Anemia in Rheumatoid Arthritis: Association with Polymorphism in the Tumor Necrosis Factor Receptor I and II Genes

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ABSTRACT. ***Objective.*** To investigate whether polymorphisms in the tumor necrosis factor receptor I (TNFRSF1A) and receptor II (TNFRSF1B) genes are associated with the anemia observed in rheumatoid arthritis (RA).

Methods. We studied a group of Caucasian patients (n = 160) with established RA on whom longitudinal data of hemoglobin (Hb) levels over 5 years were recorded. A second group of patients (n = 102) with early RA was used for a confirmation study. Polymerase chain reaction restriction fragment length polymorphism analysis was used to genotype patients for the A36G polymorphism in the TNFRSF1A gene, and the T676G polymorphism in TNFRSF1B. Serum levels of ferritin were determined by ELISA and used to differentiate between iron deficiency anemia (IDA) and anemia of chronic disease (ACD). Data were analyzed by Kruskal-Wallis analysis of variance and logistic regression analysis.

Results. The TNFRSF1A GG genotype was associated with lower 5-year mean area under the curve Hb levels compared with other genotypes (p = 0.01). Analysis of anemic status showed an increased frequency of anemia in patients carrying a G allele, with the highest frequency in GG homozygotes. The TNFRSF1A GG genotype was significantly associated with IDA in established RA (OR 4.3, p = 0.01), and this was confirmed in a group of patients with early RA (OR 4.8, p = 0.04). Analysis of the combined groups also showed a weak association of the G allele with ACD (OR 2.2, p = 0.04). No association was found between TNFRSF1B variants and anemia when the cohorts were analyzed separately, but an association between carriage of the T allele and ACD was found when the 2 groups were combined (OR 11.5, p = 0.01).

Conclusion. Our data suggest that polymorphisms within the TNFRSF1A and TNFRSF1B genes are associated with IDA and/or ACD in patients with RA. (J Rheumatol 2005;32:1673–8)

Key Indexing Terms:
RHEUMATOID ARTHRITIS   TUMOR NECROSIS FACTOR-α   ANEMIA
TUMOR NECROSIS FACTOR RECEPTOR   POLYMORPHISM

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints that ultimately leads to joint destruction. Despite recent advances, the precise etiology of the disease remains to be elucidated. Anemia is a common extraarticular manifestation in RA, with up to 50% of patients developing the condition during the disease course[1]. The main causes of this are iron deficiency anemia (IDA) and anemia of chronic disease (ACD), the development of which is poorly understood. The high frequency of anemia observed in these patients suggests that components of the inflammatory response such as cytokines may be important in the pathogenesis of this condition.

Tumor necrosis factor-α (TNF-α) is now considered a major factor in the development and chronicity of RA[2]. This has led to the development of new biologic therapies targeted at this cytokine[3,4]. In patients with RA, anti-TNF-α therapy has been shown to produce significant improvements in hemoglobin (Hb) levels[3,5,6]. Further, serum TNF-α concentrations have been found to correlate inversely with Hb[7,8]. These findings suggest an important role for TNF-α in the anemia associated with RA.

Erythropoiesis is the process of erythrocyte development and maturation. It has been demonstrated that TNF-α is able to suppress this process both *in vitro*[9,10] and *in vivo*[11], although this inhibition may be an indirect effect mediated via TNF-α–induced production of ß-interferon[9]. The major hormone in this process is erythropoietin (EPO), the mRNA levels of which appear to be inhibited by TNF-α[12]. However, anti-TNF-α administration does not result in recovery of EPO concentrations[5], suggesting that EPO pro-

tein levels are not a specific target of TNF-α mediated inhibition of erythropoiesis. The bone marrow has been proposed as an alternative target for this inhibition, where local production of TNF-α is increased in RA patients with ACD[13] and where TNF-α is thought to induce apoptosis of erythroid progenitor cells[6].

TNF-α may also have direct effects on the homeostatic regulation and metabolism of iron, a key component of the oxygen-carrying group heme. It has been shown that TNF-α inhibits macrophage iron release *in vitro*[14] and significantly reduces both the incorporation of plasma ^{59}Fe into newly synthesized erythrocytes and erythrocyte survival[15]. The importance of TNF-α in erythropoiesis and anemia is therefore apparent, perhaps more so in RA since the levels of this cytokine are significantly elevated[16,17].

Two distinct cell-surface receptor molecules, termed TNF receptor (TNFR) superfamily 1A (TNFRSF1A, p55) and TNFRSF1B (p75), transduce the TNF-α signal upon ligand-binding[18,19]. The extracellular domains of these receptors can also be shed by proteolytic cleavage, and act as natural inhibitors of TNF-α activity. The gene for TNFRSF1A has been mapped to 12p13 and consists of 10 exons, while that for TNFRSF1B, also consisting of 10 exons, maps to 1p36[20,21]. Analysis of these genes has identified multiple polymorphisms[22-24]. One of these in TNFRSF1B at nucleotide 676 (T → G; T676G) has been shown to be associated with susceptibility to familial RA, although this was not found in sporadic RA[25-27]. Similarly, no association has been found between TNFRSF1A polymorphism and sporadic RA[25,27], although a recent study has suggested that polymorphism at nucleotide 36 (A36G) has a protective role in familial RA[28].

We investigated whether the T676G polymorphism in the TNFRSF1B gene and the A36G polymorphism in TNFRSF1A are associated with the anemia observed in RA.

MATERIALS AND METHODS

Patients. The association of TNFR polymorphisms with Hb levels and anemia was studied in 160 patients with established disease (≥ 5 yrs). All the patients were of Caucasian origin and were resident in North Staffordshire, England. The demographic data are displayed in Table 1. All patients had previously been diagnosed with RA, and met the 1987 American College of Rheumatology criteria[29]. Patients with established disease were receiving various disease modifying antirheumatic drugs (DMARD), the frequencies of which have been described[30]. A second group of 102 patients with early RA (≤ 2 yrs) was used for confirmation of findings in patients with established disease (Table 1).

Genomic DNA isolation. Fresh peripheral blood samples of about 4 ml were obtained from each patient and stored in EDTA tubes at –20°C. When required, blood samples were thawed at 37°C and the genomic DNA isolated using a DNAce MegaBlood Kit procedure (Bioline Ltd., London, England). Briefly, this is a standard ethanol-based procedure for the isolation of genomic DNA from whole blood, and was performed as directed by the manufacturer.

Polymerase chain reaction (PCR) primers. Amplification of a 183 bp fragment containing the A36G polymorphism in exon 1 of the TNFRSF1A gene was performed using the following primer sequences: forward: 5'-GAG CCC AAA TGG GGG AGT GAG AGG-3'; and reverse: 5'-ACC AGG CCC GGG CAG GAG AG-3'[22].

The primer sequences used to amplify a 242 bp fragment containing the T676G polymorphism in exon 6 of the TNFRSF1B gene were: forward: 5'-ACT CTC CTA TCC TGC CTG CT-3'; and reverse: 5'-TTC TGG AGT TGG CTG CGT GT-3'[31].

PCR amplification. The fragment of interest from each of the TNFR was amplified using an identical reaction mixture as described[30]. All amplification reactions were performed in a Flexigene Thermal Cycler unit [Techne (Cambridge) Limited, Cambridge, England] using a 96 well full-skirt heating block. The reaction conditions used for amplification of the DNA fragments containing the TNFRSF1A and TNFRSF1B polymorphisms have been described[30]. Following amplification, the products were stored at 4°C until required for restriction fragment length polymorphism analysis[30].

Criteria for diagnosis of anemia. Patients were considered to be anemic if they had Hb levels below 13.3 and 12.0 g/dl for men and women, respectively. In the established RA group the 5-year mean area under the curve (AUC) Hb level was used to identify anemic patients. This was calculated from Hb measurements taken every 6 months. In the early RA group the baseline Hb level at first clinic visit was used. To differentiate between ACD and IDA, standard ELISA methodology was used to determine the serum concentrations of ferritin. Assays were performed according to the manufacturers' instructions (ICN Pharmaceuticals, Ltd., Basingstoke, England).

During inflammation, ferritin acts as an acute phase protein and is thus present at elevated levels over normal. Previous studies have determined that a ferritin cutoff point of 60 ng/ml is appropriate for differentiation between IDA and ACD in anemic patients with inflammatory disease (IDA < 60 ng/ml; ACD ≥ 60 ng/ml)[32-34]. A classification of IDA or ACD was assigned to each patient only if the cutoff points for Hb and ferritin were appropriately satisfied.

Statistical analysis. Associations between the TNFR genotypes and Hb levels were assessed using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks. Correction for multiple comparisons was carried out using the Kruskal-Wallis Z value test. Contingency tables and Fisher's exact test were used to examine the frequency of different genotypes in patients with IDA and ACD. In addition, logistic regression analyses were used to adjust for age, sex, and disease duration when examining associations between the TNFR genotypes and anemia. All data were analyzed using the Number Cruncher Statistical Software package for Windows (NCSS 2000). A p value < 0.05 was considered significant.

RESULTS

Association of TNFR genotypes with Hb levels and anemia. The genotype frequencies for each of the TNFR polymorphisms, together with the respective Hb levels in patients with established RA, are displayed in Table 2. The TNFRSF1A A36G and TNFRSF1B T676G allele and genotype frequencies are similar to those reported for Caucasian sporadic RA/control populations[25,26,28,30,35]. The observed

Table 1. Characteristics of the 2 rheumatoid arthritis populations.

	Established RA	Early RA
No.	160	102
Male/female, no.	70/90	44/58
Age, mean ± SD, yrs	57.8 ± 10.5	54.8 ± 13.8
Age at onset, mean ± SD, yrs	46.1 ± 11.2	54.1 ± 13.8
Disease duration, mean ± SD, yrs	11.8 ± 4.8	0.7 ± 0.5
Rheumatoid factor positive (%)	108/158 (68.4)	52/93 (55.9)
Nodule positive (%)	30/160 (18.8)	3/101 (3.0)

frequencies for both polymorphisms were found to be in Hardy-Weinberg equilibrium. Analysis of the TNFR genotypes with patient Hb levels by Kruskal-Wallis ANOVA and correction for multiple comparisons revealed that RA patients homozygous for the G allele of TNFRSF1A had significantly lower mean AUC Hb levels than either of the other genotypes individually, or when combined (Table 2). Stratification by sex revealed the same trend in men and women [12.5 vs 13.7, and 12.1 vs 12.5 g/dl (GG vs AA + AG), respectively], although this did not achieve significance in the individual groups (p = 0.08 and 0.1, respectively). There was no association of TNFRSF1A genotype with mean AUC erythrocyte sedimentation rate (ESR) (data not shown), suggesting that the association between TNFRSF1A GG genotype and Hb levels was independent of disease activity. No association was found between the TNFRSF1B polymorphism and mean AUC Hb levels.

The frequency of anemia was 38.8% in the established RA population. Analysis of anemic status revealed a significant association with the TNFRSF1A but not the TNFRSF1B polymorphism (Table 3). Anemia was more frequent in patients carrying a TNFRSF1A G allele, with the highest frequency in GG homozygotes. Logistic regression analysis with correction for age, sex, and disease duration indicated that patients with this genotype were significantly more likely to be anemic than the remainder [55.6 vs 35.3%, odds ratio (OR) 2.6, 95% confidence intervals (CI) 1.1–6.2, p = 0.03].

Association with IDA and ACD. In the established RA group, serum samples were available on 41 anemic patients for measurement of ferritin levels. Of these, 21 (51.2%) were identified as having ACD and 20 (48.8%) as having IDA.

A significant association was found between the TNFRSF1A polymorphism and IDA, with a trend of increasing IDA frequency with G allele dosage (p = 0.01; Table 3). Logistic regression analysis with correction for age, sex, and disease duration indicated that patients with the GG genotype were significantly more likely to have IDA

than the remainder (36.8 vs 13.1%; OR 4.3, 95% CI 1.3–13.8, p = 0.01). The association remained significant (p = 0.02) when the ESR mean AUC was included in the logistic regression analysis, indicating that it was independent of the degree of inflammation.

Confirmation of an association between the TNFRSF1A polymorphism and IDA was obtained in a separate group of patients (n = 102) with early RA. Of these, 38 (37.2%) were anemic, with 12 classified as IDA and 26 as ACD. As in the established group, logistic regression analysis with correction for age, sex, and disease duration revealed that IDA was more frequent in patients with the GG genotype than in those without it (40.0 vs 12.3%; OR 4.8, 95% CI 1.04–22.6, p = 0.04). The significance of the association was increased when analysis was carried out on the 2 groups of patients combined (37.9 vs 12.8%; OR 4.6, 95% CI 1.9–11.4, p = 0.001).

When the 2 RA cohorts were analyzed separately, no significant association was found between the TNFRSF1A polymorphism and ACD, although there was a trend toward increased frequency of ACD with increasing G allele dosage of TNFRSF1A (p = 0.07) in patients with established disease (Table 3). When analysis was carried out on the 2 groups combined there was a weak but significant association between ACD and carriage of the G allele (OR 2.2, 95% CI 1.02–4.7, p = 0.04, corrected for age, sex, and disease duration).

No association was found between the TNFRSF1B genotypes and IDA in either group of patients. Separate analyses of the cohorts also revealed no association between the TNFRSF1B polymorphism and ACD. However, it was noteworthy that in both groups, ACD was found only in patients

Table 2. TNFRSF1A A36G and TNFRSF1B T676G genotype frequencies and mean area under the curve (AUC) hemoglobin (Hb) levels in patients with established RA (n = 160).

Genotype	No. (%)	Mean AUC Hb (SD), g/dl
TNFRSF1A		
AA	50 (31.2)	13.1 (1.2)
AG	83 (51.9)	13.1 (1.6)
GG	27 (16.9)	12.2 (1.1)*
TNFRSF1B		
TT	94 (58.7)	13.0 (1.6)
TG	51 (31.9)	12.9 (1.2)
GG	15 (9.4)	13.2 (1.2)

* p = 0.01 (Kruskal-Wallis one-way ANOVA). The TNFRSF1A GG genotype was significantly different vs both the AA and AG genotypes after correction for multiple comparisons (Kruskal-Wallis Z-value test).

Table 3. TNFRSF1A A36G and TNFRSF1B T676G genotype frequencies in established RA patients with iron deficiency anemia (IDA) and anemia of chronic disease (ACD). * Characterization of IDA and ACD status was possible on 41/62 anemic patients. Significant association was found between the TNFRSF1A polymorphism and anemia, with a trend of increasing anemia frequency with G allele dosage (p = 0.01; logistic regression analysis corrected for age, sex, and disease duration). A similar trend was seen in the association of TNFRSF1A with IDA (p = 0.01). A nonsignificant trend was seen with ACD (p = 0.07). IDA was significantly more frequent in TNFRSF1A GG homozygotes than in the remainder (36.8 vs 13.1%; OR 4.3, 95% CI 1.3–13.8, p = 0.01). No association was found between TNFRSF1B and anemia, IDA, or ACD.

Genotype	Non-anemic n	Anemic n	IDA* n	ACD* n
TNFRSF1A				
AA	36	14	3	4
AG	50	33	10	13
GG	12	15	7	4
TNFRSF1B				
TT	56	38	12	12
TG	32	19	6	9
GG	10	5	2	0

who carried a T allele. Analysis of the combined groups indicated that patients with a T allele were significantly more likely to have ACD than patients lacking this allele (24.6% vs 0%; OR 11.5, 95% CI 1.05–59.9, p = 0.01).

DISCUSSION

Anemia is one of the most common extraarticular manifestations observed in RA[1]. The cytokine TNF-α may have a significant role in the development of this anemia, since it has effects on Hb levels, erythropoiesis, and iron metabolism[7-15]. The biological functions of TNF-α are transduced through the TNF receptors TNFRSF1A and TNFRSF1B, so polymorphisms in these genes represent attractive candidates for involvement in the anemia of RA.

We have identified an association between homozygosity for the G allele of the TNFRSF1A A36G polymorphism and low mean AUC Hb levels in patients with established RA. We chose to calculate the mean AUC Hb levels over a 5 year period in these patients in order to achieve a more robust measure of their anemic status over the course of the disease. Using this information we observed an increasing trend in anemia frequency with TNFRSF1A G allele dosage in these patients. This association appears to be principally with IDA, although there is a similar nonsignificant trend of increasing ACD frequency with G allele dosage. We confirmed an association between the TNFRSF1A polymorphism and IDA in a separate group of patients with early RA.

Although we found no associations between ACD and the TNF receptor polymorphisms in separate cohorts, we did find significant associations when data from the 2 groups were combined. Thus, ACD was significantly increased in patients carrying a TNFRSF1A G allele, as well as in those carrying a TNFRSF1B T allele. Multivariate logistic regression analysis indicates that the TNFRSF1A and TNFRSF1B associations are independent of each other (data not shown). These data clearly need to be treated with caution, since significance was only achieved after combining data from the 2 cohorts. This may reflect a problem of achieving sufficient patient numbers to demonstrate an effect, and further studies on large groups of patients will be needed to clarify the situation.

How variations in the TNFRSF1A and TNFRSF1B genes might influence development of IDA and/or ACD is unclear at present, but it is known that differences exist in the expression and function of these 2 receptors on erythroid progenitor cells. It has been shown that TNF-α directly inhibits the *in vitro* growth of committed erythroid progenitor cells in response to multiple cytokine combinations, and that TNF-α-induced inhibition of burst-forming unit-erythroid colony formation is mainly mediated through TNFRSF1A[36]. There is also evidence to suggest that erythroid progenitors become resistant to the inhibitory effect of TNF-α as they mature, and that this is dependent on altered expression of TNF receptors at various stages of differentiation[37]. Thus, detectable expression of TNFRSF1A has been shown to be transient in the early phase of erythroid differentiation, whereas TNFRSF1B is expressed throughout the entire course of differentiation.

A number of single nucleotide polymorphisms (SNP) of the TNFRSF1A gene have been reported, including several mutations that are dominantly inherited in the TNFR-associated periodic syndrome (TRAPS)[38,39]. This is a condition characterized by attacks of fever, arthralgia, myalgia, rash, and occasionally, systemic amyloidosis. The TNFRSF1A A36G polymorphism in exon 1 is nonfunctional, so the results of our study suggest that anemia may be associated with a nearby functional polymorphism in linkage disequilibrium. The A36G polymorphism is the closest frequent exonic polymorphism to all the known TRAPS-associated mutations found on exons 2–4, which encode the TNFRSF1A extracellular domain. Alterations in the binding of TNF-α and/or receptor shedding have been associated with some of these mutations[39]. It might be speculated, therefore, that such a functional variant in linkage disequilibrium with the A36G polymorphism is responsible for the association with anemia. Further genotyping of known TNFRSF1A SNP will be necessary to determine if such a putative mutation is in linkage disequilibrium with the A36G polymorphism, and whether anemia is associated with a particular haplotype containing the G allele.

We and others have previously failed to find an association between the TNFRSF1A A36G polymorphism and disease severity in RA[30,35], although a weak trend toward increased radiographic and Health Assessment Questionnaire scores with the GG genotype was noted[30]. This may be consistent with an association with anemia, since higher disease severity scores have been found in such patients compared with non-anemic patients (unpublished observations). It is interesting that a recent study has suggested that the TNFRSF1A AA genotype has a protective role in familial RA[28], since this particular genotype is associated with the lowest incidence of anemia in our study.

The TNFRSF1B T676G polymorphism results in a nonconservative amino acid substitution (methionine → arginine; M196R) within the fourth extracellular domain of the folded protein receptor. Functional analysis of this polymorphism in transfected HeLa cells found no effects on the production of soluble receptor, although there was increased interleukin 6 (IL-6) production in cells with the 196R polymorphism[40]. ACD has been associated with elevated concentrations of IL-6 in RA[13,41], so an association with the TNFRSF1B G allele (encoding the 196R variant) rather than the T allele might have been expected. However, one study found no pathogenic role for IL-6 in ACD, and reported a stimulatory effect of this cytokine on erythroid growth[41]. A positive correlation has also been found between IL-6 and EPO levels in ACD secondary to infection, which also suggests a pro-erythropoietic action of IL-6 in anemia[42].

Our data suggest that polymorphisms within the TNFRSF1A and TNFRSF1B genes are associated with IDA and/or ACD in patients with RA. The association between the TNFRSF1A GG genotype and IDA has been confirmed in 2 separate patient cohorts, but the association of the TNFRSF1A and TNFRSF1B polymorphisms with ACD has been shown only by combining patient groups.

Independent studies on other populations of RA patients are therefore needed to confirm these findings.

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