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Experimental study on the co-expression of double genes AdvCD40L-IRES2-ICOSL in mouse breast cancer model

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Summary

Objective: This study aims to investigate the therapeutic effect of CD40L-IRES2-ICOSL adenoviral vector on mouse breast cancer model. *Materials and Methods:* CD40L, ICOSL, and CD40L-IRES2-ICOSL adenoviral vectors were constructed by gene transfection technique. Eighty BALB/c mice were selected, in which animal models of mouse breast cancer were established with the human MCF-7 breast cancer cell line. A total of 72 successful model in mice. Mice were randomly divided into four groups: control group, CD40L group, ICOSL group, and CD40L-IRES2-ICOSL group, with 18 mice in each group. Two weeks later, breast cancer cells transfected with empty vector and recombinant vectors were injected into the tumor body of mice. Histopathological changes, tumor volumes and changes in weight, and survival time of tumor bearing mice were observed. *Results:* The T infiltration of lymphocytes in tumors significantly increased in the CD40L, ICOSL, and CD40L-IRES2-ICOSL groups. Compared with the control group, tumor growth was retarded, volumes were reduced, and survival times were prolonged in tumor bearing mice in both the CD40L and ICOSL groups, and the differences between these two groups were statistically significant ($p < 0.05$). In particular, this difference was statistically significant in the CD40L-IRES2-ICOSL group ($p < 0.01$). However, there was no significant difference between the CD40L and ICOSL groups. *Conclusions:* In the treatment of the animal model of mice with breast cancer, the co-expression of double genes of Adv CD40L-IRES2-ICOSL can inhibit tumor growth and prolong the survival time of the MCF-7 human breast cancer bearing mice.

Key words: Breast cancer; Gene therapy; AdvCD40L-IRES2-ICOSL.

Introduction

Breast cancer is one of the most common malignant tumors, which has a serious impact on women's physical and mental health, and is even life-threatening. Each year, approximately 1.2 million women have been diagnosed with breast cancer worldwide, with a mortality accounted for approximately 0.5 million, and its incidence rate has gradually increased annually [1]. In China, the incidence of breast cancer has significantly increased, and it has become the most common malignant tumor in women. At present, the main treatment methods of breast cancer are surgery, radiotherapy, chemotherapy, and immunotherapy. Surgical resection is the most commonly used method, but this operation is difficult to completely remove off lesions, and brings pain to the patient. Furthermore, radiotherapy and chemotherapy are limited due to significant side effects on the human body. Therefore, the authors' main task at present was to determine an effective way to clear tumor cells thoroughly, while minimizing side effects on the body.

Immunotherapy is a new approach to the treatment of tumors following surgery, radiotherapy, and chemotherapy. This treatment has received widespread concerns due to minimal side effects, and its theoretically complete tumor cell killing in vivo. However, at present, no expected effects have been obtained in clinical applications. The key reason is that tumor immune escape plays an important role. T lymphocytes are the main effector cells in tumor immune response, and the activation of T cells dependent on co-stimulatory signals generated by the interaction of T cells and co-stimulatory molecules. Studies have shown that the expression of the major histocompatibility complex (MHC) class I molecule on the tumor cell surface is much lower than that in professional antigen presenting cells. Furthermore, T lymphocytes cannot effectively proliferate during the antitumor immune response phase. In addition, the expression of co-stimulatory molecular ligands on the tumor cell surface is significantly reduced compared to that in normal tissues around the tumor. All these contribute to the immune escape of tumor cells. CD40/CD40L is an important pathway for inter-cellular signal transmission, which can regulate cellular immunity and humoral immunity through the induction of cell activation and cytokine production, participate in intra-cellular signal transduction pathways, and play important roles in the occurrence and development of diseases such as immunodeficiency disease, atherosclerosis, tumor immunity, and inflammatory reactions [2-4]. Inducible co-stimulatory molecule (ICOS) is a new member of the CD28 family that can combine with

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its ligand (ICOSL) and enhance all base reactions of T cells against exogenous antigens. The ICOS-ICOSL co-stimulatory pathway plays an important role in the cellular immunity and humoral immunity [5, 6]. Foreign scholars have carried out much research in the field of organ transplantation against B7 and ICOS, and revealed some certain advantages in the induction of T cell proliferation, as well as in the reduction of T cell response threshold to antigenic cells. Researchers tend to focus on a single pathway to induce immune tolerance. Since the effect has not been satisfactory, tumor immune rejection response has rarely been researched and reported. Furthermore, there are no reports on comprehensive studies in China. In this experiment, tumor cells were transfected with the CD40L-IRES2-ICOSL adenoviral vector previously constructed, and histopathological changes, tumor volume and weight changes, and survival time in tumor bearing mice were observed. The effect of the CD40L-IRES2-ICOSL adenoviral vector on mouse breast cancer was investigated.

Materials and Methods

The CD40L-IRES2-ICOSL adenoviral vector was constructed and identified. MCF-7 human breast cancer cell lines were offered as a gift by the Department of Immunology of Liaoning Medical College. Eighty female BALB/c mice, age between 4-6 weeks and weighing 18-22 grams, were included.

MCF-7 human breast cancer cells were cultured in RPM I-1640 complete medium containing 10% calf serum with 5% CO2 and saturated humidity at 37°C. Cells were conventionally passage cultured. A 40 microliter cell suspension was used in 10 edged up 0.4% phenol blue staining microscopy and counted. MCF-7 human breast cancer cells in the logarithmic growth phase were injected into the armpit of the left lower limb of BALB/c mice at the concentration of 3×106 cells. Two weeks later, average tumor diameter reached 0.8 ± 0.1 cm, and body mass was 32 ± 2.30 grams. Equivalent amounts of 3×106/0.2 ml of breast cancer cells transfected with the empty vector. CD40L, ICOSL, and CD40L-IRES2-ICOSL adenoviral vectors were injected into the center points of the tumors in animals, respectively. Then, tumor growth conditions were periodically observed.

On days 14, 28, and 40 of post-vector injection, three mice were taken from each group. The tumors were cut off and isolated after anesthesia. Long and short diameters of subcutaneous transplanted tumors were determined with compasses and vernier calipers, and tumor volume was calculated according to the general formula: [V (mm^3) = length × $width^2$ × 0.52]. Tumors were weighed on an electronic balance, and tumor inhibition rate was calculated according to the following formula: Tumor inhibition rate (%) = (average tumor weight post-treatment in the control group - average tumor weight post-treatment in the treatment group) / average tumor weight post treatment in the control group × 100%. Tumor specimens were fixed with 10% formaldehyde, and the survival time of mice was observed.

The expression of CD40L and ICOSL in the paraffin sections were detected after SP staining, according to immunohistochemistry protocols. Cells stained with brown yellow particles on the cell membrane were considered positive, five visual fields under the microscope of 10×40 times magnification were selected randomly from each slice. The negative control was unstained, 100 tumor cells were counted in each field, and results were determined according to the percentage and color depth of positive cells. The 9-point scoring system was adopted: according to the percentage of positive cells: 10% was assigned as 1 point, 10%-50% was assigned as 2 points, > 50% was assigned as 3 points; according to the intensity of staining: negative was assigned as 0 point, weak staining was assigned as 1 point, moderate staining was assigned as 2 points, and deep staining was assigned as 3 points. Then, total scores were calculated in accordance with the results of "positive cell scores × staining scores", in which it was negative when total scores were < 3 points, and positive when total scores were > 3 points.

Total RNA was extracted by the Trizol method, and the purity of the total RNA was determined using a UV spectrophotometer. The sequence of the CD40L upstream primer was 5'-GACGTCAGCATGATAGAAACATACAGCCAACCT-3', and the downstream primer was 5'-GCCGAATTCTCAGAGTTT-GAGTAAGCCAAAAGA-3'. The expected amplified fragment length was 783 bp. The sequence of the ICOSL upstream primer was: 5'-AGGAAGTCAGAGCGATGGTAG-3' and the downstream primer was: 5'-AGGCTGTTGTCCGTCTTATTG-3'. The expected amplified fragment length was 469 bp. The sequence of the actin upstream primer was: 5'-AGAGGGAAATCGTGCGT-GAC-3', and the downstream primer was: 5'-CAATAGTGAT-GACCTGGCCGT-3', and the expected amplified fragment length was 138 bp. Operated strictly according to the RT-PCR kit, cDNA was synthesized by reverse transcription, followed by PCR amplification. Reaction conditions were: pre-degenerated at 94°C for five minutes, and degenerated at 94°C for 40 seconds, annealed at 60°C for 40 seconds, extended at 72°C for two minutes, for a total of 40 cycles, then extended at 72°C for five minutes. Five (one of amplified products was obtained to run the electrophoresis in 2% agarose gel at a voltage of 110 V. Electrophoresis was run for 35 minutes, scanning analysis of the strip was carried out by the gel imaging system, the gray value was calculated, and the expression level of the target band was expressed with the ratio of gray values of the target band and the β-actin band.

Data were analyzed by SPSS 13.0 software, and expressed with mean values ± standard deviation (x ± SD). Single factor analysis of variance was used to compare multi-sampled mean values, and the test level was α=0.05.

Results

This experiment successfully established the mouse breast cancer model of human breast cancer cell line MCF-7, in which eight mice failed in the establishment of this model. Tumor formation rate was 90%, and the mean diameter of the tumor reached 0.9 ± 0.3 cm two weeks later, and the mean body weight of mice were 35 ± 2.30 grams. Mice were randomly divided into four groups, with 18 mice in each group and the differences in body weights and tumor volumes of tumor bearing mice were not statistically significant ($p > 0.05$). This guaranteed the balance in each group before vector injection.

On the seventh day of post-injection of the vector, there was no significant difference in the volume and weight of tumors ($p > 0.05$). On the 14^{th} day, when the CD40L, ICOSL, and CD40L-IRES2-ICOSL groups were compared with the control group, there were differences in both tumor volume and tumor weight ($p < 0.05$), but there were no significant differences among the CD40L, ICOSL, and

Table 1. — *The effect of the tumor weight and the inhibitory rate after injection of carrier.*

Group	Time				Inhibitory rate (%) (40 days)
	7 days	14 days	28 days	40 days	
Control	0.63±0.12	1.13±0.25	2.76±0.33	4.32±0.56	—
CD40L	0.59±0.06	0.95±0.14	1.85±0.27$^{\Delta}$	2.43±0.17$^{\Delta}$	43.75
ICOSL	0.61±0.14	1.06±0.42	1.68±0.23$^{\Delta}$	2.36±0.54$^{\Delta}$	45.37
CD40L-IRES2-ICOSL	0.58±0.07	0.89±0.31	1.31±0.22$^{\Delta}$	1.65±0.32$^{\Delta}$	61.81

Δ *compared with control group, the difference was statistically significant ($p < 0.05$).*

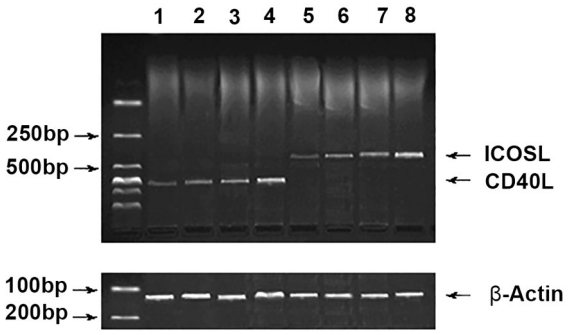

Figure 1. — Expression of mRNA in every group of CD40L and ICOSL. 1, 2, 3, and 4 are respectively CD40L in the control group, CD40L, ICOSL and CD40L - IRES2 - ICOSL mRNA expression in the group; 5, 6, 7, and 8 are ICOSL in the control group, CD40L, ICOSL group, and CD40L - IRES2 - ICOSL mRNA expression in the group.

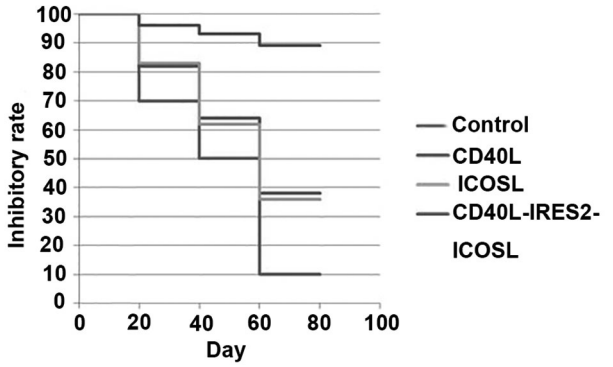

Figure 2. — Tumor injection of carrier effect on breast cancer survival in mice.

CD40L-IRES2-ICOSL groups. With the extension of time, the differences between these groups increased. On the 40th day, when the CD40L, ICOSL, and CD40L-IRES2-ICOSL groups were compared with the control group, there were differences in both tumor volume and tumor weight ($p < 0.01$). Furthermore, there were significant differences between the CD40L group and the ICOSL and CD40L-IRES2-ICOSL groups ($p < 0.01$). However, there was no significant difference between the CD40L and ICOSL groups ($p > 0.05$, Table 1).

CD40L was mainly expressed on the cell membrane of breast tumor cells, and positive cells expressed with yellow brown granules. ICOSL was mainly expressed on the cell membrane and cytoplasm of breast tumor cells, and this expression dominated on the cell membrane. On the seventh day, the staining of specimens in each group was not obvious. CD40L only revealed a weak positive staining in the control group (positive staining rate was 2.3%), while there were no obvious staining in the other groups. The differences between these groups were not statistically significant ($p > 0.05$). ICOSL was weakly stained in the ICOSL group (staining rate was 3.1%), no obvious staining were found in the other groups, and the difference between these two groups was not statistically significant ($p > 0.05$).

With the extension of time, the staining gradually deepened in each group. In specimens of the 40th day, CD40L positive rate in each group was 17.3%, 45.6%, 42.7%, and 75.5%, respectively, and there were significant differences between the experimental and the control groups ($p < 0.05$). Furthermore, there was a significant difference between the CD40L-IRES2-ICOSL, and CD40L groups and the ICOSL group ($p < 0.01$). However, there was no significant difference between the CD40L and ICOSL groups ($p > 0.05$). The ICOSL positive rate in each group was 14.6%, 38.2%, 41.8%, and 78.3%, respectively, and there was significant differences in the comparison between the experimental and control groups ($p < 0.05$). Furthermore, there was a significant difference between the CD40L-IRES2-ICOSL and CD40L groups, and the ICOSL group ($p < 0.01$), but there was no significant difference between the CD40L and ICOSL groups ($p > 0.05$).

RT-PCR was carried out on the fresh tumor tissue taken on the 40th day. RT-PCR detection results revealed that the size of the mRNA fragments of CD40L and ICOSL were 783 bp and 469 bp, respectively (Figure 1), and were consistent with the expected size of the target fragment. The mRNA expression level of CD40L in the CD40L-IRES2-ICOSL group significantly increased, compared with the other three groups. Furthermore, the ratio of CD40L/β-Actin was 0.56 ± 0.07, and the ratios in the other three groups were 0.08 ± 0.01, 0.21 ± 0.06 and 0.23 ± 0.04, respectively. The difference was statistically significant when

compared between the experimental and control groups ($p < 0.05$), and there was significant difference between the CD40L-IRES2-ICOSL and CD40L groups, and the ICOSL group ($p < 0.01$). However, there was no significant difference between the CD40L and ICOSL groups ($p > 0.05$). The mRNA expression level of ICOSL in the CD40L-IRES2-ICOSL group was significantly higher than in the other three groups. The ratio of CD40L/β-Actin was 0.63 ± 0.06, while ratios in the other three groups were 0.11 ± 0.03, 0.28 ± 0.05, and 0.31 ± 0.04, respectively, and the difference was statistically significant between the experimental and control groups ($p < 0.05$). Furthermore, there were significant differences between the CD40L-IRES2-ICOSL and CD40L groups, and the ICOSL group ($p < 0.01$). However, there was no significant difference between the CD40L and ICOSL groups ($p > 0.05$). The expression of PCR products of the internal reference β-actin had no significant differences in these two groups ($p > 0.05$, Figure 1).

The average survival time of mice in each group were as follows: 52.34 ± 4.01 days for the control group, 72.32 ± 3.23 days for the CD40L group, 73.54 ± 6.21 days for the ICOSL group, and 93.42 ± 9.25 days for the CD40L-IRES2-ICOSL group. The differences were significant when the CD40L and ICOSL groups were compared with the control group ($p < 0.05$), and the difference was extremely significant when the CD40L-IRES2-ICOSL group was compared with the control group ($p < 0.01$, Figure 2).

Discussion

The activation of antigen specific T cells requires two independent and synergistic signals [7, 8]. The first signal is provided by the combination of antigen presenting cells (APCs), MHC-antigen peptide complexes, and TCR-CD3 on T cells. The second signal is the co-stimulatory signal, which is produced by the interaction of co-stimulatory molecules expressed on the APC surface and the corresponded receptors of T cells. Studies have shown that the co-stimulatory signal could not only promote the growth, differentiation and cytokine production of T cells, but also provide a survival signal for the prevention of apoptosis of T cells. The deficiency of co-stimulatory signals would lead to T cell anergy. Furthermore, immune response can be enhanced or terminated by the regulation of co-stimulatory signal pathways [9-11].

CD40/CD40L is the most widely studied co-stimulatory pathway at present, and it is one of the necessary pathways for APC activation [12]. Dendritic cells (DCs) and B cells are the main components of APCs. APCs can recognize, process and present antigens in the immune response, and activate the immune response.

Signals produced in the interactions of CD4O-CD40L play important roles in the survival, proliferation, germinal centers formation, and memory formation of B cells, as well as the transformation of immunoglobulin types. In recent studies, it has been found that CD40L could stimulate DCs to produce pre-survival signals, which could upregulate the expression of co-stimulators (MHC11, CD58, CD80/CD86), and improve the antigen-presenting abilities of CD40+DCs. Consequently, the expression of the IL-2 receptor increased, the CD40L+ helper T cells (Th) and cytotoxic T cells (CTL) were activated, and MHC I and MHC II dependent tumor reactive T cells were amplified [13, 14]. With the activation of CD4OL+ cells, APCs further enhanced the expression of TNF-α, MIP-1α (macrophage inflammatory protein) and IL-2. As a result, the cellular immunity was promoted.

The currently known costimulatory molecules are mainly the B7 family members and CD28 family members. Interactions of the B7 family members and CD28 family members constitute an essential co-stimulatory pathway for T cells, which can trigger the antigen specific humoral and cellular immune response. Co-stimulator B7 occupies the core position in the co-stimulatory signal pathway. B7 is expressed on the surface of APCs, which is expressed simultaneously, while the APCs process antigenic peptides. It can combine with antigenic peptides, and the antigenic peptide-co-stimulator complex can combine with the corresponded T cell receptors to active T cells [15-17]. Costimulatory molecule ICOS is newly discovered in recent years, and it is essential in costimulatory signal pathways. ICOS is only expressed on the surface of activated T cells and its ligand, and ICOSL can be expressed on the surfaces of B cells, macrophages, and non-lymph node tissues. ICOSL, specifically B7h (B7 homologue), is a novel protein encoded by the full length cDNA, which was isolated from the iELcDNA library of mouse by Yashinga *et al.* [18-20]. This protein is a type 1 transmembrane glycoprotein with 20% and 19% homology to B7-1 and B7-2 at the amino acid level, respectively. It has an IgV-like and an IgC-like regions outside the cell membrane, and a hydrophobic transmembrane region, as well as a tail region in the plasma. There are conserved cysteine residues at the homologous region of 138, 185, and 242 sites, as its full-length and the relative position of the transmembrane are similar to the B7 molecule; hence, it is named as B7 homologous protein (B7h). Just like CD40/CD40L, when ICOS combines with ICOSL, not only the clonal proliferation of cytotoxic T lymphocyte can be promoted, but also the killing of tumor cells can be significantly promoted, and the transformation of Th1 to Th2 can also be induced. A series of cytokines are secreted, and the ability of NK cell activation is enhanced [21].

In this study, breast cancer cells alone and breast cancer cells transfected with CD40L and ICOSL genes were injected into breast tumor cells of mouse, their effects on tumor killing and the survival time of tumor bearing mice were observed. These results revealed that the tumor volume and weight in the CD40L and ICOSL groups de-

creased compared with the control group, and the difference was statistically significant ($p < 0.05$). The therapeutic effect in CD40L-IRES2-ICOSL group was more obvious compared with the former two groups, and the difference was statistically significant ($p < 0.01$). The result of immunohistochemistry was consistent with RT-PCR results, the survival time of tumor bearing mice was also significantly prolonged, and all these confirmed that the double genes CD40L-IRES2-ICOSL have a therapeutic effect in mice with breast cancer. From the immunohistochemical sections, the present authors found that there was much lymphocytic infiltration in CD40L, ICOSL, and CD40L-IRES2-ICOSL groups, and the infiltration was especially significant in the CD40L-IRES2-ICOSL group. This mainly caused CD40L to improve the antigen-presenting ability of CD40+DCs, and co-stimulator ICOSL provided these co-stimulatory signals. These two work together to promote T cell growth, differentiation, and cytokine production. Indeed, the immune response mechanism is complex, and the detailed mechanism needs further in-depth studies.

Through the animal experiments, the present authors observed that the double genes of AdvCD40L-IRES2-ICOSL not only inhibits the growth of mice breast cancer tumor, but also prolongs the survival time of tumor bearing mice, and this effect was more obvious than that in single gene importing. In this study, the authors successfully induced the specific immune response of mice to breast cancer cells, and provide a new strategy for the rational design of T-based tumor specific immunotherapy in clinic.

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