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Conformational Fingerprinting of Angiotensin-Converting Enzyme in the Blood in Health and Disease

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Abstract—The binding pattern of the set of 16 monoclonal antibodies to the different epitopes on the surface of two domains of angiotensin-converting enzyme, i.e., conformational fingerprinting, allows to reveal in blood the presence of an enzyme that is not produced by lung endothelial cells, but rather by other cells, e.g., Gaucher's cells and sarcoid granuloma cells. The existence of angiotensin-converting enzyme with changed conformation in the blood of patients with uremia has been shown; this enzyme was characterized by enhanced activity towards angiotensin I and decreased ability to be inhibited by specific inhibitors. The prospects of discovering conformationally changed enzyme in blood of patients with atrial fibrillation have also been discussed.

Keywords: angiotensin-converting enzyme, monoclonal antibodies, conformation, tissue specificity
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Angiotensin-converting enzyme (ACE, peptidyldipeptidase A, EC 3.4.15.1) is a zinc-dependent peptidase that metabolizes various biologically active peptides (angiotensin I, bradykinin, Ac-SDKP, etc.) and plays a key role in the regulation of blood pressure in patients with most cardiovascular pathologies. Moreover, this enzyme is involved in the neuropeptide metabolism, immune and reproductive functions, signal transduction into the cell, and other functions [1–3].

ACE is a glycoprotein and an integral type-I membrane protein. The main ACE functions in the organism are fulfilled by the membrane-bound form of the enzyme on the surface of endothelial, epithelial, and neuroepithelial, as well as immune cells, macrophages, and dendrite cells. Upon the action of still unknown enzyme (some metal-dependent secretase), ACE transforms into the soluble state through the cleavage of anchoring sequence [4, 5]. This soluble ACE is present in blood, seminal fluid, and other biological fluids. The concentration of ACE in these fluids is an important parameter for clinical observation. Healthy individuals reveal stable ACE blood level [6], while during the development of some diseases, the significant increase in this level could be observed (e.g., sarcoidosis [7, 8] or Gaucher's disease [9]); in addition, the changes of the ACE concentration in biological fluids due to the alteration of ACE gene expression could be observed in cases of the polymorphism of the gene of this enzyme [10].

The somatic (tissue) form of ACE consists of the two homologous domains (N and C) within single polypeptide chain; each of these domains contains an active center [11]. Precise 3D structure of the somatic ACE is still unknown; however, a few models have been proposed [12–14] based on the solved crystal structures of separate ACE domains [15, 16], epitope mapping of ACE [12], and the electron microscope data of full-sized enzyme [13].

The set of about 40 monoclonal antibodies (mAbs) directed to the different amino acid sequences of ACE polypeptide chain or to the conformational epitopes on the surface of the rat, murine and human enzyme had been obtained previously [12, 17–25]. These mAbs had been successfully applied for the quantitation of ACE by ELISA and flow cytometry [26, 27]. Moreover, the set of 16 mAbs directed to the various epitopes on the surface of both domains of human ACE allowed to obtain new data on the structure and mechanism of the action of ACE [12, 17, 20, 21, 28, 29], as well as to reveal carriers of new ACE gene mutations [30–34].

Structural studies demonstrated that the pattern (conformational fingerprinting) that characterizes the binding efficiency of the mAbs set directed to the different surface epitopes on both ACE domains is a very sensitive marker of local enzyme conformation that could change upon denaturation, inhibitors' binding, etc. [21, 25]. Because all epitopes for the antibodies

from this set have been characterized, the change in the binding of certain antibody with ACE indicates the place on the protein surface where the changes occurred.

The pilot study of the phenotyping of the human ACE derived from different tissues (blood plasma/serum, lungs, kidney, spleen, heart) demonstrated that the binding of the mAbs set directed to the various epitopes on the ACE surface was different for ACE from different tissues. The data allowed us to conclude that the ACE surface conformations also differed among different cells/tissues.

Normally, 75% of ACE in the blood plasma originates from the endothelium of lung capillaries [35]. Almost 100% of the lung capillaries demonstrate ACE expression, while only 10–15% of the capillaries of the vascular bed produce ACE [36]. As we have already mentioned, the level of ACE in the blood of healthy people is quite stable, while in sarcoidosis or Gaucher's disease, a significant increase in the blood ACE level was reported.

Sarcoidosis and Gaucher's disease are systemic diseases difficult for diagnosing. The cause of Gaucher's disease is the decrease of the activity of the lysosome enzyme, acid β-D-glucosidase, that leads to the accumulation of nonhydrolyzed β-D-glucocerebroside in all cells of the organism, preferentially in macrophages. The affected macrophages are activated and stimulate ACE synthesis. Sarcoidosis is a systemic granulomatous disease in which the lungs are mainly affected. It was shown that in lymph nodes containing granulomas the ACE expression was 12 times higher than in normal ones [37]. Sarcoid granulomas were suggested to be the source of the elevated ACE concentration in the blood. Thus, it could be expected that, during the development of these diseases, in the blood could be both lung-derived endothelial ACE and enzyme from other sources with different surface conformations.

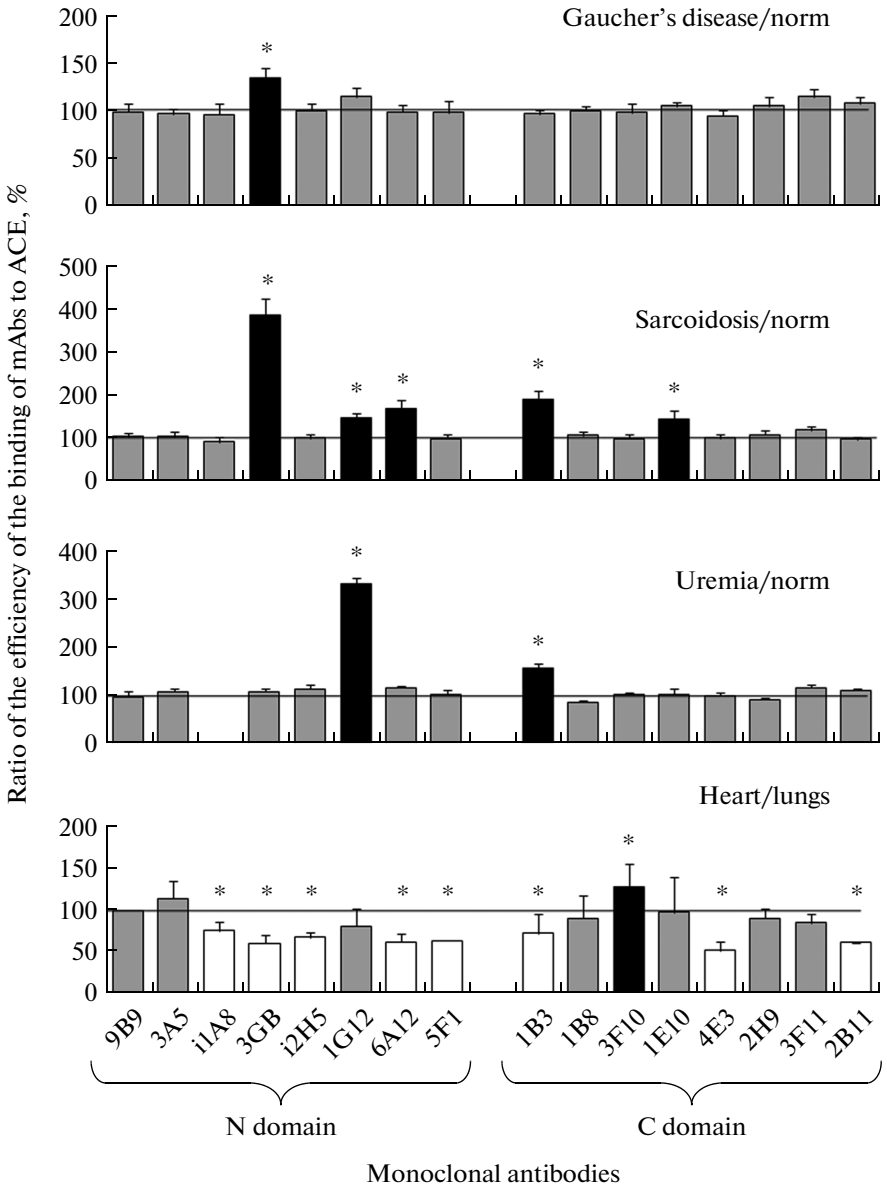
We conducted studies aimed at identifying the presence of ACE with altered surface topography in the blood of the patients with Gaucher's disease (samples were kindly provided by the Research Center of Medical Genetics, Russian Academy of Science) and with sarcoidosis (samples were kindly provided by the Research Institute of Phthisiopulmonology, Moscow Medicine Academy). The conformational fingerprinting of ACE from the plasma of healthy donors (48 samples) had been made. It was shown that the binding of mAbs varied insignificantly among these 48 samples (data not shown). The analysis of the plasma of the patients with Gaucher's disease demonstrated that the ACE activity level was two to seven times higher than in normal controls. The analysis of the mAbs binding to ACE from the plasma of the patients with Gaucher's disease revealed no major differences when compared with the binding to ACE from healthy donors. However, there was a statistically significant increase of the binding of a single mAb (3G8) to the N domain of the ACE from the patients' plasma (figure).

An analysis of the binding of mAbs to ACE from the plasma of sarcoidosis patients (ACE activity level was increased two to four times compared with the controls) had revealed a few mAbs with the binding differed from that of the donors' (figure). A statistically significant increase in the affinity of the 3G8, 1G12, and 6A12 mAbs to the N domain of the enzyme and 1B3 and 1E10 mAbs to the C domain had been demonstrated.

What are the possible reasons for these differences? First of all, the differences in the enzyme surface could be caused by the substitution of amino acid residues in the polypeptide chain of the enzyme molecule. However, the ACE synthesis is coded by a single gene and the known ACE mutations in its protein part are quite rare and could not be associated with the differences in the surface of the enzyme expressed in various tissues of the same person. It is much more likely that the observed differences are caused by the protein globule post-translational modification, which could be called "functional decorating." Post-translational modifications could include phosphorylation, sulfation, methylation, acetylation, etc. ACE is a typical glycoprotein with high carbohydrate content, and its most common post-translational modification is glycosylation. This process could differ in the proteins synthesized in different tissues by the glycosylation level and by the glucans' structure.

Human ACE molecule is known to have 17 potential glycosylation sites [11], and the real glycosylation of human two domain ACE is poorly characterized. The thorough and intensive investigation of the ACE glycosylation was only performed for the recombinant single domain testicular ACE (C domain) expressed in the ovary cells of the Chinese hamster [38]. These data certainly could not be extrapolated on the human ACE. There are very limited data on the glycosylation of human ACE. It was reported that in the somatic ACE from the seminal fluid of 7 out of 17 potential sites are indeed glycosylated (in particular Asn9). Two oligosaccharides are the high mannose structures and the other five are structures of a complex type, mainly biantennary [39]. Furthermore, for the somatic human kidney ACE [38], 6 out of 17 potential glycosylation sites have been shown to be glycosylated (Asn9, Asn25, Asn82, Asn117, Asn480, and Asn913). All glycosylation sites (apart from Asn913) were located in the N domain, and one site (Asn1196) located in the C domain of ACE was not glycosylated. There are no data about the glycosylation status of the other ten sites. Human proteome researches have demonstrated that blood plasma ACE contains three glycosylated sites, Asn480, 666, and 685 [40].

Thus, ACEs produced by the lung tissue and altered macrophages during Gaucher's disease or sarcoid granulomas during sarcoidosis most probably differ by glycosylation. It should be noted that the epitope



Conformational fingerprinting of ACE. Ratio of the values of the binding efficiency of mAbs directed to different epitopes on two domains of the enzyme in blood plasma from patients with different pathologies (Gaucher's disease, sarcoidosis, uremia) to the values of the binding efficiency of the same mAbs to plasma ACE from healthy donors. Ratio of the efficiency of the binding of mAbs to ACE from homogenates of heart and lung tissues. In each case, the results are represented as the mean of three to nine independent experiments and, in each experiment, the estimation of the efficiency of the binding of the same mAb with the same ACE was conducted twice. Asterisks indicate the statistically significant differences, $p < 0.05$.

for mAbs 3G8 (mAb with altered binding to ACE in the blood in both Gaucher's disease and sarcoidosis) contains two potential glycosylation sites (Asn25 and Asn82) in the N domain of the enzyme. The epitopes for mAbs which binding changed in sarcoidosis contain the following potential glycosylation sites: Asn416 (mAbs 1G12 and 6A12) in the N domain of the enzyme, Asn666 (mAb 1E10), and Asn1196 (mAb 1B3) in the C domain. Thus, it could be concluded that the differences in the glycosylation level and/or in the structure of oligosaccharide chains at the above-mentioned sites are the basis for the observed differences in the binding of mAbs with blood ACE from healthy people and from patients with the aforementioned pathologies. These results could serve as a basis for the development of clinical laboratory biochemical methods for diagnosing the Gaucher's disease and sarcoidosis.

We also analyzed the probability of the presence of the conformationally changed ACE in the blood of uremia patients. This disease is characterized by extremely high levels of toxins in the blood. Plasma

samples from the uremia patients before hemodialysis were kindly provided by Moscow State Medicine and Dentistry University. It turned out, that in this case, the efficiency of the binding of two mAbs to ACE from the blood plasma of uremia patients had differed from the efficiency of the binding of the same mAbs to ACE from the blood plasma of healthy donors (figure). The statistically significant differences in the efficiency of the binding of mAb 1G12 to the N domain and mAb 1B3 to the C domain were described for 4 patients out of 20. The characteristic of the conformationally changed ACE from the blood plasma of uremia patients had demonstrated that not only protein surface, but catalytic functions had been changed. Conformationally changed ACE was characterized by the increased activity (up to four times) towards its natural substrate angiotensin I, by less effective inhibition by the specific inhibitor enalaprilat (the analog of tripeptide wildly used in clinical practice as a hypotensive drug) and by the almost complete absence of inhibition by the specific inhibitor nonapeptide teprotide.

The analysis of the blood plasma from healthy donors (48) and patients without diagnosed uremia (63) have revealed eight more cases of the conformationally changed ACE similar to that described for uremia patients.

Thus, it seems that patients with this conformationally changed ACE in the blood in case of hypertension should be treated with ACE inhibitors more intensively or with another class of hypotensive drugs.

It is important, that the conformational fingerprinting of ACE in the blood of the patients with different diseases (Gaucher's disease, sarcoidosis, uremia) allows one to detect the presence of the enzyme with the surface topography specific to each disease by the individual pattern of the efficiency of the binding of the panel of 16 mAbs. This confirms the possibility of using the method in clinic.

In conclusion, the possibility of applying conformational fingerprinting in cardiology should be discussed.

It is known that patients with atrial fibrillation are characterized by increased expression of ACE in the heart tissue (atrium in particular) [41]. Earlier, it was demonstrated that ACE expression increased in the hypertrophied heart and in small myocardium vessels in patients who experienced sudden death (Bohle, Danilov, et al., unpublished work). The important role of the heart ACE in the development of cardiopathology was demonstrated in studies conducted on transgenic mice whose level of ACE expression in the heart was elevated by 100 times compared to the controls. In these mice, the volumes of both atriums (left and right) turned out to be increased by three times. ECG had demonstrated atrial fibrillation. Moreover, these mice had a high predisposition to sudden death [42, 43]. So, the increase in ACE expression in the heart could be the reason for the development of atrial fibrillation and ultimately lead to sudden death.

The proportion of the ACE produced in the heart among total ACE concentration in the blood is no more than 1%. Thus, even a threefold increase in the heart ACE in the blood could not exert a significant influence on the total enzyme activity detected in blood. However, the proportion of the heart ACE that enters the blood and complements ACE from the lungs could be estimated and, thus, patients with increased ACE expression in the blood could be revealed if it were possible to isolate antibodies specific to human heart ACE.

We have compared the phenotypes of the ACE from the lungs, which are the main source of the enzyme in the blood and of the heart ACE, the concentration of which in the blood could be increased by an arrhythmia. The comparative conformational fingerprinting of these two types of ACE is presented on figure. It was shown that the efficiency of the binding of some antibodies to the N and C domains of the heart enzyme statistically significant differed from the efficiency of the binding of the same antibodies to the lung ACE. The differences in the binding efficiency of these mAbs could be explained by the differences in the glycosylation of the following potential glycosylation sites: Asn25 and Asn289 in the N domain of the enzyme, and Asn666 and Asn731 in the C domain.

The demonstrated differences could serve as a basis for isolating the mAbs that specifically detect ACE produced by different types of cells/organ, heart tissue in particular, for the further development of the clinical laboratory method of the early diagnostics of arrhythmia.

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REFERENCES

1. Sturrock, E., Anthony, C., and Danilov, S., Peptidyldipeptidase A/angiotensin I-converting enzyme, in *Handbook of Proteolytic Enzymes*, Oxford, 2012, 3rd ed.
2. Ehlers, M. and Riordan, J., *Biochemistry*, 1989, vol. 8, p. 5311.
3. Bernstein, K., Ong, F., Blackwell, W., et al., *Pharmacol. Rev.,* 2013, vol. 65, p. 1.
4. Ramchandran, R., Sen, G., Misono, K., et al., *J. Biol. Chem.*, 1994, vol. 269, p. 2125.
5. Parkin, E., Turner, A., and Hooper, N., *Protein Pept. Lett.*, 2004, vol. 11, p. 423.
6. Alhenc-Gelas, F., Richard, J., Courbon, D., et al., *J. Lab. Clin. Med.*, 1991, vol. 117, p. 33.
7. Rohrbach, M. and Deremee, R., *Mayo Clin. Proc.*, 1982, vol. 57, p. 64.
8. Lieberman, J. and Sastre, A., *Chest*, 1986, vol. 90, p. 869.

9. Sylverstein, E. and Friedland, J., *Clin. Chim. Acta*, 1977, vol. 74, p. 21.
10. Rigat, B., Hubert, C., Alhenc-Gelas, F., et al., *J. Clin. Invest.*, 1990, vol. 86, p. 1343.
11. Soubrier, F., Alhenc-Gelas, F., Hubert, C., et al., *Proc. Natl. Acad. Sci. U. S. A.*, 1988, vol. 85, p. 9386.
12. Naperova, I., Balyasnikova, I., Schwartz, D., et al., *J. Proteome Res.*, 2008, vol. 7, p. 3396.
13. Chen, H., Lunsdorf, H., Hecht, H., et al., *Micron*, 2010, vol. 41, p. 674.
14. Danilov, S., Gordon, K., Nesterovich, A., et al., *PLoS One*, 2011, vol. 6, e25952.
15. Natesh, R., Schwager, S., Sturrock, E., et al., *Nature*, 2003, vol. 421, p. 551.
16. Corradi, H., Schwager, S., Nchinda, A., et al., *J. Mol. Biol.*, 2006, vol. 357, p. 964.
17. Danilov, S., Jaspard, E., Churakova, T., et al., *J. Biol. Chem.*, 1994, vol. 269, p. 26806.
18. Balyasnikova, I., Metzger, R., Visintine, D., et al., *Pulm. Pharmacol. Ther.*, 2005, vol. 18, p. 251.
19. Balyasnikova, I., Metzger, R., Sun, Z., et al., *Tissue Antigens*, 2005, vol. 65, p. 240.
20. Skirgello, O., Balyasnikova, I., Binevski, P., et al., *Biochemistry*, 2006, vol. 45, p. 4831.
21. Balyasnikova, I., Skirgello, O., Binevski, P., et al., *J. Proteome Res*, 2007, vol. 6, p. 1580.
22. Danilov, S., Watermeyer, J., Balyasnikova, I., et al., *Biochemistry*, 2007, vol. 46, p. 9019.
23. Gordon, K., Balyasnikova, I., Nesterovitch, A., et al., *Tissue Antigens,* 2010, vol. 75, p. 136.
24. Balyasnikova, I., Metzger, R., Franke, F.E., and Danilov, S.M., *Tissue Antigens*, 2003, vol. 61, p. 49.
25. Balyasnikova, I., Sun, Z., Berestetskaya, Y., et al., *Hybridoma*, 2005, vol. 24, p. 14.
26. Danilov, S., Savoie, F., Lenoir, B., et al., *J. Hypertens.*, 1996, vol. 14, p. 719.
27. Nikolaeva, M.A., Balyasnikova, I.V., Alexinskaya, M.A., et al., *Am. J. Reprod. Immunol.*, 2006, vol. 55, p. 54.
28. Kost, O., Balyasnikova, I., Chemodanova, E., et al., *Biochemistry*, 2003, vol. 42, p. 6965.
29. Balyasnikova, I., Woodman, Z., Albrecht, R., et al., *J. Proteome Res.*, 2005, vol. 4, p. 258.
30. Danilov, S., Deinum, J., Balyasnikova, I., et al., *Clin. Chem.*, 2005, vol. 51, p. 1040.
31. Nesterovich, A., Hogarth, K., Adarichev, V., et al., *PLoS One*, 2009, vol. 4, e8282.
32. Danilov, S., Kalinin, S., Chen, Z., et al., *PLoS One*, 2010, vol. 5, e10438.
33. Danilov, S., Gordon, K., Nesterovich, A., et al., *PLoS One*, 2011, vol. 6, e25952.
34. Danilov, S., Wade, M., Schwager, S., et al., *PLoS One,* 2014, vol. 9, e88001.
35. Ching, S., Hayes, L., and Slakey, L., *Arteriosclerosis*, 1983, vol. 3, p. 581.
36. Metzger, R., Franke, F., Bohle, R., et al., *Microvasc. Res.*, 2011, vol. 81, p. 206.
37. Silverstein, E., Friedland, J., Lyons, H., et al., *Ann. N. Y. Acad. Sci.*, 1976, vol. 278, p. 498.
38. Yu, C., Sturrock, E., Wu, Zh., et al., *J. Biol. Chem.*, 1997, vol. 272, p. 3511.
39. Ripka, J., Ryan, J., Valido, F., et al., *Biochem. Biophys. Res. Commun.*, 1993, vol. 196, p. 503.
40. Lui, T., Qian, W.-J., Gritsenko, M., et al., *J. Proteome Res.*, 2005, vol. 4, p. 2070.
41. Goette, A., Staack, T., Rocken, C., et al., *J. Am. Coll. Cardiol.*, 2000, vol. 35, p. 1669.
42. Xiao, H., Fuchs, S., Campbell, D., et al., *Am. J. Pathol.*, 2004, vol. 165, p. 1019.
43. Kasi, V., Xiao, H., Shang, L., et al., *Am. J. Physiol.: Heart Circ. Physiol.,* 2007, vol. 293, p. H182.

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