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ORIGINAL ARTICLES

Analysis of Adhesion Proteins from Neural Eye Tissues of Eight-day-old Chick Embryos

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Abstract—Two groups of proteins were isolated from the retina and pigment epithelium of eight-day-old chick embryos. Experiments with suspension cultures of retinal cells demonstrated that only the retinal extracts and the fraction of its acidic proteins can stimulate cell aggregation in vitro. Analysis by the method of high-performance liquid chromatography showed that fractions of acidic and basic retinal proteins, which markedly differ in their electric charge and biological activity, have similar composition. To study the effect of these proteins on the morphological and functional state of pigment epithelium *in vitro*, a new experimental model is proposed, with the posterior segment of the newt (*Pleurodeles waltl*) eye used as a test tissue. The fraction of basic proteins isolated from the chick embryonic pigment epithelium stabilized cell differentiation in the newt pigment epithelium. The analyzed proteins proved to be biologically active at extremely low doses, corresponding to 10^{-12} M solutions.

Key words: adhesion proteins, retinal pigment epithelium, differentiation, newts

Cell adhesion plays an important role in morphogenetic processes, providing for the cooperative interaction of cells and their proper arrangement in tissues (Reichardt, 1993). The phenomenon of cell adhesion is based on molecular mechanisms and mediated by various adhesion molecules (Anderson, 1990; Turner, 1992). Scientists have identified many adhesion proteins, analyzed their primary structure and tissue localization, and isolated the genes encoding them (Kreis and Vale, 1993).

Tissues of neural origin have a special place in studies on the mechanisms of cell adhesion, as adhesion-mediated cell interactions in neural tissues are involved in the processes of signal transduction, which provide for the transmission of nerve impulses, the main functional property of these tissues (Reichardt, 1993).

Previous studies demonstrated that the neural retina and retinal pigment epithelium (RPE) of eight-day-old chick embryos contain acidic glycoproteins capable of stimulating cell aggregation in suspension cultures of both retinal and RPE cells (Dol'nikova *et al.*, 1985a, 1986). Tissue extracts containing these adhesion proteins were obtained by means of mechanical homogenization and the treatment of tissues with enzymes and Ca^{2+} chelators (Dol'nikova *et al.*, 1985a, 1985b).

Later, adhesion proteins were isolated from the mammalian liver and lungs by another method, which did not involve mechanical homogenization and treatment with enzymes (Yamskova *et al.*, 1990). A similar approach, which excluded tissue damage, was used for isolating the S-100 protein secreted in the brain (Shashoua *et al.*, 1984).

In the present work, we studied adhesion proteins isolated from the neural retina and RPE of eight-day-old chick embryos without treatment with any agents or mechanical dissociation. They were tested for the adhesion-stimulating effect by the conventional method of cell aggregation in suspension cultures (Moscona, 1961), as in our previous studies (Dol'nikova *et al.*, 1985a, 1985b). In addition, we employed an original model of the organ–tissue culture of the posterior segment of the newt *Pleurodeles waltl* eye. This approach was motivated by the unique capacity of newt RPE to transdifferentiate both *in vivo* and *in vitro* (Reyer, 1954; Okada, 1991). As we are the first to use the organ–tissue culture of the posterior eye segment for analyzing the biological effect of adhesion proteins isolated from neural tissues, considerable attention is given to the description of this model.

MATERIALS AND METHODS

We used 90 chick embryos of the Brown breed incubated for 8 days at the Bratzevskaya factory farm (Moscow oblast).

Adhesion proteins were isolated from the retina and RPE by extraction in saline (0.15 M NaCl, 1 mM $CaCl_2$) at 4°C for 2 h. The extracts were centrifuged at 3000 g for 10–15 min and fractionated by isoelectric focusing (IEF) in a sucrose density gradient using an LKB-440 column and ampholytes pH 3.5–10.0 (Serva,

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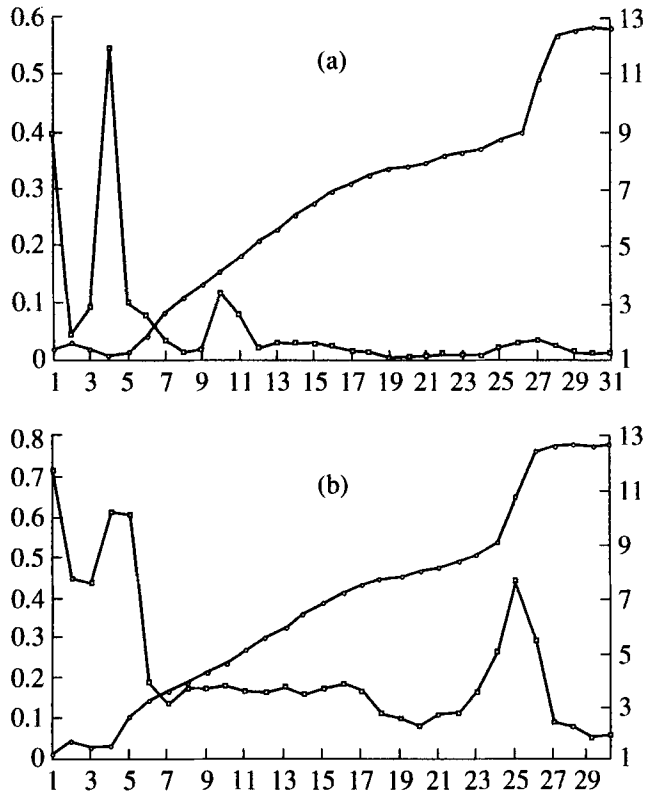

Fig. 1. Isoelectric focusing of proteins extracted from the (a) neural retina and (b) RPE of eight-day-old chick embryos. The abscissa shows the fraction number; the right ordinate (○), pH; the left ordinate (□), optical density.

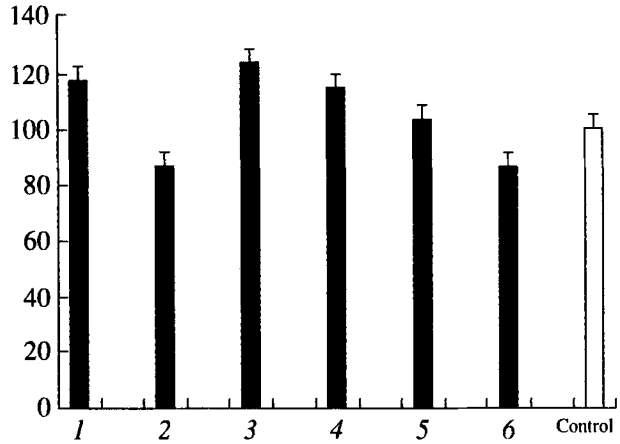

Fig. 2. Effect of test substances on cell aggregation in suspension cultures of embryonic retina, %; (*1*) extract of eight-day embryonic chick retina, (*2*) extract of RPE, (*3*) acidic protein fraction of retinal extract, (*4*) basic protein fraction of RPE extract, (*5*) basic protein fraction of retinal extract, (*6*) basic protein fraction of RPE extract; $p < 0.05$.

Germany). IEF was performed at 500–2000 V for 72 h at 4°C. Individual protein fractions detected at 280 nm were collected in vials (3–4 ml), and their pH was determined. The protein concentration in solutions was measured spectrophotometrically (Darbre, 1989).

The composition of protein fractions was studied by reverse-phase high-performance liquid chromatography (HPLC) using equipment manufactured by Gilson (France) and a C-18 column (250 × 40 mm). Measurements were made at 280 nm and 25°C. Samples (50 µl) were applied to the column and eluted with the water–acetonitrile mixture (0–50% acetonitrile gradient) at a rate of 0.8 ml/min. To remove sucrose, ampholytes, and acetonitrile after IEF and HPLC, fractions were dialyzed against 10^{-3} M $CaCl_2$ solution in water using Spectrapor membranes 8 kDa (USA).

Cell aggregation in suspension cultures was studied as described previously (Dol'nikova *et al.*, 1985a, 1985b). Protein preparations were added in amounts equivalent to 10^{-7} to 10^{-8} M solution.

For experiments with organ–tissue cultures, ten adult newts were obtained from the aquarium of the Institute of Developmental Biology. The animals were anesthetized in a 2% urethane solution in 0.65% NaCl saline. The eyes were removed and placed in a 0.1 M phosphate buffer, pH 7.4, containing 0.23 M sucrose. The posterior segment of the eye was isolated under a dissecting microscope. The eye was cut along the equator, the anterior portion and the retina were removed, and the posterior segment was cut in halves through the point where the optic nerve leaves the eye. These halves were used as test and control tissues in the same experiment. We performed four series of experiments, five cultures in each; likewise, 20 control cultures were analyzed.

The isolated fragments of the posterior eye segment were placed in vials containing 10 ml of the culture medium prepared by adding 0.003 ml of 1 M HEPES (Sigma, United States), 0.02 ml of 4% gentamycin, and 3 ml of deionized water to 7 ml of the Leibovitz L-15 medium (Flow Laboratories, Scotland). Test proteins (0.1 ml) were added to a final concentration of 10^{-12} M. After two days of incubation at 22°C, the cultures were fixed in a Bouin mixture for 3 h, dehydrated, and embedded in paraffin; Serial 5-µm sections were stained with Carracci's hematoxylin and analyzed under a microscope at different magnifications (ocular lens ×10, objectives ×40 and ×100).

RESULTS AND DISCUSSION

We previously isolated and analyzed acidic proteins from chick embryonic retina and RPE using the methods of mechanical tissue dissociation and treatment with enzymes and Ca^{2+} chelators (Dol'nikova *et al.*, 1985a, 1985b, 1986). This work is the continuation of a series of studies on the molecular bases of spatial and functional organization of the cell microenvironment in the retina and RPE of poikilothermal organisms. In this case, we analyzed proteins extracted from tissues without exposing the latter to any influences disturbing cell adhesion. Hence, one of our purposes was to study both the physicochemical and biological properties of these

adhesion proteins isolated from the neural tissues of the chick embryonic eye under the aforementioned physiological conditions at 4°C and to compare the results with data obtained in previous experiments (Dol'nikova *et al.*, 1985a, 1985b).

The initial retinal extract (5 ml) contained approximately 2.4 mg of protein, and the RPE extract (2 ml), 0.24 mg. In IEF, each extract formed two major fractions, acidic (migrating toward the anode) and basic (migrating toward the cathode). Thus, four fractions of acidic and basic proteins from the retina and RPE were obtained (Fig. 1). The protein yield was as follows: acidic retinal proteins, 0.2 mg in 5 ml; basic retinal proteins, 0.45 mg in 2 ml; acidic RPE proteins, 0.1 mg in 2 ml; and basic RPE proteins, 0.27 mg in 2 ml.

In tests on suspension cultures of retinal cells of eight-day-old chick embryos, only the retinal extract and its acidic fraction stimulated cell adhesion; the other fractions demonstrated no biological activity (Fig. 2). This result principally differs from those obtained in previous studies on adhesive proteins from the neural eye tissues of eight-day-old chick embryos (Dol'nikova, 1985a, 1985b). The fact that extraction in this case was performed without deliberate tissue damage provides evidence that test fractions consisted mainly of proteins secreted by cells (Shashoua *et al.*, 1984). On the other hand, phase transition in the supermolecular protein structure of the extracellular matrix could occur at 4°C, entailing the possible release of matrix components into the medium (Yamskova and Yamskov, 1999).

The reverse-phase HPLC analysis of acidic and basic retinal fractions showed that their composition was similar: each contained a peak accounting for no less than 80% of the total protein, which emerged from the column after the same period of time (Fig. 3). The fact that major components of both fractions were eluted with high concentrations of acetonitrile provided evidence for their hydrophobic properties. At the same time, their molecules obviously carried an electric charge and, hence, contained polar groups. A molecular structure with both highly hydrophobic and hydrophilic domains is typical of active surface substances. Therefore, the proteins studied should demonstrate a pronounced tendency to form molecular associations and micelles in aqueous solutions. Studies on other proteins isolated in a similar way from mammalian tissues showed that they are indeed capable of forming stable, high-molecular associations and rarely occurred in aqueous solutions as monomers (Yamskova and Reznikova, 1991). The basic protein fractions identified in both extracts are of special interest: as follows from the combination of their physicochemical properties, these proteins may constitute a novel group of adhesion molecules.

The results of experiments with suspension cultures provide evidence for tissue specificity of the biological activity of adhesion proteins isolated from the retina and RPE of eight-day-old chick embryos. However, in our opinion, this evidence alone is insufficient to fully explain the role of these agents in cell adhesion. The conventional method of suspension cultures is sufficiently demonstrative for assessing the contribution made by proteins of cell microenvironments to adhesive interactions between cells. On the other hand, the

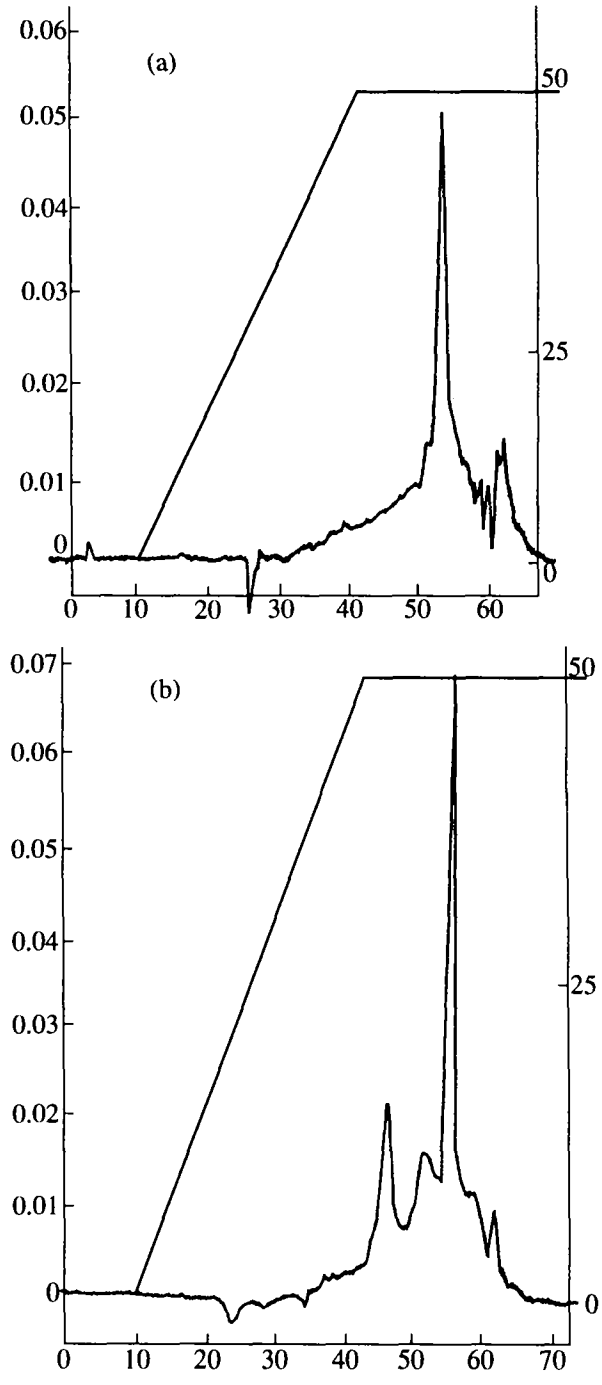

Fig. 3. High-performance liquid chromatogram of (a) acidic and (b) basic proteins of eight-day-old embryonic chick retina. The abscissa shows time, min; the left ordinate, optical density; the right ordinate, acetonitrile concentration, %.

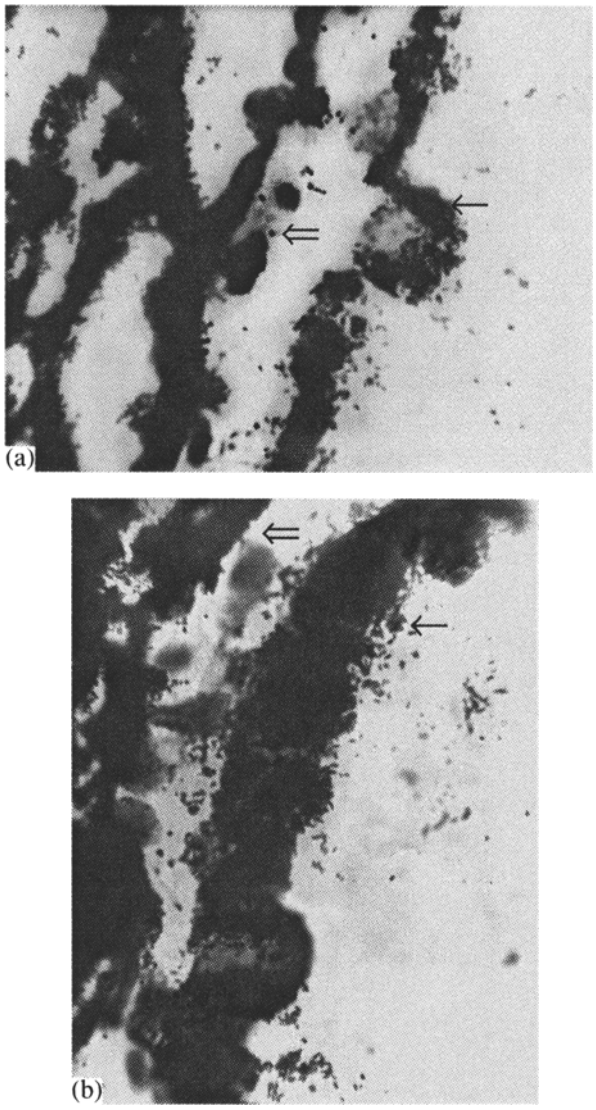

Fig. 4. Transverse sections of posterior segments of the newt (*Pleurodeles waltl*) eye: control (a), experiment (b) (RPE (←) and choroid coat are shown (⇐)). Magnification: ocular lens ×10, objective ×100.

procedure for preparing these cultures is accompanied by damage to the components of the microenvironment and to the cell membranes, which partially recover *in vitro* but never reach the state observed *in vivo*. Indeed, our previous data show that proteins isolated in a similar way from the liver and blood serum can have various biological effects in hepatocytes only in cultures preserving the tissue structure of the liver, remaining inactive in monolayer cultures (Yamskova *et al.*, 1994). Taking into account that the biological activity of the studied adhesive proteins is not species-specific (Yamskova and Reznikova, 1991), we decided to use the tissue–organ culture of the posterior segment of the newt (*P. waltl*) eye as a test model. The purpose of these experiments was to study the effect of isolated proteins on the regeneration capacity of RPE and the morphofunctional interactions of its cells after retina removal.

After cultivating the fragments of the posterior eye wall for two days under the conditions described above, we found that RPE remained viable: no cases of its dissociation or cell necrosis were revealed in experimental and control cultures.

In control cultures, the RPE layer consisted of partially depigmented, loosely packed cells (Fig. 4). As the remaining pigment concentrated in the apical zone of the cytoplasm, the nuclei were easily discernible in the basal zone. They were of almost regular round shape, and their light staining distinguished them from the nuclei of adjacent blood cells. The apical cell surface lacked the processes typical of RPE cells *in vivo* (Fig. 4a).

Cell loosening in the RPE layer was probably accounted for by the loss of lateral contacts without detachment from Bruch's membrane. Such cells acquired a round or oval shape and tended to shift toward the conventional eye cavity. Some cells, mainly in the peripheral zone, did leave the RPE layer and emerged on its apical surface (Fig. 4a). These events indicate the beginning of cell migration from the RPE, which is the usual response of this tissue to detachment from the retina under experimental conditions or in various pathological states in both lower and higher vertebrates (Keefe, 1973; Korte *et al.*, 1994; Grigoryan *et al.*, 1996). Cells leaving the RPE acquired the morphological features of melanophages described by Keefe (1973), i.e., RPE cells transformed into freely migrating cells functioning as phagocytes. No mitotic RPE cells were found, as the period of cultivation was short. Thus, we observed changes in RPE cell behavior and morphology, indicating the initial stages of cell transformation in control cultures. This is evidence for the destabilization of RPE cell differentiation under the cultivation conditions used in this study.

In experimental cultures with retinal and RPE proteins added to the medium, the topological relationships both among cells within the RPE layer and between the RPE and the underlying tissues were preserved better than in the control. Morphologically, the arrangement of cells relative to each other and their differentiation status were maintained most efficiently in experiments with basic proteins isolated from the RPE of eight-day-old chick embryos: the structure of newt RPE in these cultures did not differ from the norm (Fig. 4b). In radial and tangential sections, their cells formed continuous mosaics of elongated spindle-shaped and hexagonal elements, respectively. The cells were firmly attached to each other laterally, and cases of cell detachment were very rare. The nuclei were located basally, as in the control, but were often surrounded with pigmented cytoplasm. No dividing nuclei were found. Pigment in the cytoplasm was so abundant that individual granules were sometimes difficult to distinguish. It concentrated in the apical zone, but its content

in the basal zone was still higher than in the cells of control cultures (Fig. 4b). As the content of pigment characterizes the degree of RPE cell differentiation, the almost normal pigmentation of cells in this experiment is evidence for the maintenance of their differentiated state. We found no cases of cell depigmentation, migration from the RPE layer, or any other signs of morphological transformation. This fact confirms that the basic protein fraction isolated from the RPE of eight-day-old chick embryos can stabilize cell differentiation.

This also applies to the basic retinal protein and the acidic RPE protein, but only partially, as cases (although rare) of cell depigmentation and migration from the RPE layer have been observed in corresponding experiments. Nevertheless, the preliminary answer to the question as to whether these proteins have effects similar to that of the basic RPE protein is that they do. A different situation was observed in experiments with the acidic protein fraction isolated from the retina of eight-day-old chick embryos: newt RPE in these cultures deteriorated, as followed from the partial destruction of the cytoplasm and the release of pigment, and any effects of the fraction on the state of the cells were impossible to assess.

Thus, studies on the model of the organ–tissue culture of the posterior segment of the *P. waltl* eye have allowed us to analyze in detail, compare, and describe the morphology of RPE cells in different experimental series and control cultures. On this basis, we have demonstrated that proteins isolated from the neural retina and RPE of eight-day-old chick embryos have a stabilizing effect on the differentiation of newt RPE cells.

REFERENCES

Yamskova, V.P. and Reznikova, M.M., A Low-Molecular Blood Serum Polypeptide of Poikilotherms: Effect on Cell Adhesion and Proliferation, *Zh. Obshch. Biol.*, 1991, vol. 52, no. 2, pp. 181–191.

Yamskova, V.P. and Yamskov, I.A., The Mechanism of Biological Action of Physicochemical Factors at Extremely Low Doses, *Ross. Khim. Zh.*, 1999, vol. 43, no. 3, pp. 74–79.

Yamskova, V.P., Tumanova, N.B., and Loginov, A.S., Comparative Analysis of the Effects of Liver Extracts from C57Bl and CBA Mice on Hepatocyte Adhesion, *Byull. Eksp. Biol. Med.*, 1990, no. 3, pp. 303–306.

Yamskova, V.P., Nechaeva, N.V., Tumanova, N.B., *et al.*, Effects of Macromolecular Adhesion Factors Isolated from Mammalian Blood and Liver on the Rate of Protein Synthesis and ATP Content in Hepatocytes *in vitro*, *Izv. Ross. Akad. Nauk, Ser. Biol.*, 1994, no. 2, pp. 190–196.

Anderson, H., Adhesion Molecules and Animal Development, *Experientia*, 1990, vol. 46, pp. 2–13.

Darbre, A., *Practical Protein Chemistry: A Handbook*, Chichester: Wiley, 1986. Translated under the title *Prakticheskaya khimiya belka*, Moscow: Mir, 1989, pp. 300–302.

Dol'nikova, A.E., Yamskova, V.P., Sologub, A.A., and Stroeva, O.G., Biochemical Characterization of Cell Adhesion Factors from Chick Embryonic Retina, *Ontogenez*, 1985a, vol. 16, no. 5, pp. 497–506.

Dol'nikova, A.E., Sologub, A.A., Stroeva, O.G., and Yamskova, V.P., Roles of Calcium-Dependent and Calcium-Independent Mechanisms in Adhesion of Pigment Epithelial and Retinal Cells of Chick Embryos, *Ontogenez*, 1985b, vol. 16, no. 2, pp. 149–155.

Dol'nikova, A.E., Yamskova, V.P., Sologub, A.A., and Stroeva, O.G., Studies on Specific and Unspecific Adhesion in the Course of aggregation of Chick Embryonic Retinal Cells, *Ontogenez*, 1986, vol. 17, no. 6, pp. 620–626.

Grigoryan, E.N., Ivanova, I.P., and Poplinskaya, V.A., Identification of New, Intrinsic Sources of Neural Retina Regeneration after Detachment in Newts. Morphological and Quantitative Analysis, *Izv. Ross. Akad. Nauk, Ser. Biol.*, 1996, no. 3, pp. 319–332.

Keefe, J.R., An Analysis of Urodelean Retinal Regeneration, *J. Exp. Zool.*, 1973, vol. 184, pp. 185–257.

Korte, G.E., Perlman, J.I., and Pollack, A., Regeneration of Mammalian Retinal Pigment Epithelium, *Int. Rev. Cytol.*, 1994, vol. 152, pp. 223–263.

Kreis, T. and Vale, R., *Guidebook to the Extracellular Matrix and Adhesion Proteins*, Oxford: Oxford Univ. Press, 1993.

Moscona, A.A., Rotation-Mediated Histogenetic Aggregation of Dissociated Cells, *Exp. Cell Res.*, 1961, vol. 22, pp. 455–465.

Okada, T.S., *Transdifferentiation*, Oxford: Clarendon, 1991.

Reichardt, L.F., Extracellular Matrix Molecules and Their Receptors, *Guidebook to the Extracellular Matrix and Adhesion Proteins*, Kreis, T. and Vale, R., Eds., Oxford: Oxford Univ. Press, 1993, pp. 3–11.

Reyer, R.W., Regeneration of the Lens in the Amphibian Eye, *Ouart. Rev. Biol.*, 1954, vol. 29, pp. 1–46.

Shashoua, V.E., Hesse, G.W., and Moore, B.W., Proteins of the Brain Extracellular Fluid: Evidence for Release of S-100 Protein, *J. Neurochem.*, 1984, vol. 42, no. 6, pp. 1536–1541.

Turner, M.L., Cell Adhesion Molecules: A Unifying Approach to Topographic Biology, *Biol. Rev.*, 1992, vol. 67, pp. 359–377.