厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

放射線感受性ナノバイオ・ウイルス製剤の開発と難治性固形癌に対する臨床応用の検討

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研究代表者　藤原　俊義

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【研究要旨】
最近のゲノム科学やナノテクノロジーの進歩により、癌の悪性形質の発現に関わる分子機構が明らかとなり、癌に特異的な標的分子を定めることが可能となってきた。p53はヒト悪性腫瘍で最も高頻度に異常がみられる癌抑制遺伝子であり、その正常型の遺伝子導入により放射線感受性増強をはじめとする多彩な作用機序を介した抗腫瘍効果が認められる。しかし、非増殖型ウイルスベクターでは腫瘍内へのウイルス拡散や遺伝子導入効率に限界があり、根治を目指した治療には至っていない。ウイルスは本来、ヒトの細胞に感染して複製・増殖することで細胞を破壊する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを殺傷する抗癌剤として用いることが可能となる。本研究では、テロメラーゼ依存性に増殖し、細胞死を誘導するOBP-301（Telomelysin）をさらに武装化（arming）し、ウイルス増殖による細胞死とともにアポトーシス誘導分子p53を発現することで強力な抗腫瘍活性を発揮する新規ナノバイオ・ウイルス製剤OBP-702を開発する。平成20年度は、OBP-702とOBP-301、さらに*p53*遺伝子を発現する非増殖型アデノウイルスAdvexinとの抗腫瘍活性の比較検討を行い、p53標的遺伝子発現を検証することで分子機構の解析を行った。また、OBP-702のOBP-301より強力な放射線増感作用を*in vitro*において検証した。

A．研究目的

難治固形癌に対する新たな抗癌剤開発は、分子標的薬剤の開発などにより積極的に進められており、その治療成績の向上も現実のものとなってきている。しかし、副作用や耐性の出現など解決すべき問題点は多く、新たな治療戦略の開発は必須と考えられる。本研究の目的は、ベクターとして多くの遺伝子治療で使用され、その安全性が確認されてきたアデノウイルスのゲノムを改変し、より強力な抗腫瘍活性を有する武装化（armed）ナノバイオ・ウイルス製剤を開発することである。

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、ウイルスを癌細胞のみを殺傷する抗癌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型を基本骨格とし、80-90%のヒト悪性腫瘍で極めて高い活性がみられる不死化関連酵素テロメラーゼの構成分子である*hTERT* (human telomerase reverse transcriptase)遺伝子のプロモーターでウイルス増殖に必須の*E1A*および*E1B*遺伝子を制御することで、癌細胞のみで増殖する腫瘍融解アデノウイルス（Oncolytic adenovirus）を構築する。さらに、放射線感受性プロモーターEgr-1で強力なアポトーシス誘導機能を持つ*p53*癌抑制遺伝子を駆動する発現カセットを、ウイルスの*E3*遺伝子領域に搭載する。

このナノバイオ・ウイルス製剤OBP-702は、癌細胞で選択的に増殖することにより標的細胞死を引き起こす機能を有する。また、放射線によるアポトーシス誘導でもp53は重要なシグナル伝達経路であり、OBP-702によるp53の過剰発現により放射線感受性自体も増強されると考えられる。さらに、原発腫瘍内に局所投与されたOBP-702は、周辺のリンパ節にも到達してリンパ節転移巣でも増殖するため、臨床的には所属リンパ節を含めた放射線治療との強力な相乗効果が期待される。

平成19年度はウイルス構築と*in vitro*における抗腫瘍効果の確認を行ったが、平成20年度はOBP-301および*p53*遺伝子を発現する非増殖型アデノウイルスAdvexinとの抗腫瘍効果の比較検討を行い、分子機構の解析を含めたOBP-702の優位性を検証した。また、OBP-702の放射線感受性増強作用を検討し、OBP-702の放射線感受性ナノバイオ・ウイルス製剤としての機能を検証した。

B．研究方法

1）OBP-702によるアポトーシス誘導の比較検討

テロメラーゼ活性依存性に癌細胞で増殖し、ウイルスによる細胞死誘導機能（oncolysis）により癌

細胞を破壊するOBP-301（Telomelysin）、アポトーシス誘導機能を有する*p53*癌抑制遺伝子を発現する非増殖型アデノウイルスAdvexin、および本研究で開発したOBP-301にp53遺伝子を搭載したOBP-702を用いて、ヒト非小細胞肺癌細胞株H358、ヒト食道扁平上皮癌細胞株T.Tnにおける抗腫瘍効果を比較検討した。アポトーシス誘導能に関しては、活性化caspase 3の発現をBD FACSArray バイオアナライザーにて解析した。

また、その分子機構を解析するために、*p53*遺伝子産物の標的分子であるp21およびBaxの発現、らにアポトーシスの指標であるcaspase 3およびpoly（ADP-ribose）polymerase (PARP)のcleavageをウエスタンブロット解析にて検討した。

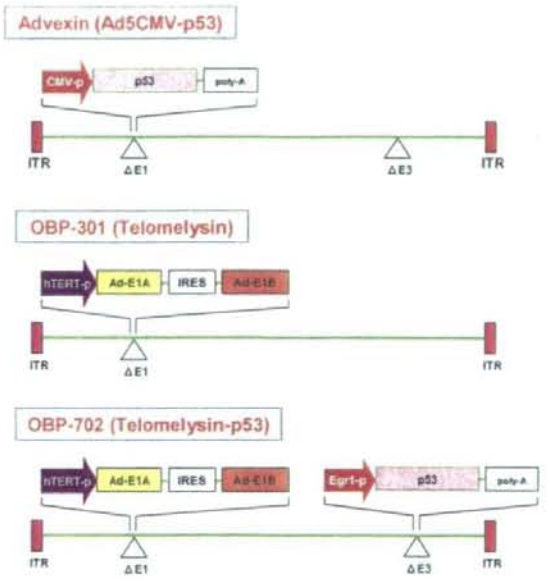

図１　ウイルス製剤の構造

2）*In vivo*におけるOBP-702の抗腫瘍効果の検討

H358ヒト非小細胞肺癌細胞をBALB/cヌードマウスの背部皮下に移植し、5-7 mm大の腫瘍形成がみられる14日目から10^8 plaque forming units (PFU)のAdvexin、OBP-301、OBP-702製剤を3日おきに3回の腫瘍内投与を行い、腫瘍サイズの変化を経日的に観察した。

3）OBP-702と放射線照射による相乗効果の検討

H358ヒト非小細胞肺癌細胞およびT.Tnヒト食道扁平上皮癌細胞に0、0.1、1、5、10 multiplicity of infection (MOI)のOBP-301あるいはOBP-702を感染させ、24時間後に0、2、5、10、20 Gyの放射線を照射し、5日後にXTTアッセイにて生細胞数を比較検討した。また、Combination Index (CI)をCalcuSyn software (ver. 2)にて計算して、相乗効果の解析を行った。

4）OBP-702製剤の大量製造

遺伝子・細胞治療センターにWave 20バイオリアクターおよびAKTAカラムクロマトグラフィー装置を設置し、HeLa細胞を用いてOBP-702の大量製造を行った。

（倫理面への配慮）

本研究は「大臣確認実験」となるため、「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、研究計画実施の承認を得ている。

C．研究結果

1）OBP-702によるアポトーシス誘導の比較検討

H358ヒト非小細胞肺癌細胞にOBP-301、OBP-702を10 MOIで感染後48時間でcaspase 3陽性細胞を比較したところ、OBP-301で6.1%に対してOBP-702では23.5％と明らかに多くのアポトーシス細胞が確認された。また、Advexinでは100 MOIで24.4％であり、OBP-702はAdvexinの約10倍のアポトーシス誘導能を有すると考えられる。また、T.Tnヒト食道扁平上皮癌細胞はOBP-301、Advexinに抵抗性であったが、OBP-702ではアポトーシス細胞が誘導された。

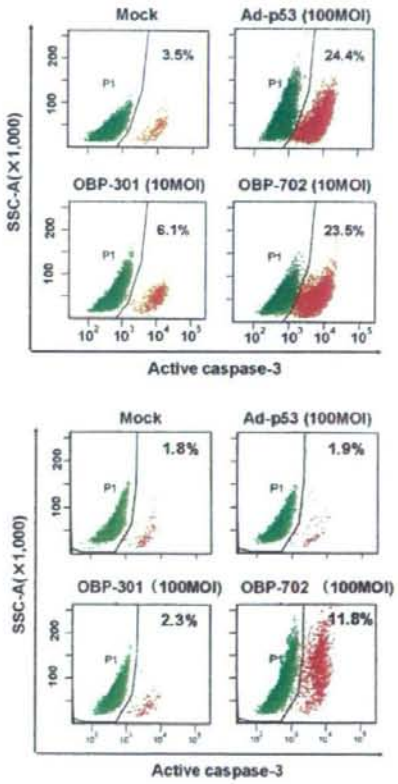

図2　OBP-702感染によるアポトーシス誘導能
（上段　H358細胞、下段　T.Tn細胞）

また、H358細胞でのp53の標的遺伝子発現を比較した。5 MOIのAdvexin感染後、p21およびBaxの発現が増強し、72時間後にPARPのcleavageが確認された。一方、OBP-702 5 MOIの感染後24時間で一過性にp21の発現がみられたが、その後に減弱し、Baxの発現増強とともにcaspase 3、PARPのcleavageが認められた。OBP-301の感染ではp21、Baxいずれれの発現増強もみられず、caspase 3、PARPにも明らかな変化はなかった。

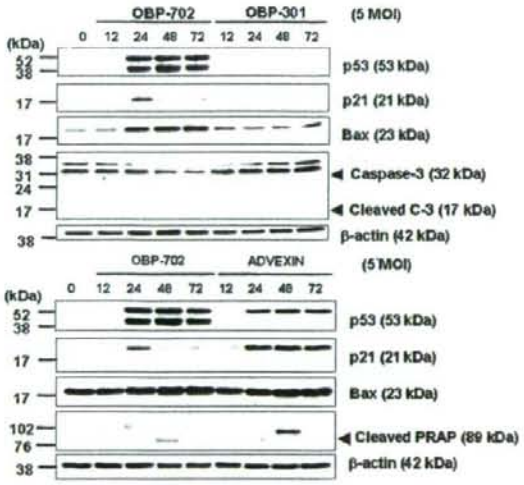

図3　OBP-702感染によるp53標的遺伝子発現

2）*In vivo*におけるOBP-702の抗腫瘍効果の検討

H358背部腫瘍へのAdvexinの腫瘍内投与はコントロール群に比べて軽度の増殖抑制効果を示したが、OBP-301およびOBP-702投与群ではさらに有意な抗腫瘍活性が認められた。

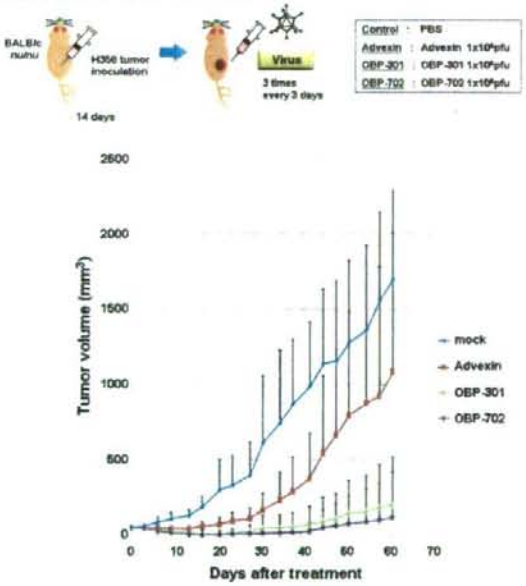

図4　*In vivo*でのOBP-702による抗腫瘍効果

3）OBP-702と放射線照射による相乗効果の検討

H358ヒト非小細胞肺癌細胞およびT.Tnヒト食道扁平上皮癌細胞において、OBP-301、OBP-702いずれも容量依存性の放射線との増強効果が認められた。しかし、CIの算出では、OBP-301はH358細胞においてのみ、またOBP-702はH358、T.Tn細胞いずれにおいても相乗効果が確認された。

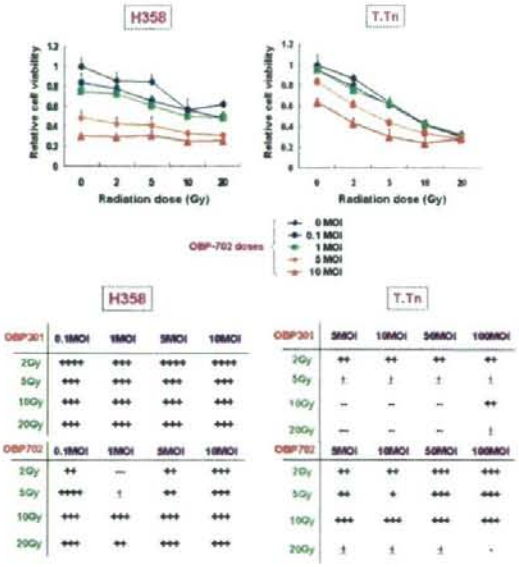

図5　OBP-702に放射線感受性増強効果
（上段 容量反応曲線、下段 Combination Index）

さらに、OBP-702と放射線によるアポトーシス誘導能を活性化caspase 3の発現で比較したところ、OBP-301やAdvexinに比べてOBP-702と放射線の併用でもっとも高率にアポトーシス細胞の増強が確認された。

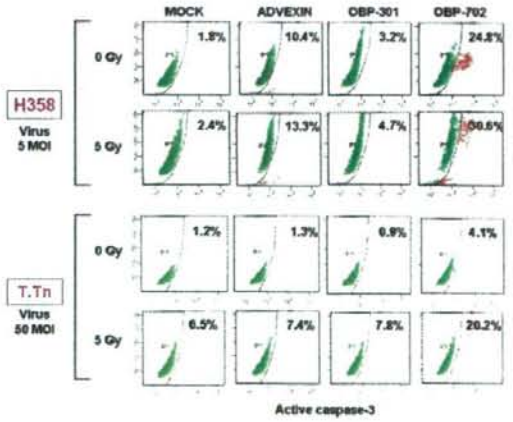

図6　OBP-702と放射線によるアポトーシス誘導

4）OBP-702製剤の大量製造

Wave 20バイオリアクターにてHeLa細胞を大量培養し、OBP-702の種ウイルスを感染させ、細胞およびウイルスが十分増殖した時点でTween 20にて細胞を融解した。安全キャビネット内でフィルトレーションを繰り返してウイルスを濃縮し、最後にAKTA100カラムクロマトグラフィーでウイルス分画を回収して高品質ウイルスストックを作成した。最終的には、2.2 x 10^{12} virus particle (vp)/ml濃度のウイルス液が54 ml採取できており、1.2 x 10^{14} vpという高濃度のウイルスストックが調達できた。

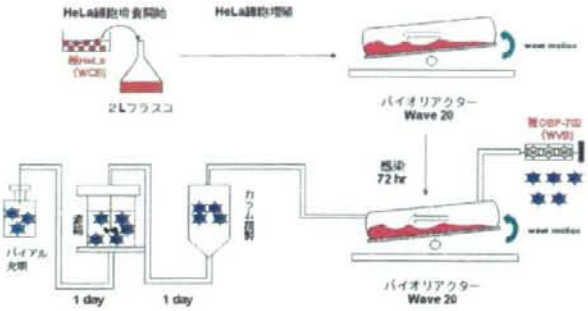

図7　Wave 20 Bioreactorによる製造工程

D．考察

OBP-702は、*hTERT*遺伝子のプロモーターでアデノウイルス*E1*遺伝子を駆動し、放射線感受性プロモーターEgr-1でヒト正常型*p53*遺伝子を発現する腫瘍融解ウイルスである。平成19年度には、肺癌、大腸癌、肝臓癌、食道癌、頭頸部癌、乳癌、悪性中皮腫などの癌細胞で選択的に増殖することによりOBP-702は標的細胞死を引き起こすと同時に、*p53*遺伝子を発現して強力なアポトーシス誘導を介した抗腫瘍効果を発揮することが明らかになった。Egr-1プロモーターの放射線制御性の根拠は得られなかったが、サイトメガロウイルス・プロモーターなどに比べて活性が低いため、十分なウイルス増殖が可能となり、その相乗効果が顕著にみられたと考察できる。

平成20年度には、*p53*遺伝子を持たないOBP-301および*p53*遺伝子発現非増殖型アデノウイルスAdvexinとの比較を行い、OBP-702はAdvexinの約10倍のアポトーシス誘導効果を有することが明らかとなった。これは、Advexinではp21が持続的に誘導されるため細胞周期停止が優位となるが、OBP-702感染では一過性のp21発現の後に減弱するためアポトーシスが強く誘導されると推測される。

また、ヌードマウスの背部に移植したH358ヒト肺癌腫瘍にOBP-702を腫瘍内投与したところ、同容量のAdvexinに比べて有意に強力な*in vivo*抗腫瘍効果が認められた。ただ、OBP-702とOBP-301の効果の差は顕著ではなく、今後、OBP-301に抵抗性の細胞株を用いた*in vivo*実験を予定している。

さらに、OBP-702の放射線感受性増強効果を検討したところ、OBP-301に感受性のあるH358ヒト肺癌細胞では同等の、またOBP-301に抵抗性のT.Tnヒト食道癌細胞では明らかに強力な放射線増感作用が観察された。フローサイトメトリーによるcaspase 3活性の測定では、いずれの細胞株でも放射線併用により2~5倍のアポトーシス誘導が認められており、OBP-702は放射線増感作用を有する生物製剤と言える。OBP-702の持つE1B55kDaによるDNA修復抑制がその作用機構のひとつと推測されるが、さらにp53の関与についても検討していく。今後は、*in vivo*におけるOBP-702の腫瘍内投与と放射線との併用効果を検証し、前臨床研究としての毒性試験や体内動態の分析を進めることで、OBP-702によるトランスレーショナルリサーチの実現を目指す。

E．結論

*p53*遺伝子を搭載したテロメラーゼ特異的増殖アデノウイルスOBP-702製剤は、基盤となった*p53*遺伝子を持たないOBP-301製剤や*p53*遺伝子を発現する非増殖型アデノウイルスより強い抗腫瘍活性を示し、その有用性が示された。また、放射線感受性増強効果も明らかであり、今後のトランスレーショナルリサーチが期待される。

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A Novel Antiangiogenic Effect for Telomerase-Specific Virotherapy through Host Immune System[1]

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Soluble factors in the tumor microenvironment may influence the process of angiogenesis; a process essential for the growth and progression of malignant tumors. In this study, we describe a novel antiangiogenic effect of conditional replication-selective adenovirus through the stimulation of host immune reaction. An attenuated adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of E1 genes, could replicate in and cause selective lysis of cancer cells. Mixed lymphocyte-tumor cell culture demonstrated that OBP-301-infected cancer cells stimulated PBMC to produce IFN-γ into the supernatants. When the supernatants were subjected to the assay of in vitro angiogenesis, the tube formation of HUVECs was inhibited more efficiently than recombinant IFN-γ. Moreover, in vivo angiogenic assay using a membrane-diffusion chamber system s.c. transplanted in *nu/nu* mice showed that tumor cell-induced neovascularization was markedly reduced when the chambers contained the mixed lymphocyte-tumor cell culture supernatants. The growth of s.c. murine colon tumors in syngenic mice was significantly inhibited due to the reduced vascularity by intratumoral injection of OBP-301. The antitumor as well as antiangiogenic effects, however, were less apparent in SCID mice due to the lack of host immune responses. Our data suggest that OBP-301 seems to have antiangiogenic properties through the stimulation of host immune cells to produce endogenous antiangiogenic factors such as IFN-γ. *The Journal of Immunology*, 2009, 182: 1763–1769.

Angiogenesis is the development of new capillaries from preexisting capillary blood vessels and is necessary for the growth of solid tumors beyond 1–2 mm in diameter (1). Targeting the angiogenic process is therefore regarded as a promising strategy in cancer therapy. Angiogenesis consists of dissolution of the basement membrane, migration and proliferation of endothelial cells, canalization, branching and formation of vascular loops, and formation of a basement membrane (2). These steps might be regulated by the local balance between the amount of angiogenic stimulators and inhibitors (3–5). As cells undergo malignant transformation, angiogenic mitogens such as vascular endothelial growth factor (VEGF),[3] basic fibroblast growth factor, platelet-derived epithelial cell growth factor, and TGF become dominant, causing the aberrant angiogenesis. In contrast, many endogenous angiogenic inhibitors such as platelet factor 4, thrombospondin 1, angiostatin, endostatin, various antiangiogenic peptides, hormone metabolites, and cytokines constitutively suppress angiogenesis in normal tissues (6). These scenarios suggest the possibility that endogenous angiogenic inhibitors that outweigh the stimulators could turn off the angiogenic switch.

Recent studies have demonstrated that the tumor microenvironment, which orchestrates with the host immune system, is a critical component of both tumor progression and tumor suppression (7). Indeed, the production of cytokines at tumor sites can either stimulate or inhibit tumor growth and progression (8). These findings provide a unique therapeutic opportunity based on selective and locoregional production of endogenous antitumor mediators such as angiogenic inhibitors. We reported previously that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, induced selective E1 expression and efficiently killed human cancer cells, but not normal human fibroblasts (9–12). Although the precise molecular mechanism of OBP-301-induced cell death is still unclear, the process of oncolysis is morphologically distinct from apoptosis and necrosis. We found that tumor cells killed by OBP-301 infection could stimulate host immune cells more efficiently compared with chemotherapeutic drug-induced apoptotic cells and necrotic cells by freeze/thaw, thus enhancing the antitumor immune response (13). These results suggest that oncolytic virus is effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that could modify the tumor microenvironment.

In the present article, we explored whether OBP-301-infected oncolytic cells can activate host immune cells and influence tumor

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; MLTC, mixed lymphocyte-tumor cell culture; MOI, multiplicity of infection.

cell-mediated angiogenesis in vitro and in vivo. Antineoplastic effect of intratumoral administration of OBP-301 on s.c. murine colon tumors transplanted was compared in syngenic immunocompetent mice and SCID mice. Finally, we examined the effect of neutralizing anti-IFN-γ Ab on OBP-301-mediated antiangiogenic potential in vivo.

Materials and Methods

Cell lines and reagents

The human colorectal carcinoma cell lines SW620 (HLA-A02/A24) and the murine colon adenocarcinoma cell line Colon-26 were maintained in vitro in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Recombinant human IFN-γ was purchased from Peprotech.

Mice

Female BALB/c (BALB/cAnNCrlCrlj), BALB/c *nu/nu* (CAnN.Cg-*Foxn1*^{nu}/CrlCrlj), and SCID (CB17/Icr-*Prkdc*^{scid}/CrlCrlj) mice, 5–6 wk of age, were purchased from Charles River Japan Breeding Laboratories. Animals were housed under specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Adenovirus

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, was constructed and previously characterized (9–12). The virus was purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation.

Cell viability assay

XTT assay was performed to measure cell viability. Briefly, cells were plated on 96-well plates at 5×10^3 per well 24 h before treatment and then infected with OBP-301. Cell viability was determined at the times indicated by using a Cell Proliferation kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

Mixed lymphocyte-tumor cell culture (MLTC) and cytokine production assay

For MLTC, SW620 tumor cells were infected with OBP-301 at a multiplicity of infection (MOI) of 10, washed three times in PBS 72 h after infection, and then cocultured with PBMC at a ratio of 1:40. The supernatant was collected at the indicated times and stored at −80°C until assay. The concentration of IFN-γ was measured with ELISA kits (BioSource International).

In vitro angiogenesis assay

In vitro angiogenesis was assessed based on the formation of capillary-like structures by HUVECs cocultured with human diploid fibroblasts according to the instructions provided with the angiogenesis kit (Kurabo). In brief, the HUVECs were incubated in a medium containing the diluted supernatants of MLTC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The medium was replaced at days 4, 7, and 9. At day 11, the HUVECs were fixed and stained by using an anti-human CD31 Ab (Kurabo) according to the instructions provided. The formation of the capillary network was observed with a microscope at a magnification of ×40.

In vivo assay for tumor angiogenesis

In vivo angiogenesis was determined using the dorsal air-sac method (14). Briefly, 2×10^6 SW620 cells were suspended in PBS containing the diluted supernatants of MLTC or control medium, and placed into round-shaped chambers that consisted of a ring covered with cellulose ester filters (pore size, 0.45 μm; Millipore) on both sides. These chambers were implanted into a dorsal air sac produced in female BALB/c *nu/nu* mice by the injection of 10 ml of air. Five mice in each group were sacrificed on day 5, and the formation of a dense capillary network in s.c. regions was examined under a dissecting microscope. The neovascularization was assessed semiquantitatively by counting the number of cork screw vessels. For each slide, a total of three fields at a magnification of ×4 were selected at random, and the scores were averaged.

In vivo tumor growth and determination of microvessels

Female BALB/c and SCID mice were s.c. implanted with 2×10^6 Colon-26 cells. When tumors grew to ~5–6 mm in diameter, the mice were randomly assigned into three groups and a 100 μl of solution containing 1×10^8 PFU of dl312 or OBP-301, or PBS was injected into the tumor on days 1, 3, and 5. Tumors were measured for perpendicular diameters every 3 or 4 days, and tumor volume (in cubic millimeters) was calculated using the following formula: $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. For histological analysis, 2 wk after treatment, the tumors were harvested, embedded in Tissue Tek (Sakura), cut into 5 μm-thick sections, and assessed by a standard H&E and immunohistochemical staining using a rat anti-mouse mAb against CD31 (BD Pharmingen). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

In vivo inhibition of IFN-γ with neutralizing Abs

For neutralizing IFN-γ, mice were i.p. administered 200 μg of rat anti-mouse IFN-γ mAb (XMG1.2; BD Pharmingen) 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 (BD Pharmingen).

Statistical analysis

Determination of significant differences among groups was assessed by calculating the value of Student's t test using the original data analysis. Statistical significance was defined at $p < 0.01$.

Results

Effect of OBP-301-infected human colorectal cancer cells on PBMC in vitro

First, we examined whether OBP-301 infection affects the viability of human colorectal cancer cells using the XTT assay. SW620 cells were either mock-infected with culture medium or infected with OBP-301 at an MOI of 1 or 10. As shown in Fig. 1*A*, OBP-301 infection induced death of SW620 cells in a dose-dependent manner. Next, we examined the ability of OBP-301-infected oncolytic cells to stimulate PBMC in MLTC. For this purpose, SW620 cells (HLA-A02/A24) treated with 10 MOI of OBP-301 for 72 h were cocultured with HLA-matched PBMC obtained from HLA-A24$^+$ healthy volunteers at a ratio of 1:40. The production of IFN-γ in the supernatants was then explored by ELISA analysis at the indicated time points. PBMC incubated with OBP-301-infected oncolytic SW620 cells secreted large amounts of IFN-γ as early as 24 h after MLTC, whereas PBMC alone induced little IFN-γ secretion (Fig. 1*B*). The maximum level of IFN-γ was ~250 pg/ml. We previously confirmed that addition of OBP-301 alone without target tumor cells did not affect the cytokine secretion from PBMC into the supernatant, indicating that infection of OBP-301 itself had no apparent effect on PBMC (13). These results suggest that PBMC stimulated with oncolytic tumor cells preferentially secrete high-level IFN-γ.

Inhibition of in vitro and in vivo angiogenesis by MLTC supernatants with OBP-301-infected human tumor cells

In the next step, we investigated the effects of MLTC supernatants with oncolytic SW620 tumor cells and HLA-matched PBMC on VEGF-induced angiogenesis in vitro. The addition of VEGF enhanced the formation of vascular-like structures of HUVECs, although tubule formation was almost absent without VEGF. This VEGF-induced angiogenesis was completely impaired by the addition of MLTC supernatants even at 1/4 dilution (Fig. 2). In contrast, although MLTC supernatants were confirmed to contain ~250 pg/ml IFN-γ, 10-fold more concentration of recombinant IFN-γ was needed to attenuate the tubule formation close to basal levels. The supernatants of PBMC

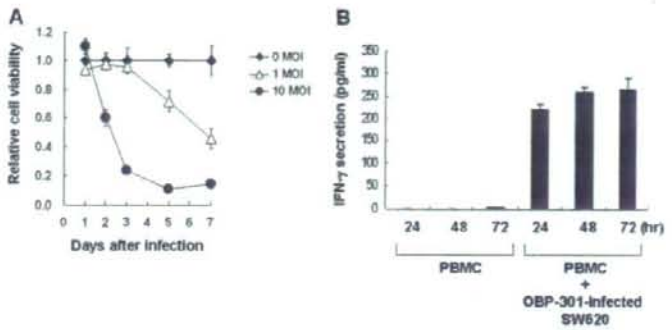

FIGURE 1. In vitro cytopathic effects of OBP-301 and IFN-γ secretion by oncolytic cell-stimulated PBMC. *A*, SW620 human colorectal cancer cells were infected with OBP-301 at indicated MOI values, and surviving cells were quantitated over 7 days by XTT assay. The cell viability of mock-treated cells on day 1 was considered 1.0, and the relative cell viability was calculated. Data are mean ± SD of triplicate experiments. *B*, IFN-γ concentrations in the supernatants of MLTC analyzed by ELISA. SW620 cells were treated with 10 MOI of OBP-301 for 72 h, and then cocultured with PBMCs obtained from HLA-A24$^+$ healthy volunteers for the indicated time periods in MLTC. The culture supernatants were harvested and tested by ELISA for IFN-γ concentrations. As a control, the supernatants of PBMC alone were also examined. Data are mean ± SD of triplicate experiments.

alone had no effect on in vitro angiogenesis. These results suggest that MLTC supernatants may contain more antiangiogenic factors in addition to IFN-γ.

We also assessed whether MLTC supernatants inhibited in vivo angiogenesis induced by human cancer cells. SW620 cells in PBS containing supernatants of OBP-301-infected SW620 cells, PBMC, or both, which were packed into membrane chambers, were implanted into a dorsal air sac produced in *nu/nu* mice. The chambers consisted of membranes that allowed the passage of macromolecules such as IFN-γ, but not cells. Five days after implantation, neovascularization, as demonstrated by the development of capillary networks and curled microvessels in addition to the preexisting vessels, occurred in the dorsal subcutis touched by the chamber, which contained SW620 cells alone. The addition of MLTC supernatants, however, reduced the size and tortuosity of the preexisting vessels, and significantly reduced the development of curled microvessels (Fig. 3). Although the preexisting vessels became thinner by supernatants of OBP-301-infected SW620 cells or PBMC, the number of curled microvessels, which is characteristic of tumor neovasculature, was consistent in these two groups with that in the group compared with SW620 cells alone. Thus, MLTC supernatants exhibited a profound antiangiogenic activity in vivo.

Involvement of host immune activity on antiangiogenic effect of OBP-301

The finding that OBP-301-infected tumor cells stimulated PBMC to produce antiangiogenic factors prompted us to study whether immunodeficiency of host animals could affect the antitumor effect of OBP-301 in vivo. When 2×10^6 Colon-26 murine colon adenocarcinoma cells were inoculated s.c. into BALB/c and SCID mice, palpable tumors appeared in 100% of the mice within 2 wk after tumor injection. Fourteen days after tumor inoculation, animals bearing Colon-26 tumors with a diameter of 5–6 mm were treated with the direct intratumoral injection of 10^8 PFU OBP-301 every 2 days for three cycles. As shown in Fig. 4, treatment with OBP-301 resulted in a significant growth suppression compared with tumors injected with PBS at least for 12 days starting on day 4 after last virus injection ($p < 0.01$) in BALB/c mice; however, OBP-301-mediated antitumor effect was partially impaired in SCID mice, as significant inhibition was observed only for 6 days starting on day 10. Intratumoral injection of replication-deficient dl312 adenovirus had no effect on the tumor growth in BALB/c or SCID mice (data not shown). These results indicate the partial involvement of the host immune system in the OBP-301-mediated antitumor effect.

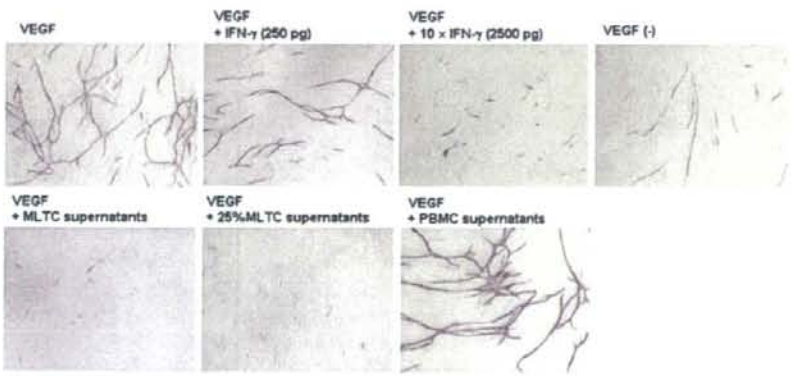

FIGURE 2. Inhibition of in vitro angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. HUVECs were incubated in a medium containing the supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The formation of the capillary network was confirmed by staining with anti-human CD31 Ab on day 11. Representative images depicting formation of capillary-like tube structures by HUVECs are shown. Original magnification is at ×40.

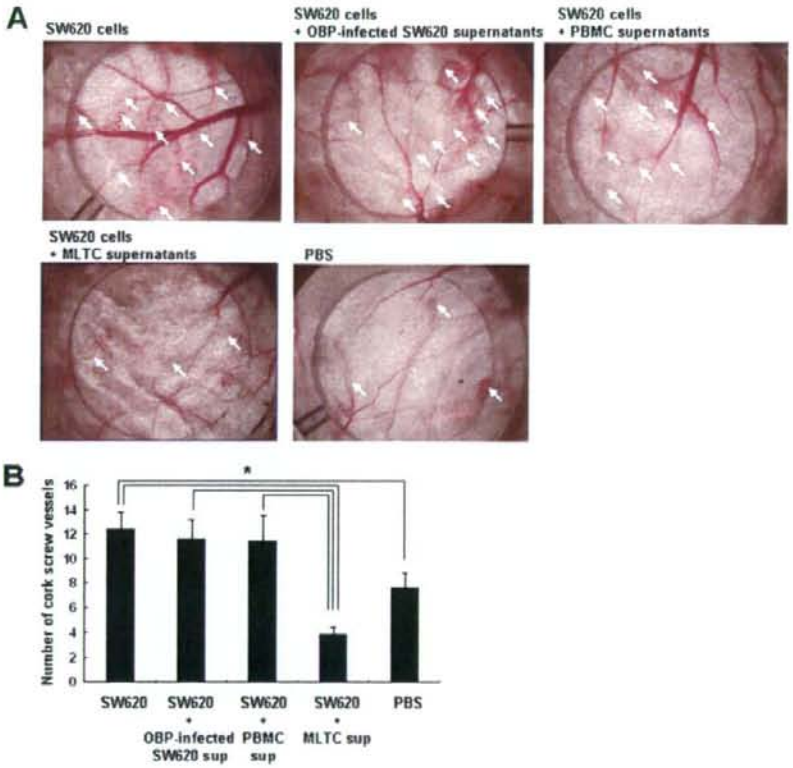

FIGURE 3. Inhibition of tumor cell-mediated in vivo angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. *A*, SW620 human colorectal tumor cells at a density of 2×10^6 were placed in a diffusion chamber in PBS containing the diluted supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or control mediums, and it was implanted into a dorsal air space produced in BALB/c *nu/nu* mice on day 0. Mice were sacrificed on day 5, and the chamber was removed from the s.c. tissue. A new ring without filters was placed on the same site to mark the position of the chamber. The capillary networks developed inside the rings were photographed to determine the effect of treatments. Representative images of treatment groups are shown. Curled microvessels are shown (arrow). *B*, The number of cork screw vessels was semiquantitatively counted to assess the neovascularization. Data are mean ± SD. *, $p < 0.01$. Similar results were observed in two independent experiments conducted in triplicate.

Antiangiogenic effect of OBP-301 on syngenic and immunodeficient murine tumor models

When Colon-26 s.c. tumors implanted in BALB/c mice were injected with PBS, replication-deficient dl312 adenovirus, or OBP-301. Macroscopically, tumors treated with OBP-301 were consistently smaller than those of the other two cohorts of mice 14 days after last virus injection (Fig. 5*A*). Furthermore, a reddish area was noted on the tumor surface on two of six mice treated with OBP-301, indicating virus-induced intratumoral necrosis of tumor cells in vivo.

To better understand the mechanisms underlying the induction of necrosis following OBP-301 treatment, histologic and immunohistochemical analyses were performed on Colon-26 tumors harvested 14 days after last injection. A standard H&E staining demonstrated the presence of many vessels in tumors injected with PBS or dl312. However, OBP-301-treated tumors showed few vessels. In addition, massive tumor cell death and cellular infiltrates at the central portions of the tumors were observed where OBP-301 was injected (Fig. 5*B*). Immunohistochemical staining of tumor sections with the Ab for CD31 Ag, an endothelial cell marker, also revealed that Colon-26 tumors injected with OBP-301 displayed very few and extremely small blood vessels (Fig. 5*C*). In contrast, OBP-301 injection could not apparently reduce the vessel numbers on Colon-26 tumors implanted in SCID mice (Fig. 5*D*). These in vivo studies demonstrated that inhibition of angiogenesis due to the stimulation of host immune system might be an important mechanism of OBP-301-mediated in vivo antitumor effect.

Contribution of in vivo IFN-γ production to the OBP-301-mediated antiangiogenic effects

Finally, to determine whether IFN-γ is involved in OBP-301-mediated antiangiogenic effects, in vivo neutralizing experiments were performed by using anti-IFN-γ mAb or isotype-matched control mAb. Angiogenesis was reduced by intratumoral injection of

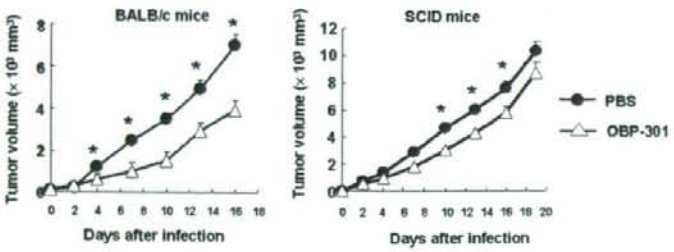

FIGURE 4. Antitumor effects of intratumorally injected OBP-301 against Colon-26 murine colon adenocarcinoma tumors in syngenic immunocompetent BALB/c and immunodeficient SCID mice. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of mice. OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days. PBS was used as a control. Six mice were used in each group. Tumor growth was expressed by tumor mean volume ± SD. *, $p < 0.01$.

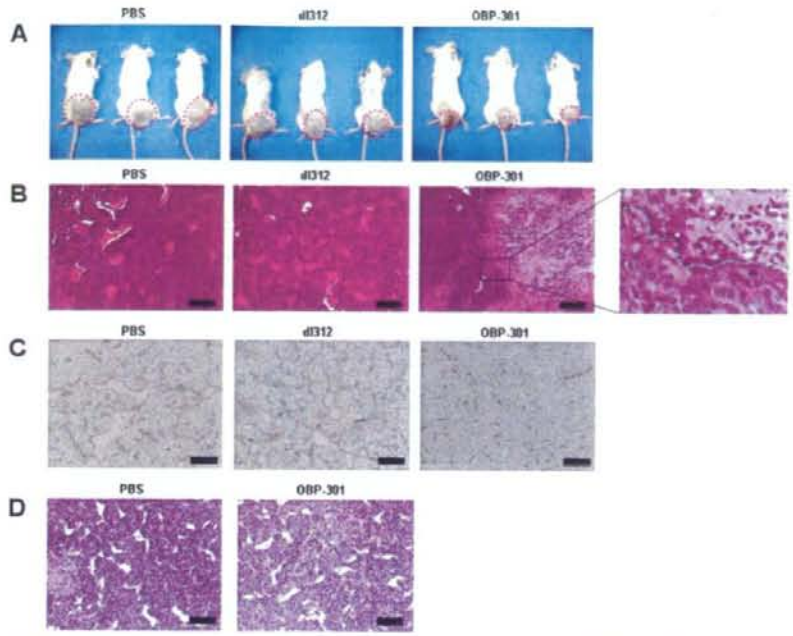

FIGURE 5. Macroscopic and histopathological analysis of Colon-26 tumors treated intratumorally with OBP-301. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and SCID mice and OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. *A*, Macroscopic appearance of Colon-26 tumors on BALB/c mice 14 days after treatment. Note the reddish area on the tumor surface in two mice treated with OBP-301. *B*, Tumor sections were obtained from BALB/c mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 μm, and magnification is $\times$100. Magnified view of the boxed region in *B* is shown. The area with cellular infiltrates is indicated with the green dotted line. *C*, Blood vessel formation in Colon-26 tumors injected with OBP-301. Frozen sections of the tumors were also probed with an Ab against CD31. Scale bar represents 50 μm, and magnification is a $\times$200. *D*, Tumor sections were obtained from SCID mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 μm, and magnification is at $\times$100 magnification.

OBP-301 on Colon-26 tumors; this antiangiogenic effect, however, could be partially inhibited in the presence of anti-IFN-γ mAb (Fig. 6). Treatment with control IgG1 had no effect on the antiangiogenic effects of OBP-301. These results suggest that IFN-γ may be one of the important factors for OBP-301 to inhibit angiogenesis in vivo.

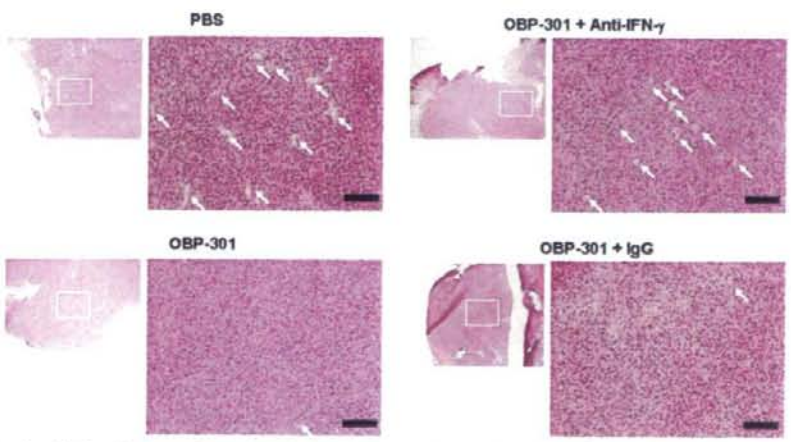

FIGURE 6. Effects of anti-IFN-γ Abs on angiogenesis in Colon-26 tumors. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. Mice were administered 200 μg of anti-IFN-γ mAb (XMG1.2) i.p. to neutralize IFN-γ 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 or PBS. Frozen sections of tumors obtained 14 days after final administration of OBP-301 were stained with H&E. Magnified view (*right*) of the boxed region (*left*). Microvessels are shown (arrow). Scale bar represents 50 μm, and magnification is at $\times$200.

Discussion

The tumor vasculature provides a new and attractive target for cancer therapy because of the reliance of most tumor cells on an adequate vascular supply for their growth and survival. Although the beneficial effects of novel antiangiogenic agents such as bevacizumab have been recently shown (15), regulation of endogenous antiangiogenic mediators may be another approach to inhibit angiogenesis. In the present study, we showed that OBP-301 infection and replication induced cytolysis of tumor cells with subsequent stimulation of host immune cells, which in turn inhibited tumor angiogenesis in vivo. Treatment of established murine colon tumors with intratumoral injection of OBP-301 resulted in a significant antitumor response characterized by extensive necrosis and reduced vascularity.

We reported previously that wild-type *p53* tumor suppressor gene transfer by a replication-deficient adenovirus vector (Advexin) could have antiangiogenic effects. The effects could be through down-regulation of angiogenic factor VEGF and up-regulation of antiangiogenic factor BAI1 because tumor p53 protein is a potent transcriptional factor (16, 17). In contrast, OBP-301 contains no therapeutic genes such as *p53* and, therefore, its infection may not directly influence the angiogenic property of infected tumor cells. However, because viral infection is known to trigger innate and adaptive immune responses presumably through the release of proinflammatory cytokines (18–20), local administration of OBP-301 might affect the tumor microenvironment, thus explaining the potential therapeutic benefit on tumor angiogenesis. In fact, dying tumor cells infected with OBP-301 promoted the production of Th1 cytokines by PBMC such as IFN-γ, which is one of the most potent antiangiogenic factors (21, 22) (Fig. 1). Viral infection itself has been reported to activate dendritic cells to secrete pro- or anti-inflammatory cytokines (23); our preliminary experiments, however, demonstrated that OBP-301 alone had no effect on cytokine production by PBMC (13), indicating that OBP-301 itself may be less infective or stimulatory to PBMC. The result is consistent with our previous finding that OBP-301 attenuated replication as well as cytotoxicity of human normal cells (9, 10). Moreover, OBP-301-infected tumor cells, but not untreated tumor cells, enhanced IFN-γ-inducible proteasome activator PA28 expression in the presence of PBMC (13), indicating that only dying tumor cells could trigger IFN-γ production by PBMC.

IFN-γ has been also known to inhibit tumor angiogenesis through the subsequent stimulation of secondary mediators, including monokine induced by IFN-γ and IFN-inducible protein 10 (24). Indeed, the observation that the supernatants of PBMC cocultured with OBP-301-infected human colorectal cancer cells exhibited a more profound antiangiogenic effect than recombinant IFN-γ (Fig. 2) suggests that other factors in addition to IFN-γ, which may not be related to IFN-γ, play important roles in inhibition of tumor cell-mediated angiogenesis. For example, we also found that oncolytic cells stimulated PBMC to secrete IL-12, which is an inducer of IFN-γ as well as an antiangiogenic factor, into the culture supernatants (13). The supernatants of neither virus-infected tumor cells alone nor PBMC alone were more antiangiogenic compared with those of MLTC in vivo (Fig. 3). Therefore, the interaction of oncolytic cells and PBMC is required to produce antiangiogenic mediators and to inhibit in vivo angiogenesis following OBP-301 treatment. The question what kind of cells produce mediators for antiangiogenic effects is of interest. We reported previously that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human tumor cells, which in turn stimulated dendritic cells to produce IFN-γ as well as IL-12 into the supernatants (13). The amount of IFN-γ produced by dendritic cells was ~40 pg/ml, although 250 pg/ml IFN-γ was detected in the MLTC supernatants (Fig. 1*B*), indicating that other cell types may contribute to IFN-γ production. Lymphocytes that promote innate immunity (i.e., NK cells) as well as classical $CD4^+$ and $CD8^+$ T cells are also know to produce IFN-γ (25). Thus, dendritic cells represent one of the sources of IFN-γ; however, IL-12 secreted from dendritic cells activated with OBP-301-infected tumor cells might trigger these cells to produce IFN-γ.

To more directly evaluate the antiangiogenic effect of OBP-301, we used a syngenic BALB/c model established by s.c. inoculation of Colon-26 murine colon adenocarcinoma cells. OBP-301 is reported to have high infectivity and the potential to induce cell death in a variety of human cancer cells (9–12), whereas murine cells are relatively refractory to adenovirus infection due to the low expression of the coxsackievirus and adenovirus receptor. We have confirmed previously that telomerase-specific oncolytic adenovirus could infect and replicate in Colon-26 cells (12). Intratumoral administration of OBP-301 significantly inhibited the growth of Colon-26 tumors in syngenic immunocompetent BALB/c mice, although the magnitude of suppression was much less when compared with that in human tumor xenografts (9, 10). The finding that tumor growth suppression by OBP-301 was partially inhibited in immunodeficient SCID mice (Fig. 4) indicates that the host immune system could be partially responsible for the antitumor effect of OBP-301. Histopathologic analysis revealed that the presence of the immune cell infiltrates and the massive necrosis in Colon-26 tumors are exclusively due to the tumor-specific viral replication because dl312-injected tumors showed neither cellular infiltrates nor tissue damages (Fig. 5*B*). In view of the fact that a cellular infiltration could be still observed as late as 14 days after the last OBP-301 injection, immune responses are likely to be induced by oncolytic tumor cells. Furthermore, as expected, tumors injected with OBP-301 formed less blood vessels than mock- or dl312-treated tumors (Fig. 5, *B* and *C*), suggesting that inhibition of angiogenesis by infiltrating cell-secreted mediators partially elicits the antitumor activity of OBP-301. In contrast, antiangiogenic effect of OBP-301 was impaired in SCID mice (Fig. 5*D*), indicating that host immune cells are necessary for this function of OBP-301. Moreover, IFN-γ is considered to be partially responsible for the antiangiogenic effects of OBP-301 because in vivo neutralization of IFN-γ by anti-IFN-γ mAb increased angiogenesis on Colon-26 tumors (Fig. 6).

It remains to be studied whether OBP-301-infected oncolytic cells are capable of inhibiting the growth of distant tumors. Circulating inhibitors of angiogenesis such as angiostatin and endostatin can suppress the growth of remote metastases (26). The observation that none of mice treated with OBP-301 showed signs of viral distress (ruffled fur, weight loss, lethargy, or agitation) as well as histopathologic changes in any organs at autopsy (data not shown) suggests that the cytokine secretion by oncolytic cell-stimulated immune cells might be local rather than systemic. Thus, it is unlikely that locally produced antiangiogenic factors interfere with the distant tumor growth, although the circulating virus itself can infect and replicate in metastatic tumors. This question is being currently investigated in our laboratory.

In conclusion, we provide for the first time evidence that oncolytic virotherapy induces novel antiangiogenic effect by stimulating host immune cells to produce antiangiogenic mediators such as IFN-γ. Our data suggest that the antitumor effect of OBP-301 might be both direct and indirect.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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Telomerase-specific virotherapy in an animal model of human head and neck cancer

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Abstract

Telomerase-specific replication-competent adenovirus, Telomelysin (OBP-301), has a human telomerase reverse transcriptase promoter that regulates viral replication and efficiently kills human cancer cells. The objectives of this study are to examine the effects of OBP-301 in squamous cell carcinoma of the head and neck cells *in vitro* and in xenografted animals *in vivo*. OBP-301 was found to be cytotoxic to the YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, HN12, and KCCOR891 cell lines *in vitro*. However, the level of cytotoxicity is not correlated with the expression levels of adenoviral receptors, which may be required for adenoviral infection in squamous cell carcinoma of the head and neck cells. OBP-301 shows remarkable antitumor activity against established s.c. KCCT873 tumors in immunodeficient animals in a dose-dependent manner. In addition, no significant toxicity was observed in animals receiving treatment. These results suggest that OBP-301 is a novel therapeutic agent with promise for the treatment of human head and neck cancers. [Mol Cancer Ther 2009;8(1):171–7]

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) accounts for 5% of newly diagnosed adult cancers in the United States and 8% of cancers worldwide (1). Most patients are treated with various combinations of surgery, radiotherapy, and systemic agents (2). Despite major advances in the treatment of locoregionally advanced SCCHN, such as the introduction of novel chemotherapy regimens and inhibitors of the epidermal growth factor receptor, treatment fails in about half of the patients (3). The median survival of patients with recurrent or metastatic SCCHN who undergo chemotherapy is 6 to 9 months (4). Therefore, a considerable number of patients with SCCHN need additional treatment as the disease progresses.

Virotherapy, the approach to treat cancer with virus, has been done in some clinical trials; for example, clinical trials primarily using *p53* gene replacement (INGN-201; a replication-competent adenoviral-based vector expressing wild-type *p53*) have provided the basis for the design of ongoing randomized gene therapy clinical trials in SCCHN patients in the United States (5). Although systemic administration is probably required in the case of micrometastatic disease, virotherapy has some promise when tumor is limited to the head and neck. SCCHN is a particularly attractive model because most primary and recurrent lesions are easily acceptable to direct injection (6). Potential usage of virotherapy may include the perioperative application in the surgical wound and the addition of intratumoral (i.t.) virotherapy to current standard options, such as radiotherapy and/or chemotherapy.

Telomerase is a ribonucleoprotein complex responsible for the complete replication of chromosomal ends (7). Many studies have shown the expression of telomerase activity in >85% of human cancers (8) but only in a few normal somatic cell types (9). Telomerase activation is considered to be a critical step in carcinogenesis, and its activity is closely correlated with human telomerase reverse transcriptase (hTERT) expression (10). Therefore, the hTERT proximal promoter can be used as a molecular switch for selective expression of target genes in tumor cells. Replication-selective tumor-specific adenoviruses are being developed as novel anticancer therapies (11–14). In this context, an adenoviral vector that drives E1A and E1B genes under the hTERT promoter has been developed, termed Telomelysin or OBP-301 (15). OBP-301 can replicate in and lyse only cancer cells but not normal cells, and its strong cytotoxic activity were shown in a variety human cancer cells (15–17). Also, OBP-301-mediated oncolysis induces uric acid production as a danger signal and stimulates CTL activity via proteasome activator up-regulation (18).

The infection efficiency of recombinant adenoviral vectors varies widely depending on the expression of the primary receptor, the coxsackie adenovirus receptor (CAR); the secondary receptors, integrin $\alpha_V\beta_3$ and integrin $\alpha_V\beta_5$; and the tertiary receptor, heparan sulfate glycosaminoglycans (HSG; refs. 19, 20). The first step is the attachment of

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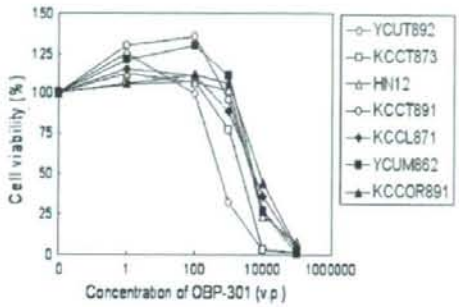

Figure 1. Sensitivity of SCCHN cells to OBP-301 *in vitro*. Cytotoxic activity of OBP-301 on 13 SCCHN cell lines was evaluated by XTT assay. Cells were cultured with various concentrations of OBP-301 (0-100,000 vp/mL). Mean ± SD of quadruplicate determinations. The assay was repeated three times.

the virus to the cell surface through CAR (20). Following attachment, the internalization of the virus into cells occurs through the integrin receptors $\alpha_V\beta_3$ and $\alpha_V\beta_5$ that are expressed in most cell types (19).

Previously, OBP-301 has been reported to induce cell death of human non-small cell lung, colorectal, and prostate cancers *in vitro* and *in vivo* (15, 17). The present study investigates the cytotoxic activity of OBP-301 in 13 SCCHN cell lines and the association between cytotoxic activity and adenoviral receptor expression. We also assessed the *in vivo* antitumor activity and toxicity and tolerability of OBP-301 in an athymic nude mouse model with KCCT873 SCCHN tumors.

Materials and Methods

Adenovirus

The recombinant replication-selective, tumor-specific adenoviral vector OBP-301 was provided by Oncolys Biopharma. The hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosome entry site (15). The virus particle (vp) titer-to-infection titer (plaque-forming units) ratios were 110:3.

Cells

The human non-small cell lung cancer cell line H1299 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L HEPES (Nacalai Tesuque), 100 μg/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesuque). The SCCHN cell line HN12 was grown in MEM containing 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The SCCHN cell lines YCUT892, KCCT873, KCCT891, KCCL871, YCUM862, KCCOR891, YCUL891, YCUM911, YCUMS861, YCUT891, 012SCC, and Wmm-SCC (21) were cultured in RPMI 1640 containing 10% fetal bovine serum, 1 mmol/L HEPES, 100 μg/mL penicillin, and 100 μg/mL streptomycin.

Cell Viability Assay

The XTT assay was done to measure cell viability. Briefly, cells were plated on 96-well plates at 1×10^3 per well 24 h before viral infection. Cells were then infected with 1 to 1×10^5 multiplicity of infection (vp) of OBP-301 and further cultured for 120 h. Cell viability was determined using the Cell Proliferation Kit II (Roche Diagnostics) according to the protocol provided by the manufacturer.

Flow Cytometry

Cells (1×10^5) were labeled with mouse monoclonal anti-CAR (RmcB; Upstate Cell Signaling Solution), anti-integrin $\alpha_V\beta_3$ (Chemicon), anti-integrin $\alpha_V\beta_5$ (Chemicon), or anti-heparan sulfate (Seikagaku) for 60 min at 4°C, incubated with FITC-conjugated goat anti-mouse IgG secondary antibody (Chemicon), and analyzed by the FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Control cells were incubated with anti-mouse IgG primary antibody (BD Bioscience) and FITC-conjugated goat anti-mouse IgG secondary antibody. G-means were calculated by the following formula: (G-means of antibody-treated cells) - (G-means of control cells). Correlation coefficients were obtained between the expression levels of CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, HSG, and the ID_{50} of OBP-301 in 7 SCCHN cell lines.

Quantitative Real-time PCR Analysis

Total RNA from cultured cells was obtained using the RNeasy Mini kit (Qiagen). Total RNA (~0.1 μg) was used for reverse transcription. Reverse transcription was done at 22°C for 10 min and then at 42°C for 20 min. The *hTERT* mRNA copy number was determined by real-time quantitative reverse transcription-PCR using a LightCycler instrument and a LightCycler DNA TeloTAGGG kit (Roche Diagnostics). PCR amplification was run with activation at 95°C for 15 s, annealing at 58°C for 10 s, and extension at 72°C for 9 s.

Athymic Nude Mouse Models of Human Head and Neck Cancer

Five- to 6-week-old female athymic nude mice (BALB/c *nu/nu*) were obtained from SLC. Animal care was in

Table 1. Cytotoxic activity of adenoviral receptors on head and neck cancer cell lines

Cell line	Origin	ID_{50} (vp)*
YCUT892	Tongue	759
KCCT873	Tongue	4,279
HN12	Lymph node	6,943
KCCT891	Hypopharynx	7,025
YCUM862	Oropharynx	7,512
KCCL871	Larynx	7,599
KCCOR891	Oral floor	9,204
YCUL891	Larynx	ND
YCUM911	Oropharynx	ND
YCUMS861	Maxillary sinus	ND
YCUT891	Tongue	ND
012SCC	Unknown	ND
Wmm-SCC	Unknown	ND

Abbreviation: ND, not done.
*ID_{50}, infection dose of OBP-301 at which 50% inhibition of cell viability is observed compared with untreated cells.

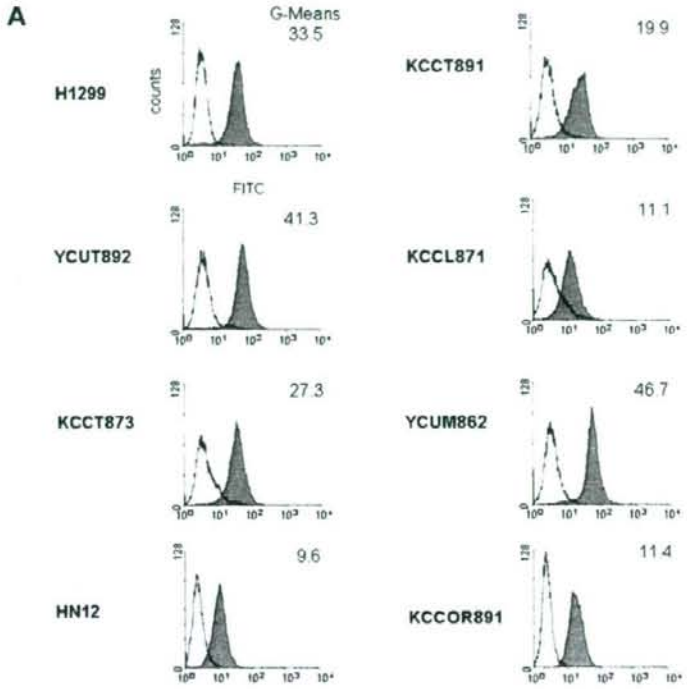

Figure 2. Expression of the CAR in SCCHN cell lines. **A,** cells were incubated with mouse monoclonal anti-CAR (RmcB) followed by detection with FITC-labeled secondary antibody. *Gray histogram,* staining with anti-CAR antibody treatment. H1299 human lung cancer cells were used as a positive control. **B,** correlation between CAR expression in SCCHN cells and the ID_{50} of OBP-301 for these cells. **C,** correlation between integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ and HSG expression in SCCHN cells and the ID_{50} of OBP-301 for these cells. The experiment was repeated three times.

C

Cell line	αVβ3	αVβ5	HSG
H1299	5.16	9.6	27.1
YCUT892	0.5	7.7	102.4
KCCT873	31.1	10.8	9.6
HN12	1.9	4.4	7.0
KCCT891	1.6	5.5	7.7
KCCL871	2.9	4.6	105.4
YCUM862	1.5	1.3	20.7
KCCOR891	2.0	8.8	156.5
correlation coefficient	**-0.2**	**-0.3**	**0.1**

accordance with the guidelines of the Kyoto University School of Medicine. A SCCHN model was established in nude mice by s.c. injection of KCCT873 tumor cells (5×10^6) in 150 μL PBS into the flank. Palpable tumors developed within 3 to 4 days. Tumors were measured by vernier calipers. Six to 7 mice were used for each group.

Toxicity Assessment

Blood samples and organs were collected from athymic nude mice at day 10 or 17 after i.t. administration of OBP-301 (3×10^{10} vp/d for days 5-9). Organs from the experimental animals were fixed in 10% formalin, and 5 μm tissue sections were prepared and stained with H&E.

Statistical Analysis

Tumor volume on a given day was calculated by the following formula: (length of the tumor) × (width of the tumor)^2 / 2. The statistical significance of tumor regression was calculated by the Student's *t* test.

Results

Cytotoxic Activity of OBP-301 to Various SCCHN Cell Lines

We first examined the effect of OBP-301 infection on the viability of SCCHN cell lines assessed by the XTT assay.

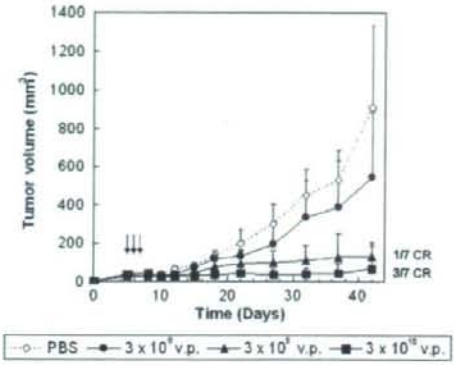

Figure 3. OBP-301 acted in a dose-dependent manner in KCCT873 tumor-bearing mice. Athymic nude mice received s.c. implantation of 5×10^6 KCCT873 cells on day 0. Animals then received injections of OBP-301 at the doses of 3×10^8 (●), 3×10^9 (▲), or 3×10^{10} vp (■) on days 5 to 7 (total of three injections). Each group had 7 animals, and the injection volume was 30 μL in each tumor. *Arrows*, day of injections. *Bars*, SD. The experiment was repeated two times.

Because OBP-301 showed slightly cytotoxic activity against 7 of 13 cell lines at a dose of 1,833 vp (50 plaque-forming units/cell), we assessed the ID_{50} of OBP-301 using these 7 SCCHN cell lines (Fig. 1; Table 1). As shown in Table 1, OBP-301 shows modest to strong cytotoxic activity in the 7 cell lines tested, with the ID_{50} varying from 759 to 9,204 vp. The cytotoxic activity of OBP-301 in these cell lines shows dose dependence (Fig. 1). YCUT892 cells were most sensitive to OBP-301 followed by KCCT873, HN12, KCCT891, YCUM862, KCCL871, and KCCOR871 cells, suggesting that 2 of the SCCHN cell lines are most sensitive to OBP-301.

Expression of Adenovirus Receptors in SCCHN Cell Lines

Because the cytotoxic activity of OBP-301 was anticipated to be correlated with efficiency of adenoviral infection through CAR, integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, or HSG receptors (22), we then assessed the expression levels of CAR on SCCHN cells using flow cytometry. As shown in Fig. 2A, all 7 SCCHN cell lines have been found to express detectable levels of CAR. However, as shown in Fig. 2B, correlation between the cytotoxic activity of OBP-301 and the expression level of CAR was not significant (correlation coefficient = −0.5). In addition, we assessed the expression of integrin $\alpha_V\beta_3$, integrin $\alpha_V\beta_5$, and HSG in SCCHN cell lines; however, the expression levels were not correlated with cytotoxic activity of OBP-301 (Fig. 2C). We also assessed *hTERT* expression using the quantitative PCR method and found that all of the SCCHN cell lines express detectable levels of *hTERT* mRNA; however, there was no correlation between the expression levels and ID_{50} of OBP-301 in these cells (data not shown). These results suggest that, although 7 of 13 SCCHN cell lines are sensitive to OBP-301, its ability to enter the cell is not necessarily correlated with the degree of cytotoxicity.

Antitumor Effect of OBP-301 in SCCHN-Bearing Animals

To assess the antitumor effect of OBP-301 in the animal model of human SCCHN, KCCT873 cells were implanted s.c. in athymic nude mice (6, 23) to examine the effect of OBP-301 at a variety of dosages *in vivo*. Mice received i.t. injections of OBP-301 at 3×10^8, 3×10^9, or 3×10^{10} vp for 3 days from days 5 to 7 after tumor implantation (Fig. 3). Tumors grew to mean tumor volume of 32.2 ± 4.5 mm^3 at day 5. As shown in Fig. 3, the 3×10^8 dose of OBP-301 treatment was less effective against KCCT873 tumor growth. The mean tumor volume was 549 mm^3 on day 42, which is comparable with control tumor volume (910 mm^3). Higher doses of OBP-301 led to superior antitumor activity. The mean tumor volume of treated tumors was 130 mm^3 at 3×10^9 vp and 67 mm^3 at 3×10^{10} vp, which is significantly smaller compared with the control tumor at day 42 ($P < 0.0001$). Remarkably, in addition to a 93% inhibition in tumor volume in mice receiving a 3×10^{10} vp dosage, 3 of 7 tumors completely disappeared by day 37, which persisted through day 42. These results suggest that OBP-301 shows a remarkable antitumor effect in a dose-dependent manner in KCCT873 SCCHN tumors. Based on these findings, OBP-301 at a dosage of 3×10^{10} vp per injection shows the maximum tumor reduction effect.

Optimization of OBP-301 Injection Times in KCCT873 SCCHN Tumors

We next evaluated the treatment schedule of OBP-301 in s.c. xenografted KCCT873 tumor-bearing mice. Mice were treated i.t. with OBP-301 for 1, 3, or 5 subsequent days. The

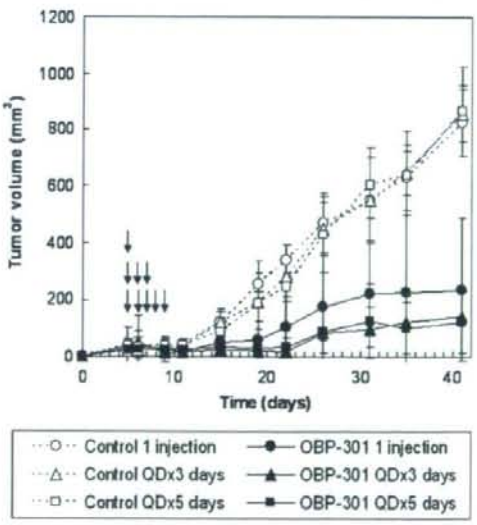

Figure 4. Regression of KCCT873 tumors by i.t. treatment of OBP-301. Athymic nude mice receiving s.c. KCCT873 implantation were treated with OBP-301 (3×10^{10} vp) for 1 (●), 3 (▲), or 5 (■) days. Injections were made on consecutive days (*QD*). Each group had 6 animals, and the injection volume was 30 μL in each tumor. *Arrows*, day of injections. *Bars*, SD. The experiment was repeated two times.