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

Yoshihiro Ikeda,* Toru Kojima,* Shinji Kuroda,* Yoshikatsu Endo,* Ryo Sakai,* Masayoshi Hioki,* Hiroyuki Kishimoto,* Futoshi Uno,* Shunsuke Kagawa,*† Yuichi Watanabe,‡ Yuuri Hashimoto,‡ Yasuo Urata,‡ Noriaki Tanaka,* and Toshiyoshi Fujiwara2*‡

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.

A ngiogenesis 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, thrombospordin 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
cell-mediated angiogenesis in vitro and in vivo. Antineoplastic ef-
fect of intratumoral administration of OBP-301 on s.c. murine co-
lon tumors transplanted was compared in syngenic immunocom-
petent 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
Foxn1nu/CrlCrlj), and SCID (CB17/Icr-Pkdcscid/CrlCrlj) mice, 5–6 wk
of age, were purchased from Charles River Japan Breeding Laborato-
ries. 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 transcrip-
tase 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 step gradient ultra-
centrifugation followed by CsCl2 linear gradient ultracentrifugation.

Cell viability assay

XTT assay was performed to measure cell viability. Briefly, cells were
plated on 96-well plates at 5 × 104 per well 24 h before treatment and then
infected with OBP-301 at a multiplicity of infection (MOI) of 10. After 72 h
infection, the supernatants of PBMC stimulated with oncolytic tumor cells preferentially secrete
PBMC in vitro.

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 10. As shown in Fig. 1A, OBP-
301 infection induced death of SW620 cells in a dose-dependent
manner. Next, we examined the ability of OBP-301-infected on-
colytic 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 se-
cretion (Fig. 1B). 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 superna-
tants 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 im-
paired by the addition of MLTC supernatants even at 1/4 dilu-
tion (Fig. 2). In contrast, although MLTC supernatants were
confirmed to contain ~250 pg/ml IFN-γ, 10-fold more concen-
tration of recombinant IFN-γ was needed to attenuate the tubule
formation close to basal levels. The supernatants of PBMC

In vivo tumor growth and determination of microvessels

Female BALB/c and SCID mice were s.c. implanted with 2 × 106
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 × 104 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 × b²/2, 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.
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 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 become thinner by supernatants of OBP-301-infected SW620 cells or PBMC, the number of curled microvessels, which is characteristic of tumor neovascularity, 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 d312 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.
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. 5A). 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 virus injection (Fig. 5A). 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.

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 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 $2 \times 10^6$ 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.

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 ($2 \times 10^5$ cells/each) were injected s.c. into the right flank of mice. OBP-301 ($1 \times 10^6$ 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.
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 ×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 cytolyis 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 (Advecin) could have antiangiogenic effects. The effects could be through down-regulation of angiogenic factor VEGF and up-regulation of antiangiogenic factor BA11 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/H9253, 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. 1B), 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 known 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 syngeneic 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 syngeneic 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 d312-injected tumors showed neither cellular infiltrates nor tissue damages (Fig. 5B). 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 d312-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. 5D), 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.
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Disclosures
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