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Anti-tumor Effects of pNEgr-mIL-12 Recombinant Plasmid Induced by X-irradiation and Its Mechanisms

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Objective To study the effect of gene radiotherapy combining injection of recombinant plasmid pNEgr-mIL-12 with local X-irradiation on cancer growth and to elucidate the mechanisms of tumor inhibition. **Methods** Alkaline lysis was used to extract the plasmid and polyethylene glycol 8000 (PEG 8000) was applied for further purification of plasmids. Enzyme-linked immunosorbent assay (ELISA) was used to detect the expression of IL-12 protein. C57BL/6J mice were subcutaneously inoculated with B16 melanoma cells and the plasmid was injected directly into the tumor. Gene-radiotherapy combining pNEgr-mIL-12 recombinant plasmid with X-irradiation was given three times to C57BL/6J mice bearing B16 melanoma. Changes in immunologic parameters of tumor-bearing mice were detected with relevant immunologic assays. **Results** Results showed a significant decrease in tumor growth rate (P<0.05-0.001) after 3 times of gene-radiotherapy with IL-12 and X-irradiation. Immunologic studies showed a significant increase in CTL and NK cytolytic activity (P<0.05-0.001) and an up-regulated secretion of IFN-γ and TNF-α (P<0.01-0.001). Moreover, the expression of mIL-12 in B16 melanoma cells of the treated tumor-bearing mice was found to be higher than that of control. **Conclusion** pNEgr-mIL-12 plasmid combined with X-irradiation can increase tumor control and the mechanism of increased tumor inhibition is related to the enhancement of anticancer immunity in tumor-bearing mice.

Key words: Interleukin 12; Gene radiotherapy; Anticancer immunity

INTRODUCTION

IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits linked by a covalent disulfide bond. Many studies have demonstrated that IL-12 is an important factor in immune response and has an effective anti-tumor function[1]. IL-12 could induce the maturation of Th1 cells from an uncommitted T cell pool and promote NK cell activity and enhance CTL maturation. In addition, the production of IFN-γ and TNF-α by NK cells is always induced. Wang *et al.* injected IL-12 cDNA into the peritumoral area of immunocompetent 129/J mice with primary vascular tumors with gene gun therapy[2]. It was demonstrated that local application of IL-12 cDNA had an anti-cancer effect on the basis of both its anti-angiogenic and immunologic effects. Moreover, local IL-12 gene therapy using retroviral vector and adenovirus vector was applied with a significant inhibition of tumor

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growth[3,4]. The injection of DNA plasmid directly into tumor has been shown to induce anti-cancer immunity in the host. The highly purified plasmid DNA could be prepared routinely and conveniently with no risk of virus infection. In the present study, naked plasmid DNA of pNEgr-mIL-12 was used in tumor gene-radiotherapy and its mode of action was studied. The preliminary results suggest that gene-radiotherapy with pNEgr-IL-12 recombinant plasmid might be a promising tool in clinical tumor treatment.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice, weighing 18±2 g, were purchased from the Animal Center of Beijing University.

Irradiation Conditions

A deep X-ray machine (X.S.S.205 (FZ) of fixation type, made in China) was used with 200 kV, 10 mA and filters of 0.5 mm Cu and 1.0 mm Al. The dose rate was 0.287 Gy/min and the distance between the X-ray source and animals was 50 cm. Local irradiation of the implanted tumor was administered with other parts of the body shielded with lead 3 mm in thickness.

Extraction and Purification of Plasmid

The pNEgr-mIL-12 expression vector that includes Egr-1 promoter and mouse interleukin 12 cDNA sequences was constructed in this laboratory[5]. The p35 and p40 subunits were linked with IRES. Alkaline lysis was used to extract the plasmid and polyethylene glycol 8000 (PEG 8000) was used to further purify the plasmid[6]. DNA concentration was determined by absorption at 260 nm and confirmed by agarose gel electrophoresis with DNA standards. Plasmid DNA was stored at -20℃ in 10 mmol/L Tris-HCl (pH 8.0) and 0.1 mmol/L EDTA. Before injection the plasmid DNA was precipitated in ethanol, washed with 70% ethanol, and dissolved in normal saline. The pNEgr control plasmid (only including Egr-1 promoter) was also constructed and purified as described above.

Enzyme-linked Immunoadsorbent Assay

Purified hamster and rat anti-mouse IL-12 p40/p70 antibody (Ab1) and biotinylated rat anti-mouse IL-12 p35/p70 antibody (Ab2) (Parmingen) were used to detect IL-12. Ab1 was diluted to 5 $\mu g \cdot mL^{-1}$ and added to a 96-well microplate, which was left at 4℃ overnight, washed three times with PBS (containing 0.1 % Tween-20), and 1 % bovine serum albumin was used to block the nonspecific binding sites for 1 h. The samples were added to the wells, washed three times after 30 min before adding Ab2 and incubation at 37℃ for 1 h. HRP-streptavidin was applied with 5 $\mu g \cdot mL^{-1}$ followed by washing three times with PBS after 30 min. OPD was added to each well and 50 μL H_2SO_4 (2 $mol \cdot L^{-1}$) was used to stop the reaction. Absorbance was measured at 490 nm.

Tumor-bearing Mice Model

B16 melanoma (5×10^5 cells/mouse in 100 μL saline) was implanted subcutaneously in the right-hind leg of C57BL/6J mice. When the diameter of the tumor reached about 3-5 mm,

the mice were divided into groups randomly. The mice receiving gene radiotherapy were injected with pNEgr-mIL-12 plasmid and irradiated locally 24 h after injection and the control mice were given saline, pNEgr plasmid or pNEgr-mIL-12 plasmid respectively as above. Tumor growth was monitored by measuring with a caliper the tumor diameters in two dimensions. The tumor volumes were calculated as follows: L (long diameter)×S^2 (short diameter)/2. The mice used for observation of changes in the immunologic parameters were sacrificed 1 d after the last irradiation.

Animal Groups

Experiment of tumor growth inhibition *in vivo* included five groups: normal control (mice transplanted B16 melanoma only), injection of pNEgr plasmid (only containing Egr-1 promoter and no IL-12 genes), injection of pNEgr-mIL-12 plasmid only, irradiation with 2 Gy and injection of pNEgr-mIL-12 plasmid combined with 2 Gy irradiation. Each group had 5 or 6 mice. Tumor growth rates were observed.

Detection of immunologic function *in vivo* included seven groups: normal control (mice transplanted B16 melanoma only), injection of pNEgr plasmid, injection of pNEgr-mIL-12 plasmid only, irradiation with 2 Gy only, injection of pNEgr-mIL-12 plasmid combined with 2 Gy irradiation, irradiation with 5 Gy only, and injection of pNEgr-mIL-12 plasmid combined with 5 Gy irradiation. Each group had 5 or 6 mice.

Injection of Plasmid Into Tumor

C57BL/6J mice bearing B16 melanoma were injected plasmid DNA directly into the tumors in 50 μL saline at three sites with a 0.25 mL syringe and a 30-gauge needle. The dose of plasmid DNA was 50 μg each time and the injection was repeated every other day for a total of three times. The control mice were injected the same volume of saline.

IFN-γ Assay

Single-cell suspensions of splenocytes from the mice treated with plasmid or saline were prepared, washed and resuspended in 10% FCS-RPMI 1640 medium. Cells (1×10^7•mL^{-1}) were cultured with ConA (20 μg/mL) for 48 h. Supernatants were collected and stored at -20℃. The activity of IFN-γ was detected with inhibition assay of cell pathological changes[7].

Induction and Detection of TNF-α

Peritoneal macrophages from mice were collected and the concentration of cells was adjusted to 2×10^6•mL^{-1}. Peritoneal macrophages (2×10^6 cells in 1 mL) were added into the 24 wells plate and cultured for 2 h, at 37℃, in 5% CO_2, washed three times with Hank's solution after culture, the non-adherent cells were discarded and the remaining cells on the surface of plates were macrophages. Macrophages were cultured with LPS (20 μg/mL) for 40 h and supernatants were collected and stored at -20℃. TNFα activity was measured with L929 cytolysis assay[7].

NK Cytotoxicity Assay

Single-cell suspensions of splenocytes from mice were prepared, washed and resuspended in 10% FCS-RPMI 1640 medium at 1×10^7•mL^{-1}. ^{3}H-TdR releasing assay was used to measure the cytotoxicity. 2×10^6 Yac-1 cells growing in a logarithm period were

co-cultured with ^{3}H-TdR (740 kBq/20 μL) at 37℃ in 5% CO_2 for 4 h, shaken once every 0.5 h and washed three times with Hank's solution after co-incubation. Cytotoxicity assay using ^{3}H-labeled YAC-1 as target cells was performed with an E: T ratio of 100:1. Triplicates were set for each sample and cpm was recorded 18 h after co-incubation with a LKB 1214 scintillation counter (Sweden).

The formula for calculating cytotoxicity is as follows: Cytotoxicity (%) = (1-[experimental cpm/self-releasing cpm]) ×100.

CTL Cytotoxicity Assay

The method was essentially the same as above. Cytotoxicity assay with ^{3}H-labeled B16 melanoma target cells was performed with an E:T ratio of 100:1. Triplicates were set for each sample and cpm was recorded 18 h after co-incubation with a LKB 1214 scintillation counter (Sweden).

The formula for calculating cytotoxicity is as follows: Cytotoxicity (%) = (1-[experimental cpm/self-releasing cpm]) ×100.

Statistics

Results were analyzed with Student's *t* test and Chi-square test.

RESULTS

The expression of pNEgr-mIL-12 recombinant plasmid *in vitro* was reported in a previous paper demonstrating that X-irradiation could activate the Egr-1 promoter to induce the expression of the downstream genes[5]. In the present paper the *in vivo* effect of gene radiotherapy with pNEgr-mIL-12 plasmid on tumor growth was studied. Fig. 1 shows that the tumor growth rate was significantly decreased (P<0.05-0.001) with a reduction of tumor volume in mice receiving gene radiotherapy to about 1/6 of the control. Moreover a decrease in death rate, an increase in surviving time (P<0.001) and a reduction of tumor weight at autopsy (P<0.05-0.01) were observed after three times of treatment with pNEgr-mIL-12 plus X-irradiation (data not shown).

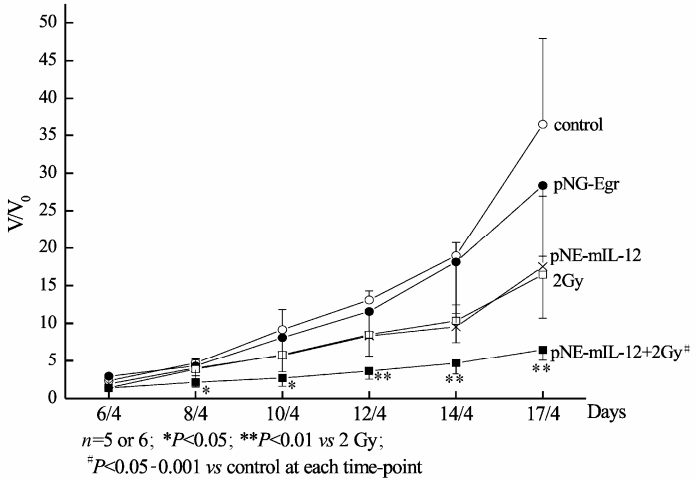
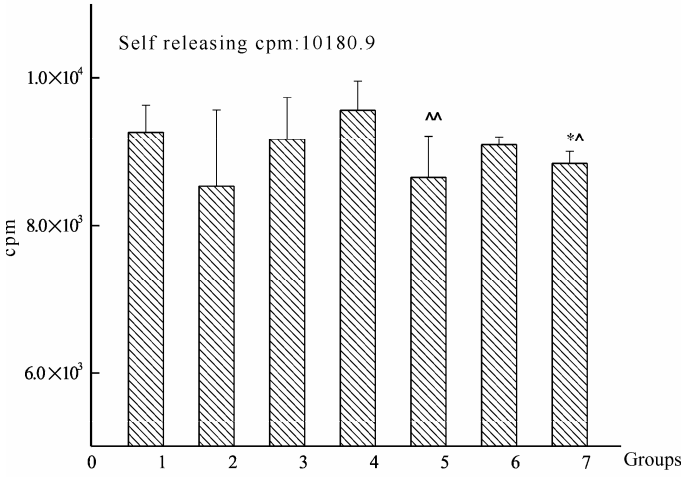


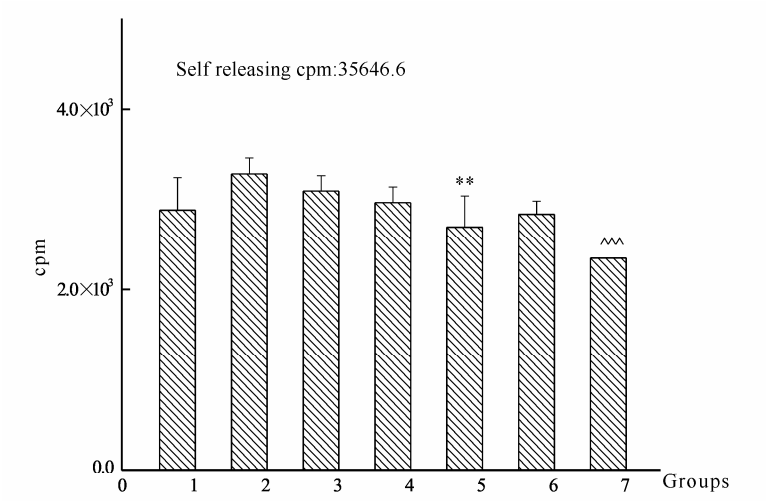
FIG.1. Effect of three times of gene-radiotherapy with pNEgr-mIL-12 and X-irradiation on tumor growth of B16 melanoma implanted in C57BL/6J mice.

Studies on the mechanism of tumor inhibition showed that NK and CTL cytotoxicity was increased significantly (P<0.05-0.001) 1 d after gene radiotherapy in comparison with control as well as with radiotherapy alone (Figs. 2 and 3).



1 Control; 2 pNG-Egr; 3 pNE-mIL-12; 4 2Gy; 5 pNE-mIL-12+2Gy; 6 5Gy; 7 pNE-mIL-12+5Gy; n=6; *P<0.05 *vs* control; ^P<0.05; ^^P<0.01; pNE-mIL-12+2 Gy *vs* 2 Gy; pNE-mIL-12+5 Gy *vs* 5 Gy

FIG. 2. Changes of CTL cytotoxic activities of C57BL/6J mice implanted B16 melanoma 1d after three times of gene-radiotherapy with pNEgr-mIL-12 and X-irradiation.



1 Control; 2 pNG-Egr; 3 pNE-mIL-12; 4 2Gy; 5 pNE-mIL-12+2Gy; 6 5Gy; 7 pNE-mIL-12+5Gy; n=6; **P<0.01 *vs* control; ^^^P<0.01; pNE-mIL-12+2Gy *vs* 2Gy; pNE-mIL-12+5Gy *vs* 5Gy

FIG. 3. Changes of NK cytotoxic activities of C57BL/6J mice implanted B16 melanoma 1d after three times of gene-radiotherapy with pNEgr-mIL-12 and X-irradiation.

Moreover, the increase of IFN-γ secretion by splenic lymphocytes and up-regulation of TNF-α by peritoneal macrophages were observed in the gene radiotherapy groups (Figs. 4 and 5). Gene-radiotherapy combined with 5 Gy irradiation showed a higher stimulatory

effect than that with 5 Gy irradiation alone ($P<0.05$).

The expression of mIL-12 (measured with ELISA 1 d after gene-radiotherapy) in the extracts of B16 melanoma cells (pooled from 3 mice) is shown in Table 1, in which higher mIL-12 contents in B16 melanoma cells from the groups with gene radiotherapy plus X-irradiation were demonstrated. It implicated that pNEgr-mIL-12 plasmid could be activated by X-irradiation with increased expression of mIL-12 to exert its anti-tumor effect.

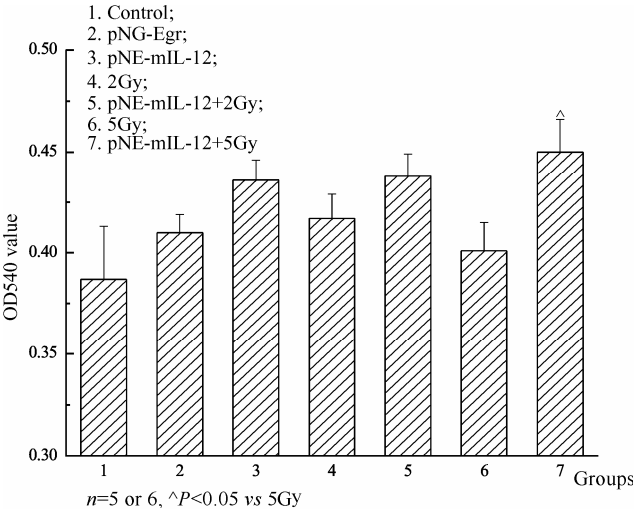


FIG. 4. Changes in IFN-γ secretion by splenocytes of C57BL/6J mice implanted B16 melanoma 1d after three times of gene-radiotherapy with pNEgr-mIL-12 and X-irradiation.

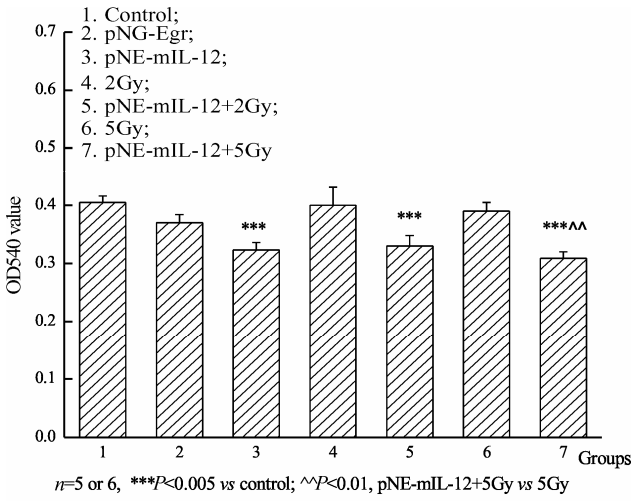


FIG. 5. Changes in TNF-α secretion by peritoneal macrophages of C57BL/6J mice implanted B16 melanoma 1d after three times of gene-radiotherapy with pNEgr-mIL-12 and X-irradiation.

TABLE 1

Expression of mIL-12 in B16 Melanoma Implanted in C57BL/6J Mice 1 d After Three Times of Gene-radiotherapy With pNEgr-mIL-12 and X-irradiation (pg/mL)

Groups	OD 490	Concentration of mIL-12 ($pg \cdot mL^{-1}$)	Weight of Tumor (mg)	Concentration of mIL-12 ($pg \cdot mg^{-1}$ Tumor)
Control	0.243	236.34	0.870	271.66
pNEgr	0.229	217.00	0.593	365.94
pNEgr-mIL-12	0.220	204.83	0.490	418.02
2 Gy	0.174	146.16	0.313	466.96
pNEgr-mIL-12+2Gy	0.122	87.69	0.061	1437.54
5 Gy	0.117	82.57	0.124	665.89
pNEgr-mIL-12+5Gy	0.189	164.62	0.062	2655.16

Note. n=3, Standard curve is as follows: y= 0.695X-2.264.

DISCUSSION

Interleukin 12 is a cytokine that has been shown to possess potent anti-tumor activity in a variety of tumor systems[8,9]. Direct injection of plasmid into tumors may be a safe and simple approach in cancer therapy. In this study, we have demonstrated that gene radiotherapy following injection of the recombinant plasmid pNEgr-mIL-12 was effective in inhibiting tumor growth and could induce an anti-tumor immune response. In 1990, Wolff first reported that intramuscular injection of DNA could result in local expression of the corresponding genes[10]. In addition, gene gun technique has obviously increased the efficiency of transfection of naked DNA into tissue. Wang has demonstrated that gene gun is a rapid, painless technique for *in vivo* gene introduction and could avoid the expensive and time-consuming *in vitro* gene introduction and cell selection[2]. Moreover, many scientists have tried to modify tumor cells with cytokine *in vitro* and to inject the modified tumor cells into mice, and the results also showed an effective inhibition. Application of retrovirus and adenovirus vectors is a hot spot in gene therapy of cancer and some meaningful results have been reported, but the safety factor is a fatal problem in using virus vectors. Now there are enough data[10,11] to show that injection of naked DNA would be a promising way to treat tumor, and data have shown that naked DNA is effective in rapidly dividing cells, and the expression of genes could be detected by immunohistochemistry assay locally. Gene medicine is different from classical gene therapy; for instance, in cancer therapy it does not need all tumor cells to be transfected with genes and only 2%-5% cells taking up genes to have the products of expression exerting the effect via autocrine or paracrine mechanism or through circulation. Moreover, one injection may have a relatively long-standing effect and injections could be repeated if necessary[12-14].

It has been demonstrated that interleukin 12 protein amplifies the immune response by stimulating the proliferation of natural killer and activated T cells, augmenting cytolysis response, and inducing the secretion of cytokines, particularly of IFN-γ by natural killer cells and T cells and TNF-α by T cells and monocytic cells. Experimental results in the present study showed that gene-radiotherapy with IL-12 could inhibit tumor growth and enhance immune functions beginning from 1 day after the treatment. The expression of mIL-12 could be detected in the B16 melanoma cells treated with pNEgr-mIL-12 plasmid and irradiation and the concentration of IL-12 was about 5.2-10 times as high as that of control. Moreover, on the basis of data from C57BL/6J mice experiment, Kunming mice-

bearing S180 sarcoma were also used to testify the results and the immunologic parameters were measured 1, 7, and 14 days after gene-radiotherapy with similar results. Furthermore, the CTL and NK cell activity in mice receiving gene-radiotherapy were maintained at a higher level until the 14th day after gene-radiotherapy and the levels of IFN-γ and TNF-α were also higher than those of the control. It was found that the treatment program of pNEgr-mIL-12 plasmid combined with 5 Gy irradiation was more effective than that of pNEgr-mIL-12 plasmid combined with 2 Gy irradiation (data not shown). It is suggested that a larger dose of radiation might be able to sustain the expression of IL-12 genes for a longer time in addition to its higher tumor-killing effect. In some reports a much larger dose, such as 20 Gy, was used to stimulate the Egr-1 promoter and its downstream genes with an anti-tumor effect[15,16]. However, 20 Gy may be too large for human patients. In the present study, the dose of 2 Gy was tried since it was the routine dose used in clinical radiotherapy and results showed that pNEgr-mIL-12 combined with 2 Gy irradiation exerted an obvious anti-tumor effect. This study would provide an experimental basis for clinical tumor gene therapy.

IL-12 gene-radiotherapy has some advantages in comparison with IL-12 protein therapy and IL-12 gene therapy. First, recombinant IL-12 protein is expensive and the dosage has to be optimized carefully since a too low dose may not be effective in tumor control and a larger dose usually has toxic or even lethal effects[17]. Second, gene therapy is generally an effective measure to inhibit tumor growth, but as a serious problem, some genes may produce toxic effect when expressed constantly after *in vivo* injection into the tissues. The Egr-1 radiosensitive promoter could solve this problem since it is possible to have the downstream gene expressed timely and locally. In other words, the target gene only expresses in response to local irradiation and so the expression is more easily controlled. Third, IL-12 gene radiotherapy has a double-killing effect both from the radiation and the immune activation to inhibit tumor growth. Finally, *in vitro* studies have shown that IL-12 protein could be detected in the supernatants of COS-7 cells and B16 melanoma cells transfected with pNEgr-mIL-12 plasmid after very low dose irradiation (0.05 Gy caused high expression of IL-12)[5]. Since total or half body irradiation with low doses has been shown to increase the efficacy of conventional radio- and chemotherapy of cancer in experimental and clinical studies[18,19], combination of low dose total body irradiation with conventional local radiotherapy of tumors with injection of pNEgr-mIL-12 plasmid should be studied in experimental trials in order to open new avenues.

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