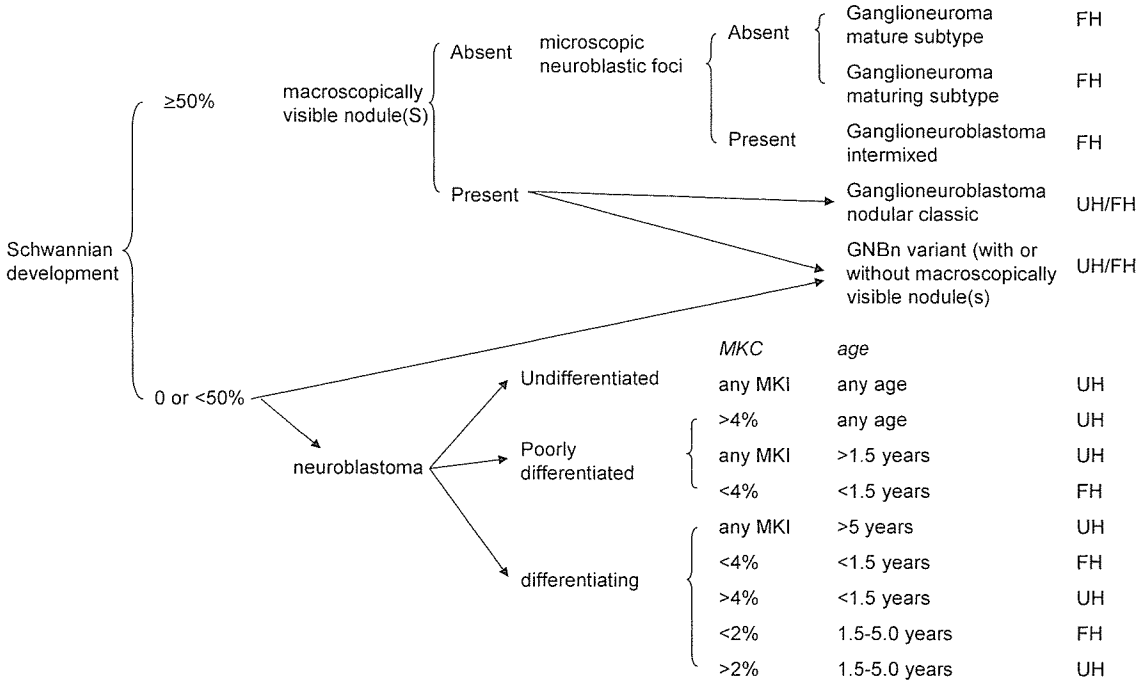

図 1 Revised international neuroblastoma pathology classification
(Peuchmaur ら, 2003)[4]

裂）もしくは karyorrhexis（核崩壊）を示す細胞数の合計をカウントして MKI（mitosis-karyorrhexis index）を算出し，100 未満のものを low，100 から 200 のものを intermediate，200 以上のものを high と定義する。腫瘍の分化度と MKI に年齢因子を加味した INPC のシステムにより，個々の腫瘍は良好な予後が期待されるもの（favorable histology：FH）と，予後不良のもの（unfavorable histology：UH）に分類される。なお年齢が 5 歳以上の神経芽腫については全例 UH に分類される（**図 1**）。

1）Undifferentiated subtype

腫瘍細胞に全く分化傾向を認めず，光学顕微鏡で神経細線維形成を確認することができないため，免疫組織学的・分子生物学的手法を用いなければ他の小円形細胞腫瘍との鑑別が困難なものを undifferentiated subtype と定義する。本腫瘍は神経芽腫群腫瘍の 1～2% と頻度は低いが，年齢にかかわらず予後不良であると考えられ，UH に分類される。この subtype に属する腫瘍細胞は分化するための生物学的能力を欠くと推測されている。

2）Poorly differentiated subtype

乳児神経芽腫に多くみられるタイプで，腫瘍細胞は N/C 比が高く小型であるが，光顕レベルで神経細線維の形成が認められ神経芽細胞への分化傾向は明らかである。この腫瘍は分化，成熟度は低いが，患児年齢がそれに対応して低く（1.5 歳未満），かつ low または intermediate MKI を示すものは FH に分類される。しかし年齢にかかわらず MKI が high の場合，または年齢が 1.5 歳を超えたものは MKI にかかわらず UH に分類される。

3）Differentiating subtype

腫瘍が成熟するにつれて，分化型神経芽細胞（differentiating neuroblasts）の出現をみるようになる。分化型神経芽細胞では細胞質・核がともにその大きさを増し，細胞質は直径が核の 2 倍以上となり，また核は細胞質内に偏在しかつ明瞭な核小体を有するようになる。分化型神経芽細胞が全体の 5% 以上を占めるものを differentiating subtype と定義する。分化型神経芽細胞は活発に

神経細線維を形成するため，エオジンに染まる細線維部分の腫瘍組織内に占める面積が増加する。このsubtypeでは，一部に間質Schwann細胞の増生を認め，次に述べる神経節芽腫への分化をうかがわせる腫瘍も存在する。このsubtypeの腫瘍では，患児年齢が1.5歳未満でMKIがlowまたはintermediateの場合，または1.5歳から5歳でMKIがlowの場合にFHと分類され，それ以外の年齢とMKIの組み合わせの場合はUHに分類される。

2．神経節芽腫(Ganglioneuroblastoma, Intermixed)—Schwannian stroma-rich

長年にわたり神経節芽腫の定義は不明瞭であったが，INPCは神経芽腫と神経節芽腫，また神経節芽腫と神経節腫の明確な組織学的区別を可能とした。神経芽腫から神経節芽腫さらに神経節腫への分化過程において，神経芽細胞は神経節細胞へ成熟するが，組織学的変化のうちで最も顕著なものはSchwann細胞よりなる間質の増生である。ここに注目し，INPCでは組織全体の50%以上がSchwann細胞で占められた腫瘍を神経節芽腫と定義した。腫瘍内には間質Schwann細胞成分と混在するように顕微鏡的な神経芽腫成分の巣(microscopic neuroblastic foci)が散見される。この神経芽腫成分は神経細線維を背景にした種々の分化段階にある神経芽細胞からなる。個々の神経芽腫成分の巣は周囲の間質Schwann細胞と連続性を有する細い間質によって分割されているのが特徴である。巣がある程度以上に大きくなると後述する結節型神経節芽腫との鑑別に注意を要することがある。神経節芽腫(ganglioneuroblastoma, intermixed)は年長児に多くみられ，大部分はstage 1か2の限局性腫瘍である。本腫瘍は全例FHに分類される。

3．神経節腫(Ganglioneuroma)—Schwannian stroma-dominant

神経芽腫群腫瘍のうち，分化の最終段階に達した腫瘍である。腫瘍全体が一様にSchwann細胞よりなる間質で占められており，その中に神経節細胞へ分化した腫瘍細胞が個々に点在する。完全に成熟した神経節細胞はsatellite細胞に包まれるようになる。腫瘍細胞がすべて完全に成熟した腫瘍をmature subtypeとし，一部に未熟な神経節細胞をみる場合にはmaturing subtypeとする。神経節腫(ganglioneuroma)は年長児にみられ，時に成人で診断される場合もある。年齢にかかわらず全例FHに分類される。

4．結節型神経節芽腫(Ganglioneuroblastoma, Nodular)—Composite, Schwannian stroma-dominant/stroma-rich and stroma-poor

生物学的特性の異なる2つ以上の腫瘍クローンからなる混成型の腫瘍である[9]。典型的には神経節芽腫もしくは神経節腫のクローンと，明らかにこれとは組織学的に移行性を欠く神経芽腫クローンの増殖をみる。後者のクローンはしばしば出血を伴い，肉眼的に結節形成を示すので，この名称がつけられた。当初INPCではこの腫瘍はすべてunfavorable histologyに分類されていた。しかし1999年に患児の予後が腫瘍内の神経芽腫クローンの性格により規定されることが報告され[10]，2003年に改訂されたINPCでは結節型神経節芽腫(ganglioneuroblastoma, nodular)をfavorable subset (FH) とunfavorable subset (UH)に区別することとなった[4]。この2つのsubsetは，神経芽腫に使われている分類法（年齢を加味した腫瘍の分化度とMKIの評価）を神経芽腫クローンに当てはめて区別する。

II．組織分類と予後との関係

INPCのシステムによって神経芽腫群腫瘍を分類し，良好な予後の期待できるグループ(favorable histology：FH)と予後不良と考えられるグループ(unfavorable histology：UH)を区別するためのフローチャートを図1に示す[4]。個々の腫瘍については，上述のとおりである。FHの代表的な組織像を**図2**に，UHの代表的組織像を**図3**に示す。

米国CCG (Children's Cancer Group)の3881/3891神経芽腫スタディでは，診断時年齢，病期(Evans分類[11])，*MYCN*遺伝子増幅の有無，血清フェリチン値とともに組織分類が予後予測因子として採用され，リスク分類に用いられた。INPCによる組織評価が可能だった神経芽腫群腫瘍

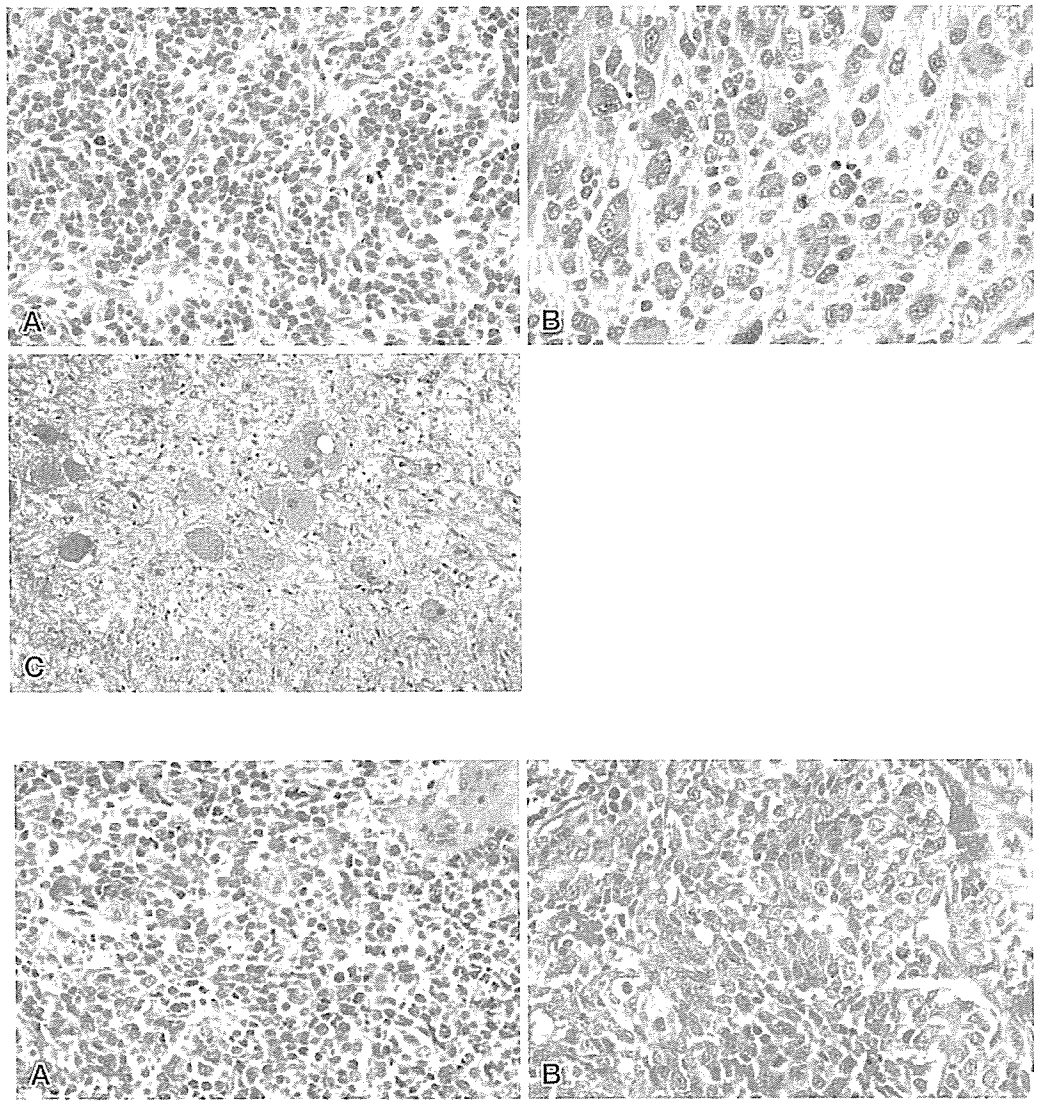

図 3 unfavorable histology の代表的な組織像

A. 1 歳 5 カ月男児：腹部腫瘤・左水腎症。stage 3, poorly differentiated, high MKI, MYCN 遺伝子増幅あり。

B. 1 歳 2 カ月女児：下肢痛・腹部腫瘤，マススクリーニング陰性例。stage 4A, undifferentiated, intermediate MKI.

630 症例中，FH と評価された 364 例の EFS/OS はそれぞれ 90.8/98.0%であったのに対し，UH と評価された 266 例の EFS/OS は 31.2/40.8%であり，病理組織分類が有意差をもって予後に反映することが示された[12]。病理所見はその後のスタディにおいても重要な予後予測因子として採用されており，現在進行中の COG (Children's Oncology Group) の神経芽腫グループスタディにおいても診断時年齢，病期 (INSS 分類)，MYCN 遺伝子増幅の有無，DNA index とともに INPC が患児のリスク決定に重要な役割を果たしている。CCG・COG 1,559 例で検討された EFS を Kaplan-Meier 曲線で図 4 に示す。

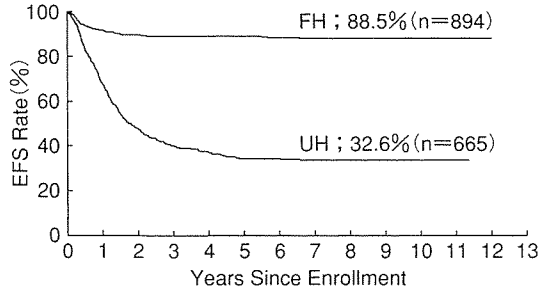

図 4 CCG, COG 登録症例 1,559 例の EFS

III. 組織分類と MYCN 遺伝子の関係

MYCN 遺伝子は 2 番染色体の短腕に存在し，胎児期の中枢神経系の発達に関与していると考えられている[13]。*MYCN* 遺伝子の増幅した神経芽腫群腫瘍が病期の進行した例に多くみられ，患児の予後が不良で[7]急速に進展すること[14]などが 1984 年以降相次いで報告され，強力な予後予測因子として位置づけられるようになった。*MYCN* 遺伝子が増幅すると，腫瘍細胞の分化は抑制され，細胞の核分裂・核崩壊が促進されることが報告されている[15,16]。

MYCN 遺伝子増幅は神経芽腫群腫瘍の 15～20%にみられ，組織学的にはほとんどが UH に分類される。一方，UH に分類される神経芽腫のうち，半数以上は MYCN 非増幅例であり，これらもまた予後不良である。

おわりに

予後の良好な神経芽腫は，低年齢では細胞の分化が不十分であっても，自然消退するか，加齢に伴い腫瘍細胞の分化，間質 Schwann 細胞の増生をみるようになる。腫瘍組織の分化・成熟は神経芽腫 poorly differentiated subtype から神経芽腫 differentiating subtype を経て，神経節芽腫，さらには神経節腫へと進むものと考えられる。一方，年齢に応じた分化を遂げられない，もしくは年齢不相応な核分裂・核崩壊像を持った神経芽腫が予後不良と判断される。結節型神経節芽腫においては結節内の神経芽腫クローンの評価によって患児の予後予測が可能である。INPC によって FH か UH かを判定することが，神経芽腫群腫瘍患児の予後を予測し，治療戦略を立てる上で必須である。

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Histopathology Classification (INPC) of Peripheral Neuroblastic Tumors

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The International Neuroblastoma Pathology Classification, established in 1999 by adopting the system of the original Shimada Classification and partly modified in 2003, distinguishes favorable histology (FH) and unfavorable histology (UH) groups among cases with peripheral neuroblastic tumors (Neuroblastoma-NB ; Ganglioneuroblastoma, Intermixed-GNBi ; Ganglioneuroma-GN ; Ganglioneuroblastoma, Nodular-GNBn). In the categories of NB and GNBn, prognostic distinction is made by applying a concept of age-appropriate framework for evaluating prognostic impacts using morphologic indicators such as grade of neuroblastic differentiation and mitosis-karyorrhexis index. GNBi and GN are usually diagnosed in older children, and are always classified into the FH group. The classification has been used as one of the most powerful front-end prognostic factors for patient stratification and protocol assignment in this disease.

* * *

特集　新たなる発想からEvidenceへ

12. 成人期に達した小児外科手術症例の現状*

金子道夫　北野良博　鈴木達也
川岸直樹　秋岡清一　竹内　功**

〔要旨〕わが国で小児外科の診療が始まって約半世紀になる．小児外科疾患の治療成績は大幅に向上し，長期生存例は確実に増加したが，不完全治癒や晩期障害を克服できない難治性疾患があることも事実である．2006年の日本外科学会で成人期に達したこれら小児の外科的疾患の問題点がシンポジウムで取り上げられることとなった．成人期に達した患者の多くは社会生活を問題なく過ごしているが，高位鎖肛，胆道閉鎖，食道閉鎖，重症の先天性心疾患では原病や手術からくる後障害に悩まされており，再手術や移植を必要とすることもある．また，成長や妊娠に伴って病状が悪化する例もあった．胆道閉鎖では移植を成人期になってから必要となる例もあり，小児期とは異なった問題点がみられた．先天性心疾患では再手術やペースメーカー植込みなどを必要とすることもある．今後，さらに重症の疾患が成人期に達するにつれ新たな問題が小児の外科的疾患に生じると考えられ，その対策が必要である．

はじめに

わが国で小児外科の診療が始まって約半世紀になる．この間の医療の進歩により，小児外科疾患の治療成績は大幅に向上し，長期生存例は確実に増加している．しかし，治療が進歩しても不完全治癒や晩期障害を克服できない難治性疾患があることも事実である．思春期以降成人後も学業，就職，結婚，妊娠，出産，子育てなどの社会的活動を行ううえで原疾患に直接あるいは間接的に関連するさまざまな医学的問題を抱える方が多いことはむしろ当然であるといえる[1]．2006年の日本外科学会で成人期に達したこれら小児の外科的疾患の問題点がシンポジウムで取り上げられる．このシンポジストとなった8名のうち5名の発表内容を中心に，現状について明らかにする．

キーワード：成育医療，成人期の小児外科，小児外科，先天性心疾患

* Late effects of pediatric surgical diseases in adulthood

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I. 成人期に達した小児外科疾患

「成人期の小児の外科」としては，成人期に

なってはじめて診断された先天性疾患，たとえば低位鎖肛，Hirschsprung病，先天性心疾患，小児期腫瘍がある．これらは小児期とは違った問題点を抱えており検討を要する領域である．しかし，今回は新生児乳児期に手術され，それが成人期まであるいは成人期になって問題となる疾患について考察することとした．それらには，食道閉鎖，高位鎖肛，Hirschsprung病，短小腸などの新生児期小児外科疾患，胆道閉鎖，複雑な先天性心疾患，小児癌，幼小児期腎不全がある．さらに，肝移植・腎移植が日常臨床になった現在，それらの成人期での問題点を考慮する必要がある．たとえば，成人先天性心疾患患者(ACHD)の増加に伴い，欧米では成人期に達した症例の抱える問題点を解決する新たな領域としてACHD外来が多くの施設に設立されている．本邦でも成育医療センターの設置はその一つであろう．

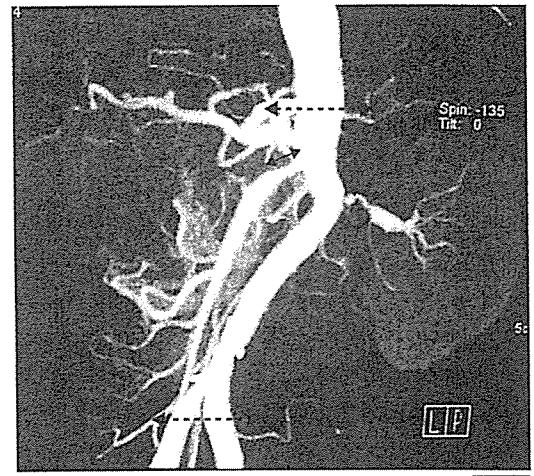

図1．点線両矢印間が照射により狭くなった腹部大動脈
腹部大動脈から出る腹部主要分枝の起始部は著しい狭窄になっている．太矢印：左腎動脈，細矢印：上腸間膜動脈の狭窄部

II. 小児癌

小児癌は死にいたる病であったのが，最近では治癒率が70%を超えるまでになった．化学療法を中心とした厳しい集学的治療が主因であるが，治療成績向上とともに多くの後障害を抱える疾患群でもある．次にその1例をあげて考察する．

症　例　24歳，女

1986年筑波大学初診の多発骨転移を有する左後腹膜原発の神経芽腫病期4の5歳女児で，腫瘍のため左水腎症となっていた．当時骨転移を有する1歳以上の神経芽腫患者に生存例はほとんどなかったが，1985年に開始された厚生省がん助成金の研究班プロトコール[1]で治療され，術中照射および外部照射を受け，幸いにも両側腎を温存して現病は治癒した．しかし，2005年11月に父親が事故で入院したさい，たまたま自分で血圧を測定し，220/126 mmHgと著しい高血圧であることが判明した．CTにて腹部血管を再構築すると(図1)，照射野に沿って腹部大動脈，腹腔動脈，上腸間膜動脈，両側腎動脈の狭窄が認められた．動脈狭窄にはバルーン拡張術が有力な方法であるが，狭窄が長いこと，放射線の後障害と考えられることから拡張は困難と判断され，両側自家腎移植が予定されている．

進行神経芽腫は現在でも治癒率は40%以下であるが，当時はほとんど0%であった．照射線量は現在では後障害を防止する意味からも減量されており，このような後障害は少なくなると考えられるが今後も予想され，当時としてはやむをえない後障害と考えられる．

難治性の小児癌の後障害としては，2次癌の発生，放射線による変形や成長障害，リンパ節郭清による神経障害などが成人期の問題点であり，変形の治療が外科的には重要である．

III. 小児外科疾患

北野は成育医療の問題点を解決すべく新たに開設された国立成育医療センターの患者について，成人期に達した小児外科患者の問題を明らかにするため，2002年3月～2005年7月に同センター外科で診療した15歳以上の症例について後方視的に検討した．小児に特有とはいえない疾患，症候群(結節性硬化症，von Recklinghausen病，先天性免疫不全症など)に合併する外科的問題を除く386例を小児外科疾患の術後患者と考えた．原

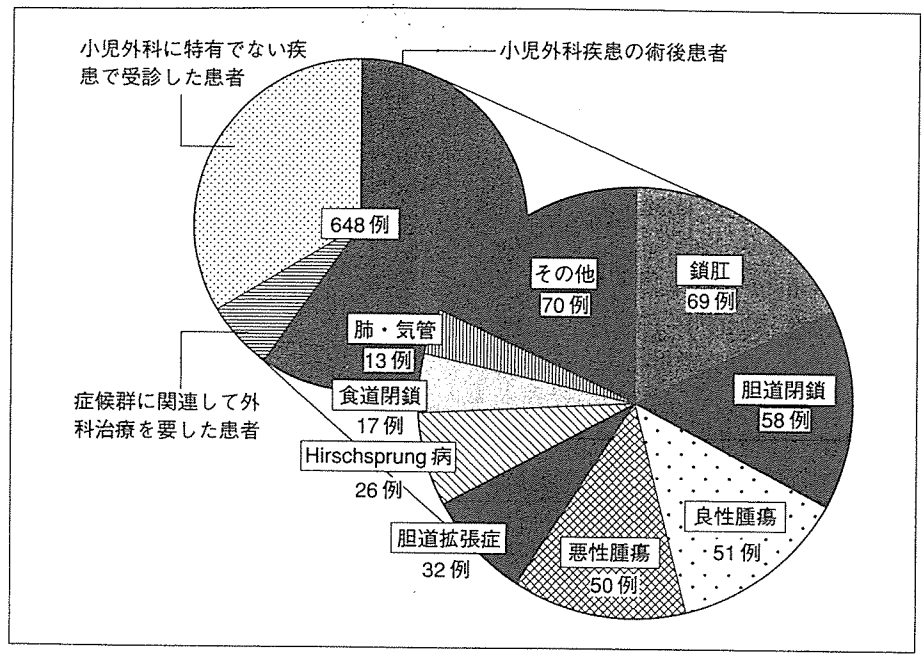

図2. 国立成育医療センターでの小児外科疾患の成人期患者の内訳

疾患は，鎖肛69例，胆道閉鎖58例，良性腫瘍51例，悪性腫瘍50例，胆道拡張症32例，Hirschsprung病26例，食道閉鎖17例，肺・気管疾患13例，その他70例であった(図2)．15歳以降に施行した手術は，骨盤内嚢胞性病変の手術が12件でもっとも多く，次いで腟形成(8件)，肝移植(8件)，直腸粘膜脱手術(7件)，腸切除(6件)などであった．高位鎖肛の術後では，卵管水瘤，腟狭窄，妊孕性などの泌尿器・婦人科的問題が大きな比重を占めていた．胆道閉鎖症例ではすでに9例(16%)が肝移植後であった．その他，慢性膵炎(胆道拡張症や十二指腸狭窄の術後)，逆流性食道炎(食道閉鎖・狭窄の術後)など成人後も医療ケアを必要とする病態が多数見受けられた．

京都府立医大の木村の報告では，同病院で治療された直腸肛門奇形症例で成人に達しフォローアップ可能であった27例でのアンケート調査では中間位型で男2例，女1例に軽度の排便障害を認めた．高位型では男9例中2例に軽度の排便障害が認められた．排尿障害は全例認められなかった．生殖機能では，男で泌尿器合併症を有した高位型の3例に射精が認められなかった．女性での既婚者は4例で，うち3例に分娩歴があった．成人期に達した直腸肛門奇形術後例において，排便・排尿機能はおおむね良好で，社会生活活動では一般成人とかわらず職に就いて活躍していた．

鎖肛と胆道閉鎖は，小児外科で扱う疾患の中でキャリーオーバーとなることが多い代表的疾患で，この2疾患で全体の1/3を占めていた．鎖肛に合併する内性器の奇形は思春期以降に卵巣嚢腫，卵管水腫，子宮留血腫などのかたちで発症するので注意を要する．内性器異常があると外科的治療を要することも多く，内分泌科や婦人科と連携をとりながら治療にあたる必要がある．心理的問題が見え隠れするケースでは，心療内科との協力も必要となる[2]．胆道閉鎖症のおよそ1/3では，葛西手術により10年以上の長期生存が得られるが，成人後も門脈圧亢進症や肝硬変が悪化して移植にいたるケースがある．今回の検討でも8人が15歳以降に移植を受けていた．とくに思春期は肝機能の予備力低下が顕性化する時期で，女性では月経や二次性徴発現の異常が肝障害と関連があ

るといわれている[3]．逆にこの時期を乗り切ることができれば，その後の妊娠・分娩にいたる経過は比較的安定するようである[4]．

今回，胆道閉鎖症と鎖肛の他にも，成人後も医療ケアを必要とする患者が多いことが確認された．より重症例，複雑例が救命されている現状を考えると，今後より高度の医療を要する成人例が増加することは間違いない．小児外科医は長期予後に基づいた術式の改善に努力すると同時に，泌尿器科，婦人科，成人内科，心療内科などの関連各科と連携して診療にあたることが求められる．そのためには，大学病院，成人総合病院，小児病院を超えた大きな枠組みで小児患者に継続的に医療を提供するシステムを考案する必要がある[5]．

鈴木らは20年以上経過した食道閉鎖症術後long gap症例を検討した．1985年までに名古屋市立大学で治療した本症は24例で，このうち生存5例(A型3例，B型2例，全例男性)を対象とした．生後5～6ヵ月に端々吻合による食道再建術が施行された．現在の年齢は，36歳，31歳，28歳，21歳および20歳である．術後の入院歴および外来通院による経過観察の状況，自他覚症状の推移，日常生活に対する影響の有無について，外来診療記録および電話あるいは面談による聞き取り調査をもとに検討した．

全例術後に胃食道逆流現象を認め，2例でそれぞれ1歳11ヵ月，5歳時に噴門形成術が施行されていた．1例に吻合部狭窄，他の1例に中部食道の狭窄にブジーを必要とした．現在も消化器症状を認める症例は2例，21歳の1例ではつかえ感が持続し，19歳時に食物が食道につまり内視鏡的摘出を受けた．36歳の1例は，つかえ感が持続し，ときどき胸焼け，嘔吐を認め，食後すぐに横になると胃内容の逆流を自覚し，ときにむせることがある．しかし，この2例を含め著しい栄養障害は認められておらず，胸郭変形を1例に，軽度の脊柱側彎を3例に認めている．全例学生あるいは社会人として通常の社会生活を送っている．

IV. 肝移植と腎移植

東北大学の川岸らは成人胆道閉鎖症に対する生体肝移植の問題点を検討した．東北大学小児外科では，1980年代以降胆道閉鎖症の約70％が10年以上生存しており[1]，20歳以上の長期生存例も40例を超えた[2]．成人の胆道閉鎖症に対する肝移植手術適応とその成績，および移植後成人に達した症例の問題点についてまとめた．

1991年7月～2005年9月に東北大で施行された胆道閉鎖症に対する54例の移植のうち成人以降に移植した3例と移植後成人となった3例を対象として検討した．胆道閉鎖症に対する肝移植適応基準は，①黄疸が消失しない，②黄疸消失後再び出現した，③繰り返す胆管炎，④門脈血流の消失，逆流を認める，⑤門脈圧亢進による消化管出血，⑥肝肺症候群による低酸素血症，⑦栄養，成長障害，⑧肝臓の線維化の進行である[3]．

胆道閉鎖症全体の5年生存率は82.1％(平均観察期間4.5年)で，2001年以降移植した18例は全例生存している．成人以降に移植した3例では，右葉グラフトを用いた2例生存中であるが(1年，8ヵ月)，左葉グラフトの1例は肺高血圧症，多臓器不全で死亡した．肝移植適応としては，肝不全状態が2例，食道静脈瘤破裂が1例であった．移植後成人となった3例は，移植時17歳，16歳，12歳で現在は26歳，24歳，21歳であり，外来フォロー中である．肝移植適応としては肝不全が2例(1例は血液型不適合)，肝不全＋肝肺症候群が1例であった．術後長期経過では2例が胆管-空腸吻合部狭窄でステント留置をしており，1例は移植後糖尿病となりインスリン療法中である．上記生存例5例はいずれも成人後小児慢性特定疾患の適応をはずれ通常の保険診療となっている．葛西手術による長期生存例が増加するとともに[4]，肝機能の悪化する例も増え，成人の胆道閉鎖症に対する生体肝移植も実施されるようになっている[5]．成人例に対する生体肝移植においては右葉グラフトを用いる施設が増えてきた．当科での右葉グラフトによる成人の胆道閉鎖症症例に大きな合併症はない．しかし，成人症例では長期の罹病による腎症，肝肺症候群などの合併症，ドナー高齢化の問題などクリアしなければならない要素が

小児に比べ多くなる．とくにドナー選定では，小児期移植よりも医学的，社会的にドナー候補者が限られ，やむなく脳死登録をする症例も散見される．小児慢性特定疾患の年齢制限のため，成人になった途端に医療費が増え，移植後入退院を繰り返した場合，医療費の負担額が未成年期と比較して非常に増加する．今後，胆道閉鎖症に対する生体肝移植においては，キャリーオーバー症例が増加すると考えられる．しかし，就学期，就職時期に入退院を繰り返すと経済的に自立した社会人にはなりにくく，医療補助の面からも新たな対策が必要である．

一方，京都府立医科大学の秋岡らは小児腎移植症例の成人後の問題点を検討した．対象は2005年までの移植時年齢15歳以下の症例39例で，移植時の平均年齢は10.6±2.9歳であった．成人に達するまで移植腎が生着し良好な腎機能が得られた症例は24例で，身長は，移植時に－1.2±0.9 SDであったが，移植後最終身長は－2.2±1.2 SDで成長障害の偏位がすすんでいた．体重は，移植時－1.0±0.5 SDから，移植後－0.8±1.5 SDと発育障害が残存したが，成長の偏位は変化がなかった．移植後満足度を調べたアンケート調査の回答者14例中13例(92.9%)が，腎移植後の生活に満足していたが，9例(69.2%)では，免疫抑制薬の副作用の不安やその発症(とくにステロイドの副作用)や易感染性など，長期間の免疫抑制療法に対する合併症などによりなんらかの苦痛や不安を感じていた．とくに2例(15.4%)が移植医療に対する周囲の知識不足や理解不足から，就学や就業に苦悩していた．米国の217例の報告[4]では，95%が満足し82%が就業し社会活動しており，京都府立医大の症例でも90%以上の満足が得られているものの，身体的問題や精神面や社会的面でのサポートを含む移植医療に対する問題などから70%近い症例では悩んでいた．今後も患者や周囲への啓蒙をすすめ，移植医療を一般化することがこれらの問題の解決に重要であると考えられた．

V. 小児循環器疾患における問題点

この30年間に先天性心疾患の治療法およびその治療成績は格段に向上した．それに伴い逆に成長に伴って新たな問題点が生じ，手術を要する疾患も増加した．これらの点について，成育医療センターの竹内は1970～1991年に国立小児病院で手術を行い成育医療センターで経過観察中(f/u)のFallot四徴症(TOF)・大血管転位症(TGA)症例81例について検討した．TOF 56例(平均年齢25±4歳)，根治術時年齢は5±2歳．合併症はHCV 3，ペースメーカー植込み(PMI) 4，再介入は6例で，f/u中央値は18.6年(最高35年)，最新のNYHAも良好である．本邦では不整脈が少ない[7]．右室流出路狭窄(>30 mmHg) 6例，中等度以上の肺動脈弁逆流18例，三尖弁逆流1例で左室駆出率(LVEF) 63±7%，2例が肺動脈弁置換術待機中である．TGAの当院f/u患者は24名〔Mustard(M)：12，Senning(S)：11，他2〕，房室ブロック2，洞不全症候群6，HCV 2例で認めPMIは7(M：6，S：1)例．現在，年齢は28±4歳でf/u中央値は26.4年(最高34年)．Mでは6例でPMI施行，残る6例中4例が結節性調律＋徐脈を呈し，SではP波の減高あるものの9/11例で洞調律である．三尖弁逆流は少なく心胸郭比(CTR)は小さいが体心室の駆出率(EF)はわるい〔<50%：16例(うち<30%：4例)〕．TOFではQOLがよいが長期的には右室流出路の問題が多く，改良が必要と考えられる．TGA-M手術は問題が多々あり，S手術も心房・心室機能の低下を伴い十分な観察が必要と考えられる．

順天堂大学の川崎らは先天性心疾患の手術後20年以上経過して再手術を行った症例11例について調査した．再手術年齢は22～43歳．再手術までの期間は平均24±5.9年で，遺残奇形は，心室中隔欠損症遺残：4，肺動脈弁狭窄症＋肺動脈弁閉鎖不全症遺残：2，肺動脈弁狭窄症遺残：1，動脈管開存症遺残：1，左室流出路狭窄遺残：1，三尖弁閉鎖不全症遺残：1であった．全例に術前に心臓カテーテルを行い手術適応を認めた．TGA I型の1例とTOFの1例を適応不全で失っ

たが，術前診断は困難であった．右心不全の1例に肝硬変が遷延したが，他の8例の経過は順調であった．長期にわたり存在した遺残心奇形は，時期を含めた再手術の適応と方法の決定に注意を要すると考えられた．

おわりに

成人期に達した小児の外科的疾患についてシンポジストの発表内容を中心にまとめた．現在も治療困難な疾患に対する外科的挑戦は続いており，今回は取り上げなかった疾患，たとえば短小腸と小腸移植，先天性気管狭窄症，総排泄腔異常，左心低形成をはじめとする複雑先天性心疾患などでも今後大きな外科的問題が予想される．救命できればよしとされた疾患も，より低侵襲で，長期的に良好な機能を目指して今後の努力が必要である．

◆ ◆ ◆ 文献 ◆ ◆ ◆

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Superior Protective and Therapeutic Effects of IL-12 and IL-18 Gene-Transduced Dendritic Neuroblastoma Fusion Cells on Liver Metastasis of Murine Neuroblastoma[1]

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Fusion vaccine of dendritic cells (DCs) and tumor cells has the advantage of inducing an immune response against multiple tumor Ags, including unknown tumor Ags. Using the liver metastasis model of C1300 neuroblastoma cells, we assessed the protective and therapeutic effects of fusion cells transduced with the IL-12 gene and/or the IL-18 gene. Improving the fusion method by combining polyethylene glycol and electroporation increased loading efficiency. In the A/J mice vaccinated with fusion cells modified with the LacZ gene (fusion/LacZ), IFN-γ production and CTL activity increased significantly compared with that of DCs/LacZ, C1300/LacZ, or a mixture of the two (mixture/LacZ). With the transduction of IL-12 and IL-18 genes into the fusion cells (fusion/IL-12/IL-18), the level of IFN-γ increased more than five times that of other fusion groups. In addition, NK cell activity and CTL activity increased significantly compared with that of mixture/LacZ, fusion/LacZ, DC/LacZ, or C1300/LacZ. In the protective and therapeutic studies of fusion cell vaccine, mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18 showed a significant decrease in liver metastasis and a significant increase in survival compared with mice given a mixture/LacZ, DCs/LacZ, or C1300/LacZ. In particular, the mice receiving fusion/IL-12/IL-18 vaccine showed a complete protective effect and the highest therapeutic effects. The present study investigates the improved loading efficiency of fusion cells and suggests that the introduction of IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects on liver metastasis of neuroblastoma. *The Journal of Immunology,* **2006, 176: 3461–3469.**

Neuroblastoma, a common malignancy in children, is derived from sympathetic nerve lineage cells and is characterized by aggressive local growth, followed by metastasis to the regional lymph nodes, liver, bones, and bone marrow (1). The prognosis for patients with neuroblastoma relates to its dissemination through the body and has not greatly improved despite multimodal treatments (2). At the time of diagnosis, two-thirds of children with this tumor present with extensive local or distant metastatic disease. The overall survival rate of patients is reportedly only 25% (3, 4). In the absence of an effective conventional therapy for neuroblastoma, alternative biologically based strategies should be investigated. Furthermore, a distinctive scenario exists in patients <1 yr old with stage IV-S disease in which the disease is broadly disseminated but lacks amplification of the N-*myc* oncogene. Of these patients, 25% have spontaneous remissions, possibly mediated by the immune system (5). These findings prompted us to investigate immunotherapy for patients with neuroblastoma.

Dendritic cells (DCs),[3] the most potent of APCs, induce a primary antitumor immune response via direct cell-cell interactions and/or cytokine production (6, 7). This antitumor immune response of DCs has resulted in the development of DC-based tumor vaccines, which are used clinically as a form of immunotherapy (8, 9). Despite the focus on DCs, this approach has not yet resulted in any significant therapeutic benefit in neuroblastoma. DCs have been loaded with tumor-derived material in several ways, such as pulsing of synthetic peptides, tumor cell lysate, and transducing tumor-derived RNA (8–11). In many tumor cells tumor-specific Ags remain unidentified. In neuroblastoma, a few tumor-associated Ags, such as survivin, NY-ESO, and MYC-N, have been reported in humans (12–14). Most clinical tumor samples have shown heterogeneity of tumor Ag and tumor cells with immunogenicity-evading immunological surveillance. Recently, an interesting new vaccine therapy that uses a fusion of DCs and tumor cells was reported (15–21). Fusion vaccine has the advantage of inducing an immune response against multiple tumor Ags, including unknown ones. With its use, the heterogeneity of tumor cells can be overcome. When making fusion vaccines, whole tumor cells are loaded with DCs, using polyethylene glycol (PEG) or electroporation. However, the loading efficiencies of these methods are insufficient and must be improved to increase the therapeutic effect of fusion vaccine (17–21).

IL-12 is a 70-kDa (p70) heterodimer protein in which the 40-kDa (p40) and 35-kDa (p35) subunits are connected by one S-S bond (22, 23). IL-12, a potent proinflammatory cytokine, is produced primarily by professional APCs, such as DC and macrophages, and exerts pleiotropic effects on immune effector cells

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[3] Abbreviations used in this paper: DC, dendritic cell; PEG, polyethylene glycol; MOI, multiplicity of infection.

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(24). IL-12 induces Th1 differentiation from naive Th0 cells, stimulates IFN-γ production, promotes proliferation of T and NK cells, and enhances CTLs, NK cells, and lymphokine-activated T cells (25, 26). IL-18, an 18.3-kDa glycoprotein, was initially identified as a cytokine that facilitates the production of IFN-γ induced by endotoxin (27). IL-18 plays an essential role in inducing a Th1 response in vivo. It stimulates T cell proliferation, augments CTL activation, and enhances NK cell cytolytic activation, mediated primarily via the FasL-Fas mechanism (28, 29). Interestingly, IL-12 with IL-18 was shown to induce the highest level of IFN-γ in vitro and in vivo (30, 31). Cumulative evidence has shown the importance of IFN-γ in the induction of native and acquired immunity.

The aim of this study is to clarify the role of fusion vaccine for treating neuroblastoma with liver metastasis, and to evaluate the antitumor effect of the transduction of both IL-12 and IL-18 genes to fusion vaccine.

Materials and Methods

Mice and cell lines

Pathogen-free A/J (H-2^a) female mice, 8- to 10-wk-old, were purchased from Japan SLC. During the experiments, they were kept in pathogen-free animal facilities at a controlled temperature and humidity, according to the guidelines of the university. Murine neuroblastoma C-1300 originating in A/J mice was maintained in RPMI 1640 (Nissui Pharmaceutical) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. MC38 adenocarcinoma cells (H-2^b) were provided by Dr. J. Primus (Vanderbilt University Medical Center, Nashville, TN), and YAC-1 lymphoma was purchased from the DNA Bank, BioResource Center, RIKEN.

Generation of bone marrow-derived DCs and phenotype of cell surface

DCs were prepared from bone marrow as described previously (32). To analyze the surface phenotype, DCs were stained with PE or FITC-conjugated mAb, including H-$2K^k$, I-A^k, CD11, CD80, CD83, and CD86 (BD Pharmingen), and expression of the surface markers was examined by FACSCalibur (BD Biosciences).

Recombinant adenoviral vectors

A mature IL-18 cDNA molecule was isolated by PCR, using full-length IL-18 cDNA expression vector plasmid (provided by Dr. M. Okamura of Hyogo College of Medicine, Hyogo, Japan). First, the PCR fragment of the mature IL-18 was cloned into a *HindIXho*I site of the pSecTag2B eukaryotic expression plasmid vector, which contains the Igκ leader sequence as described previously (Invitrogen Life Technologies) (33). Then, mature IL-18 gene combined with a leader sequence was cloned by PCR and inserted into the cosmid vector pAxCAwt using an adenovirus expression vector kit (Takara Biomedicals) to generate the pAxCAmIL-18 constructs. The subunit of the IL12p35 and IL12p40 genes was inserted into the pIRES vector (BD Biosciences). Then, the IL-12 gene (p35 and p40) was cloned by PCR and inserted into the cosmid vector pAxCAwt to generate the pAxCAmIL-12 constructs. pAxCALacZ-encoding β-galactosidase was also constructed using the same cosmid vector. To isolate the recombinant adenovirus, each construct was cotransfected with restriction enzyme-digested DNA-terminal protein complex into 293 cells (DNA Bank, BioResource Center, RIKEN). The recombinant virus was purified through ultracentrifugation in cesium chloride step gradients, and its titer was determined by PFU on the 293 cells, according to standard protocols (34). The resultant adenovirus vectors were named Ad-IL-18, Ad-IL-12, and Ad-LacZ.

Transduction of IL-12 and IL-18 genes by adenovirus vector

DCs were cultured with Ad-IL-12, or Ad-LacZ adenovirus (multiplicity of infection (MOI) 100), and C1300 neuroblastoma cells were cultured with Ad-IL-18, or Ad-LacZ adenovirus (MOI 100). The gene was transduced by centrifuging the culture plates for 2 h at 700 × *g*. The gene transduction efficacy of DCs and C1300 neuroblastoma was assessed by intracellular staining, using the X-gal staining assay kit (Gene Therapy System). After gene transduction, DCs and tumor cells were prepared for cell fusion. Forty-eight hours after culture, the production of IL-12 and IL-18 protein by the gene-transduced DCs (5×10^5), C1300 tumor cells (5×10^5), and fusion cells (5×10^5) was measured with an ELISA Kit (p70 IL-12; BD Pharmingen) (IL-18; Medical and Biological Laboratories).

Loading of tumor Ags

Three fusion methods of PEG (Sigma-Aldrich) treatment, electrofusion, and a combination of PEG and electrofusion were examined. DCs and irradiated (100 Gy) C1300 neuroblastoma were mixed at a ratio of 2:1 (DC:tumor cells) and centrifuged at1500 rpm for 5 min. For fusion by PEG, 50% PEG solution was added to the cell pellet and treated for 1 min, then diluted PEG was added and incubated at 37°C, 5% CO_2. For electrofusion, a mixture of DCs and tumor cells were resuspended in hybrid medium (0.25 M glucose with 0.1 mM $Ca2^+$ and 0.1 mM $Mg2^+$) and dielectrophoretically aligned to form cell-cell conjugates by alternating current (100 V/cm, 20 s). Then, a fusion pulse of direct current was applied to break down the membrane and to form hybrid cells (1.2 kV/cm, 30 μs), and finally, an alternating current of postfusion was applied to solidify the conformation of the hybrid cells (100 V/cm, 3s) (Nepa Gene). To improve the loading efficiency, we examined a two-step fusion procedure combining PEG treatment and electrofusion. In the first step, the mixture of DCs and irradiated tumor cells was treated with 50% PEG as described previously, and the cells were incubated at 37°C, 5% CO_2. In the second step, nonadherent cells, which are mostly unfused cells, were collected and refused by electrofusion. PEG fusion cells and electrofusion cells were gathered and prepared to examine loading efficiency. To determine the loading efficiency, DCs and tumor cells were prestained with the fluorescent dyes DiO (green fluorescence; Molecular Probes) and DiI (red fluorescence; Molecular Probes), respectively, and analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). The form of the loading cell was confirmed by an inverted system fluorescence microscope (Olympus).

Animal studies

Experimental groups for the immunization of each cell were as follows: 1) PBS; 2) DCs transduced with the LacZ gene (DC/LacZ); 3) C1300 tumor cells transduced with the LacZ gene (C1300/LacZ); 4) C1300 tumor cells transduced with the IL-12 gene (C1300/IL-12); 5) C1300 tumor cells transduced with the IL-18 gene (C1300/IL-18); 6) C1300 tumor cells transduced with the IL-12 and IL-18 gene (C1300/IL-12/IL-18); 7) a mixture of DCs/LacZ and C1300/LacZ (mixture/LacZ); 8) a mixture of DCs/IL-12 and C1300/IL-18 (mixture/IL-12/IL-18); 9) fusion of DC/LacZ and C1300/LacZ (fusion/LacZ); 10) fusion of IL-12 gene-transduced DCs and LacZ gene-transduced C1300 tumor cells (fusion/IL-12); 11) fusion of LacZ gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-18); and 12) fusion of IL-12 gene-transduced DCs and IL-18 gene-transduced C1300 tumor cells (fusion/IL-12/IL-18). To examine the protective effect, each vaccine (5×10^5 cells/mouse) was administered s.c. to the inguinal region on days 7 and 14, before the i.v. administration of C1300 tumor cells (1×10^6 cells/mouse). To examine the therapeutic effect, each vaccine (5×10^5 cells/mouse) was administered s.c. to the inguinal region on days 3 and 10 after i.v. inoculation of the C1300 tumor cells. At 21 days after the tumor injection, the livers were collected and the metastases enumerated. Survival time and rates were observed until 90 days after tumor inoculation.

Cytokine production and cytotoxicity assay

The spleens were collected 2 wk after the administration of each vaccine, and cytokine production and cytotoxicity assay (NK and CTL) were examined. Splenic NK cells were isolated from spleen cells with the NK cell isolation kit (Miltenyi Biotec) for NK assay, and T cells were separated using a T Cell Immunocolumns Kit (Cedarlane Laboratories) for cytokine production and CTL assay. To characterize the effector cells, CD4- and/or CD8-positive cells were eliminated by AutoMACS separation system (Miltenyi Biotec). Splenic T cells were restimulated in vitro with irradiated (50 Gy) fusion cells at 37°C under 5% CO_2 for 48 h, and levels of IFN-γ, IL-4, and IL-10 were detected using a Mouse CBA Kit (BD Pharmingen). For cytotoxicity assay, NK or T cells were restimulated in vitro with irradiated (50 Gy) fusion cells in the presence of 10 U of IL-2 (Sionogi) and 10 U of IL-7 (Genzyme) for 5 days. Viable cells were then separated with Lympholyte-M (Cedarlane Laboratories) and prepared as effector cells. As target cells, YAC-1 cells and C1300 were used for the NK assay, and C1300 and allogeneic MC38 cells were used for the CTL assay. The target cells labeled with Na_2 ^{51}Cr O4 (DuPont-NEN) were cultured with each of the effector cells for 4 h in NK assay and 6 h in CTL assay. Spontaneous and maximum release was measured by adding medium and 0.045 M NH4OH instead of effector cells. Each sample was assayed in triplicate.

The percentage of cytotoxicity was determined by calculating the percentage of specific ^{51}Cr release according to the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. All determinations were made in triplicate.

In vivo depletion of T cell subsets and NK cells

Mice received injections i.v. with rat azide-free anti-mouse CD4 mAb (YTS191.1; Serotec), rat anti-mouse CD8 mAb (YTS169.4; Serotec), rabbit anti-mouse Asialo GM1 polyclonal Ab (Wako Chemical), or normal rat IgG (Sigma-Aldrich) at 1 mg/mouse 1 day before fusion/IL-12/IL-18 vaccine inoculation and once every 5 days thereafter for an additional 20 days. Depletion of the corresponding NK, CD4, or CD8 T cells was >80%, as confirmed by FACS analysis of spleen cells from treated mice.

Statistical analysis

Values were presented as means ± SD. Multiple groups were evaluated by ANOVA and the posthoc Scheffe multirange test. Survival estimates were determined using the Kaplan-Meier method, and data were compared by the log-rank test. A probability of <0.05 was considered significant.

Results

Comparing the loading efficiency and antitumor effect after improving the fusion method

The loading efficiency of electrofusion, PEG, and PEG followed by electrofusion (the two-step method) was compared using the DCs and C1300 tumor cells, which were labeled with DiO and DiI intracellular fluorescent dyes, respectively (Fig. 1). DCs loaded by whole tumor cells were characterized by the emission of both colors in the *upper right* of the dot plot analysis. Double-positive cells increased from 12.9 ± 3.2% using electrofusion or 34.1 ± 2.6% using PEG treatment to 51.6 ± 2.1% using the two-step method (Fig. 1*A*). This suggests that the loading efficiency of the two-step fusion method increased 1.5 times and is 4 times higher than with PEG treatment and electrofusion, respectively. In the two-step method, the loading efficiency of gene-transduced fusion cells was 45.2 ± 2.3% in fusion/LacZ, 48.1 ± 2.2% in fusion/IL-18, 49.7 ± 4.1% in fusion/IL-12, and 50.7 ± 3.5% in fusion/IL-12/IL-18, and there were no significant differences among them (data not shown).

The same samples of FACS analysis were observed under a fluorescent microscope (Fig. 1, *A* and *B*). In fluorescent micrographs of fusion cells, a unity of cell membranes of multinuclear cells was recognized. Using this method, cell viability was >80%, which was achieved by the trypan blue dye exclusion test. In contrast, simple repetition of PEG treatment or electrofusion caused a significant decrease in cell viability (<50%; data not shown).

Next, we compared the ability for CTL production of these fusion cells generated by the different protocols (Fig. 1*C*). The cytotoxicity of splenic lymphocytes from mice vaccinated with two-step fusion cells was significantly higher than that of PEG treatment or electroporation alone. These results show the usefulness as cancer vaccines of fusion cells generated by the two-step method.

Cell surface phenotypes of DCs, tumor cells, and fusion cells

The cell surface markers of each vaccine were analyzed by FACS (Fig. 2). C1300 tumor cells transduced with the LacZ gene (C1300/LacZ) showed only the expression of MHC class I Ags (H-2K) but not MHC class II (I-A), CD11c, or costimulatory molecules (CD80, CD83, CD86). DCs transduced with the LacZ gene (DCs/LacZ) expressed high levels of MHC class II Ags and CD11c, CD80, and CD86, and a low level of CD83. Almost the same levels of DC-derived markers were identified on the surface of the fusion cells transduced with the LacZ gene (fusion/LacZ) or the IL-18 gene (fusion/IL-18). In contrast, transduction with the IL-12 genes enhanced the expression of CD83 molecule on the surface of the fusion cells, and a high level of CD83 was shown in fusion/IL-12 and fusion/IL-12/IL-18.

Cytokine production by gene-transduced DCs and tumor cells

At a MOI 100, the gene transduction efficiency for IL-12 in DCs was 61%, and that for IL-18 in C1300 tumor cells was 68% (data not shown). Fig. 3*A* shows the IL-12 protein levels in the medium of each group. Significant levels of p70 IL-12 proteins were produced in DCs or fusion cells following transduction with the IL-12 gene. The p70 IL-12 levels of the DCs/LacZ, fusion/LacZ, DCs/

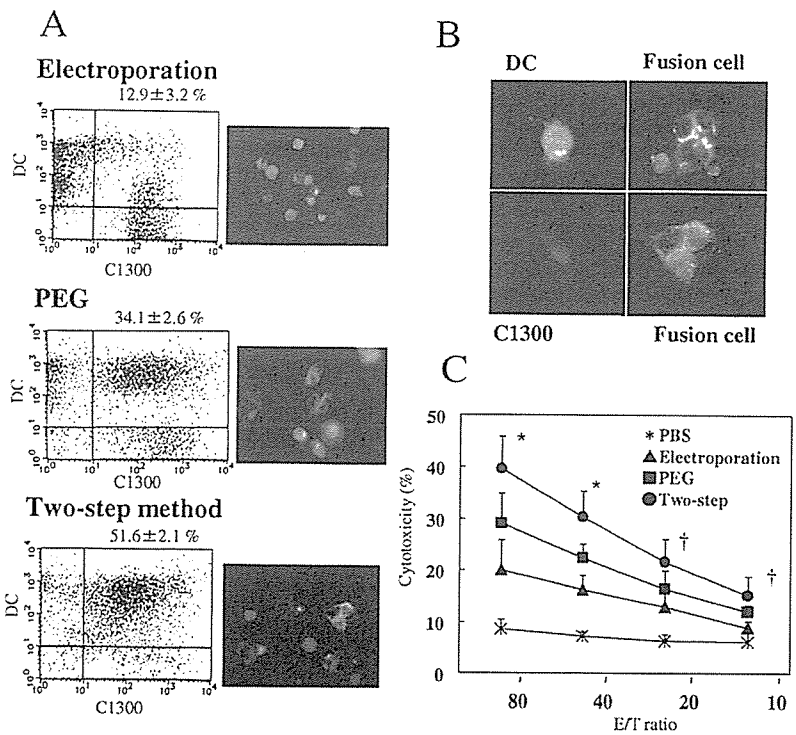

FIGURE 1. Comparison of loading method of C1300 neuroblastoma to DCs and CTL activities of fusion cells generated by different protocols. *A*, DiI-labeled C1300 neuroblastoma was loaded to DiO-labeled DCs by electroporation, PEG-treatment, or a combination of PEG and electroporation (two-step method), and loading efficiency was examined by FACS analysis. Each experiment was done in triplicate. The figures in the *upper right panel* show the mean percentages of double-positive cells (mean ± SD). Morphology of cells analyzed by FACS was observed by fluorescent microscopy (×100). *B*, Magnified fluorescent micrographs show the DC, C1300 tumor cells, and fusion cells (×250). *C*, Splenic T cells were collected from mice vaccinated with DC/1300 fusion cells prepared by electroporation, PEG treatment, or the two-step method, and cytotoxicity was measured against C1300 tumor cells. *, $p < 0.05$ (two-step vs PEG, electroporation, PBS). †, $p < 0.05$ (two-step vs electroporation, PBS. Mean ± SD ($n = 5$).

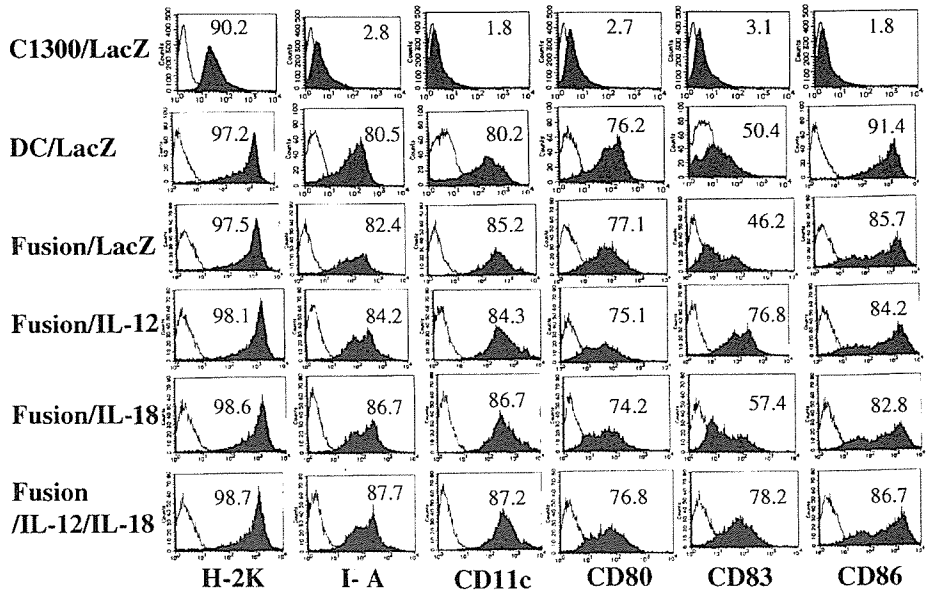

FIGURE 2. Cell surface phenotypes of DCs, C1300 tumor cells, and fusion cells. LacZ gene-transduced C1300 tumor cells (C1300/LacZ), LacZ gene-transduced DCs (DCs/LacZ), LacZ gene-transduced fusion cells (fusion/LacZ), IL-12 gene-transduced fusion cells (fusion/IL-12), IL-18 gene-transduced fusion cells (fusion/IL-18), and IL-12 and IL-18 gene-transduced fusion cells (fusion/IL-12/IL-18) were stained with PE or FITC-labeled mAb (anti-H-2K, anti-I-A, anti-CD11c, anti-CD80, anti-CD83, and anti-CD86). Surface markers were analyzed by FACS. Numerical values show the percentage of positive cells.

IL-12, fusion/IL-12, and fusion/IL-12/IL-18 were 166 ± 51 pg/ml, 185 ± 65 pg/ml, 3210 ± 702 pg/ml, 2820 ± 568 pg/ml, and 2672 ± 468 pg/ml, respectively. There were no significant differences in IL-12 levels between the DCs/IL-12, fusion/IL-12, and fusion/IL-12/IL-18. Fig. 3*B* shows the IL-18 protein levels in the medium of each group. Significant levels of IL-18 proteins were produced in C1300 cells or fusion cells by transduction with the IL-18 gene. The IL-18 levels of C1300/LacZ, fusion/LacZ, C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18 cells were 58 ± 25 pg/ml, 78 ± 35 pg/ml, 1280 ± 308 pg/ml, 1036 ± 202 pg/ml, and 1006 ± 282 pg/ml, respectively. No significant differences in IL-18 levels were found between the C1300/IL-18, fusion/IL-18, and fusion/IL-12/IL-18.

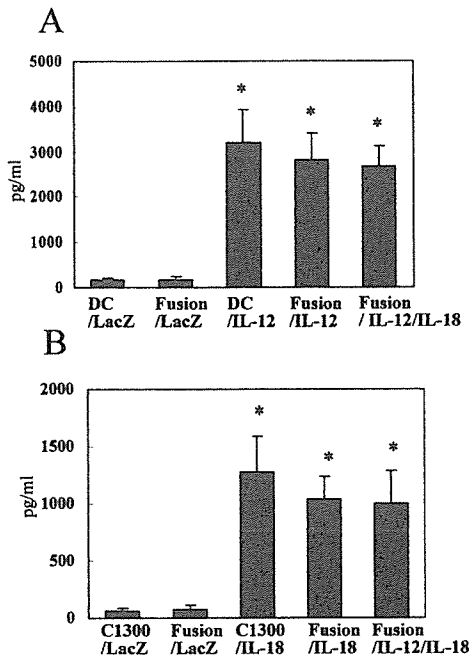

FIGURE 3. Cytokine production by gene-transduced DCs, C1300, and fusion cells. DCs and C1300 tumor cells were infected with LacZ, IL-12, or IL-18 recombinant adenovirus vector and fused. Forty-eight hours after infection, IL-12 (*A*) and IL-18 (*B*) protein levels in the supernatant were examined by ELISA. *, $p < 0.01$ (DCs/IL-12, fusion/IL-12, fusion/IL-12/IL-18 vs DCs/LacZ, fusion/LacZ). *, $p < 0.01$ (C1300/IL-18, fusion/IL-18, fusion/IL-12/IL-18 vs C1300/LacZ, fusion/LacZ). Mean ± SD ($n = 5$).

Cytokine production by splenic T cells

Production of IFN-γ by splenic T cells collected from mice immunized with each vaccine and cell surface phenotypes of effector cells in IFN-γ production were examined (Fig. 4). IFN-γ levels in the culture supernatant of splenic T cells were significantly higher in the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 than with the mixture of DC/LacZ and C1300/LacZ (mixture/LacZ), DCs/LacZ, C1300/LacZ, or PBS (Fig. 4*A*). The fusion/IL-12/IL-18-vaccinated group showed the highest levels of IFN-γ compared with that of fusion/LacZ, fusion/IL-12, and fusion/IL-18. In contrast, IL-4 and IL-10 levels from splenic T cells were very low in all groups, and there were no significant differences between these groups (data not shown). To characterize the T cells that produce the IFN-γ, $CD8^+$ T cells and/or $CD4^+$ T cells were depleted by negative selection of MACS (Fig. 4*B*). In the mice vaccinated with fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, production of IFN-γ was significantly inhibited by depletion of splenic T cells expressing not only $CD4^+$ but also $CD8^+$ T cells. These results suggest that a fusion vaccine itself has the ability to induce a Th1 immune response, and cotransduction of the IL-12 and IL-18 genes causes a strong shift to a Th1 response by markedly increasing production of IFN-γ by $CD4^+$ and $CD8^+$ T cells.

Cytotoxicity of splenic NK cells and splenic T cells

Productivity of cytotoxic splenic NK cells against NK-sensitive YAC-1 cells and C1300 cells was examined (Fig. 5*A*). Splenic NK cells of fusion/LacZ-vaccinated mice showed a significant increase of cytotoxicity against YAC-1 cells compared with mice vaccinated with DC/LacZ, C1300/LacZ, and PBS. Furthermore, NK activities were increased by transduction of IL-12 and/or IL-18 genes. Splenic NK cells of the mice vaccinated with fusion IL-12/IL-18, fusion/IL-12, fusion/IL-18, or nonfused mixture/IL-12/IL-18 showed a significant increase of cytotoxicity against YAC-1 cells and C1300 cells compared with that of mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS-administered mice. The group vaccinated with fusion/IL-12/IL-18 showed the highest NK activity in

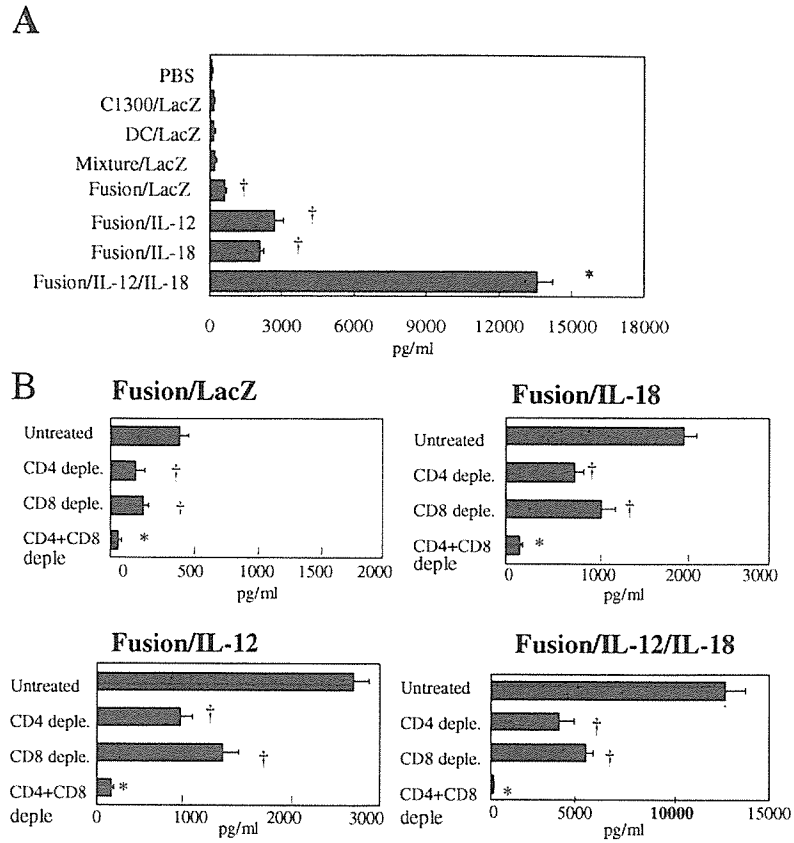

FIGURE 4. Production of IFN-γ by splenic T cells from mice immunized with each vaccine, and cell surface phenotypes of effector cells in IFN-γ production. *A*, IFN-γ production. Mice were immunized with each vaccine (PBS, C1300/LacZ, DC/LacZ, mixture/LacZ, fusion/LacZ, fusion/IL-12, fusion/IL-18, fusion/IL-12/IL-18). On day 14 after immunization, splenic T cells were separated and incubated for 48 h with irradiated DC/C1300 fusion cells. IFN-γ levels in culture medium were measured by ELISA. *, $p < 0.0001$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.01$ (fusion/LacZ, fusion/IL-12, fusion/IL-18 vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *B*, Cell surface phenotypes of effector cells in IFN-γ production. Mice were immunized with fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18, which have the ability to induce IFN-γ. CD4 and/or CD8 T cells were depleted by MACS, and the productivity of IFN-γ was compared with the untreated group. *, $p < 0.01$ ($CD4^+CD8$ depletion vs untreated). †, $p < 0.05$ (CD4 depletion, CD8 depletion vs untreated). Mean ± SD ($n = 5$).

all groups. The NK activity against YAC-1 cells was higher than that of C1300 cells. These results suggest that the fusion vaccine has the ability to induce NK activity, and that transduction of IL-12 and IL-18 genes greatly increase the NK activities.

Next, we examined the CTL activities of splenic T cells against parental C1300 tumor cells and allogeneic MC38 tumor cells (Fig. 5*B*). In the mice transduced with the LacZ gene, CTL activity against C1300 tumor cells was significantly higher in the mice vaccinated with fusion cells compared with those vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, and PBS. The CTL activities of splenic T cells in mice vaccinated with fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18 were higher than that of mice vaccinated with fusion/LacZ, and mice vaccinated with fusion/IL-12/IL-18 showed the highest level of activity in these groups. In contrast, mice vaccinated with nonfused mixture/IL-12/IL-18 or mixture/LacZ did not show a significant level of CTL activity. These results suggest that the formation of fusion cells is important for CTL induction. Cytotoxic activity against allogeneic MC38 adenocarcinoma was not detected in any group.

We then determined the surface phenotype and the location of MHC restriction of the cytotoxic effector cells induced by each fusion vaccine (Fig. 5*C*). The cytotoxic activity in all fusion groups (fusion/LacZ, fusion/IL-12, fusion/IL-18, and fusion/IL-12/IL-18) was significantly inhibited by the depletion of $CD8^+$ T cells but not by $CD4^+$ T cells. In the blocking assay of MHC Ags, CTL activities of all fusion groups were significantly decreased by treatment in anti- $H\text{-}2K^K$ mAb. However, treatment with anti-$I\text{-}A^K$ mAb did not affect cytotoxicity. These results indicate that fusion cells could induce MHC class I-restricted $CD8^+$ CTLs.

Protective effects of fusion vaccine

The protective effect induced by each vaccine was assessed by the number of liver metastases at 21 days and the survival rates 90 days after tumor inoculation (Table I and Fig. 6*A*). Each vaccine was administered on days 7 and 14, before the i.v. inoculation of C1300 tumor cells. In mice vaccinated with fusion/LacZ, a significant decrease in the number of liver metastases was observed compared with that of mice vaccinated with mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. Transduction with IL-12 or IL-12 and IL-18 gene into fusion cells led to a significantly lower liver metastasis number compared with mice vaccinated with C1300/IL-12, C1300/IL-18, mixture/LacZ, DCs/LacZ, C1300/LacZ, or PBS. In particular, fusion/IL-12/IL-18 vaccine showed a dramatic decrease of liver metastasis, and all mice were tumor-free.

In a comparison of survival rates, the mice vaccinated with fusion/LacZ showed a significant increase compared with mice vaccinated with mixture/LacZ, C1300/LacZ, DC/LacZ, or PBS (Fig. 6*A*). Transduction with the IL-12 and/or IL-18 gene into fusion cells led to a higher survival rate compared with that of fusion/LacZ. In particular, fusion/IL-12/IL-18 vaccine showed a complete protection, and all mice remained tumor-free for at least 90 days. In contrast, mice vaccinated with mixture/IL-12/IL-18, C1300/IL-12/IL-18, C1300/IL-12, and C1300/IL-18 showed partial protection compared with that of fusion/IL-12/IL-18. These results demonstrate that the fusion cells transduced with the IL-12 and IL-18 genes lead to a dramatic protective effect and that conformation of fusion cells is important for strong tumor protection.

Therapeutic effects of fusion vaccine

To assess the therapeutic effects, each vaccine was administered after tumor inoculation, and the number of liver metastasis and survival rates were examined (Table II and Fig. 6*B*). In the study of liver metastasis, the mice vaccinated with fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, or mixture/IL-12/IL-18 demonstrated a significant decrease in liver metastasis number compared with that

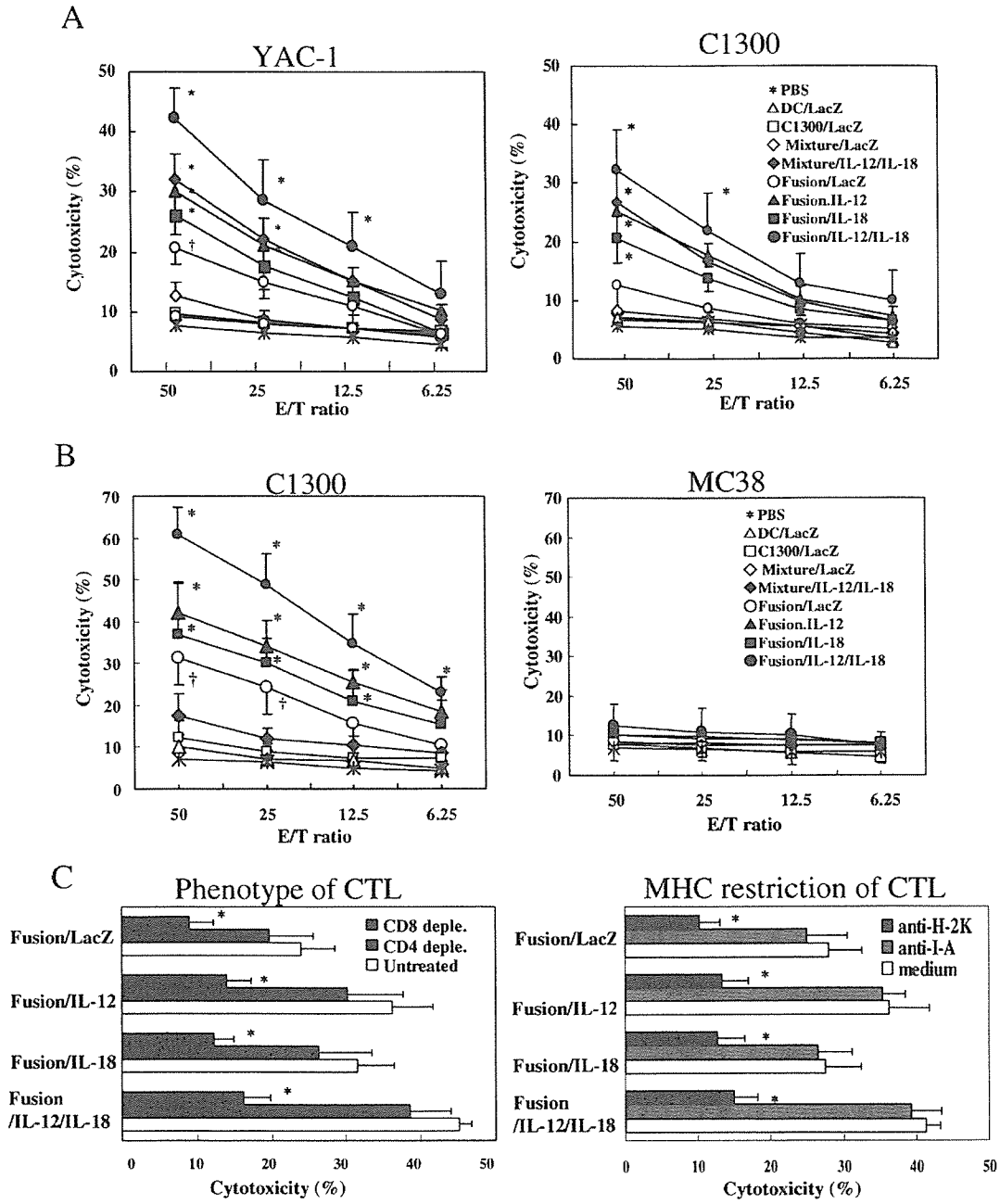

FIGURE 5. NK and cytotoxic T cell activities of splenic NK cells or T cells from mice immunized with each vaccine. *A*, NK activity. On day 14 after vaccination by DC/LacZ, C1300/LacZ, mixture/LacZ, mixture/IL-12/IL-18, fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18, NK cells were isolated from the spleen and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against YAC-1 and C1300 tumor cells. *, $p < 0.05$ (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18 vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, $p < 0.05$ (fusion/LacZ vs DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *B*, CTL activity. On day 14 after vaccination by each vaccine, T cells were separated from spleen cells and cocultured with irradiated DC/C1300 fusion cells, and cytotoxicity was measured against parental C1300 tumor cells and MC38. *, $p < 0.05$ (fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18 vs mixture/IL-12/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). †, $p < 0.05$ (fusion/LacZ vs mixture/LacZ, DC/LacZ, C1300/LacZ, PBS). Mean ± SD ($n = 5$). *C*, Phenotype and MHC restriction of CTL induced by fusion vaccines. On day 14 after immunization of fusion vaccines (fusion/LacZ, fusion/IL-12, fusion/IL-18, or fusion/IL-12/IL-18), splenic T cells were collected and cocultured with irradiated DCs/C1300 fusion cells, and phenotype and MHC restriction were examined. To examine the phenotype of CTL, $CD8^+$ and/or $CD4^+$ cells were depleted by MACS, and CTL activity was compared with that of undeleted T cells. The ratio of E:T cells was 25:1. *, $p < 0.01$ (CD8 deple vs CD4 deple, untreated). Mean ± SD ($n = 5$). To examine the MHC restriction of effector cells, C1300 target cells were pretreated with anti-H-$2K^k$ mAb, anti-I-A^k mAb or medium, and cytotoxicities were measured. The ratio of E:T cells was 25:1. *, $p < 0.01$ (anti-H-$2K^k$ vs anti-I-A^k, medium). Mean ± SD ($n = 5$).

of mice vaccinated with mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. Fusion/IL-12 showed a significant decrease in the number of liver metastases compared with C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS. In contrast, transduction of the IL-12 and IL-18 genes into fusion cells showed the lowest number of liver metastases in all groups, and this level differed significantly from that of C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ, or PBS.

Table I. *Protective effects of each vaccine on liver metastasis*[a]

Immunogen	Tumor-Free Mice	No. of Liver Metastasis
PBS	0/10	43.5 ± 9.2
DC/LacZ	0/10	37.3 ± 9.8
C1300/LacZ	0/10	40.6 ± 7.1
C1300/IL-18	1/10	24.5 ± 14.2[b]
C1300/IL-12	1/10	20.3 ± 11.9[b]
C1300/IL-12/IL-18	2/10	10.8 ± 9.6[c]
Mixture/LacZ	0/10	34.0 ± 10.5
Mixture/IL-12/IL-18	3/10	7.0 ± 5.3[c]
Fusion/LacZ	2/10	12.2 ± 9.6[d]
Fusion/IL-18	3/10	7.3 ± 7.8[e]
Fusion/IL-12	5/10	4.1 ± 5.1[e]
Fusion/IL-12/IL-18	10/10	0.0 ± 0.0[e]

[a] Mean ± SD (n = 10). The data presented were pooled from two separated experiments, and the reproducibility of results was shown.
[b] $p < 0.05$ (C1300/IL-12, C1300/IL-18 vs. C1300/LacZ, PBS).
[c] $p < 0.05$ (C1300/IL-12/IL-18, mixture/IL-12/IL-18, fusion/IL-18 vs. C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[d] $p < 0.05$ (fusion/LacZ vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).
[e] $p < 0.05$ (fusion/IL-12, fusion/IL-12/IL-18 vs. C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

In the study of survival rates, all of the mice vaccinated with fusion/LacZ died within 57 days of tumor inoculation (Fig. 6*B*). The mice vaccinated with fusion/IL-12, fusion/IL-18, or mixture/IL-12/IL-18 had increased survival rates; however, their levels were <20%. In contrast, mice vaccinated with fusion/IL-12/IL-18 showed a significant increase of survival rates compared with that of all other groups, and 60% of mice remained tumor-free for at least 90 days. The survival rates of mice vaccinated with mixture/IL-12/IL-18 decreased significantly compared with that of mice vaccinated with fusion/IL-12/IL-18, suggesting the significance of conformation of fusion cell for strong therapeutic effect. These results demonstrate that the therapeutic effect of the fusion cells transduced with the IL-12 and IL-18 genes is superior to that of any other group.

Next, we investigated the participation of immune cell subsets in the generation of the therapeutic effects of the fusion/IL-12/IL-18 vaccine (Fig. 7). The NK, CD8 T cells, or CD4 T cells of mice were depleted by the administration of anti-asialoGM1, anti-CD4, or anti-CD8 mAb. The depleted NK cells, CD8 T cells, or CD4 T cells showed a significant increase of liver metastasis compared with normal IgG-injected mice. These results suggest that NK, CD8 T cells, and CD4 T cells are necessary and are associated with the therapeutic effects of the fusion vaccine transduced with the IL-12 and IL-18 genes.

Discussion

In the present study, we demonstrated the protective and therapeutic effect of DC/tumor fusion cells on neuroblastoma with liver metastasis. The transduction of both the IL-12 and the IL-18 genes to fusion cells induced the highest levels of IFN-γ, NK cell activity, and CTL activity. Furthermore, the fusion vaccine transduced with IL-12 and IL-18 genes showed complete protective and highly significant therapeutic effects on liver metastasis of neuroblastoma in mice.

Immunotherapy has been put forward as a feasible strategy for treating neuroblastoma based upon the observation that some aggressive neuroblastomas spontaneously regress (5). Although clinical trials of DC-based immunotherapy were investigated, its effects on several carcinomas were limited by the low number of defined tumor-associated Ags and the heterogeneity of tumor cells. In contrast, a new strategy using a DC-based tumor vaccine reported that DCs loaded with whole tumor cells or tumor lysate have the advantage of inducing antitumor immunity to multiple

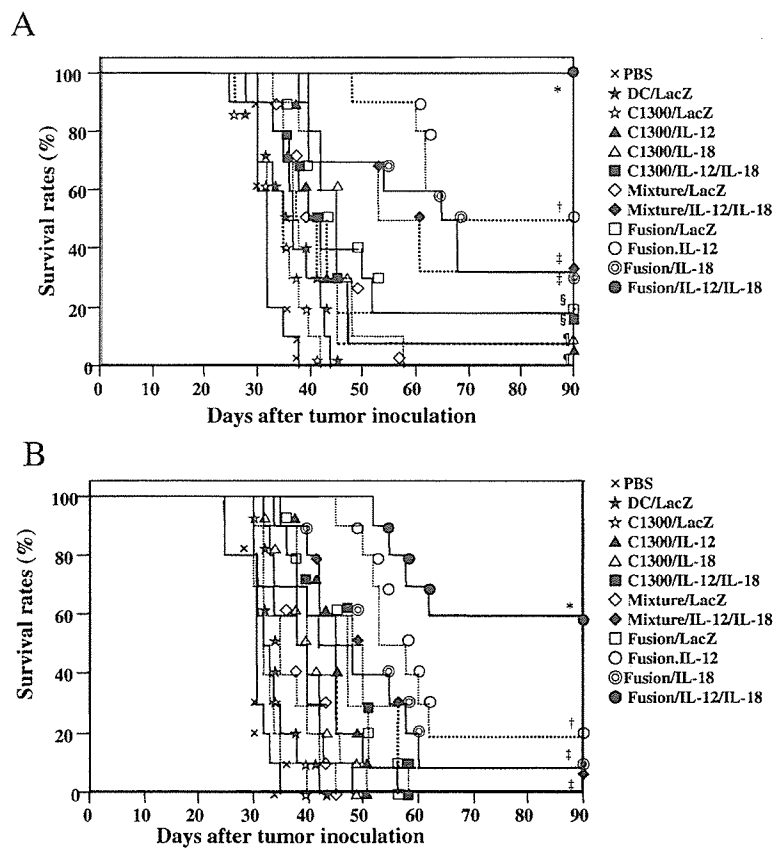

FIGURE 6. Protective and therapeutic effects of each vaccine on survival rates. *A*, Protective effect. On day 7 and day 14 before the i.v. inoculation of C1300, mice were vaccinated with fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, fusion/LacZ, mixture/IL-12/IL-18, mixture/LacZ, C1300/IL-12/IL-18, C1300/IL-12, C1300/IL-18, C1300/LacZ, DCs/LacZ, or PBS. The data presented were pooled from two separate experiments, and the reproducibility of results was demonstrated. *, $p < 0.05$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.05$ (fusion/IL-12 vs all groups except fusion/IL-18 and mixture/IL-12/IL-18). ‡, $p < 0.05$ (fusion/IL-18, mixture/IL-12/IL-18 vs fusion/IL-12/IL-18, C1300/IL-12, C1300/IL-18, mixture/LacZ, C1300/LacZ, DCs/LacZ or PBS). §, $p < 0.05$ (fusion/LacZ, C1300/IL-12/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, mixture/LacZ, C1300/LacZ, DCs/LacZ or PBS). ¶, $p < 0.05$ (C1300/IL-12, C1300/IL-18 vs fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, mixture/IL-12/IL-18, C1300/LacZ or PBS). *B*, Therapeutic effect. On days 3 and 10 after i.v. inoculation of C1300, mice were vaccinated with fusion/IL-12/IL-18, fusion/IL-12, fusion/IL-18, fusion/LacZ, mixture/IL-12/IL-18, mixture/LacZ, C1300/IL-12/IL-18, C1300/IL-12, C1300/IL-18, C1300/LacZ, DCs/LacZ, or PBS. The data presented were pooled from two separate experiments, and the reproducibility of results was demonstrated. *, $p < 0.05$ (fusion/IL-12/IL-18 vs other all groups). †, $p < 0.05$ (fusion/IL-12 vs all groups expect fusion/IL-18). ‡, $p < 0.05$ (fusion/IL-18, mixture/IL-12/IL-18 vs C1300/IL-12, C1300/IL-18, mixture/LacZ, C1300/LacZ, DCs/LacZ, or PBS)

Table II. *Therapeutic effects of each vaccine on liver metastasis*[a]

Immunogen	Tumor-Free Mice	No. of Liver Metastasis
PBS	0/10	48.0 ± 23.2
DC/LacZ	0/10	46.8 ± 21.6
C1300/LacZ	0/10	47.9 ± 21.4
C1300/IL-18	0/10	38.5 ± 10.0
C1300/IL-12	0/10	30.2 ± 11.2
C1300/IL-12/IL-18	0/10	19.6 ± 5.9[b]
Mixture/LacZ	0/10	41.3 ± 14.5
Mixture/IL-12/IL-18	1/10	16.8 ± 9.3[b]
Fusion/LacZ	0/10	20.1 ± 5.0[b]
Fusion/IL-18	1/10	18.7 ± 14.1[b]
Fusion/IL-12	2/10	9.7 ± 6.2[c]
Fusion/IL-12/IL-18	6/10	2.6 ± 3.4[d]

[a] Mean ± SD (n = 10). The data presented were pooled from two separated experiments, and the reproducibility of results was shown.

[b] $p < 0.05$ (fusion/LacZ, fusion/IL-18, C1300/IL-12/IL-18, mixture/IL-12/IL-18 vs. mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

[c] $p < 0.05$ (fusion/IL-12 vs. C1300/LL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

[d] $p < 0.05$ (fusion/IL-12/IL-18 vs. C1300/IL-12, C1300/IL-18, mixture/LacZ, DC/LacZ, C1300/LacZ and PBS).

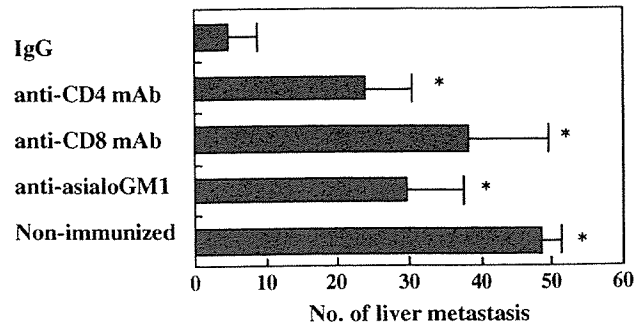

FIGURE 7. Participation of CD4, CD8, and NK cells in fusion/IL-12/IL-18 vaccine-induced immunity. After C1300 tumor inoculation, mice were administered fusion/IL-12/IL-18 vaccine or PBS (nonimmunized). Anti-CD4 mAb, anti-CD8 mAb, anti-Asialo GM1 Ab, or normal rat IgG was injected i.v. before vaccination and once every 5 days thereafter for an additional 20 days. On day 21, the number of liver metastasis was counted. *, $p < 0.01$ (nonimmunized, anti-asialo GM1, anti-CD8 mAb, anti-CD4 mAb vs IgG). Mean ± SD (n = 10).

tumor Ags, including unidentified tumor Ags. A phase I clinical trial of tumor lysate-pulsed DC treatment for patients with neuroblastoma has been conducted and a stable clinical response demonstrated (35). In contrast, our preliminary data have shown that the fusion vaccine of DCs and tumor cells induce a stronger antitumor immunity than that of tumor lysate (our unpublished observations). In previous experimental models using mice, Ag presentation and the antitumor effects of the fusion vaccine were reported in adenocarcinoma, plasmacytoma, hepatocellular carcinoma, and melanoma (15–23). Recently, we reported not only an increase in the antitumor effect, but also protective and therapeutic effects of an IL-2 gene-modified fusion vaccine in mice with pulmonary metastasis of fibrosarcoma (36). In clinical trials of the fusion vaccine in patients with melanoma, glioma, and renal cell carcinoma, complete remission in melanoma, a partial clinical response in glioma, and stability in renal cell carcinoma were demonstrated (37–39).

Although the chemical agent PEG and electrofusion have been used for the fusion vaccine, the fusion process of these methods is different. In PEG treatment, the lipid bilayer of the cell membrane is thought to be broken down through the dehydration action of PEG, followed by an increase in the fluidity of the cell membrane. In contrast, the fundamental step in electrofusion is reversible membrane breakdown. When short-duration, direct current electric impulses applied to the cell membrane exceed a critical threshold, that membrane will become transiently but highly permeable through the formation of micropores. Moreover, the adjacent process of touching cells may form channels and lead to the formation of new spherical hybrid cells. The loading efficiency of PEG and electrofusion was reported to be 17.0–35.0% and 5.0–25.0%, respectively (15–21). Cell fusion is known to be influenced by the characteristics of the cell membrane, and loading efficiency differs markedly among tumor cells. To increase and stabilize loading efficiency, we improved the fusion method by combining PEG and electrofusion. By this two-step method, tumor-loading efficiency was increased 1.6 times that of PEG treatment and 4 times that of electrofusion, and allowed higher CTL activities. We expect that the combination method of PEG and electrofusion may have beneficial effects on the stable loading of various types of tumor cells.

Previous immunotherapeutic approaches to neuroblastoma have focused on the use of IL-2 to activate both T cell-dependent and -independent cytotoxic immune responses (40, 41). Recent strategies for treating neuroblastoma have incorporated advances in molecular biology to engineer a tumor cell for the induction of a more efficient immune response. Yoshida et al. (42) showed that murine neuroblastoma retrovirally transduced with the IL-2 or GM-CSF gene exhibited tumor-specific acquired immunity. Shimizu et al. (43) and Redlinger et al. (44) reported that DCs transduced with the IL-12 gene by adenovirus could induce an antitumor immune response in an established murine neuroblastoma. However, the efficacy of DC transduced with IL-18 gene has not been reported in the murine neuroblastoma model. It has been reported that IL-18 could induce high-level expression of IFN-γ by T cells, NK cells, B cells, and monocytes and play an important role in CTL activation and enhancement of NK cell cytotoxic activity (27, 28). Furthermore, IL-18 acts synergistically with IL-12, but not IL-2 or GM-CSF, in inducing IFN-γ production by T cells (30, 31). Both IL-12 and IL-18 may be required for an effective differentiation into Th1 cells. In this study, we evaluated the Th1 immune response, CTL activity, and NK activity using fusion cells modified with both IL-12 and IL-18 genes. The production of IFN-γ by $CD4^+$ and $CD8^+$ T cells was markedly increased by the vaccination of fusion cells transduced with IL-12 and IL-18 genes. Moreover, transduction with the IL-12 and IL-18 genes in fusion cells showed a significant increase of MHC class I-restricted $CD8^+$ CTL activity and NK activity.

Our results showed that fusion cells of DCs and neuroblastoma, but not a mixture of DCs and neuroblastoma, significantly reduced liver metastasis and significantly increased survival rates compared with DCs or tumor cells alone, suggesting that the formation of fusion cells is important in inducing strong antitumor immunity. Orentas et al. (17) reported the protective effect of electrically prepared fusion cells on mice with Neuro-2a neuroblastoma cells. However, in his paper the loading efficiency by electrofusion was only 5–10%. In contrast, the loading efficiency of our two-step fusion method showed 45–52% efficiency. By improving loading efficiency, we demonstrated not only the protective effects but also the therapeutic effects of the fusion cells themselves. Furthermore, our study showed that the transduction of the fusion cells with both IL-12 and IL-18 genes produced complete protective effects and highly significant therapeutic effects on liver metastasis and survival. The relative importance of the NK cell, $CD4^+$, and $CD8^+$ T cell subsets for the therapeutic effect of the fusion/IL-12/IL-18 vaccine was demonstrated. To the best of our knowledge, this is the first study to demonstrate the protective and therapeutic effects

of an IL-12 and IL-18 gene-modified fusion vaccine on murine neuroblastoma with liver metastasis.

In conclusion, we demonstrated that improved loading efficiency may provide a basis for using a fusion vaccine and that introducing both IL-12 and IL-18 genes can induce extremely strong protective and therapeutic effects.

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Disclosures

The authors have no financial conflict of interest.

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