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Analysis of the treatment of gliomas with SEC therapy combined with radiochemotherapy

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Abstract. – OBJECTIVE: To investigate the anti-glioma effects of staphylococcal enterotoxin C (SEC) combined with surgery, radiotherapy and chemotherapy, and the IL-2 of lymphocytes activated by SEC.

PATIENTS AND METHODS: A total of 92 patients with gliomas which had been surgically removed, were randomly divided into a control, and treatment groups A (systemic application of SEC) and B (local application of lymphocytes activated by SEC). The treatment effects were observed on a CT scan of the head. The SEC's induction on the IL-2 secretion by lymphocytes was determined using immunohistochemistry and RT-PCR methods.

RESULTS: Clinical materials showed that the effective rate (CR and PR) in the control group was 32.0%. In the treatment group A, the effective rate was 51.6% and 63.6% in the group B. The IL-2 of lymphocytes secreting activity activated by SEC began to secrete IL-2 on the 1st day and reached a peak during the 3rd day and the 5th day.

CONCLUSIONS: As a superantigen, SEC combined with surgery, radiotherapy and chemotherapy can improve treatment. The lymphocytes activated by SEC can release IL-2.

Key Words:
Superantigen, Glioma, Combined modality therapy, Staphylococcal enterotoxin C.

Abbreviations

SEC = staphylococcal enterotoxin C; APAAP = alkaline phosphatase-anti-alkaline phosphatase; RPMI = Park Memorial Institute medium; PBS = phosphate buffered saline; DEPC = diethyl pyrocarbonate; DTT = dithiothreitol; M-MLV = Moloney Murine Leukemia Virus; RNase H = Ribonuclease H; PBMC = peripheral blood mononuclear cell; CTL = cytotoxic T lymphocyte; PBL = peripheral blood lymphocytes.

Introduction

Biotherapy is recognized as the fourth cancer treatment method in addition to surgery, radiotherapy and chemotherapy. As a superantigen[1], SEC has demonstrated a strong anti-tumor effect in animal testing and clinical trials[2]. The superantigen, being an excellent immunomodulator and an efficient cytokine inducer, can activate T lymphocytes thousands of times more than those by the common antigen[3-12]. In this paper, SEC's induction on the IL-2 secretion by lymphocytes is observed through the analysis of the therapeutic effects of SEC combined with surgery, radiotherapy and chemotherapy in the treatment of gliomas.

Patients and Methods

In total there were 92 patients, among whom 37 were patients of the Department of Neurosurgery of The Second Affiliated Hospital of Soochow University during April 1998 and December 1999. Fifty-five were patients of the Department of Tumor and Radiotherapy of No.1 Hospital of Anhui Medical University during October 2001 and June 2004. All patients suffered from supratentorial grade II-III gliomas by pathological sections, with a lesion ≤ 5 cm according to the findings of CT scan (see Table I for details).

Treatment Methods

A total of 92 patients were randomly divided into a control group and a treatment group by simple random sampling. Traditional comprehensive treatment was applied to the control group, in which complete excision was performed on the field visible to the naked eye. Tineposide 100 mg q.d. was given by drip phleboclysis for three days, ten days later, and lumostine 100 mg was administered at draught. Whole brain irradiation with DT of 40 Gy/20F was given one month after the surgery, and then irradiation on the reduced field with DT up to 60 Gy was performed. The treatment group was divided into group A and group B, where group A underwent surgery +

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Table I. Clinical data of cases of the two groups.

Item	Treatment group		Control group
	Group A	Group B	
Number of cases	31	11	50
Male	17	7	22
Female	14	4	28
Age (years)	23-70	22-72	14-73
Mean age	46.5	46.7	40.8
Type and grading			
(II-III) Astrocytoma (II-III)	15	5	23
(II-III) Glioblastoma (II-III)	8	2	12
Oligodendroglioma (II-III)	5	3	12
(II-III) Ependymoma (II-III)	3	1	3

chemotherapy + radiotherapy by the same method as that for the control group, and was also given SEC 1000 U by drip phleboclysis once every other day for seven days. Group B underwent surgery + chemotherapy + radiotherapy by the same method and had lymphocytes isolated by the method as specified in section 1.3.3. The lymphocytes were put in RPMI-1640 complete medium containing SEC 10 U/ml for activation and expansion, from which 2×10^7 activated lymphocytes were taken and injected into the tumor bed through Ommya tube once every other day for seven days.

Experiment

The instruments used were as follows: Phase contrast microscope: Nikon, Tokyo, Japan. CO_2 incubator, ESOEC-BAN-311-type, Tokyo, Japan. Centrifuge: LD4-2A, Beijing Medical Centrifuge Factory. DNA thermal cycler: PE Company. Electrophoresis apparatus: TS600, Shanghai Fudan Bioengineering Institute.

The main reagents used were as follows: Staphylococcal Enterotoxin C SEC, produced by Shenyang Xiehe Biopharmacy Corporation. Ficoll-Hypaque lymphocyte separation medium with specific gravity of 1.077±0.022, Shanghai No. 2 Reagent Factory. RPMI-1640 medium (Sigma Company, St. Louis, MO, USA). Calf serum, Hangzhou Sijiqing Bioengineering Material Institute. RNA extraction reagent Trizol and cDNA synthesis kit, products of GIBCO BRL Company, Grand Island, NY, USA. High fidelity Taq polymerase and dNTPs, products of Sigma Company. IL-2-secreting cell detection kit, purchased from Bejing Bangding Biomedicine Company.

The patient's venous blood 20 ml (heparin 1250 U) was diluted with PBS 50 ml, added to the lymphocyte separation medium with an equal volume, and centrifuged for 20 min at a rate of 2000 cycles/min. From the middle layer of which the peripheral blood mononuclear cell layer was sucked out, it was diluted with PBS with a volume 5 times as much as the one above. It was centrifuged for 5 min at a rate of 1000 cycles/min, in which the supernatant was removed and the precipitate was added to RPMI-1640 complete medium (containing 20% calf serum and penicillin-streptomycin, 100 U/ml respectively) for culture at 37°C, 50% CO_2 and saturated humidity. The SEC was added into the medium to make its final concentration become 10 U/ml, 5 U/ml and 2.5 U/ml. The medium was centrifuged before administering the drugs and one, three, five and seven days thereafter, in which the supernatant and lymphocytes were collected and respectively put in –20°C and –80°C refrigerators for preservation and lymphocyte smears were prepared for later use.

IL-2-secreting Cell Detection by APAAP Method

A lymphocyte smear prepared was fixed with acetone for 10 min after being dried, washed three times with pH7.2 PBS 0.01 ml, and added with primary antibody 20 µl. It was, then, placed in a humidified box at 37°C for 30 min, washed three times with pH7.2 PBS 0.01ml, added with goat anti-mouse IgG secondary antibody 20 µl, and placed in the humidified box at 37°C for 30 min. Then it was washed three times, added with APAAP compound 20 µl, placed in the humidified box at 37°C for 30 min, washed three times, added with fast red substrate solution to cover the sample for 30 min at 37°C and with a drop of hematoxylin solution for 1 to 2 min counterstain-

ing, washed with running water and observed with high power lens to count the lymphocytes.

RT-PCR detection of cytokines and cytokine expression. 1) Total RNA extraction: the lymphocytes were washed with PBS two times, in which 1×10^6 cells were added with lysate 1 ml, pipetted and placed at room temperature for 5 min. Then, chloroform 0.2 m1 was added and the mixture was vibrated, vigorously for 15 s, placed in a static condition at room temperature for 3 min, and centrifuged at 13,500 g for 15 min at 4°C. T the aqueous phase was transferred, added with isopropanol 0.5 ml and mixed evenly, placed in a static condition at room temperature for 10 min. It was, then, centrifuged at 13,500 g for 10 min at 4°C, and after removing the supernatant, 1 ml of 75% alcohol was added, vibrated, mixed, and centrifuged at 7,500 g for 5 min at 4°C. Following this, the supernatant was removed, dried in the super clean bench for 5 min, and DEPC-treated water 50 µl was added for dissolution at 55-60°C. A water bath for 10 min followed, then it was analyzed and quantitated with a spectrophotometer and after determining RNA quality by formaldehyde denaturing agarose gel electrophoresis, and stored at –80°C for later use. 2) cDNA synthesis: total DNA 3 µl, OligodT 1 µl (0.5 µg/ml) and DEPC-treated water 12 µl was immersed in 70℃ water bath for 10 min cooled on ice for 3min, mixed with PCR reaction mixture 7 µl (10 × reaction buffer 2 µl, 25 mmol/L magnesium chloride 2 µl, 10 mmol/L dNTP 1 µl, 0.1 mol/L DTT 2 µl), placed at 42°C for 5 min, added with M-MLV reverse transcriptase (200 U/ml) 1 µl, mixed evenly for 50 min reaction at 42°C, placed at 72°C for 15 min to terminate the reaction, cooled on ice for 10 min, centrifuged to collect reaction solution which was, then, added with Rnase H 1 µl for 20 min reaction at 37°C and stored at –20°C for later use. 3) Primer synthesis: product of Shanghai SANGER Company. IL-2 F: 5'- ATG TAC AGG ATG CAA CTC CTG TCT T-3'; R: 5'-AGT CAG TGT TGA GAT GAT GCT TTG ACA- 3'. 4) PCR expansion: in the reaction system 50 µl, 10 × PCR buffer solution 5 µl was added, also magnesium chloride (25 mmol/L) 2 µl, dNTP (10 mmol/L) 1.2 µl, sense and antisense primers (10 mmol/L), 2 µl respectively, template cDNA 2 µl and Taq plus DNA polymerase 1 µl. Cycling condition: 30 cycles of denaturation at 94°C for 7 min, 94°C for 60 s, 57°C for 30 s and 72°C for 90 s, with final cycle extending 7 min at 72°C. 2% agarose gel electrophoresis.

Statistical Analysis

Computer program SPSS (SPSS Inc., Chicago, IL, USA) was used to perform Chi-Square (X^2) test and ANOVA analysis to discuss whether there was any significant difference between treatment group and control group. p values less than 0.05 were considered statistically significant.

Results

Short-term Therapeutic Effect

All 92 patients took CT scan of head again at the end of radiotherapy and one month after to observe the therapeutic effects, in which the CT images were compared with preoperative ones and the therapeutic effects were determined according to the WHO solid tumor scoring criteria. The control group, namely the group receiving traditional surgical operation + chemotherapy + radiotherapy, has an effective rate of 32.0%, while the treatment group (including groups A and B) being given SEC on the basis of traditional surgery + chemotherapy + radiotherapy has an effective rate of 54.8%. According to statistical analysis ($X^2 = 4.84$, $p<0.05$), there are differences between the control and the treatment group, in which treatment group A, has an effective rate of 51.6% while group B has an effective rate up to 63.6% without any worsening (PD) (Table II).

In treatment group A, two patients had fever with body temperature below 38°C during three days before being administered SEC, accounting for 6.5% of all cases in this group, and the fever spontaneously subsided with the continuance of the administration. One patient had local redness and swelling where intravenous injection was performed, which subsided after symptomatic treatment. In the treatment group B, the lymphocytes activated by SEC were injected into the tumor bed through Ommaya tube and there was no discomfort.

Follow-up

Patients should be reexamined every three months within one year from the end of the treatment and once every six months thereafter. For all 37 patients of the Department of Neurosurgery of The Second Affiliated Hospital of Soochow University, follow-ups occur within a period up to five years. While for 55 patients of the Department of Tumor and Radiotherapy of

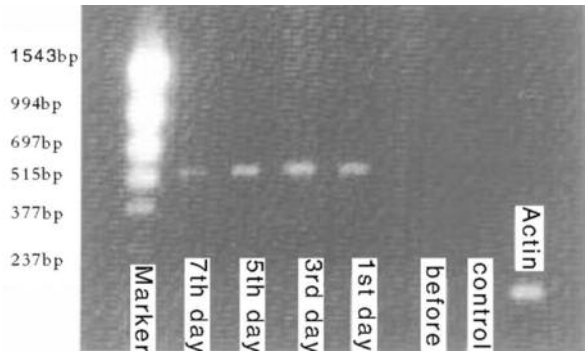

Figure 1. RT-PCR electrophoresis results of IL-2 expression in lymphocytes activated by SEC.

No. 1 Hospital of Anhui Medical University, one, three and five-year follow-ups are being conducted. The results are as follows: the one, three and five-year survival rates of the treatment group are 85.7% (36/42), 66.7% (18/27) and 45.0% (9/20), in which those of group A are 83.9% (26/31), 66.7% (12/18) and 41.6% (5/12) respectively and those of group B are 90.9% (10/11), 66.7% (6/9) and 50% (4/8), while those of the control group are 82.0% (41/50), 60.0% (18/30) and 41.2% (7/17). The survival rates of the treatment group are higher than those of the control group but there is no significant difference between the both ($p > 0.05$).

SEC's Induction on the IL-2 Secretion by Lymphocytes

The peripheral blood lymphocytes of the clinical test volunteers which were isolated through continuous density gradient centrifugation were marked by APAAP method, in which the marked ones per 100 cells were counted under a microscope. Before the activation by SEC, the frequencies IL-2-secreting cells were relatively low, and the count of the marked IL-2-secreting cells rose quickly on the first day after the administration, peaked on the third day and slowly dropped on the fifth and seventh days (see Table III for results).

The variance analysis were as follows: The differences between groups according to time: $F = 360.65$, $p < 0.01$. Q test for comparison: differences between the 3rd day and the 1st day, the 3rd day and the 7th day, the 3rd day and the 5th day, the 5th day and the 7th day, the 5th day and the 1st day: $p < 0.01$; difference between the 7th day and the 1st day: $p > 0.05$. Differences between groups according to the dosage: $F = 49.74$, $p < 0.01$. There were differences between the groups.

mRNA Expression of Lymphocyte Activated by SEC

By taking the RT-PCR method, we have tested IL-2 on the lymphocytes before and after administering SEC (10 U/ml) (before administration and the 1st, 3rd, 5th and 7th days thereafter), and found IL-2 mRNA expression in all lymphocytes activated by SEC, which were most apparent on the 1st, 3rd and 5th days (Figure 1).

Discussion

Since the concept of the superantigen was put forward, most studies on the superantigen have been relevant to the pathogenicity of the exotoxin which may result in such symptoms such as food poisoning and toxic shock. However, the superantigen, is an excellent immunomodulator and efficient cytokine inducer, and has provided a new option for human anti-tumor treatment. The superantigen is involved with anti-tumor immune response through the following mechanism: (1) *Staphylococcus aureus* enterotoxin (SE)-dependent cell-mediated cytotoxicity (SDCC). The SDCC has been found in five types (A, B, C, D and E) of SE[10]. The SE is a potential and efficient T cell agonist, which combines with the MHC-IItarget cell and activates the cytotoxic T lymphocyte (CTL) to kill the target cell. The SE at pictogram level can cause sensitized T lymphocyte to kill tumor cell within several minutes[5]; (2) The

Table II. Curative effect comparison of cases between treatment group and control group.

Group	Number of cases	Curative effect					Effective-rate (%)
		CR	PR	MR	NC	PD	
Control group	50	0	16	22	6	6	32.0
Treatment group A	31	3	13	5	7	3	51.6
Treatment group B	11	2	5	3	1	0	63.6

Table III. IL-2-secreting cells for activation by SEC ($\bar{x} \pm s$) %.

Group	Before administration	The 1st day	The 3rd day	The 5th day	The 7th day
10 U/ml	2	18.0±1.2	42.3±2.1	29.7±2.0	17.3±1.1
5 U/ml	0	9.2±0.6	28.5±1.7	21.4±1.8	10.2±0.7
2.5 U/ml	1	9.8±0.5	25.7±1.7	18.4±1.6	9.6±0.4
Control	2	3	1	2	3

T lymphocyte activated by the superantigen releases multiple cytokines. Through ELISA, it has been found that the lymphocyte activated by the superantigen can secrete multiple cytokines – IL-1, IL-2, IL-6, IL-16, TNF-α, β and IFN-γ[6,7]; (3) LAK-like cytotoxic activity. It has been found that independent application of SE can also kill MHC-II-negative target cell, ruling out the action of SDCC, where SE acts through NK cell, which is not activated by SE directly but by SE's induction on the releasing of multiple cytokines by T lymphocyte[11].

According to the clinical data, surgery, radiotherapy and chemotherapy have an effective rate of 32.0%, while the biotherapy combined with SEC has an effective rate up to 51.6% and 63.6% respectively, and its effect is better in group B (local administration). Only a very small amount of the superantigen which is an immunomodulator and cytokine inducer can activate a large number of T lymphocytes, secretes multiple sufficient cytokines, and even cause immune disorder. Besides, T lymphocytes activated often have immune tolerance to restimulation with the superantigen after they proliferate. The best solution to this problem is to limit the activity of the superantigen so that it can only act in the tumor bed, which can not only avoid the risk of disease caused by the activation of the superantigen of a large number of T lymphocytes in the whole body, but also can continuously supplement the peripheral blood T cells that have not come into contact with the superantigen. Therefore, we suggest that the superantigen be applied locally.

The test results can be proved by the proteins detected by immunohistochemistry and the mRNA by RT-PCR. Stimulated by SEC, PBMC began to secrete IL-2 on the first day, and the secretion could last for 5 to 7 days, and reached its highest level on the 3rd to 5th day. Over the years, many oncologists, in particular tumor immunologists, have conceived the idea of application of tumor-specific CTL to the prevention and treatment of tumors[8], which is very desirable since it is better than chemotherapy because the main effector cells for killing tumor cells in the body are CTL, which have specificity and thus can play a powerful role in killing tumor cells and damage no normal cells. Since the invention of IL-2, it has been used for *in vitro* culture with tumor cells or tumor antigens and lymphocytes, which can not only induce tumor-specific CTL to kill the tumor cells, but also realize the exponential amplification of CTL. The CTL injected into the body can be up to 5×10^8 without any toxicity reaction. Lotze et al of the U.S. National Cancer Institute[9] had cultured PBL ($1\text{-}2\times10^5$) of three sarcoma patients who had lung metastasis in IL-2 and sarcoma cells, and after 3 to 4 weeks, the CTL proliferated to 1×10^8 - 10^9 and could continue to grow for several months, and their killing effect on the sarcoma cells had a 200% to 300% increase. For example, the killing effect of fresh lymphocytes on tumor cells was 85.0±1.9 CPM and that of CTL induced by IL-2 increased to 253.6±15.4 CPM. Furthermore, IL-2 can enhance NK cell's activity and its anti-tumor effect.

Conclusions

The superantigen is a potential and efficient T lymphocyte activator. The T lymphocytes activated by the superantigen, namely CTL, have a lot of perforin and granzymes accumulated in cytoplasm, which are secreted by the tumor cells through exocytosis and aggregate to form pores, and some effector molecules such as granzymes, TNF, secretory ATP and polyadenylic acid-binding protein TIA-1 can enter the tumor cells, leading to the death of the cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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