AUTOANTIGEN COMPONENTS RECOGNIZABLE BY SCLERODERMA SERA ARE EXPORTED VIA ECTOCYTOSIS OF FIBROBLASTS

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SUMMARY

Previously, we have demonstrated that ectocytosis, a unique cell trafficking process to export a specific subset of cellular proteins in the form of membrane vesicles, can be triggered from human skin fibroblasts cultured in a three-dimensional collagen lattice upon stress relaxation. The same culturing system was employed in the present study using fibroblasts isolated from patients with systemic sclerosis (SSc). To see whether any putative intracellular autoantigens causing SSc might escape out of cells by way of ectocytosis, the same stress-relaxation method was used to induce a synchronized ectocytosis among cultured cells. Membrane vesicles released by scleroderma fibroblasts were subsequently isolated, resolved on SDS–PAGE and immunoblotted with sera from 89 patients with various autoimmune diseases and 11 normal volunteers. Three major polypeptides with apparent mol. wts of 12–14, 32–34 and 70–80 kDa are prominent bands on both SDS–PAGE and immunoblots.

The 32–34 kDa polypeptide has been further identified as a member of the annexin protein family, while the 70–80 kDa protein has been shown to be topoisomerase I, as judged by its reactivity to patients’ sera and a rabbit polyclonal antibody, and as also judged by a functional assay. In conclusion, our results suggest that ectocytosis might be one of the potential pathways for cells to export intracellular antigens and subsequently cause autoimmune reactions.

KEY WORDS: Autoantigens, Scleroderma, Ectocytosis, Annexin I, Topoisomerase I, Stress-relaxation method, Fibroblasts.

Two cardinal features that scleroderma [systemic sclerosis (SSc)] shares with other systemic autoimmune diseases are, namely, the pleiotropy in autoantibodies and the antigen-driven nature of the autoimmune response [1–3]. Different autoantigens have been shown to be residents in different subcellular domains, including the cell nucleus [4–6]. Moreover, most of the intracellular autoantigens do not have the signal peptide sequences required for secretion. Therefore, it is difficult, if not impossible, to envisage the aetiological and pathogenic mechanisms by which multiple autoantibodies against diverse intracellular antigens can be induced in a single disease. A further question is how those intracellular and intranuclear autoantigens escape out of the cells.

A plausible hypothesis intended to explain the diversity of autoantibodies in a single disease has been proposed, suggesting that aggregates of a host of autoantigen molecules form distinctive subcellular particles before they get out of the cells [2]. Although such a cluster could explain, in part, the disease-specific prevalence of certain autoantibodies, it fell short of illustrating the elusive pathway of autoantigens.

To address specifically the exodus of autoantigens, it has been shown that cultured human keratinocytes are capable of dumping out autoantigens by way of induced programmed cell death (apoptosis) [3, 7].

Unlike endocytosis or exocytosis, ectocytosis [8] is a less well known process by which cells export sorted polypeptides lacking signal peptide sequences [9] required for secretion in the form of enclosed membrane vesicles [10]. It is a normal cellular secretion process that has been reported to serve many important physiological functions in a variety of cells [11]. The stress-relaxation model is an in vitro model system of wound contraction developed using fibroblasts cultured in a three-dimensional collagen matrix [10]. Our previous study has shown that upon tension released from the collagen matrix, the embedded fibroblasts underwent a markedly accelerated ectocytosis. Furthermore, the right-side-out, annexin-containing membrane vesicles that discharged from fibroblasts via a budding process were entrapped by collagen lattice in the vicinity of the cells. The composition of entrapped membrane vesicles can easily be isolated from the collagen matrix for further studies [10].

In an effort to look for a more subtle method, other than self-destruction, for cells to exteriorize intracellular autoantigens, the unique feature of amplified ectocytosis provided by the stress-relaxation model was studied. Vesicles released by fibroblasts derived from patients with SSc were collected and resolved on SDS–PAGE. They were subsequently screened by immunoblotting with sera from autoimmune patients. Various polypeptides were recognized by sera from SSc patients, among which two SSc-specific autoantigens, annexin I and topoisomerase I, were further identified. Our results suggested that ectocytosis might be at least one of the possible pathways by which intracellular...
lar autoantigens escape from cells. Details of our findings are reported here.

MATERIALS AND METHODS

Sera
The sera used for this study were collected from 89 out-patients of both sexes who had been diagnosed with major typical systemic autoimmune diseases, including 49 scleroderma, 23 systemic lupus erythematosus (SLE), 10 Sjögren’s syndrome (SS), seven primary biliary cirrhosis (PBC). Sera from 11 healthy individuals were also collected as controls. A polyclonal rabbit anti-topoisomerase I antibody was also used.

Skin biopsies and cell culture
Punch biopsies were taken from clinically involved skin of the forearms of three patients with scleroderma. All the diagnoses of scleroderma met the criteria set by the American Rheumatism Association. Primary cultures of fibroblasts were set up from skin biopsies and were cultured at 37°C in a humidified atmosphere of 5% CO2, in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. After 2–3 weeks, the outgrowth of fibroblasts reached confluence, and the cells were then passaged by 0.25% trypsin/0.53 mM EDTA treatment and subcultured as above. Only fibroblasts between three and six passages were used in our experiments.

Hydrated collagen matrix cultures
The preparation and maintenance of hydrated collagen matrices from Vitrogen 100 collagen solution (Celtrix Labs, Palo Alto, CA, USA) were described in detail previously [10]. Briefly, fibroblast suspension was mixed with the neutralized collagen solutions (1.5 mg/ml) at a concentration of 5 × 105 cells/ml. Aliquots of 0.2 ml of the cell/collagen mixture were placed in Costar 24-well culture plates. It took 60 min and 50 mg/ml of ascorbic acid were added to each well.

Triggering ectocytosis and harvesting vesicles
Collagen matrices containing fibroblasts were incubated in the same condition as that for monolayer cultures described above for 48 h, during which time the fibroblasts contracted the matrix surrounding them but could not shorten the diameter of individual collagen gel since the gels attached to the substratum firmly. Thus, mechanical stress developed among cells and consequently tension was built up. To initiate stress relaxation, matrices were gently dislodged from the underlying tissue culture substratum using a spatula. In response to the stress relaxation, the cells rapidly and transiently released a large number of membrane vesicles from the cell surface. To recover membrane vesicles from the surrounding matrix, a three-step procedure was conducted as described previously [10]. In brief, stress-relaxed collagen gels were first digested with 0.05% trypsin/0.53 mM EDTA (Gibco) at room temperature for 10 min. Type I collagenase from bacteria (Sigma, C-0130) in 150 mM NaCl, 10 mM calcium acetate, 20 mM HEPES (pH 7.2), at a concentration of 5 mg/ml was added directly in the trypsin/EDTA for 30 min to break down collagen fibrils completely. Fibroblasts were collected by centrifugation at 200 g for 10 min at ambient temperature. All the membrane vesicles once entrapped by the collagenous meshwork were now suspended in the cell-free supernatant and designated as the matrix fraction in some of the following SDS–PAGE experiments. The matrix fraction was subjected to high-speed centrifugation at 100 000 g at 4°C for 60 min. At the end of high-speed centrifugation, membrane vesicles were spun down into the pellet which was designated as the vesicle fraction and subjected to the following experiments.

SDS–PAGE and immunoblotting
Samples of cells and vesicles were denatured in 62.5 mM Tris-HCl buffer (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol, and boiled for 5 min. Afterwards, samples were subjected to SDS–PAGE on a 5% stacking/15% running gel using a mini-gel apparatus (Bio-Rad). To screen individual polypeptides that can be recognized by patients’ sera, samples were then electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper with transferred proteins was cut into strips and blocked for 30 min in 5% non-fat dry milk in phosphate-buffered saline (PBS). Antisera diluted with 5% non-fat dry milk in PBS were used to react with the strips and incubated for 1.5 h at room temperature. The strips were washed three times with PBS–Tween 20, each for 10 min. The antibody bound to the specific antigen was detected by autoradiography following a 1 h incubation with 125I protein A, or alkaline phosphatase-conjugated goat anti-human IgM + IgG + IgA, or goat anti-rabbit IgG (Bio-Rad). Subsequently, they were developed with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) by following instructions provided by the manufacturer.

Pre-absorption assay for identifying annexin I
To find out the identity of the 32–34 kDa polypeptide, a pre-absorption assay was conducted. Briefly, immediately before immunoblotting, sera from SSc patients were pre-incubated with pure annexin (Sigma) at different concentrations, as indicated, at 37°C for 30 min. The pre-absorbed sera were clarified by centrifugation at 3000 r.p.m., at room temperature for 10 min. Only the supernatant was kept and used in the subsequent steps in the immunoblotting.

Topoisomerase I assay
The presence of native topoisomerase I in the vesicle fraction was detected by its ability to relax supercoiled pBR322 DNA on agarose gel electrophoresis as described previously [12]. Vesicles harvested from...
16 gels were lysed by 100 ml buffer A [140 mM NaCl, 10 mM Tris (pH 7.5), 1.5 mM MgCl2, 0.5% NP-40] for 10 min on ice, and then centrifuged at top speed for 10 min. The supernatant obtained by centrifugation was used for the assay of the relaxation of the supercoiled DNA. The 20 μl of assay mixture contained 50 mM Tris–HCl (pH 7.5), 120 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 30 mg/ml bovine serum albumin (BSA), 0.2 mg pBR322 DNA and 2 μl of topoisomerase I or 2 μl buffer A or 5 μl vesicles (from 0.8 gel equivalents). After reaction for 5, 30 and 60 min at 30°C, samples were subjected to a 0.7% agarose gel electrophoresis.

Detection of DNA fragmentation in the membrane vesicles

To distinguish the ectocytosis from the well-known apoptotic process, we isolated DNA from both the cell fraction and the vesicle fraction using the method previously described [26]. To avoid the possibility that a false-negative result might be obtained due to the DNA associated with the vesicle fraction being too low to be detected, we prepared DNA from 24 collagen gels, i.e. equivalent to the vesicles released from 2.4 \times 10^6 cells which we believed would be more than an adequate amount of DNA, if there is any, to give a positive reaction on agarose electrophoresis. For the same reason, DNA from 4.5 \times 10^6 cells was extracted and loaded onto the agarose gel electrophoresis.

RESULTS

Analysis of vesicles from scleroderma fibroblasts

As documented in our previous study, the mechanical release of tension from collagen gels triggered a synchronized ectocytosis response of fibroblasts within the matrix, featuring the budding and pinching off of membrane vesicles from the cell surface [10]. We also showed that those membrane vesicles maintained the plasma membrane intact and were accumulated in the collagen matrix near the site of release [10]. After digesting the matrix by trypsin/collagenase treatment, membrane vesicles from fibroblasts of sclerodema patients and normal subjects were harvested by high-speed centrifugation. Figure 1 shows the polypeptide profiles of cell fractions, matrix fractions and vesicle fractions of both normal and patients’ fibroblasts that were resolved on SDS–PAGE. Noticeably, there are differences in almost every fraction of the two fibroblast populations. This result suggests that fibroblasts from scleroderma patients themselves differ from their counterparts from normal subjects in biosynthesis, secretion and ectocytosis of proteins. Although these are interesting phenomena that deserve further studies, we focused our attention on ectocytotic vesicles for the time being.

Screening of putative autoantigens present in ectocytotic vesicles

Most autoantigens identified so far are intracellular or intranuclear molecules, except those membrane-bound or cell surface antigens. Under normal conditions, the autoantigens cannot be exported to the extracellular milieu by normal cell secretion processes due to the lack of signal sequences in their primary structures. Since ectocytosis provides a means by which cells can export those cytoplasmic proteins, we wondered whether any intracellular autoantigens can also be exported to the exterior of cells by the ectocytosis mechanism, either by chance or deliberately. To confirm our hypothesis, we conducted a large-scale screening of membrane vesicles released by scleroderma fibroblasts to see whether there were any putative autoantigens that can be recognized by autoantibodies in sera of patients with autoimmune dis-

![Fig. 1.](image1.png) Polypeptide profiles of various fractions isolated from normal (lanes 2, 4 and 6) and SSc (lanes 3, 5 and 7) fibroblasts. After digesting away the collagen lattice, samples were fractionated into cell (lanes 2 and 3), matrix (lanes 4 and 5) and vesicle fractions (lanes 6 and 7). Solubilized proteins from about one gel equivalent were loaded onto each lane and separated by SDS–PAGE, and stained with Coomassie blue. Lane 1 was low range molecular weight markers from Bio-Rad.

![Fig. 2.](image2.png) Screening of possible autoantigens present in ectocytosis vesicles. Membrane vesicles released by SSc fibroblasts were separated on SDS–PAGE, transferred onto nitrocellulose paper and immunoblotted with sera from patients with SSc (strips 1–8). Three prominent bands (80, 32 and 14 kDa) appeared in almost all strips blotted with SSc sera, whereas there was no band interacting with sera from a normal subject (lane 9).
eases. Isolated membrane vesicles were subjected to SDS–PAGE and immunoblotting was carried out using patients’ sera. Figure 2 shows that there are conspicuous polypeptides recognized by all three patients’ sera used, whereas not a single protein can be recognized by sera from a normal subject. Figure 2 also shows that three major polypeptide bands with apparent mol. wts of 12–14, 32–34 and 70–80 kDa, respectively, are predominant bands interacting with all the sera used. To verify that the three polypeptides are SSc specific, we checked the occurrence of the three interacting bands with sera from 49 SSc patients. Our result shows the high frequencies of autoantibodies to the three major polypeptides in the scleroderma sera with a percentage of 88% (43/49) for 70–80 kDa, 72% (35/49) for 32–34 kDa and 43% (21/49) for 12–14 kDa, respectively. This high correlation suggests that these three polypeptides are SSc-related autoantigens. It also strongly supports the notion that at least a part of the intracellular autoantigens can be exported to the exterior of cells by way of ectocytosis.

Detecting the identity of the 32–34 kDa polypeptide

Our previous study [10] has shown that ectocytotic vesicles from fibroblasts contain actin, annexin II, annexin VI and other components. Hirata et al. [13] and others [14–16] have also shown that autoantibodies against both annexin I and other members in the annexin family were detected in patients suffering from inflammatory or some autoimmune diseases, including rheumatoid arthritis, SLE, SS and even malignant melanoma. In addition, several members of the annexin family have a mol. wt in the range 32–36 kDa. To check the identity of 32–34 kDa polypeptide in the vesicles, we carried out a pre-adsorption assay in which the sera were pre-treated with commercially purchased pure annexin I, before they were blotted with patient’s sera. As shown in Fig. 3, the intensity of the 32–34 kDa bands decreased with increasing amount of pure annexin I used. The decrease in band intensity was further checked using a densitometer (data not shown). This result demonstrates that like the above-mentioned autoimmune diseases involving connective tissues, autoantibody against annexin I is also present in sera of SSc. Moreover, our result indicates that molecules of annexin I are exported by the ectocytotic mechanism.

To verify whether anti-annexin I autoantibody is SSc specific, a similar screening procedure was carried out using sera collected from 23 patients with SLE, 10 patients with SS, and seven patients with PBC, and sera from 11 normal volunteers. Although the presence of anti-annexin I autoantibody is not absolutely SSc specific, a much higher frequency (72% positive) of this autoantibody was present in sera of SSc compared with other diseases. Figure 4 shows a representative immunoblot showing positive binding at the 32 kDa position with sera from SSc, SLE and...
PBC, whereas normal subjects show only negative results.

Detecting the identity of the 70–80 kDa polypeptide

Previously, we have shown that there is a high prevalence of anti-DNA topoisomerase I (anti-Scl 70) autoantibodies in 88% (43/49) of patients with diffuse scleroderma [12]. To check the identity of the 70–80 kDa polypeptide present in vesicles, the lysates from vesicles were immunoblotted with three reference sera which had shown strong titres of anti-topoisomerase I autoantibodies. A rabbit polyclonal antibody specifically directed against topoisomerase I (lane P in Fig. 5) and sera from normal subjects (lane N in Fig. 5) was also used as a control. Figure 5 shows that the 70–80 kDa polypeptide present in vesicles can be recognized by all three reference sera and a rabbit polyclonal antibody against topoisomerase I. This result suggests that a second autoantibody specifically associated with SSc can be delivered to the outside of cells by way of ectocytotic vesicles.

To confirm further the identity of the 70–80 kDa polypeptide in vesicles, a functional assay measuring the activity of topoisomerase I was conducted [12]. Figure 6 shows that even being enclosed in vesicles, the activity for nicking–closing supercoiled DNA molecules is well preserved by topoisomerase I.

Detection of DNA fragmentation in the membrane vesicles

As shown in Fig. 7, only two thin bands appeared at the very top in lane 1, which is characteristic of intact genomic DNA, whereas there were no bands whatsoever in lane 2 onto which DNA extracted from vesicles was loaded. The absence of a DNA ladder in either fraction reassures that the process of ectocytosis is different from the apoptotic process.

DISCUSSION

Our results show that fibroblastic ectocytosis might be a potential source of some autoantigen components of SSc and/or other systemic autoimmune diseases. By using the stress-relaxation model, more than 12 polypeptides recognizable by sera from patients with SSc are discharged by fibroblasts in the form of membrane-enclosed vesicles. By using immunoblotting and functional assay, two of those interacting autoantigens were identified as annexin I and topoisomerase I. Although annexin I is mainly localized in the submembrane cortex [10] and topoisomerase is a DNA-processing nuclear enzyme [17–19], both have been demonstrated to be SSc-associated autoantigens.

Ectocytosis is a slow, sublytic secretory process, probably designed for cells to export a special subset of polypeptides that lack the signal sequences required for secretion. The ectocytotic process bears a great resemblance to the release of viral particles from an infected cell. The morphological sequence begins with surface blebbing, cytoplasmic contraction, budding with connecting stalks, and pinching off of small membrane-bound vesicles with a diameter of 50–300 nm. In a typical transmission electron micrograph, structures of various intermediary steps, including freed membrane vesicles in the vicinity of cells, can be observed simultaneously. Similar phenomena have been reported to take place either spontaneously or inducibly by appropriate triggers both in vivo and in vitro in many types of cells. These cells include platelets [20, 21], neutrophils [8], fibroblasts [10, 22], myoblasts [9] and chondrocytes [23–25]. Under the name of matrix vesicles, the release of membrane vesicles has been most extensively studied in chondrocytes. Different physiological functions of these membrane vesicles have also been suggested.

Despite its physiological importance, ectocytosis has gone unnoticed for years. It is easily overlooked for a process at such a slow rate and the tiny vesicles it generates in vivo. It is also difficult to study the process in vitro, because all the vesicles released are

Fig. 6.—Neutralization of DNA topoisomerase I activity by vesicle contents indicating that topoisomerase I was one of the molecules present in the vesicles. The supercoiled pBR322 DNA (I) was used as control (lane 1) and the relaxed pBR322 DNA (II) was the product of the nicking–closing activity of DNA topoisomerase I added in lane 2. Lane 3 was the initial control with no DNA topoisomerase I or vesicle lysate added. The activity of DNA topoisomerase I is well preserved in vesicles, as shown in lane 4. Lysates from ~0.8 collagen gels were loaded onto lane 4.

Fig. 7.—Agarose gel electrophoresis of DNA obtained from scleroderma cells (lane 1; 4.5 x 10⁶ cells) and vesicles (lane 2; released from 24 collagen gels).
immediately highly diluted and washed away from the cells by the liquid medium. A three-dimensional, histotypic culture system of fibroblasts growing in collagen matrix provides an excellent in vitro model for studies of ectocytosis. Tension is built up in the collagen matrix as a result of the retractile force generated by migratory fibroblasts. In response to the tension, fibroblasts develop stress. Upon the relaxation of stress, a synchronized ectocytotic process is triggered among most fibroblasts within the collagen matrix. As pointed out in our previous study [10], the rate of synchronized ectocytosis is markedly speeded up to almost 100-fold that of fibroblasts in normal resting condition. Moreover, all the membrane vesicles released are entrapped and thus accumulated in the surrounding collagen lattice. This allows a significant amount of materials enclosed in membrane vesicles to be isolated for subsequent studies.

By taking advantage of the stress-relaxation model, we found more than 12 polypeptides interacting with sera from SSc patients are contained in the vesicles ectocytosed from scleroderma fibroblasts. The identities of two of these have been further established as annexin I and topoisomerase I. Different annexins, up to 12 members, consist of a family of calcium- and phospholipid-binding proteins implicated in mediating membrane-related processes such as secretion, signal transduction, membrane fusion and ion channel activity. Previously, Hirata et al. [13] and Goulding et al. [14] reported that autoantibodies against annexin I were detected in patients suffering from inflammatory or autoimmune diseases. Kraus et al. [15] also found that anti-annexin autoantibodies were detected in inflammatory, autoimmune disease and malignant melanoma patients. Misaki et al. [16] reported that anti-annexin XI autoantibodies are present in sera from patients with various autoimmune diseases, predominantly in sera from patients with rheumatoid arthritis, SLE, or SS.

Topoisomerase I is an enzyme that breaks and rejoins only one of the two DNA strands. It plays important roles in chromatin organization, mitosis, DNA replication, recombination and transcription [19]. Autoantibodies directed against DNA topoisomerase I in sera of patients with scleroderma have been described by several investigators [12–15]. In the present study, our finding of the presence of annexin I and topoisomerase I in ectocytosed vesicles from scleroderma fibroblasts as targeter autoantigens specifically by scleroderma and other autoimmune sera is consistent with other studies.

Before we can claim that ectocytosis is a possible alternative pathway for autoantigens to be exported, we need to double check that all the above-mentioned observations are different from the apoptotic process. In Fig. 7, we did not observe the biochemical feature, DNA fragmentation, of apoptosis. In our previous results, we did not see any of the morphological features of apoptosis either, such as explosive membrane blebbings or cytoplasm contraction [10]. In addition, those cells harvested from relaxed collagen gels maintain viability as high as 95% (data not shown). Therefore, we are confident that ectocytosis is different from apoptosis per se.

Taken together, our results provide the first evidence that autoantigens of different sources, one from cytosol, the other from the nucleus, can be exported simultaneously in the same vehicle: the ectocytotic vesicle. Our results also support the notion that, in addition to apoptosis, ectocytosis might be a potential source of autoantigen components. Further studies to verify the identities of the other interacting polypeptides present in the ectocytosed vesicles from scleroderma fibroblasts will reveal more insights and are under way in our laboratory.

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