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ORIGINAL RESEARCH PAPER

In vivo induction of necrosis in mice fibrosarcoma via intravenous injection of type B staphylococcal enterotoxin

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Abstract The bacterial superantigen staphylococcal enterotoxin B (SEB) is a potent inducer of cytotoxic T-cell activity and cytokine production in vivo. We investigated the possibility of the therapeutic application of SEB in patients with fibrosarcoma. The anti-tumor effect of SEB in mice with inoculated fibrosarcoma (WEHI-164) was examined by intravenous (IV) and intratumoral (IT) injection and the sizes of the inoculated tumors, IFN-γ production, and CD4+/CD8+ T cell infiltration were determined. The inoculated tumors were also examined histologically. In the mice in the IV-injected group, a significant reduction ($P < 0.02$) of tumor size was observed in comparison with mice in the IT-injected and control groups. Furthermore, the mice in the IV-injected group showed significantly higher levels of IFN-γ ($P < 0.009$) and CD4+/CD8+ T cell infiltration when compared with the other groups ($P < 0.02$). A significantly higher frequency of necrosis in tumor tissues was also observed in mice in the IV-injected group ($P < 0.05$). Our present findings suggest that tumor cell death is caused by increased cytotoxic T-cell activity and cytokine levels in response to the IV injection of SEB and that SEB may be a good option for use as a novel therapy in patients with fibrosarcoma.

Keywords Anti-tumor effect · Cytokine · Mouse fibrosarcoma · Staphylococcal enterotoxin B (SEB) · WEHI-164

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Introduction

Staphylococcal enterotoxins, especially type B (SEB), are classic models of superantigens (SAgs) (Stiles and Krakauer 2005). SAg is one of the most powerful T cell mitogens: less than 0.1 pg bacterial superantigen/ml is sufficient to stimulate T lymphocytes (Proft and Fraser 2003). SEB forms a complex with MHC class II molecules on antigen-presenting

cells, binds to the outside of the peptide-binding groove and then sequentially binds the T cell receptor (TCR) via the variable region of TCR β-chain (Choi et al. 1989; Kappler et al.1989). SEB activates all T cells expressing a defined set of Vβ-TCR, irrespective of their actual antigen specificity. As a consequence, up to 25% of the total peripheral T cell pool becomes activated (Si et al. 2006). Both CD4+ and CD8+ T cells proliferate in response to this superantigen. In addition, this high level of activation of T cells is accompanied by an increased production of Th1 cytokines, such as interferon-γ (IFN-γ), interleukin-2 (IL2), and tumor necrosis factor-α (TNF-α) (Kominsky et al.2001; Perabo et al. 2005). This hyperactivation of T cells usually occurs within 48 h after superantigen exposure. T cells have a crucial role in eliminating host cells that contain intercellular pathogens and those that have undergone malignant transformation (Dalpke and Heeg 2003). One of the major goals of tumor immunotherapy is to generate a tumor-specific response that contributes to the eradication of the tumor. Fibrosarcoma often avoids presenting its own antigens to T cells and is usually resistant to the natural immune system (Ostrand-Rosenberg 1994; Shimizu et al. 2003). Our study assessed the immunotherapeutic effectiveness of SEB by direct intratumoral injection and indirect intravenous injection into mice with fibrosarcoma.

Materials and methods

Cell culture

WEHI-164, a BALB/c-originated methylcolanthrene-induced mouse fibrosarcoma cell line (Panthel et al. 2006), was purchased from the Pasteur Institute (Tehran, Iran). The WEHI-164 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Sigma) and incubated at 37°C in 5% CO_2 with appropriate humidity.

Mice and tumor models

Female inbred BALB/c mice (6–7 weeks old) were purchased from the Pasteur Institute (Tehran, Iran). WEHI-164 cells, 10^6 in 100 µl of PBS, were injected subcutaneously into the right flanks of mice to form tumors (Panthel et al. 2006). Palpable tumors developed by day 7. Tumor sizes were measured every 48 h according to the following formula: $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter) (Ma et al. 2004). Animal experiments in this study were done in compliance with Tarbiat Modares University institutional guidelines.

Preparation of SEB

Staphylococcal enterotoxin type B (SEB) was purchased from Sigma-Aldrich (Munich, Germany) and diluted with PBS. Mice were divided into two groups, an intratumoral injection group (IT-SEB) and an intravenous injection group (IV-SEB); corresponding control groups created by injecting PBS instead of SEB, termed IT-PBS and IV-PBS, respectively, were also analyzed. To monitor the adverse side effects of SEB, mice without tumors were also injected intravenously with the same amount of SEB (IV-SEB-NT). Each group consisted of 6 mice. Aliquots of 10 ng SEB were injected from day 10, and the SEB challenge was performed every 72 h for two weeks (Mondal et al. 2002).

Flow cytometric analysis of the T-lymphocyte subpopulations

Animals were sacrificed 24 h after the last treatment. The tumors were removed and cut into small pieces, rinsed twice with phosphate buffered saline (PBS), and minced with forceps and scalpels. The suspensions were passed through a 100 µm stainless steel mesh, and the cells were washed twice and labeled separately with FITC-conjugated anti-CD4 and anti-CD8 monoclonal antibodies (Becton Dickinson Labware, Franklin Lakes, NJ). All the stainings were washed with a buffer consisting of PBS supplemented with 1% heat-inactivated FBS, 0.1% sodium azide, and 2 mM EDTA. After determining the viability of the cells by Trypan Blue exclusion, the cells were washed twice in the washing buffer. Each sample was immunostained with anti-CD4 and anti-CD8 for 45 min at 4°C. The cells were washed again in washing buffer and fixed with 2% paraformaldehyde. Flow cytometric analyses were performed using an EPICS flowcytometer (Beckman Coulter, Fullerton, CA). These analyses

focused on the lymphoid areas of the forward and side scatters. Single-stained cells were analyzed using Beckman Coulter software.

Enzyme-linked immunosorbent assay (ELISA) for measuring cytokines

The spleen from each mouse was isolated, cut into small pieces, rinsed twice with PBS, and minced with a forceps and scalpel. The suspensions were passed through a 100-μm stainless steel mesh to obtain a single cell suspension. Erythrocytes were lysed at room temperature using ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.001 mM Na_2-EDTA). The cells were washed and resuspended in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. The cells, 10^5/100 μl, were cultured with SEB at 10 ng/well. After 3 days, the supernatants were collected and tested for cytokines (IFN-γ and IL-4) by sandwich-based ELISA kits from R&D (Minneapolis, MN) according to the manufacturer's instructions.

Histopathology experiments

Tumor tissues, approx $0.5 \times 0.5 \times 0.5$ cm, were isolated, transferred into an automatic processing machine for 12 h, and then embedded in paraffin. Five μm thick sections were then stained with hematoxylin and eosin (HE) for histopathological examination; necrosis was also determined.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptosis was studied by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay using in situ Cell Death Detection Kits AP from Roche Diagnostics GmbH (Mannheim, Germany) according to the manufacturer's recommendations. The sections were then incubated with anti-fluorescein-alkaline phosphatase (Vector Red Alkaline Phosphatase Substrate Kit) (Axxora, San Diego, CA) for 60 min at 37°C in a humidified chamber, rinsed in PBS, and incubated with Fast Red TR/Naphthol AS-MX (Sigma-Aldrich) for 10 min. Cells were counterstained with hematoxylin, mounted, and analyzed by bright-field microscopy. TUNEL-positive cells appeared red and exhibited cell shrinkage, nuclear margination, chromatin condensation, and nucleolar disintegration, whereas TUNEL-negative nuclei appeared blue. About 2,000 tumor cells were counted, and the count was expressed as a percentage of "apoptotic" positive cells (Macluskey et al. 2000). One negative and one positive control were included in each experiment. The negative control was incubated without TdT, and the positive control was incubated with DNase for 10 min at 15–25°C to induce DNA strand breaks.

Statistical analysis

All experiments were performed four times, and the mean ± sd was calculated. Statistical analyses were performed using the two-tailed Mann Whitney non-parametric test, and a P-value of $P < 0.05$ was considered as statistically significant. All statistical analyses were conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL).

Results

Tumor size after the SEB injection

Either SEB or PBS was challenged in the three groups of mice on day 1, and the changes in the tumor volumes on were observed day 17. The comparisons of tumor sizes are summarized as illustrated in Fig. 1; tumors of IV-SEB group grew more slowly than those in the IV-PBS (control) group ($P < 0.02$). On the other hand, IT-SEB treatment of did not suppress the

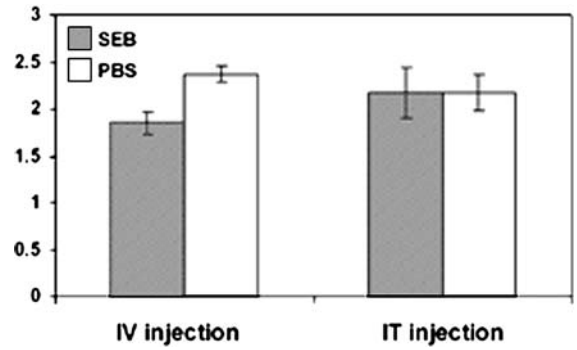


Fig. 1 Changes in tumor size. Treatment of the mice with IV-SEB significantly suppressed the tumor growth when compared to the IT-SEB and the control (IV-PBS and IT-PBS) groups ($P < 0.02$)

tumor growth. IV-SEB-NT, the control involving SEB treatment of mice without inoculated tumors, did not show any notable side effects.

Lymphocyte subpopulation after SEB injection

The effects of the SEB on the tumor-infiltrating lymphocytes, the CD8+ and CD4+ subpopulations, and the CD4+/CD8+ ratio were measured in tumors on day 17. As shown in Fig. 2, there was a significant increase in the CD4+/CD8+ ratio in the IV-SEB group when compared with IV-PBS and IT-PBS as well as with the IT-SEB groups ($P < 0.02$). No other significant differences were observed.

Levels of IFN-γ and IL-4 after the SEB injection

Splenocytes were challenged and compared for their ability to produce IFN-γ and IL-4. The results are shown in Fig. 3; the IV-SEB group showed a significantly higher level of IFN-γ producing ability ($P < 0.009$). This high ability of IFN-γ production was not observed in the IV-SEB-NT group. On the other hand, significantly higher levels of IL-4 production were observed in the IV-SEB groups, irrespective of tumor ($P < 0.02$).

Tumor necrosis and apoptosis after the SEB injection

To assess the decreased tumor size in the IV-SEB group, we studied the incidences of necrosis and apoptosis. Tumors were collected, fixed in formalin, and embedded in paraffin. Histological examinations used HE stained sections; the results are shown in Fig. 4a, b, and the summary is illustrated in Fig. 4c. A significantly increased induction of necrosis was evident in the IV-SEB group ($P < 0.05$). To elucidate the involvement of apoptosis, we performed a TUNEL assay. The results are shown in Fig. 4d, e, and the summary is illustrated in Fig. 4f; no significant differences were observed in induction of apoptosis.

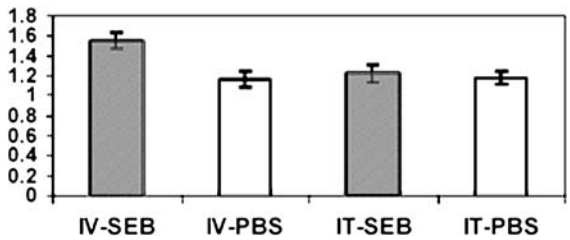


Fig. 2 The CD4+/CD8+ ratio in infiltrating lymphocytes. These results indicate that there was a significant increase in the CD4+/CD8+ ratio in the IV-SEB group ($P < 0.02$) when compared with the IV-PBS and IT groups by Mann Whitney non-parametric test

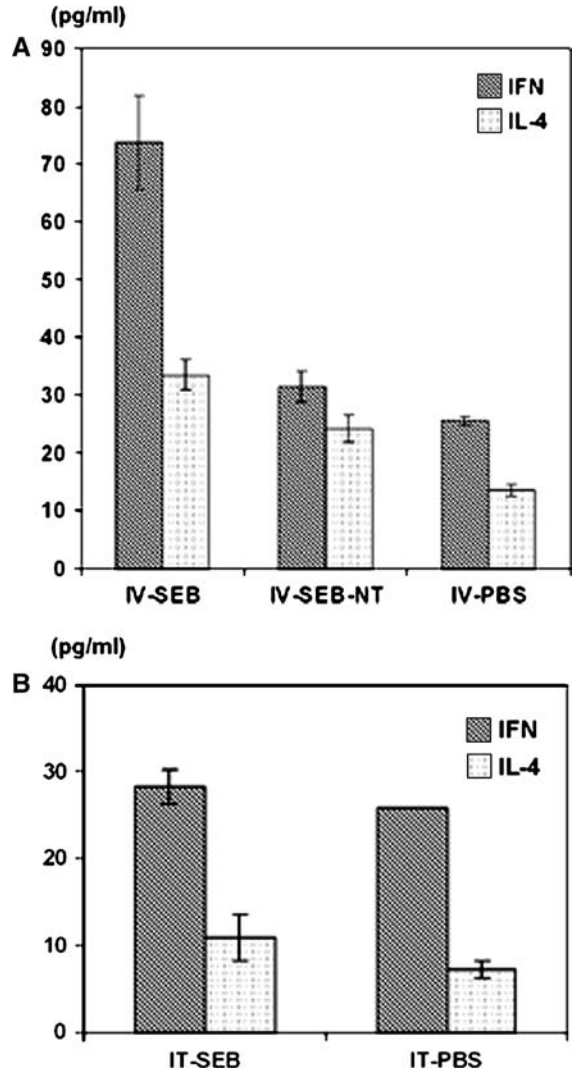
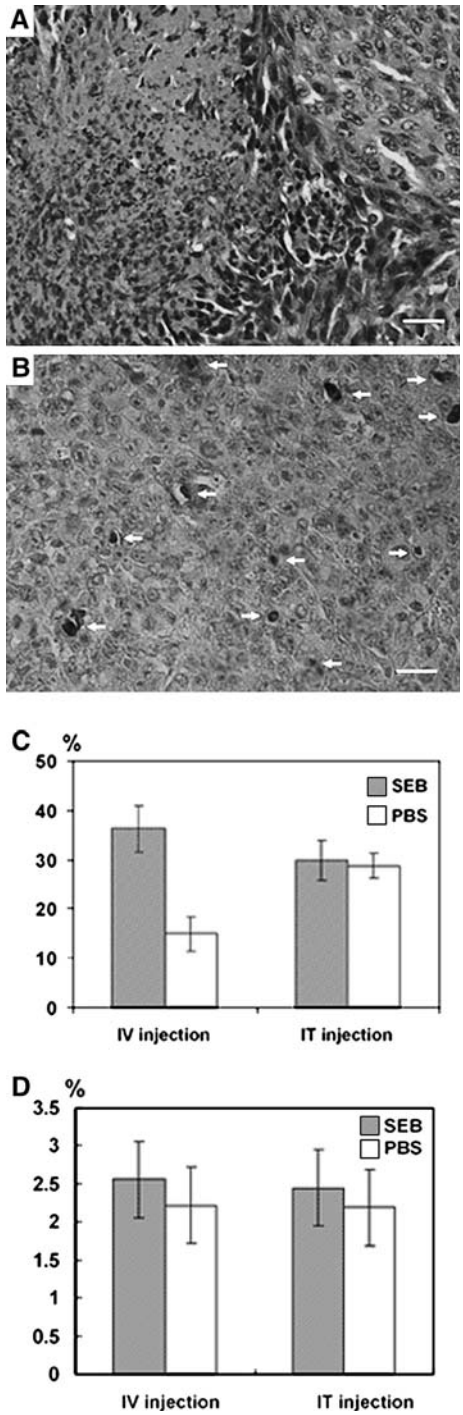


Fig. 3 Production of IFN-γ and IL-4 by splenocytes in mice in the IV-SEB (**a**) and IT-SEB (**b**) groups. Splenocytes were cultured in vitro with SEB or PBS. The supernatant of each group was harvested after 72 h of culture, and released IFN-γ and IL-4 were measured by ELISA. These experiments were run in triplicate. A significant difference was observed in IFN-γ production in the IV-SEB group by Mann Whitney non-parametric test ($P < 0.009$)



◀**Fig. 4** (**a**) Typical example of the HE-stained tumor tissue. Many dead cells are observed that the tissue is not recognizable. Many nuclei have become pyknotic and the cytoplasm and cell borders are not recognizable. Bar = 20 μm. (**b**) Typical example of the results of TUNEL assay in the tumor tissue; all slides are counterstained with hematoxylin. Arrows indicate TUNEL+ cells. Bar = 20 μm. (**c**) The percentages of necrotic cells after treatment with SEB. These results indicate that tumor cells in mice in the IV-SEB group showed an increased rate of necrosis ($P < 0.05$) when compared with the control (IV-PBS) group by Mann Whitney non-parametric test. On the other hand, mice in the IT-SEB group did not show any difference. (**d**) The percentage of apoptotic cells after treatment with SEB. No significant differences were observed by Mann Whitney non-parametric test

Discussion

Although tumors in cancer patients, treated by surgery, chemotherapy and radiotherapy regress, in many cases, they also often metastasize. Activation of the patients' own immune system is one of several promising therapeutic methods for controlling cancer progression. However, patients with malignant tumors are usually resistant to the anti-tumor activity of the immune system. Because tumor cells often avoid presenting their own antigens to T cells (Ostrand-Rosenberg 1994), one of the major goals of tumor immunotherapy is generating tumor-specific T cells that eventually contribute to the eradication of tumors. Superantigens (SAgs) are bacterial and viral proteins that can activate a large number of T cells irrespective of their antigen specificity, resulting in a massive release of cytokines from T cells and monocytes (Fischer et al. 1990; Gjorloff et al. 1991); they increase the antitumor activity of the immune system and prevent both tumor growth and metastasis (Fidler and Kripke 1980; Kreitman and Pastan 1998). Accordingly, stimulation of a stronger anti-tumor T cell response is one of the crucial steps in successful immunotherapy. In this study, the effects of SEB, one of the strongest bacterial SAgs, were examined using the mice fibrosarcoma cell line WEHI-164. Fibrosarcomas form distant metastases through blood vessels in many organs, including the lungs (Poste and Fidler 1980; Fischer et al. 1990). Significantly, these cancerous cells are more resistant to the immune system in comparison with other cancerous cells (Shimizu et al. 2003). Therefore, we chose these cells as candidates for evaluating the SEB activation of the immune system.

Hamad et al. (1997) reported that transcytosis of SEB into cells had no effect on tumor cells. Their results are in good agreement with our findings but our results using IV injection were quite different from those involving IT injection; IFN-γ and

infiltration of CD4+ T cells surrounding the tumor may have played important roles in the suppression of the tumor progression, mainly by induction of necrosis. In contrast to the report by Lando et al. (1991), SEB in our study stimulated the proliferation of cytotoxic T-cells (mainly CD4+ in this study) and produced cytotoxic cytokines (mainly INF-γ in this study) that seemed to play specific roles in tumor cell lysis and necrosis. Detailed mechanisms for the induction of necrosis remain elusive, but it is likely that the cellular injuries are caused by the induced CTLs as well as the produced cytokines.

The increase in IFN-γ may be a characteristic feature of SEB treatment. A Th1 type response has been shown to be biologically beneficial to the host (Balkwill and Burke 1989; Sinha et al. 1999). Mondal et al. found a synergistic effect between Staphylococcus enterotoxin type A and protein A (SEA + PA), but SEA alone can not stimulate the immune system; it always requires other substances or antibodies (Mondal et al. 2002). We examined the effects of SEB alone and found similar or better results than with SEA + PA. Although the tumor cells used differ between these studies, the antitumor effects of SEB may still make it a good candidate for treatment of cancer.

CD4+ T cells killed tumor cell in cooperation with macrophages (Goding 1978). Considering the fact that Vβ of T cells are specific receptors of SEB and that Shimizu et al. (1996) have shown that the proliferation and antitumor activity of T cells increases after binding of SEB to Vβ, our results with IV injection were not unexpected.

Our evaluation of an IT and IV SAg therapy may open a new approach for clinical management of patients with fibrosarcomas. This in vivo investigation indicates that SEB could be useful, but further investigations including detailed studies searching for side effects are warranted.

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