Purification of Fibroblast-Derived Celiac Disease Autoantigen Molecules¹

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ABSTRACT. We have recently purified autoantigen polypeptides reacting with celiac disease patient sera IgA from the extracellular noncollagenous matrix compartment of fetal lung tissue. These molecules trigger the production of different tissue antibodies, the so-called antireticulin and antiendomysium antibodies in celiac disease. In the present report, we show that fibroblasts synthesize and secrete celiac disease autoantigen molecules. The secretion product, reactive with IgA from celiac disease patients, is a large-molecular-weight protein aggregate. When the protein complex was treated with 4 M guanidinium hydrochloride and 0.1% SDS, 11 monocomponent polypeptides could be detected by PAGE. Of these, four single polypeptides with molecular weights of 17 000–39 500 and isoelectric points of 5.0–7.0 were observed to react with IgA separated from sera of children with celiac disease. The polypeptide molecules produced by fibroblasts *in vitro* bound to antireticulin and antiendomysium antibodies but not to antigliadin antibodies. The present observations show that tissue antibodies found to be specifically associated with celiac disease are generated against a synthesis product of fibroblasts, a cell-type known to synthesize a number of biologically active polypeptides. The fibroblast-derived extracellular matrix proteins and the formed autoantibodies may be important in the pathogenesis of gluten-sensitive enteropathy. (*Pediatr Res* 34:420–423, 1993)

Abbreviations

ARA, antireticulin antibody
AGA, antigliadin antibody
CD, celiac disease
CDAP, celiac disease autoantigen protein
EMA, antiendomysium antibody
NCRC, noncollagenous reticulin component

The etiology and pathogenesis of CD, or gluten-sensitive enteropathy, is not well understood, despite a large body of information accumulated in recent decades (1, 2). However, we have recently proposed a new way of thinking and hypothesized that the gluten-sensitive enteropathy might be attributable to an autoimmune mechanism in which antibodies are generated against the patient's own tissue material. We have identified extracellular matrix noncollagenous protein molecules that specifically react with CD patient sera IgA (3). In CD, these identified human protein molecules trigger the production of tissue anti-

bodies known as ARA and EMA (3). The occurrence of these autoantibodies is highly sensitive and specific for gluten-sensitive enteropathy in CD and dermatitis herpetiformis (4–7). Positivity for these antibodies is also genetically determined (8).

CD and dermatitis herpetiformis patient sera IgA detect molecules not only in rodent but also in primate tissues, including human jejunum, in a pattern very closely resembling the connective tissue fibrillar staining pattern produced by silver-impregnation staining (9, 10). Kárpáti *et al.* (10) showed that in these molecules the reticulin antigen was not detected directly on collagen fibers but was associated with amorphous material surrounding these fibers (6).

In close contact with the intestinal epithelial cells, separated from them by the basement membrane, lies a continuous sheath of fibroblasts that forms part of the connective tissue infrastructure of the lamina propria (11–13). Fibroblasts are also known to synthesize extracellular matrix proteins, collagen and procollagen (14, 15), and noncollagenous proteins, many of which have autocrine or paracrine functions (16–19). Berman *et al.* (20) showed that fibroblasts produced extracellular fibers that stained positively with silver-impregnation reticulin stain. Fibroblasts have also been shown to express reticulin molecules (21).

In the present study, we hypothesized that CDAP are synthesized by fibroblasts, and our results show that fibroblasts do synthesize noncollagenous proteins that, in genetically determined individuals, become autoantigens when gluten ingestion commences. We further show the CDAP molecules to be secreted by fibroblasts as part of a large protein complex.

MATERIALS AND METHODS

Fluoroimmunostaining of cultured fibroblast layer. Fibroblasts were removed from fetal lung tissue (3) and stored in liquid nitrogen. Approximately 0.5×10^6 cells were cultured in 4-mL culture vessels in 1.5 mL of cell culture medium containing 10% of FCS. The culture was incubated at 37°C in atmosphere containing 5% CO₂ for 2 d. The medium was then discarded, and the cells were acetone-fixed and fluoroimmunostained using purified CD patient sera IgA as previously described (21). For control purposes, fluoroimmunostaining was also performed using IgA purified from sera from healthy subjects.

Evaluation of fibroblast synthesis product accumulation into culture medium. Approximately 5×10^6 fetal lung fibroblasts were divided into five culture vessels of 50 mL and cultured for 4 d as described above with tritiated amino acid mixture (Amersham, Buckinghamshire, England) with a total radioactivity of 2 μ Ci. Samples of 0.5 mL were removed from the media once a day to evaluate the appearance of tritiated protein material in the culture medium. The samples were gel filtrated in 9×1.1 -cm Sephadex G-25 columns (Pharmacia, Uppsala, Sweden) to remove tritiated amino acids from protein material of the culture medium. ³H radioactivity of the protein material was counted.

Separation of fibroblast-synthesized protein molecules reacting with CD patient sera IgA. After 4 d, the fibroblast culture media were collected and concentrated by dialyzing against 60 mM

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¹ This paper is dedicated to Professor S. Auricchio on his 60th birthday.

potassium phosphate buffer, pH 6.9, containing 40% (wt/vol) sucrose. Sucrose and the remaining free titrated amino acids were removed from the dialysate by gel filtration with a 9×1.1 -cm Sephadex G-25 gel filtration column. The concentrated fibroblast culture medium was then gel filtered using HPLC equipment fitted with a 600×21.5 -mm Spherogel TSK HPLC gel filtration column (Beckman, Fullerton, CA), and 60 mM potassium phosphate buffer, pH 6.9, as eluent. Fractions of 3.5 mL were collected and 0.5 mL from each fraction was used to count ^3H radioactivity. Thereafter, 0.5 mL of each fraction was diluted to yield a protein concentration of $20 \mu\text{g/mL}$, using the method of Lowry for quantitative protein determination (22). For detection of reactivity with CD patient sera IgA of the ^3H -protein material in the fractions, a Nunc Maxisorb ELISA plate (Nunc, Roskilde, Denmark) was coated with $100 \mu\text{L}$ of each diluted fraction/well overnight at 4°C and blocked by 1% gelatin solution for 1 h at 37°C . After washing, the plate was incubated with IgA (5 g/L) purified from sera from a child with CD with extremely high EMA titer, using the Jacalin (Pierce, Rockford, IL) method (23). Alkaline phosphatase-conjugated, α -chain-specific anti-human IgA (Orion Diagnostica, Helsinki, Finland) was used as the second antibody, and $100 \mu\text{g}$ of phosphatase substrate/well were used for color development. OD was measured after 45 min incubation at 37°C . The rest of the fractions that gave an OD higher than 0.2 were then pooled and concentrated by dialyzing against 40% sucrose solution for additional analysis. For control measurement, ELISA analysis was performed similarly using a plate coated with $100 \mu\text{L}$ of corresponding diluted fraction of medium per well without fibroblast culturing, treated and gel-filtered equally. The value of the OD of the control plate was subtracted from the corresponding analysis value.

Purification of monocomponent IgA reactive polypeptides. The pooled fractions, containing ^3H -labeled CD patient sera IgA reactive fibroblast-synthesized material, were concentrated by dialyzing against 40% sucrose solution, incubated overnight at 55°C after addition of guanidinium hydrochloride (4 M) and SDS (0.1% of the protein content) and adjusting pH to 8.8 by NaOH. An aliquot of $150 \mu\text{L}$ of the mixture was then removed for isoelectric focusing, the rest of the mixture was gel-filtered by HPLC, and fractions were collected as above. The reactivity of the material in each fraction with CD patient sera IgA was analyzed by ELISA as described above. The fractions giving an OD value higher than 0.2 in the ELISA were pooled for affinity chromatography. A column of 2.5 mL of AffiGel 10 affinity chromatography gel (BioRad, Richmond, CA) linked with 3 mg of Jacalin-purified (23) CD patient sera IgA was produced according to the instructions of the manufacturer of the gel. The pooled fractions with IgA-reactive material were run through the column at a flow rate of 0.05 mL/min at room temperature. The column was washed with 150 mM PBS until no proteins were detected in the eluate by a detector fitted to the column by a peristaltic pump. Then the material bound to IgA linked to the gel was removed by eluting with 0.25 M citrate buffer, pH 3.5, and neutralized to pH 7.4. An aliquot of $150 \mu\text{L}$ of the eluate was removed for isoelectric focusing.

Binding of IgA reactive polypeptides to ARA, EMA, and AGA. To evaluate binding of the purified IgA reactive polypeptides to ARA, EMA, and AGA, $100\text{-}\mu\text{L}$ samples of sera from five children with active CD and known titers of AGA, ARA, and EMA were applied to affinity chromatography columns consisting of AffiGel 10 gel linked with crude gliadin and a mixture of the four IgA reactive protein molecules (0.5 mg of ligand to 0.5 mL of gel). Unbound material was eluted with 1 mL of PBS and collected for measurement of AGA by a standard ELISA method and of ARA and EMA by indirect immunofluorescent assay (5, 6).

Isoelectric focusing. A sample of the material treated with guanidinium hydrochloride and SDS and a sample of the material after affinity chromatography were applied on Ampholine isoelectric focusing gel with a pH range from 3.5 to 9.5 (Pharmacia Diagnostics, Uppsala, Sweden) and run at 25 W for 3 h.

Fixing and staining were performed according to the manufacturer's instructions.

RESULTS

Fluoroimmunostaining of cultured fibroblast layer. Using acetone-fixed fibroblast layer and highly purified CD patient sera IgA in the test, almost 100% of the cell monolayer stained positively (Fig. 1). Control sera IgA and the sera absorbed with Jacalin gave negative staining.

Synthesis and secretion of CD autoantigen molecules by fibroblasts. Continuous accumulation of ^3H -labeled fibroblast-synthesized proteins into the medium was observed during culture. The radioactivity of the protein material was 180 dpm/mL after 4 d of culturing. The gel filtration curve in Figure 2 shows that about 30% of the secreted ^3H -labeled protein was found to be in complex form with a molecular weight higher than 1 million. This complex, collected with fractions 3, 4, and 5, was highly reactive with CD patient sera IgA. Other secreted proteins did not react significantly with IgA (fractions 1, 2, and 6–23).

In drastic conditions, a major part of the tightly bound monocomponent polypeptides could be dissociated from the protein complex (Fig. 3). After gel filtration of the dissociated material, most of the radioactivity (indicating presence of fibroblast synthesis products) was detected in the fractions of the lower-molecular-weight region (fractions 15–19). The material in these fractions was also reactive with patient sera IgA analyzed by ELISA. The mixture of dissociated material contained at least 11 different polypeptides with isoelectric points from 4.5 to 7.8 and molecular weights from 16.5 to 39.5 (determined using HPLC gel filtration) (Figs. 3 and 4). When that mixture was applied to the affinity chromatography column with CD patient

Fig. 1. Indirect immunofluorescent staining of human embryonic lung fibroblasts by purified IgA. Fibroblasts were cultured in EBME medium (Flow, Irvine, Scotland). After 2 d the medium was removed and the fibroblast layer fixed with acetone and incubated with CD-dependent IgA (top) and with IgA separated from serum from a healthy subject (bottom). Fluorescein-conjugated anti-human IgA was used as the second antibody. The time of exposure was considerably longer in the bottom stain than in the top stain.

Fig. 2. Gel filtration chromatogram of ^3H -labeled proteins from fibroblast culture and their binding to IgA isolated from CD patient sera. Concentrated fibroblast culture medium was gel-filtrated by HPLC and fractions were collected. ^3H radioactivity of the material in each fraction was counted and binding to CD patient sera IgA was tested using the conventional ELISA method. The curve represents ^3H radioactivity as dpm and the bars represent binding to IgA measured as OD.

Fig. 3. Gel filtration chromatogram and binding to CD patient sera IgA of ^3H -labeled IgA-reactive material after guanidine hydrochloride treatment. The fractions with high binding to CD-dependent IgA presented in Figure 2 were pooled, concentrated by dialyzing against 40% sucrose, and treated with guanidium hydrochloride and SDS at 55°C . The material was then fractionated by HPLC gel filtration and reactivity of the material in each fraction with CD-dependent IgA was tested using ELISA. The curve represents ^3H radioactivity and the bars represent binding to IgA presented as OD in ELISA.

sera IgA as the ligand, four single polypeptide molecules were observed to bind to the purified IgA (Fig. 4).

The antigenic specificity of the polypeptides was ensured by ELISA using sera from 10 children with untreated CD (OD values: mean 770, range 405–1610) and 10 subjects excluded for CD showing normal jejunal mucosal architecture (OD values: mean 190, range 77–380).

Binding of IgA-reactive polypeptides to ARA, EMA, and AGA. Reaction of IgA-reactive polypeptides with ARA, EMA, and AGA in affinity chromatography is summarized in Table 1. The purified molecules produced by fibroblasts *in vitro* absorbed most of the ARA and EMA from the patient samples, whereas crude gliadin absorbed none of them.

DISCUSSION

We have now shown that fibroblasts expressing molecules detected by CD patient sera IgA in immunofluorescence studies

Fig. 4. Isoelectric focusing of CD-dependent IgA-reactive material after dissociation with guanidinium hydrochloride and SDS (lane A) and purification of IgA-reactive components by affinity chromatography (lane B).

synthesize and secrete *in vitro* a large protein complex reacting with the same IgA. The secreted large protein complex was very stable, but it could be dissociated to monocomponent polypeptides, four of which specifically and separately reacted with untreated CD patient sera IgA. In conventional immunofluorescence tests, the same sera contain so-called R1-type ARA and EMA (5, 6). We recently succeeded in purifying from human fetal lung tissue six extracellular noncollagenous CD-specific autoantigen molecules, which in combination acted as a true antigen to ARA and EMA (3). Here we show that the purified four IgA-reactive polypeptides produced by fibroblasts *in vitro* bind to ARA and EMA, but not to AGA. Our hypothesis that these autoantigens were synthesized by fibroblasts was confirmed. All four IgA-reactive fibroblast-derived polypeptides were of the same molecular weight range as the six polypeptides purified from fetal lung tissue. The isoelectric points of three polypeptides from both groups were coincidental. The differences of purification products may be caused by differences in concentrations of single polypeptides in the starting materials or CDAP degradation in tissue.

In 1973, Pras and Glynn (24) isolated an NCRC from kidney tissue. Rabbit antisera raised against these pig and human kidney molecules gave immunofluorescent staining patterns closely resembling those for ARA (25). Later studies with the NCRC confirmed that histologic reticulin is not a single entity but a compound of fibrous structure and at least one additional noncollagenous glycoprotein (21). Further studies showed that ARA found in CD did not react with collagen type III, fibronectin, or NCRC (26), and NCRC preparations produced a heterogeneous and variable product (27). Maury and Teppo (28) described a protein extracted from a rare skin tumor that had similarities to NCRC. However, this epithelial extracellular 90-kD glycoprotein seemed not to be the antigen recognized by ARA (29).

Table 1. Binding of IgA AGA, ARA, and EMA to gliadin and CDAP in affinity chromatography

Patient	Antibody titer*								
	Patient serum			Eluate from gliadin column			Eluate from CDAP column		
	AGA	ARA	EMA	AGA	ARA	EMA	AGA	ARA	EMA
1	5.59	4000	2000	1.01	4000	2000	5.72	100	50
2	7.46	2000	1000	1.91	2000	1000	7.07	100	50
3	4.88	500	500	0.30	500	500	5.10	5	<5
4	>10	500	500	2.03	500	500	>10	<5	<5
5	>10	1000	500	2.26	1000	500	>10	50	5

* For AGA, ELISA units/mL; for ARA and EMA, reciprocal of highest dilution that gave positive fluorescence.

Parker *et al.* (13) showed that the jejunal subepithelial fibroblast population replicates and migrates in parallel and in approximate synchrony with the replicating and migrating epithelial cells. This might be an important physiologic observation in terms of knowledge today, *i.e.* the known close epithelial cell-fibroblast interaction in normal and diseased states (15, 16, 18, 19). In gluten-sensitive enteropathy, subepithelial extracellular noncollagenous matrix proteins are the targets for the so-called ARA (3, 10). Here we also show the targets to be fibroblast-derived molecules. Our four identified proteins, proteins that become under certain conditions self-peptides triggering the production of reticulins, endomysium, and jejunal antibodies, might have important villus contractility structural or epithelial cell paracrine functions; thus, also the formed autoantibodies could have a role in CD pathogenesis.

We suggest that the gluten-sensitive enteropathy may arise as a consequence of an autoimmune mechanism, and in such a model, gliadin would be the specific etiologic agent and the fibroblast-derived CDAP molecules the putative autoantigen. These hypothetical autoimmune mechanisms, including the role of CD-specific susceptible MHC genes (30–32), need to be studied further.

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