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ANTITUMOR EFFECTS OF HUMAN IL-15 GENE MODIFIED LUNG CANCER CELL LINE*

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Human IL-15 cDNA fragment, which contains all codons encoding the human IL-15 mature protein and signal peptide was transducted into the human lung squmouse cancer cells (PG cells) and murine lung adenocarcinoma cells (LA795 cell lines) . Two IL-15 highly expressed cell clones PG1 and LA795A were used to inoculate the nude mice and the T739 syngeneic mice respectively. PG1 cell express higher level of class I MHC molecule on their surface than PG cells. It was shown that the modified LA795A tumor cells grew slowly in T739 mice and induced high levels of CTL/NK/LAK activity *in vivo* as well, compared with the case of inoculation with LA795 or LA795neo. No significant difference in the tumor growth was observed in groups of the nude mice inoculated by PG1, PG and PGneo cells respectively, except the gene modified cells could not show the lung metastasis of tumors. The supernatants derived from the LA795A cell culture could promote CTL/NK/LAK activity from the whole splenocytes and the CD4-/CD8-deleted splenic cells in vitro. The results indicated that the IL-15 gene transfected tumor cells play important roles in the process of antitumor or antitumor metastasis.

Key words: Interleukin-15; Gene therapy; Tumor; Gene transduction; Cytotoxicity

INTRODUCTION

Interleukin-15 (IL-15) is a novel cytokine that shares similar biological activities with IL-2. IL-15 not only promotes the proliferation and differentiation of T cells, natural killer (NK) cells and B cells,[1-3] but also induces T cells and NK cells to produce IFN-γ.[4-6] Besides, IL-15 could promote NK/cytotoxic T lymphocyte (CTL)/lymphokine-activiated killer (LAK) cell activity.[5-7] In mouse, it has been shown that IL-15 is three to four times more potent than Interleukin-2 (IL-2) in promoting NK activity, but is approximately one -third as potent as IL-2 in inducing CTL activity. The dose of IL-15 required to develop pulmonary vascular leak in mouse is six times higher than that of IL-2 required, which indicates that therapeutic index of IL-15 is superior to that of IL-2.[7] IL-15 could have significant antitumor role, therefore, IL-15 gene modified tumor cells might be a hopeful cancer vaccine to generate antitumor effect.

Recently, great progress has been made in the cytokine gene therapy for cancers, demonstrating that tumor cells transfected with cytokine genes may greatly improve their immunogenicity, hence induce the local or systemic immune response against the tumors *in vivo*. But up to date, there's no such reports

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on IL-15 yet. Here we report significant tumor growth inhibition of mice injected by IL-15 gene-modified lung cancer cell lines.

MATERIALS AND METHODS

Mice

T739 syngeneic mice were purchased from Institute for Cancer Research, Chinese Academy of Medical Sciences (CAMS). Female Babl/c mice (4-6 weeks of age) were housed in the Institute of Experimental Animals, CAMS.

Cell Lines

PG cell line is derived from human lung squmouse cancer and was provided by the department of pathology, Beijing Medical University. LA795 is a cell line of murine lung adenocarcinoma, syngeneic to T739 mice, and was provided by professor GAO Jing in our institute. Murine tumor YAC-1 and P815 lines and human tumor K562 and Daudi cell lines were preserved in our laboratory.

Genetic Modification of Tumor Cell Lines

IL-15 cDNA was amplified by RT-PCR from mRNA extracted from LPS-activated human adherent peripheral blood monocytes,[8] which contains all codons encoding the amino acids of human IL-15 mature protein and the signal peptide, and subcloned into the EcoR I and BamH I sites of the reverse virus vector pLxSN, termed pL-IL-15-SN. The pL-IL-15-SN was transfected into PG and LA795 cell lines respectively by means of lipofectin method. Bulk cultures of transducted cells were selected in the RPMI-1640 medium with 400μg/ml of G418 and cloned by the limiting dilution method. Two cell clones highly expressing IL-15, human PG1 cell and murine LA795A cell were measured to secrete 201U/ml/10^6 cells/24 hours and 178U/ml/10^6 cells/24 hours respectively by the determination of the growth of CTLL-2 cell line depended on IL-15. Those were used in the following experiments in vitro and *in vivo*.

Tumorigenicity Assessment and CTL Induction

Balb/c nude mice were inoculated subcutaneously with 0.2 ml of 2×10^6 wild type PG cells, PG-neo (vector pLxSN modified) or PG1 (pL-IL-15-SN modified) cells respectively. After 45 days of breeding in a pathogen-free state, the nude mice were killed and tumor weights were measured. T739 syngeneic mice were injected subcutaneously with the wild type LA795, LA795-neo (vector pLxSN modified) or LA795A (PL-IL-15-SN modified) cells in 0.2 ml of 1×10^6 cells per mouse. Mice were killed at day 30 for the measurement of tumor weights. Tumor growth was monitored twice per week in two perpendicular dimensions. For the induction of murine splenic CTLs, T739 syngeneic mice were challenged with type LA795 cell again in following week after inoculated with this line subcutaneously. 24 hours after last immunization, the mice were killed and the splenic cells were isolated and the cytotoxicity was measured.

Preparation of Human PBMCs and Murine Total or CD4- and CD8-Deleted Splenic Cell Populations

Human PBMCs were separated by Ficoll-Hypaque as usual. Murine splenic tissues were gently teased and passed through a stainless steel mesh to obtain single cell suspensions. Murine CD4- and CD8-deleted cell subsets were prepared via panning. Briefly, anti-CD4 (L3T4) or anti-CD8(Lyt-2,3) monoclonal antibodies (mAb, 1μg/ml, Department of Beijing Medical University) were immobilized in 24-well plate (1ml/well) at room temperature for 40 minutes respectively. Murine splenic cells were subsequently added to the wells coated by antibodies and incubated for one hour at 4 ℃. The cells not fixed on the bottom of the wells were collected after washes. For high purification, this process was repeated 2 — 3 times. Immunofluorescence analysis revealed that $CD4^+$ cells were less than 5% and $CD8^+$ cells accounted for 47% in CD4-deleted cell population, while $CD8^+$ cells were less than 7% and $CD4^+$ cells were about 60% in CD8-deleted cell populations. So CD4-deleted cells were $CD8^+$ cell enriched population, whereas CD8-deleted cells were $CD4^+$ cell enriched population.

Immunofluorescence

Tumor cells were analyzed for major histocompatibility (MHC) class I and II molecules by indirect immunofluorescence assay. HLA-A, B, C and anti-HLA-DR mAbs were kindly provided by Dr.

A. Bensussan from Unit 448, INSERM, France. Cells (1×10^6) were incubated with anti-HLA-A,B,C and anti-HLA-DR mAb for 20 minutes on ice. After two washings, cells were stained with Fluorescence isothiocyanate(FITC)-labeled $(Fab')_2$ fragment of goat anti-mouse IgG+IgM (Immunotech) for 20 minutes on ice, washed twice, and then analyzed on FACScan (BD). Murine splenic cells were stained with anti-CD4- or anti-CD8-FITC by direct immuno-fluorescence assay.

Cytotoxicity Assay

Cytolytic activities of the spleen cells of the T739 mice inoculated with LA795, LA795-neo or LA795A cells, the total or CD4-/CD8 deleted splenic cells stimulated with or without the supernatants of cultured LA795A cells and human peripheral blood mononuclear cells (PBMCs) stimulated with or without the supernatants of cultured PG1 cells *in vitro* were evaluated by MTT 〔 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide 〕 method. Briefly, 1) the induction of LAK cells: 1×10^6 murine splenic cells or human PBMCs were cultured with the supernatant of LA795A cells or the supernatants of PG1 cells in 5% CO_2 at 37 ℃ for 72 hours, and collected to measure LAK cell activity; 2) target cells were as follows: YAC-1 and K562 cells for NK activity determination; P815 and Daudi cell lines for LAK activity to be detected; LA795 cells for measuring CTL . The effector cells were freshly isolated human PBMCs or murine splenic cells (for NK activity test), human or murine LAK cells and murine CTLs respectively. 5×10^3 tumor cells were cultured with 2.5×10^5 effector cells at effector/target (E/T)=50 in a total volume of 0.2 ml/well in 96-well flat-bottomed plate at 37 ℃, 5% CO2 and 90% relative humidity for 4 hours to detect NK cell activity or for 24 hours to measure LAK and CTL activities. Meanwhile, the cultures containing only effector or target cells were set. After the incubation, 20μl MTT was added to each well. In following 4 hours of incubation, the cultures were added by 100μl of the solution containing 50% DMF and 20% sodium dodecyl sulfate (SDS) per well followed by the incubation overnight. The formazan crystals were dissolved in SDS, and the absorbance was read at 540 nm in ELISA reader as OD value. The percentage of cytolysis was calculated as:

{1−[(E+T OD value−E OD value) ÷T OD value]×100%}.

Statistical Analysis

Student's test was used for the statistical analysis.

RESULTS

High Expression of Class Ⅰ MHC Molecule on IL-15 Gene Modified Tumor Cells

With immunofluorescenc analysis, HLA-A ,B, C antigens were found to be higher expression on PG1 cells with relative percentage of 23% compared with PG (13%) and PGneo (15%) cells, while expression of class Ⅱ MHC antigen on these cell lines showed no difference in relative percentage ranged from 6 — 10% among three cell lines.

Tumorigenicity of Genetically Modified Tumor Cells

Firstly, the test for tumor growth kinetics *in vitro* has shown that there is no statistical difference between the gene transducted and non-transducted LA795 and PG lines (data is not shown). Secondly, the Tumorigenicity induced by these lines were then observed *in vivo*. As shown in Figure 1, tumor diameters in mice inoculated by LA795 and LA795neo are at same level ($P>0.05$) during 30 days of observation, but ones in LA795A-induced tumor are much less than those in other two groups ($P<0.05$). At day 30, we measured tumor weight. The result demonstrates that tumor weight (1.62±0.92 g) in LA795A cell-inoculated mice was much less than those in LA795 cell- (3.66±0.72 g) or LA795neo-(3.71±1.15 g)inoculated mice ($P<0.05$). On the other hand, we observed tumor growth in nude mice induced via the inoculation with PG1(pL-IL-15-SN transfected PG cells), PGneo (pLXSN transfected PG cells) and wild type PG tumor lines during 45 days. The result shows that tumor weights in all three groups were at same level ($P>0.05$, PG: 24.6±3g; PGneo: 24.1±4.4g; PG1: 22.6±1.9g). Histologic examination (Data in details will be reported in another paper) found that there were no metastases in three groups of T739 mice. The wild type PG cells or PGneo cells could invade lung tissue in nude mice , but the metastases in PG1 cell- inoculated nude mice have not be observed.

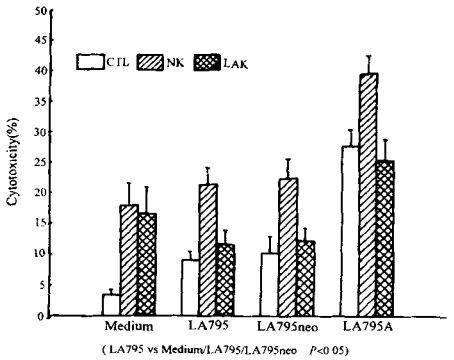

Fig 1. The growth of tumor cells in T739 mouse. T739 mouse were inoculated with 1×10^6 LA795A (pL-IL-15-SN transfected LA795 cells), LA795neo (pLXSN transfected LA795 cells) or wild type LA795 cells in the back subcutaneously. All of mice developed tumors 10 days after tumor inoculation. Data were expressed as mean tumor diameters from 7 mice in each group. Standard deviations were < 10% of the mean values.

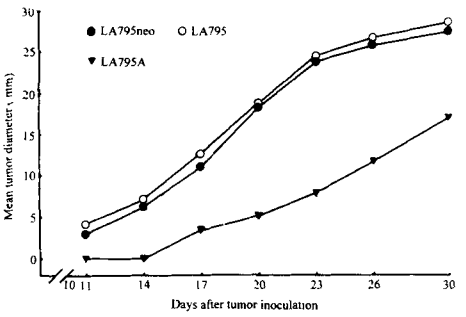

Fig 2. CTL/NK/LAK activity of murine splenic cells in mice immunized by IL-15 gene-modified tumor cells. T739 mice were immunized twice with LA795, LA795neo, LA795A cells intracutaneously respectively to induce CTL for measurement of Cytolytic activities. Data were expressed as mean percentages of cytotoxicities ± standard deviations from 7 mice in each group.

Supernatants from LA795A / PG1 Cell Culture Promote Cytotoxicities *in Vitro*

As shown in Table, either human PBMCs or murine total spleen cells in the presence of the supernatants from IL-15 gene-modified tumor cell lines manifested greater NK and LAK cell activities than those in other conditions. CD4-deleted splenic cells showed a little bit decreased NK cell activity, but no significant change in LAK cell activity compared with those from total splenic cells. However, both NK and LAK cell activities were influenced to some degree in the case of deletion of $CD8^+$ cells among splenic cells.

Generation of Cytotoxicity *in Vivo*

The T739 syngeneic mice were immunized twice with LA795, LA795neo, LA795A cells intracutaneously to induce CTL. As shown in Figure 2, much stronger CTL/NK/LAK activity could be elicited in LA795A immunized T739 mice *in vivo* compared to other groups ($P<0.05$).

DISCUSSION

IL-15 gene modified lung cancer cell lines could induce an antitumor effect *in vitro* and *in vivo*. It could depend on rather effect of these lines on immuneresponse in host than the alteration in tumorigenicity of these lines themselves due to no change of tumor growth in either IL-15 cDNA transfected or non-transfected tumor cell lines *in vitro*. Nevertheless, the fact that IL-15 cDNA tranfected (PG1) line highly express class I MHC molecule also indicates that immunological mechanism might involve in experimental model we used. The gene modification of tumor cells via IL-15 cDNA transduction indeed inhibits the development of tumor in T739 mice. However, this manifestation does not occur in the case of nude mice model. Even so, lung metastasis of IL-15 gene modified tumor cells in nude mice has not be found, but reverse results happened to the groups with wild type tumor or vector-transfected tumor cells. Because nude mouse is T cell-free, IL-15 gene modified tumor cells might stimulate NK cells to prevent tumor-bearing mice from tumor metastasis via blood stream.

The results of the cytotoxicities further support our hypothesis. IL-15 gene modified lung cancer cell lines could induce marked CTL/NK/LAK cell activity in both human and murine systems *in vitro and in vivo*. Several reports have demonstrated that IL-15 is potent cytokine to induce LAK cell activity and promote CTL/NK cell activity.[5-7] Our result suggests that IL-15 gene modified tumor cells might exert the influence on CTL/NK/LAK cell activity of the body via production of IL-15. In term of such obvious alteration in NK/LAK cell activity following by deletion of

$CD8^+$ T cell in murine splenic cell population, $CD8^+$ T cells might play a major role in the model of IL-15 cDNA modified tumors. Without doubt, Our results indicate that the antitumor mechanism of IL-15 gene modified tumor cells is at least T cell-dependent. Taken together, these findings suggest that the IL-15 gene is likely to be a hopeful new cytokine gene for the tumor gene therapy.

Table. Supernatants from LA795A / PG1 cell culture promote cytotoxicities of human PBMCs/murine total/CD4-deleted/CD8-deleted splenic cells in vitro[1]

	NK cell activity (%)[2]	LAK cell activity (%)[2]
Human PBMCs		
Medium	16± 1	8± 2
PG	12± 1	8± 1
PGneo	15± 2	7± 3
PG1	30± 2*	18± 2*
Murine total splenic cells		
Medium	13± 6	10± 1
LA795	13± 2	11± 3
LA795neo	16± 3	11± 2
LA795A	28± 3*	19± 2*
CD4-deleted splenic cells		
Medium	10± 2	8± 1
LA795	11± 4	9± 1
LA795neo	14± 4	9± 1
LA795A	21± 2#	18± 3*
CD8-deleted splenic cells		
Medium	11± 1	6± 1
LA795	12± 2	9± 1
LA795neo	10± 2	7± 1
LA795A	24± 5*	12± 1*

[1] Human PBMCs and murine total or CD4-/CD8- deleted splenic cells were cultured with RPMI-1640 medium or the supernatants of cultured PG1 cells or PGneo or PG cells and LA795A or LA795neo or LA795 cells respectively for indicated time as described in Materials and Methods for the determination of the cytotoxicities.

[2] Data were expressed as mean percentages of cytotoxicities ± standard deviations from three experiments.

*$P<0.05$, when compared with Medium, PG /PGneo and LA795/LA795neo. # LA795A vs Medium, $P<0.05$. * LA795A vs Medium or LA795neo, $P<0.05$.

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