・井廻道夫

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Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNα gene to mice

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Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. To evaluate the therapeutic efficacy and mechanisms of IFNα on hepatic metastasis of tumor cells, we hydrodynamically injected naked plasmid DNA encoding IFNα1 (pCMV-IFNa1) into Balb/cA mice having 2 days hepatic metastasis of CT-26 cells. Single injection of pCMV-IFNa1 efficiently enhanced the natural killer (NK) activity of hepatic mononuclear cells, induced production of IFNγ in serum and led to complete rejection of tumors in the liver. Mice protected from hepatic metastasis by IFNα therapy displayed a tumor-specific cytotoxic T cell response and were resistant to subcutaneous challenge of CT-26 cells. NK cells were critically required for IFNα-mediated rejection of hepatic metastasis, because their depletion by injecting anti-asialo GM1 antibody completely abolished the antimetastatic effect. To find whether NK cells are directly activated by IFNα and are sufficient for the antimetastatic effect, the responses to IFNα were examined in SCID mice lacking T cells, B cells and NKT cells. IFNα completely rejected hepatic metastasis in SCID mice and efficiently activated SCID mononuclear cells, as evidenced by activation of STAT1 and a variety of genes, such as MHC class I, granzyme B, tumor necrosis factor-related apoptosis-inducing ligand and IFNγ, and also enhanced Yac1 lytic ability. Study of IFNγ knockout mice revealed that IFNγ was not necessary for IFNα-mediated NK cell activation and metastasis protection. In conclusion, IFNα efficiently activates both innate and adaptive immune responses, but NK cells are critically required and sufficient for IFNα-mediated initial rejection of hepatic metastasis of microdisseminated tumors.

Key words: DNA; innate; adaptive; immunity NK

The liver is the most common site of metastatic malignancy and the status of this organ is an important determinant of survival in patients with advanced disease. The risk of hepatic metastasis remains high in many patients after potentially curative surgery at primary sites.[1] This suggests that the spread of tumor cells can occur in the liver even when they cannot be detected by current diagnostic modalities. To suppress the incidence of liver metastasis, whole liver therapy against microdisseminated tumors should be considered.[2] Since the liver contains an abundance of immune cells, the cytokine-meditated activation of these cells may be a promising approach toward this end.[3,4]

Interferon (IFN) α is a pleiotropic cytokine acting as an antiviral substance, cell growth inhibitor and immunomodulator. IFNα as well as IFNγ are primarily induced in response to viral infection of cells and ligate a cognate receptor for the Type 1 IFN expressed on target cells.[5] On the other hand, Type 2 IFN, IFNγ, is produced predominantly by T lymphocytes, natural killer (NK) cells and NKT cells and uses a distinct receptor. IFNα-mediated antiviral activity includes induction of 2′-5′ oligoadenylate synthetases, double-stranded RNA-activated protein kinase (PKR) and Mx proteins. IFNα can exert direct effects on tumor cells by inhibiting proliferation, inducing apoptosis and inhibiting the release of proangiogenic factors such as vascular endothelial growth factor.[6] IFNα-mediated immunomodulation includes dendritic cell maturation, NK cell activation, MHC Class I induction and cytokine production.[7] Most, if not all, of these actions are mediated by the Jak-STAT signaling pathway downstream of the Type 1 IFN receptor.[8–10] Type 1 IFN receptor upon ligand ligation phosphorylates Jack1 and then phosphorylates STAT1, which activates a variety of IFN-regulated genes. IFNα and IFNβ have been shown to elicit antitumor effects in various murine models of cancer.[11–14] IFNβ was also shown to be effective for retarding metastatic tumor growth in murine liver, but the underlying mechanisms have not been elucidated.[15]

In the present study, we investigated the efficacy of hydrodynamics-based expression of IFNα in the liver against a murine model of hepatic metastasis of CT-26 colon cancer cells and the mechanisms of an IFNα-mediated therapeutic effect of hepatic metastasis. Mice treated with IFNα completely rejected hepatic metastasis and became resistant to rechallenge by CT-26 cells. Although IFNα induced a variety of host responses including increased NK activity, increased IFNγ production and tumor-specific T cell responses, the initial rejection of hepatic metastasis was solely dependent on NK cells. Our study has shed light on NK cell activation as an important mechanism by which IFNα ablates microdisseminated tumors in the liver.

Material and methods

Mice

Specific pathogen-free female Balb/cA mice, SCID mice and their wild-type control mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/cA background was kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo).[16] All mice were used at the age of 5 to 8 weeks. They 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 the study protocol complied with the institution's guidelines.

Tumor models

Intrasplenic injection of tumor cells was used to establish microdisseminated liver tumors in mice.[17] CT-26 colon cancer cells originating from Balb/cA mice were maintained in DMEM supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 150 μl of PBS and injected into the spleen. For subcutaneous tumor models, CT-26 cells (5×10^5) were injected into the back of the mice under light anesthesia.

NK cell depletion

For depletion of NK cells *in vivo*, anti-asialo GM1 antibody (Wako, Osaka, Japan) was intraperitoneally administered.[17] We

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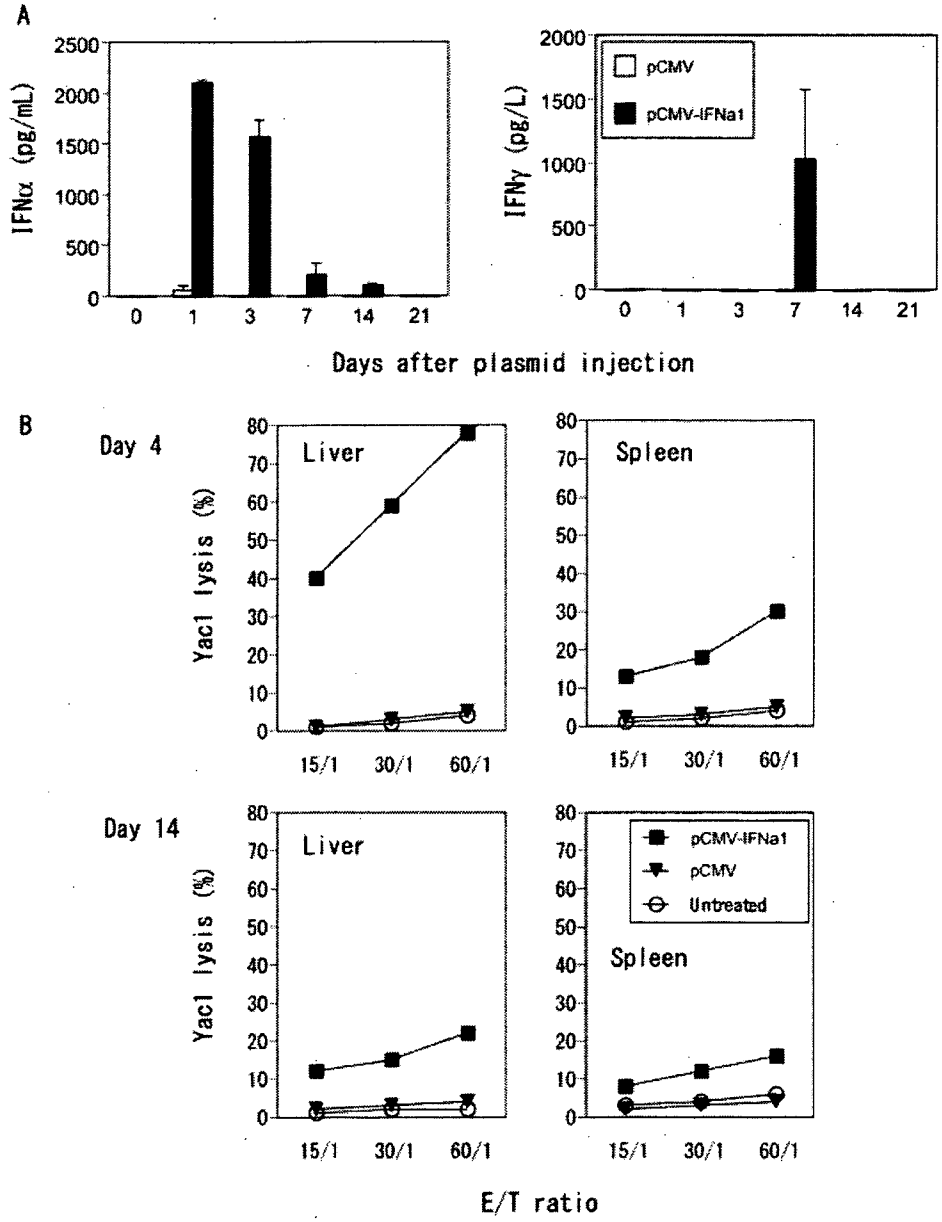

FIGURE 1 – Effects of hydrodynamic injection of IFNα-expressing plasmid. (*a*) Serum IFNα and IFNγ concentration. Balb/cA mice were hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) and bled at indicated time points to measure the levels of serum IFNα and IFNγ. The results are indicated as mean and SD (n = 3/group). Shown are representative data for 2 independent experiments. (*b*) Yac1 lytic ability. Hepatic or splenic mononuclear cells were isolated from naive Balb/cA mice (open circles) and those injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Yac1 lytic ability was measured by a standard chromium-release assay at indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown.

determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. Injection of this dose of anti-asialo GM1 antibody depleted more than 95% of DX-5 positive, TCRβ-negative cells (NK cells) in the liver. NKT cells were less affected than NK cells, because 40% of Cd1d-tetramer positive cells, which are invariant NKT cells, still remained in the liver after the treatment. Anti-asialo GM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Injection of naked plasmid DNA

A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr. Daniel J. J. Carr (University of Oklahoma, Health Science Center).[18] Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described.[19] In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 sec.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IFNα and IFNγ were measured using commercially available ELISA kits (Biomedical Laboratories for murine IFNα; Endogen for murine IFNγ).

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described.[20] The NK activity of mononuclear cells was assessed with standard 4-hr ^{51}Cr-releasing assay using Yac1 cells as targets. To examine CT-26-specific responses, splenocytes were stimulated with CT-26 cells for 5 days in the presence of 30 U/ml

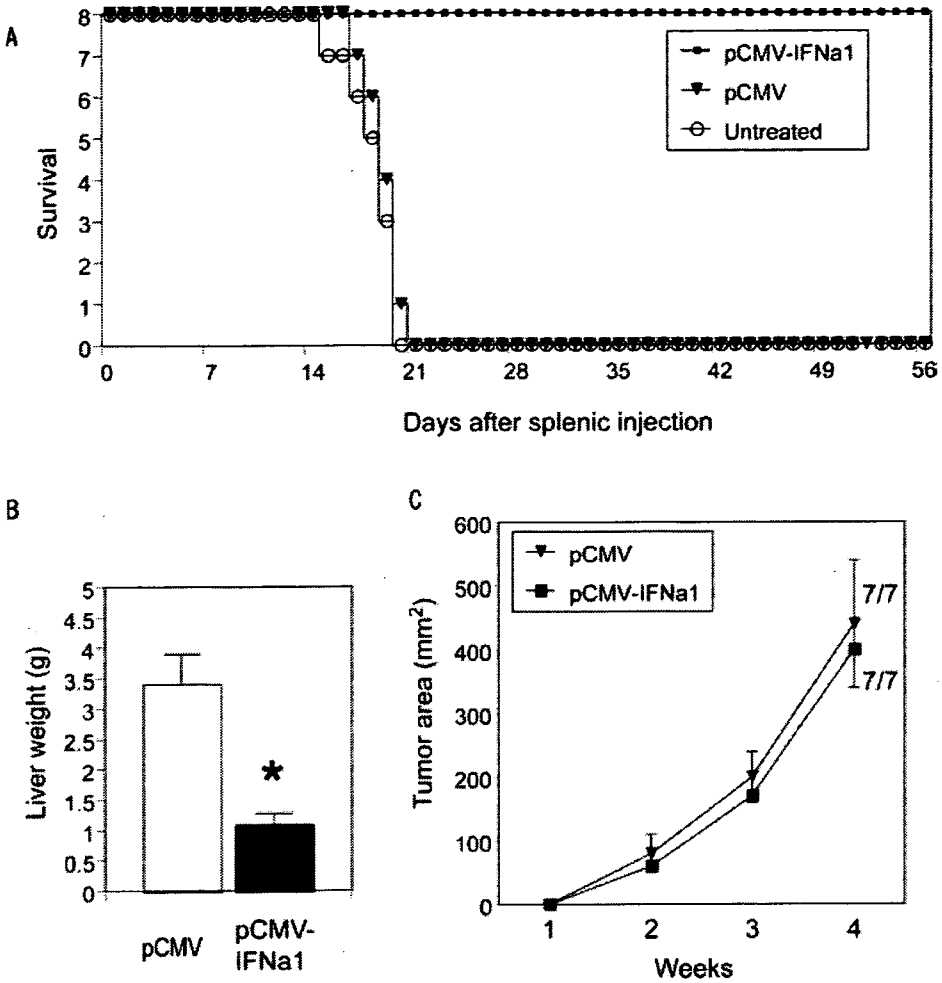

FIGURE 2 – Anti-tumor effects of IFNα therapy. (*a*) Survival. Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomly assigned to 3 groups and received hydrodynamic injection of either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) or untreated (open circles). The number of survivors in each group was monitored. (*b*) Anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. At 14 days after the splenic injection, the mice were sacrificed to examine liver tumor development by measuring liver weight. All experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*c*) Anti-tumor effects on subcutaneous tumors. Balb/cA mice were subcutaneously injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles) 2 days later. Tumor growth was examined every week. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 4 weeks is shown in the figure.

of murine IL-2 and subjected to analysis for lytic activity against CT-26 cells or BNL A.7 murine hepatoma cells by 4-hr ^{51}Cr-releasing assay. In some experiments, mononuclear cells were separated into CD90-positive cells (T cells) and CD90-negative cells (non-T cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Western blotting

Mouse recombinant IFNα was generously provided by Fujisaki Institute, Hayashibara Biochemical Laboratories (Okayama, Japan). Mononuclear cells were treated with or without IFNα. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was stained with anti-STAT1 antibody (Upstate Biotechnology, Lake Placid, NY) or antiphospho-sepcific STAT1 (Y701) antibody (Upstate Biotechnology) and visualized by chemiluminescence. The specificities of STAT1 and phosphorylated STAT1 signals were confirmed by siRNA experiment using BNL A.7 cells in the presence or absence of IFNα treatment (data not shown). Anti-STAT antibody recognizes STAT1α, whereas antiphospho-STAT1 antibody recognizes phosphorylated form of both STAT1α and STAT1β.

Microarray analysis

Total RNA was isolated from cultured SCID splenocytes in the presence or absence of IFNα by ISOGEN. RNA was analyzed using the GeneChip Mouse Genome Array 430 2.0 (Affymetrix, Santa Clara, CA). Analysis of difference expression was performed by GeneChip Operating Software Ver. 1.1. Genes were considered to be significantly upregulated according to the following criteria: (*i*) the mean fold increase was more than 4-fold; (*ii*) the expression of a gene was significant in NK cells after IFNα treatment; (*iii*) a significant increase was registered based on the algorithm of the software.

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction or ANOVA for experiments with more than two subgroups. *Post hoc* tests were done using the Bonferroni's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Single intravenous injection of IFNa1 gene enhances NK activity and completely rejects hepatic metastasis of CT-26 cells

Hydrodynamics-based gene delivery establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes.[21,22] Serial measurement of serum IFNα demonstrated that pCMV-IFNa1 injection led to substantial IFNα production on Day 1. The levels of serum IFNα then declined but were still detectable at Day 14 (Fig. 1*a*). To examine biological effects of the produced IFNα, we evaluated the NK activity of mononuclear cells from the liver and spleen. pCMV-IFNa1 injection, but not

control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells and, to a lesser extent, splenic mononuclear cells at 4 days. The levels of Yac1 lytic activity declined but were still higher at 14 days after the injection (Fig. 1*b*). We also measured IFNγ production in serum, since IFNα is known to activate IFNγ production.[23,24] pCMV-IFNa1 injection, but not pCMV injection, increased serum IFNγ at 7 days (Fig. 1*a*). Since serum IFNγ increased relatively at a later time point, it may be an indirect effect rather than a direct effect of IFNα. These data indicated that hydrodynamic injection of pCMV-IFNa1 efficiently produced biologically active IFNα for a while in mice.

To evaluate the therapeutic effects of IFNα, Balb/cA mice were intrasplenically injected with CT-26 cells. Two days later, the mice were randomized into 3 groups and intravenously injected with either pCMV-IFNa1 or pCMV or were not treated. All pCMV-injected mice or untreated mice died within 3 weeks (Fig. 2*a*). They exhibited massive liver tumor in the liver. In contrast, all mice receiving pCMV-IFNa1 survived more than 2 months. To evaluate tumor metastasis, we sacrificed another cohort of mice at 2 weeks after tumor inoculation. There were no macroscopic or microscopic liver tumors in the pCMV-IFNa1-injected mice. In contrast, livers from pCMV-injected mice had massive tumors and were significantly heavier than those from pCMV-injected mice (Fig. 2*b*).

These results clearly indicated the striking therapeutic effects of IFNα on hepatic metastasis of CT-26 cells. To examine this therapeutic effect at a site other than the liver, Balb/cA mice were subcutaneously injected on the back with CT-26 cells and hydrodynamically injected 2 days later with pCMV-IFNa1 or pCMV. No difference in tumor growth was noted between pCMV-IFNa1-injected mice and pCMV-injected mice (Fig. 2*c*).

Mice protected from hepatic metastasis by IFNα gene therapy were resistant to subcutaneous challenge of CT-26 cells and exhibited a tumor-specific T cell response

We next investigated the possibility of IFNα-mediated rejection of hepatic metastasis being followed by induction of an adaptive immune response to the original tumor. To this end, we subcutaneously injected CT-26 cells into the mice that had been protected from CT-26 hepatic metastasis by IFNα therapy. The mice were rechallenged with CT-26 cells 1 month after the initial splenic injection. The controls were naïve Balb/cA mice as well as those receiving pCMV-IFNa1 but not CT-26 splenic inoculation. The incidence of tumor formation was lower in mice that had rejected hepatic metastasis by IFNα therapy than in the control mice. Even if they developed subcutaneous tumors, tumor size was significantly smaller than in the control mice (Fig. 3*a*).

To examine the tumor-specific response, splenocytes were isolated 3 weeks after tumor inoculation and restimulated *in vitro* with CT-26 cells. Splenocytes isolated from CT-26 bearing mice treated with IFNα showed significant levels of killing ability against CT-26 cells, but not against BNL A.7 cells (Fig. 3*b*). When mice were intrasplenically injected with UV-irradiated CT-26 cells, the splenocytes did not show significant killing activity regardless of the subsequent IFNα therapy (Fig. 3*c*). Separation of effector cells into T cells and non-T cells based on CD90 expression revealed that this killing ability was mediated by T cells, but not by non-T cells (data not shown). Thus, a tumor-specific cytotoxic T cell response was established in mice that had rejected hepatic metastasis of CT-26 cells by IFNα therapy.

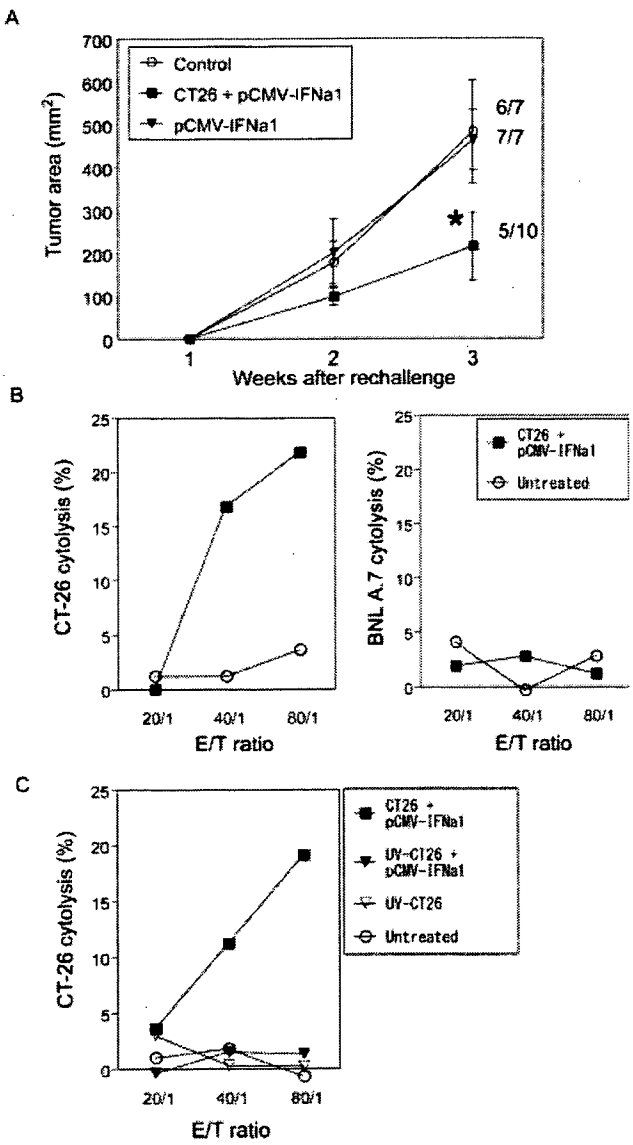

FIGURE 3 – Systemic immunity and tumor-specific T cell response. (*a*) Anti-tumor effects on rechallenged tumors. Balb/cA mice that had rejected hepatic metastasis of CT-26 cell by IFNα (closed squares), those treated with IFNα alone (closed triangles), and naïve mice (open circles) were challenged with subcutaneous injection of CT-26 cells 1 month after the previous treatment. Subcutaneous tumor growth was examined every week by measuring tumor area. Tumor size was expressed as the mean tumor size of only those mice bearing tumors. Each data point represents the mean tumor size and SD. The ratio of the number of mice bearing tumor/the number challenged for each treatment group at 3 weeks is shown in the figure. *, $p < 0.05$ *vs.* control or pCMV-IFNa1 injection only group. (*b*) *In vitro* tumor-specific killing ability. Balb/cA mice were intrasplenically injected with CT-26 cells and then treated with pCMV-IFNa1 2 days later. Splenocytes were isolated from CT-26 plus pCMV-IFNa1-injected mice at 3 weeks (closed squares) or naïve mice (open circles), restimulated with CT-26 cells for 5 days and then subjected to analysis for the lytic ability against CT-26 cells (left) or BNL A.7 cells (right). Shown are representative data for 3 independent experiments. (*c*) Requirement of CT-26 cells and IFNα on induction of tumor-specific killing ability. Balb/cA mice were intrasplenically injected with live CT-26 cells (squares) or UV-irradiated CT-26 cells (triangles) and then treated with (closed symbols) or without (open symbols) pCMV-IFNa1 2 days later. Splenocytes were isolated from mice at 3 weeks, restimulated with CT-26 cells for 5 days and then subjected to the analysis for the lytic ability against CT-26 cells. Mice injected with live CT-26 cells without following injection of pCMV-IFNa1 did not survive for 3 weeks naïve mice were included as controls (open circles). Shown are representative data for 3 independent experiments.

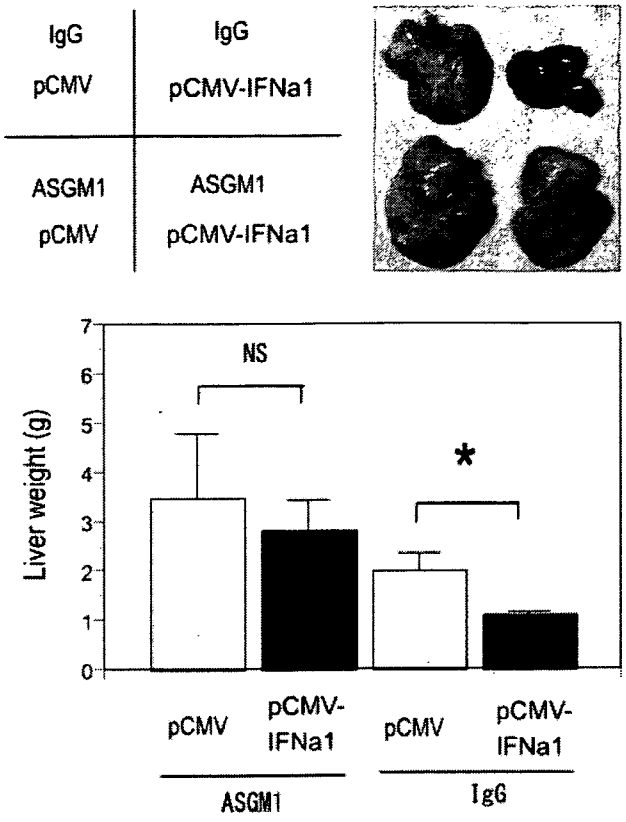

FIGURE 4 – Requirement of NK cells on IFNα-mediated anti-metastatic effects. Balb/cA mice were intrasplenically injected with CT-26 cells, intraperitoneally injected with either anti-ASGM1 or control IgG at 1 day, and hydrodynamically injected with either pCMV-IFNa1 (closed bars, n = 8/group) or pCMV (open bars, n = 7/group). Mice were sacrificed at 14 days to examine tumor growth in the liver. Top, a representative picture of the liver in each group. Bottom, comparison of liver weight among treatment groups. Experiments were performed at least 3 times and representative data are shown. *, $p < 0.05$. NS, not significant.

NK cells are required for IFNα-mediated initial rejection of hepatic metastasis

To examine whether the observed increase in NK activity of hepatic mononuclear cells is involved in the complete rejection of hepatic metastasis, we induced depletion of NK cells by injecting anti-asialo GM1 antibody. pCMV-IFNa1 injection completely abrogated hepatic tumor formation in control immunoglobulin-injected mice. In sharp contrast, pCMV-IFNa1 injection did not offer antimetastatic effects in anti-aialo GM1 antibody-injected mice, suggesting the critical contribution of NK cells to the anti-metastatic effects of IFNα (Fig. 4). We examined the possibility that hepatic mononuclear cells can serve as direct effectors cells for CT-26 eradication. Although CT-26 cells were more resistant to hepatic mononuclear cells than Yac1 cells, pCMV-IFNa1 injection clearly enhanced the killing ability of hepatic mononuclear cells against CT-26 cells (data not shown). This result indicated that CT-26 is potentially susceptible to hepatic mononuclear cells upon IFNα therapy.

IFNα directly activates NK cells

IFNα is known to be able to activate a variety of immune cells. To examine whether NK cells can be directly activated by IFNα, we analyzed SCID mice that lack T cells, B cell and NKT cells due to spontaneous DNA-dependent protein kinase point mutation.[25] SCID or wild-type splenocytes were cultured with IFNα and examined for STAT1 phosphorylation, which peaked at 30 min and declined at 6 hr after IFNα stimulation in both mice (Fig. 5*a*). However, the signals of STAT1 phosphorylation were weaker in SCID splenocytes than in wild-type cells. Of interest is the finding that STAT1 expression was reduced in SCID cells compared to wild-type cells. Similar data were also obtained from experiments on Rag2 KO mice, another model of deficiency for T cells, B cells and NKT cells. To examine the reasons for SCID or Rag2 KO cells expressing low levels of STAT1, we separated wild-type splenocytes into T cells and non-T cells based on CD90 expression. The levels of STAT1 expression were weaker in non-T cells than in T cells (Fig. 5*b*). Taken together, the difference in the levels of STAT1 expression among lymphocyte subsets could explain the reduced phosphorylation signals after IFNα treatment in SCID or Rag2 KO cells.

To examine the gene profiles activated by IFNα in NK cells, we used Affymetrix DNA array analysis on SCID hepatic mononuclear cells. Six hours treatment of IFNα (1,000 U/ml) upregulated 243 of 45,101 genes in SCID cells by more than 4-fold. They included well known IFNα-regulated genes such as H2, 2′-5′ oligoadenylate synthetases, Mx1, IRF and suppressor of cytokine signaling (SOCS). Among the effector molecules for cytotoxicity, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and granzyme B were activated. Various cytokines such as IL-15 and IFNγ were also upregulated. These data revealed that NK cells upon IFNα stimulation produced well-characterized IFN-inducible genes and others that are relatively specific to killer cells or immune cells.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in SCID mice

We examined the *in vivo* effects of IFNα in SCID mice. In agreement with SCID cell activation *in vitro*, pCMV-IFNa1 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in SCID mice (Fig. 5*c*). To examine whether NK cells are sufficient for IFNα-mediated rejection of hepatic metastasis, we injected pCMV-IFNa1 or pCMV into mice that had been intrasplenically injected with CT-26 cells 2 days earlier. pCMV-IFNa1 completely suppressed tumor formation in the liver (Fig. 5*d*). As described in the *Material and methods* section, anti-asialo GM1 injection reduces the number of NKT cells. However, this SCID experiment clearly showed that NKT cells are not required for NK cell activation by IFNα and its antimetastatic effects.

pCMV-IFNa1 injection completely suppressed tumor formation in the liver in GKO mice

IFNγ has been established as an endogenous inhibitor of tumor development and progression.[26] Exogenous administration of IFNγ suppresses tumor formation in a variety of models.[15,27] To examine the possibility of IFNγ being involved in antimetastatic effects on IFNα, we injected pCMV-IFNa1 or pCMV plasmid into GKO mice exposed to 2 days of metastasis of CT-26 cells. IFNα treatment led to complete rejection of CT-26 cells in GKO mice (Fig. 6*a*). pCMV-IFNa1 injection, but not pCMV injection, augmented the Yac1 lytic ability of mononuclear cells (Fig. 6*b*).

Discussion

Here we report that a single injection of pCMV-IFNa1 could lead to complete rejection of preexisting hepatic metastasis of colon cancer cells. This partly agrees with a previous report by Kobayashi et al.,[15] who hydrodynamically injected IFNβ- or IFNγ-expressing plasmid into CT-26 bearing mice and reported the antimetastatic effects of IFNβ or IFNγ. In contrast to our study, all mice died within 45 days due to metastasized tumor growth even if plasmid injection was began one day after tumor inoculation and repeated every other day. The complete protection against hepatic metastasis observed in the present study allowed

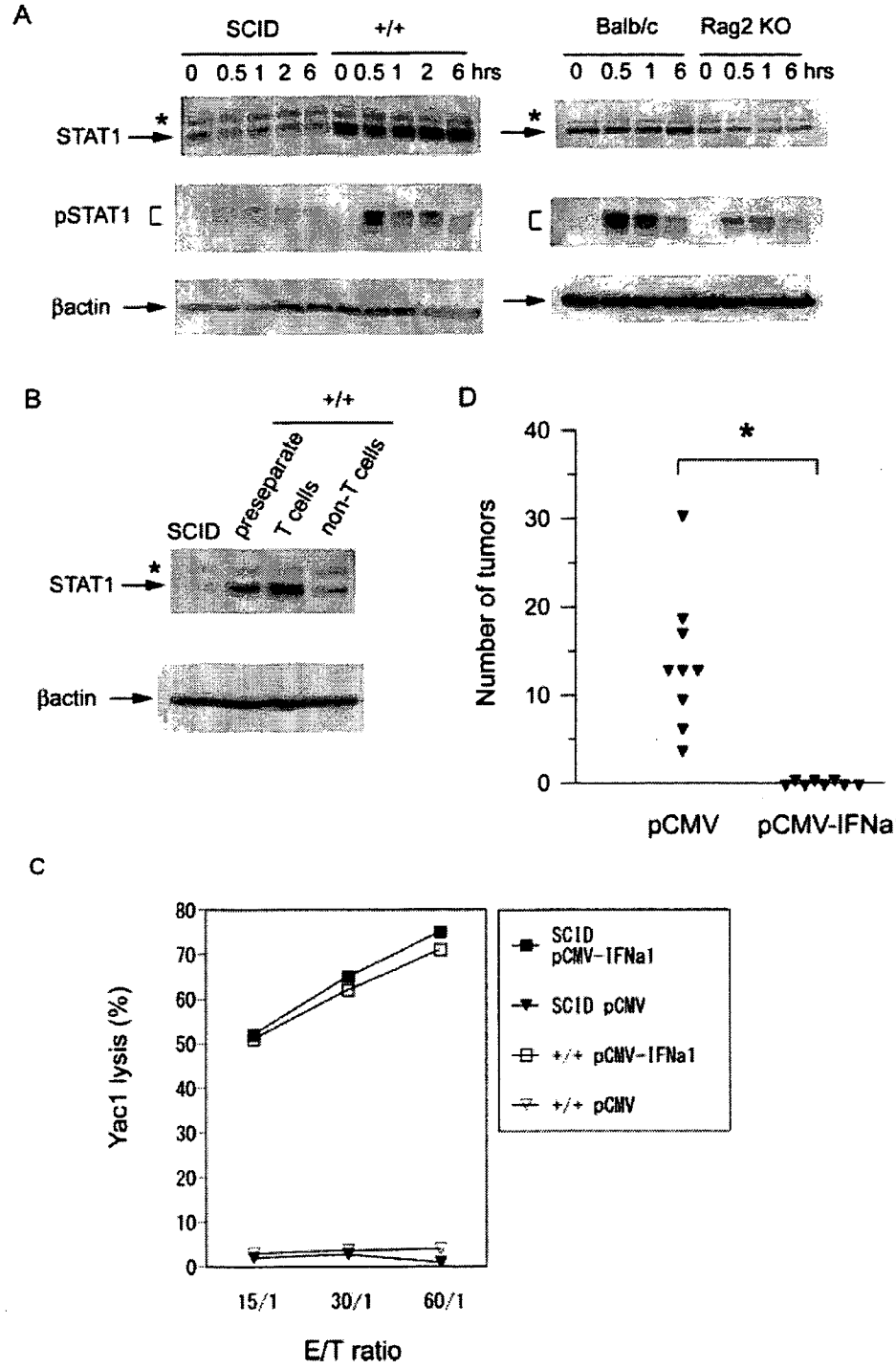

FIGURE 5 – IFNα-mediated NK cell activation and anti-metastatic effects in SCID mice. (*a*) STAT1 and phospho-STAT1 expression of splenocytes from SCID or Rag2 KO mice. Splenocytes were treated with 1,000 U/ml of IFNα and lysed at indicated time points (0 to 6 hr). Expression of STAT1 and phospho-STAT1 (pSTAT1) was analyzed by Western blot. +/+ and Balb/c indicate wild-type controls for SCID and Rag2 KO mice, respectively. *, non-specific band (see the *Material and methods* section). (*b*) STAT1 expression in T cells and non-T cells. Splenocytes from wild-type mice (+/+) were separated into T cells and non-T cells based on expression of CD90. Expression of STAT1 was analyzed by Western blot. SCID and preseparated wild-type splenocytes were included as controls. *, non-specific band (see the Material and methods section). (*c*) Yac1 lytic ability. SCID mice (closed symbols) or wild-type mice (open symbols) were hydrodynamically injected with either pCMV-IFNa1 (squares) or pCMV (triangles). Four days later, splenocytes isolated form the mice were examined for the lytic ability for Yac1 cells. Experiments were done at least 3 times and representative data are shown. (*d*) Anti-metastaic effects. SCID mice or wild-type mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 or pCMV 2 days later. After 14 days, mice were sacrificed to examine tumor development in the liver. The numbers of hepatic tumors were compared among the groups. Experiments were performed 3 times and representative data are shown. *, $p < 0.05$.

us to investigate the adaptive response after antimetastatic effects. Mice that had rejected CT-26 cells by IFNα showed a tumor-specific T cell response and suppressed tumor growth of rechallenged skin tumor. Therefore, pCMV-IFNa1 injection not only caused initial rejection of metastasized tumors but also induced durable and systemic adaptive immunity. Interestingly, splenic injection of UV-irradiated CT-26 cells, even if followed by pCMV-IFNa1 injection, did not elicit significant tumor-specific T cell responses. Therefore, the efficient induction of adaptive T cell responses requires IFNα-mediated rejection of live tumor cells and cannot be recapitulated by simple injection of dead tumor cells and IFNα.

NK cells are present in a high percentage in the liver.[28] In the present study, we focused on NK cells which were rapidly activated by IFNα to examine the cellular mechanisms of protection against hepatic metastasis. Critical requirement of NK cells was demonstrated by anti-asialo GM1 antibody-injected mice which did not show protection against CT-26 metastasis. In contrast, T cells, B cells or NKT cells were dispensable for IFNα-mediated antimetastatic effects since IFNα therapy did show antimetastatic

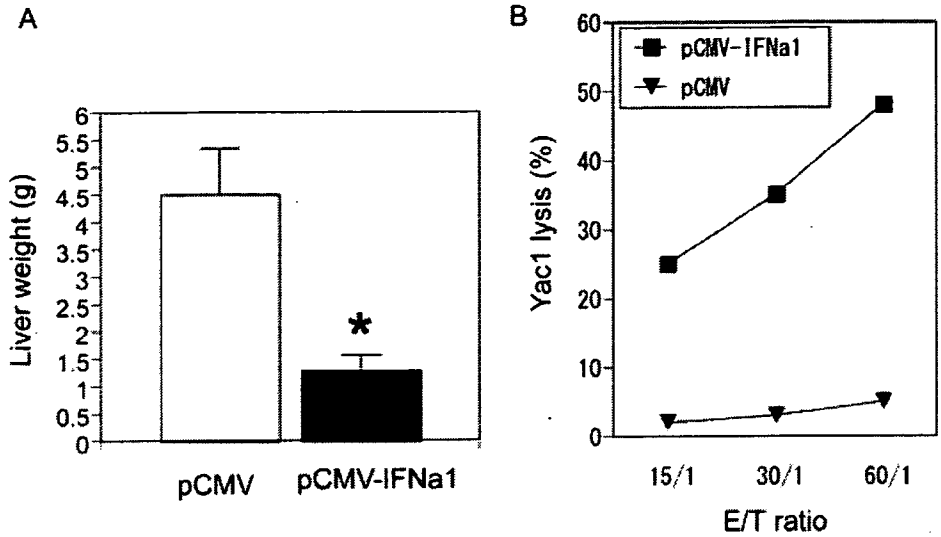

FIGURE 6 – NK cell activation and anti-metastatic effects in GKO mice. (*a*) Anti-metastatic effects. GKO mice were intrasplenically injected with CT-26 cells and hydrodynamically injected with either pCMV-IFNa1 (closed bars) or pCMV (open bars) 2 days later. After 14 days, the mice were sacrificed to examine tumor development in the liver. The liver weight was compared between the groups ($n = 8$/*group*). Experiments were performed 3 times and representative data are shown. *, $p < 0.05$ *vs.* pCMV injection group. (*b*) Yac1 lytic ability. GKO mice were hydrodynamically injected with either pCMV-IFNa1 (closed squares) or pCMV (closed triangles). Four days later, splenocytes isolated from the mice were examined for the lytic ability for Yac1 cells. All experiments were performed at least 3 times and representative data are shown.

effects in SCID mice. Research using a variety of murine models has revealed the direct effects of tumor cells[11] and the CD8 T cell response[12–14] involved in the antitumor effects of IFNα. A recent study[29] using STAT1-deficient animals and STAT1-deficient tumor cells revealed that IFNα activation of host cells, but not tumor cells, is required for antitumor effects in a peritoneal model of melanoma. They also showed the involvement of NK cells in their model. Our data demonstrated that NK cells are critically required and sufficient for IFNα-mediated protection from liver metastasis. However, NK cells are not effective for controlling tumor growth at extrahepatic sites, because IFNα activated splenic (systemic) NK activity but did not elicit antitumor effects against subcutaneously injected CT-26 cells. Subcutaneous tumor growth appeared to be controlled by adaptive immunity rather than innate pathway.

The reason that IFNα-mediated activation of NK cells leads to such a strong antitumor effect in the liver but not under the skin is not known. In the present study, we applied hydrodynamic injection of the IFNα gene to obtain efficient and stable expression of IFNα. Since the hydrodynamic procedure leads to predominant expression of foreign genes in the liver, the concentration of IFNα may be greater in the liver than in circulation. This may be related to the observed strong antitumor effects in the liver. Another possibility is that NK cells are more numerically abundant and functionally potent in the liver than in other organs.[30] In any case, the hydrodynamic injection of the IFNα gene led to higher activation of the NK lytic ability of hepatic mononuclear cells than that of systemic mononuclear cells. This may be related to the stronger antitumor activity in the liver.

An earlier study on STAT1 knockout mice revealed that STAT1 is a critical signaling molecule for IFNα in macrophage and T cells.[9] STAT1-deficient mice showed impairment of NK activity.[31] STAT1-deficient splenocytes did not show increase in NK lytic activity upon IFNα stimulation.[29] Therefore, STAT1 should also play an important role in IFNα-mediated NK cell activation. However, the significance of STAT1 in NK cells on IFNα action had not been fully proven, because splenocytes consist of a variety of lymphocyte subsets. In the present study, we found that NK cells express lower levels of STAT1 than T cells, which is associated with lower levels of STAT1 expression in SCID splenocytes than those in wild-type splenocytes. Importantly, IFNα phosphorylated STAT1 in SCID splenocytes with similar kinetics to that in wild-type splenocytes even if the signal intensities in the former were lower than those in the latter. In agreement with this, IFNα was capable of activating a variety of genes in SCID mononuclear cells. Thus, IFNα does not require other lymphocyte subsets to activate NK cells and to induce NK cell expression of IFN-regulated genes.

IFNγ was shown to be produced in lymphocytes upon IFNα administration, which is dependent on STAT4 signaling.[32] In the present study, IFNγ was produced in serum after pCMV-IFNa1 injection. Furthermore, the IFNγ gene was activated in SCID NK cells upon IFNα stimulation. However, IFNγ is not necessary for NK cell activation in terms of killing ability as well as an IFNα-mediated antimetastatic effect. NK cells, upon IFNα stimulation, expressed well-established IFN-regulated genes[33] as well as killer cell-specific molecules granzyme B or TRAIL. Although our data showed that hepatic mononuclear cells from mice receiving IFNα can kill CT-26 cells *in vitro*, it remains unclear whether NK cells serve as direct effector cells for ablating CT-26 cells *in vivo*. Further study is needed to find whether killer cell-specific molecules are actually involved in the antimetastatic effects of IFNα.

IFNα has achieved a long record of clinical use in the treatment of hematological malignancy and solid tumors such as melanoma, renal cell carcinoma and Kaposi's sarcoma.[34,35] In therapy for colon carcinoma, special attention has been paid to the use of IFNα in the combination with 5-FU, since IFNα has been shown to modulate 5-FU metabolism and to enhance its cytotoxic activity.[36] Although several clinical trials have evaluated the 5-FU plus IFNα combination for adjuvant therapy of colon carcinomas with encouraging results,[37,38] recent randomized trials revealed that addition of IFNα to 5-FU + levamisole marginally increased the recurrence-free survival time compared to 5-FU + levamisole alone, but did not alter the over-all survival.[39] Therefore, use of IFNα as a modulator of 5-FU activity may have some limitations in future clinical use. In the present study, we demonstrated that

IFNα activates both innate and adaptive immunity and ablates microdisseminated colon carcinoma cells in the liver. There may be a variety of reasons which can explain the difference between the present study and the clinical use in the therapy of metastasizing colon cancer. We found CT-26 far less sensitive to NK cells than Yac1 cells but human colon carcinoma cells might be more resistant to NK cells activated by IFNα in a clinical setting. Systemic administration of recombinant IFNα may be less effective than enforced expression of IFNα gene in the liver. In any way, we used CT-26 cells just as a murine model of hepatic metastasis and observed similar therapeutic effect of the IFNα gene when using another cell line such as BL6 melanoma cells in a C57/BL6 background (our unpublished data). Our study raised the possibility that IFNα therapy may be a promising approach for developing future adjuvant therapy for metastatic liver tumors arising from various organs. Immunological aspect of IFNα is important when considering antimetastatic effect of this cytokine.

In conclusion, IFNα-mediated protection of CT-26 hepatic metastasis critically requires NK cells. NK cells, upon IFNα stimulation, do not require other immune cells such as T cells, B cells and NKT cells for their activation and protection against hepatic metastasis. NK cell production of IFNγ is not involved in the increase in NK activity and antitumor effect. Our study has shown NK cells to be important mediators in ablating microdisseminating tumors in the liver in IFNα therapy. Eradication of microdisseminated tumor cells by IFNα led to long-lasting adaptive immune responses which may be important for suppressing tumor growth in extrahepatic sites and overall antitumor effects.

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