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非ヒト霊長類造血器腫瘍モデル作出と悪性腫瘍モデル作出に向けた基盤技術の開発に関する研究(H19-生物資源-一般-002)

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総括研究報告書

非ヒト霊長類造血器腫瘍モデル作出と悪性腫瘍モデル作出に向けた基盤技術の開発に関する研究

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研究要旨
サルに疾患モデルを作出することは分子標的薬剤を中心とする新規薬剤を開発する上で必要となる前臨床動物系確立の観点から極めて重要である。成人T細胞白血病/リンパ腫(ATLL)は日本に多く、極めて予後不良のHTLV-1ウイルスを病因とする悪性造血器腫瘍であり、新規治療法開発においてATLLサルモデルは大変重要になるものと考えられる。本研究においてはまずATLLモデルカニクイザルの作出を目的に、HTLV-1産生細胞（CM-1細胞）を妊娠カニクイザル２頭に静脈投与しその後の経過を観察した。母体末梢血中に短期的にHTLV-1の存在が観察され、その後一過性に抗HTLV-1抗体の出現を認めた。また新生仔にも抗HTLV-1抗体の出現を一過性に認めたものの、HTLV-1の存在は末梢血中には認められなかった。今後免疫抑制剤などの投与を併用し、まずHTLV-1キャリアーカニクイザルの作出を行うことが重要であると考えられた。この結果を基に、飼育が比較的簡便でコスト的にも経済的な小型霊長類コモンマーモセットを対象とした検討を行うこととし、コモンマーモセット2個体にHTLV-1産生細胞(MT-2細胞)を、腹腔内もしくは経静脈的に投与した。腹腔内投与個体において1年間以上にわたりHTLV-1抗体価の上昇とプロウイルスの検出を認めており、キャリア状態の作出に成功した。今後免疫抑制剤を投与することで、ATLL発症誘導の可能性についても検討する予定である。
同様に、造血器悪性腫瘍の中でも治療予後の悪いフィラデルフィア染色体陽性急性リンパ性白血病に対する新規治療法開発においても、コモンマーモセットモデルは極めて有用になるものと考えられる。コモンマーモセットに急性リンパ性白血病モデルを作出する目的で、レンチウイルスベクターを用いたp190 bcr/abl遺伝子導入自家末梢血幹細胞移植ならびに骨髄内への同レンチウイルスベクター直接注入を行い、後者において持続的にコモンマーモセット末梢血ならびに骨髄細胞内にp190遺伝子持続発現を認めた。しかし、その後p190遺伝子は検出できなくなり現在に至っている。白血病発症を加速させるべく、免疫抑制剤の投与やp190以外の付加的な遺伝子の投与等を検討している。
コモンマーモセット造血および免疫系細胞をより科学的に同定・解析することはコモンマーモセット白血病ならびにATLLモデルの解析上きわめて重要となるため、細胞同定および分離に必須となるコモンマーモセット単クローン抗体を作製するとともに、その特性を検討した。また、これらの研究に供するためのコモンマーモセット骨髄細胞の供給支援体制を確立した。
遺伝子ノックイン技術を応用し悪性腫瘍高発症コモンマーモセットを作出することを最終目的にコモンマーモセット ES 細胞への p53 遺伝子への変異導入を行うため、コモンマーモセット p53 ゲノム解析とそれを基に Zinc finger nuclease の設計・構築を行った。

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A. 研究目的

1. 成人T細胞性白血病・リンパ腫(ATLL)は、HTLV-1 (human T-cell leukemia virus type 1) ウイルスの感染後、長期間のキャリア状態を経て発症する予後不良の造血器悪性腫瘍であり、未だ有効な治療法は確立されておらず特に本邦に多くのHTLV-1キャリアおよび患者が集積していることから、新たな治療法の導入が急務である。候補となる新薬の安全性と有効性を検討する前臨床動物としてげっ歯類のATLLモデルはすでに報告されているが、ヒトと遺伝学的背景がより近い霊長類のモデルは未だ確立されていない。本研究では、ATLLモデル作出をカニクイザルならびに小型霊長類コモンマーモセットを用いて行い、いずれが適切なHTLV-1感染モデル動物系として前臨床試験に用いることができるかについて検討する。
2. Ph(フィラデルフィア)染色体陽性成人急性リンパ性白血病（ALL）は、9番染色体と22番染色体の長腕間相互転座t(9;22)(q34;q11)(Ph1染色体)から生じるp190 bcr/abl融合遺伝子を主病因とする予後不良の造血器悪性腫瘍である。未だ有効な治療法は確立されておらず、造血細胞移植後においても再発率が高いことより、新たな治療法の導入が急務である。候補となる新薬の安全性と有効性を検討する前臨床動物としてマウス ALL モデルはすでに報告されているが、ヒトと遺伝学的背景がより近い霊長類のモデルはまだ確立されていない。本研究では ALL モデル作出を小型霊長類コモンマーモセットを用いて行い、前臨床試験に用いることができるかを検討する。
3. サルを実験動物として用いることにより、より精度の高い前臨床研究を行うことが可能となる。特に近年、急速に開発が進められている抗体医薬や遺伝子治療薬を含む分子標的治療薬の有効性・安全性の検討にはサルを用いた前臨床研究が必須と考えられる。そこで、小型霊長類コモンマーモセットを用いた疾患モデル作出を目指して、マーモセットES細胞への遺伝子変異導入法の検討を行った。またコモンマーモセットを用いたフィラデルフィア陽性急性リンパ性白血病モデルの作出には大量のマーモセット骨髄が必要となるが、当研究所と九州大学とは遠隔地であるため、骨髄を輸送する必要がある。
そこで骨髄を劣化させることなく輸送する方法についても検討した。
4. マーモセットはライフスパンが 20 年程度有り、他のげっ歯類実験動物モデルと比較して著しく長期である。従って、白血病遺伝子の導入に成功した場合

に対する単クローン抗体産生クローンを単離するとともに、これらの特性をヒトおよびコモンマーモセット血球細胞を用いて検討した。

6.Zinc finger nuclease (ZFNs)法を用いたp53ノックイン技術開発：サル悪性腫瘍疾患を作出する目的で、代表的ながん抑制遺伝子であるp53遺伝子に着目し、p53遺伝子変異導入サルES細胞の作製実験に着手した。p53遺伝子はヒトがん細胞において極めて高頻度にその変異が確認され、またマウス個体モデルにおいても同遺伝子の欠失及び変異が腫瘍形成を誘導することが報告されている(Lawrence et al.Nature. 356:215-221. 1992)。本研究ではコモンマーモセットp53遺伝子をノックアウトする目的で、先ずコモンマーモセットp53遺伝子へのノックイン技術を開発する。

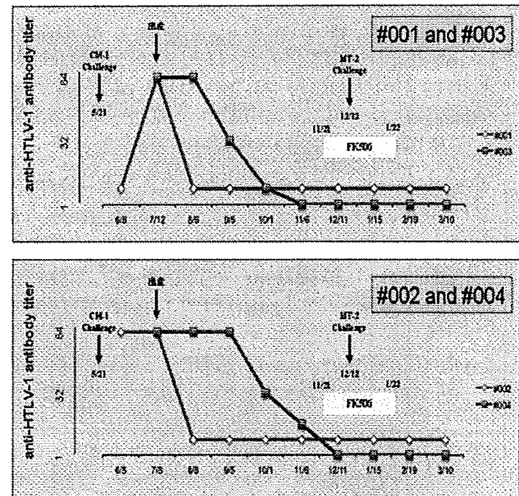
(1)ジンクフィンガーヌクレアーゼ (Zinc finger nuclease：ZFNｓ) 設計を目的として、コモンマーモセットES細胞のp53ゲノム構造を解析し、塩基配列を決定した。次にこの解析結果とヒトp53ゲノム構造を比較し、ヒト腫瘍において高頻度に検出される一塩基多型Arg175, Arg248, Arg273に対応するコモンマーモセット塩基部位を決定した。

(2)(1)の多型部位をターゲットとするZFNsの設計をSangamo社に依頼しZFNｓ発現プラスミドを作成した。そして正常なp53遺伝子配列であることを確認したコモンマーモセット線維芽細胞にZFNs発現プラスミドを電気穿孔法により遺伝子導入し、その機能効率を検討した。

(3)Sangamo社により設計されたZFNs（コモンマーモセットp53Arg177、またはArg250 の変異を作製する）ペアをヒト腫瘍細胞株293T細胞に遺伝子導入（生細胞への導入効率＞90%、導入効率は免疫抗体染色法にて確認）し、導入後4日目の細胞よりゲノムを抽出し各ZFNsペアの切断効率をCelⅠアッセイ法にて検討した。さらにこれらZFNsペアとともに蛍光タンパク質を発現する非ゲノム挿入型レンチウィルスベクター（IDLV-ZFN-IRES-Venus）を作製し、コモンマーモセットaorta-gonad-mesonephros領域より採取した細胞（AGM細胞）に遺伝子導入（生細胞への導入効率＞90%、蛍光発現にて確認）し、同様の方法でZFNsペアの切断効率を検討した。

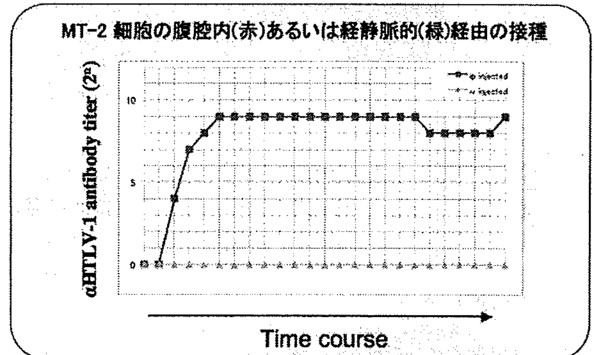
（倫理面への配慮）
本研究の実施に際しては「研究開発等に係る遺伝子組換え生物等の第二種使用等に当たって執るべき拡散防止措置等を定める省令」（一部旧組換えDNA 実験指針）と各施設内動物実験に関する指針「九州大学動物実験に関する指針」「東京大学動物実験マニュアル」にのっとり各施設内委員会において承認後（承認済み）、安全かつ倫理的に研究を実施した。

C.研究結果
1.ATLLモデル：
(1)ATLLモデルカニクイザルの作出研究：CM-1細胞の接種後母体の血漿中において抗HTLV-1抗体価の一過性の上昇を観察したが、接種3ヶ月後までには陰性レベルにまで低下した。一方、MT-2細胞投与に起因した抗HTLV-1抗体価の推移は確認されなかった。新生仔においても出生後3および4ヶ月間にわたって抗HTLV-1抗体価の上昇が観察されたもののそれ以降は陰性となった。さらに、実験期間を通じて母体の母乳と末梢血および新生仔の末梢血においてHTLV-1ウィルスの存在はPCRレベルで検出されなかった。さらに、全個体を屠殺して各組織をPCR法にて解析したがHTLV-1ウイルスの存在は確認できなかった。

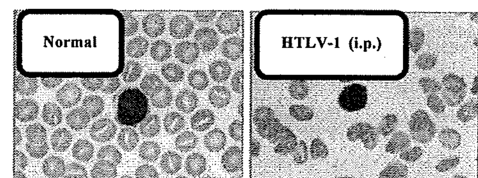


(図1)HTLV-1感染細胞(CM-1, MT-2)静脈投与後の母体(#001,#002)ならびに新生仔(#003, #004)血中抗HTLV-1抗体の推移

(2)ATLLモデルコモンマーモセットの作出研究：経静脈的投与個体では、HTLV-1抗体価の上昇を認めなかった。一方、経腹腔内投与個体においては、投与後より現在まで1年以上、HTLV-1抗体価の持続的な上昇を認めた(図２)。いずれの個体の血液細胞ゲノムDNAからもHTLV-1プロウイルスが持続的に検出された。定量PCRではプロウイルス量は1%未満であった。末梢血スメアでは、ATLLに典型的な花弁用核を持つ細胞(flower cell)は、現在まで認められていないが、核に軽度の切れ込みをもった細胞が散見された(図３)。以上より、抗体価に違いがあるものの、これらコモンマーモセット個体においてHTLV-1キャリア状態を作出することができた。



(図2)MT-2細胞を経静脈的もしくは経腹腔内に投与されたコモンマーモセット個体における血中HTLV-1抗体価の推移



(図3) MT-2細胞を経腹腔内に投与されたコモンマーモセット個体末梢血中に認められた異形リンパ球

2 .Ph染色体陽性ALLモデル：
(1)p190 遺伝子導入造血前駆細胞の自家移植後、2 個体の末梢血で 4 週目と 8 週目に、骨髄では 13 週目に p190 遺伝子 mRNA の発現が確認されたが、以降は検出されなかった。
(2)大腿骨髄内に直接ウイルスベクターを注入する方

基が全てコモンマーモセットにおいても保存されていることが明らかとなった。

b)コモンマーモセットp53ゲノム(エクソン、イントロン)構造と塩基配列決定:

上記で決定したcDNA配列を参考に十数組のプライマーセットを作製し、コモンマーモセットp53ゲノム(エクソン、イントロン)構造を決定した。PCRクローニング法により計約15kbのゲノム断片を単離し、p53全ゲノム構造及び塩基配列決定を完了した。その結果、ヒトとコモンマーモセット間において極めて高度にゲノム構造が保持されていることが明らかとなった。

c)p53遺伝子の発現解析:

コモンマーモセットES細胞及び分化誘導したEB細胞においてp53遺伝子の発現解析をRT-PCR法により行った結果、未分化ES細胞でp53遺伝子の発現が観察された。

d)ZFNs活性のin vitroでの検討:

Arg177およびArg250変異作製ZFNペア各3種類について、293T細胞へ遺伝子導入し切断効率を検討したところ2日目より遺伝子導入細胞において細胞死が確認されたが、4日目には浮遊、接着両細胞においてArg177に対するZFNsペア3種類のみZFNsペアが作用したときに確認できる断片（非切断；392bp、切断断片;190bp）を確認できた。この3種類のうち、切断効率の高かった2種類のIDLV-ZFN-IRES-Venus（切断部位；14734/14735、14742/14744）を293T細胞およびAGM細胞に遺伝子導入したところ、AGM細胞は2日目より細胞のパイルアップが確認でき、4日目においてZFNsによるゲノムの切断が（非切断；392bp、切断；287bp）確認できた（293T細胞；図６（左）、AGM細胞；図６（右））。

現在3つのp53変異コモンマーモセットES細胞を作製している。すなわち2つのIDLV-ZFN-IRES-Venusのみを遺伝子導入しArg177を除いたES細胞、ストップコドンを組み込むことにより作製されるArg177以下のp53ゲノム配列を除いたES細胞、そしてヒト悪性腫瘍に高頻度に検出される一塩基多型を持つES細胞を作製する予定である。

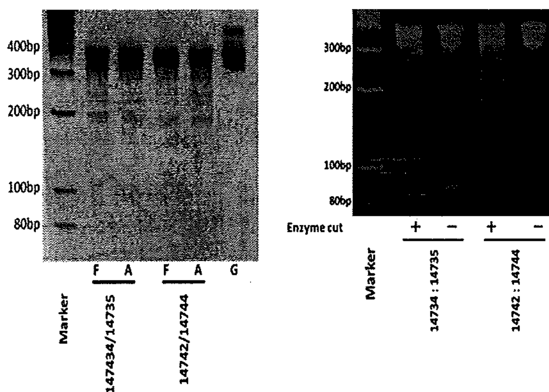


図6. (左) 293T細胞のCel1アッセイの結果
F；浮遊細胞、A；接着細胞、G；モックベクターを遺伝子導入した細胞ZFNペアによる切断断片は190ｂｐに確認できた

図6. (右) AGM細胞のCel1アッセイの結果
＋；ZFNsペアを遺伝子導入細胞、—；モックベクターを遺伝子導入した細胞ZFNsペアは287bpに確認できた

D.考察

(1)ヒト成人T細胞性白血病・リンパ腫の作出

カニクイザル母仔HTLV-1感染モデル結果から母体ならびに新生仔において一過性の抗体上昇を認めたものの明らかなHTLV-1キャリアー状態の誘導はできなかったことに加え、屠殺した個体からリンパ節などの免疫組織を回収し検討した結果からもHTLV-1感染は証明できなかった。原因として投与したCM-1細胞接種数がまだ少ない可能性と、抗HTLV-1抗体の産生が考えられた。本系における今後の検討課題として投与細胞数を増加させると共に、抗HTLV-1抗体誘導を防ぐ為の免疫抑制剤の前投与を考えることが重要であると考えられた。これらのカニクイザルでの飼育経験をもとに小型霊長類コモンマーモセット新生仔に大量のMT-2細胞を経静脈もしくは腹腔内投与したところ、経静脈投与個体においてはHTLV-1抗体価の上昇を認めなかったが、腹腔内投与個体においては、投与後より現在まで1年以上、HTLV-1抗体価の持続的な上昇を認めた。コモンマーモセットでは、いずれの個体の血液細胞のDNAからもHTLV-1プロウイルスが持続的に検出されたことより、キャリア化モデルとしては適切な非ヒト霊長類であるものと考えられた。今後ATLLの発症については長期的に経過観察する必要があるものの、費用ならびに時間的制約があるため、免疫抑制剤の投与による発症までの時間的短縮が可能であるかについて検討予定である。なお、臨床現場においても、臓器移植後、FK506を投与されたHTLV-1キャリアにおいて、ATLLへの進行が報告されており、この方法は有用であると期待される。

(2)フィラデルフィア陽性急性リンパ性白血病(Ph1-ALL)モデルの作出

Ex vivo遺伝子導入個体においては長期的なp190遺伝子導入血球の検出はできなかった。以前のわれわれの研究結果から、コモンマーモセット末梢血単核球に多剤耐性（MDR1）遺伝子をレトロウイルスベクターにて導入して自家移植した場合には約3年後にもMDR1遺伝子導入血球が末梢血中に検出された。今回の結果と比較した場合、Ex vivoでのコロニーアッセイ結果から遺伝子導入効率の間には大きな差がなかったことから、長期的な導入遺伝子検出の差異は単に遺伝子導入ベクターの違いによるものか、導入された遺伝子によるものかについては不明である。レンチウイルスベクターはレトロウイルスベクターと異なり、転写遺伝子近傍への遺伝子挿入がなされる確率は一般的に低いとの報告があり、より導入した遺伝子自身の発現効果を検討する上で（すなわち、用いるベクターによる2次的影響を除外できることから）適しているものと考えられることより、レンチウイルスベクターを用いる上でいくつかの検討が今後必要となるものと考えられる。たとえば、G-CSF（顆粒球コロニー刺激因子）を個体に投与して造血幹・前駆細胞を末梢血中に動員し、CD34陽性細胞をFACS法により分収してコロニーアッセイを行い、多分化能を有した造血細胞にそれぞれの白血病細胞が導入されているかを確認するとともに、標識タンパク質として導入したVenusを指標として、リンパ球に導入遺伝子が組み込まれているかについて各種細胞表面抗体を用いながらFACS解析を行う予定である。さらに、免疫抑制剤を用いてそれぞれの白血病関連融合遺伝子導入個体における病態の発症を試みる。また、これら一連のコモンマーモセット個体を用いた研究と並行して、免疫不全マウス（NOGマウス）を用いたex vivoでの遺伝子導入造血幹・前駆細胞の分化能と白血病化についても検討することも有効であると考えられる。すなわち、ライフスパンの長いコモンマーモセットと比較

究にコモンマーモセットを用いている。我々は、それぞれの研究のために安楽死を行った動物の有効利用を目指して、多くの異なる領域の研究者に生体材料を提供するシステムを構築した。これにより、コモンマーモセットを用いる研究の相互的推進が可能となった。この生体材料の分与システムは、研究を推進するのみではなく、実験動物のガイドラインに記載された３Rのうち、Reductionにあたる動物実験の数の低減にも有効であると考えられた。
今後の新たな造血器腫瘍を含む悪性腫瘍モデル作出法としてZFNs法の有用性は高いものと考えられp53遺伝子変異導入サルES細胞ならびに受精卵の作製に必要な試料が準備できたことにより今後in vivoにおける研究を推進できるものと期待される。
以上の様に、小型霊長類コモンマーモセットにいくつかの方法で造血器悪性腫瘍を中心とした悪性腫瘍モデル系を作出すべく研究を実施してきており、以上の成果を上げることができた。引き続き研究を発展させることで、社会への高い貢献度が十分に期待できる前臨床試験モデルサルの作出が可能になるものと考えられた。

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G．知的財産権の出願・登録状況

1．特許取得

1.発明の名称：Novel erythrocyte differentiation inducing method and novel erythrocytedifferentiation marker 5 α-reductase inhibitor
出願番号：61/310353, 2010-03-04
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2.発明の名称：Therapeutic agent for malignant tumor using oncolytic measles virus, and therapeutic method using the agent
出願番号：61/308477, 2010-02-26
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3.発明の名称： NOVEL ONCOLYTIC VIRUS FOR LUNG CANCER
整理番号　QP090217-US
代表発明者:谷　憲三朗
出願番号:61/315129
出願日:2010-03-18

2．実用新案登録
なし

3.その他
なし

研究成果の刊行に関する一覧表レイアウト

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Inhibition of tumor growth through suppression of angiogenesis by brain-specific angiogenesis inhibitor 1 gene transfer in murine renal cell carcinoma

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Abstract. This study was designed to elucidate the therapeutic effect of transfering the brain-specific angiogenesis inhibitor 1 (BAI1) gene to a mouse renal cell carcinoma cell line (Renca). Female BALB/c mice were inoculated subcutaneously with wild-type Renca (Renca/Wild) cells or Renca cells transfected with the BAI-1 (Renca/BAI-1) or LacZ (Renca/LacZ) gene. Tumor growth was observed every other day from 3 to 35 days after implantation. Moreover, the intratumoral injection of the adenovirus vector containing the gene encoding BAI1 was conducted at two-day intervals from 11 to 31 days after implantation of the Renca/Wild or Renca/BAI1 tumor. Tumor blood flow was measured by colorimetric angiogenesis assay (CAA). The concentration of the vascular endothelial growth factor (VEGF) in the cell culture supernatants was determined by enzyme-linked immunoassay. The size of the Renca/BAI1 tumor was significantly ($p<0.01$) suppressed compared to the Renca/Wild and Renca/LacZ tumors 21 days after tumor implantation. The injection of the BAI1 viral vector at 2-day intervals significantly inhibited the growth of both the Renca/Wild and Renca/BAI1 tumors. The blood volume measured by CAA and microvessel density was significantly lower in the Renca/BAI1 than in the Renca/Wild and Renca/LacZ tumors ($p<0.01$ and $p<0.05$, respectively). A significant ($p<0.01$) reduction in VEGF concentration in the supernatant was demonstrated in the Renca/BAI1 compared with the Renca/Wild and Renca/LacZ cell cultures. These observations suggest that the transfer of the BAI1 gene to Renca can suppress the tumor growth via the inhibition of angiogenesis. The down-regulation of VEGF production in tumor cells contributes to this anti-tumor effect.

Introduction

New vascular formation is required for tumor growth, invasion and metastasis (1,2). Various factors that regulate angiogenesis have been elucidated successively over the years, and cancer treatments that target these factors have been devised (1,2). Radical nephrectomy or nephron-sparing surgery is the main-stay of curative treatment for localized renal cell carcinoma (RCC), which is unresponsive to radiotherapy and also refractory to chemotherapy (3). Immunotherapies using interferons and interleukin 2 have been used for advanced RCC. However, the two therapies alone as well as in conjunction have demonstrated disappointing success rates of 20% or less (4). RCC is a typical hypervascular tumor, and neovascularisation is thought to play a principal role in tumor progression (5). The inhibition of angiogenesis could lead to promising developments of new treatment modalities.

A brain-specific cancer-regulating p53 target gene was isolated and identified as a new angiogenesis inhibitor, and was named the brain-specific angiogenesis inhibitor 1 (BAI1) gene (6). The BAI1 gene encodes a 1584-amino-acid product containing five thrombospondin (TSP) type 1 repeats. A recombinant protein analogous to the TSP type 1 repeats of this gene product has been shown to inhibit angiogenesis induced by the basic fibroblast growth factor (bFGF) in the rat cornea (6). Although BAI1 mRNA was initially reported to be expressed specifically in the brain, this gene has subsequently been demonstrated in the neoplastic and/or nonneoplastic tissues of the colon, stomach and lung, and its expression correlates inversely with vascular density (7-9).

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Abbreviations: BAI1, brain-specific angiogenesis inhibitor 1; RCC, renal cell carcinoma; TSP, thrombospondin; VEGF, vascular endothelial growth factor

Key words: gene therapy, angiogenesis, brain-specific angiogenesis inhibitor 1, renal cell carcinoma

The overexpression of BAI1 inhibited tumor growth by suppressing tumor angiogenesis in human pancreatic adenocarcinoma cells (Panc-1) (10). Moreover, the overexpression of BAI1 induced cell death in human umbilical vein endothelial cells (10,11).

We designed this study in order to elucidate the possibility of gene therapy by BAI1 gene transfer in RCC. Gene transfer in a mouse RCC cell line (Renca) was conducted using replication-deficient recombinant adenoviral vectors encoding BAI1 (10). Tumor growth was observed after the subcutaneous inoculation of Renca cells transfected with the BAI1 gene and compared with wild-type cells and cells transfected with LacZ. In addition, we evaluated whether the anti-tumor effect of the BAI1 gene transfer is associated with the inhibition of new vascular formation.

Materials and methods

Reagents. A commercial MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraslium bromide] colorimetry kit was purchased from Chemico (Temecula, CA, USA). X-gal staining solution was from Gibco BRL (Tokyo, Japan) and used at 37˚C. An RNeasy Mini Kit was purchased from Qiagen (Tokyo, Japan). The commercial ELISA kits (human bFGF ANALYZA ELISA and mouse VEGF ANALYZA ELISA) were from Genzyme (Minneapolis, USA).

Cell lines and animals. The mouse RCC cell line (Renca) was kindly provided by Dr R.H. Wiltrout (N.C.I., Fredrick, MD), which was maintained *in vivo* by serial intrarenal passage. *In vitro* culture of the Renca cells was performed in RPMI-1640 containing 10% fetal cow serum (FCS) at 37˚C under 5% CO_2 (13). Female, euthymic BALB/c mice were housed in a special pathogen-free facility and used routinely at 8 to 10 weeks of age when they weighed 22 to 24 g.

Gene transfection and in vitro tumor growth. Gene transfer in the Renca cells was conducted using the replication-deficient recombinant adenoviral vector encoding the full length cDNA of BAI1 (Renca/BAI1) or LacZ (Renca/LacZ), at a multiplicity of infection (MOI) of 20 for 72 h as described previously (10,12). Renca cells not transfected with any gene (Renca/Wild) were used as the controls.

For the *in vitro* cytotoxicity test, Renca cells (1×10^5/well) and the viral vector at an MOI of 1, 2, 10, 20, 50, 100, 200 and 300 were added to a 96-well microplate. After incubation for 72 h, the MTT reagent was added and the cells were incubated for a further 4 h. The reaction was then stopped by the addition of isopropanol hydrochloride, and the absorbance (540 nm) was measured.

In order to confirm the vector transfer efficiency, Renca cells were transfected with the control vector (AdexCA-LacZ) at an MOI of 20 for 72 h and ß-galactosidase was assayed using X-gal staining. The Renca/LacZ cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS(-) for 10 min at 4˚C. The fixative was removed, and then X-gal staining solution was added. After 3 h of reaction at 37˚C, the cells were examined under a light microscope.

In order to measure the *in vitro* cellular proliferation capability, Renca cells (1×10^5) inoculated in a 25 cm^2 tissue culture flask were transfected with the virus vector (AdexCA-LacZ or pAdex1Cawt-BAI1) at an MOI of 20, and cell counts were performed on days 1, 3, 5 and 7.

Confirmation of the BAI1 gene transfer using RT-PCR. RNA was extracted from Renca/Wild, Renca/LacZ and Renca/BAI1 cells using the RNeasy Mini Kit, and subjected to RT reaction using oligo(dT)12-18 and SuperScript II (Gibco BRL) at 37˚C for 90 min. PCR was then performed using Taq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan) and the primers given below. The thermal conditions were 95˚C for 10 min, followed by 35 cycles (94˚C for 30 sec, 55˚C for 30 sec, at 72˚C for 60 sec), and a final extension at 72˚C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and examined under UV light. The primers used were G3PDH, 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (sense) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (anti-sense); BAI1 (6), 5'-ACTCATCCTGCGACGGTGTG-3' (sense, E1S) and 5'-TCCCTCAGGTCCTTCATGCG-3' (anti-sense, E1A). The RT-PCR products obtained in the above manner were fluorescent dyed using the BigDye Terminator (Applied Biosystems), and the base sequences were determined using the ABI PRISM310 Genetic Analyser.

Assay of bFGH and VEGF in cell culture supernatant. Supernatants were obtained from the Renca/Wild, Renca/LacZ and Renca/BAI1 (n=5) cell cultures on days 1, 3, 5 and 7. Concentrations of bFGF and the vascular endothelial growth factor (VEGF) were determined using the commercial ELISA kits mentioned above.

In vivo tumor growth. Renca/Wild, Renca/LacZ or Renca/BAI1 cells (1×10^6 cells in 0.05 ml) were inoculated subcutaneously into the dorsal skin of mice (n=10). The tumors were observed on consecutive days for 35 days, and the long and short diameters of the tumors were measured. Tumor volume was estimated according to the following formula, and used as a measure of the antitumor effects: Tumor volume (mm^3) = (short diameter)^2 x (long diameter) x 0.5 (13).

In order to examine the changes in BAI mRNA expression during the experimental period, we obtained Renca/BAI1 tumor tissues on days 3, 5, 9, 15, 21, 29. RNA was extracted from these tissues and subjected to RT-PCR using the same method used for the cultured Renca cells.

Tumor angiogenesis assay. Tumor neovascularisation activity and intratumor blood volume was determined using *in vivo* colorimetric angiogenesis assay (CAA) (13). Renca/Wild, Renca/LacZ or Renca/BAI1 cells (2×10^6 cells in 0.05 ml per site) were inoculated subcutaneoulsy into 4 sites of the dorsal skin of a mouse (n=8 per group). After 72 h, 0.25 ml 1% Evan's blue solution was injected into the caudal vein, and the tumors were removed 2 min later. The specimens of these tumors were dissolved in 0.35 ml sodium sulfate/acetone, and the absorbance (620 nm) of the supernatant thus obtained was measured with an ELISA reader. Mouse blood collected after the injection of Evan's blue was dissolved in the same solvent and serially diluted. The blood volume was then calculated using the absorbance of the

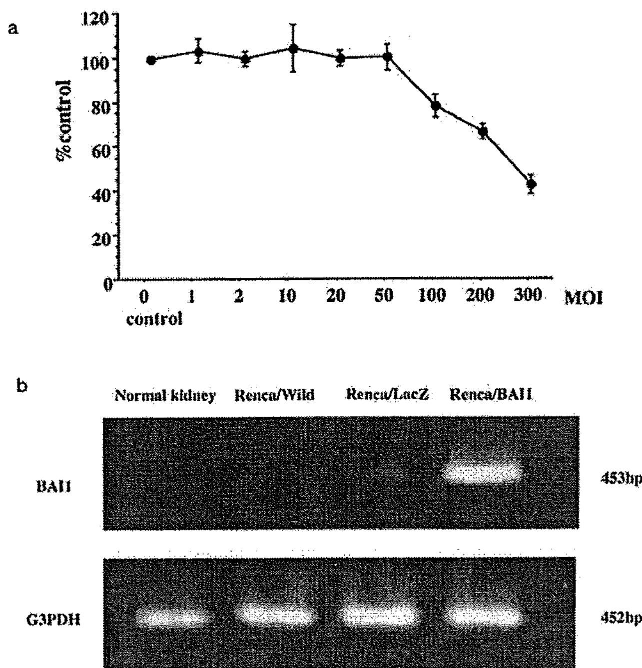

Figure 1. (a) *In vitro* cytotoxic effect of the viral vector at various multiplicities of infection (MOI) on the Renca cell culture. MTT assay was performed in duplicate. No cytotoxicity was observed when virus vectors were added at MOI 50 or less. (b) RT-PCR of the three Renca cell lines and normal kidney tissue. The expression of BAI1 was detected only in the Renca cells transfected with BAI1.

supernatant obtained from serially diluted mouse blood samples as the standard. The total blood volume of 2 transplant sites was defined as the tumor neovascularisation activity. Normal samples were obtained from non-inoculated sites in order to measure the blood volume in the non-tumor regions.

Immunohistochemistry and quantification of microvessel density. Immunostaining for CD31 was performed on 3-5 μm sections from paraffin-embedded tumor tissues (n=6) on day 15 using rat anti-mouse CD31 (BD Pharmingen, San Diego, USA).

Intratumoral microvessel density (MVD) was determined by light microscopy after immunostaining the sections with anti-CD31 antibody. The MVD was expressed as the average number of the 5 highest areas identified within a single x200 per fields.

Intratumor administration of BAI1 gene vector. Twenty microliters of pAdex1Cawt-BAI1 (1.7×10^8 pfu/ml) or PBS(-) were injected into the subcutaneously implanted Renca/Wild or Renca/BAI1 tumor, starting on the 11th day after implantation and continuing on every other day for a total of 11 doses (n=10 for each group). Tumor volume was estimated using the same formula as described above.

Statistical analysis. The Mann-Whitney U test was used for the statistical analysis of the comparative data. Values of $p<0.05$ were considered significant.

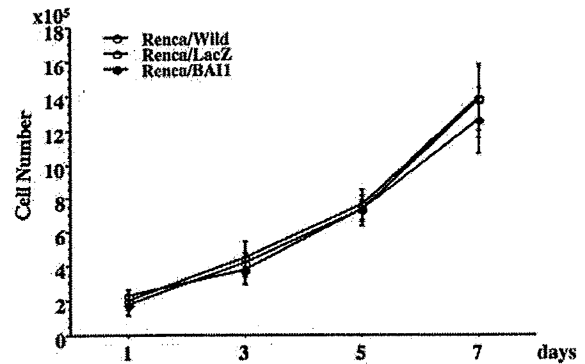

Figure 2. *In vitro* proliferation capability of Renca/Wild, Renca/LacZ and Renca/BAI1 cells. No significant difference was observed among the three cell lines.

Results

In vitro tumor growth. No cytotoxicity was observed by the addition of the virus vectors at MOI 50 or less, but toxicity was seen at MOI 100 or higher (Fig. 1a). Therefore, subsequent viral vector transfection experiments were performed using an MOI of 20 in order to eliminate the possibility of vector cytotoxicity. Beta-galactosidase staining of the Lac Z transfectants (MOI 20) confirmed a very high infection rate (almost 100%) of tumor cells by adenovirus-mediated transfer. The results of RT-PCR of the RNA samples obtained from normal mouse kidney tissues (n=3) and the three Renca cell lines are shown in Fig. 1b. A strong 453 bp band was seen only in the RNA sample of Renca/BAI1 cells, signifying that the BAI1 gene was transferred to the Renca cells. The base sequence for the PCR products obtained from the Renca/BAI1 cells exactly matched that for the human BAI1 gene (6).

Fig. 2 shows the *in vitro* proliferation capability of the Renca/Wild, Renca/LacZ and Renca/BAI1 cells. The cell counts increased ~7-fold in all three cell cultures by the 7th day. No significant difference in the proliferation rate was found among the Renca/Wild, Renca/LacZ and Renca/BAI1 cells.

Concentrations of bFGF and VEGF in cell culture supernatant. Assays of angiogenic factors in the cell culture supernatant are shown in Fig. 3 (n=5 for each group). VEGF levels on days 3, 5 and 7 post-infection with the viral vector were significantly ($p<0.01$) lower in the Renca/BAI1 cells than in the Renca/Wild or Renca/LacZ cells (Fig. 3a). In contrast, no significant differences in the bFGF level were observed among the three cell lines throughout the experimental period (Fig. 3b).

In vivo tumor growth and angiogenesis. Renca/Wild and Renca/LacZ tumors demonstrated rapid growth, as shown by the tumor volume measured up to day 35 (Fig. 4a). In contrast, the growth of the Renca/BAI1 tumor was significantly ($p<0.01$) suppressed from days 27 to 35 compared with the Renca/Wild or Renca/LacZ tumor. The expression of BAI1 mRNA was maintained in the Renca/BAI1 tumors until day 15. However, the attenuation of BAI1 mRNA expression was observed on days 21 and 29 (Fig. 4b).

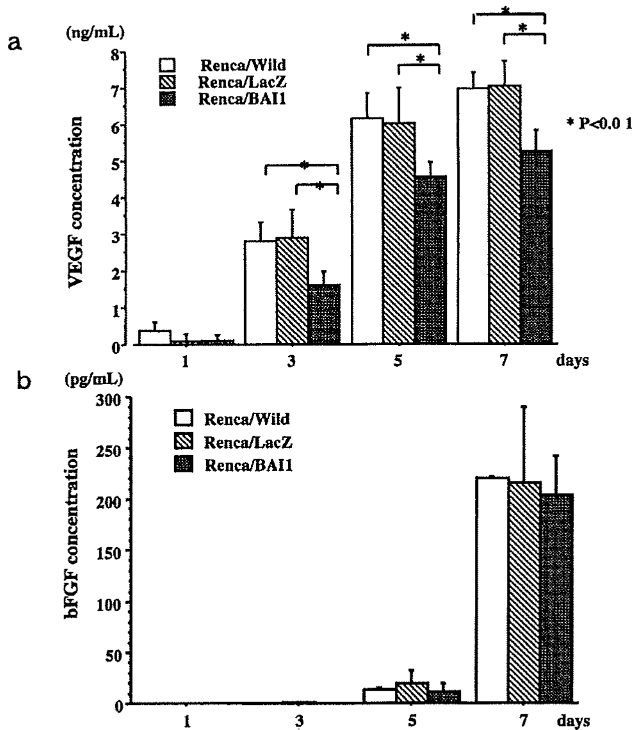

Figure 3. Concentrations of VEGF (a) and bFGF (b) in the cell culture supernatants. The levels of VEGF were significantly lower in the Renca/BAI1 cell culture than in the Renca/Wild and Renca/LacZ cell cultures on days 3, 5 and 7 post-infection (a). No significant differences in the level of bFGF were noted among the three cell cultures (b).

Intratumor blood volumes as measured by the CAA method are shown in Fig. 5a. The blood content (mean ± standard deviation; in μl/66.4 mm^2) of tumors inoculated into the mouse dorsal skin was 10.02±2.97, 10.87±3.06 and 5.44±2.48 in Renca/Wild, Renca/LacZ and Renca/BAI1 tumors, respectively (Fig. 5b). The intratumoral blood volume was significantly ($p<0.01$) lower in the Renca/BAI1 tumor compared with the Renca/Wild or Renca/LacZ tumor. The blood content of normal skin was 1.75±0.88.

Microvessels stained with CD31 were attenuated in the Renca/BAI1 tumors compared with the Renca/Wild and Renca/LacZ tumors (Fig. 6a). MVD was significantly lower in the Renca/BAI1 than in the Renca/Wild and Renca/LacZ tumors ($p<0.05$) (Fig. 6b).

Intratumor BAI1 viral vector injection. Tumor growth was inhibited significantly in the Renca/Wild tumors injected with the BAI1 viral vector compared with the tumors injected with PBS(-) from days 21 to 31 (p=0.004 on day 31) (Fig. 7). In addition, the same antitumor effect was found in the Renca/BAI1 tumor injected with the BAI1 viral vector compared with the tumors injected with PBS(-) from days 25 to 31 (p=0.003 on day 31) (Fig. 7).

Discussion

Tumor growth and metastasis depend on angiogenesis, and the suppression of new vascular formation is one of the pivotal strategies for inhibiting tumor progression (1,2). A variety of pro- and anti-angiogenic molecules regulates new vascular formation. Angiogenesis is switched off when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules. In contrast, a net balance tipped in favor of angiogenesis begins the angiogenesis process. Tumors themselves produce some angiogenesis activators and stimulate new vascular formation. Blocking angiogenesis could be an efficacious therapy for preventing tumor growth (1,2). VEGF is one of the principal members of pro-angi-

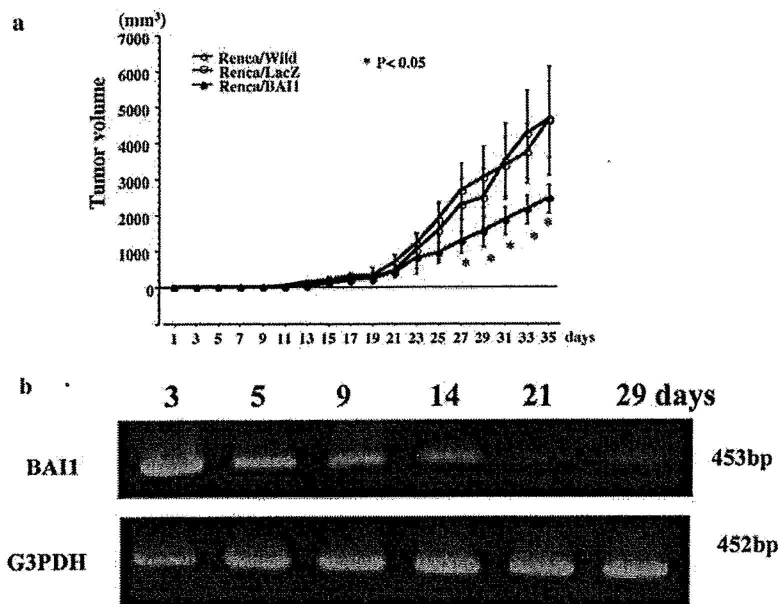

Figure 4. *In vivo* tumor growth up to day 35 after the subcutaneous inoculation of tumor cells (a) and changes in BAI1 mRNA expression in the Renca/BAI1 tumors during the experimental period (b). The volume of the Renca/BAI1 tumor was significantly reduced compared with the Renca/Wild and Renca/LacZ tumors from day 27 onward ($p<0.05$). The expression of BAI1 mRNA was maintained until day 15 (b). On days 21 and 29, BAI1 mRNA expression was attenuated.

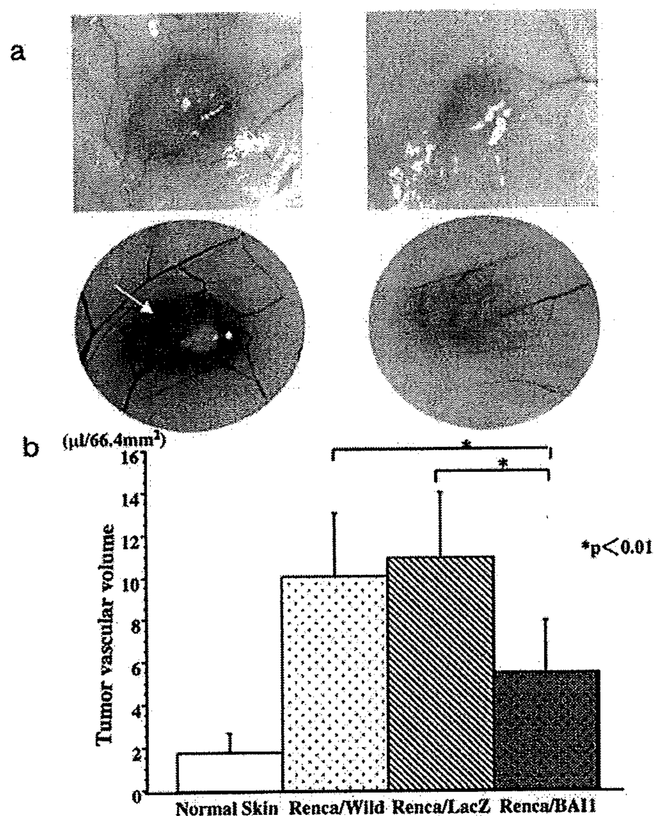

Figure 5. Tumor neovascularisation. (a) Macroscopic findings of tumor vascularization 72 h after subcutaneous inoculation. Angiogenesis is not promoted by Renca/BAI1 cells. In contrast, a stable vascular network is established by Renca/LacZ (arrow). (b) Intra-tumor blood volumes determined by the colorimetric angiogenesis assay method. A significantly lower blood volume was observed in the Renca/BAI1 tumor compared with the Renca/Wild and Renca/LacZ tumors ($p<0.01$).

ogenic molecules and the effectiveness of anti-VEGF therapy in inhibiting tumor growth has been reported (2). On the contrary, BAI that was originally isolated as a p53-target gene possessing an extracellular domain containing TSP type 1 repeats has been considered an inhibitor of angiogenesis (6). *In vivo* experiments have revealed that the transfection of the BAI1 gene into human pancreatic adenocarcinoma cells (Panc-1) results in suppressed tumor growth by inhibiting angiogenesis (10). *In vitro* studies have detected no difference in tumor growth between parental Panc-1 cells and BAI1 or LacZ transfectants, although an antiproliferative effect was found in human umbilical vein endothelial cells after the transfection of the BAI gene. In our *in vivo* study, the Renca/BAI1 tumor implanted subcutaneously into female BAL/c mice demonstrated significantly suppressed growth, while similarly implanted Renca/Wild and Renca/LacZ tumors proliferated rapidly. Intratumor blood volume and MVD were significantly reduced in the Renca/BAI1 tumors compared with the Renca/Wild and Renca/LacZ tumors. Our *in vitro* study showed no difference in cell growth between Renca/BAII and Renca/LacZ, consistent with the observation in human pancreatic adenocarcinoma cells (10). Since the BAI1 gene was expressed only in Renca/BAI1 cells and not in Renca/Wild or Renca/LacZ cells on RT-PCR, the suppression of tumor growth in Renca/BAI1 could be attributed to the reduction in vascular formation as a consequence of the BAI1 overexpression.

A peptide containing TSP type 1 repeats suppresses bFGF-induced neovascular formation (6). BAI-1 is a transmembrane protein and has five TSP type 1 repeats and an integrin binding site (RGD motif) in its extracellular domain (6). BAI-1 is proteolytically cleaved at a conserved G-protein-coupled receptor proteolytic cleavage site (GPS), releasing its 120 kDa extracellular domain (14). This extracellular fragment has a potent anti-angiogenic action (11,14). The definite mechanism for the anti-angiogenic effect of BAI1 is being studied, but recent study has reported that the secret extracellular domain of BAI1 exerts anti-proliferative action on the surrounding endothelial cells by blocking $\alpha v \beta 5$ integrin partly through activating the caspase (11). In a previous *in vitro* study, a reduction in VEGF and matrix metalloproteinase-1 (MMP-1) expression has been demonstrated in a human pancreatic adenocarcinoma cell line (Panc-1) after BAI1, but not after LacZ transfer (10). The present study also showed a reduction in VEGF concentration in the supernatant of the Renca/BAI1 but not the Renca/LacZ cell culture. From these observations, BAI1 shows anti-angiogenic activity not through a direct action on endothelial cells but through indirect effects by inhibiting other factors such as VEGF and MMP-1.

The expression of the BAI1 gene was initially considered to be specific to brain tissue (6). A lack of this gene is thought to be the cause of chaotic vascular proliferation in glioblastoma. However, BAI1 gene expression has subsequently been demonstrated in lung, gastric and colorectal cancer, suggesting that BAI1 plays an important role in the inhibition of angiogenesis associated with a variety of neoplasia other than cerebral tumors (7-9). A significantly lower MVD was demonstrated in lung cancers expressing BAI1 mRNA compared with those not expressing this mRNA (9). In colorectal cancers, the expression of BAI1 mRNA was significantly lower in malignant than in non-malignant colorectal tissues (7). BAI1 gene expression was inversely related to vascular invasion as well as metastasis, and also inversely correlated with vascular density. These two studies suggest that the expression of BAI1 can inhibit new vascular formation and/or tumor metastasis in lung and colorectal cancers. The expression of BAI1 mRNA was also reduced in gastric cancer (8). A significant prolonged survival period after gastrectomy has been demonstrated in patients with good BAI1 expression in the adjacent normal mucosa compared with those with poor BAI1 expression.

In the present study, the transferring of the BAI1 gene to mouse RCC Renca cells via an adenovirus vector resulted in inhibited tumor growth and suppressed tumor angiogenic activity *in vivo*, although tumor cell growth was not inhibited *in vitro*. Transfection of the human pancreatic adenocarcinoma cell line Panc-1 with the BAI gene also resulted in suppressed tumor growth *in vivo* but had no effect on *in vitro* cell growth (10). The inhibition of *in vivo* growth of the BAI-transfected Panc-1 tumor was associated with the suppression of angiogenesis, as was also observed in the BAI1-transferred Renca tumors in the present study. As mentioned above, BAI1 could be one of the principal factors in the suppression

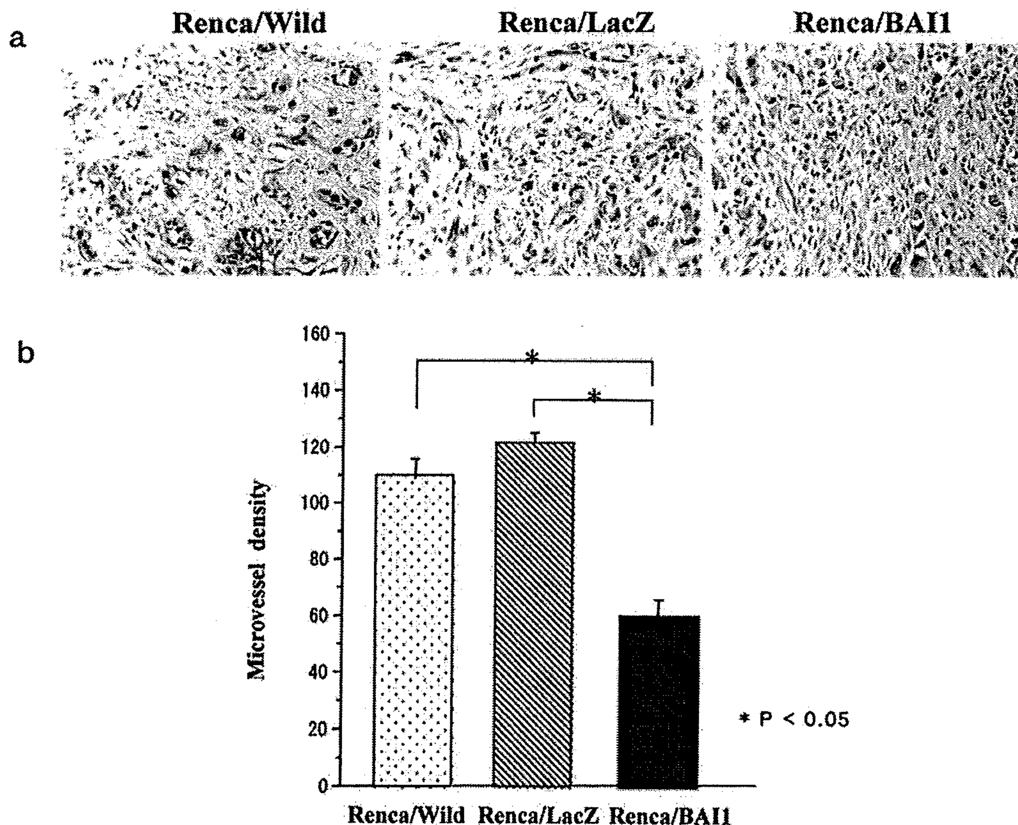

Figure 6. Immunostaining for CD31 (a) and microvessel density (MVD) (b) in Renca/Wild, Renca/LacZ and Renca/BAI1 tumors. The number of microvessels stained with CD31 was lower in the Renca/BAI1 than in the Renca/Wild and Renca/LacZ tumors (a). MVD was significantly lower in the Renca/BAI1 than in the Renca/Wild and Renca/LacZ tumors ($p<0.05$).

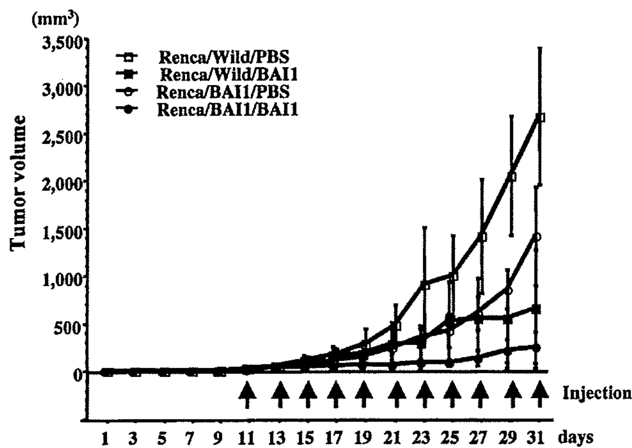

Figure 7. *In vivo* tumor growth after intratumoral injection of the BAI1 viral vector. Repeated BAI1 viral vector injection significantly suppresses tumor growth not only in the Renca/wild tumor but also in the Renca/BAI1 tumor, compared with the PBS(-) injection.

of tumor growth via the inhibition of angiogenesis and should be considered for gene therapy.

In order to examine the possibility of BAI1 as a candidate for gene therapy in RCC, we injected the BAI1 gene vector into subcutaneously implanted Renca/Wild and Renca/BAI1 tumors from the 11th day after implantation at 2-day intervals. The intratumor injection of the BAI1 gene vector significantly inhibited the growth of the Renca/Wild tumor compared with the PBS injection instead of the BAI1 gene. In addition, a significant reduction in the subcutaneously implanted Renca/BAI1 tumor was also achieved by the intratumor injection of the BAI1 gene, in comparison with the PBS injection. These observations could indicate the possibility of BAI gene therapy or the development of efficient drugs based on the extracellular fragment of BAI1 not only for RCC with decreased BAI1 expression but also for those with good BAI1 expression.

We investigated the antitumor effects of the BAI1 gene transfer into the murine RCC cell line, Renca, using replication-deficient recombinant adenovirus vectors encoding the full-length cDNA of BAI1. Significant suppression of both tumor growth and vascular formation was demonstrated in the Renca/BAI1 tumor compared with the Renca/Wild and Renca/LacZ tumors. The concentrations of VEGF were lower in the supernatant of the Renca/BAI1 cell culture compared with the Renca/Wild and Renca/LacZ cell cultures, suggesting that the suppression of VEGF production is involved in the anti-angiogenic effect of BAI1. Growth was inhibited in both the Renca/Wild and Renca/BAI1 tumors upon tumoral injection of a viral vector encoding BAI1, suggesting the possibility of the clinical application of this gene therapy.

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BASIC–ALIMENTARY TRACT

A Critical Role of CD30 Ligand/CD30 in Controlling Inflammatory Bowel Diseases in Mice

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Background & Aims: **A CD30-ligand (CD30L) is a 40-kilodalton, type II membrane-associated glycoprotein belonging to the tumor necrosis factor family. Serum levels of soluble CD30 increased in inflammatory bowel diseases (IBD), suggesting that CD30L/CD30 signaling is involved in the pathogenesis of IBD. In this study, we investigated the role of CD30L in oxazolone (OXA)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis in CD30L knockout (KO) mice.** ***Methods:*** **Colitis was induced by OXA or TNBS in CD30LKO mice with BALB/c or C57BL/6 background, respectively, and diverse clinical signs of the disease were evaluated. Cytokine production from lamina propria T cells of the colon was assessed by enzyme-linked immunosorbent assay. Anti-interleukin (IL)-4 monoclonal antibody (mAb) or agonistic anti-CD30 mAb was inoculated in mice with colitis induced by OXA or TNBS.** ***Results:*** **CD30LKO mice were susceptible to OXA-induced colitis but resistant to TNBS-induced acute colitis. The levels of T helper cell 2 type cytokines such as IL-4 and IL-13 in the LP T cells were significantly higher, but the levels of interferon γ were lower in OXA- or TNBS-treated CD30LKO mice than in wild-type mice. In vivo administration of agonistic anti-CD30 mAb ameliorated OXA-induced colitis but aggravated TNBS-induced colitis in CD30LKO mice.** ***Conclusions:*** **These results suggest that CD30L/CD30 signaling is involved in development of both OXA- and TNBS-induced colitis. Modulation of CD30L/CD30 signaling by mAb could be a novel biologic therapy for IBD.**

Human inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by inflammation in the large and/or small intestine associated with uncontrolled innate and adaptive immunity against normal constituents, including commensal bacteria and various microbial products.[1–4] The responding T cells exhibit a T helper cell (Th) 1 phenotype capable of producing interferon (IFN)-γ in CD, whereas Th2 cytokines are closely associated with UC.[5–7] Among various experimentally induced colitis models in mice, spontaneous colitis in interleukin (IL)-10-deficient mice,[8] colitis in recombination-activating gene (RAG)-deficient mice transferred with $CD4^+CD45RB^{high}$ T cells,[9] and hapten-induced colitis in mice caused by intrarectal administration of trinitrobenzene sulfonic acid (TNBS)[10–13] are thought of as a Th1-type colitis animal model resembling CD. On the other hand, spontaneous colitis in IL-2-deficient mice[14] or in T-cell receptor (TCR) α-deficient mice[15] resembles UC with Th2-like responses. Another hapten-induced colitis caused by intrarectal administration of oxazolone (OXA) is characterized by inflammation with increased Th2-type cytokine secretion and is thought to be associated with Th2-like responses mediated by $CD4^+$ T cells including natural killer (NK) T cells.[16,17]

A CD30 ligand (CD30L, CD153) is a 40-kilodalton, type II membrane-associated glycoprotein belonging to the tumor necrosis factor (TNF) family[18] and is expressed on both $CD4^+$ Th1 and Th2 cells, although there are several lines of evidence for expression on macrophages, dendritic cells (DC), and B cells.[19–22] CD30, a receptor for CD30L, is expressed preferentially by activated or memory Th2 cells but not by resting B or T cells.[23–26] There are several lines of evidence showing that the CD30L/CD30 signaling is involved in Th2 cell responses and Th2-associated diseases.[23–26] However, a number of recent studies suggested that CD30L/CD30 signaling is also linked to Th1 cell responses and Th1-associated diseases.[27–30] Serum levels of soluble CD30 (sCD30) increased in UC, suggesting that CD30L/CD30 signaling is involved in the pathogenesis of IBD.[31] However, little is known about the roles of CD30L/CD30 signaling in IBD.

In the present study, to verify the roles of CD30L in IBD, we examined susceptibility of CD30L knockout

Abbreviations used in this paper: Ag, antigen; APC, allophycocyanin; CD, Crohn's disease; CD30L, CD30 ligand; DC, dendritic cell; KO, knockout; LPL, lamina propria lympocyte; mAb, monoclonal antibody; OXA, oxazolone; TNBS, trinitrobenzene sulfonic acid; UC, ulcerative colitis.

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