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PHYSIOLOGY

The Effect of Hexapeptide Glutoxim on Tissue Explant Development in Organoid Cultures

L. A. Kozhemyakin*, N. I. Chalisova, O. S. Ketlinskaya*, V. A. Penniyainen**, A. V. Komashnya**, and Academician A. D. Nozdrachev****

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The biological integrity of an organism is regulated at the cellular level by humoral factors termed cytokines. They belong to an large class of para- and autocrine polypeptides that mediate signal transduction between different cell types. These secretory proteins maintain a complex equilibrium between integrative physiological processes, cell proliferation and physiological cell death or apoptosis. The cytokine family includes a series of interleukins and peptide growth factors, i.e., polypeptides with low molecular weight. Currently, the theory and methodology of drugs with specified properties are being developed. Glutoxim®, a chemically synthesized biologically active compound, is a peptide analog of the key metabolites [1]. Glutoxim is a hexapeptide with a stabilized disulfide bond (sodium bis-γ-*L*-glutamyl)-*L*-cysteinyl-bis-glycinate); the summary formula is ($C_{20}H_{32}O_{16}N_6S_2$). Glutoxim belongs to Thiopoetins® and regulates intracellular thiol metabolism, which plays an important role in cellular and tissue genetic and metabolic processes. Glutoxim is used as an immunomodulator capable of modifying the cell response during proliferation, differentiation, and apoptosis, as well as in case of various conditions and diseases when the endogenous production of cytokines and hemopoietic factors should be activated. As shown by histochemical methods, glutoxim stimulates a cascade of mechanisms underlying phosphate modification of the key proteins of the signal-transduction systems. This agent triggers the cytokine system, including the endogenous production of IL-1, IL-6, TNF, IFN, and erythropoietin and mimicking the effect of IL-2 [1, 2]. Organoid tissue cultures are the most adequate model for studying the direct effect of biologically active substances on cells [3, 4]. In this model, nervous, humoral, and other effects that take place in the whole organism are eliminated, whereas the "hierarchic" relationships between various cell populations are retained.

In this work, the effect of glutoxim on tissue explant development was studied using the organoid cultures of various tissues and organs, including the cerebral cortex, liver, heart, kidney, and lung, which were obtained from one-day-old immature and one-month-old mature rats. In one-day-old rats, glutoxim at concentrations of 0.05–400 ng/ml either inhibited the explant growth or had no effect. Conversely, in one-month-old rats, glutoxim used at effective concentrations stimulated the growth of all tissue explants. Thus, glutoxim has a stimulating effect on mature tissues but inhibits immature tissues, in which the processes of programmed cell death are pronounced.

The organoid cultures were maintained as described previously [4–6]. Explants of the cerebral cortex, liver, heart, kidney, and lung (300 explants of each) were obtained from one-day- or one-month-old Wistar rats. The fragments of rat tissues isolated under sterile conditions were divided into smaller fragments of about 1 mm^3 in size. The latter were placed into Petri dishes with a collagen-coated bottom. The nutrient medium contained 35% of Eagle's medium, 35% of Hanks solution, 25% of fetal calf serum, and 5% of chicken embryonic extract. The medium was supplemented with glucose (0.6%), insulin (0.5 U/ml), and gentamycin (100 U/ml). Glutoxim was added to the culture medium at concentrations ranging from 0.001 to 600 ng/ml. The Petri dishes were kept in a temperature-controlled chamber at 37°C for three days and then examined under a phase-contrast microscope. The area index (AI), i.e., the ratio of the entire explant area, including the zone of migrating cells, to the central area of the explant was calculated in arbitrarily units.

The explants were examined using a series 10 MTN-13 television attachment to the microscope (Alfa-Telecom, Russia). The AI was calculated using the PhotoM 1.2 software. The cell composition of the explant growth area was determined by hematoxylin–eosin staining. The effect of each substance was assessed by examining 20–25 experimental and the same number of control explants. Control explants grew in the medium without additions; glutoxim was added to the experimental explants at various concen-

* *Institute of Bioregulation and Gerontology, Russian Academy of Medical Sciences, pr. Dinamo 3, St. Petersburg, 197110 Russia*

** *Pavlov Institute of Physiology, Russian Academy of Sciences, nab. Makarova 6, St. Petersburg, 199034 Russia*

trations. Significant differences between the AIs of control and experimental explants were estimated using Student's *t* test. The AI was expressed in percent; the control AI value was taken to be 100%.

During the first day of cultivation, the explants spread over the collagen support. A typical peripheral zone of explants of various tissues contained a marked peripheral growth area and an explant capsule consisting of two layers of fibroblasts. The capsule surface was covered with mesothelium that formed a discontinuouslayer. Mesothelium cells often detached from one another and from the basal membrane and became rounded. Instead of the detached cells, large gaps were formed, through which a portion of cells migrated out of the explant. Among the migrating and proliferating cells, there were macrophages, fibroblasts, and specialized cells corresponding to the explant tissue type, for example, alveolar epithelium in lung explants and hepatocytes in liver ones. These cells formed the peripheral growth zone, which was measured to determine the AI of each explant. If an increase in growth zone was stimulated, the AIs of experimental explants exceeded those of control explants after three days. In the case of growth deceleration or suppression, the AIs of experimental explants decreased compared to those of control explants.

In the first series of experiments, tissue explants from one-month-old rats were studied. In cerebral-cortex cultures, migrating neurons with large central nuclei appeared in the growth zone, as well as the proliferating glial cells and fibroblast-like elements. The addition of glutoxim (20 ng/ml) into the culture medium of the cerebral-cortex explants led to a statistically significant increase in AI by 37 ± 7% compared to the control explants (n = 20 and n = 19, respectively; $p < 0.05$). However, higher glutoxim concentrations (up to 100 ng/ml) eliminated this effect, and the AI of the experimental explants was similar to this parameter in the control (Fig. 1). At glutoxim concentrations of 200 and 400 ng/ml, proliferation was slightly inhibited.

In cultures of liver fragments, the growth zone was represented by migrating hepatocytes, mast cells, and fibroblasts. At a concentration of glutoxim in the culture medium as low as 0.005 ng/ml, AI significantly ($p < 0.05$) increased by 20 ± 5% (n = 18) compared to control explants (n = 20) (Fig. 2). Further increase in glutoxim concentration resulted in a statistically insignificant inhibition (AI was reduced by 15% at a glutoxim concentration of 0.1 ng/ml). At a glutoxim concentration of 20 ng/ml, AI increased by 12%; at higher concentrations, there was no significant difference between the experiment and control AIs. However, at a drug concentration of 400 ng/ml, proliferation was significantly increased. Thus, two peaks of proliferative activity were observed.

In experiments on the cultivation of kidney fragments, the migration of fibroblast-like elements and of cells from the core layer and parenchyma was

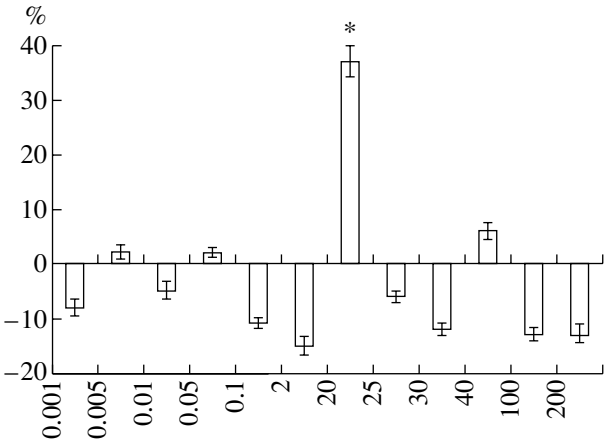

Fig. 1. The effect of glutoxim on the cerebral-cortex explants obtained from one-month-old rats. *Y* axis, AI; *X* axis, glutoxim concentration, ng/ml. Here and in Fig. 2: vertical segments indicate 95% confidence intervals for mean values; * significant difference from control values ($p < 0.05$).

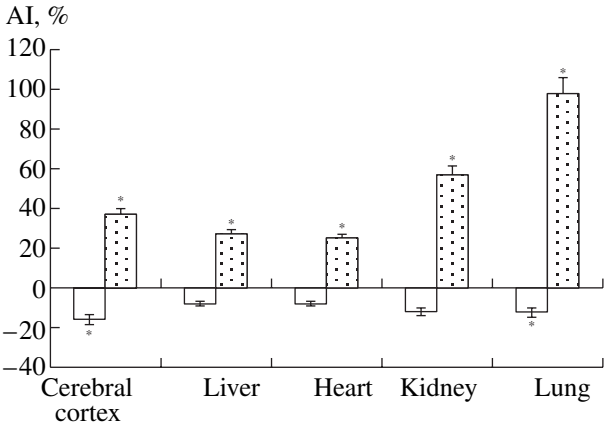

Fig. 2. The effect of glutoxim on explants from the cerebral cortex, liver, heart, kidney, and lung of one-day-old (light columns) and one-month-old (hatched columns) rats.

observed. At a glutoxim concentration of 2 ng/ml, AI increased by 30 ± 7% (n = 20, $p < 0.05$); at higher concentrations, there was no significant difference between the AIs of the experimental and control cultures (Fig. 2). At drug concentrations of 100, 200, and 400 ng/ml, AI increased significantly by 28–57%.

In the cultured heart fragments, the growth zone contained migrating cardiomyocytes and fibroblasts. The maximum decrease in proliferative activity was observed at a drug concentration of 0.005 ng/ml, whereas a significant peak of AI (as high as 16 ± 3%; n = 18, $p < 0.05$) was observed at a concentration of 2 ng/ml (Fig. 2). At concentrations of 400 and 600 ng/ml, proliferation was inhibited.

In the culture of lung fragments, the growth zone contained alveolar epithelium cells and numerous fibroblasts that formed a monolayer over the entire

perimeter of the explants. A statistically significant increase in AI was observed at drug concentrations of 2 and 100–400 ng/ml (Fig. 2). For example, at a glutoxim concentration of 100 ng/ml, AI was increased by 98 ± 11% ($n = 21, p < 0.05$) compared to the control ($n = 22$).

Thus, effective glutoxim concentrations were determined that caused the maximum increase in the proliferative activity of cultured mature tissues of the cerebral cortex, liver, kidney, heart, and lung.

In the second experimental series, the explants of immature tissues obtained from newborn one-day-old rats were cultivated. The effective glutoxim concentrations determined in the first series caused the opposite effects after addition to the cultures of immature tissues. The growth of all cultured explants from various tissues of one-day-old rats was inhibited and AI values were reduced (Fig. 2). The growth zone of the brain cortex explants was significantly reduces by 20 ng/ml glutoxim (the dose that significantly increased AI in explants from one-month-old rat), with the AI of immature explants being 16 ± 5% lower than in the control ($n = 23$ and $n = 22$, respectively; $p < 0.05$). At a glutoxim concentration of 100 ng/ml, the AI of the lung immature explants was 22 ± 7% lower ($n = 25; p < 0.05$) than in the control ($n = 20$). The effect of a glutoxim concentration of 20 ng/ml on kidney explants resulted in an AI decrease by 12 ± 3% ($n = 20; p < 0.05$) compared to the control ($n = 18$). In heart and liver explants from newborn rats, the growth zone was also reduced by glutoxim at concentrations of 2 and 0.005 ng/ml, respectively. The same concentrations stimulated the development of heart and liver explants obtained from one-month-old animals.

Thus, the results of our study suggest that glutoxim regulates cell proliferation and apoptosis at different stages of individual development. This phenomenon depends on the stages of tissue maturation, which differ in the degree of programmed cell death or apoptosis [7–10]. We may conclude that glutoxim inhibits the development of explants obtained from various immature tissues of newborn animals, because it enhances programmed cells death. In the mature tissues of three-week-old animals, in which programmed cell death is less intense, glutoxim enhances the proliferative cell potential.

In our previous studies [3, 4], differently directed effects of four polar amino acids (lysine, arginine, asparagine, and glutamic acid) and synthetic thymus dipeptides (Lys–Trp and Lys–Glu) on both nervous and lymphoid tissues were also shown to depend on the degree of tissue maturation. The data on glutoxim effect also confirm the current view that short peptides regulate key cellular processes, including cell proliferation and cell death [4, 11–14].

Glutoxim belongs to a new generation of immunomodulating biologically active substances. It may be effective when used in combination with antibacterial, antiviral, anti-tuberculosis, and anti-tumor chemotherapy, because it eliminates the toxic effect of the latter. Here, we have demonstrated that glutoxim stimulates cell proliferation in mature tissues, which testifies to the lack of neuro-, nephro-, hepato-, or cardiotoxicity of this drug. The fact that glutoxim stimulates apoptosis in immature nondifferentiated tissues holds much promise for the use of this drug in tumor treatment. Thus, the short synthetic peptide glutoxim exhibits specific properties. It has opposite effects on mature differentiated cells and immature cells (and tumor cells are immature). The new methodology of a combination of anti-tumor chemotherapy and synthetic peptides may become a promising approach in modern biology and medicine.

REFERENCES

1. Kozhemyakin, L.A. and Balazovskii, M.B., RF Patent 2 089 179, *Byull. Izobret.*, 1997, no. 25.
2. Kozhemyakin, L.A., Belokhvostov, A.S., Antonov, V.G., *et al.*, *Vopr. Onkol.*, 1999, vol. 45, no. 6, pp. 89–94.
3. Chalisova, N.I., Penniyainen, V.A., and Khaaze, G., *Ross. Fiziol. Zh.*, 2002, vol. 88, no. 5, pp. 627–633.
4. Chalisova, N.I., Khavinson, V.Kh., and Nozdrachev, A.D., *Dokl. Akad. Nauk*, 2001, vol. 379, no. 3, pp. 411–413.
5. Levi-Montalchini, R. and Angeletty, P., *Physiol. Rev.,* 1982, vol. 48, pp. 534–569.
6. Kalyunov, V.N., *Biologiya faktora rosta nervnoi tkani* (The Biology of Nerve Growth Factor), Minsk: Nauka i Tekhnika, 1986.
7. Novikov, V.S., Bulavina, D.V., and Tsygan, V.N., in *Programmirovannaya kletochnaya gibel'* (Programmed Cell Death), St. Petersburg: Nauka, 1998.
8. Zamyatnin, A.A., *Dokl. Akad. Nauk SSSR,* 1987, vol. 292, no. 5, pp. 1261–1264.
9. Miller, J.F., *Immunologist*, 1996, vol. 1, pp. 9–15.
10. Arai, T., Hiromatsu, K., and Nishimura, H., *Biochem. Biophys. Res. Comm.*, 1995, vol. 213, no. 2, pp. 600–607.
11. Canete, M., Juarranz, A., Lopez-Nieva, P., *et al.*, *Acta Histochem.*, 2001, vol. 103, no. 2, pp. 117–126.
12. Belokrylov, G.A., Popova, O.Ya., and Derevnina, O.N., *Drug Dev. Ind. Pharm.*, 1998, vol. 24, no. 2, pp. 115–127.
13. Shataeva, L.K., Khavinson, V.Kh., and Ryadnova, I.Yu., *Peptidnaya samoregulyatsiya zhivykh sistem* (Peptide Self-regulation of Living Systems), St. Petersburg: Nauka, 2003.
14. Pauling, L., *Science,* 1961, vol. 134, no. 1, p. 15.