Neuroprotective Effect of Ultra-Low Doses of Antibodies against S100 Protein in Neuroblastoma Culture during Oxygen and Glucose Deprivation

T. M. Pankova, M. V. Starostina, M. B. Shtark, and O. I. Epstein*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 9, pp. 260-263, September, 2007
Original article submitted July 5, 2007

Antibodies against S100 protein applied in high and ultra-high dilutions possess neuroprotective activity and maintain survival of neuroblastoma C-1300 cells under conditions of oxygen and glucose deprivation. The examined antibody preparations stimulated differentiation in neuroblastoma culture thereby demonstrating pronounced neurotrophic activity.

Key Words: *antibodies; neuroblastoma; oxygen and glucose deprivation; neuroprotection*

A large body of investigations focused on the effects produced by extremely low doses of antibodies against bioactive chemicals promoted the development of a new class of therapeutic preparations [3]. For example, antibodies against S100 protein in ultra-low doses exhibit anxiolytic and antidepressant activity [2]. In addition, they demonstrate neuroprotective properties in experimental ischemic damage to the brain [1].

Transplantable cultures of human and animal neuroblastoma are widely employed to examine the mechanisms of action of various damaging factors such as glutamate toxicity, oxidative stress, and oxygen-glucose deprivation. In addition, they are used for the search for substances with neuroprotective properties.

Our aim was to study the effects of preparations based on high and ultra-high dilutions of antibodies against S100 protein in C-1300 neuroblastoma culture subjected to oxygen-glucose deprivation (OGD).

Institute of Molecular Biology and Biophysics, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk; *Research-and-Production Company "Materia Medica Holding", Moscow

MATERIALS AND METHODS

The experiments were carried out on C-1300 mouse neuroblastoma obtained from Institute of Human Morphology (Russian Academy of Medical Sciences). The cells were cultured at 36°C in a CO_2-incubator (5% CO_2, 80% humidity) in DMEM supplemented with 5% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The medium was changed every 2-3 days.

The study used preparations based on ultra-high dilutions of antibodies against S100 protein prepared using routine homeopathic technique (C6, C1000, and C12+C30+C200 mixture) and potentiated water in the same dilutions.

For evaluation of the effects of the above preparations on cell differentiation, the neuroblastoma cells were transferred to 24-well culture plates (5000 cells in 1 ml) in standard medium. After 24 h, the medium was replaced with a medium containing 2% serum. The test substances (or potentiated water) were added to the wells in 20 μl aliquots (2 wells for each agent); 20 μl medium was added to control cultures.

The cells were cultured for 4 days; thereafter the numbers of differentiated and undifferentiated cells were counted under an inverted Biolam-1 mi-

0007-4888/07/1443-0288

croscope (LOMO) at ×200. The cells were counted in 4 visual fields for each substance (~200 cells per a well). By definition, differentiated cells had processes with a length of no less than 2 cell diameter or more than 2 processes. Counting was performed rapidly to prevent changes in medium pH and temperature.

To study the effect of the test preparations on survival of neuroblastoma cell subjected to OGD, the medium was replaced with RPMI with the same admixtures in two last passages before testing. The cells were transferred into 24-well plates (20,000 cells in 3 ml per a well) into RPMI medium with admixtures, but without glucose, which was preliminary aerated with oxygen-free gas mixture (95% N_2, 5% CO_2, 1.5-2 h for 50 ml medium). The examined preparations (20 μl per well) were added simultaneously with the cells. The plates were placed for 20 h in a sealed chamber filled with the same gas mixture. The control cultures remained in the CO_2-incubator in standard RPMI medium containing glucose and all necessary admixtures.

In a special series of experiments, antibodies in C6 dilution were added to the culture for 20 h before hypoxia. During OGD, some cultures were maintained in the above-mentioned medium with antibodies, while others were placed into the antibody-free medium.

After 20 h, the numbers of live and dead cells were counted under an inverted microscope [8]. To this end, the medium in plates was replaced with 0.2% trypan blue on Hank's solution and the plates were placed for 5 min into CO_2-incubator. Then the dye was replaced with Hank's solution. The count of live and dead (colored) cells was performed in no less than 5 visual fields (200 cells on average).

The data were analyzed statistically using Origin 7.5 software and Student's *t* test.

RESULTS

OGD modeled *in vitro* in the transplanted cultures or in primary neuron cultures is a widely used model of brain ischemia. In various papers, the duration of hypoxia varied from 3-4 h to several days, which can be explained by different sensitivity of cultures and by specificity of methods used to record the examined effects. For example, assessment of cell survival in various neuroblastoma lines subjected to near-hypoxic oxygen deficiency showed that SK-N-DZ cells died after 24 h, SH-SY5Y cells lived for 4 days, while SK-N-AC could withstand hypoxia for 12-14 days [5]. Glucose deficiency in culture media potentated the effects of hypoxia [13]. In preliminary experiments, we determined

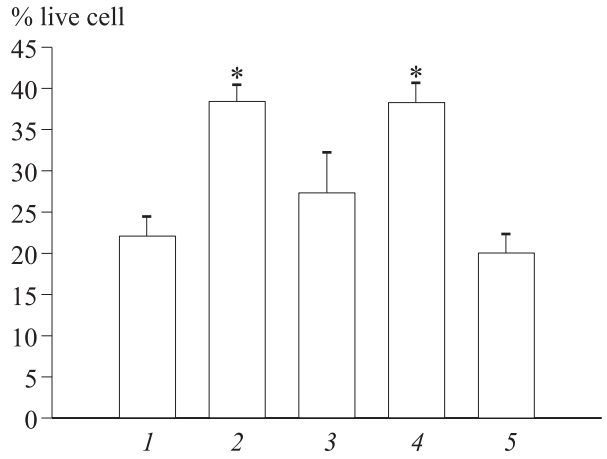

Fig. 1. Neuroprotective effects of ultra-low doses of antibodies against S100 protein in C-1300 neuroblastoma culture subjected to OGD. *1*) 20 h OGD, culturing without antibodies; *2*) OGD in the presence of antibodies in C1000 dilution; *3*) OGD in the presence of potentiated (C1000) water ; *4*) OGD in the presence of antibodies C12+C30+C200; *5*) OGD in the presence of potentiated water C12+C30+C200. Here and in Figs. 2 and 3: the mean data were calculated from independent experiments (n=4). *p<0.05 compared to cultures incubated without antibodies.

the exposure period necessary to reveal OGD effects in C-1300 neuroblastoma culture. To this end, we changed the exposure time from 1 to 24 h. The most clear and reproducible results were obtained in cultures subjected to OGD for 20 h, which can be explained by relative tolerance of neuroblastoma cells used in this study. These data agree with those obtained in human neuroblastoma line [8,15].

In this series, cell survival in control cultures attained 85-90%. By contrast, a great percent of cells died in cultures subjected to OGD (Fig. 1). The number of survived cells depended on the ini-

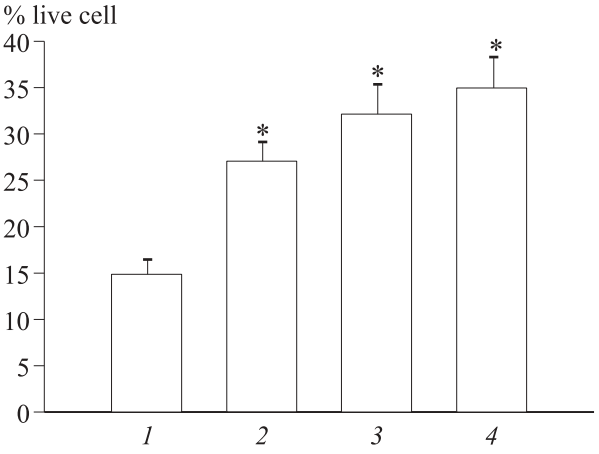

Fig. 2. Neuroprotective effects of antibodies against S100 protein in high dilution (C6) in C-1300 neuroblastoma culture subjected to OGD. *1*) 20 h OGD, culturing without antibodies; *2*) OGD in the presence of antibodies; *3*) 20 h culturing with antibodies before OGD and without antibodies during OGD; *4*) 20 h culturing with antibodies before and during OGD.

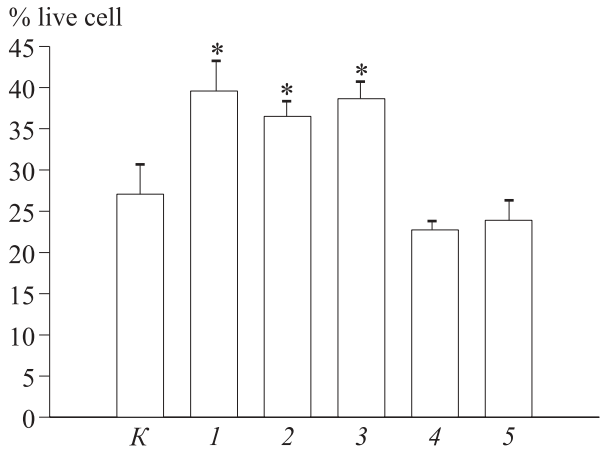

Fig. 3. Stimulation of neuroblast differentiation in C-1300 neuroblastoma culture by ultra-low doses of antibodies against S100 protein. *C*) control culture; *1*) culturing in the presence of antibodies (C6 dilution); *2*) antibodies in C1000 dilution; *3*) C12+C30+C200; *4*) culturing in the presence of potentiated water; *5*) potentiated water C12+C30+C200.

tial state of the culture and was 14-20%, therefore each series included the corresponding control.

All examined dilutions of antibodies significantly increased survival of neuroblastoma cells subjected to OGD (Figs. 1, 2). These effects were comparable to the action of vascular endothelial growth factor, whose neuroprotective effect is comprehensively examined on neuroblastoma culture [8]. Potentiated water produced no effect (Fig. 1); hence, the neuroprotective effects was produced by antibodies in high dilutions.

In a special series of experiments, antibodies in C6 dilution were added to standard medium 1 day before OGD (Fig. 2). The neuroprotective effect was observed in all cases, and was most pronounced when antibodies were present in the medium both before and during OGD (Fig. 2). The fact that antibodies present in the culture media only during the period preceding OGD still promoted cell survival attests to long-term protective action of the drug.

A large body of research demonstrated a correlation between hypoxia and the degree of cell differentiation in neuroblastoma. Clinical studies showed that the number of undifferentiated cells increases in solid neuroblastoma regions where vascularization is poor or absent, and this phenomenon is closely related to tumor malignancy [4,8, 9,14]. Hypoxia belongs to factors capable to induce *in vitro* cell differentiation in neuroblastoma cultures [5,7]. Oxygen deficiency directly affects activity of genes responsible for differentiation of neuroblasts [12,14]. The factors stimulating differentiation of neuroblasts exhibit also protective properties during hypoxia and other adverse exposures [7].

C-1300 neuroblastomacells used in this study can differentiate spontaneously. Under normal conditions, the culture contains about 25% cells with morphological signs of differentiated neurons. Examination of cultures subjected to OGD revealed the absence of the cells with projections, while cultures protected with ultra-low doses of antibodies contained differentiating neuroblasts. In a special experimental series, we analyzed neuritogenic activity of the test preparations. All three preparations significantly increased the number of differentiated neurons in neuroblastoma culture. At the same time, the effects of potentiated water did not differ from the control (Fig. 3).

Thus, all examined preparations of antibodies against S100 protein used in ultra-low doses demonstrated both the neuroprotective and differentiating (neuritogenic) effects in C-1300 neuroblastoma culture. It is noteworthy that S100 protein isolated from the brain also possesses neurotrophic activity and promotes survival of neurons and differentiation of neuroblasts [6,11]. Therefore, the effects of low doses of antibodies against S100 are similar to those exerted by the protein itself.

REFERENCES

1. G. A. Romanova, T. A. Voronina, *et al*., Sib. Vestn. Psikhiatr. Narkol., **1**, No. 27, 123-125 (2003).
2. O. I. Epshtein, S. A. Sergeeva, *et al*., in: *Proproten-100. Ultra-low Doses of Affinity-Purified Antibodies against S-100 Protein* [in Russian], Moscow (2002), pp. 108-112.
3. O. I. Epshtein, M. B. Shtark, *et al*., *Pharmacology of Ultra-low Doses of Antibodies against Endogenous Functional Regulators* [in Russian], Moscow (2005).
4. H. Axelson, E. Fredlund, M. Ovenberger, *et al*., *Semin. Cell Dev. Biol.*, **16**, No. 4-5, 554-563 (2005).
5. F. Hedborg, E. Ulleras, L. Grimelius, *et al*., *FASEB J*., **17**, No. 6, 598-609 (2003).
6. C. W. Heizmann, G. Fritz, and B. W. Schafer, *Front. Biosci.*, **7**, dl356-dl368 (2002).
7. J. C. Hoehner and K. Prabhakaran, *J. Pediatr. Surg.*, **38**, No. 7, 1069-1074 (2003).
8. K. L. Jin, X. O. Mao, and D. A. Greenberg, *Proc. Natl. Acad. Sci. USA*, **97**, No. 18, 10242-10247 (2000).
9. A. Jogi, I. Ora, H. Nilsson, *et al*., *Ibid.*, **99**, No. 10, 7021-7026 (2002).
10. A. Jogi, I. Ora, H. Nilsson, *et al*., *Cancer Lett.*, **197**, No. 1-2, 145-150 (2003).
11. A. Kleindienst and M. Ross Bullock, *J. Neurotrauma*, **23**, N 8. P. 1185-1200 (2006).
12. T. Lofstedt, A. Jogi, M. Sigvardsson, *et al*., *J. Biol. Chem.*, **279**, No. 38, 39223-39231 (2004).
13. H. Nilsson, A. Jogi, S. Beckman, *et al*., *Exp. Cell. Res.*, **303**, No. 2, 447-456 (2005).
14. S. Pahlman, M. T. Stockhausen, E. Fredlund, and H. Axelson, *Semin. Cancer Biol*., **14**, No. 5, 365-373 (2004).
15. I. F. Smith, J. P. Boyle, and P. F. Vaughan, *J. Neurochem*., **79**, No. 4, 867-874 (2001).