Systemic delivery of full-length C/EBPβ / liposome complex suppresses growth of human colon cancer in nude mice

Li SUN, Bei Bei FU, Ding Gan LIU*

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

ABSTRACT

C/EBPβ (CCAAT/enhancer-binding protein β) is an important transcription factor involved in cellular proliferation and differentiation. Overexpression of the full-length C/EBPβ protein results in cellular growth arrest and apoptosis. Using a nonviral liposome as carrier, we delivered the full-length C/EBPβ expression plasmid, pCN, into nude mice bearing CW-2 human colon cancer tumors *via* tail vein. Southern blots revealed that the major organs and tumors were transfected. Experimental gene therapy showed that a strong suppression of tumor growth was observed in the pCN-treated mice, and such suppression was due to the overexpression of C/EBPβ, leading to the increased apoptosis in tumors of pCN-treated mice. No apparent toxic effects of pCN/liposome complex were observed in the animals. Thus, C/EBPβ has tumor suppression effect *in vivo* and may be used in gene therapy for cancers.

Keywords: tumor supression, C/EBPβ, colon cancer, apoptosis.

INTRODUCTION

Colorectal cancer is one of the most common cancers of the gastro-intestinal tract. About one million cases of the colorectal cancer are diagnosed worldwide every year, and a half of them die [1]. Treatments currently being applied to this malignant cancer, such as operation, chemotherapy and radiotherapy, are not satisfactory; therefore, development of novel, more effective therapies remains a task for molecular biologists.

C/EBPβ, also called NF-IL6, is a member of the CCAAT-enhancer binding protein (C/EBP) family of transcription factors [2-4]. These transcription factors are known to be involved in the regulation of cell growth and differentiation of several cell types [5-7]. They are expressed in a time-dependent pattern in the gastro-intestinal tract during embryogenesis in mice [8] and in the differentiation of enterocytes in adult mice [9]. C/EBPβ is also involved in antioxidant- or deoxycholic acid-induced apoptosis of colorectal cancer cells [10, 11].

It was demonstrated that the C/EBPβ protein is essential for lymphocyte differentiation [5], and is necessary for the antitumor cytotoxicity of murine macrophages [12]. Transfection of C/EBPβ to murine abdominal resident macrophages significantly enhanced their cytotoxicity to tumor cells [13]. An important property of C/EBPβ is that C/EBPβ has no intrinsic transformation ability [14, 15], as they cannot transform even such cells as NIH-3T3 [14], one of the most easily transformable cell lines. Overexpression of exogenous C/EBPβ can induce apoptosis in various malignant cells [5, 16]. Moreover, Fas-induced apoptosis in mouse hepatocytes is dependent on C/EBPβ [17]. Nevertheless, until recently, as we know, there is no report about the effects *in vivo* of the exogenous C/EBPβ on human tumors transplanted to nude mice.

In this work, we showed that systemic administration of pCN, an expression plasmid harboring the full-length wild type C/EBPβ coding region, significantly suppressed the growth of nude mice-borne CW-2 human colon tumors, but had no apparent toxic effects on the animals. We also showed that this suppression was probably due to the highly expressed exogenous C/EBPβ protein, which induced the apoptosis of tumor cells.

MATERIALS AND METHODS

Animals, cell lines, and tumor model

All animal experiments were performed under the supervision of

*Correspondence: Ding Gan LIU
TEL: +86-21-54921135; FAX: +86-21-54921011;
E-mail: dgliu@sibs.ac.cn

the Committee on Ethics in Life Sciences, Shanghai Institutes for Biological Sciences.

The Balb/c *nu/nu* nude mice, 4~5-week-old, were purchased from the Shanghai Center for Experimental Animals, Chinese Academy of Sciences, and were kept in a sterile room of the Animal facility of our Institute. Human colon cancer cell line CW-2, originally deposited in Riken Cell Bank (RCB0778), Japan, was obtained from Cell Bank of Chinese Academy of Sciences, Shanghai, and maintained in RPMI 1640 medium supplemented with 10% newborn calf serum (Invitrogen), 0.3% glutamine, and antibiotics at 37ºC in a humidified atmosphere of 5% CO_2. Each nude mouse was injected with 8×10^6 CW-2 cells subcutaneously on the right flank. When tumors reached 40~50 mm^3 in size, the animals were randomized into groups and then used in experiments.

Plasmid construction

The expression plasmid pCN was constructed by ligating a full-length human C/EBPβ cDNA (harbored in the plasmid pBlue610, a kind gift from Shizuo AKIRA in 1992) with the eukaryotic expression vector pSVL, subjecting it under the control of SV40 promoter, and the ampicillin resistance gene was substituted with chloramphenicol resistance gene. The control plasmid pCN-ND was built by deleting the C/EBPβ cDNA insert from pCN.

Liposome and DNA/liposome complexes

DOTAP:Cholesterol(Chol) (Sigma) liposomes were prepared as described in literature [18]. Briefly, DOTAP:Chol (20 mM) stock solution and stock DNA solution diluted in 5% glucose were gently mixed in equal volumes to give a final concentration of 4 mM DOTAP: Chol-100 μg DNA in 200 μl final volume, at room temperature. The complex suspension was used for injection in 2-3 h.

Delivery test of C/EBPβ/ liposome complex

In 30 tumor-bearing mice, 24 were injected *via* tail vain with 200 μl of DNA:liposome complex suspension, containing 100 μg of DNA, using a 30-gauge syringe needle (Sigma). The remaining six mice did not undergo injection, as control. Mice were sacrificed by cervical dislocation at selected time points (2 h, 6 h, 12 h, 24 h, 48 h) postinjection, and the liver, heart, lung, spleen, kidney as well as tumors were harvested for Southern blots. The sequence of the specific probe for C/EBPβ gene is (double stranded; only one strand is shown):

5'-AGA AGA AGG TGG AGC AGC TGT CGC GCG AGC TCA GCA CCC TGC GGA ACT TGT TCA AGC AGC TGC CCG AGC CCC TGC TCG CCT CCT CCG GCC ACT G-3'.

In vivo tumor suppression effect of systemically delivered C/EBPβ / liposome complexes

Tumor-bearing mice were divided into four groups, fifteen in each group, and treated as follows: no treatment; injection of liposome only; injection of pCN-ND/liposome complex; and injection of pCN/ liposome complex. All injections were in 200 μl volume and the amounts of DNAs were 100 μg. Injections were performed *via* tail vain, one dose every three days, for a total of eight doses. At 48 h after the last injection, five mice of each group were sacrificed, the main organs and tumors were collected. And the remaining animals were kept for the observation of tumor growth. Tumor measurements were performed by using an electronic vernier caliper every third day, and tumor volumes were calculated by using the formula [19]:

$V\ (mm^3) = a \times b^2/2$,

a is the largest diameter;

b is the diameter perpendicular to a.

When the observations ended, the animals were sacrificed, the tumors were dissected and weighed.

Immunohistochemistry and histopathology

Organs and tumors were fixed in 10% neutral formalin buffer, embedded in paraffin, and sectioned by using standard methods [20]. Sections were stained by hematoxylin and eosin. C/EBPβ expression was observed by immunostaining using ImmunoCruz staining system kit with a polyclonal anti-C/EBPβ primary antibody (Santa Cruz Biotech). The tumor sections stained positive for C/EBPβ were analyzed under a bright field microscope and the positive cells were counted without the knowledge of the groups. Three fields (×200) were randomly counted for each tumor sample and the numbers were averaged. For histopathological examinations, medical experts (Shanghai Medical College, Fudan University) were entrusted with observations of the slides.

In situ apoptosis assay

Apoptotic cells were identified by TUNEL assay using a DeadEnd™ Colorimetric TUNEL System kit (Promega) according to manufacturer's instructions. The numbers of CW-2 cancer cells undergoing apoptosis were determined by counting the number of apoptotic cells per field (×200): three fields (×200) were randomly counted for each tumor sample and the numbers were averaged.

Statistical analyses

Statistical analyses were carried out using Sigmaplot 2000 software. Significance levels were determined by using Student's t-test (two-side analysis). $P<0.05$ was considered significant.

RESULTS

Distribution of pCN/liposome complex in tumor-bearing nude mice

Distribution of liposome/plasmid complex in major organs and tumors was assessed following a single injection of DOTAP:Chol/pCN in tail vein using Southern blot analysis. As shown in Fig. 1, at 2-6 h postinjection, significant amounts of exogenous C/EBPβ DNA were detected in all organs checked, as well as tumors. The majority of pCN was accumulated in the lungs, and the tumor contained similar amount of pCN as that in the liver, slightly less than in lung, and the heart had lower amount. At 48 h the amounts of pCN in tumor remained high. Therefore, the pCN/liposome was capable of delivering the C/EBPβ plasmid to every tissue of the animals in fact.

Growth suppression of subcutaneous CW-2 tumors on nude mice by systemic delivery of C/EBPβ expression plasmid

To test the tumor suppression effects of C/EBPβ gene delivered by *i.v.* injection of pCN / liposome, we divided the nude mice bearing subcutaneous CW-2 tumors into four groups as described in Materials and Methods. We com-

pared the tumor growth curve (Fig. 2A), tumor growth rate (Fig. 2B) and tumor weight (Fig. 2C) at the end of the experiment. The pCN/liposome-treated group showed the most significant tumor suppression: the tumor sizes, the overall tumor growth rates and the tumor weights were all markedly decreased, compared with controls. The decreases in tumor volume, tumor growth rate and tumor weight in the experimental group compared with the no-treatment control were 88.7%, 90.2%, and 79.9%, respectively (P <0.001); compared with the pCN-ND/liposome treated control, those decreases were 71.7%, 68.6% and 68.1%, respectively (P<0.001). Therefore, the tumor suppression after systemic injection of the pCN/liposome complex was mainly exerted by the C/EBPβ. Interestingly, tumor suppression effect was also observed in the pCN-ND/liposome group, compared with the liposome only group and no-treatment group, though this effect was clearly weaker than that of pCN/liposome. And the liposome only had no antitumor activity.

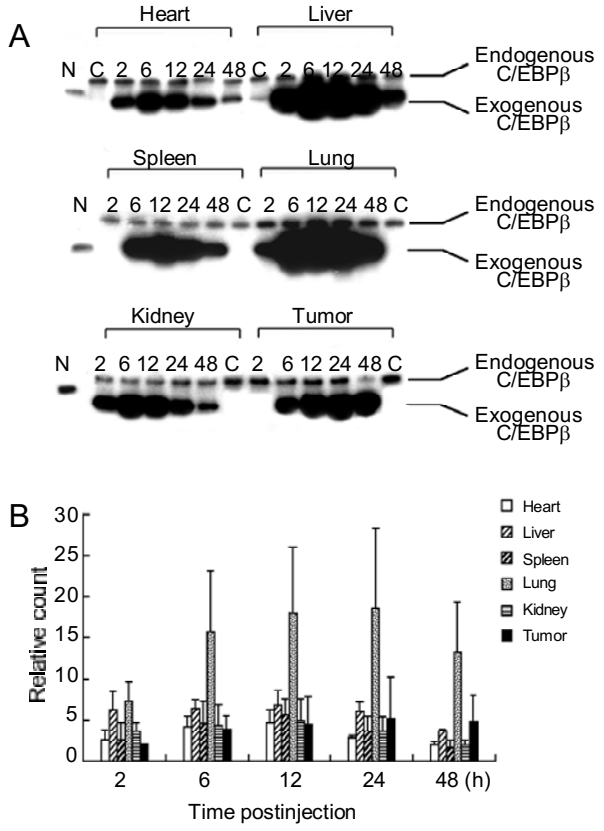

Fig. 1 Distribution of systemically injected C/EBPβ cDNA in main organs of nude mice and tumors. **(A)** Southern blots of *Eco*RI and BamHI-digested DNA from the organs and tumors (10 μg/per lane). N, copy number marker (5 copies/per cell). C, non-treatment control. **(B)** Changes with time of relative contents (copies per cell) of exogenous C/EBPβ in nude mice organs and tumors (mean±SD, n=5).

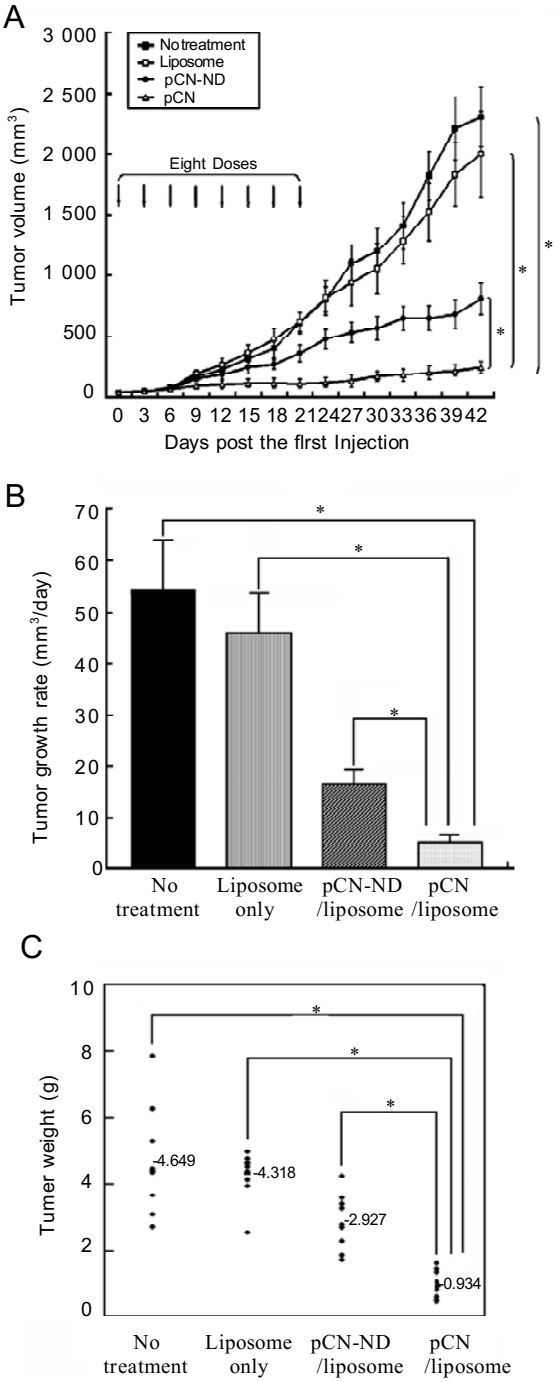

Fig. 2 *In vivo* suppression of CW-2 colon cancer tumor growth by systemic delivery of pCN/liposome complex. CW-2 tumor-bearing mice were divided into 4 groups and injected with one dose every three days, for a total of eight doses (100 μg DNA/dose). **(A)** Tumor growth curves. **(B)** Tumor growth rates calculated as tumor volume (mm^3)/day. **(C)** Comparison of tumor weights at the end of experiment. Horizontal lines represent mean values. (mean±SD, n=10). *, P<0.001.

Expression of exogenous C/EBPβ is mainly responsible for the tumor suppression

Non-specific DNA/liposome complex are reported to induce antitumor activity when administered systemically [21-23]. This is in agreement with the result of vector control group in our work. In the pCN/liposome group, however, the tumor suppression was much stronger than the pCN-ND/liposome control group; this was difficult to be explained if the DNA itself, but not the expressed protein, is assumed to cause tumor suppression. Therefore, we performed immunohistochemical assay to detect the expression of C/EBPβ protein, and the TUNEL assay to detect apoptotic cells, in the tumors 48 h after the last injection (Fig. 3). The C/EBPβ protein was clearly present in the nucleus of tumor cells treated by pCN/liposome complexes, but not in that treated by pCN-ND/liposome complexes (Fig. 3A). So, nonspecific activation of endogenous C/EBPβ by the injected DNA was ruled out. And much more apoptotic cells were detected in the tumors of pCN/liposome group than in the pCN-ND/liposome control group: at 48 h after the last injection, the average apoptotic rate (percentage of apoptotic cells) was 20.1% in tumors of pCN/liposome group, compared with 3.3% for the tumors of pCN-ND/liposome control group (Fig. 3B). As the C/EBPβ protein can induce apoptosis in malignant cells [5, 16], the co-existence of C/EBPβ protein and apoptotic cells in the same tumor strongly suggests the involvement of C/EBPβ protein in the apoptosis of cancer cells in the tumor.

In addition, on histopathological observations, the tumors that were dissected at the end of experiment all appeared as malignant, formed by the injected CW-2 cells, sharing similar histopathological characteristics. However, significant fibroblast infiltration was observed in the tumors of experimental group (Fig. 3).

The C/EBPβ/ and vector/liposome complexes did not appear toxic to the nude mice

We also observed whether the injected plasmid/liposome complexes had any toxic effects to nude mice. In the whole course of the therapy experiments, no animal that received the injections died. At the end of experiments, histopathologic observations were performed for main organs. It was found that there were no significant pathological changes in all injected animals, especially in the lung (Fig. 4), where the expression of C/EBPβ was the highest. As the loss of body weight is a generally accepted standard for the toxicity *in vivo* [24, 25], we compared the body weights of the nude mice in different groups. The result showed that no apparent body weight loss, and even some gain in body weight, was observed in plasmid/liposome-treated groups (Tab. 1). Therefore, toxic effects of the DNA/lipo-

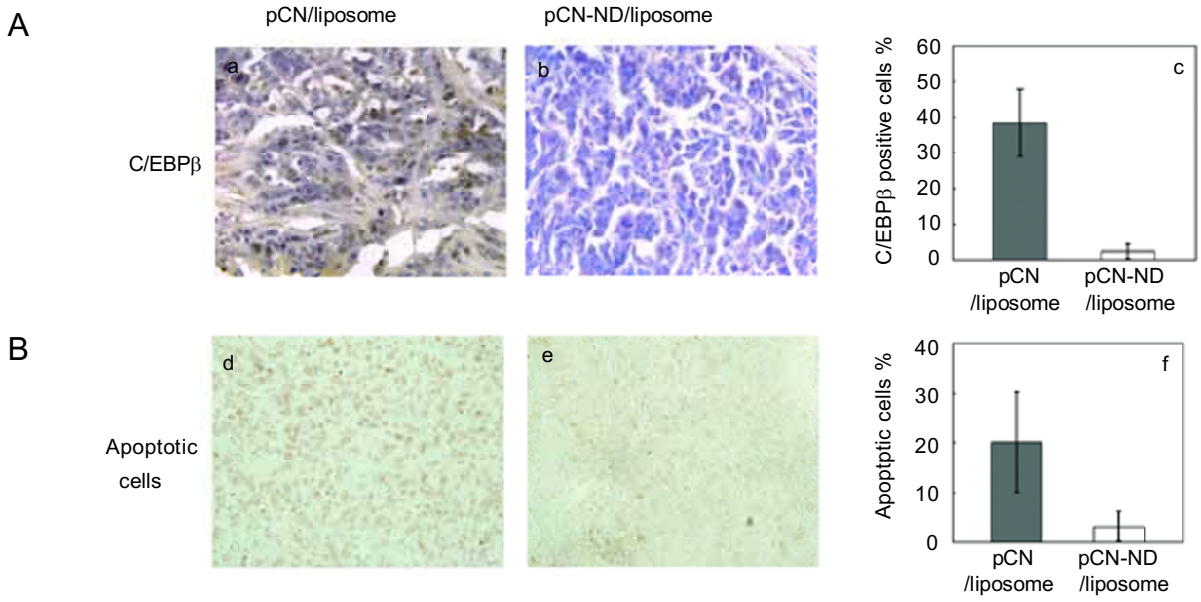

Fig. 3 Detection of expression of C/EBPβ and of apoptotic cell death in pCN/liposome complex-treated tumors. Tumors injected with pCN/liposome complex (a, d) or pCN-ND/liposome complex (b, e) were collected 48 h after the last injection for immunohistochemistry with an anti-C/EBPβ antibody (a, b) and apoptotic cell death by TUNEL staining (d, e). The percentages of the cells that express C/EBPβ (38.4%) (c), or that undergo apoptotic cell death (20.1%) (f), in tumors treated by pCN/liposome complex were significantly higher than those in tumors treated by pCN-ND/liposome complex ($P<0.001$) (mean±SD, $n=5$).

Tab. 1 Body weights of mice in different groups before and after the therapy experiment.

Group	No treatment	Liposome only	pCN/ liposome	pCN-ND/liposomeBody
Weight (g)[a] (mean±SD, n=10)	8.6±1.9	31.3±1.5	31.4±2.0	29.8±1.4
Original Body Weight (g)[b] (mean±SD, n=40)	18.9±1.6			

[a]When the experiment was ended, the tumors were dissected from the mice; the mice with tumors removed were again weighed.
[b]Before injections, the mice with tumors (about 50 mm^3) were weighed and the mean value was calculated (the weight of tumors, estimated to be 50 mg, did not affect the value of the mean).

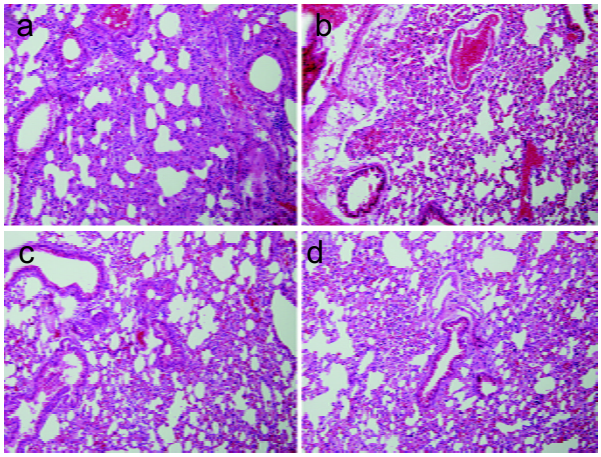

Fig. 4 Histopathologic analysis of nude mice lungs with hematoxylin-eosin stain. At the end of tumor suppression experiment, lungs were dissected for pathology analyses. **(a)**, **(b)**, **(c)** and **(d)** are, respectively, the lung from untreated mice, that from liposome-treated mice, that from pCN-ND/liposome-treated mice and that from pCN/liposome-treated mouse.

some complexes were not obviously observed.

DISSCUSION

We have shown that overexpression of the transcription factor C/EBPβ clearly suppressed the growth of human colon cancer tumor xenografts on nude mice. Furthermore, C/EBPβ overexpression induced a significant enhancement of the apoptosis in the tumors.

As our C/EBPβ cDNA was introduced into tumors and animals through a recombinant DNA plasmid, the first question is whether the tumor suppression was due to non-specific immunostimulation by the plasmid DNA; because it was well documented that plasmid DNA-liposome complex has tumor suppression effect *in vitro* and *in vivo* independently of DNA sequence [21-23, 26]. In fact, we have also observed this phenomenon in our empty vector/liposome control group. However, in our experimental group that was injected with the C/EBPβ cDNA, the tumor suppression was reproducibly much stronger than the former (P<0.001). This means that C/EBPβ did play clearly an important role in the antitumor activity of the pCN/liposome complex, besides the non-specific immunostimulation induced by its vector.

It is now known that, by translational regulation, C/EBPβ gene gives at least two different protein products: a 38 kDa full length protein, LAP and a 20 kDa truncated protein, LIP. Most studies have shown that LAP is related to maintenance of normal cellular phenotype or tumor suppression, whereas the LIP is a functional LAP antagonist and may promote transformed phenotype [27-33]. In our work, the pCN plasmid harbors the full-length C/EBP coding sequence, and the full-length C/EBP was transcribed (data not shown); however, which was the main expressed protein remains to be determined.

C/EBPβ has no intrinsic transformation ability [14, 15], which suggests that the *in vivo* use of it as a gene drug may not have any danger of tumorigenesis. C/EBPβ is a factor involved in cellular terminal differentiation [34], and it has little influence on the expression of genes that are markers of late stage of differentiation [35]; thus one can imagine that C/EBPβ may have little or no apparent proliferative effects on the well-differentiated cells. Therefore, we are inclined to think that the application of the C/EBPβ as an anticancer drug should be safe.

To sum up, our results provide evidence that the C/EBPβ has strong *in vivo* tumor suppression effect, thus this transcription factor is promising to be used in the gene therapy of cancers in the future.

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