Role of Muscarinic-3 Receptor in Systemic Sclerosis: Correlation with Disease Duration and Effects of IVIG

Short Title: SSc duration and cholinergic transmission

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Abbreviations Used in this Paper: Ach, acetylcholine; BeCh, bethanechol; EFS, electrical field stimulation; ELISA, enzyme-linked immunosorbent assay; IFI, immunofluorescence intensity; IHC, immunohistochemistry or immunohistochemical; IgG, immunoglobulin; IVIG, pooled human immunoglobulin for intravenous use; KPS, Krebs physiological solution; NIgG, IgGs from normal subjects; M₃-RL2, second extracellular loop peptide of M₃-R; F(ab′)₂, antigen-binding fragment of IVIG; SM, smooth muscle; SMC, smooth muscle cells; MCN, myenteric cholinergic neurons, MP, myenteric neuronal plexus; SSc, systemic sclerosis or scleroderma; SScIgG, IgGs from scleroderma patients;; TMB, 3,3′,5,5′-tetramethylbenzidine

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Authors’ Contribution to the Manuscript: SK, JS in human blood samples' collection, data acquisition, analysis and interpretation; and statistical analysis; AD, SC, RK and SAJ in the enrolment and follow up of scleroderma patients; FM, SJ in the isolation and purification of SScIgGs; EB for assistance in immunohistochemistry studies; and SR in funds procurements, concept and design, interpretation of data, and study supervision

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Gastrointestinal dysmotility in Systemic Sclerosis (SSc) is associated with autoantibodies against muscarinic-3 receptor (M₃-R). We investigated the temporal course of the site of action of these autoantibodies at the myenteric neurons (MN) vs. the smooth muscle (SM) M₃-R in relation to disease duration, and determined the role of intravenous immunoglobulin (IVIG) in reversing these changes. Immunoglobulins purified from SSc patients (SScIgG) were used to assess their differential binding to MN and SM (from rat colon) employing immunohistochemistry (IHC). Effect of SScIgG on neural and direct muscle contraction was determined by cholinergic nerve stimulation and bethanechol-induced SM contraction. Effects of IVIG and its antigen-binding fragment F(ab’)₂ on SScIgG binding were studied by Enzyme-linked immunosorbent assay (ELISA) of rat colonic longitudinal SM myenteric plexus (LSMMP) lysate and to second extracellular loop peptide of M₃-R (M₃-RL2). SScIgG from all patients demonstrated significantly higher binding to MN than to SM. With progression of SSc duration, binding at MN and SM increased in a linear fashion with a correlation coefficient of 0.696 and 0.726, respectively (P< .05). SScIgG-mediated attenuation of neural and direct SM contraction also increased with disease duration. ELISA analysis revealed that IVIG and F(ab’)₂ significantly reduced SScIgG binding to LSMMP lysate and M₃-RL2. Dysmotility in SSc occurs in two sequences, beginning with SScIgG-induced blockage of cholinergic neurotransmission (neuropathy), which progresses to inhibition of acetylcholine action at the SM cell (myopathy). IVIG reverses this cholinergic dysfunction at the neural and myogenic receptors by anti-idiotypic neutralization of SScIgG.

**Keywords:** Scleroderma Autoantibodies; Muscarinic Receptor; Smooth Muscle; Myenteric Neuron
Systemic Sclerosis (SSc) is a systemic autoimmune disease characterized by skin and internal organ fibrosis, vasculopathy and immune dysregulation. Among the target organs affected by SSc, the gastrointestinal tract (GIT) is the most commonly affected internal organ. While dysmotility accounts for the vast majority of SSc-associated GIT symptoms, its pathogenesis is poorly understood (22, 26).

Recent advances in SSc pathogenesis have implicated immune dysregulation, vascular dysfunction and fibrosis as the unifying mechanism of internal organ involvement (10). Among other factors, the lack of appropriate animal models reproducing gastrointestinal manifestations of SSc has limited our understanding of the pathophysiological mechanism of dysmotility and has also hampered the development of new therapies (28).

Humoral immunity dysregulation has been recognized to play an important role in SSc pathogenesis. However, despite the fact that autoantibodies are present in more than 95% of patients with SSc, they were traditionally considered to be non-pathogenic. It is now hypothesized that anti-endothelial, anti-fibroblast, anti-MMP, and anti-fibrillin antibodies may be directly pathogenic in SSc (17). It has recently been demonstrated that IgG isolated from sera of SSc patients, targets vascular smooth muscle cells and may be responsible for pulmonary hypertension (4). Similarly, research in the last decade has shown that gastrointestinal dysmotility in SSc may in part be related to functional autoantibodies (8, 11).

Earlier studies from our laboratory have demonstrated that gastrointestinal dysmotility in SSc is associated with circulating autoantibodies against the muscarinic-3 receptor (M_3-R) (24, 25). These autoantibodies inhibited the contraction of smooth muscle cells (SMC) directly stimulated with a cholinergic agent and also blocked indirect muscle response
induced by electric field neural stimulation suggesting cholinergic blockade by M₃-R inactivation at neural and muscular levels. Of significant interest, the neural and myogenic effects of these autoantibodies were reproducibly abrogated by intravenous immunoglobulin (IVIG) strongly suggesting that the antibody could be removed from the receptor or could be neutralized in vitro (24, 25).

None of the earlier studies, however, examined the temporal sequence of neurogenic or myogenic site involvement, or investigated whether this involvement correlates with duration or severity of gastrointestinal SSc. Although treatment with IVIG has been studied in tight skin mouse models and in patients with cutaneous manifestations of SSc (1) there are no data to indicate whether IVIG would be able to restore gastrointestinal dysfunction in SSc patients at different stages of the disease.

In this study, we tested the hypothesis that SScIgG initially leads to neuropathy via inhibition of M₃-R at the myenteric cholinergic neurons (MCN) which progresses to myopathy by inhibition of M₃-R at the gastrointestinal SMC in the advanced stages of SSc.

The aims of the present study were: 1). To investigate the effect of SScIgG at different stages of SSc (defined by duration of disease) by comparison of their sites of action at the MCN vs. the SMC; and 2). To determine the role of IVIG in reversing SSc gastrointestinal manifestations, and identify its mechanism of action.

MATERIALS AND METHODS

Subjects

Ten patients meeting the 2013 American College of Rheumatology criteria for the classification of SSc were selected retrospectively (29). Patients were included in the study
if they had documented gastrointestinal symptoms attributable to SSc (determined by the UCLA SCTC GIT 2.0) along with typical esophageal manometric abnormalities. Patients on immunosuppressive and disease modifying drugs were excluded from the study. Patients were divided into two groups based on duration of SSc (skin manifestation was used to define the disease onset date). Patients with disease duration ≤ 15 years (180 months) were placed in Group I, while those in Group II had a disease duration ≥ 16 years (192 months).

Medical records for all participants were obtained to verify the diagnosis and to characterize the disease. We collected the following information about SSc patients: date of first diagnosis, extent of skin involvement (limited versus diffuse), medication regimen, SSc specific autoantibody profile, gastrointestinal symptoms, esophageal manometry findings and UCLA SCTC GIT 2.0 score (18). The study was approved by the University's Institutional Review Board.

**Isolation and purification of IgGs from SSc patients and normal volunteers**

After obtaining informed consent total IgGs were purified from plasma of the 10 SSc patients (SScIgGs) and 2 normal volunteers (NlGGs) by the use of biocompatible polypropylene columns packed with 5 ml of Protein G Sepharose from genetically modified protein G lacking albumin affinity and with high binding capacity for human IgG (Hi-trap protein G HP- GE Healthcare, Pittsburgh, PA) (13).

**Experiments Using Intact Rat Colon Smooth Muscle (SM) Strips**

*Colonic smooth muscle strip preparations and isometric tension recording*
Male Sprague–Dawley rats (300–350 g) were euthanized by decapitation, and the lower portion of the colon was removed surgically and transferred to oxygenated (95% O$_2$ + 5% CO$_2$) Krebs physiological solution (KPS) at 37°C. The composition of KPS (in mmol/L) was as follows: 118.07 NaCl, 4.69 KCl, 2.52 CaCl$_2$, 1.16 MgSO$_4$, 1.01 NaH$_2$PO$_4$, 25 NaHCO$_3$, and 11.1 glucose. The SM strips (~0.5 mm thick and 7 mm long) were prepared from the circular SM layer of the colon.

The SM strips were then transferred to 2-mL organ baths containing oxygenated KPS. Isometric tension was monitored by use of force transducers (FORT10, WPI, Sarasota, FL) and Chart 4.1.2 via PowerLab/8SP data-acquisition system (AD Instruments, Colorado Springs, CO). Each SM strip was initially stretched to a tension of 1.0 g followed by 90 min of equilibration. The basal tone in each SM strips was determined at the end of the experiment by the administration of Ca$^{2+}$-free (0Ca$^{2+}$) KPS. The experimental protocol of the study was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

**Direct muscle stimulation with bethanechol (BeCh)**

The responses of the colonic SM strips to BeCh ($10^{-7}$ to $10^{-3}$ M) were quantified before and after NlgG (1mg/ml), SScIgG (1 mg/mL), IVIG (10 mg/mL), and IVIG (10 mg/mL) + SScIgG (1 mg/mL) after incubation in the muscle bath for 15 min. To determine the selectivity of the action of SScIgG on M$_3$-R inactivation, we compared the effects of K$^+$ depolarization by KCl (2.5 to 40 mM). These contractile responses were calculated and plotted as percentage of maximal contraction by $10^{-4}$ M BeCh.
**Cholinergic nerve stimulation experiments**

To determine the effects of cholinergic nerve stimulation, electrical field stimulation (EFS; 10 V, 0.5–20 Hz, 4-sec train, each pulse of 0.5 ms) was delivered using a Grass stimulator (model S88; Grass Instruments, Co, Quincy, MA). The EFS responses (percentage maximal increase in the basal activity) were quantified before and after NlgG (1mg/ml), SSclG (1 mg/mL), IVIG (10 mg/mL), and IVIG (10 mg/mL) + SSclG (1 mg/mL).

**Acetylcholine (ACh) measurements**

To measure the release of ACh in response to EFS, we determined the effect of 30-sec train of EFS (10 V, 5 Hz, each pulse of 0.5 ms duration). The muscle bath perfusates were collected in the basal state, and before and after NlgG (1mg/ml), SSclG (1 mg/mL), IVIG (10 mg/mL), and IVIG (10 mg/mL) + SSclG (1 mg/mL). ACh measurements were made using the choline/ACh quantification kit (BioVision, Milpitas, CA) following the manufacturer’s instructions. Data were quantified using fluorometric (Fluorometer Optima Micropipette Reader; BMG Labtech, Ortenberg, Germany) analysis with MARS software (Cary, NC).

**Immunohistochemistry (IHC) of Rat Colon**

**Whole mount and sections**

Rats were euthanized by decapitation and colon was removed immediately and cleaned with 4% paraformaldehyde and kept in it overnight. Paraformaldehyde was then replaced with 70% ethanol and tissues were sent for embedding and sectioning to Thomas Jefferson University Histopathology Core facility. Sections were deparaffinized by keeping them for
30 min at 70°C and washed two times (10 min each wash) in xylene. Sections were hydrated in 2 changes of 100% ethanol for 5 min each, followed by 2 changes of 95% ethanol, 1 change of 80% ethanol, and 2 changes of 70% ethanol for 3 min each. Sections were then rinsed in 2 changes of distilled water for 5 min each. Antigen retrieval was done in Tris Buffered Saline containing tween-20 (0.05M TBS, 0.05% Tween 20, pH 9.0) in pressure cooker for 30 min. Slides were washed twice with distilled water and stained with cuprolinic blue for 30 min (15). Slides were again washed with distilled water twice and blocked with donkey serum in PBST for 1h. SScIgGs and Anti-M₃-R against rat were added on the sections in PBST and kept in humidified chamber at 4°C for overnight incubation. Then, the slides were washed with PBST and stained with anti-human IgG and Anti-goat IgG in PBST buffer and incubated at room temperature (RT) for 1h and washed three times with PBST and fixed with Vectashield mounting media (Burlingame, CA) and viewed under confocal microscope (Carl Zeiss, Germany) as well as an Evos fluorescent microscope (Life technologies) and photographs were taken and analyzed with ImageJ2 (NIH, USA).

**Immunofluorescence intensity calculation**

Immunofluorescence intensity (IFI) was calculated by using ImageJ2 (NIH). Areas of interest from the images were selected corresponding to the myenteric plexus and smooth muscle and intensity surface plots were plotted and intensity per unit area was calculated by dividing total intensity by total area selected. For co-localization studies Pearson coefficient of co-localization was calculated by using Col2 plugins and plotted as bar graph in prism software.
Isolation of longitudinal smooth muscle-myenteric plexus (LSMMP) layer

Rat colon was removed proximal to the sigmoid colon and divided into 3-4 cm segments. The lumen of these segments was flushed with KPS. These segments were then stretched over a glass rod and the mesentery carefully removed. The longitudinal muscle layer with adherent myenteric plexus was separated from the underlying circular muscle layer by gently stroking tangentially away from the mesenteric attachment with a cotton-tipped swab and peeling away the mucosal, sub-mucosal and circular SM layers (2). LSMMP was used for IHC, and its lysates prepared under fresh conditions were used for ELISA.

Enzyme-linked immunosorbent assay (ELISA)

To determine the binding affinity of SScIgG to the M3-R we performed ELISA studies with M3-RL2 synthetic peptide and LSMMP lysate. 400 µg/ml rat colonic LSMMP lysate and 400pg/ml of a peptide corresponding to the M3-RL2 (KRTVPGEFCIQFLSEPTITFGTAI, amino acids 213–237) were separately dissolved in carbonate buffer and adsorbed onto separate multiwell plates used for ELISA. 5-40 nM each of SScIgG, IVIG, F(ab')2 and SScIgG + IVIG were added to these multiwell plates and incubated at 37°C for 1h. Plates were washed three times with DPBST and incubated for 1h at RT with anti-human-HRP-conjugated secondary antibodies for SScIgG, or IVIG, washed three times with DPBST and 100 µl of TMB substrate was added and kept for 15 min at RT. Then, 100 µl of Stop solution was added to each well and absorbance was recorded at 450 nm with ELISA reader.

DRUGS AND CHEMICALS

Bethanechol, KCl, and M3-R antibody were purchased from Sigma Aldrich (St. Louis, MO). M3-R loop-2 (M3-RL2) was purchased from Peptide 2.0 Inc. (Chantilly, VA). IVIG
(Privigen®) and its pepsin-derived antigen-binding F(ab')₂ fragment were prepared as previously described (16) and obtained from CSL Behring (King of Prussia, PA).

**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 were fitted by nonlinear regression, and comparisons were made using unpaired student t test, or ANOVA, using the computer software Prism (GraphPad Software, San Diego, CA). Pearson's coefficient was determined to assess correlation between binding and disease duration.

**RESULTS**

**Characteristics of study participants**

All patients were females aged between 38-73 years (Table 1). Amongst all patients, 6 had diffuse cutaneous involvement, and 4 had limited disease. The mean ± SD disease duration of patients in Group I was 141.6 ± 35.4 months, while that of patients in Group II was 290.4 ± 95.4 months. Their gastrointestinal symptom score as determined by the UCLA SCTC GIT 2.0 questionnaire ranged from 0.75-15.1. No significant correlation was found amongst various SSc specific autoantibodies and gastrointestinal disease.

**Binding of SScIgG to myenteric plexus (MP) and smooth muscle (SM)**

Myenteric plexus in whole mount rat colonic sections was identified by selective staining with neuronal dye cuprolinic blue. SScIgG from all ten patients demonstrated positive staining with rat colonic myenteric plexus and smooth muscle (Fig. 1A). Immunofluorescence intensity (IFI) analysis revealed that the binding intensity of SScIgG to myenteric plexus was significantly higher (IFI of SScIgG bound to MP was considered as
100%) in contrast to smooth muscle immunofluorescence intensity, which was less than 50% (Fig. 1B; P < .05; n = 3). These data reveal that SSclG binds at two sites in the rat colon: myenteric plexus and the smooth muscle, but with higher binding intensity to the former.

**Co-localization of SSclG and M$_3$-R on MP and SM**

Data shows that SSclG binds specifically to the M$_3$-R on rat colonic SM and myenteric plexus (Fig. 1C). IFI analysis showed a significant co-localization of M$_3$-R and SSclG binding at the colonic myenteric plexus and smooth muscle (correlation coefficient 0.70 and 0.85 respectively) (Fig. 1D; P < .05; n = 3). This binding of SSclG at the same position corresponding with M$_3$-R antibody binding, suggests that SSclG binds at the M$_3$-R on both the smooth muscle and myenteric plexus.

**Disease duration-dependent binding of SSclG at MP and SM**

Data reveal that IgG from a patient in Group I (disease duration: 120 months) had significantly higher binding at the myenteric plexus compared to binding at the smooth muscle (IFI 0.36 vs 0.27 respectively)(Fig. 2A; P < .05; n = 3). In contrast, IgG from a patient in Group II (disease duration: 420 months), revealed significantly higher binding at the both the myenteric plexus and smooth muscle (IFI 0.53 vs 0.50 respectively), with binding intensity at the latter almost approaching the binding intensity at the myenteric plexus (Fig. 2A; P < .05; n = 3).

The IFI of SSclG binding, at myenteric plexus and smooth muscle from all patients when plotted against duration of disease, revealed a strong positive correlation coefficient of R = 0.7, P<.05 and R = 0.8, P <.05 respectively (Fig. 2B). Data suggest that binding of
SScIgG at myenteric plexus and smooth muscle increases with the duration of disease in a linear fashion accounting for the progressive nature of the disease.

**Effect of IVIG on SScIgG binding**

IVIG reversed the binding of SScIgG at both the myenteric plexus and smooth muscle as evidenced by the decrease in IFI (Fig. 2C, D; \( P < .05; n = 3 \)). This reversal effect was consistently observed with SScIgG from all ten patients.

**Effect of SScIgG on cholinergic nerve stimulation in intact rat colonic SM**

EFS caused a frequency-dependent increase in the contraction of the colonic smooth muscle. The data further revealed that SScIgG (1 mg/ml) and not IVIG (10 mg/ml) by itself, caused inhibition of EFS-induced smooth muscle contraction. Moreover SScIgG from a patient in Group II (disease duration: 420 months) nearly obliterated the EFS-induced response to 80%, compared with the effect of the SScIgG from a patient in Group I (disease duration: 120 months) which caused only 30% inhibition of EFS response (Fig. 3A, B; \( P < .05; n = 4 \)). This effect of SScIgG was partially reversed when it was administered premixed with IVIG (Fig. 3A, B; \( P < .05; n = 4 \)).

These findings signify that with progression of disease duration, greater proportion of IgG molecules within the SScIgG pool recognize the target epitope (M3-R) leading to progression of cholinergic neuropathy.

**Effect of SScIgG on EFS-evoked ACh release in intact rat colonic SM**

Measurement of ACh release from colonic smooth muscle stimulation revealed that in control experiments (in the presence of normal IgG) EFS (10 V, 5 Hz, 0.5 ms, 30 sec train) caused a significant increase in ACh release, which was attenuated by 0Ca\(^{2+}\) but remained...
unaffected by IVIG. In addition, SScIgG caused a significant decrease (*; P< 0.05); Fig. 3C) in ACh release. Notably SScIgG from patients in Group II caused an even further significant decrease (**; P< 0.05; Fig. 3C) in ACh release compared to SScIgG from patients in Group I, validating the progression of cholinergic neuropathy with advancement of disease duration. The suppressant effect of SScIgG (from patients in both groups) on ACh release was reversed by IVIG (Fig. 3C; P < .05), which by itself had no significant effects on basal release of ACh. These data suggest that IVIG could potentially reverse SScIgG-mediated cholinergic dysfunction.

Effect of SScIgG on direct colonic smooth muscle contraction by bethanechol (BeCh)

BeCh caused a concentration-dependent increase in contraction of the colon smooth muscle strips that was attenuated by SScIgG. KCl also caused concentration-dependent increase in colon smooth muscle contraction that was not affected by SScIgG (Fig. 4A, B; P < .05; n = 4) suggesting the selectivity of the suppressant effects of SScIgG on M3-R activation in the colon.

In these experiments the effect of SScIgG (1 mg/ml) from a patient in Group I (disease duration: 120 months) was compared to that of SScIgG (1 mg/ml) from a patient in Group II (disease duration: 420 months). SScIgG from the former patient with disease of less duration attenuated BeCh response to only 70% of control (Fig. 4B; P < .05; n = 4) whereas SScIgG from patient in Group II lead to much greater attenuation of BeCh response to 50% of maximal contraction (Fig. 4C; P< .05; n= 4). (Maximal contraction with BeCh (10^-4 mol/L) was regarded as 100% contraction).

The attenuation of smooth muscle contraction induced by SScIgG from both patients
was significantly reversed by pre-treatment of the smooth muscle strips with 10 mg/mL IVIG (Fig. 4 B, C; P < .05, n = 4). IVIG by itself had no significant effect on BeCh-induced smooth muscle contraction.

Assessment of SScIgG binding to M₃-R and influence of complete IVIG And 
F(ab')₂ using synthetic M₃-RL2 peptide and LSMMMP lysate

In these experiments, we used multiwell plates (used for ELISA) preadsorbed with 400 pg/mL of M₃-RL2 or 400 μg/mL of LSMMMP lysate and determined the OD after incubation with varying concentrations of SScIgGs. Data show that SScIgG (and not NIgG) binds to LSMMMP lysate significantly in a concentration-dependent manner with the maximal OD at 40 nM of SScIgG was 0.532 (*; Fig. 5A; P < .05; n = 4). Pre-treatment of the SScIgG with IVIG (SScIgG + IVIG) and it’s F(ab’)₂ fragment (SScIgG+ F(ab’)₂) caused a significant decrease in the binding of the SScIgG to the LSMMMP lysate as reflected by a decrease in the OD (to 0.148 and 0.104 respectively) (Fig. 5A; P < .05; n = 4). These data suggest that the LSMMMP has M₃-R domains that bind selectively with the SScIgGs.

ELISA with M₃-RL2 revealed similar results that showed a concentration-dependent increase in M₃-R binding with SScIgG (*; Fig. 5B; P < .05; n = 4) and not with NIgG (P > .05). Maximal OD at 40 nM of SScIgG was 0.54 (Fig. 5B; P < .05; n = 4). Pre-treatment of the SScIgG with IVIG (SScIgG+ IVIG) and it’s F(ab’)₂ fragment (SScIgG+ F(ab’)₂) caused a significant and concentration-dependent decrease in the OD (P < .05; n = 4).

When the OD obtained with SScIgG (40 nM) binding to M₃-RL2 from all patients was plotted against their duration of disease a selective and disease duration-dependent increase in the binding was observed. This binding was attenuated significantly by IVIG and also by its antigen-binding fragment F(ab’)₂ suggesting the presence of anti-idiotypic
antibodies in IVIG that neutralize the pathological activity of SScIgGs (Fig. 5C; P< .05).

DISCUSSION

In continuation form our previous work on the pathogenesis of gastrointestinal dysmotility in SSc, the current study demonstrates: 1. SScIgGs from patients with disease duration of less than 15 years display high affinity inhibition of M₃-R at myenteric cholinergic neurons (MCN); 2. Further progression of disease duration leads to combined inhibition of M₃-R at the MCN and smooth muscle (SM); and 3. IVIG attenuates the binding of these autoantibodies at the MCN and SM receptors by anti-idiotypic neutralization.

Evidence for neuropathy in gastrointestinal SSc comes from two landmark studies in 1970s. First, Cohen et al (5) demonstrated that the esophageal smooth muscle responded to direct acting agent methacholine but not to indirectly acting edrophonium. (Edrophonium increases the levels of ACh by preventing its breakdown by acetylcholinesterase, whereas methacholine similar to Ach, is a muscarinic receptor agonist.). Subsequently, DiMarino et al (7) showed that duodenal myoelectric activity was intact but there was an abnormality in intestinal activation by mechanical and hormonal stimuli in SSc patients. These data collectively suggest an early neuropathic phase characterized by dysregulation of Ach release from the myenteric cholinergic neurons. Further evidence of neuropathy in SSc is supported by studies demonstrating impaired anal sensation and rectoanal inhibitory reflex in this subset of patients (14, 28).

Multiple mechanisms were proposed to explain the mechanism of neuropathy in SSc but none could gather much scientific attention until Goldblatt et al demonstrated that sera of SSc patients contained antibodies that could inhibit cholinergic-mediated contraction of
the mouse colon (11). The strongest evidence for autoantibody-mediated dysmotility can be drawn from a study that showed that SSc patients develop manometric abnormalities in the absence of histopathological changes in the esophageal musculature (27). Studies from our laboratory confirmed subsequently that autoantibodies isolated from SSc patients inhibit direct and indirect (neurally mediated) rat colonic and internal anal sphincter smooth muscle contraction (24).

The present studies provide evidence in favor of the temporal course of SScIgG binding to the neural and myogenic M₃-R. Immunohistochemical data reveal that binding of SScIgG at the myenteric cholinergic neurons was higher in patients early in the course of their disease and further increased over time. Most notably, binding to the SM increased over time as well. These data demonstrate a positive correlation between the binding intensity of SScIgG to the myenteric neurons and smooth muscle with disease duration. Thus, greater the duration of the disease, the greater is the binding accounting for the progressive nature of GIT involvement in SSc. Similar results were shown in a study wherein esophageal stiffness and impaired muscle function on manometry correlated with disease duration (12).

Results of functional studies further reiterate the higher affinity of SScIgG to the MCN earlier and later to smooth muscle with progression of the disease. This progressive effect of SScIgGs at the M₃-R located on myenteric cholinergic neurons was shown by the marked decline in EFS-induced colonic smooth muscle contraction and actual release of ACh following pre-treatment of the smooth muscle strips with SScIgG from patients in group II compared to patients from Group I. More importantly, SScIgGs from patients with significantly advanced duration of disease in Group II inhibited BeCh-induced contraction
of the rat colonic SM to a greater extent compared to Group I, suggesting the targeting of smooth muscle M₃-R (myopathy) with disease progression.

M₃-R-specific ELISA studies revealed that SSclGgs cause a selective and disease duration-dependent increase in the binding to the M₂-RL2 peptide and whole M₃-R in LSMMMP lysates. Although, exact reason for such an unexpected increase in affinity of SSclGgs towards MCN as well as SM (observed during ELISA, and I FI studies as discussed above) is not known, a few possibilities exist. Firstly, a change in the epitope of M₃-R that is recognized by SSclG owing to structural or anatomical changes in the target tissues caused by edema or inflammation, as SSc progresses. Secondly, a phenomenon of affinity maturation of the antibody response (6, 9, 20), with temporal progression of SSc may contribute to the observed findings. This issue however merits further investigation.

Present studies provide further evidence that IVIG reverses SSclG-induced M₃-R inactivation at both the neural and myogenic sites. Evidence for the effects of IVIG comes first from IHC studies showing significant decrease in M₃-R binding intensity following SSclG, at both the myenteric cholinergic neurons and smooth muscle. Secondly, IVIG reverses both the SSclG-induced attenuation of BeCh and EFS-stimulated colonic muscle contraction. Finally, IVIG attenuates SSclG-induced increase in binding intensity to the M₃-R when multiwell plates are preadsorbed with LSMMMP lysate or M₃-RL2 peptide.

In our previous study we had demonstrated that IVIG produces its effect by directly competing with pathogenic SSclG’s (24, 25). The three main mechanisms that are likely to involve direct competition with pathologic autoantibodies are anti-idiotypic binding, FcRn saturation and complement scavenging (3, 23). Our findings that F(ab’)₂ fragments of the IVIG have the same effect as IVIG in vitro (ELISA binding studies to the M₃-RL2 peptide and
whole M₃-R in LSMP) suggest the presence of anti-idiotypic antibodies in IVIG that block
the activity of pathogenic SScIgGs (3).

Currently the treatment of gastrointestinal disease in SSc is symptomatic and
ineffective (19). In this regard IVIG offers hope for treatment of SSc-associated
gastrointestinal motility disorders. A recent study focusing on the effect of IVIG in SSc
patients, reported no significant worsening in gastrointestinal symptom score at one year
follow up as a secondary end point (21). However, the exact mechanism, and the role of
other pathways in the therapeutic efficiency and efficacy of IVIG remain to be determined.

SSc is a heterogeneous disease in which internal organ involvement and outcomes vary
considerably from patient to patient. There is a subset of patients who develop rapidly
progressive debilitating gastrointestinal symptoms within a few years of diagnosis.
Whether this subset of patients exhibit neuropathy followed by myopathy, remains to be
determined. Moreover, autoantibody-associated dysmotility does not explain the
mechanism of fibrosis seen in the gastrointestinal tract and most importantly does not
correlate with the symptomatology of gastrointestinal disease (determined by the UCLA
SCTC GIT 2.0). Another limitation of the present study is sample size in terms of limited
number of patients. In spite of these limitations, the results of our study are novel and
should be followed up with a multi-center study involving a large number of SSc patients at
different stages of the disease.

In conclusion we propose that the pathophysiological changes of the gut in SSc occur in
a staged process beginning with neuropathy and progressing to myopathy. Initially,
circulating M₃-R autoantibodies block cholinergic neurotransmission via inhibition of ACh
release at the MCN (neuropathic damage) and later lead to myopathy via inhibition of ACh
action at the gastrointestinal smooth muscle cell proper. We further suggest that SSc-
associated intestinal dysfunction at both the neuropathic and myopathic stages may be
potentially reversible with IVIG.
REFERENCES


**Table 1**: Baseline characteristics of patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (Years)</th>
<th>Disease Duration (Months)</th>
<th>SSc Subtype</th>
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Figure Legends

**Fig. 1.** (A)(i) Identification of myenteric neuronal plexus (MP) by cuprolinic blue staining on whole mount z-stack and transverse section (ii) staining of MP and smooth muscle (SM) by SScIgG; Anti human FITC-conjugated-antibody was used against SScIgG, which gives green florescence. (B) Bar graph of IFI showing that the binding intensity of SScIgG to MP is significantly higher compared to SM (P < .05; n = 3). (C) Immunohistochemical colocalization of SScIgG (FITC-conjugated; green) and M₃-R (TR-conjugated; red). Arrows indicate colocalization of both probes on SM and MP (P < .05; n = 3). (D) Bar graph data show significant coefficient of colocalization of SScIgG with the M₃-R at SM and MP (P < .05; n = 3).

**Fig. 2.** (A)(i) SScIgG from a patient with early disease duration (120 months) had significantly higher binding at the MP compared to the SM (P < .05; n=3). (ii) SScIgG from a patient with advanced SSc duration (420 months), shows progression of binding intensity at the MP and SM (P < .05; n = 3). (B) Correlation between SScIgG binding intensity at SM and MP with disease duration shows that binding intensity increases in linear fashion with disease duration (P < .05). (C) SScIgG binds to the colonic smooth muscle and myenteric plexus (i). This binding is significantly attenuated by pre-treatment of SScIgG with IVIG (ii) (P < .05; n = 3). (D) Bar graph showing the relative IFI of SScIgG binding and its attenuation by IVIG. (P < .05; n = 3).

**Fig. 3.** EFS-induced contraction of the rat colonic smooth muscle. SScIgG from a patient in Group II (disease duration 420 months) (B) causes more marked attenuation of EFS response than SScIgG from a patient in Group I (disease duration 120 months)(A) (P < .05,
n = 4). Attenuation of these EFS responses is reversed by IVIG to values not significantly different from those obtained in the control and IVIG alone experiments (P > .05). (C) Values of ACh release following EFS (10V, 5 Hz, 0.5 ms, 30 sec train) in the colonic SM strips used for the experiments for SSClGgs from Group I vs Group II patients. Data show that EFS causes a significant (P < .05, n = 4) increase in ACh release that is mitigated significantly (P < .05, n = 4) by 0 Ca²⁺ and SSClG. The effects of SSClG are reversed by IVIG pre-treatment to values not significantly different from those obtained in control and IVIG alone experiments (P > .05).

**Fig. 4.** (A) SSClG and IVIG has no significant effect (P > .05, n = 4) on KCl-induced colonic smooth muscle contraction. (C) SSClG from a patient in Group II (disease duration 420 months) causes more marked attenuation of M₃-R activation by BeCh than SSClG from a patient in Group I (disease duration 120 months) (B) (P < .05, n = 4). Attenuation of these BeCh responses is reversed by IVIG to values not significantly different from control (NIgG), and pooled hIgG (IVIG) alone experiments (P > .05).

**Fig. 5.** (A) ELISA binding studies for LSMMP lysate. OD concentration curves show that SSClG (but not NIgG) bind with M₃-R on LSMMP lysate in a concentration-dependent manner (P < .05; n = 4). IVIG and F(ab')₂ significantly decrease this binding (P < .05; n = 4). (B) Similarly, ELISA binding studies for synthetic M₃-RL2 peptide. OD concentration curves show that SSClG (but not NIgG) bind with M₃-RL2 in a concentration-dependent manner (P < .05; n = 4). IVIG and F(ab')₂ significantly decrease this binding (P < .05; n = 4). (C) Graph showing significant linear correlation of binding intensity of SSClG (40 nM) to synthetic M₃-RL2 peptide with disease duration and its inhibition by IVIG and F(ab')₂ (P < .05).
Fig. 6. Proposed pathogenesis of gastrointestinal dysmotility in SSc suggests that SScIgGs initially block cholinergic neurotransmission by inhibition of ACh release by the MCN (neuropathy). With progression of the disease, SScIgGs lead to additional myopathy via inhibition of ACh action at the gastrointestinal smooth muscle proper (myopathy). This is followed by the last stage of intestinal fibrosis by yet unknown mechanisms. Dysmotility at the neuropathic and myopathic stages may be potentially reversible with IVIG, before smooth muscle fibrosis/atrophy ensue.
Fig. 1

A

Cuprolinic Blue

Whole Mount

Transverse Section

B

Fluoresc. Int./unit area

C

SScIgG

Cuprolinic Blue

Coeficient of colocalization

D

Coeficient of colocalization

M3-R

Merge (R & G)
Fig. 2

A

Cuprolinic Blue  SSclG  M3-R  Merge (R & G)

Disease Duration (months)

B

Intensity/unit area

1.5
1.0
0.5
0.0

Disease Duration (months)

MP  SM

SM  MP

Disease Duration (months)

C

Cuprolinic Blue  SSclG

D

Rel. Intensity

SSclG  SSclG+IVIG
Fig. 3

A  EFS (120 Months)

B  EFS (420 Months)

C  ACh Release

- Control
- IVIG
- SScIgG (Group I)
- SScIgG + IVIG
- NlgG

Graphs showing the effects of different treatments on EFS and ACh release.
Fig. 4

A

KCl

- % Maximal Contraction

- Control
- SSclgG
- IVIG
- NlgG

B

BeCh (120 Months)

- % Maximal Contraction

- Control
- SSclgG (Group I)
- IVIG
- SSclgG + IVIG
- NlgG

C

BeCh (420 Months)

- % Maximal Contraction

- Control
- SSclgG (Group II)
- IVIG
- SSclgG + IVIG
- NlgG

log [bethanechol] (M)

% Maximal Contraction

KCI

BeCh (120 Months)

BeCh (420 Months)
**Fig. 5**

A. **ELISA: LSMMP Lysate**

- SSclgG
- SSclgG+IVIG
- SSclgG+F(ab')₂
- NlgG

B. **ELISA: M₃-RL2 Peptide**

- SSclgG
- SSclgG+IVIG
- SSclgG+F(ab')₂
- NlgG
Fig. 6

- **Early**: REVERSIBLE: IVIG
- **Advanced**: IRREVERSIBLE
- **End Stage**

**Neuropathy**

**Myopathy**

**Atrophy/Fibrosis**

**Systemic Circulation**

MCN: Myenteric Cholinergic Neurons

SMC: Smooth Muscle Cells

- **Early**
- **Advanced**
- **End Stage**

**Disease Duration In Years**

- $M_3\cdot R$
- SSclgG
- Acetylcholine

**Legend**

- MCN: Myenteric Cholinergic Neurons
- SMC: Smooth Muscle Cells