

Apoptosis induced by 5-flucytosine in human pancreatic cancer cells genetically modified to express cytosine deaminase

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KEY WORDS apoptosis; flucytosine; fluorouracil; pancreatic neoplasms; DNA fragmentation; flow cytometry; gene therapy; cytosine deaminase

ABSTRACT

AIM: To elucidate the pattern of 5-flucytosine (5-FC)-induced apoptosis and its role in gene therapy of human pancreatic cancer. **METHODS:** The human pancreatic cancer SW1990 cells (CEA-producing) were infected with recombinant adenoviruses (Adex1CEA-prCD or Adex1CEA-prZ). Expression of CD gene protein was examined by western blot. Apoptosis induced by 5-FC in human pancreatic cancer SW1990 cells genetically modified to express cytosine deaminase was observed by means of electron microscopy, DNA electrophoresis, and flow cytometry analysis techniques. **RESULTS:** The SW1990 cells infected with Adex1CEA-prCD were treated with 5-FC at $100 \mu\text{mol} \cdot \text{L}^{-1}$ for 48 h, and cell apoptosis was observed. Typical apoptosis morphological feature appeared and DNA ladder could be demonstrated on DNA electrophoresis. Apoptosis peak was also showed by flow cytometry. Apoptotic cells accounted for 34.6 % of the cell population. Cells in G₁, S, and G₂/M phase of cell cycle were 64 %, 11 %, and 7 %, respectively. **CONCLUSION:** The apoptosis induced by 5-FC may be a primary mechanism in CD gene therapy of pancreatic cancer.

INTRODUCTION

Carcinoma of the pancreas is the fifth leading cause of cancer death in the Westerners. Although extensive surgical operation has been combined with chemotherapy and radiotherapy, little progress has been made in overall

survival, and the 5-year survival rates for all patients is less than 5 %^[1,2]. Conventional chemotherapeutic agents are effective only at high doses. In order to effect a cure, patients may be subjected to great suffering which results from toxic side-effects of the agents. There is a clear clinical need for improved treatments of pancreatic cancer.

Gene therapy approaches may offer unique methods to achieve anticancer effects. Recently, various system of somatic gene therapy have been developed for pancreatic cancer, including genetic prodrug activation therapy, genetic immunomodulation, genetic replacement, and antisense technology. The *Escherichia coli* cytosine deaminase (*E coli* CD) gene is a new example of a suicide gene. A number of studies have reported that expression of CD in cells following gene transfer confers relative sensitivity to the prodrug 5-flucytosine (5-FC)^[3-5]. From a mechanistic standpoint, previous works have focused on the effect of a bystander-mediated cell killing^[6]. In this study, we have investigated the changes of cytomorphology and cell cycle induced by 5-FC in human pancreatic cancer SW1990 cells genetically modified with CD and provide evidence for a mechanism of 5-FC-induced apoptosis.

MATERIALS AND METHODS

Cell cultures and reagents The pancreatic cancer SW1990 cells (CEA-producing) were maintained in RPMI 1640 medium containing 10 % FBS. The transformed human embryonic kidney cell line 293 (kindly provided by Prof CHEN Shi-Shu, Shanghai Second Medical University, China) was grown in MEM containing 10 % FBS. The anti-CD monoclonal antibody (mAb 16D8F2) was kindly provided by Prof Knebel Doeberitz M V (Heidelberg University, Germany). 5-flucytosine (5-FC) and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium-bromide (MTT) were the products of Sigma.

Recombinant adenovirus and adenoviral in-

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Phn 86-20-8133-2439. Fax 86-20-81871853.

Received 1999-08-17

Accepted 1999-12-27

fection Adex1CEA-prCD (adenovirus containing a CEA promoter and CD gene) and Adex1CEA-prZ (adenovirus containing a CEA promoter and the LacZ gene) were gifted by Dr Hamada (Cancer Institute, Japanese Foundation for Cancer Research, Tokyo). The recombinant viruses were subsequently propagated in 293 cells. The viral solutions were stored at -80°C until used. Viral titers were determined by plaque assay using 293 cells. SW1990 cells 1×10^6 were infected with adenoviruses (adex1CEA-prCD or adex1CEA-prZ) at a moi of 100. 24 h later, cells were cultured in 5-FC $100 \mu\text{mol} \cdot \text{L}^{-1}$ for 48 h. Then cell apoptosis and cytotoxicity were analyzed.

Western blot analysis Transfected cells were lysed in a lysis solution. The supernatants were stored at -80°C . SDS-PAGE, electroblotting to nitrocellulose membrane, and immunoreaction were performed as described previously^[7].

Cytotoxic assay The cytotoxicity of 5-FC was measured by the MTT assay^[8].

Morphological analysis After being treated with 5-FC $100 \mu\text{mol} \cdot \text{L}^{-1}$ for 48 h, the cells were fixed in 2.5 % glutaraldehyde solution and postfixed in 2 % osmium tetroxide, then dehydrated and embedded. Thin sections were photographed by transmission electron microscope.

Assay for DNA fragmentation DNA was prepared by phenol-chloroform extraction. DNA ($5 \mu\text{g}$) was separated by 2 % agarose gel electrophoresis. The gel was then stained with ethidium bromide and photographed under a UV transilluminator.

Flow cytometry analysis Cells were collected and fixed in 70 % ethanol at 4°C overnight. Subsequently, cells were treated with Tris HCl buffer (pH 7.4) containing 1 % RNase A and stained with propidium iodide (PI) $5 \text{ mg} \cdot \text{L}^{-1}$. Distribution of cells with different DNA contents was determined by flow cytometer and the data were analyzed by multicycle DNA content and cell cycle analysis software.

Statistical analysis Results were expressed as $\bar{x} \pm s$ and analyzed by *t* test.

RESULTS

Expression of CD Western blot analysis demonstrated that there was positive expression of CD gene protein (52kDa) in SW1990/CD cells. In contrast, the protein could not be detected in either the wild type SW1990 cells or SW1990/Lac Z cells (Fig 1).

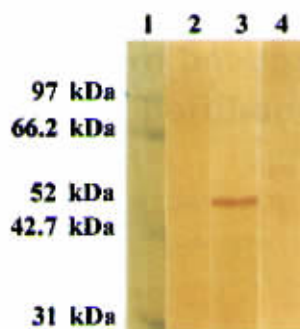


Fig 1. Expression of CD gene in SW1990 cells by Western blot. Lane 1, Marker; lane 2, SW1990; lane 3, SW1990/CD; lane 4, SW1990/Z.

5-FC mediated growth inhibition *in vitro*

The wild type SW1990 cells were used as a control in the cytotoxicity assay. The median inhibitory concentration calculated for SW1990 cells and SW1990/LacZ cells indicated resistance to 5-FC ($\text{IC}_{50} > 1000 \mu\text{mol} \cdot \text{L}^{-1}$). However, transduction of CD gene made SW1990 cells highly sensitive to 5-FC. The IC_{50} was about $50 \mu\text{mol} \cdot \text{L}^{-1}$. A profound inhibitory effect on cell growth was shown in SW1990/CD cells (Fig 2).

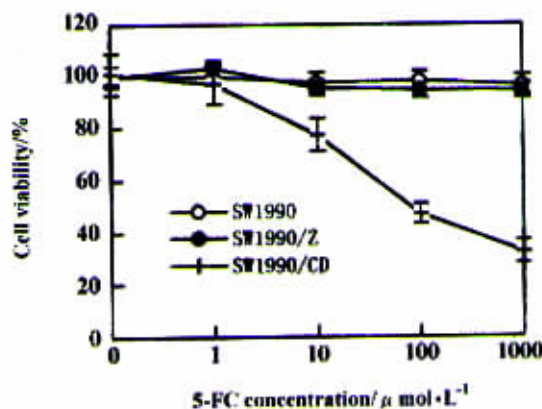


Fig 2. Effect of 5-FC on growth of SW1990 cells, SW1990/LacZ cells, and SW1990/CD cells.

Effect of 5-FC on apoptosis of transduced SW1990/CD cells The characteristic features of apoptosis exhibited in SW1990/CD cells after being cultured in $100 \mu\text{mol} \cdot \text{L}^{-1}$ 5-FC for 48 h included chromatin aggregation, nuclear material condensation and migration to the periphery. In contrast, these features were not observed in SW1990 cells and SW1990/LacZ cells treated in the same way.

Apoptosis induced by 5-FC was further examined with the DNA fragmentation assay, which is considered

as a hallmark of apoptosis. SW1990/CD cells produced a smear of different sized DNA fragments and a distinct oligosomal ladder, a typical character of cells undergoing apoptosis. In contrast, neither SW1990 cells nor SW1990/LacZ cells showed detectable DNA fragments (Fig 3).

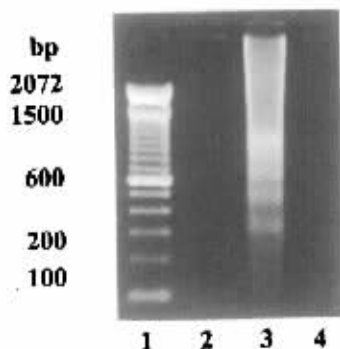


Fig 3. Effect of 5-FC $100 \mu\text{mol} \cdot \text{L}^{-1}$ on DNA fragmentation in apoptotic cells. Lane 1, 100 bp DNA marker; lane 2, SW1990; lane 3, SW1990/CD; lane 4, SW1990/LacZ.

Compared to SW1990 cells, SW1990/CD cells showed an increased apoptosis rate in flow cytometry. The apoptosis rates were 1.0 and 42.7 per cent, respectively ($P < 0.001$). An apoptosis peak was shown in SW1990/CD cells treated with 5-FC (Fig 4).

Cell cycle After treatment of SW1990/CD cells with 5-FC $100 \mu\text{mol} \cdot \text{L}^{-1}$ for 48 h, the G_1 -phase cells varied from 60 % to 64 %, and G_2/M -phase cells from 14 % to 7 %, whereas S-phase cells were markedly decreased from 21 % to 11 %. The percentage of apoptotic cells concurrently increased from 1.0 % to 34.6 % in a concentration-dependent manner (Tab 1).

DISCUSSION

Cytosine deaminase is an enzyme found in a variety of bacteria and fungi. Mammalian cells can not produce this enzyme. In our study, CD gene has been transduced into SW1990 cells by recombinant adenoviral vectors. Western blot confirmed the CD protein product at 48 h after infection. Expression of the CD gene has no intrinsic anticancer effect and must be combined with systemic administration of the nontoxic prodrug 5-FC. The CD/5-FC gene therapy is widely shown to be very effective for the treatment of a variety of transplanted tumors *in vivo*^[9,10]. However, the mechanism has not been

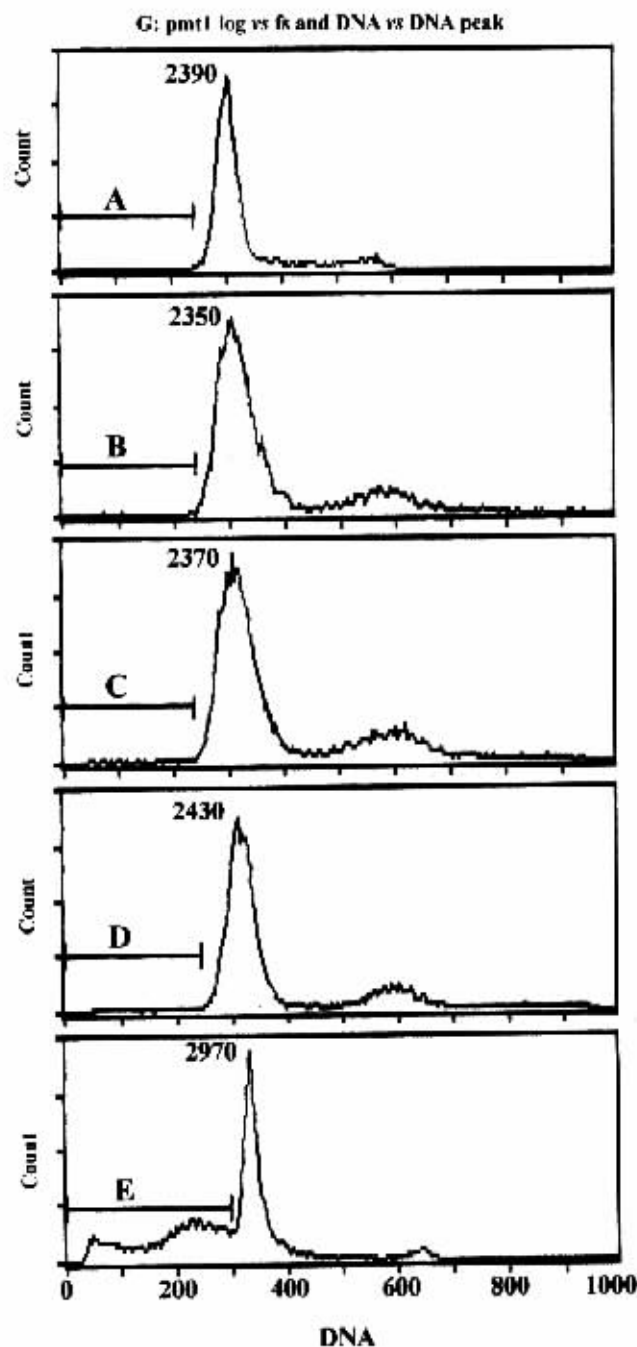


Fig 4. Induction of apoptotic marker in SW1990/CD cells as determined by flow cytometry. A SW1990, B SW1990/Z, C SW1990/CD, D SW1990/Z + 5-FC, E SW1990/CD + 5-FC.

fully known.

Apoptosis is an important physical form of cell death. It is a highly regulated process that involves the activation of a cascade of molecular events leading to cell death that is characterized by plasma membrane blebbing, shrinkage, chromatin condensation, chromosomal DNA

Tab 1. Effect of 5-FC on the cell cycle of SW1990/CD cells. $n=3$ samples (10^6 cells in each sample). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ vs control.

5-FC $\mu\text{mol}\cdot\text{L}^{-1}$	Cells cycle distribution/%			apoptosis /%
	G_1	S	G_2/M	
0	60 \pm 4	21 \pm 5	14 \pm 2	1.0
1	61 \pm 6 ^a	20 \pm 4 ^a	10 \pm 6	1.1 ^a
10	63 \pm 6 ^a	18 \pm 2 ^a	11 \pm 3	6.3 ^a
100	64 \pm 3 ^b	11 \pm 4 ^b	7 \pm 5	34.6 ^b

fragmentation, and formation of membrane-bound apoptotic bodies that are eventually phagocytized by neighboring cells⁽¹¹⁾. In the present study, we attempted to reveal the possible mechanisms by which 5-FC exerted anticancer effects on SW1990 cells genetically modified to express CD. Our study has shown that transduction of CD gene made human pancreatic cancer cell SW1990 become highly sensitive to 5-FC. The internucleosomal fragmentation of DNA, which resulted in a ladder type pattern comprising 180 ~ 200 bp intervals in gel electrophoresis, was a key molecular feature event in apoptosis. A dose-response effect of 5-FC on apoptosis has been observed in flow cytometry analysis. Furthermore, at the concentration of 100 $\mu\text{mol}\cdot\text{L}^{-1}$, 5-FC increased the percentage of cell population in the G_1 phase, but reduced it in S phase. These changes in cell cycle demonstrated that 5-FC induced cell cycle blockage in SW1990 cells transduced by CD gene.

Compared with SW1990/CD + 5-FC, neither SW1990/CD nor SW1990/5-FC produced apoptosis. However, CD has the ability to deaminate cytosine to uracil. It is also able to convert 5-FC to 5-FU, which exerts its toxic effect by interfering with DNA and protein synthesis including the substitution of uracil by 5-FU in RNA and the inhibition of thymidilate synthetase by 5-fluorodeoxyuridine monophosphate⁽¹²⁾. The apoptosis features mainly existed in the nucleus, and demonstrated that the cell death was caused by the disruption of DNA biosynthesis.

In conclusion, Our data provides strong evidence that 5-FC may induce apoptosis in human pancreatic cancer cells, genetically modified to express cytosine deaminase. The results indicate that inducing of apoptosis in

SW1990 cells is one of the mechanisms by which CD/5-FC gene therapy produces an anticancer effect on human pancreatic cancer.

REFERENCES

- Haycox A, Lombard M, Neoptolemos J, Walley T. Review article; current treatment and optimal patient management in pancreatic cancer. *Aliment Pharmacol Ther* 1998; 12: 949-64.
- Huguier M, Mason NP. Treatment of cancer of the exocrine pancreas. *Am J Surg* 1999; 177: 257-65.
- Rigg AS, Lemoine NR. Genetic prodrug activation therapy for pancreatic cancer. *Ann NY Acad Sci* 1999; 880: 319-25.
- Austin EA, Huber BE. A first step in the development of gene therapy for colorectal carcinoma cloning, sequencing and expression of *Escherichia coli* cytosine deaminase. *Mol Pharmacol* 1993; 43: 330-7.
- Zhang LH, Zheng WD, Ling WY, Bin GU, Zhi GH, Qin LY, et al. Gene therapy for human hepatocellular carcinoma with cytosine deaminase gene and prodrug flucytosine. *Acta Pharmacol Sin* 1999; 20: 440-4.
- Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994; 91: 8302-6.
- Haack K, Moebius U, Knebel Doeberitz M V, Herfarth C, Schackert HK, Gebert JF. Detection of cytosine deaminase in genetically modified tumor cells by specific antibodies. *Hum Gene Ther* 1997; 8: 1395-401.
- Dole M, Nunez G, Merchant AK, Maybaum J, Rode CK, Bloch CA, et al. Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. *Cancer Res* 1994; 54: 3253-9.
- Evoy D, Hirschowitz EA, Naama HA, Li XK, Crystal RG, Daly JM, et al. *In vivo* adenoviral-mediated gene transfer in the treatment of pancreatic cancer. *J Surg Res* 1997; 69: 226-31.
- Kanai F, Lan KH, Shiratori Y, Tanaka T, Ohashi M, Okudaira T, et al. *In vivo* gene therapy for alpha-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res* 1997; 57: 461-5.
- Steller H. Mechanisms and genes of cellular suicide. *Science* 1995; 267: 1445-9.
- Haberkm U, Oberdorfer F, Gebert J, Morr I, Haack K, Weber K, et al. Monitoring gene therapy with cytosine deaminase; *in vitro* studies using tritiated-5-fluorocytosine. *J Nucl Med* 1996; 37: 87-94.

5-FC 诱导胞嘧啶脱氨酶基因修饰的胰腺癌细胞凋亡的研究

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关键词 凋亡; 氟胞嘧啶; 氟脲嘧啶; 胰腺肿瘤; DNA 片段; 流式细胞术; 基因疗法; 胞嘧啶脱氨酶

目的: 探讨 5-氟胞嘧啶(5-FC)对 CD 基因修饰的胰腺癌细胞凋亡的影响及特征。**方法:** 以腺病毒介导的

CD 基因转染胰腺癌 SW1990 细胞, 以 Western blot 检测基因蛋白表达, 通过细胞形态学、DNA 凝胶电泳和流式细胞术观察 5-FC 对表达 CD 基因的 SW1990 细胞凋亡的影响作用。 **结果:** 以含 CD 基因的重组腺病毒转染的 SW1990 细胞, 给予 5-FC ($100 \mu\text{mol} \cdot \text{L}^{-1}$), 培养 48 h, 细胞出现典型的凋亡形态、DNA 梯带改变及凋亡峰, 细胞在 G_1 、S 和 G_2/M 各期分别为 64%、11% 和 7%, 凋亡率达 34.6%。 **结论:** 5-FC 的上述诱导凋亡作用可能是胰腺癌 CD 基因疗法的重要机制。

(责任编辑 吕 静)