Efficacy of a Cell-Cycle Decoying Killer Adenovirus on 3-D Gelfoam Histoculture and Tumor-Sphere Models of Chemo-Resistant Stomach Carcinomatosis Visualized by FUCCI Imaging

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Abstract

Stomach cancer carcinomatosis peritonitis (SCCP) is a recalcitrant disease. The goal of the present study was to establish an in vitro-in vivo-like imageable model of SCCP to develop cell-cycle-based therapeutics of SCCP. We established 3-D Gelfoam histoculture and tumor-sphere models of chemo-resistant stomach carcinomatosis visualized by FUCCI imaging. FUCCI-expressing MKN-45 stomach cancer cells were transferred to express the fluorescence ubiquinized cell-cycle indicator (FUCCI). FUCCI-expressing MKN-45 cells formed spheres on agarose or on Gelfoam grew into tumor-like structures with G\textsubscript{0}/G\textsubscript{1} cancer cells in the center and S/G\textsubscript{2} cancer cells located in the surface as indicated by FUCCI imaging when the cells fluoresced red or green, respectively. We treated FUCCI-expressing cancer cells forming SCCP tumors in Gelfoam histoculture with OBP-301, cisplatinum (CDDP), or paclitaxel. CDDP or paclitaxel killed only cycling cancer cells and were ineffective against G\textsubscript{1}/G\textsubscript{2} MKN-45 cells in tumors growing on Gelfoam. In contrast, the telomerase-dependent adenovirus OBP-301 decoyed the MKN-45 cells in tumors on Gelfoam to cycle from G\textsubscript{0}/G\textsubscript{1} phase to S/G\textsubscript{2} phase and reduced their viability. CDDP- or paclitaxel-treated MKN-45 tumors remained quiescent and did not change in size. In contrast, OB-301 reduced the size of the MKN-45 tumors on Gelfoam. We examined the cell cycle-related proteins using Western blotting. CDDP increased the expression of p53 and p21 indicating cell cycle arrest. In contrast, OB-301 decreased the expression of p53 and p21. Furthermore, OB-301 increased the expression of E2F and pAkt as further indication of cell cycle decoy. This 3-D Gelfoam histoculture and FUCCI imaging are powerful tools to discover effective therapy of SCCP such as OBP-301.
Introduction

Collagen sponge-gel histoculture was developed by Leighton [1] and enables cultured cells to form 3-dimensional structures [2].

As described in detail previously [2], osteosarcoma growing on Gelfoam®K, formed three-dimensional tissue-like structures, but did not form structures in monolayer culture and only colonies on Matrigel™ [2,3].

As also previously described in detail [3–11], Gelfoam®K can be used for tumor growth [4,5], to culture differentiating stem cells [6–8], to culture hair-producing hair follicles [9], to culture hair-growing skin [10] and to culture functional lymphoid tissue [11].

We recently observed that cancer cells in G0/G1 phase in Gelfoam®K histoculture had more extensive migration than in S/G2/M. Upon entry into S/G2/M, the cells ceased migrating. After mitosis, when the cells entered G0/G1, the cells could resume migrating. The migrating cells in G0/G1 were not sensitive to chemotherapy. Fluorescence ubiquination cell cycle indicator (Fucci) enabled monitoring of the cell cycle phase of each cell in real time as described in detail previously [12,13].

We previously showed with Fucci imaging that cancer cells in Gelfoam®K and in tumors in mice had a similar cell-cycle-phase distribution. Only the surface cells in Gelfoam®K histoculture and tumors was cycling; interior cells of tumors and Gelfoam®K histocultures were quiescent in G0/G1. In mono-layer culture, in contrast, cancer cells continuously cycle. In both Gelfoam®K histoculture and tumors in mice, chemotherapy had similar efficacy, in contrast to cancer cells in mono-layer, which were much more chemo-sensitive as described previously in detail [13–15].

In the present report, we determined the efficacy of cell-cycle decoying telomerase-dependent adenovirus OBP-301 on stomach cancer carcinoma growing on Gelfoam®K histoculture, using Fucci imaging to monitor cell-cycle changes.

Materials and Methods

Cells

MKN-45 cells (Cat. #JCRB 0254; Japanese Collection of Research Bioresources, Osaka, Japan), expressing Fucci, were cultured on plastic plates in RPMI-1640 medium to produce sufficient numbers of cells for Gelfoam®K histoculture (please see below) [12,14,16,17].

Gelfoam®K Histoculture

Gelfoam®K (Pharmacia & Upjohn, Kalamazoo, MI), was cut into 1 cm cubes and placed in 6-well tissue culture plates containing RPMI-1640 medium and placed in a 37°C incubator until the Gelfoam®K absorbed the medium. Fucci-expressing cancer cells (1×10⁶) were then carefully layered on top of the Gelfoam®K and placed in an incubator for 1 h, after which additional medium was added up to the top of the Gelfoam®K and then incubated again at 37°C with 5% CO2/95% air, as described previously in detail [2,4–6,12,18].

Establishment of Fucci-Expressing MKN-45 Cells

In order to establish Fucci-expressing MKN-45 cells, two plasmids were utilized: mKO2-hCdt1 containing an orange-red fluorescent protein and mAG-hGem, containint a green fluorescent protein (Medical and Biological Laboratory, Nagoya, Japan) [19] were sequentially transfected into MKN-45 cells with the use of Lipofectamine™ LTX (Invitrogen, Carlsbad, CA). After transfection with each plasmid, the cells were cultured for appropriate
Fig 1. OBP-301 decoys MKN-45 stomach cancer cells in G₁/G₀ to cycle in tumor spheres. A. Representative of FUCCI MKN-45 stomach cancer cells in vitro. B. Experimental setup for treatment of FUCCI-expressing MKN-45 cells in spheres with OBP-301, cisplatinum (CDDP), or paclitaxel. C. Representative images of control, OBP-301-, CDDP-, or paclitaxel-treated spheres. D. Bar graphs show the size of viable tumor spheres after each treatment. E. Histogram shows cell cycle phase of treated tumor spheres. Data are shown as means ± SD (n = 5). *P < 0.01.

doi:10.1371/journal.pone.0162991.g001
Efficacy of a Cell-Cycle Decoying Adenovirus on a 3D Histoculture Carcinomatosis Model

A

Gelfoam 3D culture (established tumor)

Sphere culture (floating aggregated cancer cells)

B

Bright field

FUCCI-Orange

FUCCI-Green

FUCCI

C

Bright field

FUCCI-Orange

FUCCI-Green

FUCCI

2500 μm

D

E

Bright field

FUCCI-Orange

FUCCI-Green

FUCCI

2500 μm

F

PLOS ONE | DOI:10.1371/journal.pone.0162991 September 27, 2016 4 / 9
periods of time and sorted for the fluorescence color corresponding to plasmid used as described previously in detail [12].

Imaging of FUCCI-Expressing MKN-45 Cells

The OV100 variable magnification fluorescence imaging system (Olympus) was used to acquire macro images as described previously in detail [20]. The FV1000 confocal laser scanning microscope (Olympus) was used to image single FUCCI-expressing cells. The FV1000 contains 473 nm and 559 nm lasers [21]. The 4× objective lens with a 0.20 numerical aperture and the 20× objective lens with a 0.95 numerical aperture were used. Fluoview software (Olympus) was used for image acquisition and Velocity 6.0 version (Perkin Elmer) [15] were used for 3D analysis. The above methods were previously described in detail [21].

Statistical Analysis

The Student’s t-test was used for comparison of 2 groups. Data are presented as means ± SD. Significance was determined via a P value of < 0.05 [12].

Results and Discussion

Cell-cycle Decoy and Killer Efficacy of Telomerase-Dependent Adenovirus OBP-301 in Comparison with Chemotherapy on Tumor Spheres

FUCCI-expressing MKN-45 stomach cancer cells cultured on agarose spontaneously formed spheres and were mostly FUCCI red, indicating they were in G₀/G₁ phase (Fig 1A). Tumor spheres were treated with OBP-301, cisplatinum (CDDP), or, paclitaxel (Fig 1B). CDDP- or paclitaxel-treated MKN-45 cells remained in the quiescent G₀/G₁ state and did not change size compared with control-spheres or spheres before treatment (Fig 1C–1E). In contrast, OBP-301 decoyed the cell cycle of tumor spheres from G₀/G₁ phase to S/G₂ phase where they expressed FUCCI green and reduced the size of the tumor (Fig 1C–1E). G₀/G₁ cancer cells in spheres were resistant to chemotherapy but could be decoyed to S/G₂ by OBP-301 which reduced their viability.

Cell-cycle Distribution of MKN-45 Stomach Cancer Cells Forming Tumors on Gelfoam®

FUCCI-expressing MKN-45 cells on Gelfoam® grew into tumor-like structures with the majority of the cells in G₀/G₁ (Fig 2) when the tumor-like structure matured. The mature tumors or spheres on Gelfoam® have the vast majority of their cells in G₀/G₁ (Figs 1E and 3E and 3F) and thereby look red or yellow. The images are of the whole tumor or sphere, not cross
Fig 3. Time-course imaging of decoy of quiescent MKN-45 cancer cells in tumor-like structures on Gelfoam® by OBP-301 and their subsequent killing. A. Experimental setup for treatment of MKN-45.
tumor-like structures growing in Gelfoam\textsuperscript{1} with OBP-301, CDDP, or paclitaxel. \textbf{B.} Time-course images of FUCCI-expressing MKN-45 cancer cells forming tumor-like structures on Gelfoam\textsuperscript{1} treated with OBP-301, CDDP, or paclitaxel. The cells in G\textsubscript{0}/G\textsubscript{1}, S, or G\textsubscript{2}/M phases appear red, yellow, or green fluorescent, respectively. \textbf{C.} Histogram shows the cell cycle phase of FUCCI-expressing MKN-45 cancer cells forming tumor-like structures on Gelfoam\textsuperscript{1} treated with OBP-301, CDDP, or paclitaxel. \textbf{D.} Representative images of control, OBP-301-, CDDP-, or paclitaxel-treated tumors. \textbf{E.} Bar graphs show the size of viable tumor after each treatment. \textbf{F.} Histogram shows cell-cycle phase of MKN-45 cells in treated and untreated spheres. Scale bars, 100 μm. Data are shown as means ± SD (n = 5). *P < 0.01. The percentage of cells in G\textsubscript{0}/G\textsubscript{1}, S, and G\textsubscript{2}/M phases are shown.

\hspace*{1cm} doi:10.1371/journal.pone.0162991.g003

sections. The yellow fluorescence is derived from the mixture of red (G\textsubscript{0}/G\textsubscript{1}) and green (cycling) cells on the surface of the sphere or tumor on Gelfoam\textsuperscript{1}.

**OBP-301 Decoyed Quiescent Cancer Cells in Tumors Growing on Gelfoam\textsuperscript{1} to Cycle and Reduced Their Viability**

Gelfoam\textsuperscript{1} histocultures of FUCCI-expressing MKN-45 cancer cells were treated with OBP-301, CDDP, or paclitaxel (Fig 3A). Only cycling MKN-45-FUCCI cells were sensitive to CDDP and paclitaxel; quiescent G\textsubscript{0}/G\textsubscript{1} MKN-45 cells were resistant to these drugs (Fig 3B). In contrast, OBP-301 decoyed the MKN-45 cells in the tumor-like structures on Gelfoam\textsuperscript{1} to cycle from G\textsubscript{0}/G\textsubscript{1} phase to S/G\textsubscript{2} phase and reduced their viability (Fig 3B and 3C). CDDP- or paclitaxel-treated MKN-45 tumor-like structure remained quiescent and did not change in size (Fig 3D and 3E). In contrast, OBP-301 reduced the size of the MKN-45 tumor-like structures on Gelfoam\textsuperscript{1} (Fig 3D–3F).

**OBP-301 Modulates Cell-Cycle Related Gene Expression**

We examined the cell cycle-related proteins using Western blotting. CDDP increased the expression of p53 and p21 indicating cell-cycle arrest. In contrast, OBP-301 decreased the expression of p53 and p21 (Fig 4). Furthermore, OBP-301 increased the expression of E2F and pAkt indicating in cell-cycle decoy (Fig 4).

\hspace*{1cm} doi:10.1371/journal.pone.0162991.g004

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**Fig 4.** \textbf{A.} Western blots show the expressions of p53, p21, E2F, and pAkt after OBP-301 or CDDP treatment. \textbf{B.} The scheme of cell cycle change.
The cell cycle decoy effect of OBP-301 is large scale, whereby the cells in untreated mature spheres or Gelfoam® tumor are almost all in G0/G1 and almost all of them cycle after OBP-301 infection. The sphere or tumor area is reduced by approximately one half after OBP-301 treatment which is not as great an effect as the extent of decoy. Future experiments will investigate the kinetics of cancer-cell killing after OBP-301 treatment.

Conclusions

SCCP growing as spheres or tumor-like structures on Gelfoam® cycle became mostly quiescent as they matured and were thereby resistant to chemotherapy. Telomerase-dependent adenovirus OBP-301 could decoy the quiescent tumor cells in spheres or tumor-like structures in Gelfoam® to begin to cycle whereby they lost viability. These results are further demonstration of the power of cell-decoy chemotherapy.

Acknowledgments

This paper is dedicated to the memory of A.R. Moossa, M.D.

Author Contributions

Conceptualization: SY RMH.
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Investigation: SY KT RMH.
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