Immunoglobulins from scleroderma patients inhibit the muscarinic receptor activation in internal anal sphincter smooth muscle cells

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Singh J, Mehendiratta V, Del Galdo F, Jimenez SA, Cohen S, DiMarino AJ, Rattan S. Immunoglobulins from scleroderma patients inhibit the muscarinic receptor activation in internal anal sphincter smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 297: G1206–G1213, 2009. First published September 24, 2009; doi:10.1152/ajpgi.00286.2009.—Systemic sclerosis (SSc) IgGs affecting the M3-muscarinic receptor (M3-R) have been proposed to be responsible for the gastrointestinal (GI) dysmotility in this disease. However, the effect of SSc IgGs on smooth muscle cell (SMC) function has not been studied. We determined the effect of SSc IgGs on the muscarinic receptor activation by bethanechol (BeCh; methyl derivate of carbachol) in SMC and smooth muscle strips from rat internal anal sphincter. IgGs were purified from GI-symptomatic SSc patients and normal volunteers, with protein G-Sepharose columns. SMC lengths were determined via computerized digital micrometry. The presence of M3-R and IgG-M3-R complex was determined by Western blot. IgGs from SSc patients but not from normal volunteers caused significant and concentration-dependent inhibition of BeCh response (P < 0.05). The maximal shortening of 22.2 ± 1.2% caused by 10−4 M BeCh was significantly attenuated to 8.3 ± 1.2% by 1 mg/ml of SSc IgGs (P < 0.05). Experiments performed in smooth muscle strips revealed a similar effect of SSc IgG that was fully reversible. In contrast to the effect on BeCh, the SSc IgGs caused no significant effect (P > 0.05) on K+ depolarization and α1-adrenoceptor activation by phenylephrine. Western blot studies revealed the specific presence of SSc IgG-M3-R complex. SSc IgGs attenuated M3-R activation, which was reversible with antibody removal. These data suggest that SSc Gl dysmotility may be caused by autoantibodies that inhibit the muscarinic neurotransmission. Future treatment of SSc patients may be directed at the removal or neutralization of these antibodies.

systemic sclerosis; rectoanal function; muscarinic receptor; autoantibodies

SYSTEMIC SCLEROSIS (scleroderma; SSc) is a systemic connective tissue disease characterized by extensive collagen deposition and sclerosis of the microvasculature and is accompanied by prominent alterations of the autonomic nervous system (14). As many as 90% of SSc patients have gastrointestinal (GI) symptoms (9, 13), usually involving the esophagus, and 50–70% may involve anorectum (21, 44). Diarrhea, constipation, fecal incontinence, and abdominal distension are commonly present in this patient population (30, 44).

Gastrointestinal dysmotility in SSc is believed to be neuropathic in etiology, with subsequent smooth muscle atrophy and fibrosis (9). The exact mechanism of neuropathic dysfunction is still unknown. Numerous autoantibodies have been found in the sera of SSc patients (14, 25).

A recent study has found a high incidence of antinuclear neuronal antibodies in the sera of SSc patients with GI symptoms (22). A follow-up study showed that passive transfer of these antibodies into a rat model significantly disrupts intestinal myoelectric activity (15), further supporting a neuropathic etiology to dysmotility in SSc patients. Similarly, paraneoplastic syndromes that cause GI dysmotility have also been associated with anti-Hu antibodies that target the myenteric plexus of GI smooth muscle (1, 28). The precise targeted neuronal antigen in these studies remains to be determined.

Functional autoantibodies in SSc have been identified that specifically inhibit animal colonic smooth muscle (7, 19). These studies have shown that the SSc IgGs inhibit the smooth muscle contraction caused by carbachol-induced activation of M3-R. The data were interpreted to indicate that M3-R antibodies from the SSc patients’ sera may lead to failure of the cholinergic neurotransmission and, in turn, result in GI motility dysfunction.

Goldblatt et al. (19) showed that serum from patients with SSc inhibited smooth muscle contraction in vitro. In another study, experimentally generated antibodies against the M3-R mimicked functional autoantibodies found in Sjögren syndrome (7).

The purpose of the present study was to characterize autoantibodies present in the serum of SSc patients that may recognize functional components of smooth muscle cell (SMC). Delineation of an antibody-mediated cause of GI dysfunction in SSc would be a major step in understanding the pathogenesis and possible treatment of this progressive and often fatal condition.

The specific aims of the study are 1) to characterize the effect of SSc IgGs on the muscarinic receptor activation in the internal anal sphincter smooth muscle cell, 2) to determine the specificity of this antibody response, and 3) to determine its potential for reversibility.

MATERIALS AND METHODS

Subjects. Seven patients, all female, ages 37 to 83 with initial cutaneous SSc as described by LeRoy et al., were studied. The study was approved by the University’s Institutional Review Board. All patients fulfilled the criteria for SSc classification of The American College of Rheumatology. Disease duration varied from 4 to 42 years. All patients had the classic esophageal manometric features of SSc. Two patients had fecal incontinence.

Isolation and purification of IgGs from SSc patients and normal volunteers. Total IgGs were purified from plasma from all SSc patients and from two normal volunteers, by use of recombinant protein G-Sepharose 4B conjugate (Zymed Laboratories, San Fran-
SSc IgGs ATTENUATE MUSCARINIC TRANSMISSION

To determine the selectivity of the action of SSc IgG on M3-R activation, we compared the effects of K+ depolarization by KCl (2.5 to 40 mM) and α1-adrenoceptor (α1-A) agonist phenylephrine (10^{-9} to 10^{-3} M).

M3-receptor identification using Western blot analyses. Dual-color infrared scanning of Western blots was used to determine the presence of M3-R and its possible association with the IgGs from SSc patients vs. normal individuals. M3-R receptor antibody from Santa Cruz Biotechnology (Santa Cruz, CA) was used for the identification of M3-R in the rat IAS. The details for the preparation of rat tissue lysates and other details for the Western blots have been described before (37). Briefly, 30 μg of the tissue lysate proteins in 30 μl were mixed with 2× Laemmli sample buffer (with final concentrations 62.5 mM Tris, 1% SDS, 15% glycerol, and 0.005% bromophenol blue, and 2% mercaptoethanol) and placed in a boiling water bath for 5 min and separated by SDS-PAGE (7.5%) as described before (38). The separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane at 100 V for 1 h at 4°C. To block membrane and antibody binding, the membranes were soaked for 1 h at room temperature in LI-COR blocking buffer (LI-COR Biotechnology, Lincoln, NE). A portion the membrane (containing lanes 2 to 7) was incubated with the IgG purified from the SSc patients whereas the other portion (containing lanes 8 and 9) was incubated with the normal IgGs. These IgGs (SSc or normal; 2 mg/μl; diluted in LI-COR buffer containing 0.1% Tween 20) served as the primary antibodies for overnight incubation at 4°C as previously described (35). M3-R antibody was added to all the membranes as positive control, as described before (26). After washing three times with Tris-buffered saline with Tween (TBS-T; 10 min each wash), the membranes were incubated with the IRDye-conjugated secondary antibodies (1:4,000 dilution anti-human IgG-IRDye 680 from Rockland, Gilbertsville, PA and anti-goat IgG-IRDye 800 from LI-COR). The membranes were washed again three times with TBS-T (10 min each wash) and finally kept in PBS buffer for 10 min with constant shaking at room temperature. The membranes were finally scanned with an LI-COR Odyssey Imaging System infrared scanner, and relative densities of M3-R and human IgG-M3-R complex were determined by line profile analysis via Image-Pro Plus 4.0 (Media Cybernetic).

Drugs and chemicals. BeCh was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Darifenacin and 4-DAMP were generous gifts from Pfizer Central Research (Sandwich, Kent, UK) and from Dr. R. B. Barlow (Department of Pharmacology, University of Bristol Medical School, Bristol, UK), respectively.

Statistical analysis. Data are presented as means ± SE of multiple experiments. P values less than 0.05 were considered statistically significant. The concentration-response curves (CRC) were fitted by nonlinear regression using the computer software Prism (GraphPad Software, San Diego, CA). E_{max} (maximum contraction) and pEC50 (negative logarithm of the concentration producing the half-maximum effect) values were calculated from the fitted curves, while PA2 (negative log concentration of the antagonist producing twofold increase in the EC50 for BeCh) values were calculated by Schild plot.

RESULTS

Influence of the selective M3-R antagonist darifenacin on the effect of BeCh on the IAS SMC. BeCh caused concentration-dependent decrease in the SMC lengths (Fig. 1). To determine whether BeCh-induced shortening of the IAS SMC is via M3-R activation, we examined the effect of the M1-R antagonist darifenacin (3, 23). Darifenacin (1 × 10^{-9}, 3 × 10^{-9}, and 1 × 10^{-8} M) caused concentration-dependent rightward parallel shift in the BeCh CRCs. Data reveal competitive antagonism of BeCh CRC with darifenacin, a concentration-dependent increase in the EC50 of BeCh without significant (P > 0.05) changes in the maximal effect (E_{max}). In control experiments,
values for the EC50 of BeCh in control and following three different concentrations of darifenacin were 4.0 \times 10^{-6}, 7.0 \times 10^{-6}, 1.0 \times 10^{-5}, \text{ and } 6.0 \times 10^{-5} \text{ M, respectively (Fig. 1). The pA2 value of darifenacin in antagonizing the responses of BeCh in the IAS SMC was 8.8. These data justify the use of BeCh as the M3-R selective agonist for the present investigation.}

**Effect of SSc vs. normal IgGs on BeCh-induced contraction of the SMC.** It has been shown before that SSc IgGs attenuate the effect of M3-R activation by carbachol in the murine colonic smooth muscle cells (SMC). A: percent decrease in the SMC lengths. B: percent maximal decrease in the SMC length. Darifenacin competitively and significantly antagonizes BeCh-induced decrease in rat IAS SMC (n = 5). These data suggest that BeCh causes shortening of the SMC by M3-R activation.

IgG obtained from SSc patients caused significant and concentration-dependent attenuation of BeCh-induced CRC in the IAS SMC (*P < 0.05; n = 5; Fig. 2A). These data were reproducible in all patients examined. A concentration of 1 mg/ml of SSc IgG attenuated BeCh (1 \times 10^{-4} \text{ M})-induced shortening of the SMC from 22.7 ± 0.45 to 9.2 ± 0.27% (*P < 0.05). However, IgGs from normal volunteers (22.4 ± 0.44) had no significant effect (P > 0.05). The data show an increase in the EC50 and a decrease in the ECmax with BeCh following different concentrations of SSc IgGs, suggesting that the possible effect of SSc IgGs on the M3-R activation by BeCh as a substitute for carbachol.

The data show that systemic sclerosis (SSc; *P < 0.05; n = 5) but not normal (P > 0.05) IgGs cause concentration-dependent antagonism of the BeCh concentration-response curves (CRC) in the IAS SMC. This effect of SSc IgGs may be nonselective inactivation of M3-R since it resembles the noncompetitive but significant (*P < 0.05) antagonism by 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP) shown in B.
nonselective inhibition by the SSc IgGs resembles that with the nonselective muscarinic antagonist 4-DAMP (Fig. 2B).

Figure 3A provides typical examples of the changes in lengths of the IAS SMC following different concentrations of normal and SSc IgG and IgGs. Figure 3B shows quantitative data on the actual SMC lengths before and after different concentrations of the IgGs. These data suggest that SSc IgGs attenuate M3-R activation caused by BeCh.

Effect of the nonselective M3-R antagonist 4-DAMP on M3-R activation by BeCh in the IAS SMC. 4-DAMP caused significant and concentration-dependent rightward shift in the BeCh CRC in the IAS SMC. However, this shift in the BeCh CRC in the IAS SMC was characteristic of nonselective antagonism, with increase in the EC50 without achieving the Emax comparable to the control experiments. The EC50 values for BeCh in the control and three different concentrations of 4-DAMP (1 × 10⁻⁹, 3 × 10⁻⁹, and 1 × 10⁻⁸ M) were 1.5 × 10⁻⁶, 4.5 × 10⁻⁶, 6.2 × 10⁻⁶, and 4.0 × 10⁻⁵, respectively. The Emax of the IAS SMC in control experiments (with BeCh 1 × 10⁻⁸ M) was 25.1 ± 2.0% and decreased significantly in a concentration-dependent manner in the presence of 3 × 10⁻⁹ and 1 × 10⁻⁸ M 4-DAMP (*P < 0.05; Fig. 2B).

Effect of SSc vs. normal IgGs on the SMC contraction by KCl (depolarizing agent) and phenylephrine (α1-adrenoceptor agonist). To determine the selectivity of BeCh-mediated M3-R inactivation by the SSc IgG on the IAS SMC, we compared the effects of K⁺ depolarization and the α1-adrenoceptor (α1-AR) agonist phenylephrine. Interestingly, in contrast with the attenuation of BeCh-induced effect, neither KCl nor phenylephrine-induced shortening of the IAS SMC was significantly modified either by SSc or normal IgGs (P > 0.05; Fig. 4; n = 4–6). These data suggest selective attenuation by the SSc IgGs of the muscarinic receptor.

Effect of SSc vs. normal IgGs on M3-R activation in the IAS tone. To determine the effect of the SSc IgGs on M3-R activation in the intact smooth muscle, we examined its effect on BeCh-induced increase in the IAS tone. Such experiments were limited to the effects of 0.3 mg/ml SSc IgG because of the limitation of the SSc IgGs available. Data show that in agreement with the effect in the SMC, the IgGs from SSc but not from normal volunteers caused rightward shift in the BeCh-induced CRC in the intact smooth muscle strips (*P < 0.05; n = 3; Fig. 5A) in a noncompetitive manner. These experiments offer a distinct advantage in determining the reversal effect following the IgG washout. The effect of SSc IgGs was reversible on rigorous washing (from 10 to 12 washes) so that the reversal values were not significantly different from the controls (P > 0.05; Fig. 5).

Association of human IgG with the M3-R in the SSc IgGs. A dual-color infrared LI-COR Odyssey Imaging System was used to determine an interaction between SSc IgGs and M3-R. The blots containing the rat tissue lysates from the IAS were simultaneously incubated with two different primary antibodies as follows: rat M3-R (as a positive control from Santa Cruz Biotechnology) and SSc IgGs from SSc patients. Secondary antibodies conjugated with two different fluorophores IRDye 680 (red) against rat (Fig. 6A) and IRDye 800 (green) (Fig. 6B) against human IgGs were used to determine the presence and binding location of the M3-R and the human IgG on the blots.

The binding of their respective antibodies at the same position in correspondence with M3-R receptor in SSc patients is expected to result in yellow fluorescence as a consequence of merging of red and green fluorescence from the respective secondary antibodies. Such merging was observed only in the case of SSc IgGs and not in the normal IgGs (Fig. 6C), suggesting an interaction of SSc IgGs with M3-R. Prestained Protein Molecular marker (New England Biolabs, Ipswich, MA) was loaded in the first lane to be visualized both by 700 and 800 infrared channels.

DISCUSSION

These data for the first time provide direct evidence that IgGs isolated from SSc patients specifically interfere with the muscarinic receptor activation in SMC and intact smooth muscle of the IAS. These findings have significant implications in the pathophysiology and therapeutic approaches in SSc patients.

Fig. 3. A: IAS SMC lengths in the basal state and following the highest concentration of the IgG from normal vs. different concentrations of the SSc IgGs. Data show a significant attenuation of BeCh responses in the presence of SSc but not normal IgGs (*P < 0.05; n = 5). Right: actual pictures of the SMC lengths showing the effect of BeCh before and after SSc IgGs in different concentrations (0.3, 0.6, and 1.0 mg/ml). B: quantitative data on the length of the SMC before and after different concentrations of the SSc IgGs alone.
Data suggest that the IgGs isolated from SSc patients (but not from normal volunteers) attenuate M_3-R activation by BeCh in freshly isolated SMC of the IAS. Data from the isolated smooth muscle strips are complementary to the SMC data. The concept of M_3-R inactivation in the IAS SMC by SSc IgGs is supported by the former studies in the murine colonic tissues by Cavill et al. (6). To implicate the site of SSc IgGs M_3-R inactivation at the level of SMC, rather than examining the effect directly at SMC, the investigators examined the effect of carbachol in the colonic tissues in the presence of different neurohumoral antagonists.

The significance of M_3-R at the SMC membrane of the GI tract in the pathophysiology and therapy of the GI disorders is well known (17, 20, 25, 36, 45). This has been confirmed by using animals with targeted deletion of the M_3-R gene (16, 46). Therefore, the effect of M_3-R attenuation by SSc IgGs in the colonic tissues may explain the underlying pathophysiological mechanisms of the intestinal manifestations of SSc (9, 14). The effect of SSc IgGs on the anorectum, a region of specific interest in the anorectal motility dysfunction in SSc, however, has not been investigated before.

Studies examining the effect of methacholine (acetyl-β-methylcholine) (4), another muscarinic agonist, in the human esophagus revealed the preservation of the effect of that agonist, whereas the effect of agents targeting the nerves was severely affected (10). There are different possibilities for these observations. First is the attenuation by the SSc IgGs of predominantly M_3-R agonists such as carbachol and bethanechol; methacholine in contrast primarily activates M_1-R (40) present not only on the SMC but also on the intrinsic nerves. Secondly, differences in the progression of the disease in two groups of patients selected, either in terms of anatomical progress (esophagus vs. large intestine) or in terms of the neuropathic vs. the myopathic stages of the disease, may explain the disparity.

In the present studies, we used BeCh in place of carbachol because of the preferential effect at M_3-R in the smooth

Fig. 4. Effect of SSc IgG and normal IgG on KCl depolarization (A)- and phenylephrine (α_1-adrenoreceptor agonist) (B)-induced shortening of the IAS SMC lengths. Data show that neither the SSc nor the normal IgGs have any significant effect on the effect of KCl or phenylephrine (P > 0.05; n = 4–6).

Fig. 5. Effect of SSc IgG and normal IgGs on the increase in the IAS tone (in the smooth muscle strips), caused by BeCh. In agreement with the data in the SMC, in the smooth muscle strips also, the IgGs from the SSc patients (A) cause significant rightward shift (*P < 0.05; n = 3) in the BeCh CRC. The IgGs from the normal volunteers (B) on the other hand have no significant effect (P > 0.05). Additionally, the effect of SSc IgG on BeCh response was reversible upon washing.
muscles of the GI tract (4, 47). In the IAS, carbachol produces primarily relaxation of the smooth muscle (24), perhaps via the activation of nicotinic receptors at the nonadrenergic, noncholinergic inhibitory neurons (4, 5, 8, 12). Additionally, our data via the use of the selective muscarinic antagonist darifenacin suggest the preferential effect of BeCh at the M3-R.

Although it is not possible to completely rule out the nonspecific effects of SSc IgGs on M3-R inactivation, the data with K+ depolarization and α1-AR activation data point toward the selectivity of action. SSc IgGs attenuate the effect of BeCh, but not those of K+ depolarization and phenylephrine (α1-AR agonist). The possibility of patient medication interfering with the M3-R activation is unlikely because none of these medications used in these patients is known to interfere with the M3-R. In addition, the suppressant effect of SSc IgGs in the IAS smooth muscle was found to be reversible. These observations suggest the possibility of neutralization of deleterious effects of the SSc IgGs following their removal or inactivation in the SSc patients. Whether attenuation of M3-R in the IAS SMC by the SSc IgGs is coincidental, is the precursor to the disease, or is a result of the smooth muscle atrophy accompanied with the fibrosis of the smooth muscle is not known.

The present data provide direct support to the hypothesis that autoantibodies targeting GI structures (neuronal and/or smooth muscle) contribute to the clinical manifestations of SSc (14). The exact mechanism of this M3-R inactivation is not known. There is evidence to suggest that these autoantibodies block cholinergic neurotransmission at the G protein-coupled M3-R, based on radioligand binding and functional studies in the colon (6, 19). It has been suggested that the second extracellular loop of M3-R may be an epitope for the IgGs as reported in the case of Sjögren’s syndrome, another systemic autoimmune disorder (31, 39). On the basis of these concepts (39), intravenous infusion of IgG (which works via the neutralization of SSc IgGs) has been used in the symptomatic treatment of SSc (6).

The observed inhibitory effect of SSc IgGs possibly on M3-R in the IAS SMC has wide implications in the GI smooth muscle dysfunction in SSc. Once activated, M3-R may initiate the smooth muscle contraction via the influx of Ca2+ and its release from the sarcoplasmic reticulum via inositol 1,4,5-trisphosphate. This activates the Ca2+/calmodulin/MLCK pathway, leading to the phosphorylation of 20-kDa myosin light chain (p-MLC20). The maintenance of the initiated...
smooth muscle contraction in response to M$_3$-R activation, however, may depend largely on the PKC and Rho kinase (RhoA/ROCK) pathways (18, 33, 34, 43). Both of these pathways converge in inhibiting myosin light chain phosphatase. This prevents the dephosphorylation of p-MLC$_{20}$. It remains to be conclusively determined whether the observed inhibitory effect of the muscarinic receptor activation is primarily at the receptor or it is the result of the downstream signal transduction dysfunction. The association of SSc IgGs with the M$_3$-R (Fig. 6) suggests the effect at the receptor, with otherwise intact SMC function.

The relevance of the SSc IgGs-mediated attenuation of the muscarinic receptor in the basal tone and neurotransmission in the IAS is not known at the present time. Initial studies using lower concentrations of the SSc IgGs induced no significant effect on basal IAS tone. A major limitation in such studies is the lack of knowledge of the exact concentrations of the IgGs circulating in the IAS region of SSc patients. Such information may provide appropriate guidelines for selecting the precise concentrations for future studies. Alternatively, despite attenuation of M$_3$-R activation, functional antagonism of M$_3$-R may be compensated by the redundant mechanisms, as was the case in the targeted deletion of M$_3$-R (32).

In conclusion, the studies reveal that IgGs from SSc patients attenuate the muscarinic receptors in the IAS SMC. These data have significant implications in the pathophysiology of rectoenteric smooth muscle. They may provide appropriate guidelines for selecting the precise concentrations of the SSc IgGs for future studies. Alternatively, despite attenuation of M$_3$-R activation, functional antagonism of M$_3$-R may be compensated by the redundant mechanisms, as was the case in the targeted deletion of M$_3$-R (32).

DISCLOSURES

No conflicts of interest are declared by the author(s).

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