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Evaluation of Metastasis Inhibition by ABD-IL-2 Compared to Human IL-2 in a Breast Cancer Mouse Model

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ABSTRACT

Background: Interleukin-2 (IL-2) is a well-known cytokine that plays a crucial role in stimulating immune cells, including natural killer (NK) cells and cytotoxic T cells. It has been studied as an immunotherapy for a variety of diseases, including cancer. However, due to its short serum half-life, high doses of IL-2 are required which can result in systemic toxicities like capillary leak syndrome.

Objective: To demonstrate the enhanced antitumor efficacy of Albumin Binding Domain-conjugated IL-2 (ABD-IL-2) at a lower dose compared to IL-2.

Methods: IL-2 and ABD-IL-2 were purified using Ni-NTA resin with a histidine sequence added to their C-terminal region for purification purpose. Peripheral blood lymphocytes were stimulated with IL-2 and ABD-IL-2 to assess their function. 4T1 cells were injected into BALB/c mice to establish a breast cancer model with metastasis evaluated in the lungs.

Results: Both recombinant proteins significantly stimulated T lymphocyte proliferation compared to the negative control (P=0.000, P=0.001). Administration of both proteins reduced the size of isolated tumors in the breast cancer mouse model. The control group had more nodules and larger lung metastatic centers (P=0.000). Metastasis to secondary lymphoid organs occurred only in the control group.

Conclusion: By using ABD-IL-2 at a one-third concentration compared to IL-2, we aimed to reduce administration toxicity associated with high doses of IL-2 in immunotherapy. This approach shows potential for improving IL-2-based treatments while minimizing adverse effects.

Keywords: Interleukin-2, Tumor, Metastasis, Breast Cancer

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INTRODUCTION

Cancer is defined as uncontrolled cell growth with metastasis being the main cause of cancer-related deaths (1). Breast cancer is the most common form of cancer and the second leading cause of cancer-related fatalities among women. In approximately 20-30% of cases, the cancer spread to other parts of the body, including the brain, liver, lungs and bones (2). While treatments like chemotherapy, radiotherapy, and surgery to remove tumor tissue are used to treat this cancer, there is currently no effective treatment for metastatic cases (3).

Cytokine-based immunotherapy is a promising field in cancer treatment because cytokines regulate the responses of the host immune system against tumor cells and induce programmed cell death in cancerous cells, through anti-proliferative and apoptosis induction. Predominantly, recombinant human interleukin-2 (rhIL-2) is applied as an immunotherapy in research and clinical studies (4-6).

Interleukin 2 is a cytokine with a molecular weight of 15.5 kD, primarily produced by TCD4+ lymphocytes in response to antigen stimulation (7). The rhIL-2 protein, commercially available as Proleukin (Aldesleukin), is used in the immunotherapy of cancers, such as melanoma and renal cancer (8-10), but Proleukin has a short half-life (13 to 80 minutes) after injection into the blood, necessitating high doses (HD) for effectiveness. These high doses are often linked to severe side effects (11, 12).

There are various strategies to increase the half-life of therapeutic proteins, including conjugation of the target protein to the albumin-binding domain (ABD) (13, 14). ABD is naturally present in the G protein of streptococcal strains. The G protein has two parts with three domains in each part. The intracellular part binds to the albumin while the extracellular part binds to the FC region of IgG. Among the three domains that bind to albumin, the C-terminal domain known as G148-ABD3 has been the focus of most studies. ABD3 has a high binding capacity to albumin (Kd~4nM) and is physically and chemically stable (13, 15, 16). The half-life of several drug proteins is increased through binding to the G148- ABD3 sequence, including the HER2-binding Fab fragment of the trastuzumab drug (17), Granulocyte-colony stimulating factor (GCSF) (18), human TNF-related apoptosis-inducing ligand (hTRAIL) (19), Interferon α (20), and Interleukin 15 (21). In 2016, Dr. Kardar and his colleagues demonstrated that in BALB/c mice, the half-life of rhIL-2 protein increased from 46±0.2 minutes to 150±1.3 minutes, three times higher after conjugation with ABD without affecting its biological activity (22). Building upon this research, the current study aimed to enhance the half-life of IL-2 by conjugating it to albumin binding domain (ABD) The study showed improved antitumor activity of IL-2 and ABD-IL-2 along with inhibition of metastasis in the 4T1 breast cancer model. this findings suggest potential for further exploration in clinical trial studies.

MATERIAL AND METHODS

Strain and Plasmid Construction

Amplification of the rhIL-2 and ABD-rhIL-2 gene fragments available in the project laboratory was performed using specific primers that contained the *NdeI* and *XhoI* restriction sites. The PCR products were then digested with *NdeI* and *XhoI* enzymes and ligated into the pET26b+ plasmid. PCR reactions were performed using Taq polymerase as follows: 95°C for 5 min, 94°C for 30 s, 50°C for 30 s, and 72°C for 9 min repeated for 35 cycles, with a final extension at 72°C for 5 min. The rhIL-2 and ABD-rhIL-2 genes were transformed into *E. coli* DH5α competent cells using $CaCl_2$ and heat shock treatments. The cloned bacteria were then cultured in Luria broth (LB) containing 50 μg/ml kanamycin at 37°C overnight/ with agitation at 180 rpm. Each plasmid was

extracted using a plasmid extraction kit (GTP, Iran) and purified using a plasmid purification kit (Biobasic, USA).

Expression of rhIL-2 and ABD-rhIL-2

Rosetta BL21 was used for periplasmic expression of rhIL-2 and ABD-rhIL-2 as outlined above. The overnight culture was added to 1 liter of culture media with the same antibiotic concentration and incubated at 37°C/ 250 rpm until the absorbance of bacteria at 600 nm reached 0.6-0.8. Expression was then induced with 1mM IPTG, and bacteria were harvested 4 and 6 hours post-induction at 37°C.

Periplasmic Extraction of rhIL-2 and ABD-rhIL-2

Protein extraction was carried out using the osmotic shock method. Cell pellets were resuspended in 25 ml of a hypertonic solution (30 mM Tris, 20% w/v sucrose, 1mM EDTA, pH 8.0) and incubated at 4 °C for 30 min. After Centrifugation at 10,000 ×g for 5 min, 25 ml of a hypotonic solution (5 mM $MgSO_4$) was added. The supernatants from both the hypertonic and hypotonic solutions were combined and dialyzed against PBS1X at 4 °C for overnight.

Purification of rhIL-2 and ABD-rhIL-2

1 ml of 50% Ni-NTA slurry (Qiagen, England) was added to clarified cell lysate and incubated for 3 h at room temperature with gentle shaking. The lysate-Ni-NTA mixture was then loaded onto a column. Washing steps were performed with 10 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, PH 8.0). The rhIL-2 or ABD-rhIL-2 was eluted ten times with 0.5 ml of an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, PH 8.0). The eluates were pooled and protein quantification was performed using the Bradford assay.

Bioactivity Assay of rhIL-2 and ABD-rhIL-2 In Vitro

To analyze the specific function of purified rhIL-2 and ABD-rhIL-2, a proliferation assay was conducted on lymphocytes isolated from peripheral blood mononuclear cells (PBMCs). Heparinized peripheral blood was obtained from a healthy volunteer. PBMCs were isolated using a Ficoll solution (Sigma, USA). After centrifugation at 1500g for 20 minutes at room temperature, the PBMCs layer was located beneath the plasma layer. The PBMCs were washed and then resuspended in RPMI-1640 medium containing a 1X penicillin-streptomycin antibiotic solution and 10% fetal bovine serum (FBS). The next day, peripheral blood lymphocytes were collected from the non-adherent cell population and cultured in a 96-well plate. Each well received 5×10^5 cells in a complete medium, resulting in a total volume of 100 µl per well. The cells were treated with three distinct concentrations of 100, 200, and 400 ng/ml for both rhIL-2 and ABD-rhIL-2, in triplicate. Additionally, 1 µg/ml of phytohemagglutinin (PHA) and 50 ng/ml of phorbol-12-myristate-13-acetate (PMA) were included as positive controls, while the negative control group was treated with medium devoid of any mitogen. The cells were incubated at 37°C in a 5% CO2 atmosphere for a duration of 72 hours. After incubation period, 10 µl of a 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) solution (Sigma, USA) was introduced, and the cells were further incubated for an additional 4 hours. Subsequently, 100 µl of dimethyl sulfoxide (DMSO) was added as a solvent, and the optical density of the cells was measured at 595 nm after 24 hours.

Mouse Tumor Model

Twelve female BALB/c mice, aged 6-8 weeks, were obtained from the Pharmacology Animal Lab at Tehran University of Medical Sciences (TUMS), Tehran, Iran. The animals were housed and treated in accordance with protocols approved by the Animal Care Committee of Tehran University of Medical Sciences. Briefly, the 4T1 mouse breast cancer cells were cultured in RPMI medium

supplemented with 10% FBS. A total of 5×10^5 4T1 cells, suspended in 100 µl of PBS, were subcutaneously injected into the third mammary fat pads on the right side of each mouse. The mice were divided into three groups, each containing of four mice: (1) the ABD-rhIL-2 group, (2) the rhIL-2 group, and (3) the negative control group, which received no treatment.

Animal Treatment Procedure

On the 13th day after tumor cell inoculation, BALB/c mice were intraperitoneally (i.p.) dosed with 0.3 µg of ABD-rhIL-2 and 1 µg/ml of rhIL-2. The same treatment was administered on days 17, 22, 24, and 27. Following treatment, the tumor area was monitored every 3 days with a caliper. Tumor size was measured twice daily and calculated using the formula length × $(width)^2$ × 1/2 body weight. Mice were sacrificed on the day 28. The mice in the treatment group received PBS as a vehicle control.

½ body weight is correct?

Histopathological Analysis

The isolated lungs from mice were fixed in 10% formalin and stained using the H&E method. The numbers and diameters of micro-metastatic nodule in lung tissues were recorded in 200-micrometer thickness by using a digital camera and analyzed with ImageJ. Five histological slides, each 5-micrometers thick, were randomly selected from sections at 500-micrometer interval for microscopic examination.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 8.0 and SPSS 23. The normality of the data was assessed with the One-Sample Kolmogorov-Smirnov test, which indicated a lack of normal distribution (P=0.071). Subsequently, one-way analysis of variance (ANOVA) was employed to compare the means across different groups. The Tukey test was then used as the post-hoc analysis to evaluate all group comparisons. A significance level of P<0.05 was forest to determine statistical significance.

RESULTS

Proliferative Effect of rhIL-2 and ABD-rhIL-2 on Lymphocytes

Both rhIL-2 and ABD-rhIL-2 induced lymphocyte proliferation. However, the highest concentration (400 ng/ml) of both rhIL-2 (P<0.001) and ABD-rhIL-2 (P<0.000) had a significant effect on lymphocyte proliferation. The comparison between rhIL-2 and ABD-rhIL-2 groups showed that the proliferation in these two groups was not significantly different (Fig. 1).

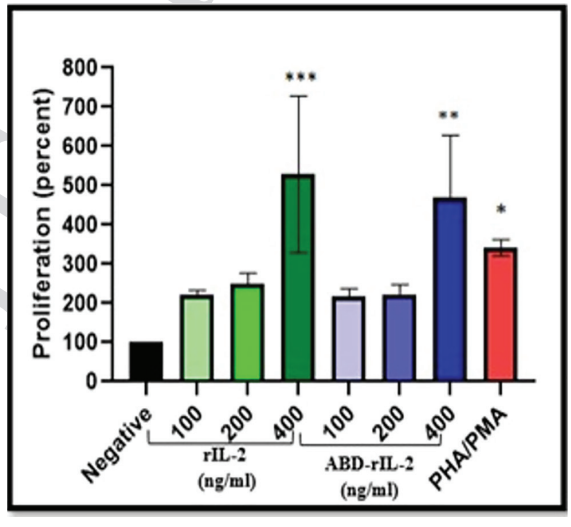

Fig. 1. Effects of rhIL-2 and ABD-rhIL-2 on lymphocyte proliferation. Data analysis was performed using the Post Hoc test and presented as Mean±SD compared to the negative control. *P<0.05, **P<0.001 and ***P<0.0001.

Please write a brief description of the experiment in the legend. Please provide definitions for all abbreviations used in the legends.

rhIL-2 and ABD-rhIL-2 Inhibit Tumor Growth in Mice

The tumor weight of mice treated with rhIL-2 and ABD-rhIL-2 was significantly lower than that of the control group (p=0.0006) (Fig. 2B). Additionally, the tumor volume of mice treated with rhIL-2 and ABD-rhIL-2 was significantly reduced compared to the control group (P=000.1) (Fig. 2C).

Metastasis Inhibition of rhIL-2 and ABD-rhIL-2

In the control group, numerous large

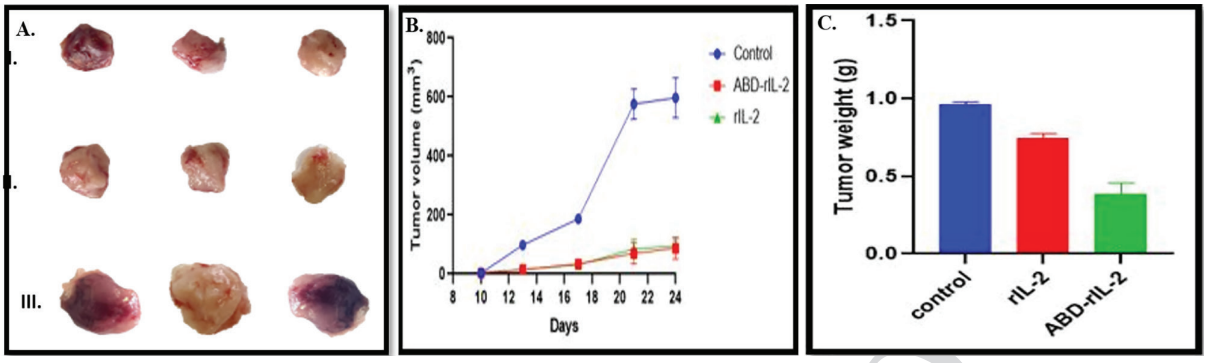

Fig. 2. A. Primary tumor masses of I. ABD-rhIL-2, II. rhIL-2 and III. Control. B. Tumor volume was measured every 3 days. Significantly, the control group had larger tumors than the two treated groups. (P=0.000 for rhIL-2 and P=0.001 for ABD-rhIL-2). C. Tumor weight was measured after isolation of mice on day 28. There is a remarkable difference between the treatment groups (P=0.000 for both) compared to the control group.

metastatic nodules (6-7 mm) and neoplastic cell infiltration were observed in the lung parenchyma. However, in the rhIL-2 and ABD-rhIL-2 treatment groups, only small metastatic nodules (1-2 mm) and a maximum of two nodules were present. In the control group, more than 90% of the alveoli were filled with RBC and fibrin secretions and the majority of the bronchioles were also filled with RBC and dead cells. In the rhIL-2 and ABD-rhIL-2 treatment groups, less than 20% of alveoli were filled with RBC and fibrin secretions and the majority of the bronchioles appeared normal. These results suggest that rhIL-2 and ABD-rhIL-2 significantly inhibit metastasis to the lungs (Figs. 3 and 4).

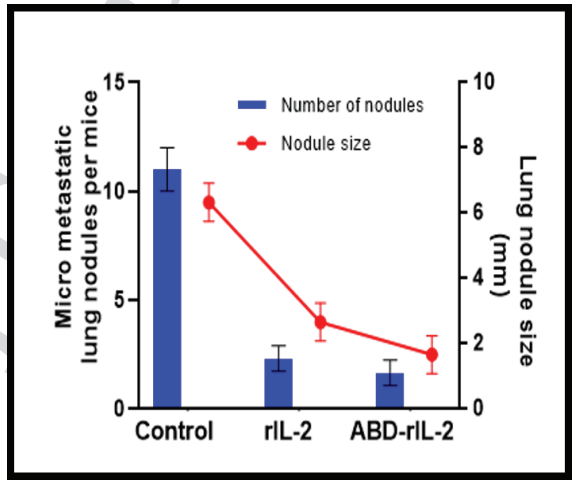

Fig. 3. The number and size of micro metastatic nodules in 200-micrometer-thick lung tissue. The average numbers and sizes of nodules were significantly lower in the treated groups in compared to in the control group (P=0.000 for both).

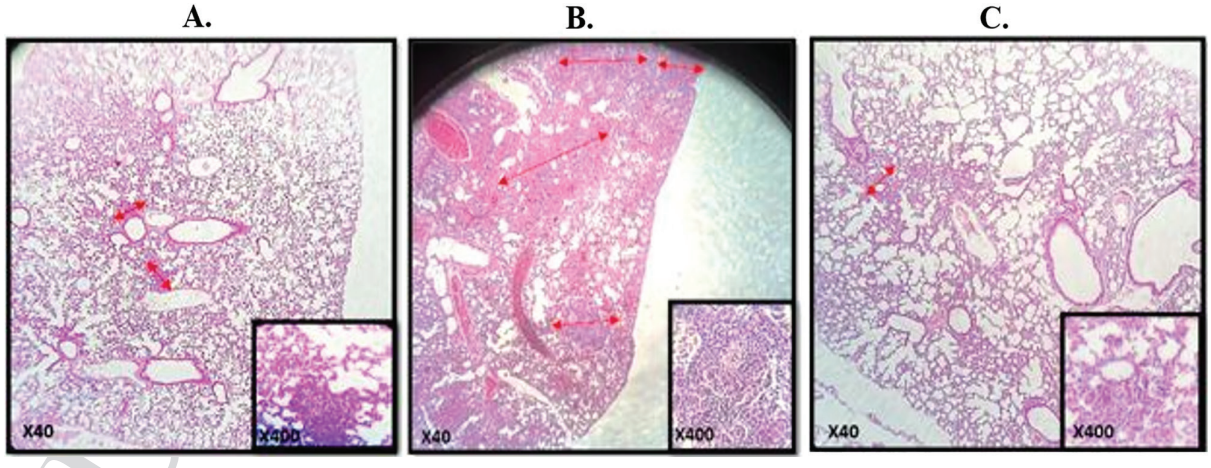

Fig. 4. The H & E staining of lung tissues obtained from mice in different groups. A. Control, B. rhIL-2 and C. ABD-rhIL-2. In the control group the alveolar space in the lung tissues is filled with infiltrated cells and fluid. However in both treated groups the alveolar space is empty. The size of the nodules is indicated by the arrow.

DISCUSSION

Interleukin-2 is a key player in cancer immunotherapy (23). The commercial form, Proleukin, has been approved for the treatment of metastatic melanoma and renal cell carcinoma (24). The primary effect of using rhIL-2 in immunotherapy is the stimulation of cytotoxic T cells and T regulatory cells due to their high affinity IL-2 receptor. Consequently, the drug is administered in high doses. Common adverse effects of high doses of rhIL-2 include a hypovolemic state, vascular leak syndrome, and fluid accumulation in the extravascular space (25). Various methods exist to extend the half-life of therapeutic proteins, such as linking the protein to the Fc domain of immunoglobulin G, using polyethylene glycol (PEG), and albumin (26). Attaching PEG polymer to interleukin 2 increases the cytokine's size and enhances its anti-tumor properties. In a murine sarcoma cancer model, the PEG conjugate of rhIL-2 (NKTR-214) was found to be 60 times more effective than rhIL-2 alone, although the charge of rhIL-2 is altered by PEG modification (27). Repeated injections of pegylated proteins may lead to cytotoxicity and the development of anti-PEG antibodies (28).

Another study found that the combining IL-2 with human serum albumin (Albuleukin) suppressed renal tumor growth, resulting in decreased tumor size and fewer metastatic liver nodules in a mouse model of melanoma. Nevertheless, further investigations are required to evaluate the potential toxicity of Albuleukin (29). Furthermore, albumin may have a negative impact on the interactions between the therapeutic protein and its target(s) (30). In 2017, Kardar et al. demonstrated that the ABD-rhIL-2 fusion protein had a longer serum half-life in mice (22). Our research suggested the potential therapeutic use of ABD-rhIL-2 at a lower concentration compared to rhIL-2 in 4T1 mouse breast cancer. The results showed a significant decrease in tumor growth and isolated tumor weight in mice treated with rhIL-2 and ABD-rhIL-2 compared to the control group. Li R et al also demonstrated that hTRAIL fusion ABD resulted in a reduction in tumor size and enhanced tumor growth inhibition in a colon cancer mouse model (19). Furthermore, Hsu et al's study on recombinant human IL-15 fused with ABD showed superior antitumor activity in terms of reducting tumor growth, inhibiting regulatory T lymphocyte accumulation, and increasing the population of TCD8+ and natural killer cells in a colon cancer mouse model (21). Similarly, c-IFN-ABD exhibited the highest efficacy in suppressing tumor growth in BALB/c nude mice with C8161 melanoma (20). Our study presented the first evaluation of the anti-metastatic properties of both ABD-rhIL-2 and rhIL-2. Hematoxylin-eosin (H&E) staining of lung tissues revealed a significantly higher number and size of lung nodules in the control group compared to the ABD-rhIL-2 and rhIL-2 groups, with no significant difference between the two treated groups. These findings were consistent with Liu et al's research on AZD4547, a small molecule inhibitor targeting fibroblast growth factor receptors (FGFRs), which significantly reduced the number of lung metastatic nodules in the 4T1 breast cancer mouse model ($P<0.001$) (31). Wang et al's study involving Arsenic sulfide (As4S4) in the 4T1 breast cancer model also demonstrated the absence of obvious lung metastatic nodules in treated mice, showcasing promising results (32).

It is understood that the population of T regulatory cells increases following IL-2 administration. However, the primary objective of inhibiting metastasis was achieved. Notably, ABD-rhIL-2 proved to be a more effective alternative to rhIL-2, capable of inhibiting cancer metastasis at a one-third concentration.

CONCLUSION

By using ABD-IL-2 at one-third the concentration of IL-2, we aimed to reduce

the administration toxicity associated with high doses of IL-2, an FDA-approved drug used in immunotherapy. This approach shows potential for improving IL-2-based treatments while minimizing adverse effects. These promising results serve as a foundation for the potential use of ABD-rhIL-2 in clinical trials for breast cancer immunotherapy.

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AUTHORS' CONTRIBUTION

MT performed the investigation, obtained the experimental data, and wrote the manuscript. AM interpreted the H & E staining data. MY contributed to the analysis and verification of the data. AR contributed to the design of the study and discussed the results. GK conceptualized and designed the study, advised scientifically, verified results, and edited the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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