Antinuclear antibodies are associated with tumour necrosis factor receptor I gene polymorphism in patients with rheumatoid arthritis

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Abstract

Objective

Antinuclear antibodies (ANA) are a common feature of autoimmune diseases such as rheumatoid arthritis (RA). Herein, we investigate the relationship between ANA and polymorphism in the tumour necrosis factor receptor (TNFR) genes.

Methods

Serum titers of ANA at diagnosis were measured in 267 patients with RA and a single nucleotide polymorphism (SNP) in each of the TNFR-I (36A/G) and TNFR-II (676T/G) genes was genotyped by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis. Circulating levels of soluble TNFR (sTNFR) and TNF-α were also measured in some patients.

Results

Our initial analyses revealed the presence of ANA was associated with the TNFR-I 36A/G SNP, with a trend of increasing ANA frequency with G allele dosage (p=0.004). ANA status was also associated with lower sTNFR-I levels and a raised sTNFR-II/sTNFR-I ratio. The TNFR-II 676T/G SNP and circulating levels of sTNFR-II and TNF-α were not associated with ANA status. In an adjusted multivariate regression model the TNFR-I 36 GG genotype (OR 7.8, p=0.008) and levels of sTNFR-I (p=0.018) were independently associated with ANA status.

Conclusion

Our findings suggest a possible link between the production of ANA and the TNF-α/TNFR-I signalling system, which may be related to the apoptosis-inducing ability of this cytokine.

Key words

rheumatoid arthritis, antinuclear antibodies, tumour necrosis factor receptor, single nucleotide polymorphism, apoptosis

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Introduction

One of the characteristic phenomena of systemic autoimmune inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is the presence of a broad spectrum of autoantibodies. These molecules are antibodies with specificity for a variety of ubiquitous endogenous antigens, although they are not restricted to disease. Rather, autoantibodies are a component of the healthy immune system (1).

Certain specificities of autoantibody are classically associated with RA. Rheumatoid factor (RF), immunoglobulins type M (IgM), A (IgA) and G (IgG) with specificity for the Fc portion of IgG, is present in 70–80% of patients (2, 3), whilst autoantibodies against the modified amino acid citrulline (anti-CCP antibodies) are highly specific to RA (>95%) (4, 5). A large class of autoantibodies with specificity for a variety of nuclear antigens, termed antinuclear antibodies (ANA), is also observed in RA. These typically occur in up to 50% of patients (6-10). The specific antigens for ANA include DNA (anti-dsDNA; present in <5% of RA patients (7-10)), ribonucleoproteins such as Ro/SSA (anti-Ro; <15% (6, 10-12)), La/SSB (anti-La; <15% (10-12)) and Sm (anti-Sm; <5% (10)), and nucleosomes/histones. Despite the common finding of ANA in patients with RA and their potential to form immune complexes, there is currently no firm evidence to indicate a direct role in disease pathogenesis.

The current view for the genesis of autoantibodies is one whereby autoantigens themselves directly drive autoantibody production (13). It is hypothesised that such autoantigens, nuclear-derived in the case of ANA, may become exposed to the immune system as a result of aberrations in apoptosis (programmed cell death) and the clearance of apoptotic material from the circulation (13, 14). Indeed, nucleosomes and other nuclear antigens have been detected in surface blebs of cells undergoing apoptosis (15, 16). Nucleosomes are also strong autoantigens for T and B cells (17), which correlates with the presence of nucleosome-specific T cells (18).

Tumour necrosis factor-α (TNF-α) is a chronically over-expressed cytokine pivotal in the pathogenesis of RA. Its actions are mediated through binding to each of two specific cell surface receptor molecules, TNF receptor I (TNFR-I; TNFRSF1A [TNFR superfamily 1A]) and TNFR-II (TNFRSF1B). Although only TNFR-I possesses a death domain (19), both receptors can mediate the signal for TNF-α-induced apoptosis (20, 21). The role of TNF-α in the induction of apoptosis is well established. Furthermore, several recent reports demonstrate an inhibitory role of TNF-α in the phagocytic clearance of apoptotic cells by macrophages (22-24), an effect which would also diminish the anti-inflammatory response initiated in macrophages following cell engulfment.

Polymorphism within the TNFR-I (36A/G; rs767455) and/or TNFR-II (676T/G; rs1061622) genes has been associated with RA susceptibility (25-27), extraarticular manifestations (28) and the response to anti-TNF-α therapy (29, 30). Furthermore, the 676T/G polymorphism in TNFR-II has been reported to increase cellular sensitivity to TNF-α-mediated cytotoxicity and to enhance apoptosis mediated through TNFR-I (31, 32). Collectively, these data implicate TNF-α-induced apoptosis as a possible factor in the genesis of ANA. This prompted us to examine the relationship between ANA and polymorphism in the TNFR genes in patients with RA.

Materials and methods

Patients

A cohort of 267 RA patients (104 male, 163 female) who satisfied the 1987 American College of Rheumatology revised criteria set for the classification of RA (33) were studied. All patients were Caucasian, were resident in North Staffordshire, England, and were attending the Staffordshire Rheumatology Centre as outpatients for the treatment of RA. The average age was 55.2±13.0 years (mean ± SD), with an average age at onset and disease duration of 49.0±14.0 and 6.3±7.7 years respectively. Approximately 60% (150/252) of the patients were RF

(ever) positive, 73% (153/205) were positive for anti-CCP antibodies and 70% (166/234) had ever smoked. Data on the level of systemic inflammation (measured by the erythrocyte sedimentation rate [ESR] and C-reactive protein [CRP] levels) and disease activity (measured using the disease activity score from 28 joints [DAS28]) were also available.

At the time of sample collection, all patients were receiving a variety of disease modifying anti-rheumatic drugs, as previously described (34). No patients were receiving anti-TNF-α treatment. Serum levels of ANA were measured by indirect immunofluorescence on HEp2 cells as part of the routine laboratory investigations, and were recorded as the lowest titer (dilution) at which ANA could be detected. Patients were considered to be ANA positive following detection of ANA at the lower dilution of 1:80 (designated ANA80). The study was approved by the North Staffordshire local Research Ethics Committee.

Genomic DNA isolation and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

Allelic genotyping of the TNFR-I 36A/G and TNFR-II 676T/G single nucleotide polymorphisms (SNP) was conducted using PCR-RFLP analysis as previously described (34). Briefly, genomic DNA was extracted from peripheral blood samples (4 ml) using the DNAce MegaBlood Kit as recommended by the manufacturer (Bioline, London, UK). Subsequently, DNA fragments containing the 36A/G and 676T/G SNP were amplified by PCR using sequence specific primer sets (35, 36), and the resultant PCR products were digested using the restriction endonucleases MspA1 I and Nla III for the TNFR-I and TNFR-II SNP, respectively. Allelic typing of each SNP was accomplished through separation of restriction fragments by agarose gel electrophoresis and subsequent visualisation of the restriction fragment pattern: for the 36A/G SNP, the A allele gave rise to a single 183-bp fragment and the G allele to fragments of 108 and 75-bp, whilst the T allele of the 676T/G SNP gave rise to fragments of 133 and 109-bp, and the G allele to a fragment of 242-bp.

Enzyme-linked immunosorbant assay (ELISA)

In addition to genotyping, serum samples were available for approximately half of the patients. The levels of soluble TNFR-I (sTNFR-I; n=138) and sTNFR-II (n=154) and TNF-α (n=83) were quantified by ELISA using the respective Duoset ELISA Development Kit, performed as directed by the manufacturer (R&D Systems, Abingdon, UK). Sera were diluted 1:10 and 1:20, respectively, for the detection of sTNFR-I and sTNFR-II, and run undiluted for detection of TNF-α. All samples were run in duplicate on 96-well microplates, in conjunction with the appropriate standards. Measurement of optical density was performed at 450 nm.

Statistical analysis

Association between the individual TNFR SNP and ANA status was assessed using logistic regression analysis with adjustment for age, sex and disease duration. This technique was also used to identify differences in soluble TNF-α and sTNFR levels between patients with and without ANA. To identify the primary association(s) with ANA status, a series of multivariate logistic regression models were analysed which incorporated TNFR genotypes and soluble levels plus various demographic and clinical data. All data were analysed using Number Cruncher Statistical System 2000 for Windows (NCSS 2000; NCSS LCC, USA). *P*-values <0.05 were considered statistically significant.

Results

ANA status and TNFR SNP

The genotype and allele frequencies for the TNFR-I 36A/G and TNFR-II 676T/G SNP are similar to those previously reported for Caucasian RA/control populations (25, 28, 30, 34, 37), and satisfied the Hardy-Weinberg equilibrium. Using the ANA80 cut-off, 54/267 patients (20.2%) were positive for ANA. This figure approximately halved with a 1:160 dilution (29 patients, 10.9%). The lowest dilution at which ANA were detected in patient sera was 1:2560.

Table I displays ANA status and genotype frequencies for each of the TNFR SNP. Logistic regression analysis corrected for age, sex and disease duration revealed a significant association between ANA80 status and the TNFR-I 36A/G SNP, with a trend of increasing ANA frequency with G allele dosage (p=0.004). Patients homozygous for the G allele were more likely to be ANA80 positive than were patients with the other genotypes combined (31.8 *vs*. 18.0%; odds ratio (OR) 2.1, 95% confidence interval (CI) 1.0–4.4, p=0.051).

Table I. TNFR-I 36A/G and TNFR-II 676T/G genotype frequencies in rheumatoid arthritis (RA) patients stratified by status of antinuclear antibody at a dilution of 1:80 (ANA80).

Genotype	ANA80 status*	
	Negative, n (%)	Positive, n (%)
TNFR-I		
AA	81 (89.0)	10 (11.0)
AG	101 (77.1)	30 (22.9)
GG	30 (68.2)	14 (31.8)§
TNFR-II		
TT	115 (80.4)	28 (19.6)
TG	79 (79.8)	20 (20.2)
GG	1 (75.0)	6 (25.0)

*ANA80 data was available for 266/267 patients.

§A significant association between the TNFR-I 36A/G SNP and ANA80 status was observed, with a trend of increasing ANA frequency with G allele dosage (p=0.004; logistic regression analysis adjusted for age, sex and disease duration). A positive ANA80 was significantly more likely in patients homozygous for the TNFR-I G allele (31.8 *vs*. 18.0%; odds ratio (OR) 2.1; 95% confidence interval (CI) 1.0–4.4; p=0.051). The TNFR-II 676T/G SNP was not associated with ANA status.

ANA status was not associated with the TNFR-II 676T>G SNP.

ANA status and soluble TNFR and TNF-α levels

We next investigated the relationship between ANA status and levels of sTNFR and TNF-α in patient sera. Table II displays these levels in ANA positive and negative patients. A negative association was found between sTNFR-I levels and ANA80 status (p=0.048), but no associations were found with sTNFR-II (p=0.8) or TNF-α (p=0.067).

Consistent with previous studies (38-40) the serum levels of sTNFR-I and II were associated with CRP levels (r_s=0.305, p=0.0008, and r_s=0.266, p=0.004, respectively; Spearman's Rank correlation), although only sTNFR-II was significantly associated with disease activity (DAS28, r_s=0.270, p=0.002). However, measures of systemic inflammation or disease activity showed no association with ANA80 status, TNFR genotypes or any combination of ANA positivity and TNFR genotype (data not shown).

As TNFR-I is considered the primary TNFR responsible for mediating TNF-α-induced apoptosis, we were interested to see whether the ratio of the two receptors may be related to the development of ANA. In logistic regression analyses we identified a significant association between the sTNFR-II/sTNFR-I ratio and ANA80 status (Table III), with an increased ratio in ANA positive patients (p=0.023; adjusted for age, sex and disease duration). This is explained by the observed decrease in sTNFR-I levels in ANA positive patients, rather than an increase in sTNFR-II (4587 *vs*. 4561 pg/ml, mean sTNFR-II levels, ANA80 negative *vs*. ANA80 positive; Table II). Examination of the ratio of TNF-α with either sTNFR did not reveal any association with ANA status (data not shown).

Association of ANA with TNFR SNP and sTNFR levels in multivariate analyses

To determine which factors displayed the primary associations with ANA status, we performed multivariate logistic regression analysis incorporating each TNFR SNP, the levels of both sTNFR and also various clinical data (age, sex, disease duration, ESR, CRP, anti-CCP status and RF status) as independent variables in the model. This allowed us to adjust for any effects on ANA80 status of other clinical parameters, including measures of inflammation. The TNFR-I 36A/G SNP was found to have the most significant association with ANA80 status (p=0.003), with the frequency of ANA80 positive patients increasing with G allele dosage. The only other factor to show independent association with ANA80 status was the level of sTNFR-I (p=0.014 [sTNFR-II, p=0.061]).

We then repeated the regression analysis but replacing the TNFR SNP genotype terms in the model with the GG genotype term for each TNFR SNP. This again revealed that the best independent predictor of a positive ANA80 was the TNFR 36A/G SNP, where patients carrying the GG genotype were significantly more likely to be ANA positive (OR 7.8, 95% CI 1.7–35.3, p=0.008). As before, the only other significant variable was the level of sTNFR-I (p=0.018 [sTNFR-II, p=0.083]). Forward stepwise selection confirmed only these two variables displayed significant independent associations with ANA80 status (Table IV).

Table II. Soluble tumour necrosis factor receptor (sTNFR) and TNF-α levels in RA patients stratified by ANA80 status.

Ligand/Receptor*		ANA80 status	
		Negative	Positive
sTNFR-I	pg/ml	1629 ± 905	1301 ± 443§
	n. (%)	101 (73.7)	36 (26.3)
sTNFR-II	pg/ml	4587 ± 2011	4561 ± 1964
	n. (%)	116 (75.8)	37 (24.2)
TNF-α	pg/ml	193 ± 331	88 ± 160
	n. (%)	58 (69.9)	25 (30.1)

*sTNFR and TNF-α levels are expressed as the mean ± S.D.
§A significant negative association between sTNFR-I levels and ANA80 was identified (p=0.048; logistic regression analysis adjusted for age, sex and disease duration). No significant associations were identified with sTNFR-II or TNF-α.

Table III. Ratio of soluble TNFR-II/TNFR-I in RA patients stratified by ANA80 status.

ANA80 status	No. (%)	sTNFR-II/sTNFR-I ratio*
Negative	99 (73.3)	3.06 ± 1.1
Positive	36 (26.7)	3.58 ± 0.9

*sTNFR-II/sTNFR-I ratios are expressed as the mean ± S.D.
Logistic regression analyses (correcting for age, sex and disease duration) revealed a significant positive association between the sTNFR-II/sTNFR-I ratio and ANA80 status (p=0.023).

Table IV. Stepwise multivariate regression analysis identifying the best predictors of a positive ANA80 in patients with RA.*

Independent variable	Regression coefficient	Standard error	Odds ratio (95% CI)	p-value
TNFR 36A/G GG	1.824	0.71	6.2 (1.5–25.0)	0.011
Serum sTNFR-I	-0.002	<0.01	–	0.005

*Forward stepwise logistic regression analysis. ANA80 was the dependent (response) variable, with TNFR-I 36A/G GG genotype, TNFR-II 676T/G GG genotype, serum levels of sTNFR-I and sTNFR-II (pg/ml), age, sex, disease duration, ESR (erythrocyte sedimentation rate), CRP (C-reactive protein), RF (rheumatoid factor) and anti-CCP (anti-cyclic citrullinated protein) as independent (predictor) variables. Only significant variables retained in the stepwise model are shown.
95% CI represents 95% confidence interval.

Discussion

The presence of antigen-specific autoantibodies remains a conundrum in both health and disease. The majority of ANA observed in RA appear to be a consequence of disease, although they may still play an as yet undefined role in disease pathogenesis. Herein, we have identified in RA patients a previously unreported association between the presence of ANA and an exonic SNP at position +36 in the TNFR-I gene, suggesting involvement of the TNF-α/TNFR signalling pathway in the genesis of ANA. We used detection of ANA at a dilution of 1:80 as indication of a positive result. Whilst this titer is lower than those typically used to confirm ANA in disease, our aim was to determine if there was an intrinsic association between ANA and TNFR polymorphisms, rather than a disease-specific relationship.

In the cohort of RA patients studied we observed a significant increasing trend of ANA frequency according to G-allele dosage for the TNFR-I 36A/G polymorphism (AA<AG<GG). In particular, patients homozygous for the G-allele were twice as likely to be ANA positive as patients without this genotype. The risk associated with carriage of this genotype was substantially increased (8-times) when we conducted multivariate logistic regression analyses to adjust for other independent variables, including ESR, CRP, RF, anti-CCP and demographic data. In fact, the 36A/G SNP, together with the levels of soluble TNFR-I, were the only variables to be independently associated with ANA. Conversely, there was no evidence of association between ANA status and the TNFR-II 676T/G SNP, a polymorphism which has previously been associated with increased cytotoxicity to TNF-α (31) and enhanced TNFR-I-induced apoptosis (32).

The 36A/G SNP occurs within the first intron of the TNFR-I gene. It is a non-functional polymorphism, with both the A- and G-alleles coding for the non-polar amino acid proline. The observed association with ANA cannot therefore be explained directly by the SNP itself. Rather, it indicates that the association may actually be with a nearby functional polymorphism(s) in linkage disequilibrium with the 36A/G SNP. Possible candidates include the known exonic mutations dominantly inherited in TRAPS (TNF receptor-associated periodic syndrome) (41, 42). The TRAPS mutations have previously been associated with modulation of TNF-α binding and/or release of soluble TNFR (42), demonstrating that sequence alterations in the TNFR-I gene can have functional consequences for TNF-α/TNFR signalling.

Supporting a role for TNFR-I polymorphism and TNF-α-induced apoptosis in the genesis of ANA is the parallel finding that polymorphism within the promoter for the death receptor Fas is associated with both the presence and induction of ANA (43, 44). Fas is a member of the TNFR superfamily and binds Fas ligand (FasL), a member of the TNF superfamily, leading to potent induction of the apoptotic pathway. Both the TNF-α/TNFR and FasL/Fas pathways require the adaptor protein FADD (Fas-associated death domain) for apoptosis signal transduction (45), culminating in caspase-mediated cell death. The distinct functional similarity between the two pathways strengthens the suggestion that a functional polymorphism in linkage with the TNFR-I 36A/G SNP may, in a similar manner to Fas polymorphism, influence the development of ANA.

Any influence of TNFR polymorphisms on ANA formation might also be derived through effects on the nuclear factor-kappa B (NF-κB) signalling pathway, the other major pathway activated by TNF-α. In addition to targeting genes with survival and pro-inflammatory actions, the NF-κB transcription factor also activates anti-apoptotic genes, such as those coding for the cellular inhibitor of apoptosis proteins (cIAP). Thus, the two pathways are coupled: as one pathway is activated, the other is suppressed. The 676T>G polymorphism in TNFR-II has previously been associated with reduced NF-κB signalling and diminished induction of cIAP (32). Whether polymorphism within the TNFR-I gene could have similar effects is unclear at present – conflicting evidence from patients with TRAPS suggests that some TNFR-I mutations convey reduced NF-κB and apoptotic signalling (46, 47), whilst others do not (46).

Accompanying the association between the TNFR-I 36A/G SNP and ANA, we identified a negative association of ANA with serum levels of sTNFR-I (but not sTNFR-II levels, which remained unchanged). The reduced levels of soluble TNFR-I in ANA positive patients may imply that the levels of membrane-bound TNFR-I, from which sTNFR-I are derived, are increased in these patients. This would be expected to favour increased TNF-α signalling through TNFR-I, including enhanced TNF-α-induced apoptosis. Of interest however, sTNFR-I have been demonstrated to induce apoptosis through reverse signalling via membrane-bound TNF-α (48), an activity independent of the traditional death receptor pathways. Further studies are required to explore the mechanism behind the association observed between sTNFR-I and ANA.

In a recent study the development of ANA was associated with age (49). We found no such association in our multivariate analyses, nor any association of ANA with disease duration or inflammatory markers. Although none of the patients studied were receiving anti-TNF-α therapy, the development of ANA is a common finding in such patients (6, 8-10). This observation opposes a direct role for TNF-α/TNFR signalling in the genesis of ANA. However, formation of ANA in anti-TNF-α treated patients may actually be related to reduced clearance of apoptotic nuclear material by CRP (50, 51), the levels of which decrease significantly with TNF-α inhibitors. Although not significant, we observed a negative trend between CRP levels and the frequency of ANA (p=0.2, adjusted logistic regression), suggesting that disturbances in apoptotic clearance by CRP may also be involved in ANA genesis in anti-TNF-α naïve individuals. It remains likely that multiple pathways and events lead to the generation of ANA and other autoantibodies, and that TNF-α is just one of many factors in this process.

Whilst ANA may develop as a consequence of apoptosis-induced antigen

exposure, ANA have also been shown to influence the shedding and activation of TACE (TNF-α converting enzyme) (52), an enzyme responsible for the release of soluble TNF-α and TNFR from the cell surface (53-55). In this respect, ANA may inhibit TNF-α/TNFR signalling by promoting the release of TNFR from the cell surface. However, our data do not support this since sTNFR levels were actually lower (sTNFR-I) or unchanged (sTNFR-II) in ANA positive individuals.

We found no association of the TNFR-II 676T/G SNP with ANA. Despite evidence of a functional role for this polymorphism (31, 32), there is no evidence of direct effects on TNF-α-induced apoptosis mediated through TNFR-II. Rather, TNFR-I is considered the primary TNF receptor mediating apoptosis. Thus, if TNF-α-induced apoptosis, through the release of nuclear antigens, does contribute to the development of ANA, polymorphism within the TNFR-II gene might not be expected to significantly influence this process.

In conclusion, we have identified a novel association between ANA and a TNFR-I exonic polymorphism (36A/G) in patients with RA. Since this polymorphism is non-functional, the association is likely with a functional polymorphism(s) in linkage and for which the 36A/G SNP is a marker. Our findings suggest that the TNF-α/TNFR signalling pathway is involved in the genesis of ANA, and that polymorphism in the TNFR-I gene modulates this process, which we hypothesise may be through affects on TNF-α-induced apoptosis. Independent studies on other disease/healthy populations will be required to confirm and extend our findings.

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