# BIOPHYSICS AND BIOCHEMISTRY

# Stabilizing Effect of Milk Angiogenin on the Crystal Structure of Biological Fluids

**G. V. Plaksina, G. S. Komolova*, A. E. Mashkov, Yu. L. Rustam'yan*, and D. A. Pykhteev**

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We revealed a new property of angiogenin to restore the crystal structure of biological fluids (human blood plasma and exudates) impaired in various pathologies.

**Key Words:** *angiogenin; blood plasma; tissue exudate; crystal structures of biological medium; inflammation*

Angiogenin (AG) belonging to the superfamily of pancreatic ribonucleases acts as a potent growth factor for blood vessels and possesses protective and homeostasis-maintaining properties [9]. This substance stabilizes polymorphonuclear leukocytes [11], modulates immune reactions [1], and exhibits bacteriostatic activity [6]. The first investigations in this area suggest that AG performs a variety of functions. AG is expressed in various mammalian cells and present in the blood and milk [9]. Recent studies indicate that AG improves reparative and regenerative processes in non-healing tissue defects, osteogenesis, homeostatic parameters, and immune protection [2,8]. The mechanisms underlying biological activity of AG are poorly understood. The maintenance of physiological systems by AG is probably realized via supramolecular structures of biological medium. It primarily concerns complex protein aggregates. Crystallographic study characterizes morphological structures in biological fluids and allows more early detection of metabolic disorders at the molecular and supramolecular levels compared to biochemical investigations [3].

Crystallographic methods for studies of biological fluids are based on the ability of some crystal substances (*e.g.*, NaCl and $CuCl_2$) to form various textures depending on the composition and density of complex protein gels. It should be emphasized that crystallization pattern depends on amino acid heterogeneity of protein substrate. Crystal optic studies employing hanging drop vapor diffusion technique demonstrate morphostructural changes in the liquid-crystal phase of the process. Polarization optical study of diagnostic preparations in crossed polarizers reveals anisotropic structures. The appearance of atypical pathological forms reflects pronounced metabolic disturbances in the complex protein composition [7].

Systemic self-organization of biological medium is determined by various factors and can be adequately evaluated by the method of wedge dehydration (dry drop or facia). The degree of harmonization depends on the severity and form of abnormalities in physiological rhythms of wave fluctuations and chemical activity of the liquid phase that undergoes changes under pathological conditions.

Here we studied *in vitro* effects of AG on crystal structures of protein complexes in blood plasma and wound exudate.

M. F. Vladimirskii Moscow Regional Research Institute of Clinical Studies; A. N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow. ***Address for correspondence:*** KomolovaGS@yandex.ru. Komolova G.S.

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## MATERIALS AND METHODS

AG was isolated from the natural source (cow milk) by ion exchange and hydrophobic chromatography [4]. Lipids were separated from milk by centrifugation, and the cationic protein fraction was obtained by chromatography on a column with carboxymethylcellulose 52 (Serva). Then, this fraction was purified on cation exchanger CM-Toyopearl (Tosoh) followed by hydrophobic chromatography on a column with Butyl-Touopearl (Tosoh). AG concentration in chromatographic fractions was measured by enzyme immunoassay using polyclonal antibodies to bovine AG conjugated with horseradish peroxidase [5]. The eluate containing AG was dialyzed against water for 1 day and lyophilized. Electrophoresis was performed in 15% polyacrylamide gel in the presence of sodium dodecyl sulfate. Nativity of AG was determined by its ability to compete with pancreatic RNase A (Serva) for the placental RNase/AG inhibitor RNasin (Promega) [10]. RNase activity was determined by changes in optical density of acid-soluble products formed after enzymatic hydrolysis of total yeast RNA (Sigma) at 260 nm.

Morphological study was performed on blood plasma and tissue exudate from 25 patients (5-14 years) with noninflammatory diseases admitted at the Children Surgery Department (M. F. Vladimirskii Moscow Regional Research Clinical Institute). The state of crystal structures was determined in blood plasma from patients with severe bronchial asthma. The effect of AG on adaptive processes was studied in a modified functional test of "skin window". This method allows evaluation of the state of cell-mediated defense and structural characteristics of complex proteins in wound exudates involved in stimulation of granulocytic and monolymphocytic cooperation. The epidermis was scarified on the skin of the forearm (1.5×1.5 cm). Filter paper (0.3 cm in diameter) was applied on the corner of the scarified region and a coverslip was placed on the skin. Dermal cells were counted in scarification imprints after 8 and 24 h. Crystallography of liquid-crystal phase was performed using closed drop technique. Diagnostic discs were placed in plastic tubes with 0.3 ml 0.9% NaCl for 2-4 h. Investigations were performed by the method of wedge dehydration and polarization optical study of the preparation in crossed polarizers. The procedure allowed us to detect liquid-crystal birefringent structures [3]. Physiological saline in an equivalent volume was added to control probes. *In vitro* measurements were repeated 3 times.

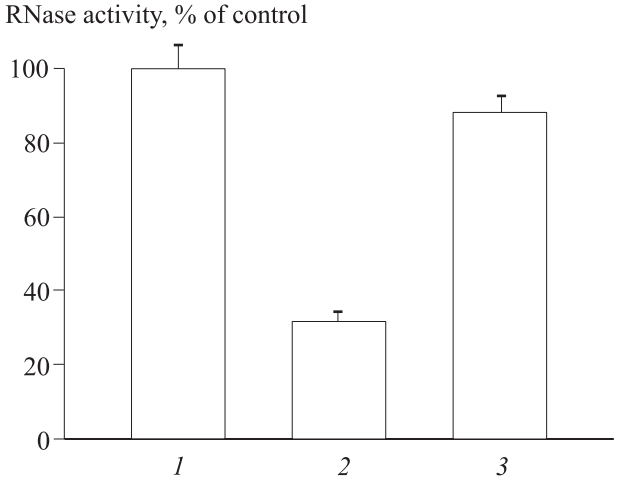


**Fig. 1.** Release of RNase A from the complex with RNasin in the presence of milk angiogenin (AG). Ordinate: RNase activity, % of control (100%). RNA and RNase (*1*); RNA, RNase, and RNasin (*2*); and RNA, RNase, RNasin, and AG (*3*).

## RESULTS

Electrophoresis showed that chromatography of cow milk yielded a homogenous protein (single protein band corresponding to a molecular weight of 17 kDa). Bovine AG has several isoforms with a molecular weight of 14.5-20.0 kDa [10,12]. Irrespective of the source, these isoforms exhibit properties typical of AG, but differ in the degree of induced reactions. Similarly to AG, the isolated protein did not hydrolyze yeast RNA, the substrate easily hydrolyzed by pancreatic RNase A. However, the protein competed with this enzyme for RNasin binding sites (Fig. 1), which is typical of AG. This test is the most adequate method for identification of AG. Therefore, the protein isolated from cow milk can be considered as native AG.

Morphological analysis of blood plasma from 25 patients with noninflammatory diseases revealed an insignificant number of liquid-crystal lines (LCL) and single small spheroliths, which corresponds to normal. Pathological morphological structures were not found. After addition of AG (in physiological saline) to native serum (8.3 μg/ml) we observed a considerable increase in the number of thin branching LCL, formation of small and intermediate spheroliths, and appearance of layered structures. The increase in the number of morphological structures reflects widening of protein composition in blood plasma, which is partially due to accumulation of defense proteins. These changes were not observed after the addition of physiological saline to the plasma (control). Studies of the native serum from 20 of 25 patients by the method of wedge dehydration revealed insignificant structural changes and the presence of concentric waves in the facia. This reflects the development of molecular and supramolecular metabolic disorders. Biochemical parameters in these patients remained unchanged, which illustrates high informativeness of morphostruc-

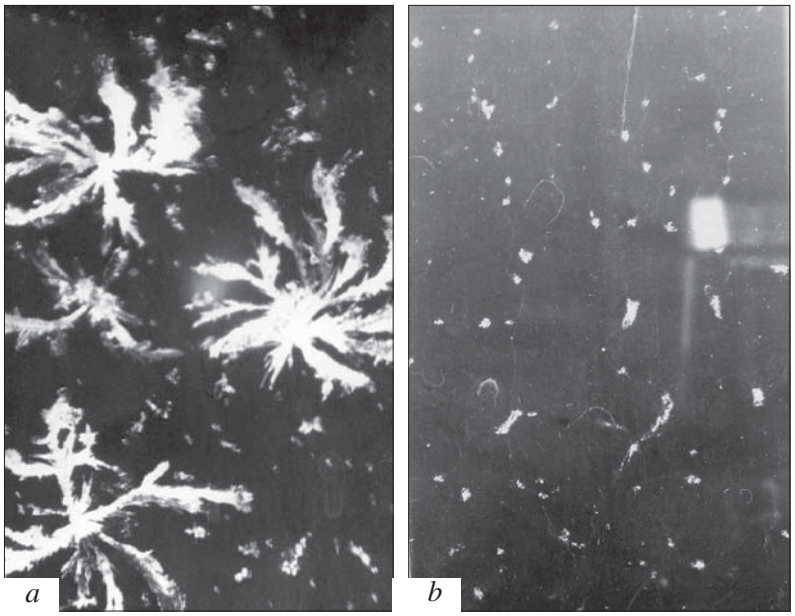


**Fig. 2.** *In vitro* effect of AG on blood plasma from patients with bronchial asthma. Large pathological anisotropic confocal domains (*a*) and small anisotropic structures (*b*).

tural analysis. Addition of AG to blood plasma harmonizes the structure of dry drop, which reflects positive effect of AG on protein and saline composition and normalization of physicochemical processes in the complex system.

AG modulated the crystal structure of blood plasma that was impaired under pathological conditions (Fig. 2). These changes illustrate the positive effect of AG on macrostructures of biological fluids. The number of atypical anisotropic pathological structures (fan-like forms) markedly increased in two patients with severe hormone-dependent bronchial asthma, which reflected pronounced conformational changes in proteins. The addition of AG to blood plasma markedly reduced the size of structures, which was related to a decrease in the ratio of pathological conformations in proteins of the biological medium. These results indicate that AG has stabilizing activity and does not induce negative structural changes in blood plasma.

The positive effect of AG was revealed during studies of morphological changes in the tissue exudate from the focus of local aseptic inflammation (Fig. 3). Scarification tests demonstrate functional relationships between cell-mediated defense and structural characteristics of complex proteins in the inflammatory focus. The granulocytic phase of defense reaction (after 8 h) developed in 20 patients. Neutrophil count in dermocytograms surpassed 80%. The range of morphological structures estimated by the crystallographic method of marginal dehydration correlated with der-

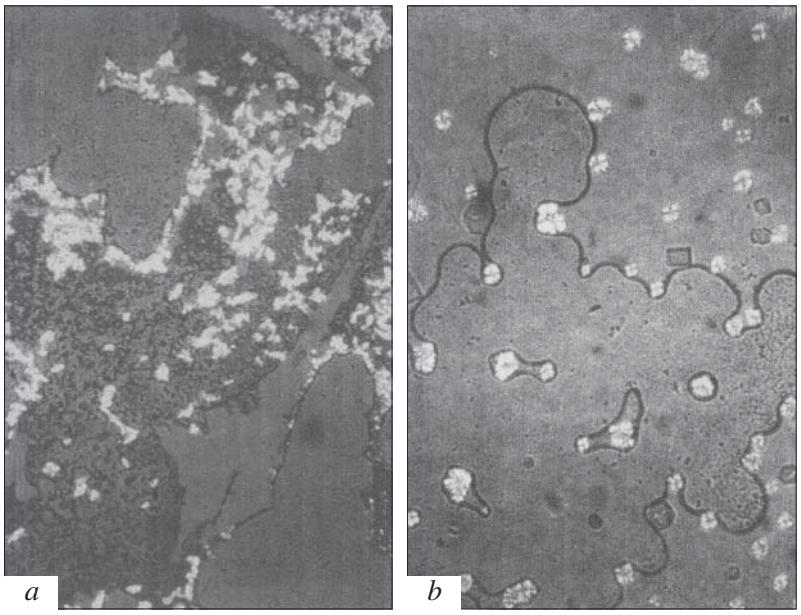


**Fig. 3.** Effect of AG on liquid-crystal structures in imprints of purulent wounds. Before therapy with AG: large number of small asymmetric pathological structures, 89% neutrophils in cytological preparations (*a*). After therapy with AG (2-3-fold treatment): agglomerates of symmetric anisotropic structures and liquid-crystal lines, single agglomerates of cells in cytological preparations and pronounced wound epithelization.

mal cell count ($r$=0.89). In 18 patients the macrophage-lymphocytic phase of inflammation was insignificant, which characterizes reduction of immune protection. The absence of anisotropic structures in tissue exudates was accompanied by weak lymphocytic and monocytic response (inflammatory stage II) and increase in the amount of catabolic products in dermocytograms. Application of a drop of the AG solution in a concentration of 8.3 μg/ml to the scarified region of the epithelium stimulated the inflammatory response in exudates from 18 patients with the reduced adaptive reaction. The macrophage-lymphocytic reaction increased after 8 h, which was accompanied by the appearance of typical spheroliths in crystallograms. Therefore, the wound exudate contained protein complexes stimulating cellular response to inflammation. These changes were not observed in the control (physiological saline without AG).

It cannot be excluded that the increase in blood AG level plays a compensatory role under pathological conditions. The influence of AG on the crystal structure of biological systems probably underlies the maintenance of homeostasis. Stabilization of supramolecular protein structures in biological systems is one of the unknown physiological functions of AG.

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