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Generation of anti-NAG-2 mAb from patients' memory B cells: implications for a novel therapeutic strategy in systemic sclerosis

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Abstract

We have previously reported that antibodies directed against the cytomegalovirus-derived protein UL94 cross react with the cell surface tetraspanin transmembrane 4 superfamily member 7 (TM4SF7 or NAG-2) molecule inducing apoptosis of endothelial cells and activation of fibroblasts in patients with systemic sclerosis (SSc). We aimed at generating a non-functional mAb directed against NAG-2 from patients' memory B cells. Direct and competitive ELISA methods have been used to evaluate the binding of antibodies from scleroderma patients' and controls' sera to the NAG-2 peptide. IgG memory B cells were sorted, EBV transformed and cloned to obtain NAG-2-specific mAbs. Endothelial cells and fibroblasts were cultured under standard conditions and used for functional assays. Anti-NAG-2-purified antibodies obtained from patients' Ig induce endothelial cell apoptosis and fibroblast proliferation. Patients' Igs depleted of the anti-NAG-2 fraction do not exert such functional activity. Therefore, the NAG-2 molecule represents a potential novel candidate for therapeutic intervention in SSc. Here, we describe the generation of a human mAb directed against the NAG-2 molecule. Such mAb does not retain any functional property and is able to block the effect of serum pathogenetic anti-NAG-2 antibodies. The majority of SSc patients present antibodies directed against tetraspanin NAG-2 and mediate both endothelial cell apoptosis and fibroblast proliferation, features of the disease. The anti-NAG-2 human mAb we have obtained blocks signal transduction and therefore may be a potential candidate for a new treatment in SSc, a disease where the current biological therapies have little or no efficacy.

Keywords: human mAb, memory B cells, NAG-2, systemic sclerosis

Introduction

Progressive systemic sclerosis (SSc) is an autoimmune disease with prominent vascular damage and multi-organ fibrosis (1). Several auto-antibodies present in the sera of patients with SSc, including anti-endothelial cells (2) and anti-fibroblasts (3–5) antibodies, may directly contribute to disease pathogenesis. The identification of pathogenetically relevant auto-antigens is a major goal in autoimmune disorders such as SSc. We have previously identified a tetraspanin transmembrane 4 superfamily member 7 (TM4SF7 or NAG-2) peptide epitope (CGVLGVGIWLAA) as a key auto-antigen target of IgG auto-antibodies in SSc (6). NAG-2 is a tetraspanin belonging to the transmembrane 4 superfamily that forms complexes with the integrins alpha3beta1 and alpha6beta1; it is highly expressed on endothelial cells and on fibroblasts and seems to have a role in cell mobility and adhesion (7). The NAG-2 peptide shares a high degree of homology with the Cytomegalovirus (hCMV)-derived protein UL94. Different infectious agents are believed to play a role in the immunopathogenesis of SSc (8–10). We provided direct evidence for a molecular mimicry mechanism by which antibodies against a hCMV-derived protein can be linked to endothelial cell damage and fibrosis in patients with SSc (6, 11). Indeed, affinity-purified anti-UL94 IgG antibodies derived from SSc patients' sera recognize NAG-2 in a whole-cell lysate and induce apoptosis of endothelial cells upon engagement of the NAG-2–integrin complex (6). The same

antibodies are also able to cause fibroblast activation and proliferation and to induce modulation of genes involved in cell apoptosis and activation (11). Therefore, we proposed that hCMV is linked to the pathogenesis of SSc through a particular subset of anti-hCMV antibodies that specifically interacts with the normally expressed cell surface receptor NAG-2 sharing similarity with the UL94 viral protein.

Based on these previous findings, our aim was firstly to evaluate the frequency of anti-NAG-2 antibodies in patients with SSc and secondly to obtain a fully human anti-NAG-2 mAb able to block the signal transduction, following a recently described method to derive antigen-specific mAbs from patients' memory B cells (12, 13). We describe here the generation of the functionally inactive mAb JB007 and propose it as a possible biological therapy in SSc.

Methods

Patients

A total of 90 patients affected by SSc were enrolled in this study: 35 patients had SSc with diffuse cutaneous scleroderma (dSSc) and 18 of them had antibodies against topoisomerase I (Scl70), 55 patients had SSc with limited cutaneous scleroderma (lSSc) and 32 of them had antibodies against the kinetochore (centromere). All patients were antinuclear antibody positive and fulfilled the American College of Rheumatology criteria for SSc (14). Moreover, all of them had IgG antibodies against hCMV, indicating a previous contact with the virus. The characteristics of the patients are summarized in Table 1. Control sera were obtained from 50 patients with rheumatoid arthritis, 50 patients with systemic lupus erythematosus (diagnosed following the American College for Rheumatology criteria), 30 patients with undifferentiated connective tissue disease and 95 normal age- and sex-matched individuals. Two of the three patients (CM and ML) used for the generation of the mAbs were affected by the diffuse cutaneous form of the disease and were Scl-70 positive, one of them (ML) had a recent onset of the disease (4 months). One patient (PD) was suffering from the limited cutaneous form of the disease and was anti-centromere positive; also, this patient had a recent onset of the disease (6 months) and had a recent contact with varicella virus.

The study was approved by the local ethical committee and written informed consent was obtained from patients and controls.

Table 1. Characteristics of the 90 patients affected by SSc enrolled in the study

Patients	Diffuse SSc	lSSc
Male/female	1/34	3/52
Age (years)	21–62	26–78
Mean disease duration (years)	7.1	6.7
Antinuclear antibody positivity	35/35	55/55
Anti-SCL70 positivity	18/35	0/55
Anti-centromere positivity	0/35	32/55
Digitals ulcers	7/35	13/55
Renal involvement	0/35	5/55
Pulmonary artery hypertension	3/35	7/55
Pulmonary fibrosis	6/35	4/55
Gastro-intestinal involvement	31/35	43/55

Cell culture and antibodies

Human endothelial cells and human dermal fibroblasts as well as their growth media were purchased from Promocell Bioscience Alive (Heidelberg, Germany). Endothelial cells were used between passages 2 and 5, while dermal fibroblasts between passages 3 and 6.

Antibodies directed against the NAG-2 epitope were obtained from pooled IgG Igs derived from 10 patients with SSc by affinity chromatography using a NAG-2 peptide–sepharose column. The flow through of the column was then used to prepare Ig depleted of the anti-NAG-2 antibody subsets by sequential absorption on a NAG-2 peptide–sepharose column until no anti-NAG-2 antibody activity could be detected in the antibody preparation by ELISA on a peptide-coated plate.

The human mAb JB007 was affinity purified using a protein A–sepharose column by standard technique.

The amount of the purified antibodies was quantified using an ELISA assay employing a standard curve of human IgG; moreover, the preparation was also checked in silver stain.

EBV B-cell immortalization

PBMC cells were isolated from SSc patients by Ficoll-Hypaque density gradient centrifugation. IgG memory B cells were purified through a combination of magnetic and fluorescence cell sorting. Briefly, CD22+ B cells were isolated with CD22 beads (Miltenyi Biotec) according to the manufacturer's instructions. Following staining for lineage (CD3, CD14, CD56; Beckman Coulter) and surface IgD (Becton Dickinson, Franklin Lakes, NJ, USA), IgM and IgA (Jackson Immunoresearch, West Grove, PA, USA), IgM, IgA and IgD B cells were sorted by FACSAria (Becton Dickinson). Sorted B cells were cultured at 30 cells per well in the presence of EBV, CpG 2006 (5′-tcgtcgttttgtcgttttgtcg tt-3′; TIB Molbiol, Genova, Italy) and irradiated mononuclear cells. Immortalized positive B-cell cultures were cloned at one cell per well in the presence of CpG 2006 and irradiated mononuclear cells.

Peptide synthesis

The NAG-2 peptide (CGVLGVGIWLAA) and the irrelevant control peptide (VTLPKDSDVELP) were manually synthesized using the standard method of solid-phase peptide synthesis, which follows the 9-fluorenylmethoxycarbonyl strategy with minor modifications (15). All the synthesized compounds were purified by reverse-phase high-performance chromatography, and the molecular weights were finally confirmed by electrospray mass spectrometry.

Enzyme-linked immunosorbent assay

The direct and competitive ELISA methods for antibody binding to the synthetic peptides have been described (6). The synthetic peptides were used at a concentration of 20 μg ml^{-1} in PBS to coat polystyrene plates (Nunc, Roskilde, Denmark). Bound antibodies were detected by an alkaline phosphatase-conjugated antiserum against human IgG (Sigma). For competitive assays, the amount of antibody that gave 50% of the maximum binding to the antigen on the

solid phase was pre-incubated with different amounts of competitors or buffer and then transferred to the antigen-coated plates. The assay was then continued as a direct binding assay. In the ELISA assay for the detection of serum antibodies directed against the peptides, 95 serum samples, diluted 1:200, from normal age- and sex-matched subjects were used as a control group. Absorbance values higher than the mean + 3 SD of such serum dilution of the control group were considered positive. The ELISA for topoisomerase I (Scl-70) and centromere was performed using commercially available kits (Delta Biomedicals, Rome, Italy). The ELISA for recall antigens used commercially available viral extracts (Virion, Ruschlikon, Switzerland). The extracts were used at a concentration of 25 μg ml^{-1} in PBS to coat polystyrene plates (Nunc). The assay was then carried on as above described.

For the detection of anti-platelet-derived growth factor receptor-alpha (PDGFRA) antibodies, plates were coated with human PDGFRA (Millipore, Billerica, MA, USA) (3.5 μg ml^{-1}) overnight at 4°C and blocking was performed with 200 μl of blocking buffer (PBS–0.05% Tween 20–1% BSA). Sera (diluted 1:5 in blocking buffer) were incubated for 2 h at room temperature. The remainder of the assay was carried on as above described.

Rabbit antiserum production

Polyclonal antibodies were generated in New Zealand White rabbits using standard techniques. Immunizations used a peptide corresponding to NAG-2 amino-acid residues 25–36 (CGVLGVGIWLAA) coupled to keyhole limpet haemocyanin. The antisera were tested by ELISA on peptide-coated plates and the anti-peptide fraction was purified by affinity chromatography on peptide-sepharose columns.

FACS analysis

For FACS analysis, cells were incubated with specific or control antibodies for 30 min on ice. As control antibodies, either a human polyclonal IgG preparation or a human monoclonal anti-tetanus toxoid IgG antibody was used and antibody binding was revealed with Cy-5-conjugated anti-human IgG antibodies (Jackson Immunoresearch). Samples were run on a FACScan flow cytometer (Becton Dickinson).

Western blot analysis

Endothelial cells and dermal fibroblasts were lysed in cold lysis buffer [0.5% NP-40, 10 mM TRIS (pH 7.4), 0.15 M sodium chloride, 5 mM magnesium chloride), and lysates were immunoprecipitated with rabbit anti-NAG-2 antibodies cross-linked to sepharose. Eluted proteins were resolved by SDS–PAGE and transferred to nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA). Blots were incubated with either rabbit anti-NAG-2 antibodies or human antibodies (10 μg ml^{-1}). The Renaissance Chemiluminescence Kit (NEN, Boston, MA, USA) was used for detection.

Measurement of apoptosis

The extent of internucleosomal DNA fragmentation was quantified using a commercially available kit (Roche Biochemical, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells (10^5 cells ml^{-1}) were cultivated for 12 h in microtiter plates in the presence or absence of apoptotic stimuli (affinity-purified polyclonal antibodies, human mAbs, control antibodies and SSc serum or 50 ng ml^{-1} tumour necrosis factor (TNF)-alpha as a positive control). To avoid any interference by serum complement components, the serum was heat inactivated. Cell pellets were then incubated in lysis buffer. The principle of this test is based on the detection of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates by using biotinylated antibodies against histone- and peroxidase-coupled antibodies against DNA. Results are expressed as % of apoptotic cells where the OD value obtained with 50 ng of TNF-alpha is considered 100% of apoptotic cell death. For inhibition experiments, endothelial cells were pre-incubated with 20 μg ml^{-1} of mAb against NAG-2 for 30 min at 37°C and cells were then washed with complete medium and either purified polyclonal anti-NAG-2 peptide antibodies (10 μg ml^{-1}) or heat-inactivated SSc patients' serum (20 μl per well) were added. The assay was then carried out as above described. A human mAb directed against tetanus toxoid of the same isotype as the anti-NAG-2 mAb was used as control.

Proliferation assay

To assess cell proliferation, fibroblasts (5000 cells per well) were cultured for various intervals of time in microtiter plates in the presence or absence of antibodies. Cell viability was assessed using the commercially available kit (Alexis Biochemicals, San Diego, CA, USA).

AKT phosphorylation

AKT phosphorylation was assessed using a commercially available kit (RACE AKT; Active Motif, Carlsbad, CA, USA). The kit detects both total and phosphorylated AKT. An increased level of phosphorylated AKT indicates cell activation. Incubation time was 30 min.

Statistical analysis

The comparison of the frequency of IgG against the NAG-2 peptide and PDGFRA between patients and controls was performed using the Mann–Whitney U-test.

The comparison of the prevalence of anti-NAG-2-specific IgG B cells producing clones with those specific for recall antigens in the three patients analysed was carried out using the Pearson's chi-square test.

Results

NAG-2 is a major autoantigen target of SSc auto-antibodies

The NAG-2 peptide is specifically recognized by serum IgG from 92% (83 of 90) of the patients studied, by both direct ELISA [absorbance (mean ± SD): 0.28 ± 0.051 for a serum dilution of 1:200] and competitive ELISA, such reactivity was not detected by the individual sera of 95 healthy age- and sex-matched controls [absorbance (mean ± SD): 0.056 ± 0.034] (Fig. 1a) and of 50 patients with rheumatoid arthritis, 50 patients with systemic lupus erythematosus and

a

	Patients	Controls	*p*
NAG peptide	83/90 92%	0/95 0%	p<0.001
PDGFRA	16/90 18%	15/95 16%	p=0.71 (n.s.)

b

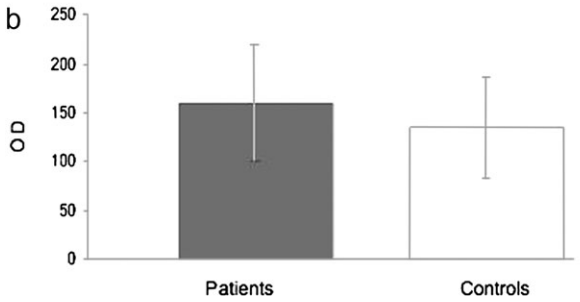

Fig. 1. IgG from SSc patients recognize the NAG-2 peptide and not the PDGFRA. (a) Frequency of IgG antibodies directed against the NAG-2 peptide and PDGFRA in patients with SSc and in normal healthy controls. (b) Comparison of anti-PDGFRA antibodies levels between healthy controls and patients with SSc. Data are presented as box plots, each plot represents the mean of the absorbance of the different sera ± the SD.

30 patients with undifferentiated connective tissue disease (data not shown). The seven patients without NAG-2 antibodies had the following characteristics: five had the lSSc and two had dSSc with a disease duration >10 years; they were not on active phase when bleeded.

These data indicate that this peptide sequence (NAG-2 peptide) contains an epitope recognized by the sera of nearly all the patients with SSc.

Since anti-PDGFRA auto-antibodies have been described in SSc (5) and these auto-antibodies have been shown to have agonistic activity on the receptor, we tested our patient's sera for reactivity against PDGFRA in ELISA. As shown in Fig. 1(a), only a small percentage (18%) of SSc patients had IgG antibodies against PDGFRA when compared with healthy control donors (16%). Moreover, the anti-PDGFRA antibody levels are similar in patients with SSc and in healthy controls (Fig. 1b). These data show that anti-PDGFRA antibodies are not typically associated with SSc.

We next purified the anti-NAG-2 peptide fraction from pooled Ig obtained from 10 SSc patients and found that the purified antibodies bound endothelial cells and fibroblasts (Fig. 2a). Moreover, these antibodies were able to induce endothelial cell apoptosis and fibroblasts proliferation (Fig. 2b and c), known to be the key features of SSc. Since AKT regulates cell survival (16, 17), we confirmed fibroblast activation and proliferation by evaluating AKT phosphorylation in fibroblasts incubated with the anti-NAG-2-specific antibodies (Fig. 2d).

To confirm that the effects observed were confined to the purified anti-NAG-2 antibody fraction, Ig obtained from the same 10 patients and completely depleted of the anti-NAG-2 antibody subset were tested for binding to endothelial cells (Fig. 2e) and fibroblasts (Fig. 2f). Binding to both endothelial cell and fibroblast cell surface was dramatically diminished in the NAG-2-negative fraction (>70% of reduction).

We then checked whether the anti-NAG-2-negative Ig had any functional effect on endothelial cells. Such Ig, even when the concentration was raised to 200 μg ml^{-1}, did not exert any effect on the cells. Indeed, no difference was observed between cells incubated with the anti-NAG-2-deprived Ig or with medium alone (Fig. 2b). We next investigated reactivity to PDGFRA in both purified fractions.

As shown in Fig. 3, the NAG-2-positive IgG fraction does not bind PDGFRA, whereas a slight binding to the receptor was observed within the IgG fraction depleted of anti-NAG-2 activity.

Moreover, we failed to detect any PDGFRA-activating mechanism both in the NAG-2 positive and in the NAG-2 negative IgG fraction (data not shown).

These data indicate that the induction of endothelial cell apoptosis and fibroblasts activation is indeed confined to the purified anti-NAG-2 antibody fraction and that Ig directed against other antigens (including PDGFRA) do not retain such capacity.

Generation of IgG human mAbs from SSc patients' memory B cells

Since we confirmed that the integrins-associated tetraspanin NAG-2 is a key autoantigen target in SSc, a possible therapeutic strategy is the generation of a mAb able to bind the NAG-2 molecule and to block the signal transduction. To this aim, we took advantage from a recently published procedure that allows to dissect the memory B-cell repertoire and to isolate with high-efficiency B-cell clones producing antigen-specific mAbs (12, 13). This method has been successfully used in the generation of neutralizing antibodies directed against severe acute respiratory syndrome coronavirus (12). We reasoned that in autoimmunity, the B-cell response directed against a pathogenetically relevant autoantigen epitope may comprise heterogeneous subsets of auto-antibodies with different functional properties. Therefore, our aim was to isolate a human mAb able to bind NAG-2 but functionally inactive, from SSc patients. The pool of IgG memory B cells was isolated by negative selection through fluorescence cell sorting from three patients with SSc. IgG + B cells were immortalized with EBV at low number of cells per well, in the presence of TLR9 agonist, CpG oligonucleotides 2006 and irradiated mononuclear cells.

IgG producing B-cell cultures were then tested for the ability to bind either the NAG-2 peptide by ELISA or endothelial cells and fibroblasts by FACS analysis or all the three antigens tested (Table 2A). Noteworthy, the frequency of clones producing IgG antibodies directed against the NAG-2 epitope was much higher than the frequency of clones producing antibodies directed against the scleroderma autoantigen Scl-70 in patient CM. Moreover, clones producing auto-antibodies directed against Scl-70 or centromere were not detected in the other two patients, suggesting that the frequency of anti-NAG-2 IgG-producing B cells is much

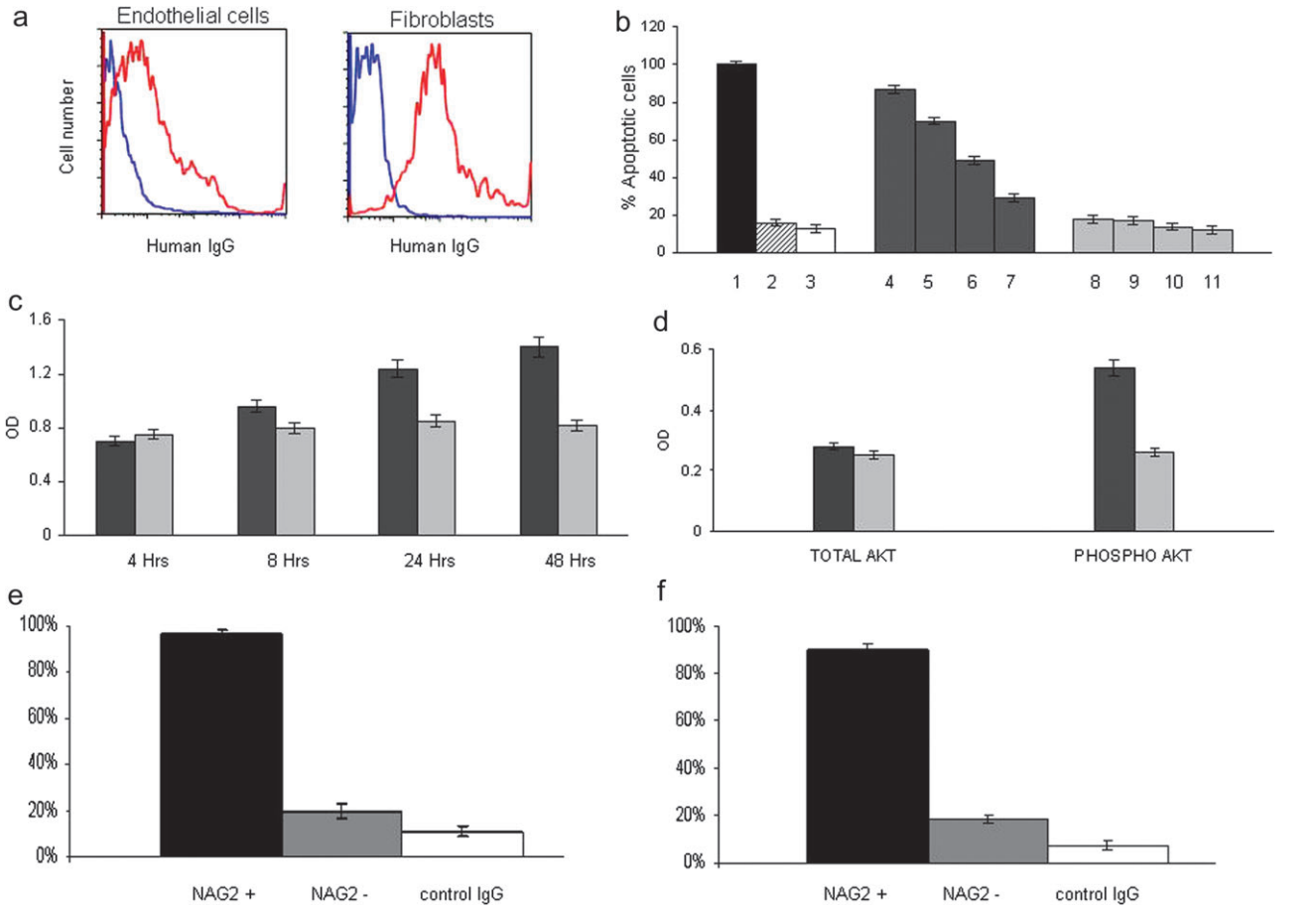

Fig. 2. Serum antibodies directed against the NAG-2 molecule induce apoptosis of endothelial cells and activate fibroblasts. (a) FACS analysis of the binding of antibodies affinity purified from patients' sera against the NAG-2 peptide (red line) to human endothelial cells and fibroblasts. Blue line is negative control (polyclonal IgG preparation). (b) Human endothelial cells incubated for 12 h with affinity-purified antibodies directed against NAG-2 (grey bars) or antibodies depleted of the anti-NAG-2 antibody activity (light grey bars) at different concentrations: 20 μg ml^{-1} (bars 4 and 8), 10 μg ml^{-1} (bars 5 and 9), 5 μg ml^{-1} (bars 6 and 10), 2.5 μg ml^{-1} (bars 7 and 11). Results are expressed as % apoptotic cells where samples treated with 50 ng ml^{-1} of TNF-alpha (bar l) are considered 100%. Bar 2 is medium alone. Bar 3 is normal polyclonal Ig (20 μg ml^{-1}). Results are the mean of three independently performed experiments ± SD. (c) Shown are proliferation levels for fibroblasts incubated for various intervals of time with affinity-purified antibodies against the NAG-2 peptide (dark grey bars) or with antibodies depleted of the anti-NAG-2 antibody subsets (light grey bars). OD, optical density at 570 nm. Results are the mean of three independently performed experiments ± SD. (d) Levels of total and phosphorylated AKT in fibroblasts treated for 30 min with affinity-purified antibodies against the NAG-2 peptide (dark grey bars) and with antibodies depleted of the anti-NAG-2 antibody subsets (light grey bars). OD, optical density at 450 nm. Results are the mean of three independently performed experiments ± SD. (e and f) Binding to both endothelial cell (e) and to fibroblast (f) cell surface is dramatically diminished in the NAG-2-negative fraction IgG and is similar to the binding of Igs obtained from normal donors (control IgG).

higher than the frequency of B cells directed against the classical scleroderma auto-antigens Scl-70 and centromere.

We have also tested the clones for reactivity against the PDGFRA; however, we could not detect any reactivity within the clones tested (Table 2A).

Surprisingly, in the three patients analysed, the frequency of the NAG-2-specific IgG-producing B-cell clones was significantly higher than the frequency of the clones producing IgG against the recall antigens tested (measles virus, varicella virus and rotavirus) (Table 2A and B and Table 3). Patient PD had high frequency IgG against varicella virus; however, she had a recent contact with the virus at the moment of the venopuncture. These data indicate that the anti-NAG-2 autoreactive B memory cells are present at high frequency in patients with SSc.

The human monoclonal JB007 binds endothelial cells and fibroblast upon interaction with NAG-2 molecule but is functionally inactive

The mAbs that bound the NAG-2 peptide, endothelial cells and fibroblasts were then tested in functional assays. Using this approach, among all the anti-NAG-2 IgG-producing clones, we selected a mAb JB007 (IgG, k) that bound NAG-2 but had no effect on endothelial cells and fibroblasts. Therefore, nearly all the anti-NAG-2 IgG produced by the generated clones were functionally active. Figure 4 shows the characteristics of the mAb JB007: (i) it binds endothelial cells and fibroblasts as shown by FACS analysis (panel a), (ii) it recognizes the NAG-2 molecule as shown in western blot (panel b) and (iii) it does not induce endothelial cell apoptosis (panel c) and fibroblast activation and proliferation (panels e and f).

Interestingly, endothelial cells pre-incubated with such mAb do not undergo apoptosis when serum from patients with SSc or polyclonal antibodies directed against NAG-2 purified from the patients are added to the culture (Fig. 4d), indicating that the mAb is able to block the activity of pathogenic serum auto-antibodies.

Discussion

In this study, we show that the NAG-2 molecule represents the main autoantigen target of pathogenic auto-antibodies in SSc. Indeed, serum IgG antibodies directed against this molecule are present in nearly all the patients with SSc; such antibodies are able to induce vascular damage and fibroblast proliferation, the two key features of the disease, whereas antibodies deprived of the anti NAG-2 activity are not pathogenetically relevant since they are unable to induce cell damage.

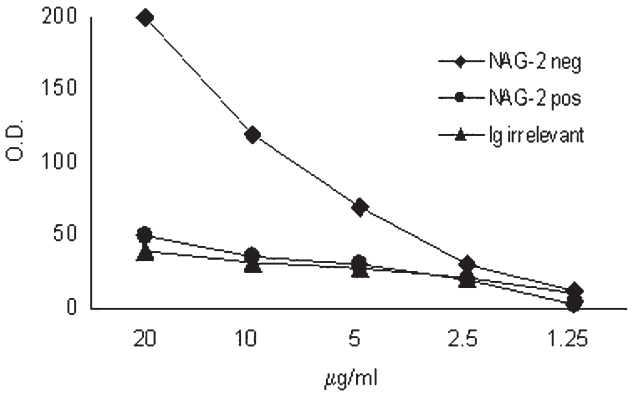

Fig. 3. NAG-2-positive IgG fraction fails to bind PDGFRA. Binding of NAG-2-positive Igs (NAG-2 pos) of the IgG fraction depleted of anti-NAG-2 activity (NAG-2 neg) and of Igs directed against the irrelevant control peptide (Ig irrelevant) to the PDGFRA.

Auto-antibodies specific to PDGFRA have been detected in the serum of patients with SSc (5). These antibodies displayed agonistic activity, as demonstrated by induction of PDGFR phosphorylation in cultured fibroblasts. We could not detect anti-PDGFRA activity in our SSc sera and only a weak reactivity against PDGFRA can be detected in the IgG fraction deprived of anti-NAG-2 activity. Importantly, this antibody population failed to activate the PDGFR. Our results are consistent with recent reports that raise questions regarding the existence and/or the significance of agonistic auto-antibodies to PDGFR in SSc (18–20).

B memory cells secreting IgG antibodies directed against the NAG-2 molecule are present at high frequency in the B-cell repertoire of patients with SSc as shown by the lower number of B-cell clones producing IgG against the recall antigens tested (measles virus, varicella virus and rotavirus) and by the nearly absence of clones producing anti-topoisomerase I and anti-centromere antibodies. We describe here the generation of the human mAb JB007 derived from a SSc patient, which specifically binds the NAG-2 molecule on endothelial cells and fibroblasts. Such antibody is functionally inactive since it does not induce endothelial damage and fibroblast proliferation indicating that in the

Table 2. Analysis of B-cell repertoire in three patients affected by SSc

A IgG-secreting positive B-cell cultures specific for auto-antigens in scleroderma patients (absolute numbers)

Donors	Total IgG-secreting cultures	NAG-2	ECs	NAG-2, ECs, fibroblasts	No functional activity	Scl70[a]/centromere[b]	PDGFRA
CM[a]	1096	84	156	24	1	10	0
ML[a]	905	80	130	25	2	0	0
PD[b]	1035	54	147	18	1	0	0

B IgG-secreting positive B-cell cultures specific for recall antigens in scleroderma patients (absolute numbers)

Donors	IgG-secreting cultures screened	Measle virus	Varicella virus	Rotavirus
CM[a]	500	18[c]	10[c]	11[c]
ML[a]	500	39	34	27[d]
PD[b]	500	26[d]	51	19[c]

EC, endothelial cell.
[a]Diffuse cutaneous form.
[b]Limited cutaneous form.
[c]$P < 0.01$ versus NAG-2 by chi-square test.
[d]$P < 0.05$ versus NAG-2 by chi-square test.

Table 3. Frequency of IgG-secreting B-cell clones specific for NAG-2 and recall antigens (see Table 2 for the absolute numbers)

	NAG-2 versus measle virus	NAG-2 versus varicella virus	NAG-2 versus rotavirus
CM	$P = 0.002$, Pearson's chi square = 9.48	$P < 0.001$, Pearson's chi square = 19.88	$P < 0.001$, Pearson's chi square = 18.31
ML	$P = 0.503$, Pearson's chi square = 0.45	$P = 0.18$, Pearson chi square = 1.79	$P = 0.02$, Pearson chi square = 5.42
PD	$P = 0.038$, Pearson's chi square = 4.31	$P = 0.117$, Pearson's chi square = 1.83	$P = 0.002$, Pearson chi square = 10.03

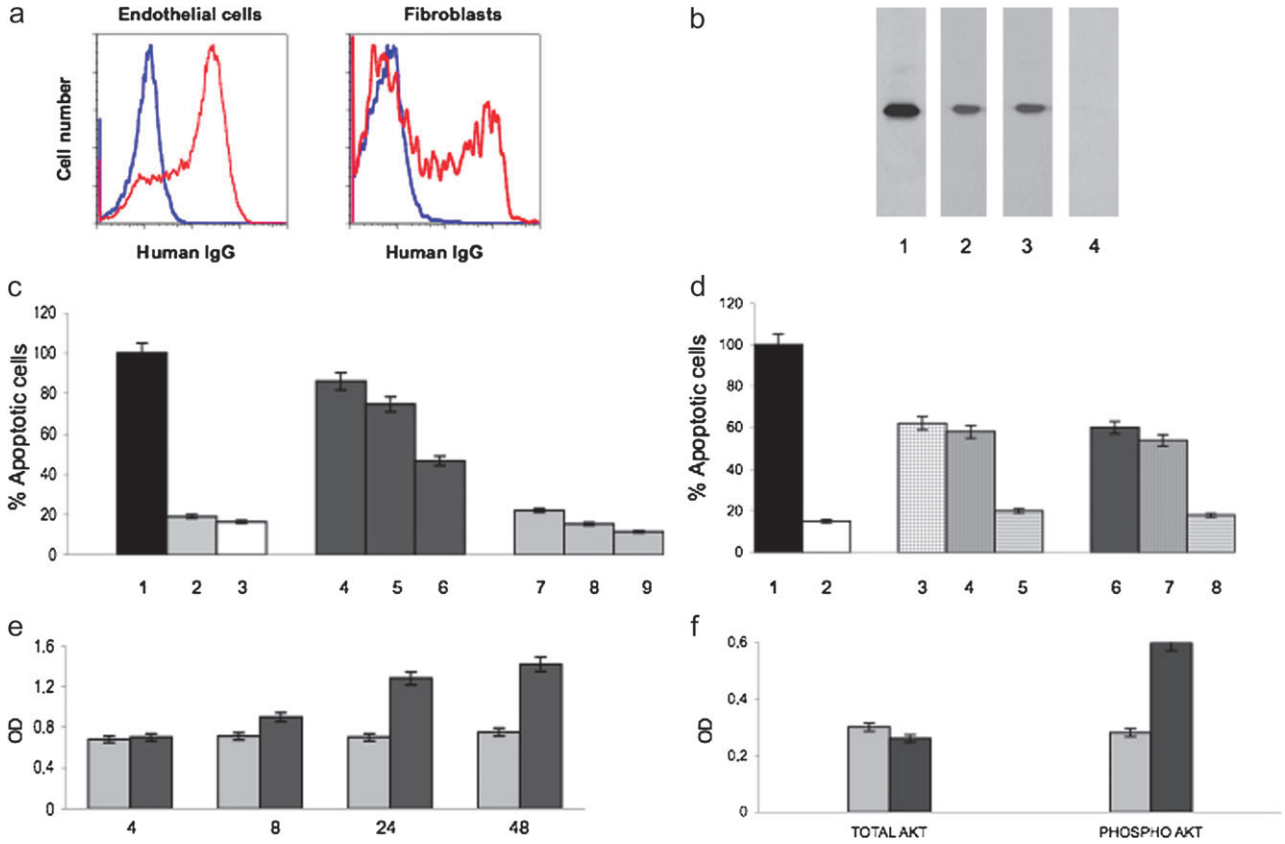

Fig. 4. The human mAb JB007 reacts with the NAG-2 molecule and is functionally inactive. (a) FACS analysis of the binding of human mAb JB007 (red line) to human endothelial cells and fibroblast. Blue line is negative control (mAb directed against tetanus toxoid). (b) Lysates from human dermal fibroblasts were immunoprecipitated with a rabbit affinity-purified anti-NAG-2 peptide antibody cross-linked to sepharose. Immunoprecipitates were resolved in SDS–PAGE and transferred to nitrocellullose. Blots were incubated with rabbit anti-NAG-2 peptide antibody (lane 1), affinity-purified antibodies directed against the NAG-2 peptide isolated from patients with SSc (lane 2), with the human mAb JB007 (lane 3) and with a human mAb directed against tetanus toxoid (lane 4). (c) Human endothelial cells were incubated for 12 h with affinity-purified antibodies directed against NAG-2 (grey bars) or with the human mAb JB007 (light grey bars) at different concentrations: 20 μg ml^{-1} (bars 4 and 7), 10 μg ml^{-1} (bars 5 and 8) and 5 μg ml^{-1} (bars 6 and 9). Results are expressed as % apoptotic cells where samples treated with 50 ng ml^{-1} of TNF-alpha (bar l) are considered 100%. Bar 2 is medium alone. Bar 3 is a human mAb directed against tetanus toxoid (20 μg ml^{-1}). Results are the mean of three independently performed experiments ± SD. (d) Human endothelial cells incubated for 12 h with affinity-purified antibodies directed against NAG-2 (bar 3) or serum obtained from a patient with SSc (bar 6). In bars 4 and 7, cells were pre-incubated with a control human mAb directed against tetanus toxoid; in bars 5 and 8, cells were pre-incubated with the human mAb JB007. Results are expressed as % apoptotic cells where samples treated with 50 ng ml^{-1} of TNF-alpha (bar l) are considered 100%. Bar 2 is medium alone. Results are the mean of three independently performed experiments ± SD. (e) Proliferation levels of fibroblasts incubated for various intervals of time with the human mAb JB007 (light grey bars) or with affinity-purified antibodies against the NAG-2 peptide (dark grey bars). OD, optical density at 570 nm. Results are the mean of three independently performed experiments ± SD. (f) Levels of total and phosphorylated AKT in fibroblasts treated for 30 min with the human mAb JB007 (light grey bars) and with affinity-purified antibodies against the NAG-2 peptide (dark grey bars). OD, optical density at 450 nm. Results are the mean of three independently performed experiments ± SD.

autoreactive B-cell repertoire directed against a particular autoantigen, auto-antibodies with different functional activity are present.

The use of biological therapies in SSc has been disappointing so far. Indeed, only a few cases have been treated with anti-TNF-alpha and anti-CD20 agents, giving conflicting results (21–23). The only biological therapy specifically designed for SSc is a mAb directed against the transforming growth factor-beta, a profibrotic cytokine thought to play a pivotal role in the pathogenesis of the multi-organ fibrosis typical of scleroderma. Unfortunately, such therapy seems to have no efficacy in the treatment of the disease (24).

Imatinib mesylate is a small molecule inhibitor that exerts selective dual inhibition of the transforming growth factor-beta and platelet-derived growth factor receptors pathways (25, 26). It seems to be useful in the treatment of skin fibrosis (27, 28); however, it does not affect the vascular damage, a pivotal feature of the disease.

Autologous haematopoietic stem cell transplantation is another possible therapeutic option in patients with early diffuse SSc with the aim to achieve remission of the disease: prospective randomized trials are in progress to evaluate the safety and efficacy of this treatment (29, 30).

In conclusion, our data suggest that the selected fully human mAb we have generated and described here may represent an ideal candidate for a novel therapeutical intervention in SSc since its target is a molecule that plays a fundamental role in the pathogenesis of the disease.

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Author contributions: ET, CL and AP conceived the idea and were the principal investigators of the Verona and Genova teams. ET performed the B-cell cloning and generated the mAb. CB screened the clones for NAG-2 specificity. MD performed some of the ELISA tests and synthesized the peptides used in the study. AP performed the FACS analysis and the mAb activity. RC advised on the preparation of the report. CL and AP wrote the article with input from ET.
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