Chinese Journal of Cancer Research 10(4): 275-280, 1998

STUDY ON THE MECHANISM OF ESCAPING IMMUNE SURVEILLANCE IN HUMAN GLIOMAS

Pu Peiyu 浦佩玉　　Xu Xiaohua 徐小华

Department of Neurosurgery, Tianjin Medical University General Hospital, Tianjin 300052

Objective: To study mechanisms by which human gliomas may escape immune surveillance. Methods: The effect of supernatant (SN) obtained from cultured media of malignant glioma cell lines on the proliferation of phytohemagglutinin-p stimulated peripheral blood lymphocytes (PBLs) from healthy subjects and patients with gliomas was examined by MTT assay. The immunosuppressive factor which might be existed in the SN was identified by neutralization method with specific antibodies and Northern blot hybridization of glioma cells. In addition, the cellular immunity of patients with gliomas and relevant hormone and catecholamine were determined. Results: It was found that the malignant glioma cells could release an immunosuppressive factor in an autocrine fashion which was further identified as the transforming growth factor β_2 (TGF-β_2). It was also demonstrated that the plasma levels of norepinephrine in glioma patients were significantly reduced and correlated well with the suppression of the patients' own cellular immunity. Conclusions: Two distinct mechanisms by which human gliomas may evade immune surveillance: 1. The secretion of an immunosuppressive factor which was identified as TGF-β_2; 2. The dysfunction of Neuro-Immune modulation in the presence of cerebral gliomas.

Key words: Glioma, Cellular immunity, Immunosuppressive factor, TGF-β_2, Neuro-immune modulation

One of the explanations of why human gliomas could evade immune surveillance was thought to be related to the autocrine of an immunosuppressive factor which has been found to be identical to TGF-β_2[1,2]. So we have engaged in study on the immunosuppression in gliomas. Besides, according to the theory of Neuro-Endocrino-Immune Network, the central nervous system (CNS) modulates the function of the immune system via some pathways mediated by hormones, neuro-peptides and neuro-transmitters. Therefore, we hypothesized that the integrity of the CNS would be disturbed in the presence of cerebral gliomas which might affect the function of neuro-immune network. In order to verify our new hypothesis, we have examined PBLs subpopulation, the blastogenic response of PBL to different concentration of PHA-P, some relevant hormones and neuro-transmitters including cortisol, growth hormone, norepinephrine, dopamine, epinephrine etc. in the patients with gliomas.

Accepted May 19, 1998

This project was supported by the National natural Science Foundation of China, No. 39170737

MATIERALS AND METHODS

Reagents

Phytohemagglutinin-p(PHA-P) was purchased from Difco, USA. Neutralizing anti- TGF-β_2 antibodies (IgG) were kindly provided by Dr. P Ramage (Sandoz Company, Basel, Switzerland). MTT< 3 -(4,5-dimethythiazol-2yl)2,5-diphenyl tetrazolium bromide >was purchased from Sigma, USA.

Cell Culture Media

Cells were incubated at 37°C in 5% CO2. The following cell culture media were used. (a) Medium for PBL: RPMI-1640 (Gibco, USA), supplemented with 15% fetal calf serum (FCS); (b) glioma cell

culture medium: Waymouth MAB 87/3 medium (Gibco, USA), supplemented with 15% fetal calf serum (Institute of Hematology, Chinese Academy of Medical Science).

Malignant Glioma Cell Lines and Collection of Supernatant (SN) from Cultured Media

The establishment and characteristics of human malignant glioma cell lines TJ 861 and TJ 905 used in this study have been described.[3,4] For collection of SN, serum-free medium for glioma cells was added to confluent cell cultures. After 3 days the spent culture media were collected and stored at -20°C until further use.

Healthy Subjects and patient Population for PBL's Collection

The PBLs of healthy subjects and patients harboring supratentorial gliomas were collected. None of the patients were receiving corticosteroids, phenytoin, chemotherapy or radiation therapy at the time blood was drawn for study. Healthy hospital employees served as controls. 8 were men and 8 were women; ages ranged from 23 to 56 years (median=34). 42 glioma patients with different grades of malignancy (24 with high grade gliomas, 18 with low grade gliomas) were studied. 24 were men and 18 were women; ages ranged from 10 to 69 yr (median=48). Heparinized venous blood obtained from healthy, medication-free control subjects and glioma patients several days before surgery was separated on a Ficoll-Hypaque gradient[5] and centrifuged at 400 G for 20 minutes at room temperature. After centrifugation, the PBLs were harvested from the density interface, washed twice with Hanks' balanced salt solution, and adjusted to 1 x 10^6 cells/ml in RPMI-1640 medium.

The Effect of Malignant Glioma-Derived Supernatant (SN) on the Proliferation of PHA-p Stimulated PBLs

PBLs were adjusted to 1 x 10^6 cells/ml in RPMI-1640 medium containing 15% FCS and plated 1 x 10^5 cells in 100 µl medium to each well of 96 well plates in triplicate. The cells were stimulated with PHA-P at a final concentration of 2.5 µg/ml and divided into seven groups: The control cell group was added with 100 µl of RPMI-1640 medium per well; the experimental groups were added with supernatants diluted with RPMI 1640 medium into SN (1: 2), (1: 4). (1: 8), (1: 16), (1:32) and (1: 64). The cells were incubated at 37°C in 5% CO_2 for 72 hours. Then the proliferative activity of the PBLs was determined by MTT assay as described previously.[6] The results were presented with percentage of control.

Neutralization of TJ861 SN with Anti-TGF-β_2 Antibodies

The PBLs of 14 glioma patients with different grades of malignancy (7 with low grade of malignancy. 7 with high grade of malignancy) were collected as above mentioned. The PBLs were adjusted to 1 x 10^6 cells/ml in serum-free RPMI 1640 medium. The cells were dispensed in 100 µl volumes into 96-well microtiter plates at a final concentration of 1 x 10^5 cells/well. The triplicate cell cultures were activated with 2.5 µg/ml of PHA-P. The experiments consisted of three groups: 1) The control group: added with 100 µl of serum-free RPMI 1640 medium per well; 2) SN group: each well was added with 1: 2 SN 100 µl, i.e. TJ861 SN diluted with equal amount of serum free RPMI 1640; 3) SN+MAb group: anti-TGF-monoclonal antibody at the concentration of 25 µg/ml was preincubated for 2 hours at room temperature with TJ861 SN, then 100 µl of neutralized 1: 2 SN was added to each well. The cultures were incubated at 37°C in 5%CO_2 for 72 hours. The proliferative activity of the PBLs was determined by MTT assay.

Avidin-Biotin Peroxidase Complex Staining

For examination of TGF-β_2 expression in normal human brain tissues & malignant glioma cell lines, the avidin-biotin peroxidase complex (ABC) technique was employed as described previously.[7] The anti-TGF-β_2 monoclonal antibodies were diluted 1: 80 with PBS at the time of use.

Northern Blot Analysis

For examination of the TGF-β_2 mRNA expression, total RNA was extracted from 4 human fetal brain tissues and TJ861, TJ905 cell lines by the acid guanidinium-phenol-chloroform method.[8] TGF-β_2 oligonucleotide probes were synthesized on a model 380A DNA synthesizer (Applied Biosystems, Foster City, CA). Northern blot hybridization was carried out

for TGF-β_2 mRNA analysis.

The Study on the Function of Neuro-Endocrino-Immune Network

43 healthy volunteers served as controls. 20 were men and 23 were women; ages ranged from 20 to 58 years (median=35). 31 glioma patients with different grades of malignancy (20 with high grade of malignancy, 11 with low grade of malignancy). 18 were men and 13 were women; ages ranged from 26 to 60 years (median=38). The PBLs subsets, the lymphocyte proliferation assay, some related neuro-transmitters and hormones were tested. The CD3, CD4, CD8, CD20 subsets of PBLs were presented with percentage of total PBLs. The lymphocyte proliferation assay was presented with stimulating index (SI). Both methods were referred to the reference.[9] The plasma levels of norepinephrine (NE), epinephrine (EPI) and dopamine (DA) were examined by high performance liquid chromatography, the detailed method was described previously.[10] The serum cortisol was determined with KCOD1 radioimmunological detection kit (DPC, USA). The serum growth hormone was detected with radioimmunological detection kit (Institute of Shanghai Biological Products, P.R. China).

RESULTS

Inhibition of PBLs Growth by SN of Malignant Glioma Cells

The effect of SN of two human malignant glioma cell lines on the response of PBLs obtained from healthy volunteers and glioma patients to PHA-p was shown in Table 1. SN of two malignant glioma cell lines was found to be inhibitory on the lectin response in a dose-dependent manner.

Table 1. Inhibitory rate of SN TJ861 and TJ905 cell lines on the response of PHA-P stimulated PBLs obtained from glioma patients and healthy subjects

Concentration of SN	PBLs obtained from healthy subjects		PBLs obtained from glioma patients	
	Inhibitory rate of TJ861 SN(%)	Inhibitory rate of TJ905 SN(%)	Inhibitory rate of TJ861 SN(%)	Inhibitory rate of TJ905 SN(%)
1: 2	39. 39 ± 4. 71[a]	33.03 ± 3. 96[a]	32. 31 ± 8. 72[a]	33. 86 ± 8. 74[a]
1: 4	25. 00 ± 4. 25[a]	16. 96 ± 2. 71[b]	23. 08 ± 7. 15[a]	23. 62 ± 6. 85[a]
1: 8	23. 21 ± 4. 18[a]	13. 39 ± 1. 88[c]	19. 23 ± 6. 15[b]	17. 32 ± 5. 20[b]
1: 16	19. 64 ± 3. 14[b]	5. 36 ± 0. 80	15. 39 ± 5. 08[b]	13. 39 ± 4. 55[c]
1: 32	17. 86 ± 3. 04[b]	4. 46 ± 0. 63	14. 62 ± 4. 82[c]	11. 02 ± 4. 00[c]
1: 64	13. 39 ± 2. 41[c]	1. 79 ± 0. 23	13. 08 ± 4. 32[c]	0
R(n=6)	0. 974	0. 990	0. 995	0. 923

Note: 1. Compared to those in control group, a: $P<0.001$, b: $P<0.01$, c: $P<0.05$.

2. "r" stands for the correlative coefficient between SN dose and inhibitory rate.

Neutralization of TJ861 SN Activity by Anti-TGF-β_2 Antibody

The effect of TJ861 SN after neutralization with anti-TGF-β_2 monoclonal antibodies on the response of PHA-p stimulated PBLs obtained from glioma patients is demonstrated in Table 2. The neutralized TJ861 SN by anti-TGF-β_2 antibodies failed to suppress significantly the PHA-p activated response of PBLs obtained from glioma patients.

Immunohistochemical Staining by Anti-TGF-β_2 Monoclonal Antibodies

Immunohistochemical staining with anti-TGF-β_2 monoclonal antibodies in two malignant glioma cell lines and normal brain tissues was shown in Figure 1–3. It was found to be positive staining in two malignant glioma cell lines, in contrast, negative in normal brain tissues.

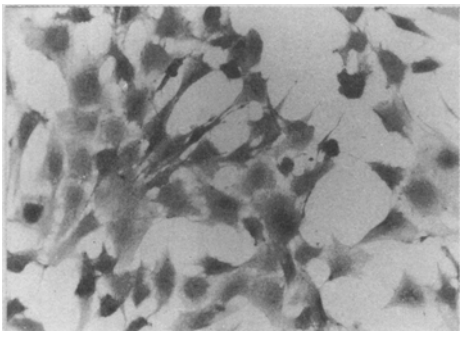

Fig 1. Positive immunostaining of the TGHF-β_2 in TJ861 cells. ABC method x 200.

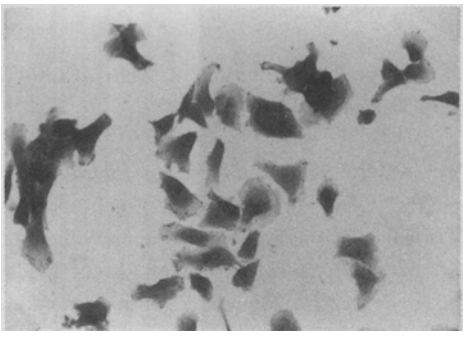

Fig 2. Positive immunostaining of the TGF-β_2 in TJ905 cells. ABC method x 200.

Table 2. Inhibitory rate of TJ861 SN after neurotralization with anti-TGF-β_2 monoclonal antibodies on the PHA-P stimulated response of PBLs obtained from glioma patients

	Control group N=14	SN (1: 2) N=14	SN (1: 2) + MAb (25 μg/ml) N=14
OD570	0. 97 ± 0. 14	0. 74 ± 0. 16	0. 88 ± 0. 15[a]
Inhibitory rate (%)		23. 73 ± 3. 79	9. 28 ± 1. 39[a]

*Compared to the group of SN (1: 2), a: $P<0.05$

Table 3. The PBL subsets (%) and PBL proliferative rate (SI) in glioma patients and healthy subjects

	Control group (n)	Glioma group (n)	*P* value
CD3 subset	64. 85 ± 2. 37 (30)	53. 93 ± 2. 14 (31)	<0.001
CD4 subset	42. 54 ± 2. 49 (30)	39. 36 ± 1. 73 (31)	<0.001
CD8 subset	23. 82 ± 2. 49 (30)	27. 07 ± 2. 00 (31)	<0.001
CD4/CD8 ratio	1. 79 ± 0. 16 (30)	1. 46 ± 0. 14 (31)	<0.001
CD20 subset	10. 47 ± 1. 80 (30)	13. 26 ± 6. 65 (31)	>0.05
PHA-p 1 μg/ml	0. 81 ± 0. 30 (18)	0. 53 ± 0. 18 (25)	<0.001
PHA-p 0.25 μg/ml	0. 54 ± 0. 24 (23)	0. 31 ± 0. 12 (25)	<0.001

Expression of Transforming Growth Factor-β_2 mRNA

Transforming growth factor-β_2 mRNA transcripts corresponding to approximate 6 kb were present in two malignant glioma cell lines. However, human fetal brain specimens did not express TGF-β_2 (Figure 4).

Lymphocyte Subsets and Lymphocyte Proliferative Rate in the Patients and Control Subjects

Table 3 showed the quantitative analysis of pan T (CD3+) cells and their subsets (CD4+, CD8+), pan B+ lymphocytes and PHA-p stimulated PBLs proliferative rate in the glioma patients and control subjects. The percentage of circulating CD3+ (pan T) cells, CD4+ cell category, CD4/CD8 ratio, the lymphocyte proliferative rate were reduced significantly ($P<0.001$), while the percentage of CD8+ cells was increased significantly ($P<0.001$), the percentage of CD20+ (pan B+) lymphocytes remained within normal limits ($P>0.05$) in the different kinds of gliomas as

compared to the values in the control subjects.

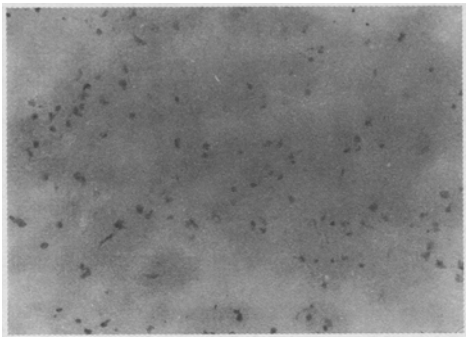

Fig 3. Negative immunostaining of the TGF-β_2 in normal brain tissues. ABC method x 200.

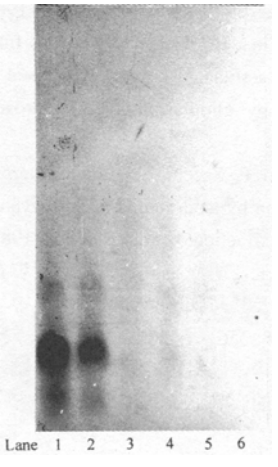

Fig 4. 6kb TGF-β_2 mRNA was found to be expressed in TJ861 and TJ905 cells, while no expression was noted in 4 normal human brain specimens.

Lane 1. TJ861 cells. Lane 2. TJ905 cells. Lane 3-6. Normal fetal brain tissues.

The Plasma Content of Catecholamine

Compared to those in the control group, the plasma levels of norepinephrine and epinephrine were decreased significantly, while the plasma content of dopamine remained within normal range in the group of 30 glioma patients as shown in Table 4.

Correlation between the Plasma Content of Catecholamine and the Index of Cellular Immunity in Glioma Patients

The plasma level of NE was correlated negatively with the percentage of CD8+ cells (r= -0.377, n=26, $P<0.05$), but positively with CD4/CD8 ratio (r=0.407, n=26, $P<0.0025$) in the peripheral blood of glioma patients. Positive correlation also existed between the level of NE and the proliferative rate of PHA-p stimulated lymphocytes in the peripheral blood of glioma patients (r=0.769, n=12, $P<0.01$).

The levels of cortisol and growth hormones were both within normal limits.

Table 4. The serum levels of catecholamine in glioma patients (ng/ml)

	Control group (n)	Glioma group (n)	P value
NE	484.80±147.60	369.20±156.24	<0.002
EPI	352.20±140.31	242.02±118.90	<0.001
DA	101.42±28.34	88.48±83.10	>0.05

DISCUSSION

In the present study, the supernatants of two malignant glioma cell lines inhibited significantly the mitogenic response of PBLs *in vitro*, either from healthy volunteers or from patients with gliomas in a dose-dependent manner. This evidence indicated that the human malignant glioma cells secreted an immunosuppressive factor which was autocrine in fashion. The anti-TGF-β_2 monoclonal antibodies could neurtralize the inhibitory effect of TJ861 SN on PHA-p activated response of PBLs obtained from glioma patients. Positive immunostaining of the TGF-β_2 existed in two malignant glioma cell lines. Accordingly, 6 kb TGF-β_2 mRNA was also expressed in the same two malignant glioma cell lines, while negative expression of TGF-β_2 peptide and its mRNA was found in normal brain or fetal brain specimens. These facts suggested that the autocrine of an immunosuppressive factor by glioma cells, consisting predominantly of TGF-β_2, may contribute to the escape of immune surveillance for the development of gliomas. The plasma levels of NE and EPI were firstly found to be reduced in glioma patients in this study. Furthermore, the plasma level of NE was negatively correlated with the percentage of CD8+ subsets in PBLs obtained from the same patient with glioma,

while it was positively correlated with CD4/CD8 ratio and lymphocyte proliferative rate in the patient's PBLs. It is well known that the norepinepherinergic nerves are distributed in the vascular system and intrinsic region of lymph organs in mammary animals; epinephrine β_2 receptor exists in T,B lymphocytes and monocytes. The immune enhancement of NE can be blocked by the β_2 receptor antagonist. Hence NE can enhance immune response by activation of the β_2 receptor. It demonstrates that the lower level of plasma NE in glioma patients may reduce its remote effect on the immune system. These preliminary results support our new hypothesis that the dysfunction of neuro-Immune Network may be another pathway which contributes to the depressed cellular immunity and the escape of immune surveillance in glioma patients. Our experiments led to the identification of two mechanisms by which human glioma cells may evade immune attack: 1. The secretion of an immuno-suppressive factor which was identified as TGF-β_2; 2. The dysfunction of the Neuro-Endocrino-Immune Network in the presence of cerebral gliomas. But a very complex network existed in the Neuro-Endocrino-Immune system, many hormones, neurotransmitters & neuropeptides involve in this network, it is necessary to study further for confirmation of this hypothesis.

REFERENCES

1. Bodmer S, Siepl C, Fontana A. Immunoregulatory Factors Secreted by Glioblastoma Cells: Glioblastoma-Derived T-Cell Suppressor Factor/Transforming Growth Factor-β_2. In: Neuroimmune Networks: Physiology and diseases. Alan R. Liss, Inc., 1989; 73.
2. Kuppner MC, Hamou MF, Sawamura Y, et al. Inhibition of lymphocyte function by glioblastoma-derived transforming growth factor β_2. J Neurosurg 1989; 71: 211.
3. 浦佩玉，钟跃，王淑兰，等。初发及复发恶性星形细胞瘤体外细胞系 TJ899 及 TJ905 的建立与其特征比较。中华神经外科杂志 1992; 8: 38
4. 王淑兰，李峰，浦佩玉，等。多形性胶质母细胞瘤体外细胞系 TJ899 及 TJ905 的建立及其特征。天津医药 1996; 24: 416.
5. Elliott LH, Brooks WH, Roszman TH. Activation of immunoregulatory lymphocytes obtained from patients with malignant gliomas. J Neurosurg 1987; 67: 231.
6. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55.
7. Black KL, Chen K, Becker DP, et al. Inflammatory leukocytes associated with increased immuno-suppression by glioblastoma. J Neurosurg 1992; 77: 120.
8. Chomozynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156.
9. 林彤，浦佩玉，胡应礼，等。胶质瘤患者细胞免疫功能的检测及其与临床指标的关系。中华神经外科杂志 1992; 8: 256。
10. 林雯，江德华。脑室内注射纳络酮对脑缺血初期单胺递质变化的影响。中华神经外科杂志 1990; 6(增刊): 42.