Use of Interferon-α-Induced Dendritic Cells in the Therapy of Patients with Malignant Brain Gliomas

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Clinical and immunological analysis of the efficiency of combined immunotherapy with the use dendritic cells for the treatment of malignant glioma of the brain was carried out. Dendritic cells generated in the presence of granulocyte-macrophage CSF and IFN-α retain their functional characteristics in patients with gliomas, which suggests the possibility of their use for the treatment of malignant tumors (glioma) of the brain. Combined therapy using interferon-induced dendritic cells was associated with generation of antigen-specific immune response during vaccinations. The results indicate satisfactory tolerance of combined immunotherapy using dendritic cells and the absence of toxic side effects at the stage of adoptive immunotherapy and at the stage of vaccinations with dendritic cells. Clinical trials showed that vaccinations with dendritic cells included into combined immunotherapy improved the quality of life and survival of patients with malignant gliomas.

Key Words: *dendritic cells; glioma; immunotherapy*

Malignant brain tumors (MBT) are a serious medical problem. Despite complex approach to their treatment including surgical intervention, radio-, and chemotherapy, the mean life span of patients with poorly-differentiated gliomas remains extremely low [19]. This necessitates the search for new therapeutic strategies, among which special attention is paid to methods of immunotherapy, particularly to combinations of different immunotherapeutic approaches [9]. Our previous data also indicate that addition of combined immunotherapy based on combination of adoptive immunotherapy and cytokine therapy to the protocols of MBT treatment significantly improves survival of these patients [3-5]. However, the development of relapses in delayed periods necessitates the maintenance of antitumor immunity after completion of immunotherapy course and calls for the creation of more effective methods for stimulation of cytotoxic lymphocyte generation for elimination of tumor cells. Dendritic cells (DC) are very interesting in this aspect [21].

Dendritic cells are antigen-presenting cells ensuring most effective recognition of various antigens by T cells, which leads to their activation [7]. Since DC can present tumor-associated antigens and trigger antitumor immune response, immunization with DC loaded with tumor antigens (DC vaccines) is regarded as a new approach in immunotherapy of malignant tumors [11,16]. The efficiency of DC vaccines in the treatment of glio-

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mas was demonstrated on experimental animals with intracerebral tumors: DC vaccines promoted generation of specific cytotoxic lymphocytes and improved animal survival [6]. Clinical use of DC also confirmed the possibility of specific immune response generation and, according to preliminary data, this treatment modality prolonged patient life span [9,10,20,21].

Traditionally, DC are obtained by culturing of the adherent fraction of mononuclear cells (MNC) with granulocyte/macrophage CSF (GM-CSF) and IL-4 with subsequent addition of maturation cytokines (IL-1, IL-6, prostaglandin E_2, and TNF-α) [13, 18]. However, it was recently shown that partially mature DC can be rapidly generated during monocyte culturing with GM-CSF and IFN-α, and this generation pathway seems to be more physiological [15]. Dendritic cells obtained by this method and called interferon-induced CD (IFN-DC) are characterized by certain advantages: rapid generation, high antigen-binding capacity, stability in the absence of cytokines, high migration activity, and effective stimulation of Th1 response [12,15]. Despite these advantages, IFN-DC have never been tried in clinical practice. Our previous findings indicate the possibility of IFN-DC generation in patients with malignant gliomas [1].

We analyzed phenotypical and functional characteristics of IFN-DC in patients with MBT and evaluated the safety and efficiency of combined immunotherapy including IFN-DC in complex treatment of MBT.

MATERIALS AND METHODS

The study included 119 patients with MBT aged 16-69 years, examined and treated at Neurosurgery Hospital of Novosibirsk Institute of Traumatology and Orthopedics in 1999-2006. The diagnosis was made in accordance with the WHO International Classification of Tumors of the Nervous System (1993) modified by V. V. Yartsev. Clinical status was evaluated by common cerebral symptoms using a 5-point scale for evaluation of clinical status of neurooncological patients and by estimation of the quality of life score using Karnovskii scale. Histologically verified glioblastoma was diagnosed in 42% patients, 3rd-degree anaplastic astrocytoma (AA) in 58% patients. The reference group consisted of 62 donors. All patients with MBT were divided into 2 groups. The main group included 39 patients, in whom surgical treatment and radiotherapy were supplemented by combined immunotherapy using DC. The reference group consisted of 80 patients, in whom visually discernible tumors were removed and radiotherapy was carried out in a standard dose (55-60 Gy). Examinations and treatment were carried out after informed consent of all patients.

Combined immunotherapy using DC was carried out as a pilot study during the postoperative period and included a course of adoptive immunotherapy and a course of DC vaccinations. Adoptive immunotherapy consisted of 2 locoregional injections of lymphokine-activated killer cells (LAK) and cytotoxic T cells into the tumor bed. MNC for LAK generation were cultured with recombinant IL-2 (50 U/ml; Roncoleukin, Biotech) for 48 h, after which LAK (250,000 U) in combination with roncoleukin was injected through a catheter into the tumor bed. Cytotoxic T cells were obtained by culturing patient MNC with DC loaded with tumor antigens in the presence of roncoleukin (50 U/ml) for 5 days, after which they were injected (250,000 U) in combination with roncoleukin into the tumor bed. Antigenic material (tumor cell lysate) for DC loading was obtained from a fragment of autologous tumor by 5-fold freezing/defrosting and was used in a concentration of 0.1 mg protein/ml. A course of vaccine therapy included 6 subcutaneous injections of DC loaded with tumor antigens in a dose of 10×10^6 per injection (in 4-6 points) every other week in combination with subcutaneous roncoleukin (250,000 U).

Delayed-type hypersensitivity *in vivo* was evaluated in 25 patients by the intensity of skin reaction to injection of 0.5 ml tumor cell lysate (0.1 mg/ml) after 48 h by the presence of papule and hyperemia at the site of injection. Delayed-type hypersensitivity *in vitro* was evaluated by modified leukocyte migration test in response to similar stimulation of the leukocyte suspension [2]. A complex of human brain tissue antigens in the same concentration served as the test antigen.

Mononuclears were isolated from heparin-treated venous blood by centrifugation in Ficoll-Verografin density gradient. Dendritic cells were generated by culturing adherent MNC fraction in culture flasks (BD Biosciences Falcon) for 3 days in RPMI-1640 (Sigma) with 0.3 mg/ml L-glutamine, 5 mM HEPES buffer, 100 μg/ml gentamicin, and 5% inactivated donor serum (AB (IV) group) in the presence of GM-CSF (Sigma; 40 ng/ml) and IFN-α (1000 U/ml; Roferone-A, Roche), with subsequent post-maturation for 24 h with 10 μg/ml lipopolysaccharide (*E. coli* 0114:B4; Sigma, for experimental models) or with leukinferon (Intekor Company) in a final dilution of 1:40 (for therapeutic procedures). Dendritic cells were phenotyped by single- or dual-color flow cytofluorometry (FACS

Calibur, Becton Dickinson) using FITC-, allophycocyanin- (APC), or phycoerythrin-labeled (PE) antibodies (CD1a, CD11c, CD14, CD25, CD83, CD123; PharMingen). Allostimulatory activity of DC was evaluated in the mixed lymphocyte culture. Donor MNC (0.1×10^6/well) served as the responder cells; they were cultured in 96-well round-bottom plates for immunological tests in RPMI-1640 with 10% inactivated donor serum (AB(IV) group) at 37°C in a CO_2 incubator. Dendritic cells in 100:1 MNC:DC ratio served as stimulators. Proliferative response was evaluated radiometrically on day 5 by incorporation of ^{3}H-thymidine (1 μCi/well) added 18 h before the end of culturing. The efficiency of antigen-specific response was evaluated on day 3 by proliferative response of patient MNC (0.1×10^6/well) cultured with tumor cell lysate antigens in concentrations of 0.1 and 0.01 mg/ml. The production of Th1/Th2 cytokines (TNF-α, IFN-γ, IL-10, IL-13) was evaluated by flow fluorometry on an automated dual-beam laser analyzer (Bio-Plex Protein Assay System, Bio-Rad) in 72-h supernatants of secondary mixed lymphocyte culture. To this end, monocyte-depleted MNC were cultured for 5 days with DC in 10:1 ratio, after which they were restimulated with the corresponding DC for the next 48 h. The results were processed by methods of descriptive, parametric, and nonparametric statistics using Statistica 5.0 software.

RESULTS

Phenotypical characteristics of DC. Comparison of the efficiency of IFN-DC generation in donors and MBT patients showed that blood monocytes of patients, similarly as donor cells, lost the capacity to adhere to the plastic after 3-4-day culturing with GM-CSF and IFN-α and acquired typical morphological features of DC. Cell viability was ≥90% in all experiments and was similar in the studied groups. The yield of dendritic cell in patients and donors was also similar (0.10±0.01 and $0.13\pm0.01\times10^6$ DC from 1×10^6 MNC). However, analysis of phenotypical markers of IFN-DC showed some specific features (Table 1). For example, the counts of cells with monocyte phenotype ($CD14^+$) and immature DC ($CD1a^+$) in patients were higher. On the other hand, the percentage of cells with mature DC marker (CD83) was lower. CD83 and CD1a markers characterize different stages of myeloid DC maturation. In contrast to donors, in whom the majority of CD83 cells did not carry the CD1a molecule, an appreciable part of patient IFN-DC expressed CD83 and CD1a molecules simultaneously or CD1a molecules alone. Increased total content of immature $CD1a^+CD83^-$ DC and $CD83^+CD1a^+$ DC of intermediate maturity was noted in patients with MBT (13.5±3.2 *vs.* 5.4± 1.9%; $p<0.05$ and 7.4±2.6 *vs.* 4.7±1.0%; $p<0.05$, respectively).

These data indicate the possibility of IFN-DC generation from the population of monocytes in MBT patients, including the possibility of obtaining the same number of mature myeloid DC with the $CD83^+CD1a^-$ phenotype as in donors. On the other hand, the increase in the $CD14^+$, $CD1a^+CD83^-$, and $CD1a^+CD83^+$ subpopulations indicates a delay of myeloid DC differentiation and maturation in patients with MBT.

Functional characteristics of DC. In order to evaluate the capacity of IFN-DC to present antigens and activate T cells, we studied the allostimulatory activity of donor and MBT patient DC in mixed lymphocyte culture. Dendritic cells from donors (n=34) and MBT patients (n=21) exhibited pronounced allostimulatory activity and induced effective proliferative response of donor MNC. The level of proliferation in mixed lymphocyte culture induced by donor and patient DC (13,280±1130 and 11,500±1400 cpm, respectively) and stimulation indexes (19.6±1.6 and 15.6±4.4) were similar and did not differ statistically. In addition, patient DC induced (in the presence of IL-2) a proliferative response of autologous patient MNC to tumor lysate antigens, while without DC patients MNC did not proliferate in response to tumor antigens (Fig. 1).

Evaluation of the capacity of patient DC to activate Th1 and Th2 cells showed that T cells restimulated in mixed lymphocyte culture with DC from MTB patients exhibited as effective production of IFN-γ as after stimulation with donor DC (Table 2). Patient DC similarly induced the synthesis of TNF-α, virtually did not stimulate the production of

TABLE 1. Phenotypical Characteristics of IFN-α-Induced DC (%; *M*±*SEM*)

Marker	Donors (n=18)	MBT patients (n=9)
CD14	8.7±1.4	17.7±2.6*
CD86	62.4±3.2	65.2±4.4
HLA-DR	90.7±3.6	86.0±2.5
CD1a	10.4±2.0	19.3±4.5*
CD83	29.4±2.9	22.6±1.9*
CD25	25.1±3.5	19.2±2.3
$CD83^+CD1a^-$	20.1±2.0	15.8±2.3*
$CD83^+CD1a^+$	4.7±1.0	7.4±2.6*
$CD83^-CD1a^+$	5.4±1.9	13.5±3.2*

Note. *$p<0.05$ compared to donors.

IL-10, but much more actively stimulated the production of IL-13. Hence, both Th1 and Th2 responses were stimulated with patient DC in secondary mixed lymphocyte culture. However, comparison of stimulatory activity indexes showed that DC capacity to induction of IFN-γ was by an order of magnitude higher than its capacity to activation of Th2 cytokines.

According to published data, donor IFN-DC effectively induce the production of IFN-γ in mixed lymphocyte culture [17]. Moreover, the production of Th2 cytokines (IL-10, IL-4) is also detected in supernatants of mixed lymphocyte culture [8], which is in line with our data. Despite more pronounced (in comparison with donor DC) Th2 stimulatory activity of IFN-DC, patients with MTB retain the capacity to activate Th1 response, which predominates in comparison with Th2 activation.

Our results demonstrated the basic possibility of IFN-DC generation in patients with malignant gliomas; judging from some phenotypical features, these cells are characterized by delayed differentiation, but nonetheless completely retain their allostimulatory activity, can present tumor antigens and activate effective Th1 response. The results indicate functional competence of DC and confirm the possibility of their clinical use in the treatment of malignant glioma of the brain.

Clinical trials of combined immunotherapy. Clinical trials of combined immunotherapy using IFN-DC were carried out in accordance with the protocol approved by the Academic Council of the Institute and the local Ethical Committee. The groups (Table 3) were matched for patient sex and age, histological variants of intracerebral tumors, initial levels of quality of life (Karnovskii scale).

For evaluation of the efficiency of antitumor response generation, we determined the intensity of immune response to tumor antigens present in tumor cell lysate before the course of vaccinations and after 3 and 6 vaccinations. Sensitization to tumor antigens manifested *in vitro* by intensification of the proliferative response of MNC and increase in leukocyte migration index during immunotherapy. Tumor cell lysate antigens initially did not stimulate the proliferation of MNC, but after 6 vaccinations proliferation increased significantly (10-fold) in response to injection of tumor antigens in a dose of 0.01 mg/ml and 7-fold in response to a dose of 0.1 mg/ml. No appreciable increase in proliferative response of patient MNC to control antigen (human brain tissue extract) was observed (Table 4). A significant increase in the leukocyte migration index was observed during vaccination. After 6 vaccinations the leukocyte migration index in the presence of tumor antigens in a dose of 0.1 mg/ml increased from 1.02±0.05 to 1.48±0.26 ($p<0.05$). Similarly as for the proliferative response, the leukocyte migration index did not increase in response to control antigen in the similar dose (1.02±0.06 initially and 0.97±0.09 after the course of vaccinations). Hence, induced immune response was aimed predominantly towards tumor-associated, but not tissue antigens.

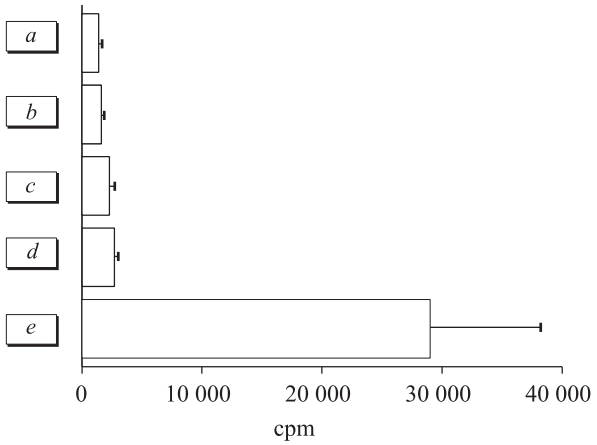

Fig. 1. Stimulation of proliferative response of autologous MNC with DC loaded with tumor antigens. *a*) incubation without stimulation; *b*) with 0.1 mg/ml tumor antigen; *c*) with autologous DC in 10:1 ratio; *d*) autologous DC loaded for 1 h with 0.1 mg/ml tumor antigen (DC+antigen); *e*) autologous DC loaded with tumor antigen and 50 U/ml IL-2 (DC+antigen+IL-2).

TABLE 2. Effects of Donor and MBT Patient DC on the Production of Th1 Proinflammatory and Th2 Antiinflammatory Cytokines in Mixed Lymphocyte Culture

Cytokine	0	+DC from donors (n=10)	+DC from patients (n=10)	Efficiency index
IFN-γ	46±17	1500±356	1116±311	558±155
TNF-α	61±19	95±21	132±27	4.0±1.4
IL-13	78±22	314±43	744±158*	15.0±4.8*
IL-10	6.0±0.2	19±3	15.6±5.0	8.0±2.4

Note. Mean values of cytokine concentrations (pg/ml) and patient DC efficiency indexes of cytokine production in 48-h supernatants of mixed lymphocyte culture are presented. *$p<0.05$ compared to donor DC.

TABLE 3. Characteristics of Patients in the Groups

Sign		Reference group	Main group
Mean age (min/max)		45.8±1.2 (15-69 years)	42.6±1.6 (16-69 years)
Sex	male	38/80 (47.5%)	22/39 (56.4%)
	female	42/80 (52.5%)	17/39 (43.6%)
Quality of life, Karnovskii score		55.2±1.9	59.3±1.9
AA/glioblastoma coefficient		1.4	1.6
Operated	initially	74/80 (92.5%)	32/39 (82.1%)
	repeatedly	6/80 (7.5%)	7/39 (17.9%)

The results of delayed-type hypersensitivity reaction *in vivo* were analyzed. Initially skin reaction to tumor antigen was negative in all patients. Positive skin tests were recorded in 7 (28%) patients after 3 immunizations and in 16 (64%) after 6 immunizations.

Clinical observation showed that combined immunotherapy was well tolerated and caused no pronounced cytokine or toxic reactions, presenting as changes in the general status and/or hemodynamic disorders. Moreover, patients receiving immunotherapy exhibited more pronounced positive clinical shifts by the moment of discharge from the hospital (after the stage of adoptive immunotherapy). Initially (on days 7-9 postoperation), common cerebral symptoms were detected equally frequently in the majority (73.8%) of patients in the main group and reference group (72.2%), while before discharge from the hospital common cerebral symptoms persisted in only 2 (5.1%) patients in the main group *vs.* 20% patients in the reference group.

Analysis of the quality of life (Karnovskii scale) showed that the patients of the two groups did not initially differ by this parameter, while 6, 12, and 24 months after surgery it was significantly higher in the main group (84.0±1.4, 86.0±1.6, and 91.0±2.3 points) than in the reference group (66.0± 1.6, 66.0±1.8, and 61.0±3.7 points, respectively).

Statistical analysis of survival values in the groups showed that the percent of survivors in the main group after 12, 24, and 36 months was significantly higher than in the reference group (Table 5). In the astrocytoma group, significant differences in survival were detected after 24 months of observation and were retained by the term of 36 months. On the other hand, the differences in survival values for patients with glioblastomas, characterized by extremely unfavorable prognosis and short life span, were recorded as early as after 12 months. After 24 months survival value in glioblastoma reduced to zero in the reference group, while in the main group 3 of 8 patients (37.5%) were alive and had no relapses.

Analysis of the relationship between antigen-specific response generation and efficiency of therapy (2-year survival) showed no differences in the subgroups of survivors (n=11) and dead patients (n=4) with AA. On the other hand, sensitization to

TABLE 4. Induction of Antigen-Specific Response during Vaccination of MBT Patients with DC

Parameter		Before vaccination	After 3 vaccinations	After 6 vaccinations
Spontaneous proliferation		425±74	321±88	666±145
Tumor antigen	0.01 mg/ml	663±154 (1.65±0.40)	363±121 (1.3±0.3)	5170±1507* (10.9±4.9)
	0.1 mg/ml	1083±280 (2.40±0.56)	554±243 (1.7±0.6)	3632±474* (7.6±3.1)
Control antigen	0.01 mg/ml	450±57 (1.10±0.35)	815±79 (2.1±0.6)	1050±245 (1.36±0.70)
	0.1 mg/ml	715±89 (1.6±0.4)	976.0±85.8 (2.5±0.8)	1293±101 (1.99±0.50)

Note. Mean values of proliferative response (cpm) of patients (n=10) MNC to specific tumor antigen in different concentrations and to control antigen (human brain tissue extract) are presented. In brackets: efficiency indexes, estimated as a quotient of division of antigen- (or control antigen)-induced proliferation by spontaneous proliferation. *p<0.05 compared to the status before vaccination (Mann—Whitney test).

TABLE 5. Analysis of Patient Survival during Therapy Using DC

Parameter	12 months		24 months		36 months	
	reference group	main group	reference group	main group	reference group	main group
All gliomas	52.5% (42/80)	74%* (29/39)	27.5% (22/80)	61%* (14/23)	19% (15/80)	50%* (4/8)
Astrocytomas	70% (33/47)	79% (19/24)	47% (22/47)	73%* (11/15)	32% (15/47)	57%* (4/7)
Glioblastomas	39% (9/33)	66.7%* (10/15)	0% (0/33)	40.0%* (4/10)	0% (0/33)	0% (0/1)

Note. *$p<0.05$ compared to the control group (χ^2).

tumor antigens in glioblastoma patients (n=10) was recorded after DC vaccination in 3 of 4 survivors (75%) and in only 2 of 6 patients (33%) who died within 24 months. These results indicate that generation of effective antigen-specific response is associated with a higher level of survival for patients with glioblastoma, though because of small sampling the differences manifested just as a trend and were not statistically significant.

The results indicate satisfactory tolerance of combined immunotherapy using DC and the absence of side toxic reactions at the stage of adoptive immunotherapy and at the stage of DC vaccination. Moreover, according to our pilot data, combined immunotherapy improves the quality of life and survival values. We found just few publications on clinical trials of DC-based vaccines. The authors reported that this approach is characterized by good tolerance and in some patients leads to generation of antitumor immune response not associated with autoimmune complications and to life span prolongation. Hybrid cells obtained by fusion of DC and tumor cells from patients with malignant glioma were tried: intracutaneous injection of hybrid autologous DC led to increase of intracellular IFN-γ expression by T cells in some patients [10]. Similar data were obtained with mature DC loaded with tumor lysate [22]. Dendritic cells loaded with proteins eluted from glioma cell surface were tried, which also led to activation of the cytotoxic activity and infiltration of the tumor by cytotoxic T-lymphocytes in the majority of patients [23]. Induction of antigen-specific antitumor response was paralleled by life span prolongation. It is noteworthy that no autoimmune neurotoxic reactions were observed in these studies. Our results are in line with these data and one more time demonstrate the safety and efficiency of immunotherapy in MBT patients. We tried a new type of DC generated in the presence of IFN-α, which are presumably superior by their functional characteristics to DC obtained by the standard protocol using GM-CSF and IL-4.

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