Peptidegic Regulation of Differentiation of Embryonic Retinal Cells

V. Kh. Khavinson[1,2], V. E. Pronyaeva[2], N. S. Linkova[2], and S. V. Trofimova[2]

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We studied Molecular mechanisms of the retinoprotective effect of short peptides AEDG and KE. The peptides stimulate differentiation of neurons and retinal pigment epithelium cells and therefore can be considered as potential retinoprotective preparations for the treatment of age-related degenerative changes in the retina.

Key Words: *retina; short peptides; cell differentiation*

Pathological processes in the retina are underlain by the molecular mechanism of inhibition of functional activity of retinal cells. Changes in the expression of differentiation marker participating in the retina ontogeny are the key factor of homeostasis disorders. For instance, changes in the synthesis of Pax6 protein expressed in neurons at the initial stages of their differentiation can result in aniridia, myopia, and foveal dysplasia and hypoplasia [3,9]. Myopia can be associated with changes in the expression of TTR, a marker of retinal pigment epithelium cells [12]. Mutations in Vsx1 gene inducing terminal differentiation of retinal bipolar cells are associated with the development of keratoconus and posterior polymorphous corneal dystrophy, while enhanced expression of Prox1 protein correlates with retinoblastoma development [7].

Complex peptide preparation Retinalamin promotes functional recovery of the retina [4]. On the basis of its amino acid composition, AEDG peptide (H-Ala-Glu-Asp-Gly-OH) was synthesized. In addition, KE peptide (H-Lys(H-Glu-OH)-OH) was synthesized. AEDG promotes regeneration of the retina in toxic injuries and abiotrophy, reduces the severity of destructive changes, and improves recovery of layer-by-layer structure of the retina and its light sensitivity in visual disadaptation [1].

KE normalizes the parameters of local immunity and microcirculation in retinal vessels. Moreover, this peptide inhibits angioneogenesis in the choroid and retina in age-related macular degeneration, which accelerates vision recovery [2].

Clinical effects of the retinoprotective activity of KE and AEDG peptides can be associated with functional activation of different types of retinal cells. It was found that AEDG and retinalamin added to the culture of polypotent cells induce differentiation of the nervous and retinal tissues, respectively [4,6].

Here we compared the effects of KE and KE+AEDG on the molecular mechanisms of retinal cell differentiation.

MATERIALS AND METHODS

Retina samples from 10-day-old chicken were obtained at the Institute of Influenza, Russian Academy of Medical Sciences (St. Petersburg). Immediately after isolation, the retina samples were placed to a sterile Petri dish, cut into explants (~1 mm^3), transferred to a collagen-coated Petri dish, and cultured in 3 ml nutrient medium (pH 7.2) containing (per 100 ml): 41 ml Hanks saline, 30 ml Eagle medium, 25 ml fetal calf serum, 1 ml 40% glucose, 2.5 ml insulin (100 U), and 0.5 ml (20 mg) gentamicin.

[1]I. P. Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg; [2]St. Petersburg Institute of Bioregulation and Gerontology, Russia. ***Address for correspondence:*** miayy@yandex.ru. N. S. Linkova

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The duration of culturing was 3 days (36.7°C, 5% CO_2), because this incubation period is required for the formation of growth zone [5].

Organotypic cultures were divided into 3 groups that were incubated with either 0.9% NaCl (group 1), or KE peptide in a concentration of 0.05 ng/ml (group 2), or a combination of KE and AEDG peptides in a concentration of 0.01 ng/ml (group 3).

Organotypic cultures were fixed with 95% ethanol cooled to -20°C. For description of cell morphology, the explant growth zone represented by cell monolayer was stained with hematoxylin and eosin [5].

Then, the organotypic cultures were stained by immunocytochemical methods using monoclonal antibodies to Brn3, Pax6, Prox1, Vsx1, and TTP (1:50, Dako), the key factors of retinal cell differentiation. Transcription factor Brn3 was chosen as the marker of differentiation of ganglion cells playing an important role in the mechanisms of electrical pulse conduction [11].

The cultures were photographed using AST-1 software. The results of immunocytochemical analysis were evaluated morphometrically using a computer-assisted microscopic image analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, and Videotest-Morphology 5.2 software.

The relative expression area was calculated as the ratio of immunopositive cell area to the total area of cells in the field of view. Optical density of the expression was calculated and expressed in arbitrary units. The relative expression area and optical density reflect the number of cells carrying the specified marker and the content of marker proteins per cell, respectively.

Statistical processing of the results included calculation of the arithmetic mean, standard deviation, and confidence interval for each sample (Statistica 6.0 software). The type of distribution was determined using Shapiro–Wilk test. Statistical homogeneity of samples was evaluated using nonparametric univariate analysis of variance (Kruskal–Wallis test).

RESULTS

Histological staining of the growth zone in control organotypic retinal culture revealed elongated and triangular cells forming a network; the majority of these cells had neuron-like morphology (Fig. 1, *a*).

In group 2 and 3 cultures, round dying cells were practically absent, which indicated improvement of their viability (Fig. 1, *b, c*). The data of histological analysis attest to the retinoprotective effect of KE and AEDG peptides, which was later confirmed by the results of immunocytochemical analysis.

The area of Pax6 expression in group 2 increased by 2 times in comparison with the control (group 1; Fig. 2) and optical density increased by 28% (Fig. 3). In group 3, the area of Pax6 expression increased by 3 times in comparison with the corresponding parameter in the control group (Fig. 2), while optical density remained unchanged (Fig. 3). Thus, KE added to the culture (group 2) produced the most pronounced effect on differentiation of neuronal precursors.

The expression area of bipolar cell marker Vsx1 in group 2 increased by 6 times in comparison with the control (Fig. 2), while optical density remained unchanged (Fig. 3). In group 3, Vsx1 expression did not differ from the control (Figs. 2 and 3).

The area of expression of final stage ganglion cell differentiation marker Brn3 in groups 2 and 3 increased by 12 and 14.5 times, respectively, in comparison with the control (Fig. 2). Optical density remained unchanged in both groups (Fig. 3).

The area of Prox1 expression (marker of mature neurons) in group 2 increased by 2.3 times (Fig. 2) and optical density increased by 23% in comparison with the control (Fig. 3). In group 3, the area of Prox1

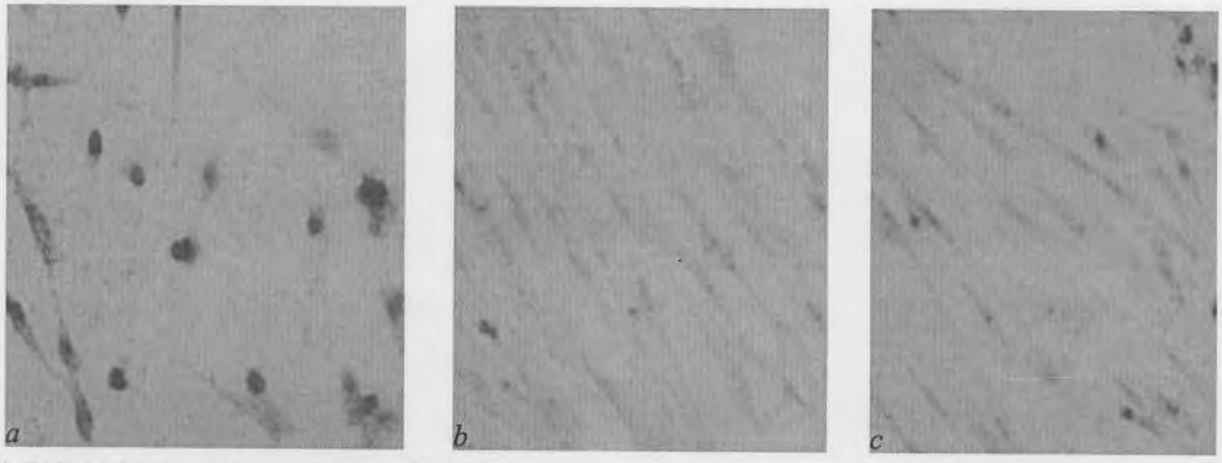

Fig. 1. Retinal cell culture. Hematoxylin and eosin staining, ×400. *a*) group 1 (control); *b*) group 2; *c*) group 3.

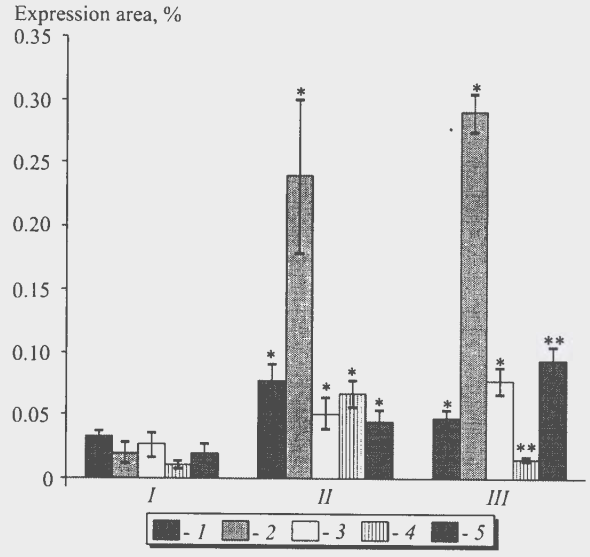

Fig. 2. Effect of peptides on expression area of differentiation markers in retinal cell culture. Here and in Fig. 3: *I*: group 1 (control); *II*: group 2; *III*: group 3. *1*) Prox1, *2*) Brn3, *3*) Pax6, *4*) Vsx1, *5*) TTR. **p*<0.05 in comparison with the control; ***p*<0.05 in comparison with group 2.

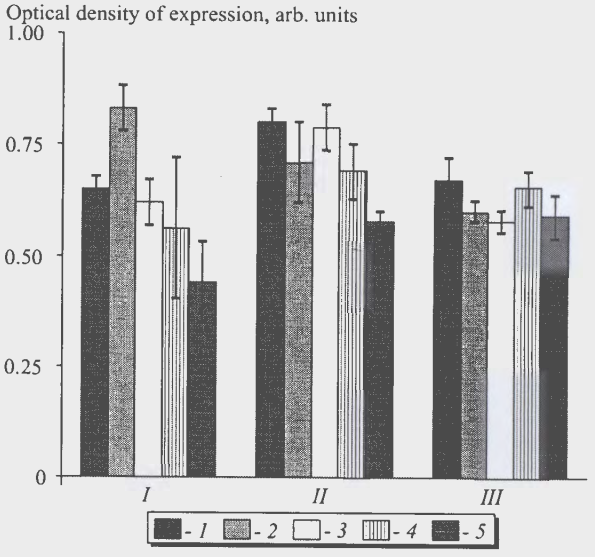

Fig. 3. Effect of peptides on optical density of differentiation markers expression in retinal cell culture.

expression increased by 45% (Fig. 2), while optical density did not change (Fig. 3).

The area of expression of retinal pigment epithelium cell marker TTR in group 3 increased by 4.9 times (Fig. 2) and optical density increased by 34% in comparison with the control (Fig. 3). In group 2, these parameters increased by 2.4 times and 32%, respectively (Figs. 2 and 3).

The results of immunocytochemical analysis suggest that peptide KE and KE+AEDG combination act as inductors of retinal cell differentiation, which agrees with the data of other investigators on the stimulating effects of hormones and peptides on functional activity of retinal cells. It is known that modulation of somatostatin expression affects differentiation of bipolar cells [8]. It has been demonstrated that short peptides ADNF-9 and NAP improved survival of ganglion cell culture and stimulated axonal growth in retinal explants [10].

Peptide KE primarily modulated the expression of retinal neuronal markers (Brn3, Prox1, Vsx1) and can be studied as the retinoprotective preparation for the treatment of tapetoretinal abiotrophy, cone-rod dystrophy, and various neurodegenerative pathologies.

Combined treatment with KE and AEDG produced the most pronounced stimulatory effect on the expression of pigment retinal epithelium cell marker TTR. Thus, the combination of these peptides can be an effective means in the treatment of age-related macular degeneration and retinitis pigmentosa.

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