GENETICS

Detection of New Epitopes of Antibodies to Filaggrin in Filaggrin Protein Molecule

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 5, pp. 552-555, May, 2011
Original article submitted March 11, 2010

Immunogenic characteristics of filaggrin protein molecule as an antigen for antibodies to filaggrin, markers of early rheumatoid arthritis, were studied. Two new peptide motives, possible epitopes for antibodies to filaggrin, were shown in the filaggrin molecule by predictive analysis using programmed algorithms. Only IMG-3 and its cyclic form IMG-4 exhibited antigenic reactivity with sera from rheumatoid arthritis patients, differing significantly from the reactivity with donor sera. The immunogenic characteristics of IMG-3 differed from the characteristics of a previously described epitope.

Key Words: *rheumatoid arthritis; filaggrin; peptides; antibodies to filaggrin; immunoreactive epitope*

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology. Until recently, the diagnosis of RA was based mainly on clinical signs and detection of rheumatoid factor, an insufficiently specific serological marker [2,3]. However, antibodies to filaggrin protein (40 kDa; AF) have been detected in the blood of RA patients in recent years. These antibodies appear even before manifestations of clinical symptoms of RA. Detection of these antibodies in patients with early RA confirms the diagnosis [4,5,13,14]. It is therefore essential to study new immunogenic characteristics of filaggrin molecule as antigen for AF, specific markers of early RA.

G. A. Shellekens, *et al.* [11,12] detected an antigenic determinant, a 19-member peptide including amino acid residues 306-324 of filaggrin molecule. A specific feature of filaggrin immunoreactive epitope is the presence of citrullin, an atypical amino acid. Citrullin forms during filaggrin processing from profilaggrin as a result of dephosphorylation, when some arginine residues transform into citrullin [6].

We searched for alternative regions of filaggrin molecule and some other proteins, which can act as immunoreactive epitopes reacting with antibodies in the sera of RA patients.

MATERIALS AND METHODS

The homology and polymorphism of protein molecules were analyzed using BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.egi) and Vector NTI Advance software (Invitrogen).

Predictive analysis of antigenic epitopes was carried out using an original programmed algorithm [7].

Five peptides reproducing fragments of filaggrin molecule were synthesized by solid phase method: IMG-1, IMG-2 (IMG-1 cyclic form), IMG-3, IMG-4 (IMG-3 cyclic form), and cyclic citrullinated peptide

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0007-4888/11/1515-0615

(CCP) described by G. A. Shellekens [11]. CCP consisted of 21 amino acids with one Arg to Cit substitution, other peptides contained 20 amino acids with two Arg residues substituted by Cit.

Blood sera were collected from patients at Department of Faculty Therapy (Head Prof. B. A. Alikhanov) of the Maimonide State Classical Academy. The study was carried out in 49 patients with verified RA and 25 donors. Rheumatoid arthritis was diagnosed by ACR criteria (1987) [1]. The group of patients included 8 (16%) men and 41 (84%) women, mean age 53.0±1.0 years, with 2-30 year history of RA. The process of the first-degree activity was found in 9, second degree in 38, and third degree activity in 2 patients. Thirty-nine patients presented with X-ray stage II, ten with X-ray stage III. First degree articular function insufficiency was detected in 13, second degree in 34, and third degree dysfunction in 2 patients. Thirty-eight patients were rheumatoid factor seropositive, eleven were seronegative.

Immunoreactivity of synthetic peptides was evaluated by EIA. Peptide solutions (1 μg/100 μl in 0.01 M carbonate-phosphate buffer, pH 9.0) were applied on polystyrene plates (Medpolymer and Costar), 100 μl solution per well. Peptides were adsorbed at 2-4°C for 16 h. After adsorption, the peptide solution was shaken and 2% BSA (Sigma) was added to each well. Incubation was carried out for 2 h at 37°C.

The tested sera were diluted 100-fold with 2% BSA and the resultant solution was pipetted into the wells (100 μl per well). Incubation was carried out for 1.5 h at 37°C. After incubation, the plate was washed 3 times with phosphate buffered saline with 0.05% Twin-20 (PBS), 250 μl per well. Rabbit IgG to human IgG conjugated with horse radish peroxidase in 2% BSA were then added to each well (100 μl solution; 1:1000) and incubation was carried out for 1 h at 37°C. After incubation the solution was shaken and the plate was washed 3 times in PBS. Tetramethylbenzidine with hydroperitum was then added into each well (100 μl per well) and incubation was carried out for 5-15 min in the darkness at ambient temperature until staining development. The reaction was stopped by adding 50 μl 50% HCl into each well. The staining intensity was measured on a vertical scanning photometer at λ=450 nm. Sera with optical density (OD) higher than 0.2 were considered positive.

Antibodies to filaggrin were also evaluated using commercial DIASTAT Anti-Cyclic Citrullinated Peptide (CCP) kit (Axis-Shield Diagnostics Limited) according to the instruction.

RESULTS

The search for new immunogenic epitopes of filaggrin molecule was carried out using programmed algorithm [7] predicting not only apparent, but also latent epitopes. Latent epitopes in this case are epitopes exposed only under certain conditions. As for RA, we can hardly imagine that apparent epitopes serve as the main cause of autoimmune disease development, because in this case the pathological process would develop in parallel with the formation of the immune system. As a result of analysis, seven amino acid motives 8 to 22 amino acids long were selected, with potential immunogenic activity, one of the sites coinciding with the previously described one [11,12]. The location of these epitopes is presented in Fig. 1.

a

Lyqvstheqsesthgqtapstggrqgsrheqarnssrhsasqdgqdnirghpgssrggrqgsyheqsvdrsghsgyhhshttpqgrsdas
hgqsgprsasrqtrneeqsgdgsrhsgsrhheastragssrhsqvgqgesagsktsrrqgssvsqdrdseghsedserrsesasrnhygssr
eqsrhgsrnprshqedrashghsaessrqsgtrhaetssggqaassqeqarsspgerhgsrhqqsadsstdsgtgrrqdssvvgdsgnrgs
sgsqasdseghseesdtqsvsahgqagphqqshqeftrgqsggrsgrsg

b

Peptide 4 ----------

Query 4 181 SREQSRHGSRNPRSHQEDRASHGHSAESSRQSGTRHAETssggqaassqeqarssPGERH 240
Query 181 SAQEQLRDGSRHPRSHQEDRAGHGHSADSSRQSGTRHTQTSSGGQAASSHEQARSSAGDR 240

Query 5 241 GSRHQQSADSSTDSGTGRRQDSSVVGDSGNRGSSGSQASDSEGHSEESDTQSVSAHGQAG 300

Peptide 6 ----------

Query 6 1741 GPHQQSHQESTRGQSGERSGRSGS 1764

Fig. 1. Filaggrin amino acid sequence with location of the repeating motive (double underlining) and potential antigenic epitopes (underlined; *a*). Candidate peptides sequence (underlined; *b*).

TABLE 1. Antibodies to Filaggrin in Sera of RA Patients and Donors and Their Reactions with CCP, IMG-1, IMG-2, IMG-3, and IMG-4 Peptides

Control sera	**AF, opt. density unit/ml	CCP binding ($OD_{450}\times10^{-2}$)	IMG-1 binding ($OD_{450}\times10^{-2}$)	IMG-2 binding ($OD_{450}\times10^{-2}$)	IMG-3 binding ($OD_{450}\times10^{-2}$)	*IMG-4 binding ($OD_{450}\times10^{-2}$)
K	76.9	197	7.6	8.7	68.2	32.7
M	100	103	15.9	19.6	85.1	16.6
S	136	113	9.1	8.4	28	-
Sa	29.3	130	8.1	7.1	20	-
Donor	—	14.3	10.9	12.4	12.3	17.8

Note. *Mean data of 5 experiments are presented. **AF was measured in sera using DIASTAT Anti-Cyclic Citrullinated Peptide (CCP) kit (Axis-Shield Diagnostics Limited).

Experimental testing of antigenic activities of all potentially variable sites is an extremely difficult complex process. Before studies of the antigenic characteristics of predicted peptides, we studied natural polymorphism of filaggrin molecules and its coincidence with predicted potential antigenic epitopes. A total of 24 sequences of filaggrin molecules belonging to humans and 3 animal species (*Macaca mulatta*, *Rattus norvegicus*, *Mus musculus*) were analyzed. Fifteen sequences were analyzed for the human species.

Analysis showed that all potential antigenic epitopes were liable to natural variability of this or that degree, and therefore, additional selection of antigenic epitopes was carried out. Two main selection criteria were the length of the epitope (>12 amino acids), with at least 5 amino acid sequences without variability within the epitope, and the presence of Arg residues. Two regions met these requirements and therefore, served as the basis for the synthesis of candidate peptides (Fig. 1; shown as peptides 4 (228-245) and 6 (1741-1764).

Based on the results of these studies, citrullin-containing peptide constructs IMG-1 and IMG-3 were synthesized, corresponding to motives 4 and 6, and their cyclic modifications IMG-2 and IMG-4, respectively.

The next step of the study was computer analysis aimed at the search for possible similar sites in the molecules of filaggrin and fibrin series proteins. This analysis was carried out using BLAST software on the basis of previous data [8-10] on AF reactions with other citrullinated proteins (fibrin-α and fibrin-β).

TABLE 2. Reactions of RA Patients and Donor Sera with IMG-3 and IMG-4 Peptides

Peptide	Experiments	Sera of RA patients			Donor sera		
		n=34			*n*=12	*n*=6	
IMG-3	1	(+)	(-)	OD_{450} mean$\times10^{-2}$	(+)	(-)	OD_{450} mean$\times10^{-2}$
		28 (82)	6	47.5*	4	8 (70)	24.3
	2	19 (56)	15	23.3	3	3 (50)	23.7
	3	26 (76)	8	21.4***	2	4 (70)	17.8
IMG-4	1	*n*=9			*n*=7		
		8 (88.9)	1	29.5	3	4 (57)	22.1
	2	8 (88.9)	1	61.5**	7	0 (100)	37.2
	3	8 (88.9)	1	30.1*	3	4 (57)	20.9

Note. *n*: number of sera in the group; (+): number of reactive sera in the group; (-): number of areactive sera in the group. Percentage of reactive and areactive sera from the total number of tested sera is shown in parentheses. OD_{450} mean: mean optical density after binding of reactive sera in the group. *p<0.003, **p<0.008, ***p<0.02 compared to donors.

Filaggrin sequence with identification No. gil3220177 served as the reference sequence. Filtration ruling out "all Homo sapiens proteins" found no positions associated with fibrin. Fibrinogen positions were found when selecting proteins by "fibrin association", but these were positions with low similarity values. Very short homology regions were detected in equalized sequences of filaggrin and fibrin molecules, presumably those were stray coincidences. Parallel analysis of filaggrin and fibrin protein molecules sequences in the Vector NTI Advance software also failed to detect significant homology between these proteins.

Hence, no appreciable homology between filaggrin and fibrin series proteins was detected by comparative analysis.

Immunoreactive characteristics of filaggrin were then evaluated by testing immunogenic activities of synthesized peptides. Experiments were carried out using 4 control sera from RA patients (K, M, S, Sa) after preliminary characterization of these sera using commercial kit and testing with synthetic CCP [11,12] and donor serum (D). All sera contained AF in high concentrations (Table 1).

The immune properties of IMG-1 and IMG-2 as antigens to AF were weak, because they inefficiently reacted with control sera (Table 1). OD remained at the baseline level. Peptides IMG-3 and IMG-4 in similar experiments exhibited a significantly higher dose-dependent immune activity (data not presented). These results suggested that IMG-3 amino acid sequence could serve as the AF antigenic epitope.

In order to verify this hypothesis, a group of RA patients and donors was selected and reactions of sera from these patients with IMG-3 and IMG-4 were studied. The study was carried out under the same conditions in 3 successive experiments for each peptide. The results (Table 2) indicated that the sensitivity of IMG-3 peptide was 56-82%, which corresponded to the data for CCP [13], while its specificity was low (30-50%). The sera from RA patients and donors differed by IMG-3 binding (p<0.003-0.020). Low OD (OD_{450}) values after binding of IMG-3 with sera from RA patients were comparable to the values in donor sera binding. In addition, the levels of binding (OD_{450}) varied from one experiment to another in the studied groups of sera and in individual sera (Table 2). Analysis of the IMG-4 characteristics showed similar regularities (Table 2).

Hence, of the two studied motives of filaggrin molecule peptide only motive 6 exhibited antigenic reactivity with sera from RA patients. Both peptides (IMG-3 and IMG-4) representing motive 6 demonstrated a significantly higher binding to immunoreactive sera compared to donor sera.

Studies of immunogenic characteristics of filaggrin molecule showed an AF affine epitope in its structure, differing from previously described one [11,12]. These molecule sites differed by the degree of immunoreactivity, presumably responsible for the heterogeneity of the AF population [6]. The use of this characteristic in practical diagnostic work will presumably lead to more precise detection of RA patients.

REFERENCES

1. F. C. Arnett, S. M. Edworthy, D. A. Bloch, *et al.*, *Arthritis. Rheum*, **31**, No. 3, 315-324 (1988).
2. L. F. Callahan and E. H. Yelin, *Primer on the Rheumatic Diseases*, Ed. J. D. Klippel, Atlanta (2001), pp. 1-4.
3. E. D. Harris, *N. Engl. J. Med.*, **322**, No. 18, 1277-1289 (1990).
4. E. J. Kroot, B. A. de Jong, M. A. van Leeuwen, *et al.*, *Arthritis. Rheum.*, **43**, No. 8, 1831-1835 (2000).
5. O. Kuru, A. Bilgici, A. Birinci, *et al.*, *Bratisl. Lek. Listy*, **110**, No. 10, 650-654 (2009).
6. T. Mimori, *Intern. Med.*, **44**, No. 11, 1122-1126 (2005).
7. A. N. Nekrasov, *J. Biomol. Struct. Dyn.*, **21**, No. 5, 615-624 (2004).
8. M. L. Pérez, M. J. Gómara, G. Ercilla, *et al.*, *J. Med. Chem.,* **50**, No. 15, 3573-3584 (2007).
9. R. Sanmarti, E. Graell, M. L. Perez, *et al.*, *Arthritis Res. Ther.*, **11**, No. 5, R135 (2009).
10. M. Sebbag, N. Moinard, I. Auger, *et al.*, *Eur. J. Immunol.*, **36**, No. 8, 2250-2263 (2006).
11. G. A. Shellekens, B. A. de Jong, F. H. van den Hoogen, *et al.*, *J. Clin. Invest.*, **101**, No. 1, 273-281 (1998).
12. G. A. Shellekens, H. Visser, B. A. de Jong, *et al.*, *Arthritis. Rheum.*, **43**, No. 1, 155-163 (2000).
13. M. A. van Boekel, E. R. Vossenaar, F. H. van den Hoogen, and W. J. van Venrooij, *Arthritis Res.*, **4**, No. 2, 87-93 (2002).
14. X. Zeng, M. Ai, X. Tian, *et al.*, *J. Rheumatol.*, **30**, No. 7, 1451-1455 (2003).