Expression of Adenovirus Type 5 E1A in the Methylotrophic Yeast *Pachia pastoris* and the Inhibitory Effect on S-180 Tumor Growth

Yewei Ma, Xiaoshan Zhou, Qingzheng Zhao,* Yanchun Li, Yuying Liu, Zheng Wang, and Yangpei Zhang

Cancer Institute, Peking Union Medical College and Chinese Academy of Medical Sciences; Beijing, 100021, China.

The human adenovirus type 5 (Ad5) early-region 1A (E1A) proteins have been shown to have strong tumor-suppressive activities in human tumor cells and to enhance the sensitivity of a variety of malignant tumors to apoptosis induced by ionizing radiation and chemotherapeutic agents. However, the inherent limitations of E1A gene therapy prevent its application, such as the efficiency of expression, precision of targeting, and toxicity of vector. This prompted us to construct an E1A expression vector (pPIC9/E1A) and express the E1A protein in the methylotrophic yeast *Pichia pastoris*. The E1A protein was purified using two steps of ion-exchange column chromatography on HiTrap Q and HiTrap SP. The analysis indicated that the E1A protein/liposome inhibited S-180 tumor growth and also rendered the S-180 tumor strongly susceptible to the anticancer drug bleomycin *in vivo*. Furthermore, tunnel assay clearly revealed that the mechanism was induction of cellular apoptosis. Importantly, the E1A protein overcame the limitations of gene therapy. Thus the E1A protein may be a useful therapeutic agent for some malignant tumors.

Key words   early-region 1A (E1A) protein; yeast expression system; liposome; S-180; apoptosis

The human adenovirus type 5 (Ad5) early-region 1A (E1A) gene, which codes for two major related proteins of 243 and 289 amino acids by alternative splicing in two exons, can activate or repress transcription of several viral and cellular genes.[1] E1A, a tumor-suppressor gene,[2,3] has been shown to have tumor-suppressor activities in human cancers both *in vitro* and *in vivo*, including breast carcinoma, ovarian carcinoma, *etc.*[4—6] E1A may suppress the malignancy of diverse human tumor cells by partially converting those tumor cells into an epithelial phenotype. Furthermore, E1A has epithelial cell master-programming activity in diverse human tumor cells of mesenchymal origin.[7] E1A apparently enhances the sensitivity of tumor cells to ionizing radiation and chemotherapeutic DNA-damaging agents such as 5-fluorouracil, etoposide, doxorubicin, cisplatin, and paclitaxel, and the sensitivity is independent of the p53 protein status and other oncogenic alterations.[8—11] These results indicate that E1A is a tumor-suppressor gene and could be utilized in the therapy of diverse human cancers.

Despite the progress in the studies of E1A gene therapy, there are many aspects for further investigation, including modifying viral vectors to reduce toxicity and immunogenicity, increasing the transfection efficiency of nonviral vectors, enhancing vector targeting and specificity, regulating gene expression, and identifying synergies between gene-based agents and other cancer therapeutics.[12] Thus E1A gene therapy might harbor the principal risk of enhancing tumor malignancy. In this paper, to overcome the limitations of E1A gene therapy, we first described the E1A eukaryotic expression vector (pPIC9/E1A) that efficiently expresses the full-length product of the adenovirus type 5 E1A 12S using the *Pichia pastoris* yeast expression system. Yeasts as host cells are considered safe, cheap, and amenable to high expression levels. Therefore they are good candidates for the production of E1A protein on an industrial scale.[13] Our studies demonstrated that the E1A protein/liposome could effectively inhibit S-180 tumor growth and enhance the sensitivity of S-180 tumors to bleomycin.

MATERIALS AND METHODS

Construction of the Recombinant Vector The E1A 12S segment was generated by PCR using Taq DNA polymerase and 5′ and 3′ primers containing XhoI and NotI sites, respectively. The specific primers used for E1A were: forward primer, 5′-CGACTCGAGATGAGACATATTATCTGC-CAC-3′; and reverse primer, 5′-ATAGCGGCCGCTTATG-GCCTGGGGCGTTTACA-3′. The PCR product (747 bp) was digested with XhoI and NotI and cloned into the corresponding sites of pPIC9. The recombinant construct was verified by sequence analysis.

Screening of Positive Yeast Strains and Expression of E1A Protein in Shaking Flask Cultures Plasmid pPIC9/E1A was propagated in *Escherichia coli* strains (DH5α) and linearized with *Bgl*II. The *P. pastoris* strains GS115 (Mut⁺, His⁻) were transformated according to the manufacturer's instructions (Invitrogen). The transformants were selected on a nitrocellulose membrane on MD plates (1.34% yeast nitrogen base, 4×10^{-5}% Biotin, 1% dextrose, 1% agar) and were cultured at 30 °C for 3 d. The nitrocellulose membrane was transferred to MM plates (1.34% yeast nitrogen base, 4×10^{-5}% Biotin, 1% methanol, 1% agar). The E1A protein was induced at 28 °C for 48 h. The positive transformants were screened using an E1A monoclonal antibody (PharMingen Co.).

The positive yeast strains were grown in 200 ml of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% Biotin, 1% glycerol) at 28 °C to an optical density at 600 nm (OD_{600}) of 10. Then the yeasts were harvested, washed once with BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% Biotin, 1% methanol), resuspended in BMMY at an OD_{600} of approximately 30, and incubated at 28 °C for 1 to 5 d to induce expression. The supernatants were concentrated 10-fold by pressure dialysis using an Amecon YM 30 membrane. The concentrated supernatants were tested by Western blot-

---

ting.

Purification of E1A Protein For the first purification scheme, HiTrap Q chromatography was used as an initial step. The supernatant fraction containing the E1A protein was dialyzed extensively against buffer A (50 mM Tris–HCl, pH 7.5 at 25 °C). The dialysate was loaded on a HiTrap Q column equilibrated with buffer A. The E1A protein fraction was eluted from the column at about 0.2 M NaCl using the NaCl concentration gradient in buffer A. HiTrap SP chromatography was used as the second purification scheme. The E1A protein fraction was pooled and dialyzed against buffer B (50 mM HAc-NaAc, pH 4.0 at 25 °C) and loaded on a Hi-Trap SP column previously equilibrated with buffer B. The fraction containing the E1A protein was collected at 0.15 M NaCl using the NaCl concentration gradient in buffer B. They were run at a flow rate of 5 ml/min. All steps in the E1A protein purification procedure were carried out at 4 °C. The yield of the E1A protein following this purification procedure was evaluated.

Sodium Dedecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting The E1A protein was separated on 10% polyacrylamide gels according to the method of Laemmli[14] using a Mini Protein II electrophoresis cell (BioRad). Gels were stained with silver. Polyacrylamide gels were electroblotted on a nitrocellulose membrane (Schleicher & Schüll) according to the method of Towbin and Staehelin.[15] The membrane was blocked for 2 h at room temperature with Tris-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20. The bands were detected by consecutive incubations overnight at 4 °C with E1A anti-mouse monoclonal antibody (1 : 1000), followed by the alkaline phosphatase-conjugated anti-mouse goat antibody IgG (1 : 200) for 2 h at room temperature and stained with BCIP/NBT as a phosphatase substrate.

E1A Protein/Liposome Inhibition of S-180 Tumor Growth *in Vivo* Twenty-five male Kunming mice (body weight: 18—20 g) were used in this study. The animals were provided with sterilized food and water and maintained at constant temperature (24 ± 1 °C) and humidity (55 ± 5%). S-180[16] carcinoma cells (1×10^6 s.c.) were inoculated into the dorsal regions of the Kunming mice. The next day, the mice were randomly allocated ($n=5$) to five groups: control group (vehicle only); liposome (Invitrogen) group (local tumor injection of liposome 1.25 mg/kg/d); bleomycin group (bleomycin 5 mg/kg/d i.p.); E1A protein group (local tumor injection of E1A protein 2.5 mg/kg plus liposome 1.25 mg/kg/d); and combination group (bleomycin 5 mg/kg and local tumor injection of E1A protein 2.5 mg/kg plus liposome 1.25 mg/kg/d i.p.). The E1A protein and liposome were gently mixed at a ratio of 2 : 1 (M/M) and incubated at room temperature for 30 min to allow formation of the E1A protein/liposome complexes before injection. The mice were treated on days 3, 5, 7, 9, 11, and 13 after tumor inoculation. Tumor diameters were measured and the tumor volume (mm^3) was calculated as (length)$\times$(width)$^2\times0.52$, as previously reported.[17] At the end of the experiment, mice were killed by decapitation. The tumors were dissected and cleaned and samples were used for tunnel assay analyses.

Tunnel Assay Tissue samples obtained as described above were fixed in 10% formalin and embedded in paraffin. After deparaffinization, the tissue samples were stripped of

protein by incubation with 20 μg/ml proteinase K (Sigma Chemical Co.) for 15 min at room temperature. Tunnel staining was performed using an apoptosis *in situ* detection kit (AP Boehringer Mannheim) according to the manufacture's directions.

RESULTS

Expression of Secreted E1A Protein The positive strains secreted E1A protein on the nitrocellulose membrane (Fig. 1). The strains that highly expressed the E1A protein resulted in an intensive brown cycle. A light brown cycle or noncycle appeared in strains that expressed low or no E1A protein. The strains with intensive brown cycles were used as engineering yeast strains. Recombinant *P. pastoris* strains (GS115-pPIC9/E1A) were examined in shaking flask cultures at 1, 2, 3, 4, and 5 d. Aliquots of the cultures were centrifuged, and the supernatants were concentrated 10-fold and tested for the expression of E1A protein. The amount of E1A protein in the culture broth reached a maximum at 3 d of induction (data not shown), and the E1A protein (3 mg/l) was secreted into the BMMY medium as active and soluble forms (Fig. 2A).

Characterization of E1A Protein from the Supernatant Fraction The E1A protein was purified from the crude induced supernatant by two steps of column chromatography, and the purified E1A protein showed a single protein band with a molecular weight of 27 kDa on a 10% SDS-PAGE gel (Fig. 2B). E1A protein was confirmed by Western blotting using E1A monoclonal antibody (Fig. 2C).
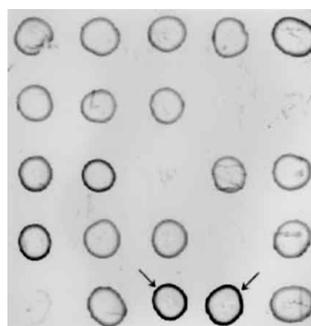
Fig. 1. Screening the High Expression-positive Yeast Strains

←: E1A protein high-expression yeast strains.
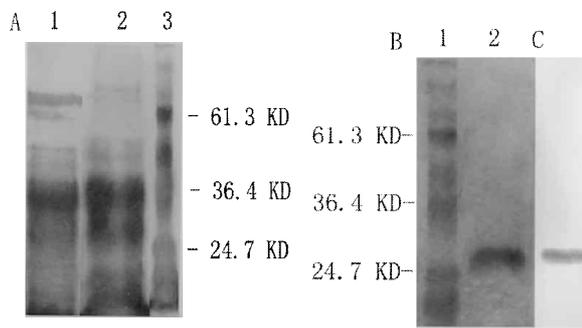
Fig. 2. SDS-PAGE and Western Blot Analysis

(A) SDS-PAGE of culture supernatant, uninduced (lane 1); induced (lane 2); marker of molecular weight (lane 3). (B) SDS-PAGE of the purified E1A protein, marker of molecular weight (lane 1); purified E1A protein (lane 2). (C) Western blot analysis of the purified E1A protein.

E1A Protein/Liposome Inhibition of S-180 Tumor Growth *in Vivo* Inoculation of 4×10^5 S-180 carcinoma cells induced tumor growth in Kunming mice, with average tumor volumes of $62 \, \text{mm}^3$ 3 d after inoculation (Fig. 3). The average tumor volume in the liposome or bleomycin group on day 15 was found to be about $970 \, \text{mm}^3$, but the differences from the controls were statistically not significant ($p > 0.05$). E1A protein/liposome injected locally into tumors every 2 d reduced the tumor volume to $610 \pm 168 \, \text{mm}^3$ (50% of the control volume). In contrast to the control, liposome, or bleomycin group, E1A protein/liposome significantly inhibited the growth of tumors ($p < 0.05$). Combination therapy consisting of E1A protein/liposome and bleomycin reduced the tumor volume to $366 \pm 98 \, \text{mm}^3$ ($p < 0.01$) compared with the mean tumor volume of $1247 \pm 203 \, \text{mm}^3$ in the control mice 15 d after inoculation. The tumor volume was the least in the combination group. The mice tolerated the treatment well and no mice died in any group.
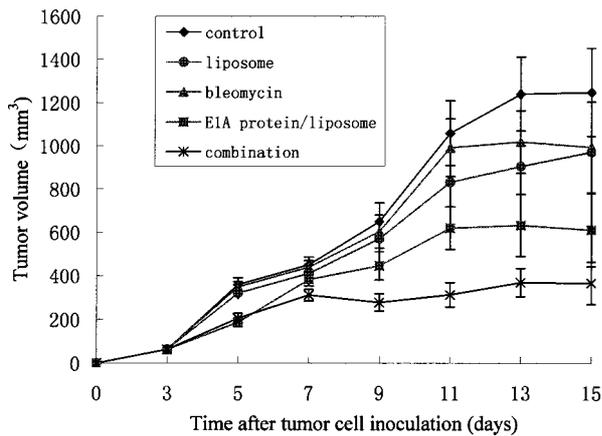
Fig. 3. E1A Protein Inhibition of S-180 Tumor Growth *in Vivo*

Injections were given on days 3, 5, 7, 9, 11, and 13 after tumor cell incubation. The data are expressed as the mean±S.D. of tumor volume ($n = 5$).

E1A Protein/Liposome Induction of Apoptosis in S-180 Cells *in Vivo* Tunnel assay clearly revealed a distinct pattern of nuclear staining. A substantial amount of cell death had already occurred in the S-180 tumors treated with E1A protein/liposome plus bleomycin or E1A protein/liposome (Figs. 4A, B), but there were more apoptotic cells in the E1A protein/liposome plus bleomycin than in the E1A protein/liposome tumor group. There were few or no cells with nuclear staining in the bleomycin, liposome, and control groups (Figs. 4C—E).

DISCUSSION

The present methods for treating tumors with the E1A gene are not satisfactory because the limitations of E1A gene therapy prevent its clinical application in inhibiting tumor growth and enhancing chemosensitivity and radiosensitivity. The E1A protein may provide a useful tool to overcome those limitations. Multiple molecular mechanisms may contribute to the tumor- and metastasis-suppression function of E1A in different cancer cell types. One attractive explanation for the tumor-suppressing function is that E1A can induce apoptosis under some conditions by either a p53-dependent or p53-independent mechanism.[18] E1A may also suppress tumors by modulating the tumor cell immune response and control cell proliferation by repressing the expression of the growth factor-inducible genes.[19]

In this study, using the yeast expression system *P. pastoris*, we expressed soluble E1A protein that could inhibit tumor growth, enhance chemosensitivity, and overcome the gene therapeutic limitations. We first examined the effects of the E1A protein/liposome alone or in combination with bleomycin in S-180 carcinoma in Kunming mice. Liposome, consisting of water surrounded by bilayered phospholipid membranes, can be used to encapsulate therapeutic agents to alter the distribution of the drug within the body. The utility of liposomes as drug-delivery systems is based on their
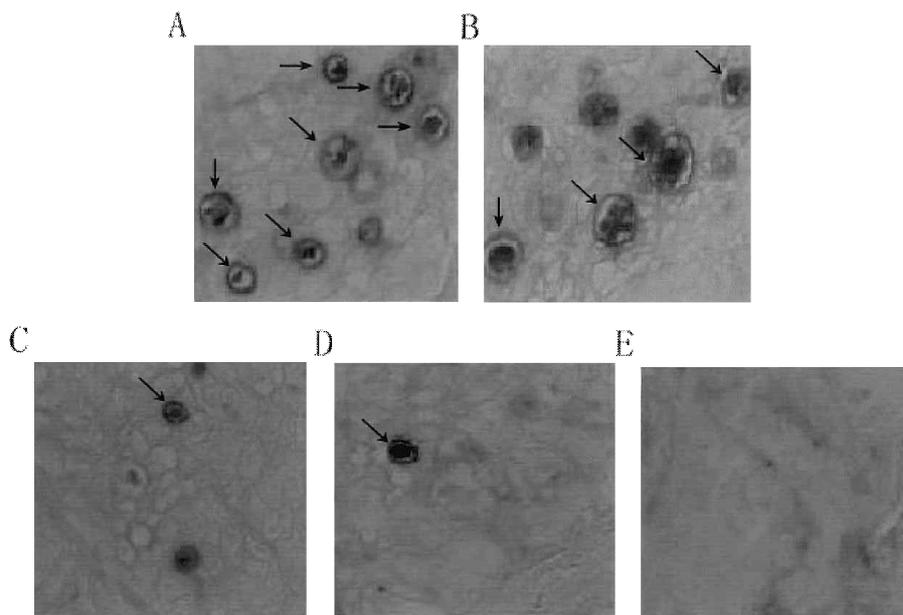
Fig. 4. Tunnel Assay of S-180 Tumor Tissue

Paraffin sections from S-180 tumors in Kunming mice treated with various regimens. (A) Combination (E1A protein/liposome plus bleomycin); (B) E1A protein/liposome; (C) bleomycin; (D) liposome; (E) Control (phosphate-buffered saline). Apoptotic cells are marked by arrows.

lipid/water structure and particulate nature. It has been shown that liposome can be used to encapsulate therapeutic agents for delivery to specific organs of the body and can be administered to the body in a variety of ways.[20] S-180 carcinoma cells growing in Kunming mice were chosen for tumor growth experiments because this tumor is relatively resistant to chemotherapy or radiotherapy. Our results suggest that the application of the E1A protein/liposome could inhibit tumor growth and convert partially or totally drug-resistant tumors into drug-sensitive tumors. Thus the E1A protein for treating cancer combined with conventional chemotherapy or radiation might be developed and applied for a greater therapeutic effect. Apoptosis is an important mechanism in the cytocidal effect of anticancer drugs or radiation. Tunnel assays indicated that the mechanism underlying this inhibition was induction of apoptosis in cells in S-180 tumor tissues. These results raise the prospect of developing applications of the E1A protein for therapy of diverse human cancers.

In summary, our results show that the E1A protein/liposome effectively inhibited the growth of S-180 tumors in Kunming mice, producing a significant reduction in final tumor volume. Thus the E1A protein from *P. pastoris* may be an appropriate choice as an anticancer therapeutic agent.

REFERENCES

1)   Moran E., Mathews M. B., *Cell*, **48**, 177—178 (1987).
2)   Chinnadurai G., *Oncogene*, **7**, 1255—1258 (1992).
3)   Frisch S. M., *Mutat. Res.*, **350**, 261—266 (1995).
4)   Yu D., Wolf J. K., *Cancer Res.*, **53**, 891—898 (1993).
5)   Chang J. Y., Xia W., *Oncogene*, **14**, 561—568 (1997).
6)   Frisch S.M., *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 9077—9081 (1991).
7)   Frisch S. M., *J. Cell. Biol.*, **127**, 1085—1096 (1994).
8)   Frisch S. M., Dolter K. E., *Cancer Res.*, **55**, 5551—5555 (1995).
9)   Sanchez-Prieto R., Quintanilla M., *Oncogene*, **13**, 1083—1092 (1996).
10)   Lowe S. W., Ruley H. E., *Cell*, **74**, 957—967 (1993).
11)   Ueno N. T., Bartholomeusz C., *Clin. Cancer Res.*, **6**, 250—259 (2000).
12)   Roth J. A., Cristiano R. J., *J. Natl. Cancer Inst.*, **89**, 21—39 (1997).
13)   Malissard M., Zeng S., *Biochem. Biophys. Res. Commun.*, **267**, 169—173 (2000).
14)   Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
15)   Towbin H., Staehelin T., *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 4350—4354 (1979).
16)   Efferth T., Volm M., *Cancer Lett.*, **70**, 197—202 (1993).
17)   Yatusnami J., Tsuruta N., *Clin. Exp. Metastasis*, **17**, 119—125 (1999).
18)   Frisch S. M., Reich R., *Oncogene*, **5**, 75—83 (1990).
19)   Whyte P., Williamson N. M., *Cell*, **56**, 67—75 (1989).
20)   Ostro M. J., *Drug Ther.*, **1992**, 61—65 (1992).