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Reduced Tumor Necrosis Factor Signaling in Primary Human Fibroblasts Containing a Tumor Necrosis Factor Receptor Superfamily 1A Mutant

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Abstract

Objective—Tumor necrosis factor receptor–associated periodic syndrome (TRAPS) is an autoinflammatory syndrome associated with mutations in the gene that encodes tumor necrosis factor receptor superfamily 1A (TNFRSF1A). The purpose of this study was to describe a novel TNFRSF1A mutation (C43S) in a patient with TRAPS and to examine the effects of this TNFRSF1A mutation on tumor necrosis factor α (TNF α)–induced signaling in a patient-derived primary dermal fibroblast line.

Methods—TNFRSF1A shedding from neutrophils was measured by flow cytometry and enzyme-linked immunosorbent assay (ELISA). Primary dermal fibroblast lines were established from the patient with the C43S TRAPS mutation and from healthy volunteers. Activation of NF- κ B and activator protein 1 (AP-1) was evaluated by electrophoretic mobility shift assays. Cytokine production was measured by ELISA. Cell viability was measured by alamar blue assay. Apoptosis was measured by caspase 3 assay in the fibroblasts and by annexin V assay in peripheral blood mononuclear cells.

Results—Activation-induced shedding of the TNFRSF1A from neutrophils was not altered by the C43S TRAPS mutation. TNF α -induced activation of NF- κ B and AP-1 was decreased in the primary dermal fibroblasts with the C43S TNFRSF1A mutation. Nevertheless, the C43S TRAPS fibroblasts were capable of producing interleukin-6 (IL-6) and IL-8 in response to TNF α . However, TNF α -induced cell death and apoptosis were significantly decreased in the samples from the patient with the C43S TRAPS mutation.

Conclusion—The C43S TNFRSF1A mutation results in decreased TNF α -induced nuclear signaling and apoptosis. Our data suggest a new hypothesis, in that the C43S TRAPS mutation may cause the inflammatory phenotype by increasing resistance to TNF α -induced apoptosis.

Tumor necrosis factor receptor–associated periodic syndrome (TRAPS; MIM no. 142680) is an autosomal-dominant inherited autoinflammatory syndrome characterized by recurrent fevers and abdominal pain associated with cutaneous, muscle, and joint inflammation. It is associated with mutations in the gene that encodes tumor necrosis factor receptor

superfamily 1A (TNFRSF1A) (1). At least 40 TNFRSF1A mutations associated with TRAPS have been reported on the INFEVERS (Internet periodic fevers) Web site (<http://fmf.igh.cnrs.fr/infevers>) (2). Initial studies suggested that the TRAPS mutations impair activation-induced shedding of TNFRSF1A (1,3), although this is not the case for all mutations (4,5) and may be dependent on cell type (6). There are no published studies on tumor necrosis factor α (TNF α)-induced activation of transcription factors or apoptosis in cells derived from patients with TRAPS. The mechanisms by which these TNFRSF1A point mutations result in the inflammatory phenotype remain unclear.

TNF α exerts its proinflammatory effects through 2 receptors, namely, TNFRSF1A (TNFRI p55) and TNFRSF1B (TNFRII p75). TNFRSF1A is widely expressed and appears to be the major receptor for soluble TNF α -induced signaling (7). Activation of these receptors recruits adapter proteins to the intracellular domain of the receptor and activates downstream signaling cascades (8). This causes the activation of NF- κ B and activator protein 1 (AP-1), which regulate the transcription of a variety of genes, including interleukin-6 (IL-6) and IL-8. Many of these TNF α -induced molecules (such as NF- κ B, IL-6, and IL-8) are elevated in inflammatory conditions, such as rheumatoid arthritis (9), making them good candidates for investigation in TRAPS. In addition, the intracellular domain of TNFRSF1A contains a death domain motif, which is involved in TNF-induced apoptosis via activation of a caspase cascade (8).

We describe a novel TNFRSF1A mutation (C43S) in a patient with TRAPS that does not impair activation-induced shedding of TNFRSF1A. We generated a primary dermal fibroblast line from this patient that showed decreased TNF α -induced NF- κ B and AP-1 activation relative to that in normal controls. However, TNF α was able to induce IL-6 and IL-8 to levels similar to those in the controls. TNF α -induced cell death and apoptosis were markedly decreased in the fibroblasts with the C43S TRAPS mutation compared with the control fibroblasts. In addition, we observed decreased TNF α -induced apoptosis in the patient's peripheral blood mononuclear cells (PBMCs). We hypothesize that this reduced apoptosis in response to TNF α may be a factor in the inflammatory phenotype of this patient.

MATERIALS AND METHODS

Generation of primary dermal fibroblast cell line

Primary skin fibroblast lines were established using an adapted method (10) after ethical permission and informed consent were granted. Lignocaine (1%) was injected intradermally to anesthetize and raise the biopsy area. A small biopsy sample ($\sim 2\text{mm}^3$) was obtained, cut into 8 small fragments, placed into 35-mm surface-modified tissue culture dishes (Primaria Easy Grip; Becton Dickinson, Mountain View, CA), and covered with a glass coverslip. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% (volume/volume) fetal calf serum (FCS), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM glutamine. The medium was changed at weekly intervals. When cells were confluent, they were transferred to tissue culture flasks and maintained in DMEM containing 10% v/v FCS, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM glutamine. Early-passage fibroblasts were stored in liquid nitrogen.

Generation of cell extracts

Early-passage fibroblasts (passages 5–10) were seeded overnight at 0.3×10^6 cells per 60-mm tissue culture dish. They were stimulated with TNF α (Calbiochem, La Jolla, CA). Stimulation was terminated by removal of DMEM and addition of ice-cold phosphate buffered saline. Cells were harvested using a cell scraper. All buffers were supplemented

with a protease inhibitor (phenylmethylsulfonyl fluoride) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). Nuclear extracts were generated and electrophoretic mobility shift assays (EMSAs) were performed as previously described (11).

Annexin V assay in PBMCs

PBMCs were isolated from fresh whole blood using a Ficoll gradient. PBMCs (2×10^6) were placed in 2 ml RPMI 1640 with 10% FCS and were left untreated or were stimulated with TNF α and cycloheximide (CHX; 50 μ g/ml) either alone or in combination. Apoptosis was assayed using the TACS annexin V–fluorescein isothiocyanate kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions, after which fluorescence was measured by flow cytometry.

RESULTS

Identification of a novel TNFRSF1A mutation (C43S)

The index patient was a 50-year-old woman of Welsh origin with recurrent attacks of fever, pharyngitis, and arthritis accompanied by a migrating skin rash and myalgia. Her first attack occurred at the age of 18 months, with attacks typically lasting 1–2 weeks. The patient's deceased father had similar recurrent episodic fevers. The patient's clinical picture was consistent with the reported TRAPS phenotype (1). Mean $\pm$ SEM plasma levels of soluble TNFRSF1A were $3,839 \pm 131$ pg/ml, which is consistent with levels reported in TRAPS patients with renal impairment (5). DNA was extracted from the patient's blood, and polymerase chain reaction (PCR) amplifications were performed, with subsequent sequencing of the PCR products. We identified a novel TNFRSF1A mutation (nucleotide 215 G $\rightarrow$ C, exon 3) resulting in the substitution of serine for cysteine at residue 43 (C43S). This amino acid substitution disrupts the disulfide bond at this position in the first extracellular domain of TNFRSF1A. This substitution was not observed in any of the 734 control chromosomes screened by genomic sequencing.

Effect of the C43S mutation on shedding of TNFRSF1A

To investigate whether TNFRSF1A shedding was affected by the C43S mutation, we measured levels of TNFRSF1A on the surface of the patient's neutrophils by flow cytometry. Following phorbol myristate acetate (PMA) treatment, membrane TNFRSF1A decreased to levels comparable with those of healthy controls (Figure 1A). To establish whether this decrease in surface TNFRSF1A was a result of internalization or cleavage of the receptor, levels of soluble TNFRSF1A were measured in the supernatant of the cells. Soluble TNFRSF1A levels increased in response to PMA, indicating that the loss of surface TNFRSF1A was a result of receptor shedding (Figure 1B). Activation-induced cleavage was therefore not impaired by the C43S mutation, and could not account for the inflammatory phenotype in this patient.

Generation of a primary TRAPS fibroblast line

To facilitate the study of the C43S TNFRSF1A mutation, we generated a primary dermal fibroblast cell line from a skin biopsy sample taken from the patient. The skin is an accessible site for obtaining fibroblasts and is also the site of the prototypical rash that characterizes TRAPS. The sample was taken from an area of normal skin during an asymptomatic period. Similar dermal fibroblast lines were generated from age- and sex-matched healthy volunteers and used as controls.

Role of the C43S TNFRSF1A mutation in NF- κ B and AP-1 activation and induction of IL-6 or IL-8

TNFRSF1A nuclear signaling was investigated using the dermal fibroblast lines. Cells were stimulated with 10 ng/ml of TNF α for 1 hour, after which nuclear extracts were generated. NF- κ B activation was determined by EMSA using radiolabeled DNA corresponding to a specific NF- κ B site. All the fibroblast lines were able to activate NF- κ B in response to TNF α . However, the activation was consistently less in fibroblasts from the patient with TRAPS compared with fibroblasts from healthy controls (Figure 2A). There was no evidence of constitutive activation of NF- κ B in the TRAPS fibroblasts. In response to IL-1 stimulation, the TRAPS fibroblasts activated NF- κ B to levels similar to those of healthy controls (data not shown). Supershift assays with antibodies to NF- κ B subunits indicated that the complexes are very similar, with both the TRAPS and control fibroblasts activating an NF- κ B complex containing p50 and RelA subunits (Figure 2B).

To determine whether the NF- κ B activation in C43S TRAPS fibroblasts was more sensitive to TNF α or more prolonged than in healthy controls, TNF α dose-response and time-course experiments were performed. TNF α -induced NF- κ B activation was consistently lower in the TRAPS fibroblasts at all doses of TNF α tested (Figure 2C). In addition, the duration of TNF α -induced NF- κ B activation was significantly shorter in the C43S TRAPS fibroblasts compared with the control fibroblasts (Figure 2D). There was also no increase in NF- κ B activation at any later time points up to 24 hours (results not shown). The C43S fibroblasts also resulted in less activation of AP-1 compared with the normal fibroblasts across a range of TNF α doses and time points (results not shown). The above results were consistent for both control fibroblast lines.

To determine whether the C43S TRAPS mutation altered TNF α -induced production of IL-6 and IL-8, the levels of these cytokines in the culture supernatants of the primary dermal fibroblasts were measured by enzyme-linked immunosorbent assay. While absolute values varied with the passage number, the induction of IL-6 (Figure 2E) and IL-8 (Figure 2F) in response to TNF α in the C43S TRAPS fibroblast line was not statistically different from that observed in the controls. Baseline levels of the cytokines were also similar in the fibroblast lines. Therefore, in spite of the reduced NF- κ B and AP-1 activation, the C43S TRAPS fibroblast line was able to produce IL-6 and IL-8 in response to TNF α .

Reduction of TNF α -induced apoptosis in dermal fibroblasts and PBMCs due to C43S TRAPS mutation

Another important TNFRSF1A-mediated effect is the induction of apoptosis. TNF α also induces antiapoptotic genes, and therefore the apoptotic response to TNF α is usually dependent on the inhibition of protein synthesis (12). Fibroblasts were stimulated with TNF α (10 ng/ml) either alone or in the presence of CHX (13). Three assays for cell survival were used. First, cells were examined by light microscopy (BX41 microscope; Olympus, Lake Success, NY) after 24 and 48 hours. Dramatic differences were observed at both time points, with more TRAPS-derived fibroblasts than wild-type cells surviving the combination of TNF α and CHX (Figure 3A). The differences observed with light microscopy were quantified using an alamar blue assay (14). This nontoxic dye is chemically reduced by the innate metabolic activity of cells, which allows quantification of cell viability by fluorometry. Figure 3B shows the effect of a range of doses of TNF α on the survival of C43S TRAPS fibroblasts and a control fibroblast line. TRAPS-derived fibroblasts were markedly less sensitive to TNF α -induced cell death. Statistically significant ($P < 0.05$) differences in cell viability were observed at doses of TNF α > 1 ng/ml. Caspase 3 activity, a measure of apoptosis, was also assayed. TNF α and CHX induced significantly less caspase 3 activity in the TRAPS fibroblasts at all time points measured (Figure 3C).

Because the effects of TNF α can vary in different cells (12), we investigated whether the C43S TNFRSF1A mutation would also result in decreased sensitivity to TNF α -induced apoptosis in circulating inflammatory cells. PBMCs from the patient with the C43S TRAPS mutation and from healthy volunteers were isolated. PBMCs were stimulated with TNF α and CHX for either 2 or 6 hours. Cells were stained with annexin V and propidium iodide (PI) and analyzed by flow cytometry. Cells in the live gate (PI negative) were analyzed for annexin V staining. Although similar percentages of unstimulated cells were positive for annexin V, after stimulation with TNF α and CHX, fewer PBMCs from the patient with TRAPS were annexin V positive at both time points (Figure 3D). The difference observed at 6 hours was statistically significant ($P < 0.05$). The C43S TNFRSF1A mutation therefore resulted in decreased TNF α -induced apoptosis in both fibroblasts and PBMCs.

DISCUSSION

This report describes a novel TNFRSF1A mutation (C43S) associated with TRAPS and characterizes the signaling abilities of this mutation in a primary dermal fibroblast line established from the patient. TNF α activated the transcription factors NF- κ B and AP-1 at reduced levels in C43S TRAPS fibroblasts but was able to induce the proinflammatory cytokines IL-6 and IL-8 to levels similar to those in healthy controls. TNF α -induced apoptosis was significantly decreased in the fibroblasts bearing the C43S TNFRSF1A mutation. This defect in TNF α -induced apoptosis was also seen in PBMCs isolated from the patient. Thus, this study demonstrates that this TRAPS mutation results in reduced TNF α -induced nuclear signaling and apoptosis in this patient.

The initial studies of TRAPS cells proposed a mechanism of impaired activation-induced cleavage of TNFRSF1A that could cause the systemic inflammation associated with this syndrome (1,3). However, impaired shedding of TNFRSF1A does not appear to be the case for all TRAPS mutations (4,5). Our results support the hypothesis that TRAPS does not require impaired activation-induced shedding of TNFRSF1A from neutrophils.

Our data show that the C43S TRAPS fibroblasts exhibit reduced NF- κ B and AP-1 activation and decreased apoptosis in response to stimulation with TNF α . However, no significant difference in the induction of proinflammatory cytokines was observed, findings consistent with those of a previous study (1). This suggests a possible hypothesis to explain the inflammatory pathology: cells survive longer because of impaired apoptosis, but remain capable of producing proinflammatory cytokines. The levels of these cytokines would be expected to accumulate and could result in an inflammatory phenotype. Apoptosis of inflammatory cells is an important homeostatic mechanism for limiting an inflammatory response once it is established (15). Our hypothesis is also compatible with the high levels of serum amyloid A and C-reactive protein, surrogate markers for serum IL-6, noted in our and other TRAPS patients (1). However, this hypothesis is based on results from a single patient, and it remains to be established whether this is also the case in other TRAPS patients.

Because both IL-6 and IL-8 are regulated by NF- κ B, the question remains of how the induction of these proinflammatory cytokines is normal, despite the reduced NF- κ B activation. We propose 2 possible explanations. The first is that the threshold for TNF α -induced cytokine production is lower than that for apoptosis. Thus, reduced NF- κ B still generates a sufficient signal to allow induction of both IL-6 and IL-8. A second explanation is that signaling via TNFRSF1B (TNFR2) may play a role. TNFRSF1B is able to activate NF- κ B, but does not contain a death domain (8) and, thus, could induce IL-6 and IL-8 without causing apoptosis. TNF α generally results in less activation via TNFRSF1B than

TNFRSF1A in vitro (16), which may explain the reduced NF- κ B activation observed in our experiments.

From the published literature, it appears likely that the different TNFRSF1A mutants may induce TRAPS by different mechanisms. Some mutations affect TNFRSF1A shedding, while others do not. It will be important to determine whether other TRAPS mutations have similarly reduced TNF α nuclear signaling and apoptosis, as is the case with the C43S mutation. Reduced TNF α signaling could explain why treatment with TNF blocking agents does not completely abolish acute attacks in TRAPS patients (17). If reduced TNF α signaling is also demonstrated for other TRAPS mutations, then approaches to block the downstream proinflammatory cytokines (such as IL-6 and IL-1), in addition to current attempts to block TNF α , might prove beneficial. It is also possible that strategies to induce apoptosis in relevant cell types may be beneficial. In summary, this study suggests that, in some patients, TRAPS may be a result of defective or reduced TNF α -induced nuclear signaling and apoptosis.

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Figure 1.

Levels of activation-induced shedding of tumor necrosis factor receptor superfamily 1A (TNFRSF1A) from neutrophils obtained from a patient with C43S TNFR-associated periodic syndrome (TRAPS). **A**, Membrane expression of TNFRSF1A (CD120a) on neutrophils from a healthy volunteer and from the C43S TRAPS patient before and after stimulation with phorbol myristate acetate (PMA). Cells were stained with phycoerythrin-conjugated anti-CD120a and analyzed by flow cytometry. **B**, After PMA stimulation, supernatants were collected, and soluble TNFRSF1A levels were measured by enzyme-linked immunosorbent assay. Values are the mean $\pm$ SEM changes in soluble TNFRSF1A levels relative to baseline levels in 3 independent experiments. Mean $\pm$ SEM absolute values at time 0 were 13.45 ± 0.2 pg/ml in the control subject and 4.77 ± 0.98 pg/ml in the TRAPS patient.

Figure 2.

Effect of the C43S TNFRSF1A mutation on NF-κB activation and cytokine production in dermal fibroblasts. **A**, Electrophoretic mobility shift assay (EMSA) showing NF-κB activation in fibroblasts from 2 controls and the patient with C43S TRAPS, stimulated for 1 hour with TNFα (10 ng/ml). Densitometric analysis revealed a mean $\pm$ SEM reduction of $65.6 \pm 2.8\%$ in the TRAPS fibroblasts compared with controls in 5 separate experiments. DNA-protein complexes are indicated (arrows). Free (unbound) radiolabeled DNA was distinct from these complexes and is not shown in any of the figures. **B**, NF-κB supershift assay. TNFα-activated nuclear extracts were incubated with antibodies to the various subunits of NF-κB prior to incubation with the radiolabeled oligonucleotide probe. DNA-protein complexes (solid arrow) and antibody-DNA-protein complexes (open arrow) are indicated. **C**, EMSA of nuclear extracts showing a TNFα dose response at 1 hour for NF-κB activation in control and C43S TRAPS fibroblasts. Densitometric analysis revealed a mean $\pm$ SEM $60.2 \pm 7.6\%$ reduction in NF-κB activation in the TRAPS fibroblasts over a range of TNF doses in 5 separate experiments. **D**, EMSA showing the time course of NF-κB activation after stimulation of fibroblasts with 10 ng/ml TNFα. All EMSA results shown are representative of at least 5 separate experiments and were similar in both controls. **E**, Production of interleukin-6 (IL-6) and **F**, production of IL-8 by the primary dermal fibroblasts, as measured by enzyme-linked immunosorbent assay (ELISA) of the culture supernatants 24 hours after stimulation with 10 ng/ml TNFα. ELISA results are the mean $\pm$ SEM of 9 observations from 3 independent experiments for IL-6 and of 6 observations from 2 independent experiments for IL-8. See Figure 1 for other definitions.

Figure 3.

TNF α -induced apoptosis in C43S TRAPS fibroblasts and peripheral blood mononuclear cells (PBMCs). Fibroblasts (5×10^4) were stimulated with TNF α (10 ng/ml) and cycloheximide (CHX; 50 μ g/ml), either alone or in combination. **A**, Light microscopy images (4x objective) of fibroblasts after 24 hours of stimulation. Cells were stained with toluidine blue. Results shown are representative of 5 separate experiments. **B**, Cell viability of C43S TRAPS and control fibroblasts after 24 hours of incubation with varying doses of TNF α in the presence of CHX, as measured by alamar blue assay. Values are the mean $\pm$ SEM percentage of baseline levels (CHX alone). * = $P < 0.05$ versus controls, by Student's t -test. **C**, TNF α -induced caspase 3 activity. Fibroblasts (5×10^5) were stimulated with TNF α (10 ng/ml) and CHX (50 μ g/ml), after which they were analyzed for caspase 3 activity at the time points shown. Values are the mean $\pm$ SEM of 5 independent experiments. * = $P < 0.05$ versus controls. **D**, Annexin V assay of PBMCs. PBMCs from the patient and 2 healthy volunteers were incubated with TNF α and CHX for the times indicated. Annexin V activity was determined by flow cytometry, with gating on live (propidium iodide-negative) cells. Results shown are the difference between the mean in cells stimulated with both TNF α and CHX and the mean in unstimulated cells. Values are the mean $\pm$ SEM of 2 independent experiments. * = $P < 0.05$ versus controls. See Figure 1 for other definitions.