別添1

厚生労働科学研究研究費補助金

萌芽的先端医療技術推進研究事業

がん特異的増殖機能を有するウイルス製剤と高感度GFP蛍光検出装置を用いた体外超早期がん診断および体内微小リンパ節転移診断システムに関する研究

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平成18年度　総括研究報告書

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(総括・分担)研究報告書

がん特異的増殖機能を有するウイルス製剤と高感度GFP蛍光検出装置を用いた体外超早期がん診断および体内微小リンパ節転移診断システムに関する研究

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【研究要旨】
近年増加を続けるがん患者の生存率や治療成績の向上には、早期発見、適格な悪性度の予知、適切な治療方針の決定が重要な因子となる。特に、微小リンパ節転移の検出は患者のQOLを重視した必要最小限の低侵襲外科手術の確立に役立ち、また微小播種病巣の検出技術はピンポイント標的療法の開発に有用である。
本研究では、テロメラーゼ活性（hTERT遺伝子発現）依存性にがん細胞で選択的に増殖し、オワンクラゲ由来の蛍光遺伝子GFP (Green Fluorescence Protein)を発現する改変アデノウイルス製剤 TelomeScan（Telomelysin-GFP、OBP-401）を標識薬剤とし、プローブ型の高感度GFP蛍光検出装置を用いた微小がん組織診断用の外科手術ナビゲーション・システムを開発する。微小リンパ節転移検出のためには、内視鏡などのアクセスを用いてTelomeScanを原発腫瘍内に局所投与することでリンパ流を経由するウイルスの所属リンパ節への拡散を促す。TelomeScanはリンパ節内の微小転移巣でがん細胞に感染・増殖して選択的にGFP蛍光を発するため、一定期間の後に開胸あるいは開腹にて転移リンパ節を可視化することができる。この技術により、微小リンパ節転移を手術中にリアルタイムに検出してリンパ節郭清範囲を同定する低侵襲外科手術が可能となる。また、胸膜あるいは腹膜播種病巣の検出には、胸腔や腹腔などの体腔内にTelomeScanを投与し、同様に一定期間の後に高感度GFP蛍光検出装置にて観察する。微小播種巣では選択的にTelomeScanが感染・増殖するため、肉眼では検出できない微小病巣を確認することができる。さらに、GFP蛍光を狙った新しいピンポイント標的療法を開発することで、効果的な先進治療技術の開発が可能となる。
平成18年度は、プローブ型の高感度GFP蛍光検出装置の第1号試作機を作成し、手術中の使用を想定た場合の操作性や検出範囲の妥当性、GFP蛍光強度と検出感度との相関などを検討してきた。

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A. 研究目的

日本人の主要な死因が感染症から成人病、いわゆる生活習慣病へと移行する中で、特に癌は1981年以来日本人の死亡原因の第1位となっており、今後の本邦の癌罹患数は2015年には男性55万4000人、女性33万6000人となると推測されている。癌患者の生存率や治療成績の向上には、早期発見、適格な悪性度の予知、適切な治療方針の決定が重要な因子となる。特に、微小リンパ節転移の検出は患者のQOLを重視した必要最小限の低侵襲外科手術の確立に役立ち、また微小播種病巣の検出技術はピンポイント標的療法の開発に有用である。

本研究では、テロメラーゼ活性依存性にがん細胞で選択的に増殖してオワンクラゲ由来の蛍光遺伝子GFP (Green Fluorescence Protein)を発現する改変アデノウイルス製剤 TelomeScan (OBP-401)を標識薬剤とし、プローブ型の高感度GFP蛍光検出装置を用いた微小がん組織診断用の外科手術ナビゲーション・システムとしての有効性を検討する。1年目には、体外診断として末梢血中の浮遊がん細胞を検出する試みも平行して行っていたが、動物モデルにおいて体内リンパ節転移診断に関する良好な研究成果が得られた(**Kishimoto *et al*, *Nature Med.*, 12:1213-1219, 2006**)。したがって、より体内診断および低侵襲手術のための外科ナビゲーション・システムの開発が現実的となってきたため、以後は体内微小転移診断システムに重点をおいて研究開発を進めている。

微小リンパ節転移検出のためには、内視鏡などのアクセスを用いてTelomeScanを原発腫瘍内に局所投与することでリンパ流を経由するウイルスの所属リンパ節への拡散を促す。TelomeScanはリンパ節内の微小転移巣でがん細胞に感染・増殖して選択的にGFP蛍光を発するため、一定期間の後に開胸あるい

は開腹にて転移リンパ節を可視化することができる。この技術により、微小リンパ節転移を手術中にリアルタイムに検出してリンパ節郭清範囲を同定する低侵襲外科手術が可能となる。肺がんや胃がんなどの進行固形がん手術の際に、TelomeScanはリンパ節内の微小転移巣で増殖しGFP蛍光を発するため、原発巣周辺の組織にプローブを接触させることで転移リンパ節を検出することができ、リンパ節廓清範囲決定の有効な指標となると期待される。また、胸膜あるいは腹膜播種病巣の検出には、胸腔や腹腔などの体腔内にTelomeScanを投与し、同様に一定期間の後に高感度GFP蛍光検出装置にて観察する。微小播種巣では選択的にTelomeScanが感染・増殖するため、肉眼では検出できない微小病巣を確認することができる。本技術は肉眼的に確認できない微小播種病巣を検出する診断用システムとして応用できるとともに、GFP蛍光を狙った新しいピンポイント標的療法を開発することで、効果的な先進治療の開発が期待される。

B. 研究方法

1) TelomeScan (OBP-401)の構造

TelomeScan は幼児の「かぜ」症状の原因となるアデノウイルス 5 型を基本骨格とし、テロメラーゼ構成成分である hTERT (human telomerase reverse transcriptase)遺伝子のプロモーターの下流にウイルス増殖に必須の E1A および E1B 遺伝子が IRES 配列で連結して組み込まれている。また、ウイルスゲノムの E3 領域に、サイトメガロウイルス（CMV）プロモーターとオワンクラゲ由来の GFP (Green Fluorescent Protein)蛍光発現遺伝子が挿入されている（図 1）。TelomeScan は癌細胞で選択的に増殖し、GFP 蛍光を発するとともに、最終的には細胞死を誘導する。一方、テロメラーゼ活性を持たない正常細胞では、その増殖は抑制され、GFP もみられず、細胞死も生じることはない。

2) TelomeScan感染ヒト癌細胞での経時的GFP蛍光発現と細胞形態の変化

H1299ヒト非小細胞肺癌細胞に10 MOI (multiplicity of infection)の濃度のTelomeScanを感染させ、タイムラプス画像解析装置を用いて経時的に細胞形態の変化と蛍光発現を観察した(図2)。

タイムラプス画像解析装置は、蛍光観察機能付き倒立顕微鏡、高感度CCDカメラ、CO_2および温度コントローラーと画像処理ソフトを備えている。顕微鏡架台上で細胞を培養しつつ、一定時間おきに位相差視野および蛍光視野で細胞を連続的に観察記録することが可能である。

3) プローブ型高感度蛍光検出装置の試作

プローブ型GFP蛍光検出装置の第1号試作機を作成した（図3）。プローブ内にインパクトロンCCDカメラと蛍光励起用青色抗原を内蔵し、プローブ先端には多重コート処理保護ガラスを装着した。このプローブユニットをカメラコントロールユニットに接続し、さらにビデオキャプチャーボードを介して画像処理用のパソコン本体に連結した。パソコンで処理された画像は、2色画像にてディスプレー上に描出される。

4) マウス同所性直腸癌モデルにおける微小転移リンパ節検出の検討

ヒト大腸癌細胞株をヌードマウスの直腸に移植して約4-6週間置くと、傍大動脈リンパ節に高率に転移が発生する（図4）。この大腸癌リンパ節転移モデルを用いて、まず原発巣に投与したウイルス液が所属リンパ節に到達可能か否かを、青色色素によって確認した（図5）。さらに、1×10^8 PFUのTelomeScanを腫瘍内に投与し、5日後に開腹して転移リンパ節のGFP蛍光を第1号試作機を用いて観察した（図6）。

（倫理面への配慮）

本研究は「ヒトのクローンに関する研究等」に該当するおそれはないと判断される。本研究に用いるTelomeScan 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（平成 16 年文部科学省・環境省令第 1 号）」の「別表第一（第四条関係）」における「へ　自立的な増殖力及び感染力を保持したウイルス又はウイロイド」に相当する。したがって、本研究は「大臣確認実験」となるため、平成 16 年 8 月に「第二種使用等拡散防止措置確認申請書」を作成、学内の担当部署での検討の後に文部科学省に申請し、研究計画の実施が承認されている。

C. 研究結果

1) TelomeScan (OBP-401)の構造

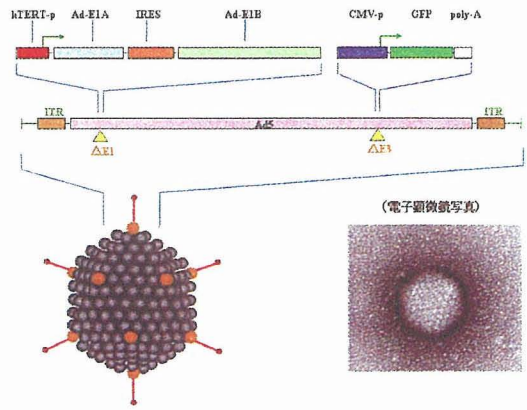


図 1　TelomeScanの構造と概観

2）TelomeScan感染ヒト癌細胞での経時的GFP蛍光発現と細胞形態の変化

ヒト培養肺癌細胞 H1299 に 10 MOI の TelomeScan を感染させ、位相差視野と蛍光視野でタイプラプス観察を行った。感染後 20 時間から GFP 蛍光発現が認められ、40 時間後にはほぼすべての細胞が GFP 蛍光陽性となった。その後、50 時間後から細胞は光沢を伴って円形化し、シャーレから浮遊して細胞死に至ることが明らかとなった。

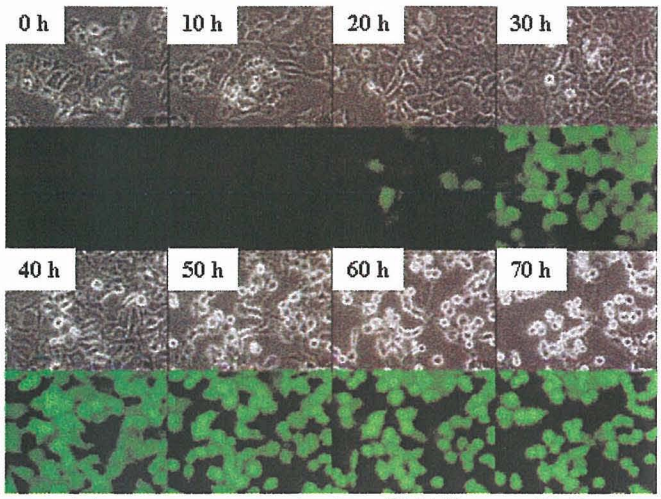
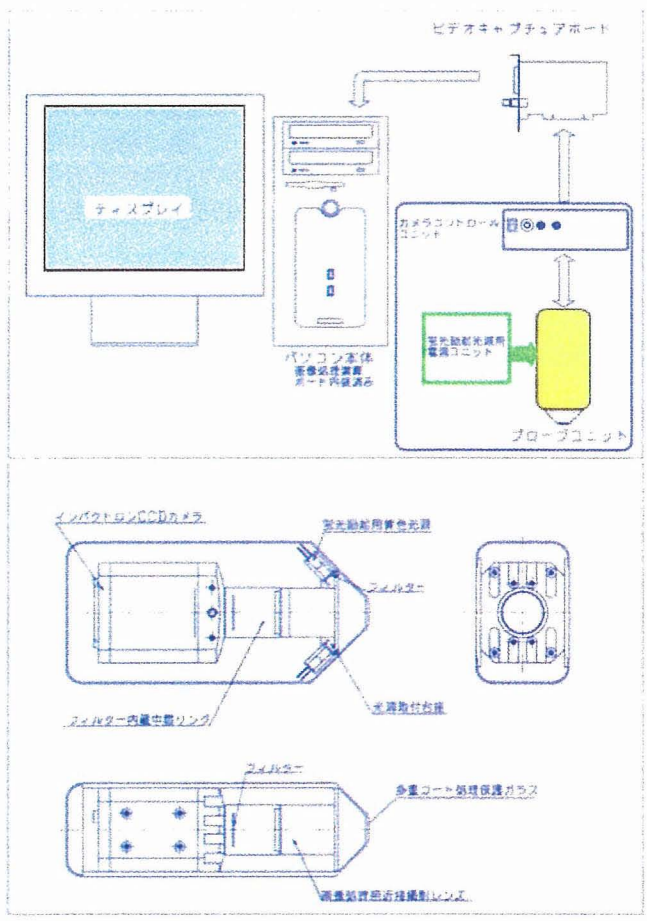


図 2　TelomeScan による選択的 GFP 発現

3）プローブ型高感度蛍光検出装置の試作



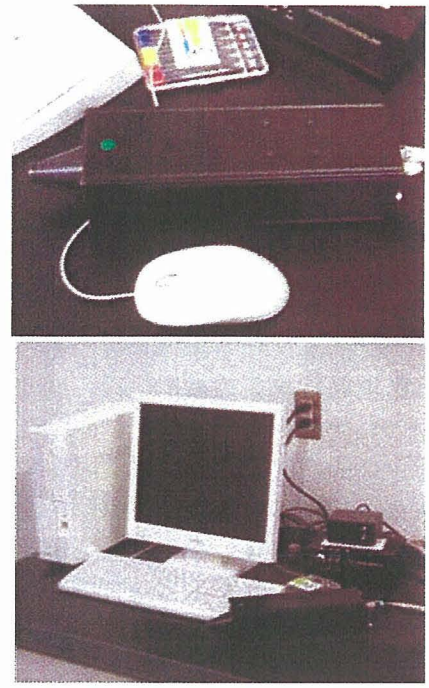


図 3　高感度 GFP 蛍光検出装置（1 号試作機）
（システム図、構造図、概観）

4）マウス同所性直腸癌モデルにおける微小転移リンパ節検出の検討

マウスモデルにおける微小リンパ節転移の検出：ヒト大腸がん細胞をヌードマウスの直腸粘膜下に移植すると、同所性直腸がんが形成され、約 4-6 週間後に大動脈周囲に高頻度にリンパ節転移が認められる。

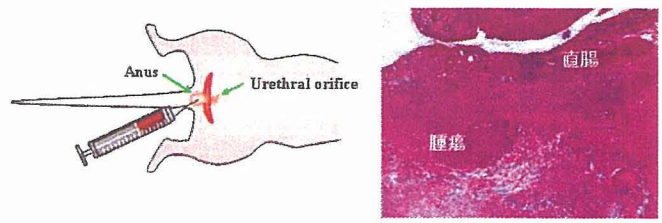


図 4　同所性ヒト直腸癌モデル

このモデルにおいて、原発巣へのウイルス投与によるリンパ管内への拡散を確認するため、青色色素の腫瘍内投与を行った。直後に開腹して腹腔内を観察したところ、リンパ節への色素流入が明らかとなった。

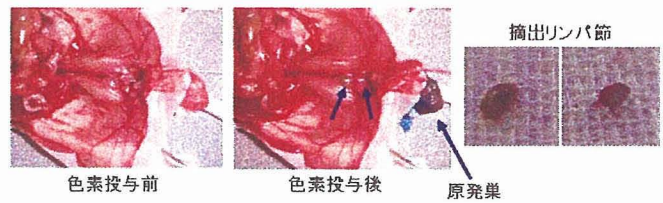


図 5　原発腫瘍への投与によるリンパ流への拡散

さらに、TelomeScan を直腸腫瘍に直接投与し、5日後に開腹し、キセノン光で蛍光を励起して高感度3CCD カメラにて観察した（図 6A）。同時に、1 号試作機によりそれぞれのリンパ節を確認したところ、GFP 陽性リンパ節を高感度に検出することが可能であった（図 6B）。

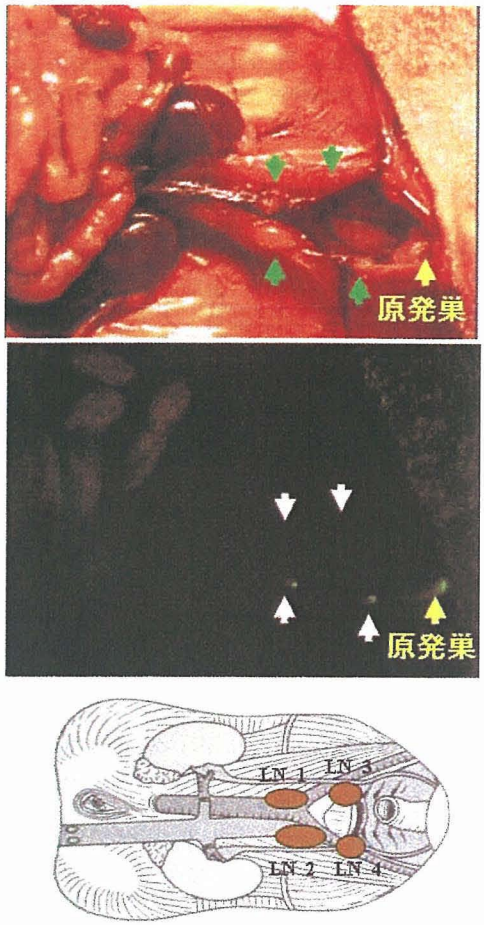


図 6A　高感度 CCD カメラによる GFP 発現

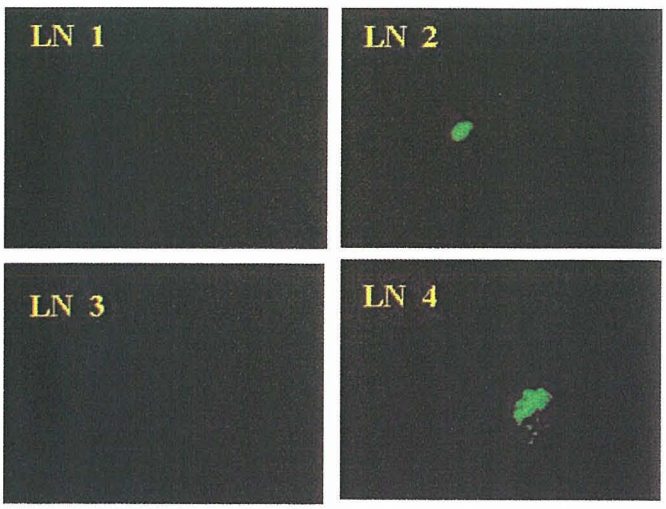


図 6B　1 号試作機プローブによる GFP 発現検出

D.　考察

ウイルスは本来ヒトの細胞に感染して、その構造蛋白質を産生することで複製・増殖する。その増殖機能に選択性を付加することにより、ウイルスをがん細胞を標識する診断用製剤として用いることが可能となる。「かぜ」症状の原因となるアデノウイルス5型を基本骨格とするTelomeScanは、ウイルス増殖に必須のE1遺伝子をテロメラーゼ構成分子であるhTERT (human telomerase reverse transcriptase)遺伝子のプロモーターで制御することで、がん細胞のみで増殖するように改変されたウイルス製剤である。さらに、E3領域にGFP遺伝子が組込まれており、がん細胞のみで緑色蛍光を発する。

われわれが標的とするテロメラーゼは80-90%の癌で極めて高い活性の上昇がみられ、正常組織で活性が検出されるリンパ球や小腸上皮での発現レベルは低いため、TelomeScanのがん選択性はかなり厳格かつ広範である。また、TelomeScanはがん細胞で複製・増殖するため、経時的なGFP蛍光強度の増強が期待できることが画期的であり、ウイルスを細胞標識に用いる点が独創的であると言える。さらに、GFPは導入遺伝子発現などの目的で多くの基礎研究で使用されているため、蛍光顕微鏡やFACSなどの検出技術の開発が進んでおり、TelomeScanのGFP蛍光を高感度に検出する装置の開発は技術的には可能と思われる。

1年目（平成17年度）のフィージビリティー・スタディーでは、TelomeScanを用いたがんの体内転移診断の可能性を検証した（原理の証明 [Proof of Concept]）。マウスモデルにおける微小リンパ節転移の検出：ヒト大腸がん細胞をヌードマウスの直腸粘膜下に移植すると、同所性直腸がんが形成され、約5-6週間後に大動脈周囲に高頻度にリンパ節転移が認められる。このモデルにおいて、TelomeScanウイルス製剤を直腸腫瘍に直接投与し、5日後に開腹し、キセノン光で蛍光を励起して高感度3CCDカメラにて観察した。最終的に組織学的に確認したところ、GFP陽性リンパ節では高頻度に微小転移が検出された。感度は、sensitivity 92.3%、specificity 86.6%であり、1 mm以下の微小転移巣を蛍光spotとして同定することが可能であった(**Kishimoto *et al*, *Nature Med.*, 12:1213-1219, 2006**)。また、TeloemScanの複製・増殖が炎症性のリンパ節腫大ではみられず、がん細胞に選択的に生じることを明らかにした。

2年目（平成18年度）にはプローブ型の高感度GFP蛍光検出装置を試作し、その有用性の評価と問題点に対する改良を行った。第1号試作機は、プローブ内にレンズとモノクロCCDカメラ、および蛍光励起のためのLEDを内蔵したため、7（縦）x 7.5（横）x 29（長さ）センチとサイズ的にかなり大型のものとな

った。同機でマウス直腸がんリンパ節転移を開腹下に観察したところ、正常組織による自家蛍光がかなりのノイズとなることが判明したため、画像解析ソフトの蛍光検知閾値を調整することで転移リンパ節のみを高感度に検知することが可能となった。しかし、サイズと重量が著しくプローブの操作性を損なっているため、励起光を独立させて術野に照射する案も検討したが、その場合は部屋を暗室状態にする必要があり、現実的ではないと判断した。これらの結果に基づき、第2号試作機ではプローブにはレンズとLEDのみを内蔵し、イメージファイバーでつないだ本体にカラーCCDカメラを設置する方法を採用した。この改良により、カメラをモノクロからカラーに変更し、またプローブを軽量化することで操作性を優先することが可能となった。また、モニターも術野外に大型のものを設置することで、鮮明な画像を得ることができる。

また、平成18年3月には、TelomeScanとGFP発現カセット以外の基本骨格を同じくする抗がんウイルス製剤 TelomelysinのIND (Investigational New Drug) applicationを米国食品医薬品庁（FDA）に提出した。8月に承認を受けた上で、10月より米国にて抗がん剤としてのTelomelysinの臨床試験を開始している。この第I相臨床試験は各種固形癌を対象として1年以内の終了を目指しており、その安全性情報はTelomeScanの臨床研究計画の立案に極めて有用な根拠となると期待される。

E. 結論

テロメラーゼ活性依存性に癌細胞で選択的に増殖して蛍光遺伝子GFPを発現する改変アデノウイルス製剤 TelomeScan は、標識薬剤として微小リンパ節転移検出外科手術ナビゲーション・システムに応用可能である。

F. 研究発表

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H. 知的財産権の出願・登録状況

特許番号：3867968、発明の名称：腫瘍細胞において選択的に増殖する腫瘍融解ウイルス、発明者：藤原俊義、白木屋佳子、川嶋健、田中紀章、京哲、出願年月日：平成14年7月8日、登録年月日：平成18年10月20日

別添5

研究成果の刊行に関する一覧表

書籍

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
藤原俊義 田中紀章	遺伝子治療の現状と展望	工藤翔二	肺癌のすべて	文光堂	東京	2006年	pp356-358

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Watanabe, T., Hioki, M., Fujiwara, T., et al.	Histone deacetylase inhibitor FR901228 enhances the antitumor effect of telomerase-specific replication-selective adenoviral agent OBP-301 in human lung cancer cells	**Exp. Cell Res.**	312	256-265	2006
Fujiwara, T., Tanaka, N., Nakamura, H., et al.	Multicenter phase I study of repeated intratumoral delivery of adenoviral p53 (ADVEXIN) in patients with advanced non-small cell lung cancer	**J. Clin. Oncol.**	24	1689-1699	2006
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藤原俊義 田中紀章	主要癌の遺伝子治療研究の現況：肺癌	**Biotherapy**	20	270-279	2006
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Research Article

Histone deacetylase inhibitor FR901228 enhances the antitumor effect of telomerase-specific replication-selective adenoviral agent OBP-301 in human lung cancer cells

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Abstract

Replication-competent oncolytic viruses are being developed for human cancer therapy. We previously reported that an attenuated adenovirus OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site, could replicate in and causes selective lysis of human cancer cells. Infection efficiency in target cancer cells is the most important factor that predicts the antitumor effects of OBP-301. The objectives of this study are to examine the effects of the histone deacetylase inhibitor FR901228 on the level of coxsackie and adenovirus receptor (CAR) expression and OBP-301-mediated oncolysis in human non-small cell lung cancer cell lines. Flow cytometric analysis revealed up-regulated CAR expression in A549 and H460 cells following treatment with 1 ng/ml of FR901228, which was associated with increased infection efficiency as confirmed by replication-deficient β-galactosidase-expressing adenovirus vector. In contrast, neither CAR expression nor infection efficiency was affected by FR901228 in H1299 cells. To visualize and quantify viral replication in the presence of FR901228, we used OBP-401 (Telomelysin-GFP) that expresses the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus promoter in the E3 region. Fluorescence microscopy and flow cytometry showed that FR901228 increased GFP expression in A549 and H460 cells following OBP-401 infection in a dose-dependent manner, but this effect did not occur in H1299 cells. In addition, OBP-301 and FR901228 demonstrated a synergistic antitumor effect in A549 cells in vitro, as confirmed by isobologram analysis. Our data indicate that FR901228 preferentially increases adenovirus infectivity via up-regulation of CAR expression, leading to a profound oncolytic effect, which may have a significant impact on the outcome of adenovirus-based oncolytic virotherapy.

Keywords: Oncolytic virus; HDAC inhibitor; Adenovirus; CAR; Lung cancer

Introduction

Replication-selective, oncolytic viruses provide a new platform to treat a variety of human cancers [1,2]. Promising clinical trial data have shown the antitumor potency and safety of mutant or genetically modified adenoviruses [3–6]. We previously constructed an adenovirus vector (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase (hTERT) promoter element drives expression of E1A and E1B genes linked with an internal ribosome entry site (IRES). We showed that OBP-301 caused efficient selective killing in human cancer cells, but not in normal cells [7]. Although OBP-301 demonstrated a broad-spec-

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trum antitumor activity, infection efficiency of the presently available adenoviral agent, which is derived from human adenovirus serotype 5, varies widely depending on the expression of coxsackie-adenovirus receptor (CAR) [8,9]. To overcome the limitation of low levels of CAR in certain tumors, we further modified the fiber of OBP-301 to contain RGD peptide. We demonstrated that this fiber-modified OBP-405 permits CAR-independent cell entry and effective destruction of tumors lacking the primary CAR [10]. This strategy has been commonly used to alter adenovirus infectivity [11,12]; the genetic modification, however, might not always be successful. An alternative approach is to modify CAR expression in target tumor cells.

FR901228 (Depsipeptide, FK228) is a novel anticancer agent isolated from the fermentation broth of *Chromobacterium violaceum*. FR901228 has been identified as a potent histone deacetylase (HDAC) inhibitor, although it has no apparent chemical structure that interacts with the HDAC

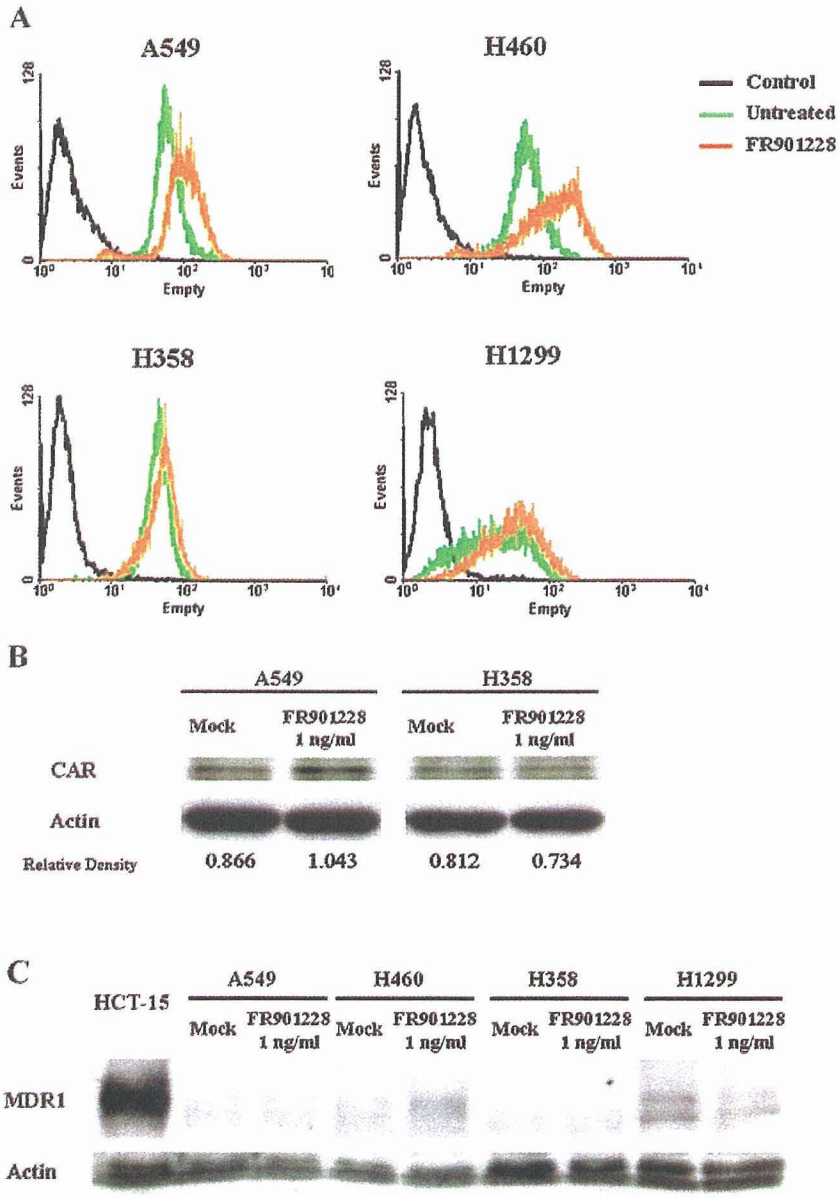

Fig. 1. (A) Expression of CAR on human NSCLC cell lines after FR901228 treatment. Cells were treated with 1 ng/ml of FR901228 for 48 h and then subjected to flow cytometric analysis. Both treated and untreated cells were incubated with mouse monoclonal anti-CAR (RmcB) followed by detection with FITC-labeled secondary antibody. An isotype-matched normal mouse IgG1 conjugated to FITC was used as a control in all experiments. The rightward shift of the histogram after exposure to FR901228 indicates increased CAR expression. (B) Western blot analysis of CAR and actin in A549 and H358 cells. Cells were treated same as above. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane, probed with anti-CAR antibody, and then visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum. CAR protein expression was quantified by densitometric scanning using NIH Image software and normalization by dividing the actin signal. (C) Western blot analysis for MDR1 expression. Equivalent amounts of protein obtained from whole cell lysates were loaded in each lane and probed with anti-MDR1 antibody with or without FR901228 treatment. The cell lysate obtained from HCT-15 human colorectal cancer cells was used as a positive control.

active-site pocket. Thus, FR901228 is structurally distinct from other known HDAC inhibitors such as the trichostatins and trapoxins [13–15]. Histone deacetylation is an important component of transcriptional control, and it has been reported that FR901228 can increase CAR gene expression in several different cancer cell lines [16–20]. Moreover, FR901228 is known to increase viral and transgene expression following adenovirus infection [16]. These findings led us to examine whether FR901228 could augment the antitumor activity of OBP-301 against human cancer cells.

In the present study, we show that FR901228 treatment up-regulates CAR levels on target tumor cells, which in turn increases the amount of cellular viral replication, thereby promoting a synergistic antitumor effect. These findings suggest that treatment with OBP-301 in combination with FR901228 is a promising strategy for human cancer.

Results

Effect of FR901228 on CAR expression in human non-small cell lung cancer (NSCLC) cell lines

To explore the combination effect of OBP-301 and FR901228, we first used flow cytometry to determine if FR901228 has an effect on the cell surface expression of CAR. CAR was expressed in all four cell lines tested: the percentages of CAR-positive cells were 99.4%, 99.4%, 99.8%, and 86.8% in A549, H460, H358, and H1299 cells, respectively. As shown in Fig. 1A, CAR expression levels apparently increased in A549 and H460 cells following 48-h exposure to 1 ng/ml of FR901228, whereas H358 and H1299 cells showed a similar expression pattern of CAR before and after FR901228 treatment. The data were calculated as the relative mean fluorescent intensity (MFI), in which the MFI from FR901228-treated cells is divided by the MFI of cells without FR901228 treatment, thereby giving a fold enhancement of expression. In A549 and H460 cells, there were 1.6- and 2.15-fold higher MFI after FR901228 treatment compared to those before treatment, respectively (MFI: 61.13 to 97.88 [A549] and 59.51 to 127.94 [H460]). Western blot analysis for CAR expression also demonstrated that FR901228 treatment resulted in a 1.2-fold increase in the CAR protein level in A549 cells, whereas CAR protein expression level in H358 cells was consistent even after FR901228 treatment (Fig. 1B).

FR901228 has been previously reported to be a substrate for multidrug resistance protein (MDR1), which mediates FR901228 resistance in human cancer cells [21]. We next examined whether FR901228 treatment could alter MDR1 protein expression in the cell lines we used. As shown in Fig. 1C, MDR1 expression was not detected in these cell lines by Western blot analysis and could not be induced even after FR901228 treatment.

Adenovirus infectivity after FR901228 treatment in human NSCLC cell lines

To evaluate the effect of FR901228 on the infectious efficiency, we compared β-galactosidase-positive cells by X-gal staining 24 h after infection with recombinant replication-deficient adenovirus carrying the β-galactosidase gene under the control of cytomegalovirus (CMV) promoter (Ad-LacZ) in the presence or absence of FR901228. A549 and H1299 cells were treated with FR901228 at the indicated concentration for 48 h. Then, cells were infected with Ad-LacZ at multiplicities of infection (MOI) of 1 or 10 after removing medium containing FR901228. The X-gal staining 24 h after infection demonstrated that FR901228 treatment increased the percentage of blue-stained β-galactosidase-positive A549 cells in a dose-dependent manner; FR901228,

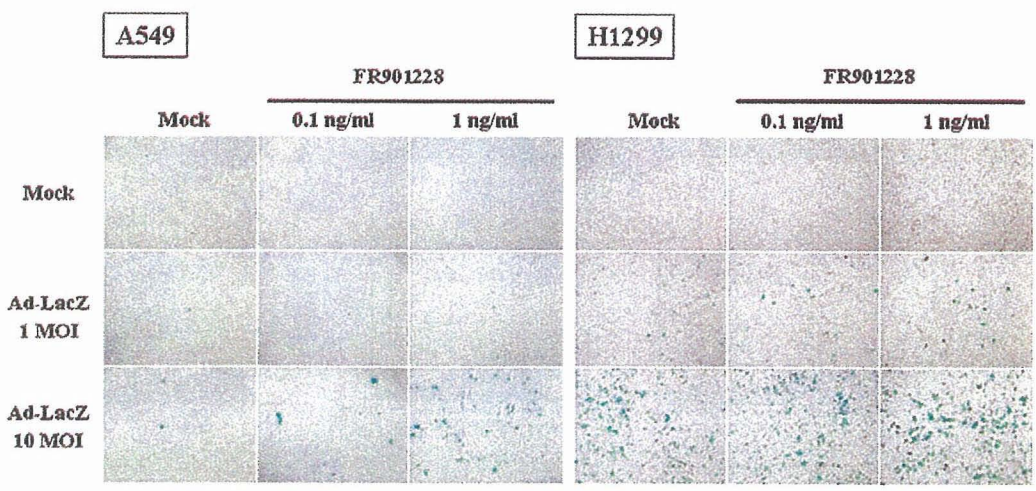

Fig. 2. Expression of β-galactosidase after Ad-LacZ infection in FR901228-treated cells. A549 and H1299 cells were treated with Ad-LacZ and FR901228 at the indicated concentrations, grown for 24 h, and then stained for β-galactosidase activity.

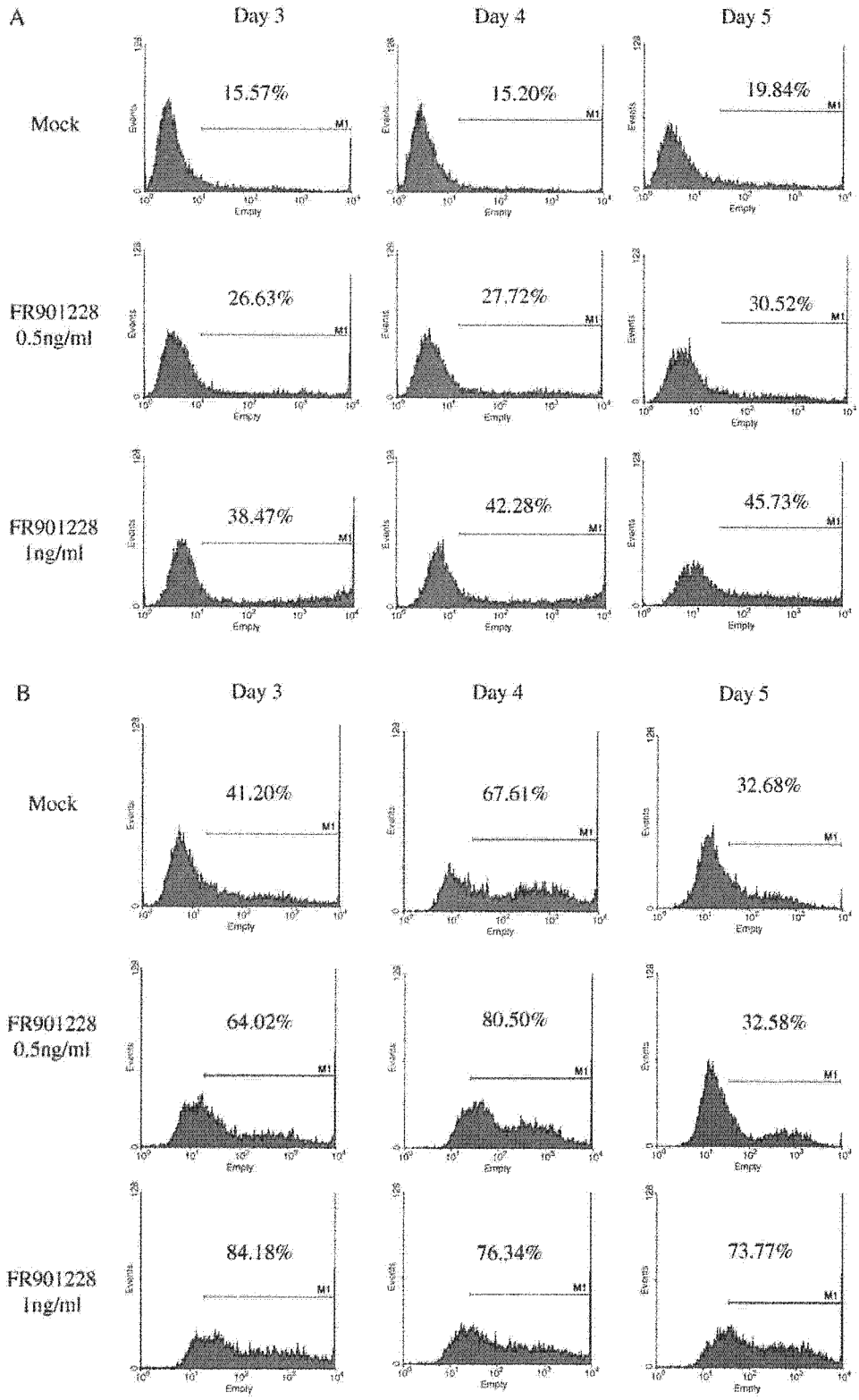

Fig. 3. Flow cytometric analysis of viral-replication-associated GFP expression following OBP-401 and FR901228 treatment. A549 (A), H460 (B), and H1299 (C) cells were infected with 0.1 MOI of OBP-401 and simultaneously treated with FR901228 at the indicated concentrations. The percentages of GFP-positive cells were assessed at different time points after treatment.

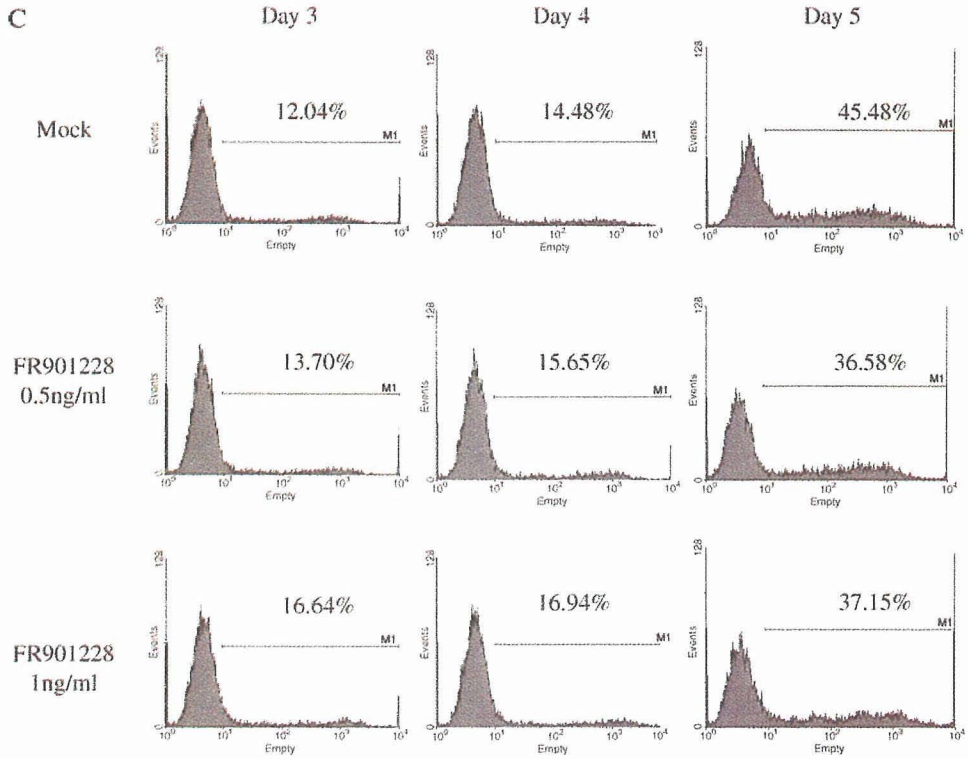

Fig. 3 (*continued*).

however, had no apparent effect on adenovirus infectious efficiency in H1299 cells (Fig. 2). These results were compatible with the effect of FR901228 treatment on CAR levels.

Oncolytic virus replication after FR901228 treatment in human NSCLC cell lines

We next examined the effect of FR901228 on oncolytic virus replication by flow cytometric analysis using telomerase-specific oncolytic adenovirus containing the GFP gene (OBP-401). The proportion of GFP-positive A549 cells increased from 15.57% (day 3) to 19.84% (day 5) after OBP-401 infection because OBP-401 can replicate in telomerase-positive tumor cells. However, the percentage of GFP-expressing cells was 45.73% in the presence of 1 ng/ml of FR901228 compared with 19.84% in cells without FR901228 at 5 days post-infection with 0.1 MOI of OBP-401 (Fig. 3A). Similarly, treatment with 1 ng/ml of FR901228 increased the percentage of GFP-expressing cells from 32.68% to 73.77% in H460 cells 5 days after infection with 0.1 MOI of OBP-401 (Fig. 3B). In contrast, there was no increase in the fraction of GFP-expressing cells after FR901228 treatment in H1299 cells (Fig. 3C). Moreover, the percentage of GFP-expressing H1299 cells decreased in the presence of 1 ng/ml of FR901228 5 days after OBP-401 infection, presumably due to the toxicity of FR901228.

To visualize viral replication in vitro, OBP-401-infected cells were photographed under a fluorescent microscope. As shown in Fig. 4, FR901228 increased the GFP fluorescence intensity in a dose-dependent manner on A549 and H460 cells 4 days after infection with 0.1 MOI of OBP-401. These findings are consistent with the results of flow

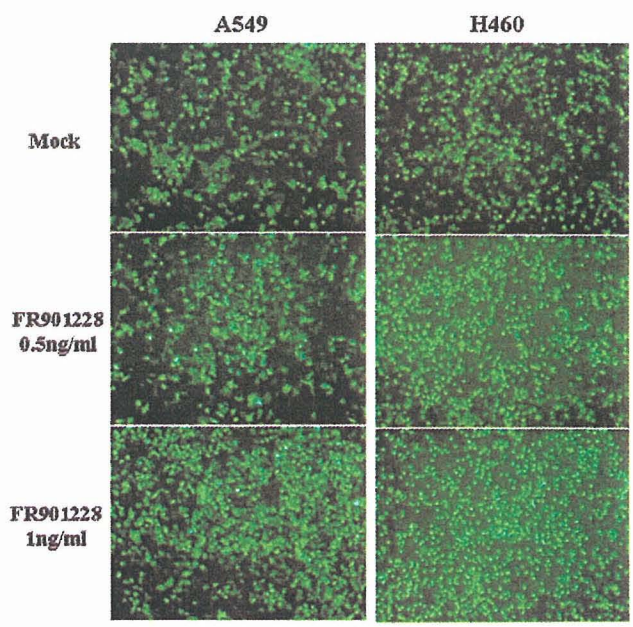

Fig. 4. Visualization of viral-replication-associated GFP expression following OBP-401 and FR901228 treatment. A549 and H460 cells were treated as described in the legend for Fig. 3. Cells were assessed for GFP expression with fluorescence microscopy 4 days after treatment.

cytometric analysis. The microphotographs also demonstrated that the number of GFP-positive cells increased in the presence of FR901228, indicating that OBP-401 could efficiently spread to the neighboring tumor cells. These results suggest that FR901228-induced up-regulation of CAR expression resulted in increased replication of oncolytic virus.

Cell cycle analysis after oncolytic virus infection and FR901228 treatment

To examine whether OBP-301 infection and FR901228 treatment result in cell cycle arrest, apoptosis, or a combination of both processes, the cell cycle distribution was determined by flow cytometric analysis of propidium-iodide-stained cells, a measure of DNA content. As shown in Table 1, neither OBP-301 infection nor FR901228 treatment affected the cell cycle distribution or the fraction of sub-G_0–G_1 apoptotic population in A549 and H460 cell lines.

Synergistic antitumor effect of OBP-301 and FR901228 in human NSCLC cells

We finally examined whether concurrent addition of FR901228 had an effect on the antitumor effect of OBP-301 in A549 cells. The cell viability with 8 doses of OBP-301 or 6 doses of FR901228 was assessed by MTT assay 4 days after treatment with concentrations that resulted in 0 to 100% cell kill when either drug was given alone (Fig. 5A). To determine whether the interaction between the two drugs exhibited synergistic cytotoxic effects, the data from the dose–response curves were examined by constructing isobolograms. Isobologram analysis indicated that the combination was synergistic across all dose levels tested, with all of the points lying into the area below the envelope of additivity (Fig. 5B). The combination index (CI) value of observed data points was 0.6823 ± 0.0761, which was smaller than 1. Thus, telomerase-specific oncolytic adenovirus OBP-301 in combination with FR901228 produces synergistic cell cytotoxicity in A549 human NSCLC cells.

Table 1
Cell cycle analysis after OBP-301 and FR901228 treatment

Cell line	Treatment	Sub-G_0/G_1 (%)	G_1 (%)	S (%)	G_2/M (%)
A549	Pre-treatment	2.04	68.74	15.35	14.18
	FR901228	1.13	74.58	10.43	13.88
	OBP-301	0.78	79.64	10.55	9.17
	FR901228 + OBP-301	2.24	73.08	11.58	13.26
H460	Pre-treatment	18.70	51.79	14.82	15.15
	FR901228	11.28	51.71	11.80	25.33
	OBP-301	1.07	69.70	14.72	14.56
	FR901228 + OBP-301	15.10	47.08	13.60	23.74

A549 and H460 cells were treated with 1 ng/ml of FR901228, 0.1 MOI of OBP-301, or both. The DNA content was determined by propidium iodide staining and flow cytometric analysis at 72 h after treatment. The percentage of cells in each stage of the cell cycle is shown.

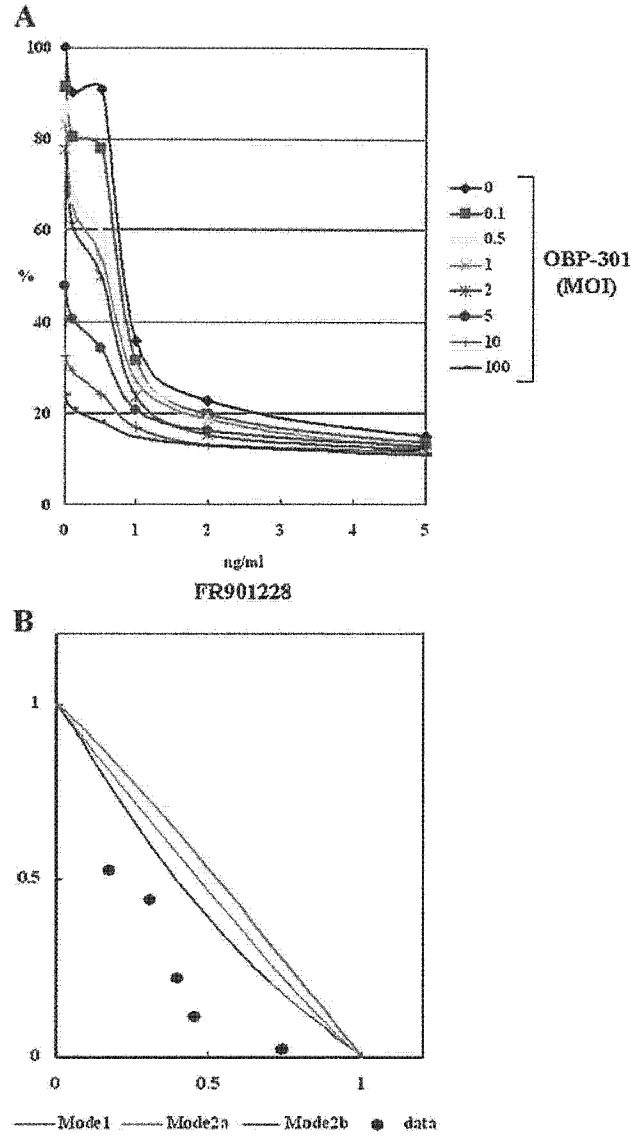

Fig. 5. Antitumor effect of OBP-301 and FR901228 on A549 NSCLC cells in vitro. (A) Effect of OBP-301 and FR901228 at various concentrations was assessed by MTT assay 4 days after treatment. Results are expressed as the percentage of untreated control. (B) The data from dose response curves were subjected to isobologram analysis. Shown is the isobologram at IC50 based on the results of MTT assays for the A549 cell line treated with combinations of OBP-301 and FR901228 added simultaneously. Points below the envelope indicate a synergistic effect.

Discussion

Oncolytic adenovirus can efficiently kill a variety of human cancer cells; the death process, however, is morphologically distinct from apoptosis that is characterized by chromosome condensation and nuclear shrinkage and fragmentation [22]. In contrast, most of conventional chemotherapeutic drugs trigger apoptosis in human cancer

cells via caspase activation. These two types of anticancer agents that use different cytotoxic machinery have been shown to induce a combination effect [23–25], although the precise mechanism is still unclear. In an attempt to establish multidisciplinary therapeutics based on the rationale, we explored the antitumor effect of oncolytic virus in combination with an HDAC inhibitor. The HDAC inhibitor is a new class of anticancer agents that can modulate transcriptional activity [13,14]. As a result, in addition to the induction of apoptosis, HDAC inhibitors are able to block the cell cycle and angiogenesis, promote differentiation, and mediate additional unknown effects in human cancer cells. In the present study, we found that the most advanced therapeutic candidate, FR901228, had a synergistic antitumor effect with telomerase-specific oncolytic adenovirus OBP-301 through up-regulation of CAR expression in certain types of human NSCLC cells. This may be a novel therapeutic intervention of HDAC inhibitors.

Gene expression is considered to be regulated by chromatin remodeling. Chromatin is intrinsically modified by histone acetyltransferase or HDAC, and alterations in these mechanisms may lead to altered gene expression [26]. It has been reported that the activation of the CAR gene promoter is modulated by histone acetylation and that FR901228 increases CAR RNA levels through histone H3 acetylation in human cancer cells, leading to an increased adenovirus transgene expression [16–18]. Compatible with these observations, we demonstrated that FR901228 treatment led to increased levels of membrane-associated CAR protein in A549 and H460 human NSCLC cell lines (Figs. 1A, B). The reason why FR901228 did not affect CAR expression in H358 and H1299 cells is unclear. As all cell lines were negative for MDR1 expression that could mediate FR901228 resistance [21] (Fig. 1C), other mechanisms such as the Raf-MEK-ERK signal transduction pathway may be involved in CAR gene expression in H358 and H1299 cell lines [27].

We also confirmed increased infection efficiency of adenovirus following FR901228 treatment using Ad-LacZ vector (Fig. 2). One of the advantages of replication-competent oncolytic adenoviruses is that less virus particles are required for treatment because viruses can be produced in tumor tissues by replication. To directly evaluate the reliability of increased virus infectivity by FR901228 for oncolytic virus, we used Ad-LacZ at an MOI of 1 or 10, although 100 MOI of the vector was commonly used in previous studies. We previously reported that the fiber-modified oncolytic adenovirus (OBP-405) containing an RGD motif in the HI loop of the fiber knob showed increased initial virus entry into the target tumor cells and resulted in the augmented viral replication [10]. Thus, FR901228 treatment is expected to enhance virus infectivity as well as replication in A549 and H460 cells. In fact, the percentages of GFP-positive cells were more than 2-fold higher in A549 and H460 cells treated with GFP-expressing OBP-401 in combination with FR901228 than those infected with OBP-401 alone (Figs. 3 and 4). Studies using a non-viral plasmid vector have found that FR901228 enhances luciferase transgene expression at the transcriptional level [28]. Therefore, it is possible that increased GFP expression reflects transcriptionally enhanced transgene expression, but not viral replication. However, the fact that FR901228 treatment did not affect OBP-401-induced GFP expression in H1299 cells suggests that FR901228 could not alter the levels of transgene expression without CAR up-regulation and that GFP expression is dependent on virus infectivity and replication. Taken together, FR901228 increases CAR expression, which in turn facilitates virus infection, thereby leading to increased viral replication in certain types of human NSCLC cells.

Adenovirus replication within a target tumor cell can cause cell destruction by several mechanisms such as direct cytotoxicity due to viral proteins and augmentation of antitumoral immunity [2]. As expected, OBP-301 in combination with FR901228 demonstrated a profound antitumor effect in vitro in A549 cells, presumably because of enhanced viral replication. Isobologram and CI analyses, which are the two most popular methods for evaluating drug interactions in combination cancer chemotherapy [29], consistently identified OBP-301 infection and FR901228 treatment as a synergistic combination (Fig. 5). Theoretically, some chemotherapeutic drugs could act to inhibit viral replication because they might affect the cell cycle; our data, however, demonstrated that FR901228 with or without OBP-301 had no effect on the cell cycle distribution (Table 1), indicating that FR901228 may be an appropriate partner for oncolytic adenovirus because it does not affect the virus life cycle. Moreover, phase I clinical trials for advanced cancer as well as leukemia have shown that FR901228 can be safely administered without any life-threatening toxicities or cardiac toxicities, although the appropriate administration schedule has to be further examined [30,31]. These observations indicate that clinical trials of OBP-301 in combination with FR901228 are warranted.

In conclusion, our data demonstrate a possible interaction between a telomerase-specific oncolytic adenoviral agent and an HDAC inhibitor. Delineating specific virus/drug combinations that are tailored to be particularly effective in human cancer may have the potential to greatly improve the already encouraging results seen in the field of oncolytic virotherapy.

Materials and methods

Cells and culture conditions

The NSCLC cell lines A549, H460, H358, and H1299 were propagated in monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Compounds

FR901228 was kindly provided by the Fujisawa Pharmaceutical Company (Tokyo, Japan).

Recombinant adenoviruses

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin) was previously constructed and characterized [7,32]. Replication-selective OBP-401 (Telomelysin-GFP) containing GFP cDNA under the control of the CMV promoter and replication-deficient adenoviral vector containing β-galactosidase cDNA (Ad-LacZ) were also used [33]. These viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. Determination of virus particle titer and infectious titer was accomplished spectrophotometrically by the method of Maizel et al. [34] and by the method of Kanegae et al. [35], respectively.

Flow cytometry

The cells (2×10^5 cells) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Biotechnology, NY) for 30 min at 4°C, incubated with FITC-conjugated rabbit anti-mouse IgG second antibody (Zymed Laboratories, San Francisco), and analyzed by the FACSCalibur (Becton Dickinson, Mountain View, CA) using CELL Quest software. The window was set to exclude dead cells and debris. Expression of the GFP gene was also assessed by the FACSCalibur.

Western blot analysis

The primary antibodies against CAR (RmcB; Upstate Biotechnology) and MDR1 (DAKO, Carpinteria, CA) and peroxidase-linked secondary antibody (Amersham, Arlington Heights, IL) were used. Cells were washed twice in cold PBS and collected then lysed in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% glycerol, 0.5% NP40] containing proteinase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4). After 20 min on ice, the lysates were spun at 14,000 rpm in a microcentrifuge at 4°C for 10 min. The supernatants were used as whole cell extracts. Protein concentration was determined using the Bio-Rad protein determination method (Bio-Rad, Richmond, CA). Equal amounts (50 μg) of proteins were boiled for 5 min and electrophoresed under reducing conditions on 6–12.5% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to a Hybond-polyvinylidene difluoride (PVDF) transfer membranes (Amersham Life Science, Buckinghamshire, UK) and incubated with the primary followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amersham) was used to detect secondary probes.

X-gal staining

Cells were seeded in 6-well plates (2×10^5 cells/well) and infected with Ad-LacZ at indicated MOIs. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed 24 h after infection according to the protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO).

MTT assay, isobologram analysis, and CI analysis

The cytotoxicity of FR901228 and OBP-301 was determined by measurement of cell viability by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated in 96-well tissue culture plates 24 h before treatment. Then, cells were treated with FR901228 and OBP-301 concurrently at the indicated concentrations and MOIs, respectively. Four days later, a medium containing 0.5 mg/ml MTT (Sigma) was added to each well after the wells were rinsed with PBS. After a 4-h incubation at 37°C, an equal amount of solubilization solution (0.04 N HCl in isopropyl alcohol) was added to each well and mixed thoroughly to dissolve the crystals of MTT formazan. Results were quantified using a Labsystems Multiskan MS at 540 nm wave length. Control absorbance was designated as 100%, and cell survival was expressed as a percentage of control absorbance. MTT results were analyzed quantitatively and statistically by plotting the observed experimental data onto the isobologram [29]. When the observed data points for a combination fell mainly within the envelope of additivity, the effect of the combination was considered as having an additive effect. When the observed data points for a combination fell into the area below the envelope of additivity, the combination effect was regarded as supra-additive (synergism). When the observed data points for a combination fell above the envelope but within the square or the cube, the combination's effect was considered as sub-additive. When the observed data points for a combination fell outside the square or the cube, the effect of the combination was considered protective. Both sub-additive and protective interactions were considered as antagonistic. CI analysis, similar to isobologram analysis, provides qualitative information on the nature of drug interaction. CI is a numerical value calculated as described previously. CIs of less than, equal to, and more than 1 indicate synergy, additivity, and antagonism, respectively. All of the analyses were performed using Statistica software (StatSoft Inc., Tulsa, Oklahoma).

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ORIGINAL REPORT

Multicenter Phase I Study of Repeated Intratumoral Delivery of Adenoviral *p53* in Patients With Advanced Non–Small-Cell Lung Cancer

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
To determine the feasibility, safety, humoral immune response, and biologic activity of multiple intratumoral injections of Ad5CMV-p53, and to characterize the pharmacokinetics of Ad5CMV-p53 in patients with advanced non–small-cell lung cancer (NSCLC).

Patients and Methods
Fifteen patients with histologically confirmed NSCLC and *p53* mutations were enrolled onto this phase I trial. Nine patients received escalating dose levels of Ad5CMV-p53 (1×10^9 to 1×10^{11} plaque-forming units) as monotherapy once every 4 weeks. Six patients were treated on a 28-day schedule with Ad5CMV-p53 in combination with intravenous administration of cisplatin (80 mg/m^2). Patients were monitored for toxicity, vector distribution, antibody formation, and tumor response.

Results
Fifteen patients received a total of 63 intratumoral injections of Ad5CMV-p53 without dose-limiting toxicity. The most common treatment-related toxicity was a transient fever. Specific *p53* transgene expression was detected using reverse-transcriptase polymerase chain reaction in biopsied tumor tissues throughout the period of treatment despite of the presence of neutralizing antiadenovirus antibody. Distribution studies revealed that the vector was detected in the gargle and plasma, but rarely in the urine. Thirteen of 15 patients were assessable for efficacy; one patient had a partial response (squamous cell carcinoma at the carina), 10 patients had stable disease, with three lasting at least 9 months, and two patients had progressive disease.

Conclusion
Multiple courses of intratumoral Ad5CMV-p53 injection alone or in combination with intravenous administration of cisplatin were feasible and well tolerated in advanced NSCLC patients, and appeared to provide clinical benefit.

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INTRODUCTION

Lung cancer is the most common cause of cancer related deaths in both men and women worldwide.[1] In 2001, 39,880 males and 15,122 females died of lung cancer in Japan, which ranked first among males and third among females in the number of cancer deaths.[2] Recent advances in molecular biology have fostered remarkable insights into the molecular basis of lung cancer,[3] and suggest that restoration of the function of critical gene products could halt or reverse cancer pathogenesis, thus having a therapeutic effect in cancer.

p53 is the most extensively studied tumor suppressor gene, and its mutation has been reported to be one of the most common genetic changes found in malignant tumors.[4] *p53* gene mutation is reported to occur in 40% to 50% of non–small-cell lung cancer (NSCLC),[5] and aberrant *p53* expression correlates with an adverse prognosis in lung cancers.[6,7] The *p53* gene product is involved in multiple pivotal cellular processes as a potent transcriptional regulator, and one of its most important roles is in the regulation of apoptosis.[8] We previously reported that the overexpression of the wild-type *p53* (wt-*p53*) gene by recombinant, replication-deficient viral vector, Ad5CMV-p53 (ADVEXIN; Introgen Therapeutics Inc, Houston, TX), triggered apoptosis in a variety of human cancer cells independent of their *p53* status.[9-13] ADVEXIN in combination with chemotherapeutic drugs, such as cisplatin, showed a profound antitumor effect in