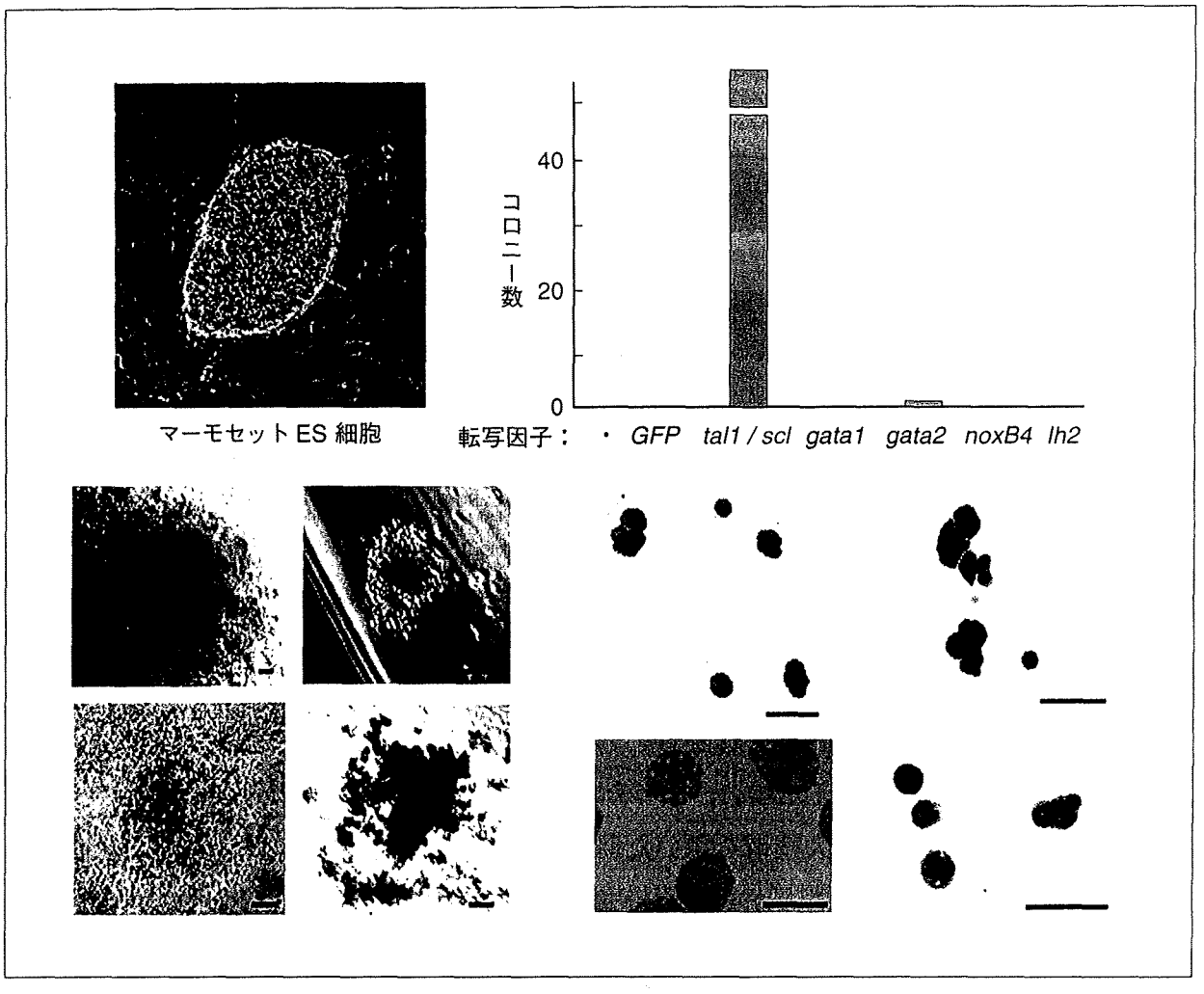
図6 *Tal1 / Scl* 遺伝子導入コモンマーモセット胚性幹（ES）細胞からの無ストローマ細胞系での高効率造血細胞分化誘導[43]



ってきていることは言うまでもない．このような観点から現在，我々は遺伝子改変などの方法を用いたコモンマーモセット疾患モデル作出研究を進めてきており，今後より理想的な実験動物としてのコモンマーモセットの意義が増すことを期待している．無論，実験動物倫理にも十分配慮した体制の中での研究の実施が必要であることは言うまでもない[44]．

謝　辞

本研究の推進に際しましては，日比野仁先生，杉山 肇先生，伊澤清子先生，佐々木えりか先生，栗田 良先生，谷岡功邦先生，野村達次先生，浅野茂隆先生，その他の共同研究者の先生方の多大なご支援をいただきました．

ここに深く感謝申し上げます．

文　　献

1) 谷岡功邦 編著: マーモセットの飼育繁殖·実験手技·解剖組織．アドスリー社，東京，1996.
2) Sasagawa A, et al: Absence of antibodies against SV5, measles and herpes simplex in indoor colony-bred cynomolgus monkeys. Jpn J Med Sci Biol 34: 381, 1981.
3) Merker H J, et al: Embyotoxic effects of thalidomide derivatives on the non-human primate Callithrix jacchus. 1. effects of 3-(1, 3-dihydro-1-oxa-2H-isoindol-2-yl)-2, 6-dioxopiperidine (EM12). Arch Toxicol 61: 165-179, 1988.
4) Neubert R, et al: Down-regulation of adhesion receptors on cells of primate embryos as a probable mechanism of the teratogenic action of thalidomide. Life Sci 58: 295-316, 19.
5) Yamaguchi A, et al: Bone in the marmoset: A resemblance to vitamin D-dependent rickets, type II. Calcif Tissue Int 39: 22-27, 1986.
6) Moro M, et al: New hyperprolactinemia and anovulation model in common marmoset (Callithrix jacchus) and effect of cabergoline. Eur J Pharmacol 368: 57-66, 1999.
7) 日比野仁，他: 医学研究へのマーモセット利用の試み－疾患モデルと遺伝子治療に向けての研究展開－．アニテックス 11: 303-309, 1999.
8) 't Hart B A, et al: A new primate model for multiple sclerosis in the common marmoset. Immunol Today 21: 290-297, 2000.
9) McFarland H L, et al: Effective antigen-specific immunotherapy in the marmoset model of multiple sclerosis. J Immunol 166: 2116-2121, 2001.
10) Mancardi G, et al: Demyelination and axonal damage in a nonhuman primate model of multiple sclerosis. J Neurol Sci 184: 41-49, 2001.
11) Fukuoka T, et al: The common marmoset (Callighrix jacchus) as a model for neuroleptic induced acute dystonia. Pharmacol Biochem Behav 58: 947-953, 1997.
12) Marshall J W B, et al: Clomethiazole protects against hemineglect in a primate model of stroke. Brain Res Bull 52: 21-29, 2000.
13) Maclean C J, et al: Naturally occurring and experimentally induced b-amyloid deposits in the brains of marmoset (Callithrix jacchus). J Neural Transm 107: 799-814, 2000.
14) Baker H F, et al: Experimental induction of beta-amyloid plaques and cerebral angiopathy in primates. Ann N Y Acad Sci 695: 228-231, 1993.
15) Geula C, et al: Amyloid-b deposits in the cerebral cortex of the aged common marmoset (Callithrix jacchus): incidence and chemical composition. Acta Neuropathol 103: 48-58, 2002.
16) Gnanalingham K K, et al: Alterations in striatal and extrastriatal D-1 and

D-2 dopamine receptors in the MPTP-treated common marmoset: an autoradiographic study. Synapse 14: 184-194, 1993.

17) Roeling T A, et al: Effects of unilateral 6-hydroxydopamine lesions on neuropeptide immunoreactivity in the basal ganglia of the common marmoset, Callithrix jacchus, a quantitative immunohistochemical analysis. J Chem Neuroanat 9: 155-164, 1995.

18) Maratos E C, et al: Both short-and long-acting D-1/D-2 dopamine agonist induce less dyskinesia than L-dopa in the MPTP-lesioned common marmoset (Callithrix jacchus). Exp Neurol 179: 90-102, 2003.

19) Kendall A L, et al: Functional integration of striatal allografts in a primate model of Huntington's disease. Nat Med 4: 727-729, 1998.

20) Hohjoh H, et al: Molecular cloning and characterization of the common marmoset huntingtin gene. Gene 432: 60-66, 2009.

21) Iwanami A, et al: Establishment of graded spinal cord injury model in a nonhuman primate: The common marmoset. J Neurosci Res 80: 172-181, 2005.

22) Iwanami A, et al: Transplantation of human neural stem cells for spinal cord injury in primates. J Neurosci Res 80: 182-190, 2005.

23) Johannessen I: Crawford DN. In vivo models for Epstein-Barr virus, (EBV)-associated B cell lymphoproliferative disease (BLPD). Rev Med Virol 9: 263-277, 1999.

24) Cox C, et al: Persistent Epstein-Barr virus infection in the common marmoset (Callithrix jacchus). J Gen Virol 77: 1173-1180, 1996.

25) Dhurandhar N V, et al: Human adenovirus Ad-36 promotes weight gain in male rhesus and marmoset monkeys. J Nutr 132: 3155-3160, 2002.

26) Ablashi D V, et al: Experimental infection of Callithrix Jacchus marmosets with Herpesvirus ateles, Herpesvirus saimiri, and Epstein Barr virus. Biomedicine 29: 7-10, 1978.

27) Wright J, et al: Susceptibility of common marmosets (Callithrix jacchus) to oncogenic and attenuated strains of Herpesvirus saimiri. J Natl Cancer Inst 59: 1475-1478, 1977.

28) Pinto M A, et al: Experimental hepatitis A virus (HAV) infection in Callithrix jacchus: early detection of HAV antigen and viral fate. Exp Toxicol Pathol 53: 413-420, 2002.

29) Kobune F, et al: Nonhuman primate models of measles. Lab Anim Sci 46: 315-320, 1996.

30) Avila M M, et al: Protection of Junin virus-infected marmosets by passive administration of immune serum: association with late neurologic signs. J Med Virol 21: 67-74, 1987.

31) Denham D A, et al: Experimental Brugia pahangi and B. malayi infections of callitrichid primates. J Helminthol 63: 84-86, 1989.

32) Mitchell G H, et al: Plasmodium vivax malaria in the common marmoset, Callithrix jacchus: adaptation and host response to infection. Parasitology

33) Lever M S, et al: Experimental respiratory anthrax infection in the common marmoset (Callithrix jacchus). Int J Exp Pathol 89: 171–179, 2008.

34) Adams A P, et al: Common marmosets (Callithrix jacchus) as a nonhuman primate model to assess the virulence of eastern equine encephalitis virus strains. J Virol 82: 9035–9042, 2008.

35) Baker H F, et al: Prion protein immunohistochemical staining in the brains of monkeys with transmissible spongiform encephalopathy. Neuropathol Appl Neurobiol 24: 476–486, 1998.

36) 小泉 均, 他: コモンマーモセットの臓器移植への応用性に関する検討. 今日の移植 11: 233, 1998.

37) Hibino H, et al: Common Marmoset as a Target Preclinical Primate for Cytokine and Gene Therapy Studies. Blood 93: 2839–2848, 1999.

38) Hibino H, et al: Haematopoietic progenitor cells from common marmoset as targets of gene transduction by retroviral and adenoviral vectors. Eur J Haematol 66: 272–280, 2001.

39) Izawa K, et al: Hematopoietic Activity of Common Marmoset CD34 Cells Isolated by a Novel Monoclonal Antibody MA24. Exp Hematol 32: 843–851, 2004.

40) Ito R, et al: Novel monoclonal antibodies recognizing different subsets of lymphocytes from the common marmoset (Callithrix jacchus). Immunol Lett 121: 116–122, 2008.

41) Wu M S, et al: MHC (Major histocompatibility Complex)-DRB genes and polymorphisms in common marmoset. J Mol Evol 51: 214–222, 2000.

42) Sasaki E, et al: Establishment of Novel Embryonic Stem Cell Lines Derived from the Common Marmoset (Callithrix jacchus). Stem Cells 23: 1304–1313, 2005.

43) Kurita R, et al: Tal1/scl gene transduction using a lentiviral vector stimulates highly efficient hematopoietic cell differentiation from common marmoset (Callithrix jacchus) ES cells. Stem Cells 24: 2014–2022, 2006.

44) Viatale A, et al: Environmental enrichment techniques in non-human primates. The case of Callitrichids. Ann Ist Super Sanita 40: 181–186, 2004.

Common Marmoset as Excellent Preclinical Research Animal for Stem Cell Research

Kenzaburo Tani[1,2]

[1] Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University

[2] Department of Advanced Molecular and Cell Therapy, Kyushu University Hospital

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com

BIOMEDICINE & PHARMACOTHERAPY

ELSEVIER MASSON

Biomedicine & Pharmacotherapy 63 (2009) 275–286

Original article

Tumor growth suppression by adenovirus-mediated introduction of a cell growth suppressing gene *tob* in a pancreatic cancer model

Hironobu Yanagie [a,b,*], Tuyoshi Tanabe [c], Hidetoshi Sumimoto [d], Hirotaka Sugiyama [b], Satoru Matsuda [e], Yasumasa Nonaka [f], Naoko Ogiwara [g], Katsunori Sasaki [g], Kensaburo Tani [h], Shinichi Takamoto [b,i], Hiroyuki Takahashi [a,b], Masazumi Eriguchi [b,j]

[a] *Department of Cardiac Surgery, Cooperative Unit of Medicine and Engineering Research, The University of Tokyo Hospital, Tokyo 113-8655, Japan*
[b] *Department of Nuclear Engineering and Management, Graduate School of Engineering, The University of Tokyo, Tokyo 113-8655, Japan*
[c] *National Institute of Advanced Industrial Science and Technology, Ibaragi 305-8562, Japan*
[d] *Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo 160-8582, Japan*
[e] *Department of Molecular Pathogenesis, Nagoya University School of Medicine, Aichi 466-8560, Japan*
[f] *Department of Surgery, Hoyo Hospital, Iwate 028-3111, Japan*
[g] *Department of Anatomy, Shinshu University School of Medicine, Nagano 390-8621, Japan*
[h] *Medical Institute of Bioregulation, Kyusyu University, Fukuoka 874-0838, Japan*
[i] *Department of Cardiac Surgery, The University of Tokyo Hospital, Tokyo 113-8655, Japan*
[j] *Department of Microbiology, Syowa University School of Pharmaceutical Sciences, Tokyo 142-8555, Japan*

Received 18 April 2008; accepted 29 April 2008
Available online 18 June 2008

Abstract

TOB (transducer of ErbB-2) is a tumor suppressor that interacts with protein-tyrosine kinase receptors, including ErbB-2. Introduction of the *tob* gene into NIH3T3 cells results in cell growth suppression. In this study, we evaluated the effect of *tob* expression in pancreatic cell lines (AsPC-1, BxPC-3, SOJ) and discuss the tumor-suppressing effects of adenoviral vector expressing *tob* cDNA. We first measured the levels of endogenous *tob* mRNA being expressed in all pancreatic cancer cell lines. Then, we examined the effect of adenoviral vector containing *tob* cDNA (Ad-*tob* vector) on cancer cell lines. The viral vector was expanded with transfection in 293 cells. The titer of the vector was 350×10^6 pfu/ml. These cancer cells were able to be transfected with MOI 20 without adenoviral toxicity. The transfection of Ad-*tob* vector results in growth suppression of SOJ and AsPC-1 cell lines. The magnitude of the expression of the Ad-*tob* gene in cancer is correlated to tumor suppressive activity. We prepared pancreatic cancer peritonitis models using a peritoneal injection of AsPC-1 cells. In this model, bloody ascites and multiple tumor nodules were seen at the mesentery after 16 days. AdCA*tob* (50×10^6 pfu/day) was administered from day 5 to day 9 after 4 days of peritoneal injection of 2×10^6 AsPC-1 cells. Tumor growth suppression occurred 10 days after peritoneal injection of AdCA*tob* compared with the control group. There were no tumor nodules in the abdomen and no bloody ascites. These results suggest that the peritoneal injection of AdCA*tob* has potential to suppress the formation of pancreatic cancer peritonitis, and can be applied for chemotherapy-resistant cancer peritonitis.
© 2008 Elsevier Masson SAS. All rights reserved.

Keywords: *Tob* gene; Tumor suppressor gene; Adenovirus vector; Gene therapy

1. Introduction

Pancreatic cancer is one of the leading causes of cancer deaths in the world. Diagnosis of pancreatic cancer is difficult,

* Corresponding author. Department of Cardiac Surgery, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 9194; fax: +81 3 5800 9195.
E-mail addresses: yanagie@n.t.u-tokyo.ac.jp, h.yanagie@gmail.com (H. Yanagie).

0753-3322/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biopha.2008.04.010

and once metastasis to the liver or peritoneal dissemination has occurred, current treatments, including surgery and chemotherapy, are difficult to induce complete remission [1].

Advances in science and technology for direct gene transfer into living animals have provided opportunities to develop treatment modalities of malignancies by somatic gene therapy [2–5].

TOB (transducer of ErbB-2) is a 45 kDa tumor suppressor that interacts with protein-tyrosine kinase receptors, including ErbB-2 [6,7]. ErbB-2 phosphorylates and interacts with Shc, which participates between active tyrosine protein kinases to the Ras signaling pathway. A point mutation or an elevated expression of ErbB-2 is commonly observed in pancreatic cancers and breast cancers. Matsuda et al. reported that the carboxy-terminal half of TOB is relevant to its interaction with ErbB-2 and the amino-terminal half is homologous to the growth suppressor protein BTG-1, and introduction of the *tob* gene into NIH3T3 cells results in cell growth suppression [6,8–10]. Expression of BTG-1 is high in quiescent cells and decreases when cells enter the growth cycle, suggesting that the gene product is inhibitory to G0/G1 progression. The *tob* is localized on chromosome 17q21, telomeric to the BRCA 1 locus.

Using the anti-proliferative function of TOB, here we evaluated *tob* expression in pancreatic cancer cell lines and discussed its potential as a useful candidate for genetic therapy of pancreatic cancer peritonitis with peritoneal (ip) injection of recombinant adenovirus vector containing the *tob* gene (AdCA*tob*) *in vitro* and *in vivo*.

2. Materials and methods

2.1. Target tumor cells, mice and antibodies

The human pancreatic carcinoma cell line SOJ and AsPC-1 producing carcinoembryonic antigen (CEA), were maintained in RPMI1640 medium (Hazleton Biologics, Inc., Kansas, USA) supplemented with 10% fetal calf serum (Cell Culture Laboratories, Ohio, USA) and 100 $\mu g\,ml^{-1}$ kanamycin. All cultures were incubated in high moisture air with 5% CO_2 at 37 °C. The medium was changed three times a week.

Male BALB/*cnu/nu* mice were obtained from Nihon SLC (Shizuoka, Japan) and used at 6–7 weeks of age. In each experiment, mice of similar age and weight were selected. Mice were housed in plastic cages and maintained in an air-conditioned room. The procedures for tumor implantation and sacrifice of the animals were in accordance with approved guidelines of the Institution's Animal Ethics Committee.

Mouse anti-human TOB monoclonal antibody (IgG 2a), 4B1, was obtained from Immuno-Biological Laboratories (Gunma, Japan).

2.2. Construction of plasmid

Expression plasmid pMIK-*tob* was constructed by inserting the 1.3 kbp *tob* cDNA fragment into pMIK vector (a derivative of pME18S, kindly provided by Dr. K. Maruyama, DNAX Res. Inst., CA, USA) [6].

2.3. Northern blot analysis

Total RNA of cancer cells was extracted by the guanidium isothiocyanate method. RNA samples (10 μg) were separated and blotted following the general protocol. One kbp *Hind* III fragment of λ *tob* cDNA was used as a probe labeled with α-^{32}P-dCTP [6].

2.4. Recombinant adenovirus preparation

Adenovirus vector containing the *tob* driven by CAG promoter (AdCA*tob*) was prepared in this study following the method described previously [11–13]. Briefly, the 1.2 kb human *tob* fragment was blunt ended and subcloned into downstream of the CAG promoter of adenovirus vector. This expression cassette was subcloned into the *Swa*I site of the pAdex1cw cosmid, resulting in pAdex1*tob*. The pAdex1cw is a 42 kb cosmid containing a 31 kb adenovirus type 5 genome lacking *E1A*, *E1B*, and *E3* genes, as described previously. The expression cosmid cassette and adenovirus DNA-terminal protein complex were cotransfected into 293 cells by calcium phosphate precipitation. The recombinant viruses were propagated with 293 cells and viral solution was stored at −80 °C. The titers of viral stocks were determined by plaque assay on 293 cells. Adenovirus containing the *lacZ* gene coding for the bacterial enzyme β-galactosidase (AdCA*lacZ*) was used as a control to measure the efficiency of tumor cell infection.

2.5. Adenovirus-mediated lac Z expression in vitro

The pancreatic cancer cell lines were plated at a density of 50×10^3 cell/well in 24-well culture plates (Iwaki Glass, Tokyo, Japan) 12 h before AdCA*lacZ* infection. Then, culture medium was replaced with medium containing varying amounts of adenovirus per cell (MOI). After 48 h, the cells were stained with X-gal (Wako Ltd., Tokyo, Japan) and the number of β-galactosidase-positive cells was counted in order to demonstrate the transfection efficiency [13].

2.6. Cell growth assay

Human pancreatic cancer cell lines (50×10^3 cell) were cultured in 60 mm tissue culture dishes (Corning Glass Works, NY, U.S.A.) for 12 h. Then, the culture medium was replaced with suspensions of AdCA*lacZ* or AdCA*tob* at an MOI of 20. After transfection, the medium was changed every other day. Cell growth was assessed by counting the number of live cells on the indicated day after transfection. The results are the means ± SD from three independent experiments.

2.7. Protein immunoblotting

Six days after transfection of AdCA*lacZ* or AdCA*tob* into the pancreatic cancer cell lines, total protein was isolated by

lysis in 0.5 ml 1% NP-40 (Sigma). The lysates (100 μg protein) were electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose (Nytran, Schleicher & Schuell, Keene, NH) [14]. Western analysis was performed with the anti-TOB monoclonal antibody using a second antibody conjugated to peroxidase. The preparations were visualized with diaminobenzidine.

2.8. In vivo tumor experiments

2.8.1. Establishment of tumors in nude mice

We prepared pancreatic cancer peritonitis models using intraperitoneal (ip) injection of AsPC-1 pancreatic cancer cells. AsPC-1 cells were trypsinized, washed once with RPMI1640 and suspended in RPMI1640 at 1, 2, 5 or 10×10^6 cells/0.2 ml; a 0.2 ml cell suspension was injected into each nude mouse peritoneally.

2.8.2. Inhibition of tumor growth in vivo

In order to examine the tumor suppressor effect of the *tob* on the formation of pancreatic cancer peritonitis, ip injections of AdCA*tob* or AdCA*lac*Z were performed in tumor-bearing mice of a cancer peritonitis model. Peritoneal injection of AdCA*tob* or AdCA*lac*Z (50×10^6 pfu/0.2 ml/day, from day 5 to day 9) after 4 days of ip injection of AsPC-1 cells (2×10^6, doubling time 2–3 days). After 16 days of tumor inoculation, the mice were sacrificed and tumor formations investigated in the abdominal cavity.

2.8.3. Pathological evaluation of tumors in nude mice

At 16 days of follow-up, tumors from AdCA*tob*-, AdCA*lac*Z-treated groups and the non-treated control group were evaluated for differences between the control and the experimental groups by analyzing the anti-TOB immunostained sections of each tumor. Formalin-fixed paraffin-embedded *in vivo* experimental tissues were cut at 4–5 μm, dried at 60 °C, deparaffinized, and hydrated with distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in PBS, followed by rinsing in several changes of distilled water and PBS. Immunochemical studies were performed using the avidin–biotin–peroxidase complex method of Hsu et al. in the following manner: sections were blocked with normal rabbit serum for 30 min at room temperature and incubated with mouse anti-human TOB monoclonal antibody (clone 4B1, 1:100 dilution) for 30 min at room temperature. HISTOFINE SAB-PO (M) kit (Nichirei Co. Ltd., Tokyo, Japan) was used to apply biotinylated anti-mouse IgG/IgA/IgM and avidin–biotin–peroxidase complexes, incubating for 10 min at room temperature. The immunoperoxidase staining reaction was visualized by using 0.5% dimethyl-aminoazobenzene in 0.61 M Tris buffer (pH 7.4) containing 0.03% hydrogen peroxidase.

3. Results

3.1. Northern blot analysis of endogenous tob transcripts in pancreatic cancer cell lines

The endogenous *tob* mRNA was expressed in the three pancreatic cancer cell lines (Fig. 1). The level of *tob* mRNA of SOJ cells was low, but that of AsPC-1 cells was high (A). The overexpression of *c-erbB-2* mRNA was shown in BxPC-3 and AsPC-1 cells (B).

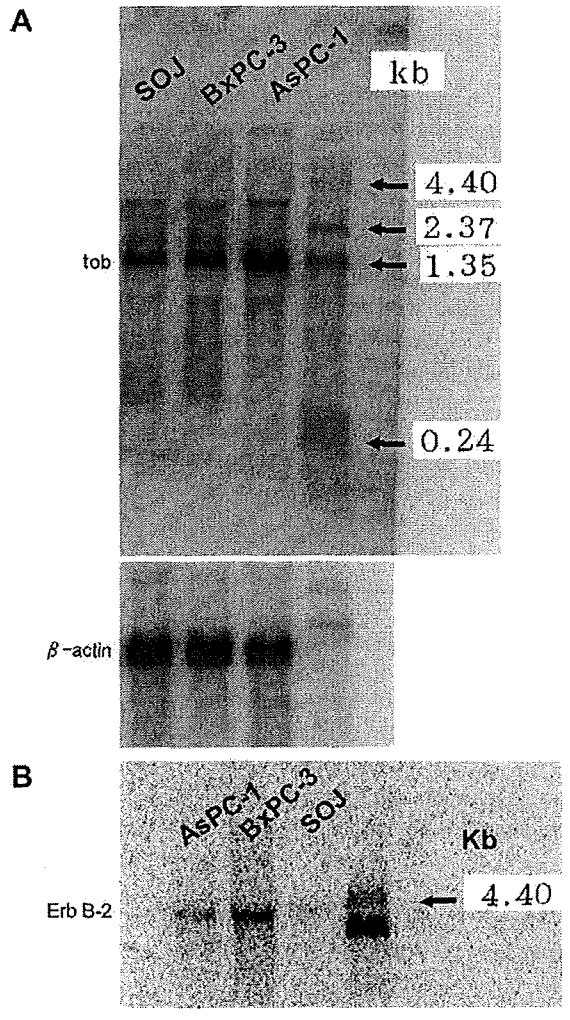


Fig. 1. Expressions of *tob*, *β-actin* (A) and *erbB-2* (B) mRNA in pancreatic cancer cell lines. Three human pancreatic cancer cell lines, AsPC-1, BxPC-3, and SOJ, were used in this experiment. (A) *tob* mRNAs are shown as a 1.3 kb band in the left side (upper panel of A). Total RNA was extracted and processed for Northern blot analysis as described in Section 2. A 10 μg of RNA were loaded in each lane. Standard molecular weights are shown on the right side as arrows. Internal control of the expression level of mRNA is shown as *β-actin* mRNA. (B) Expression level of *erbB-2* mRNAs is also compared among pancreatic cancer cell lines. Four kb bands corresponding to *erbB-2* mRNA are indicated by an arrow.

3.2. Adenovirus-directed lacZ gene transfer in pancreatic cancer cell lines in vitro

Use of a recombinant β-gal adenovirus AdCA*lacZ* allowed us to establish the measures of gene transfer efficiency in pancreatic cancer cells. Three human pancreatic cancer cell lines, AsPC-1, BxPC-3, and SOJ were used as target cells for the vector. These cell lines were transfected with AdCA*lacZ* at different MOIs (Fig. 2). The *lacZ* was expressed in all cell lines, in parallel with the increase in MOI. There was no significant difference in transfection efficacy among the cell lines, indicating that cell lines are susceptible to adenovirus transfection.

3.3. Tumor suppression by transfection of AdCAtob

In order to examine the tumor suppressor effect of the *tob* gene on the growth of cancer cells, the cells were transfected with AdCA*lacZ* or AdCA*tob* at an MOI of 20. The cells could be transfected with MOI 20 without adenoviral virulence. SOJ and AsPC-1 cell lines transfected with AdCA*tob* vector showed growth suppression (Fig. 3A). SOJ and AsPC-1 cells expressed exogenous *tob* mRNA, and were enlarged and megakaryocytic with many granules in the cytoplasm (Fig. 3B). The characteristics resembled those of senescent cells. Anti-proliferative activity of TOB seems to be well correlated with the level of its expression. In comparison with AdCA*lacZ*, the two-fold growth suppression was shown in the SOJ cell line expressing a good amount of TOB (Figs. 3A and 4).

3.4. Exogenous tob expression

TOB expression was analyzed with Western blot analysis of lysates of pancreatic cancer cell lines after transfection of AdCA*lacZ* or AdCA*tob*. As shown in Fig. 4, an exogenous 45 kDa band was detected in all transfected cell lines. Tob was successfully introduced, especially into SOJ cells, and the magnitude of the expression of TOB is correlated to tumor growth suppressive activity, as shown in Fig. 3A.

3.5. Pathological findings of tumors treated with AdCAtob vector: adenovirus-mediated tob expression in vitro

Expression of exogenous TOB (45 kDa) was confirmed by immuno-cytostaining of cells. AsPC-1 cells were plated at a density of 50×10^3 cells/well in a 24-well culture plate 12 h before AdCA*tob* infection. Then, cells were transfected with AdCA*tob* vector (MOI 20). After 72 h, cells were spinned down and stained with anti-TOB monoclonal antibody 4B1 in order to determine the expression of TOB. AsPC-1 cells showed the expression of TOB with AdCA*tob* transfection (Fig. 5).

3.6. Electronmicroscopic findings

We noted a dramatic change in the light scatter pattern of AsPC-1 cells upon induction of *tob* expression. Using transmission electron microscopy, we found that TOB-overexpressing AsPC-1 cells showed degradation of the nucleus and many autophagosomes and electron-dense cytoplasmic inclusions (Fig. 6A). The contents of these electron-dense cytoplasmic vesicles consisted of lamellar material that resembled lipofuscin, a lipid substance with auto-fluorescence properties that has been shown to accumulate with aging in the lysosomes of all vertebrates. Neither the increase inside scatter nor the appearance of lipofuscin granules was seen in AsPC-1 cells transfected with or without AdCA *mock* vector (Fig. 6B, C). Cytotoxic changes in TOB-induced AsPC-1 cells showed the degradation of autophagy.

3.7. Anti-tumorigenic effects of AdCAtob vector on AsPC-1 cell-derived tumors in nude mice: morphological findings of tumors treated with AdCAtob vector

Pancreatic cancer peritonitis model was established in nude mice using ip injection of AsPC-1 cells. Four days after the ip injection of AsPC-1 cells, the mice were ip-injected with AdCA*tob* (50×10^6 or 150×10^6 pfu/0.5 ml/day, from day 5 to day 9).

Bloody ascites and multiple tumor nodules were seen at the mesothelium after 16 days of ip injection of 2, 5 and 10×10^6 AsPC-1 cells. We designed our initial experiments to determine whether *in vivo* AdCA*tob*-mediated gene transfer would affect the formation of pancreatic cancer peritonitis after implantation of cancer cells into the abdominal cavity. Peritoneal injection of AdCA*tob* suppressed tumor nodule formation in the abdominal cavity compared with the non-treated group (Fig. 7). Several tumor nodule formations were observed in AdCA*lacZ*-treated mice. Bloody ascites was not seen in either AdCA*tob or* AdCA*lacZ*-treated mice (Table 1).

3.8. Pathological findings of tumor treated with AdCAtob vector: adenovirus-mediated tob expression in vivo

Only one tumor nodule was recognized in the abdominal cavity of AdCA*tob* (50×10^6 pfu)-treated groups, and not found in that of 150×10^6 pfu groups. The tumor continued to express TOB with staining by anti-TOB monoclonal antibody, 4B1 (Fig. 8A). On the other hand, the jejunum, mesothelium, acinal gland and pancreatic islets of Langerhans did not show the expression of exogenous TOB with AdCA*tob* transfection (Fig. 8B). AsPC-1 cells expressing exogenous *tob* mRNA were enlarged and megakaryocytic with characteristics resembling those of senescent cells. These results suggest that our adenovirus-mediated *tob* gene therapy was applicable for the specific and efficient treatment of pancreatic cancer peritonitis.

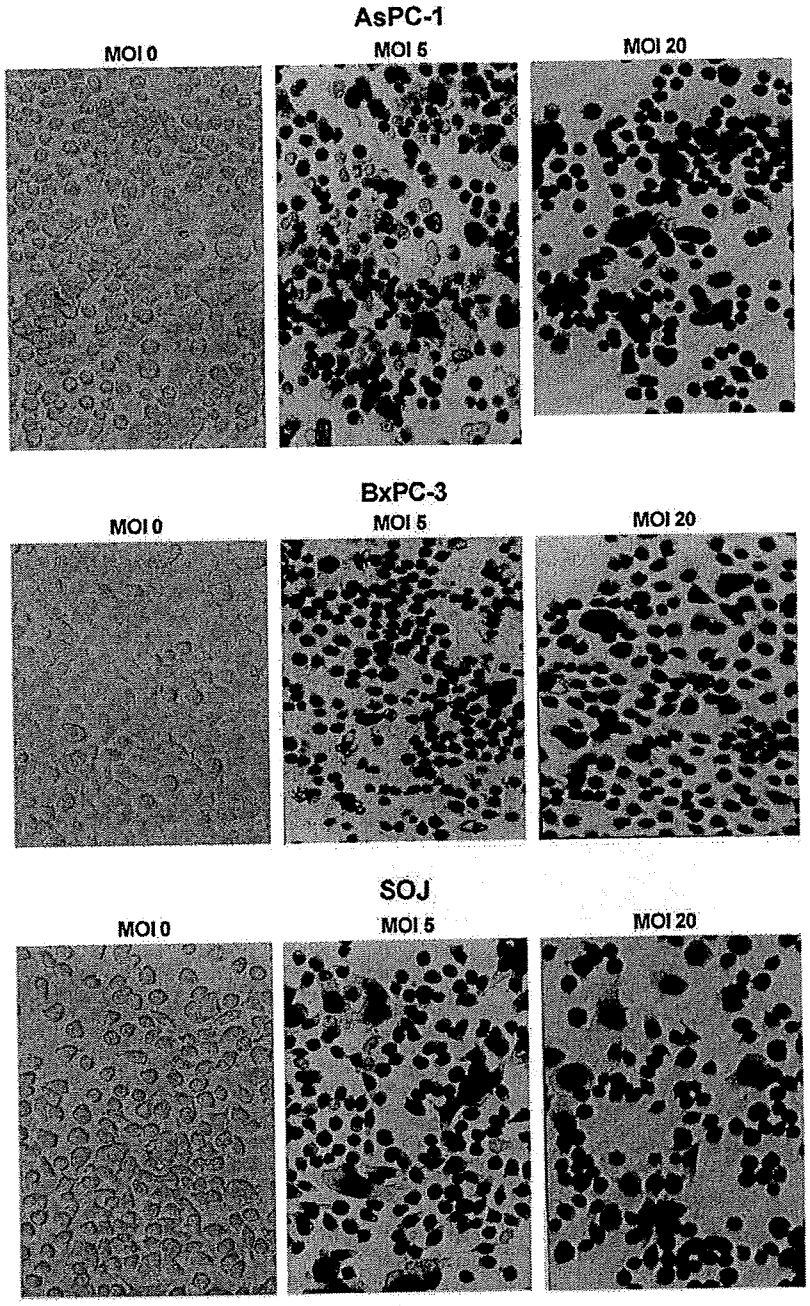


Fig. 2. Transduction efficiency of adenovirus in cancer cells *in vitro*. Optimal MOI for adenovirus-mediated gene transduction without virulence was determined in pancreatic cancer cells with control viral vector AdCA*lacZ*. AsPC-1, BxPC-3 and SOJ cells were plated on 24-well plates and transfected with AdCA*lacZ* at MOI of 0, 5, and 20. Forty-eight hours later, cells were fixed and stained with x-gal to demonstrate *lacZ* gene expression. The magnification of all photographs is ×400. No stained cells were detected at MOI 0 in the left panels, and increased transduction of *lacZ* in the right two panels of each cell line.

4. Discussion

The development of gene transfer technologies has provided new possibilities for the treatment of malignancies. Adenovirus vector systems that can produce high titers of viruses capable of efficient expression in target cells can deliver exogenous genes into a variety of cells and tissues.

Matsuda et al. reported that 185 kDa protein immunoreactive to anti-erbB-2 antibodies was detected in TOB immunoprecipitates, and a 45 kDa protein reactive to the anti-TOB antibodies was co-immunoprecipitated with $p185^{erbB-2}$, reciprocally [6,7]. TOB physically interacts with the *c-erbB-2* gene product. Exogenously expressed TOB is able to suppress the growth of NIH3T3 cells, and delivers growth inhibitory signals. These findings raise the possibility that the *tob* is a tumor suppressor gene [6,15]. Inspection of the deduced amino acid sequence of TOB revealed significant homologies (40.6%) to the BTG-1 anti-proliferative gene

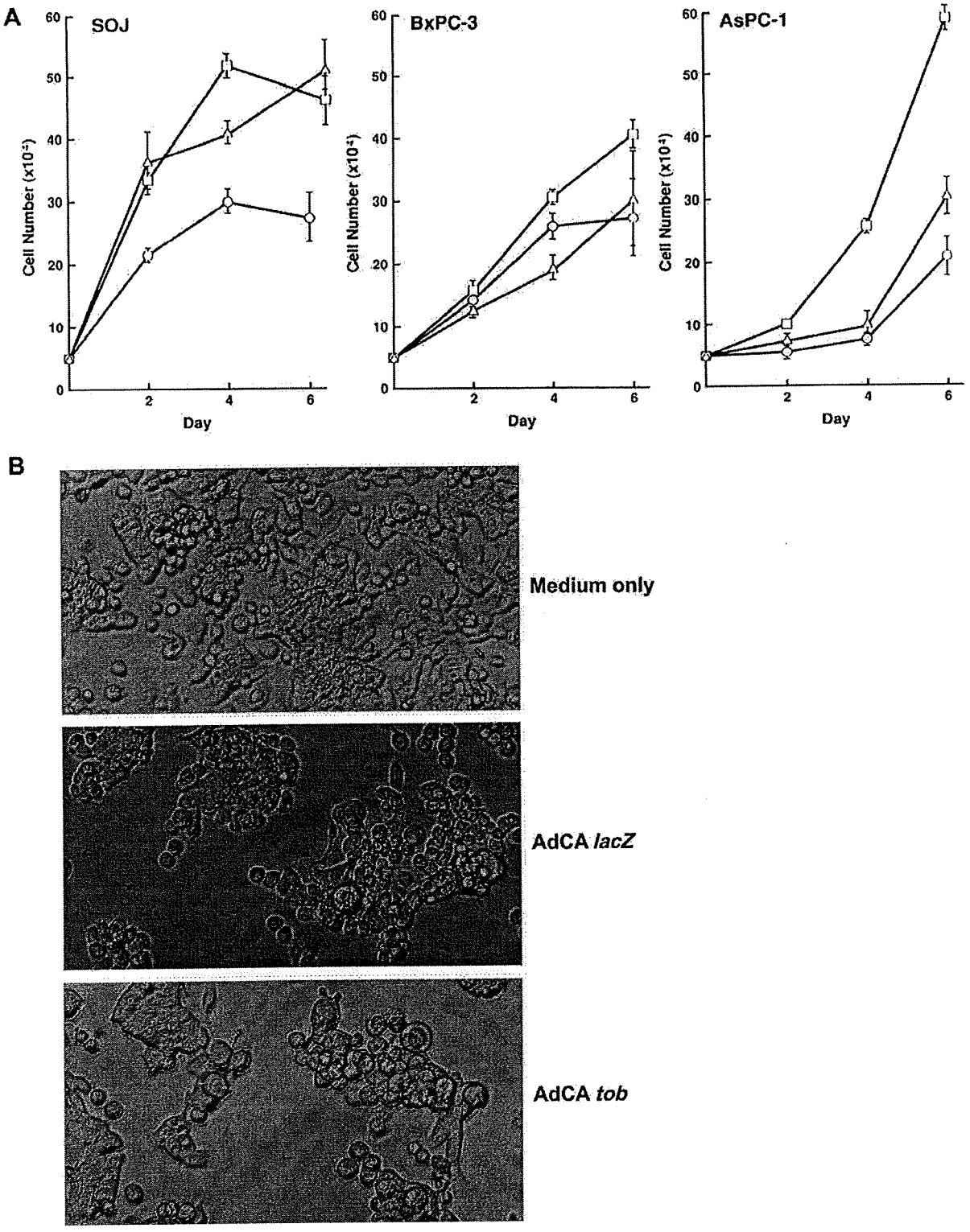


Fig. 3. (A) Tumor suppressor effect of the *tob* gene AsPC-1, BxPC-3, and SOJ cell lines were transfected with AdCA*tob* (○) or AdCA*lacZ* (△) at an MOI of 20. Medium without adenovirus vector (□) is also shown as medium only. These cancer cells could be transfected with MOI 20 without adenoviral virulence. Live cells were counted on the indicated days after transfection. Results are the means ± SD of three independent experiments. (B) Morphologies of transfected AsPC-1 cells with AdCA*tob*, AdCA*lacZ*, medium only without adenovirus vector. Enlarged megakaryotic cells appeared with many granules in cytoplasm. Photograph magnification is ×400.

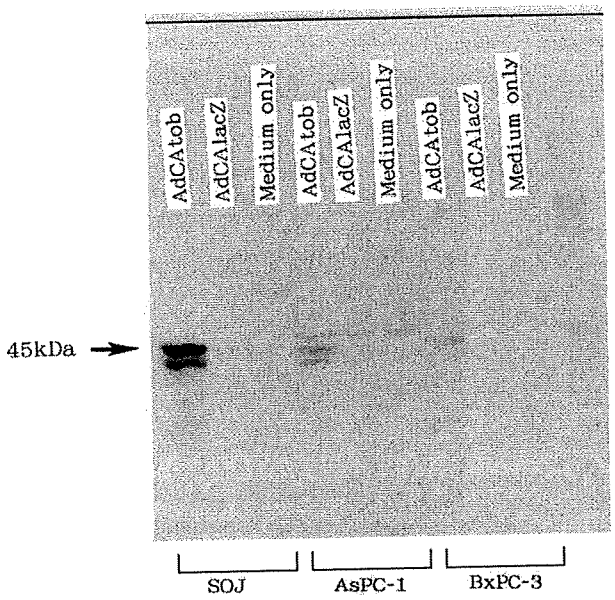


Fig. 4. Western blot analysis of lysates of pancreatic cancer cell lines after transfection of AdCA*lacZ* or AdCA*tob* using the anti-TOB monoclonal antibody Transfected adenovirus vectors are indicated above lanes. Cancer cells are indicated below lanes. Arrow on the left side of gel indicates 45 kDa band specific to exogenous TOB. Band higher than 45 kDa indicates endogenous TOB expressed in AsPC-1 cells.

product and to the PC 3 (BTG-2) gene product at its amino-terminal half. Both BTG-1 and BTG-2, having the significant homologies to TOB, suppress cell growth, and perform cell cycle control [8,9]. ANA, belonging to the anti-proliferative TOB family, interacts with CCR transcription factor-associated protein Caf1 [16,17].

NIH3T3 cells expressing exogenous *tob* mRNA were enlarged and showed swollen nuclei with many granules in the cytoplasm. The characteristics resembled those of senescent cells. Evidence of senescence change were the accumulation of lipofuscin granules, an ultrastructural change associated with aging, and flattened enlarged cell morphology. TOB exhibits anti-proliferative activity when the level of its expression is elevated and/or deregulated. The interaction of TOB with *c-erbB-2* gene products was suggested to occur through the carboxyl-terminal half of TOB. Exogenously expressed TOB also exhibits anti-proliferative activity. Protein-tyrosine kinase receptors induce the expression of G1 cyclins, which in turn interact with and activate CDK family proteins, resulting in the phosphorylation of Rb protein. It is necessary to examine whether protein-tyrosine kinases other than $p185^{erbB-2}$ could also interact with TOB in the proliferative signal transduction of cancer cells. Expression of BTG-1 is high in G0/G1 phases of the cell cycle and is down regulated when the cells enter the growth cycle, suggesting that the gene product is inhibitory to G0/G1 progression. A forced expression of exogenous BTG-1 in NIH3T3 cells resulted in the suppression of cell growth [8]. The BTG-1 gene is 60% homologous to PC3, an immediate early gene induced by nerve growth factor in rat PC12 cells. Rouault et al. named PC3 as BTG-2, and have reported that BTG-2 expression is induced through a p53-dependent mechanism and the function may be relevant to cell cycle control and cellular response to DNA damage [9].

Yoshida et al. had reported that *tob* is a member of anti-proliferative family genes. Mice lacking *tob* are prone to spontaneous formation of tumors. The occurrence rate of diethylnitrosamine-induced liver tumors is higher in $tob^{-/-}$ mice than in wild-type mice. $TOB^{-/-}$ $p53^{-/-}$ mice show accelerated tumor formation in comparison with single null mice. Expression of cyclin D1 mRNA is increased in the absence of and reduced by TOB [18]. Suzuki et al. had reported that TOB inhibits cell growth by suppressing cyclin D1 expression, which is canceled by Erk1- and Erk2-mediated TOB phosphorylation. TOB is critically involved in the control of early G1 progression [19]. Iwanaga et al. reported

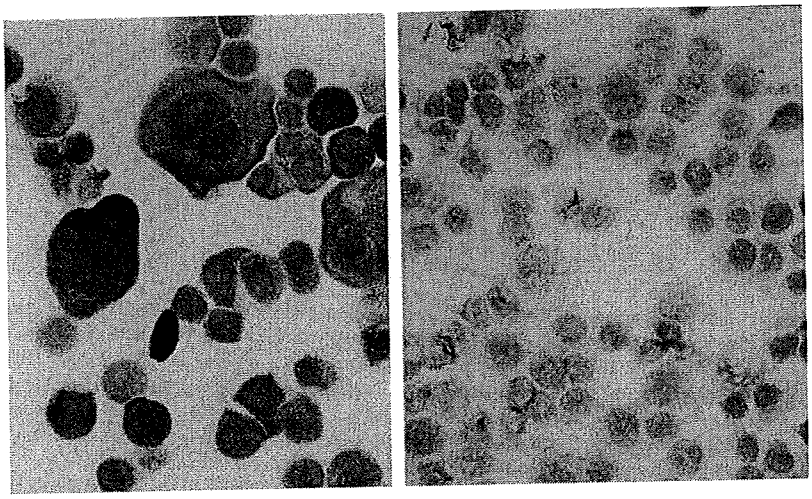


Fig. 5. AdCA*tob* vector-mediated *tob* expression *in vitro* AsPC-1 cells (50×10^3) were transfected with AdCA*tob* vector (MOI 20). After 72 h, cells were collected and stained with anti-TOB monoclonal antibody 4B1 by peroxidase immunostaining in order to determine the expression of TOB protein. Photograph magnification is ×400. Left: transfected cells were well stained. Right: non-treated naïve cells.

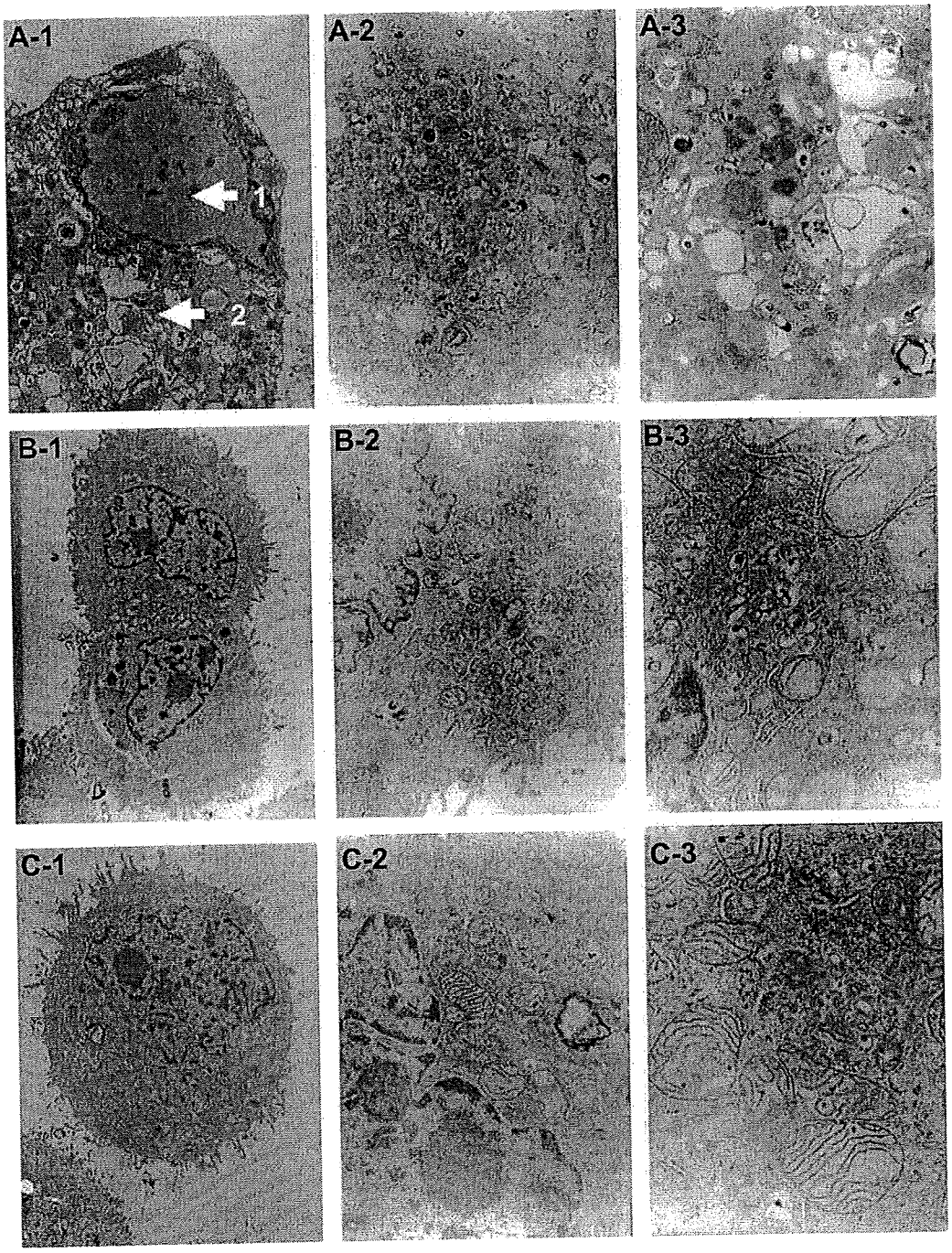


Fig. 6. Cytotoxic effect on AsPC-1 with *tob* expression by AdCA*tob* vector on electron microscopy. (A) AdCA*tob* transfectant (MOI 100), (B) AdCA *mock* transfectant (MOI 100), (C) Non-treated control. (A) AdCA*tob* transfectant (MOI 100) showed degradation of nucleus (arrowhead 1), many autophagosomes and electron-dense cytoplasmic inclusions (arrowhead 2). The contents of these vesicles consisted of lamellar material that resembled lipofuscin, a lipid substance with auto-fluorescence properties. (B) AdCA *mock* transfectant (MOI 100) showed irregular-shaped nucleus, enlargements and deformities of mitochondria, increase of endoplasmic reticulum and lysosome vesicles. No increase of lipofuscin granules. (C) Non-treated control showed irregular-shaped nucleus, enlargement of mitochondria, increase of endoplasmic reticulum. Several autophagosomes were seen in cells treated with AdCA *mock* and non-treated cells, but increase of autophagosomes were recognized in cells treated with AdCA*tob*. There was neither an increase of inside scatter nor the appearance of lipofuscin granules. A-1, B-1, and C-1 are low magnification of the photograph (×5000), A-2, B-2, and C-2 are high magnification of the photograph (×8000), and A-3, B-3, and C-3 are high magnification of the photograph (×15,000).

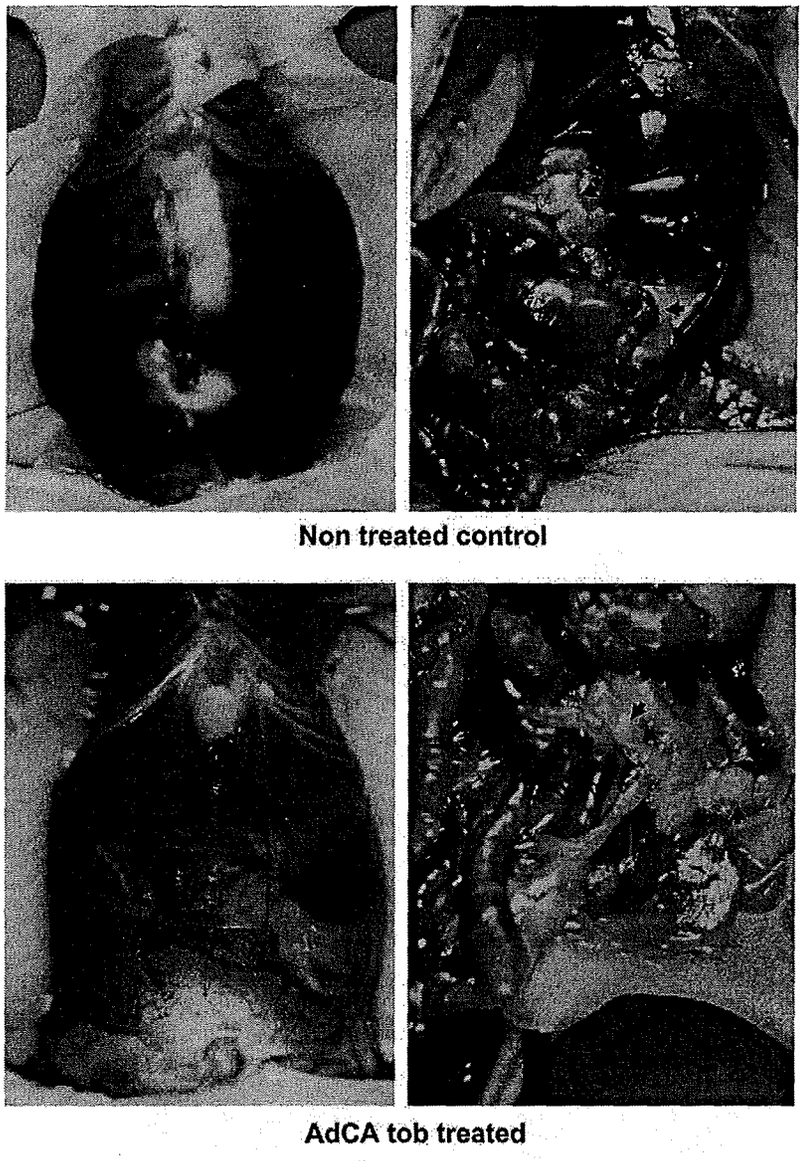


Fig. 7. Tumor growth suppression of cancer peritonitis model with peritoneal AdCA*tob* transfection. Bloody ascites and tumor growth suppression in the abdominal cavity disappeared with viral *tob* expression. (A). Non-treated control; Bloody ascites (Left) and multiple tumor nodules (Right) were seen at the mesentery after 16 days of 2×10^6 ip injection of AsPC-1 cells. (B). AdCA*tob*-treated; mice underwent ip injections of AdCA*tob* (1.5×10^8 pfu/0.2 ml/day, from day 5 to day 9) after 4 days of ip injection of 2×10^6 AsPC-1 cells. Peritoneal injections of AdCA*tob* suppressed tumor nodule formation in the abdominal cavity compared with the non-treated group (Right). Bloody ascites was not seen in AdCA*tob*-treated mice (Left).

that the phosphorylated and inactive form of TOB was detected in 76% of cancer tissues of adenocarcinoma patients, but not in normal alveolar epithelial cells [20]. Cho et al. reported that phosphorylation of myristoylated alanine-rich C kinase substrate, MARCKS, removes TOB from ErbB-2 by increasing its binding affinity with TOB, and thereby activates ErbB-2-mediated signal transduction [21]. TOB phosphorylation contributes to the progression of papillary carcinoma of the thyroid, especially in the later phase through cancellation of its anti-proliferative function [22]. Exogenous overexpression of TOB family proteins suppresses cell proliferation. Mutation in the nuclear localization signal sequence of TOB affects its nuclear localization and impairs anti-proliferative activity [23,24]. ERK phosphorylation negatively regulates the anti-proliferative function of TOB [25]. Sasajima et al. reported that the BTG/TOB family was degraded by the ubiquitin-proteasome system [26].

We evaluated the expression of *tob* mRNA and gene product in pancreatic cancer cell lines, AsPC-1, BxPC-3, and SOJ with or without *tob* transfection. The *tob* mRNA was expressed in all pancreatic cancer cell lines, and the level of *tob* mRNA of AsPC-1 cells was strongest among them. The *tob* mRNA

Table 1
Inhibition of the formation of pancreatic cancer peritonitis with Adeno-virus mediated *tob* gene transfer *in vivo*

Treatment group	Tumor nodules		Ascites	
AdCA*tob* (1.5×10^8 pfu) ($n=4$)	–	(0/4)	±	Serous
AdCA*tob* (5×10^7 pfu) ($n=3$)	+	(1/3)	±	Serous
AdCA*lacZ* (1.5×10^8 pfu) ($n=3$)	+	(2/3)	±	Serous
AdCA*lacZ* (5×10^7 pfu) ($n=3$)	+	(2/3)	+~++	Turbid
Non-treated (1.5×10^8 pfu) ($n=3$)	+++	(3/3)	+++	Bloody

Despite the observed heterogeneity for individual animals, a significant tumor growth inhibitory effects of AdCA*tob* has been noted.

expression was increased in correlation with erbB-2 mRNA expression in AsPC-1 and BxPC-3 cells, but the endogenous *tob* gene product is not increased in these cells.

Overexpression of erbB-2 and EGF-R protein was observed in pancreatic cancer cells, so the suppressive effect of endogenous TOB could be impaired by the passage of protein-tyrosine kinases as $p185^{erbB\text{-}2}$. We prepared adenoviral vector containing *tob* cDNA (AdCA*tob*). AdCA*tob* was transfected and expanded in 293 cells. The titer of the vector was 350×10^6 pfu/ml. Monitoring the viability of pancreatic cancer cells transfected with adenoviral vector containing the *lacZ* gene revealed that these cancer cells were able to be transfected with MOI 20 without adenoviral toxicity. Growth

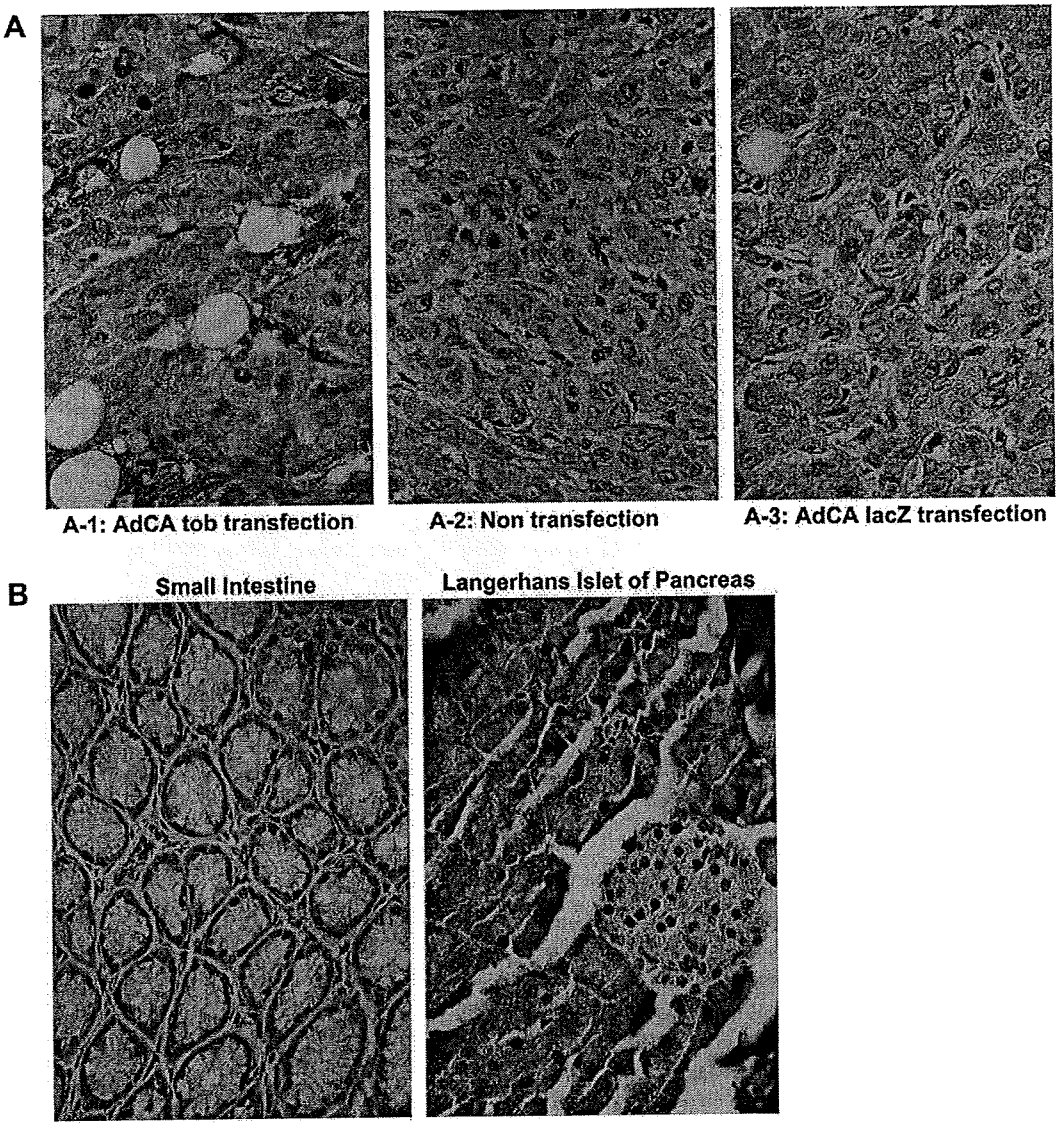


Fig. 8. Pathological findings of tumor treated with AdCA*tob* vector *in vivo*. Only one tumor nodule was recognized in the abdominal cavity in the AdCA*tob* (5×10^7 pfu)-treated group, and not found in 1.5×10^8 pfu groups. The tumor continued to express TOB with staining by anti-TOB monoclonal antibody 4B1 (A-1). Tumors of non-treated control (A-2) and AdCA*lacZ* (A-3)-transfected groups showed only a few endogenous TOB. The jejunum (B-1), mesothelium, acinal gland and pancreatic islets of Langerhans (B-2) did not show the expression of exogenous TOB with AdCA*tob* transfection.

suppression with transfection of AdCA*tob* was shown in SOJ and AsPC-1 cell lines according to *tob* expression. We evaluated the tumor-suppressing effects of AdCA*tob* in pancreatic cancer cell lines. SOJ and AsPC-1 cell lines transfected by AdCA*tob* showed growth suppression. Significant suppression was shown in SOJ cell lines after the overexpression of TOB. These results suggest that the recombinant adenovirus vector containing the *tob* gene is a useful candidate for anti-tumor gene therapy, and could be applied for cancer peritonitis. AsPC-1 cells expressing the exogenous *tob* were enlarged and megakaryocytic with characteristics resembling those of senescent cells.

We also found that TOB-overexpressing AsPC-1 cells showed degradation of the nucleus and many autophagosomes and electron-dense cytoplasmic inclusions. One cancer cytotoxic mechanism is based on autophagy. Autophagy is a cellular degradation pathway for the clearance of damaged or superfluous proteins and organelles [27,28], and a survival pathway required for cellular viability during starvation; however, if it proceeds to completion, autophagy can lead to cell death. Autophagy has emerged as a homeostatic mechanism regulating the turnover of long-lived or damaged proteins and organelles, and buffering metabolic stress under conditions of nutrient deprivation by recycling intracellular constituents. Autophagy is also a form of cell death, when allowed to proceed to excessive levels and when apoptosis-defective cells are triggered to die. It has been thought that autophagy may play an active role in programmed cell death [29]. We had observed autophagic conformation by the formulation of autophagosomes and localization of GFP-LC3 on the cytotoxicity of human pancreatic cancer cells treated with polyoxomolybdates (PM-17) [30]. Faiy et al. indicated that quercetin induced autophagy specifically in Ha-RAS-transformed cells [31]. They had reported that flavonoid quercetin drastically reduces the half-life of oncogenic Ras, and Ras protein levels in cell lines expressing oncogenic Ras proteins. Quercetin induces autophagic processes in Ha-RAS-transformed cells. Microtubule-associated protein light chain 3 (LC3) protein is localized in autophagosomes and autolysosomes membranes after processing in quercetin-treated cancer cell lines.

In this report, we also prepared pancreatic cancer peritonitis models using ip injection of AsPC-1 cells. In this model, bloody ascites and multiple tumor nodules were seen at the mesentery after 16 ip days. We administered ip injection of AdCA*tob* to mice bearing pancreatic cancer peritonitis. Tumor growth was suppressed 10 days after ip injections of AdCA*tob* compared to the control group. Our new model of gene therapy for pancreatic cancer by AdCA*tob* in the first week induced significant tumor reduction and complete tumor regression. There was no tumor nodule in the abdomen and no bloody ascites. AdCA*tob* has shown no significant toxic effect on untransformed cells. AdCA*tob* treatment produced significant growth inhibition both *in vivo* and *in vitro*.

Overexpression of wt p53 triggered a short-term cellular response leading to irreversible growth arrest and senescence [32–34]. The commitment to senescence became irreversible with in 48–72 h and no longer required p53 expression. A number of studies utilizing adenoviral or retroviral vectors have evaluated the anti-proliferative and anti-tumorigenic potential of restoration of wild-type p53 in *in vitro* as well as *in vivo* animal models of cancer [35–40]. Nielsen et al. reported that ip injection of AdCMV*wt p53* resulted in reducing the tumor burden of SK-OV-3 ovarian cancer *in vivo* [2]. We also performed ip injections of AdCMV*wt p53* (5×10^7 pfu/day, from day 5 to day 9) after 4 days of ip inoculation of AsPC-1 cells, and tumor growth was suppressed 10 days after ip injections of AdCMV*wt p53* (data not shown). Tumor suppressor gene *p53* includes the regulation of G1-associated cell growth inhibition, maintenance of genomic integrity, control of the apoptotic pathway, and regulation of inhibitors of angiogenesis. Consequently, reconstituting the normal p53 function in tumor cells with defective p53 via introduction of the *wt p53* gene may have therapeutic utility [41,42].

It has been known that the adenovirus vector-mediated gene transfer system has several limitations for *in vivo* application, including transient expression of transferred gene and immunogenic response of the host against adenovirus. As an extension of this study, we are now evaluating the *tob* gene therapeutic potential with a cationic liposomal delivery system to a pancreatic cancer model. Injection of the AdCA*tob* vector provides significant growth inhibition of tumor progression, and our study offers strong support for the gene therapeutic potential of this vector in human pancreatic cancer peritonitis. Continuous progression of these investigations in the future will be necessary for the successful development of a new treatment modality for clinical trials of gene therapy for metastatic pancreatic cancer.

Acknowledgements

The authors are grateful to Professor Alistair Renwick, Institute of Health and Community Medicine, University of Malaysia Sarawak, for comments on the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture, Japan (No. 15390389 and 15659308 to Hironobu Yanagie), a Grant from the Sato Memorial Foundation for Cancer Research (to Hironobu Yanagie), and a Grant from the Foundation for the Promotional of Cancer Research in Japan (to Hironobu Yanagie).

References

[1] Reyes G, Villanueva A, Garcia C, Sancho FJ, Piulats J, Lluis F, et al. Orthotopic xenografts of human pancreatic carcinomas acquire genetic aberrations during dissemination in nude mice. Cancer Res 1996;56: 5713–9.

[2] Nielsen LL, Gurnani M, Syed J, Dell J, Hartman B, Cartwright M, et al. Recombinant E1-deleted adenovirus-mediated gene therapy for cancer: efficacy studies with p53 tumor suppressor gene and liver histology in tumor xenografts models. Hum Gene Ther 1998;9:681–94.

[3] Fueyo J, Gomez-Manzano C, Yung WKA, Liu TJ, Alemany R, McDonnell TJ, et al. Overexpression of E2F-1 in glioma triggers apoptosis and suppresses tumor growth in vitro and in vivo. Nat Med 1998;4: 685–90.

[4] Iqbal-Ahmed CM, Sugarman BJ, Johnson DE, Bookstein RE, Saha DP, Nagabhushan TL, et al. *In vivo* tumor suppression by adenovirus-mediated Interferon α2b gene therapy. Hum Gene Ther 1999;10:77–84.
[5] Kaneko S, Hallenbeck P, Kotani T, Nakabayashi H, McGarrity G, Tamaoki T, et al. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. Cancer Res 1995;55: 5283–7.
[6] Matsuda S, Kawamura J, Ohsugi M, Yoshida M, Emi M, Nakamura Y, et al. Tob, a novel protein that interacts with p185 erbB2, is associated with anti-proliferative activity. Oncogene 1996;12:705–13.
[7] Akiyama T, Matsuda S, Namba Y, Saito T, Toyoshima K, Yamamoto T. The transforming potential of the c-erbB-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. Mol Cell Biol 1991; 11:833–42.
[8] Rouault JP, Rimokh R, Tessa C, Paranhos G, Ffrench M, Duret L, et al. BTG-1, a member of a new family of antiproliferative genes. EMBO J 1992;11:1663–70.
[9] Rouault JP, Falette N, Guehenneux F, Guillot C, Rimokh R, Wang Q, et al. Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. Nat Genet 1996;14: 482–6.
[10] Rouault JP, Samarut C, Duret L, Tessa C, Samarut J, Magaud JP. Sequence analysis reveals that the BTG-1 anti-proliferative gene is conserved throughout evolution in its coding and 3' non-coding regions. Gene 1993;129:303–6.
[11] Kanai F, Lan KH, Shiratori Y, Tanaka T, Ohashi M, Okudaira T, et al. *In vivo* gene therapy for a-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. Cancer Res 1997;57:461–5.
[12] Tanaka T, Kanai F, Okabe S, Yoshoda Y, Wakimoto H, Hamada H, et al. Adenovirus-mediated prodrug gene therapy for carcinoembryonic antigen-producing human gastric carcinoma cells *in vitro*. Cancer Res 1996;56:1341–5.
[13] Kanegae Y, Lee G, Sato Y, Tanaka M, Nakai M, Sasaki T, et al. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 1995;23: 3816–21.
[14] Zenilman ME, Magnuson TH, Perfetti R, Chen J, Shuldiner AR. Pancreatic *reg* gene expression is inhibited during cellular differentiation. Ann Surg 1997;225:327–32.
[15] Yoshida Y, Matsuda S, Yamamoto T. Cloning characterization of the mouse *tob* gene. Gene 1997;191:109–13.
[16] Yoshida Y, Matsuda S, Ikematsu N, Kawamura-Tsuzuku J, Inazawa J, Umemori H, et al. ANA, a novel member of Tob/BTG-1 family, is expressed in the ventricular zone of the developing central nervous system. Oncogene 1998;16:2687–93.
[17] Yoshida Y, Hosoda E, Nakamura T, Yamamoto T. Association of ANA, a member of the antiproliferative Tob family proteins, with a Caf1 component of the CCR4 transcriptional regulatory complex. Jpn J Cancer Res 2001;92:592–6.
[18] Yoshida Y, Nakamura T, Komoda M, Satoh H, Suzuki T, Tsuzuki KJ, et al. Mice lacking a transcriptional co-repressor Tob are predisposed to cancer. Genes & Dev 2003;17:1201–6.
[19] Suzuki T, K-Tsuzuku J, Ajima R, Nakamura T, Yoshida Y, Yamamoto T. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. Genes Dev 2002;16(11):1356–70.
[20] Iwanaga K, Sueoka N, Sato A, Sakuragi T, Sakao Y, Tominaga M, et al. Alteration of expression or phosphorylation status of tob, a novel tumor suppressor gene product, is an early event in lung cancer. Cancer Lett 2003;202:71–9.
[21] Cho SJ, La M, Ahn JK, Meadows GG, Joe CO. Tob-mediated cross-talk between MARCKS phosphorylation and ErbB-2 activation. Biochem Biophy Res Comm 2001;283:273–7.
[22] Ito Y, Suzuki T, Yoshida H, Tomoda C, Uruno T, Takamura Y, et al. Phosphorylation and inactivation of Tob contributes to the progression of papillary carcinoma of the thyroid. Cancer Lett 2005;220:237–42.
[23] Kawamura-Tsuzuku J, Suzuki T, Yoshida Y, Yamamoto T. Nuclear localization of Tob is important for regulation of its antiproliferative activity. Oncogene 2004;23:6630–8.
[24] Maekawa M, Yamamoto T, Nishida E. Regulation of subcellular localization of the antiproliferative protein Tob by its nuclear export signal and bipartite nuclear localization signal sequences. Exp Cell Res 2004; 295:59–65.
[25] Maekawa M, Nishida E, Tanoue T. Identification of the Anti-proliferative protein Tob as a MAPK substrate. J Biol Chem 2002;277:37783–7.
[26] Sasajima H, Nakagawa K, Yokosawa H. Antiproliferative proteins of the BTG/Tob family are degraded by the ubiquitin-proteasome system. Eur J Biochem 2002;269:3596–604.
[27] Omodeizorini A. Considerations on primary carcinomatous caverns of the lung. Possibility of the intervention of a phenomenon of "Autophagia of the neoplastic cells". Riforma Med 1964;78:533–50 [in Italian].
[28] Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in cancer. Nat Rev Cancer 2007 Dec;7(12):961–7.
[29] Karantza-Wadsworth Vassiliki, White Eileen. Role of autophagy in breast cancer. Autophagy 2007;3(6):610–3.
[30] Ogata A, Yanagie H, Ishikawa E, Morishita Y, Mitsui S, Yamashita A, et al. Antitumour effect of polyoxomolybdates: induction of apoptotic cell death and autophagy in *in vitro* and *in vivo* models. Br J Cancer 2008;98(2):399–409.
[31] Psahoulia Faiy H, Moumtzi Sophy, Roberts Michael L, Sasazuki Takehiko, Shirasawa Senji, Pintzas Alexander. Quercetin mediates preferential degradation of oncogenic Ras and causes autophagy in Ha-RAS- transformed human colon cells. Carcinogenesis 2007;28: 1021–31.
[32] Asgari K, Sesterhenn IA, Mcleod DG, Cowan K, Moul JW, Seth P. Inhibition of the growth of pre-established subcutaneous tumor nodules of human prostate cancer cells by single injection of the recombinant adenovirus p53 expression vector. Int J Cancer 1997;71:377–82.
[33] Sugrue MM, Shin DY, Lee SW, Aaronson SA. Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. Proc Natl Acad Sci U S A 1997;94:9648–53.
[34] Hamada K, Alemany R, Zhang WW, Hittelman WN, Lotan R, Roth JA, et al. Adenovirus-mediated transfer of a wild-type *p53* gene and induction of apoptosis in cervical cancer. Cancer Res 1996;56:3047–54.
[35] Eastham JA, Hall SJ, Sehgal I, Wang J, Timme TL, Yang G, et al. *In vivo* gene therapy with p53 or p21 adenovirus for prostate cancer. Cancer Res 1995;55:5151–5.
[36] Yang C, Cirielli C, Capogrossi C, Passantini A. Adenovirus-mediated wild-type p53 expression induces apoptosis and suppresses tumorigenesis of prostatic tumor cells. Cancer Res 1995;55:4210–3.
[37] Clayman GL, El-Naggar AK, Roth JA, Zhang WW, Goepfect H, Taylor DL, et al. *In vivo* molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 1995;55:1–6.
[38] Graber HU, Friess H, Kaufmann B, Willi D, Zimmermann A, Korc M, et al. ErbB-4 mRNA expression is decreased in non-metastatic pancreatic cancer. Int J Cancer 1999;84:24–7.
[39] Putzer BM, Bramson JL, Addison CL, Hitt M, Siegel PM, Muller WJ, et al. Combination therapy with Interleukin-2 and wild-type p53 expressed by adenoviral vectors potentiates tumor regression in a murine model of breast cancer. Hum Gene Ther 1998;9:707–18.
[40] Sandig V, Brand K, Herwig S, Lukas J, Bartek J, Strauss M. Adenovirally transferred $p16^{INK4/CDKN2}$ and p53 genes cooperate to induce apoptotic tumor cell death. Nat Med 1997;3:313–9.
[41] Harris MP, Sutjipto S, Wills KN, Hancock W, Cornell D, Johnson DE, et al. Adenovirus-mediated *p53* gene transfer inhibits growth of human tumor cells expressing mutant p53 protein. Cancer Gene Ther 1996;3: 121–30.
[42] Harada N, Gansauge S, Gansauge F, Gause H, Shimoyama S, Imaizumi T, et al. Nuclear accumulation of p53 correlates significantly with clinical features and inversely with the expression of the cyclin-dependent kinase inhibitor$^{WAF-1/CIP1}$ in pancreatic cancer. Br J Cancer 1997;76:299–305.

In: Basophil Granulocytes
Editors: Paul K. Vellis

ISBN: 978-1-60741-797-2
© 2009 Nova Science Publishers, Inc.

Chapter 3

BASOPHILIC GRANULOCYTES

Toshihisa Tsuruta* and Kenzaburo Tani†
* Department of Internal Medicine, National Hospital Organization
Kumamoto Medical Center.
†Department of Medical Genomics, Medical Institute of Bioregulation.
Kyushu University and Department of Advanced Cell and Molecular Therapy,
Kyushu, University Hospital.

ABSTRACT

Basophilic granulocytes (basophils) are a very small population of peripheral blood leukocytes. Because basophils have high-affinity immunoglobulin E receptors (FcεRI) and secrete chemical mediators that contain histamine, they are thought to be very similar to mast cells. However, research characterizing the function of basophils was slow to proceed and their unique function and importance were not established for a significant period of time. Recently, a series of studies have characterized the role of basophils in anaphylactic shock, chronic allergic reactions, and other human immunological reactions. These studies have shown that basophils are not a supplementary cell type but key players in very serious immune reactions. In this chapter we introduce the recently discovered characteristics of basophils. In addition, we consider how these aspects are clinically important and are connected with new cellular or molecular treatments for allergic reactions.

INTRODUCTION

Basophilic granulocytes (basophils) are a small population of peripheral blood leukocytes that were first described in 1879 by Ehrlich[1]. These cells contain unique cytoplasmic granules that stain with basophilic dyes, such as toluidine blue. The percentage of basophils in the peripheral blood is low (<1%), and they share physicochemical properties with other blood cells. Like mast cells, basophils possess high-affinity immunoglobulin (Ig) E receptors (FcεRI) that are cross-linked when the receptor bound IgE is engaged with the corresponding antigen ("allergen"). Receptor cross-linking results in the release of a number of mediators, which contain some elements that are common to both cell types. Based on their similarity to mast cells, basophils have been thought to play a minor and possibly redundant role as "circulating mast cells"[2, 3].

Not so long ago, some investigators thought that mice entirely lacked basophils because they could not be detected by normal hematologic staining (eg.Wright-Giemsa). In 1981, Urbina et al. showed that mouse basophils have a distinct morphology that is different from other species, which may have contributed to their inability to be detected in routine skin preparations[4]. Furthermore, the ultrastructural features of mouse basophils have been well defined[5], and many studies have been conducted on this unique cell population[6, 7].

Phenotype, Development, and Activation of Basophils

The early stages of basophil maturation and their relationship to other cell lineages are not well understood[2]. Basophils express a variety of cytokine receptors (IL-1RII (CD121b), IL-2Rα (CD25), IL-3Rα (CD123), IL-4Rα (CD124), IL-8R (CD128), GM-CSFRα (CD116), IFNRγ (CD119)), chemokine receptors [CCR1, CCR2, CCR3, CCR5 (CD195), CXCR1 (IL-8Rα), CXCR2 (IL-8Rβ), CXCR4 (CD170), CRTH2], complement receptors [CD11b (iC3bR), CD11c (C3biR), CD21 (C3dR), CD35 (C3bR, C4bR), CD45 (C3bR, C4bR), CD55 (C4b/2aR, C3b/BbR), CD59 (C5b-8R, C5b-9R)], homing receptors and related molecules [CD15s, CD62L, CD162, CD11a (LFA-1), CD18, CD29, CD44 (Pgp-1), CD49a (VLA-1), CD49d (VLA-4)], prostaglandin receptors, and Ig Fc receptors [CDw32 (FcγRIIA and B), FcεRI, FcγRIII[8-10].

Previous reports have suggested that basophils evolved from eosinophil/basophil progenitors, and this hypothesis is supported by the presence

of granulocytes with hybrid eosinophil/basophil phenotypes in patients with chronic or acute myelogenous leukemia and in cell culture[11-13]. On the other hand, the possibility that mast cells and basophils share a common lineage arises from the observation that basophils with phenotypic features that are characteristic of mast cells can be found in patients with asthma, allergies, or allergic drug reactions[14]. Therefore, the current predominant model that mast cells and basophils originate from separate lineages is still debated and may have to be revised[2].

Our understanding of basophils has been advanced by the development of basophil-specific monoclonal antibodies, Bsp-1, 2D7, BB1 and 212H6[10, 15-17]. In addition, the 97A6 monoclonal antibody has been described as an antibody specific for mature mast cells, basophils, and their progenitors[18]. 97A6 does not react with any other hematopoietic or nonhematopoietic cell types. The epitope recognized by 97A6 may therefore be associated with the commitment of the CD34 precursor to a mast cell or basophil lineage that is distinct from other lineages[2].

Among the many cytokines that stimulate basophils, IL-3 is thought to be the main growth and differentiation factor for basophils[19, 20]. SCF together with IL-3 expand the progenitor pool of most hematopoietic cell types in the bone marrow, including mast cells and basophils[20].

As with mast cells, basophils express complete and functional FcεRI receptors, and cross-linking of these receptors leads to basophil activation, granule exocytosis and mediator release[21]. C3a and C5a can also activate basophils through the C3aR and C5aR complement receptors, respectively. Activation through any of these receptors leads to histamine release, eicosanoid synthesis, and IL-4 and IL-13 gene expression. In addition, basophil activation may be associated with increased CD18 and CD63 expression and decreased Leu-8 (CD62L) expression[8].

Basophils and Anaphylaxis

Anaphylaxis is an immune reaction that is induced upon exposure to food, wasp toxins, and allergens, such as medicine and latex, and can lead to a severe generalized allergy[22, 23]. Because anaphylaxis is often associated with rapid skin urticaria, decreased blood pressure, dyspnea, consciousness disorder, etc., this condition is dangerous and sometimes fatal. Therefore, anaphylaxis is a pathosis that concerns medical professionals and both rapid diagnosis and treatment are necessary. Previous work has shown that both mast cells and