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ORIGINAL ARTICLE

Genotoxicity of Anti–Tumor Necrosis Factor Therapy in Patients With Juvenile Idiopathic Arthritis

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Objective. **To assess the possible effects of both inflammation and the anti–tumor necrosis factor agents (anti-TNF) on DNA damage with a specific assay, and their effects on the repair capacity of DNA.**

Methods. **From a group of 20 children with juvenile idiopathic arthritis (JIA), 16 patients who completed the study and 16 control subjects were evaluated. DNA damage and repair capacity were analyzed by the comet assay at the level of peripheral lymphocytes before anti-TNF (etanercept) injections and on the 15th, 90th, and 180th days after the first injection.**

Results. **The amount of damage as detected by the aforementioned assay was higher in patients with JIA compared with controls. On the 15th day after the initial anti-TNF injection, there was a decrease in the mean DNA tail length of JIA patients, however on the 90th day an increase was observed; thereafter, an upward trend was observed until the end of the study. JIA patients had a DNA repair capacity that was significantly less than that of controls.**

Conclusion. **The results of the comet technique suggests that JIA patients already have increased basal DNA damage before anti-TNF therapy; they are more sensitive to the DNA damage produced by H_2O_2, and have a less efficient DNA repair system in comparison with control cells. After an initial improvement at 2 weeks, parameters of genotoxicity worsened, and DNA repair was further impaired 6 months after the addition of an anti-TNF agent to treatment.**

INTRODUCTION

Anti–tumor necrosis factor (anti-TNF) therapy has become a cornerstone in the treatment of juvenile idiopathic arthritis (JIA) and has dramatically changed the disease course. Anti-TNF agents have an overall good safety profile in patients with rheumatoid arthritis (RA), psoriatic arthritis, ankylosing spondylitis, Crohn's disease psoriasis, and JIA (1). However, with increasing use, cases of malignancy have been reported, especially in adults with RA (2). Since RA itself has been associated with increased malignancy, it has been hard to clarify the exact role of anti-TNF treatment in these cases. The rheumatology literature also lacks studies on possible mechanisms.

The comet assay or single-cell gel test is a microgel electrophoresis technique that measures DNA damage at the level of single cells. (3). The comet assay is attractive to many researchers for many reasons, and it is a valuable tool for investigating fundamental aspects of DNA damage and cellular responses to this damage (4). The assay has been used in fundamental research for DNA damage and repair, in genotoxicity testing of novel chemicals and pharmaceuticals, environmental biomonitoring, and human population monitoring (3). The aim of this study was to investigate the DNA damage and repair capacity using the comet assay in patients with JIA before and after receiving anti-TNF therapy.

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SUBJECTS AND METHODS

Subjects and sampling. This study was performed at Hacettepe University Medical School, Department of Pediatrics and Gazi University Pharmacy Faculty, Department of Toxicology. During the enrollment period, a total of 20 children diagnosed with JIA according to Durban criteria and who still had active disease despite receiving 6 months of methotrexate (MTX; 15 mg/m^2/week) were included. All had polyarticular-onset or systemic-onset disease with a polyarticular disease course. Sample 0 rep-

resented the sample obtained after 6 months of MTX treatment (i.e., just before the start of anti-TNF treatment [etanercept; 0.8 mg/kg/week]). Samples 15, 90, and 180 were obtained 15, 90, and 180 days after the start of anti-TNF treatment, respectively. The enrolled patients were not exposed to any genotoxic agents such as radiation, chemicals, or indoor smoking. All patients were receiving concomitant nonsteroidal antiinflammatory drugs (NSAIDs) and no patient was receiving continuous oral corticosteroid treatment during the study period. The control group consisted of 16 healthy age-and sex-matched subjects with no known exposure to genotoxic agents. None of the patients and the controls or their first degree relatives had any known genetic disease.

Venous blood samples (3 ml) were collected in heparinized glass tubes. Samples were taken just before each biologic therapy session (day 0), on the 15th, 90th, and 180th days. Only 1 blood sample was drawn from the control group.

Chemicals and media. Frosted microscope slides were obtained from Menzel (Braunschweig, Germany) and all plastics were from Corning Laboratory Sciences (Corning, New York, NY), with the exception of microtubes, which were purchased from Treff Lab (Degersheim, Switzerland). All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) with the exception of RPMI 1640 medium, fetal calf serum (FCS), phytohemagglutinin (PHA), and trypan blue, which were obtained from Biochrom (Berlin, Germany).

Single-cell gel electrophoresis. The lymphocytes were isolated with Histopaque 1077 in phosphate buffered saline (PBS) on ice from heparinized blood samples. The lymphocyte suspension was then divided into 2 identical groups; group 1 was used to measure repair capacity via H_2O_2 exposure, and group 2 was used for regular single-cell gel electrophoresis. The procedure was carried out as follows: lymphocytes suspended in ice-cold PBS were exposed to 100 μM H_2O_2 for 5 minutes on ice in microcentrifuge tubes ($\sim 2 \times 10^5$ cells/ml). To examine repair of H_2O_2 damage in lymphocytes, cells were resuspended in RPMI 1640, supplemented with 20% FCS, 2 mM L-glutamine and incubated at 37°C for 1 hour (5). The single-cell gel electrophoresis assay was performed under alkaline conditions using an adaptation of the method as described previously by Singh et al (6). The lymphocytes were mixed with 100 μl of 0.65% low-melting-point agarose in PBS at 37°C and rapidly pipetted onto a frosted glass microscope slide precoated with 100 ml of 1% agarose, spread out with a coverslip, and maintained at 4°C for 30 minutes to solidify.

After removal of the coverslip, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, NaOH to pH 10.0, and 1% Triton X-100) for overnight at 4°C, to remove cellular proteins. Slides were initially placed in an electrophoresis tank containing 1 mM Na_2EDTA and 300 mM NaOH, (pH 13) for 20 minutes. Afterward, the tank was set at 25 V (1.6 V/cm, 300 mA) for 20 minutes at an ambient temperature of 4°C. The slides were then washed 3 times (for 5 minutes each), with Tris buffer (0.4 M Tris, pH 7.5), at 4°C before staining them with 65 μl ethidium bromide (20 μg/ml). Each analysis was done in duplicate and carried out immediately after sample collection without freezing or storing. Cell viability, using trypan blue, was found to be <95% at each time point of the study. Repair capacity was defined as the relative decrease of the DNA migration length (tail length) 60 minutes after hydrogen peroxide treatment (7).

Image analysis of the slides. After the staining process, 100 cells were analyzed using double slides that were selected randomly for examination at ×200 magnification under a fluorescent microscope (Zeiss-Axioskop, Oberkochen, Germany) equipped with an excitation filter 515–560 nm and a 100 WHg lamp. DNA migration (tail length), tail intensity, and tail moments were measured, using Comet Assay III image analysis system (Perceptive Instruments, Suffolk, UK) (Figure 1). All slides were coded and scored blindly.

Statistical analysis. Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, IL). Data were expressed as mean, median, minimum and maximum values, percentages, and SDs. Mann-Whitney U test, Friedman's test, and Wilcoxon's signed rank test were used to compare data between or within groups. P values < 0.05 were considered significant.

RESULTS

Four of the 20 children enrolled did not complete the study; 1 child with a diagnosis of systemic-onset JIA died due to macrophage activation syndrome and 3 patients stopped anti-TNF therapy and did not complete the study. Therefore, 16 patients (8 girls and 8 boys) completed the study. The control group also consisted of 8 girls and 8 boys. The age of the patients and the controls was mean ± SD 11.11 ± 4.29 years (range 3–18 years) and 11.32 ± 3.96 years (range 4–18 years), respectively ($P > 0.05$). There were no statistically significant differences between the height and weight values of the children ($P > 0.05$).

When mean ± SD baseline values were compared, JIA patients were found to have increased DNA damage as compared with the healthy controls (36.46 ± 7.88 versus 30.31 ± 1.83; $P = 0.029$). Comparison of the mean DNA tail length of JIA patients on the 0th, 15th, 90th, and 180th days after the initiation of anti-TNF injections are shown in Table 1. The amount of damage was higher in the JIA population when compared with controls on all studied dates ($P < 0.05$). On the 15th day after anti-TNF injection, there was a decrease in the mean ± SD DNA tail length of the patients from 36.46 ± 7.88 (day 0, baseline level) to 35.79 ± 5.56. On the 90th day, the median tail length again began to increase, after which it had an upward trend till the end of the study (Figure 2).

Patients with JIA at baseline were found to have decreased DNA repair (mean SD 41.95 ± 11.79 versus 49.73 ± 5.63; $P = 0.037$) as compared with healthy controls. Comparison of the mean DNA repair of JIA patients

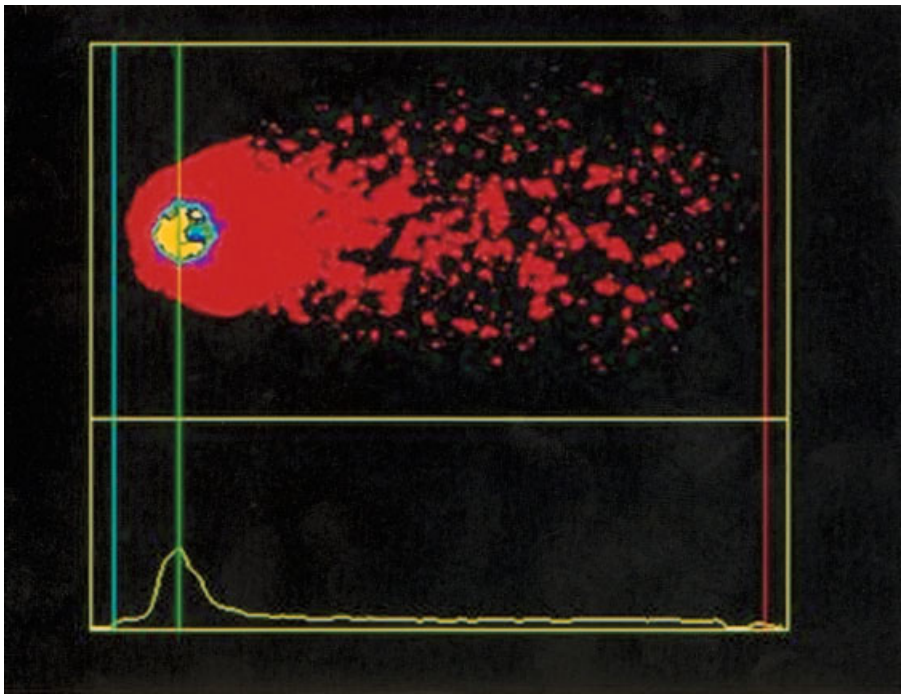

Figure 1. A comet assay image of a human lymphocyte obtained after in vitro challenge with H_2O_2 from patients with juvenile rheumatoid arthritis exposed to etancerept taken with the Comet Assay II image analysis system (Perceptive Instruments, Suffolk, UK). The tail length (μm) of cells were analyzed at baseline, after in vitro challenge with H_2O_2, and after 1 hour repair.

on the 0th, 15th, 90th, and 180th days after the initiation of anti-TNF injections are shown in Table 2. On the 15th day of anti-TNF treatment, there was no significant difference as compared with baseline (Day 0); however the difference became significant on the 90th day and thereafter (Figure 3).

DISCUSSION

This is the first study assessing the DNA damage/genotoxicity and DNA repair mechanisms in children with JIA who received anti-TNF treatment. We have demonstrated that cells of JIA patients have increased basal DNA damage, are more sensitive to the DNA damage produced by H_2O_2, and have less efficient DNA repair system in comparison with control cells. Parameters of genotoxicity were also found to be worsened and DNA repair was further impaired 6 months after the addition of an anti-TNF agent to MTX therapy.

Biologic effects of DNA-damaging agents have been detected by the use of a number of techniques. Singh et al (6) developed a simple approach, the comet assay, for sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. Single-cell gel electrophoresis has proven to be a very sensitive method to assess DNA damage induced by several agents, and to assess individual cell repair kinetics (8). To assess the DNA re-

Table 1. Mean DNA tail length values (μm) of patients with JIA during treatment*

Anti-TNF therapy duration, days	DNA tail length, mean ± SD	*P*†
0–15	(36.46 ± 7.88) – (35.79 ± 5.56)	1.0
0–90	(36.46 ± 7.88) – (40.19 ± 5.73)	1.0
0–180	(36.46 ± 7.88) – (42.49 ± 7.73)	0.47
15–90	(35.79 ± 5.56) – (40.19 ± 5.73)	0.08
15–180	(35.79 ± 5.56) – (42.49 ± 7.73)	0.02
90–180	(40.19 ± 5.73) – (42.49 ± 7.73)	0.36

* JIA = juvenile idiopathic arthritis; TNF = tumor necrosis factor.
† By Wilcoxon's signed rank test with Bonferroni corrections.

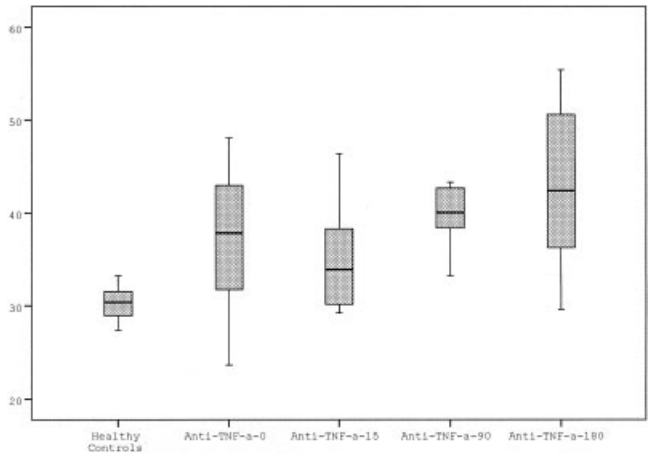

Figure 2. Median DNA tail length values (μm) of the control patients and the patients with juvenile idiopathic arthritis during treatment. Anti-TNF = anti–tumor necrosis factor.

Table 2. Mean DNA repair capacity values of patients with JIA during treatment (%)

Anti-TNF therapy duration, days	DNA repair capacity, mean ± SD	*P*†
0–15	(41.95 ± 11.79) – (47.33 ± 8.17)	0.30
0–90	(41.95 ± 11.79) – (38.19 ± 7.41)	1.00
0–180	(41.95 ± 11.79) – (38.78 ± 6.05)	1.00
15–90	(47.33 ± 8.17) – (38.19 ± 7.41)	0.03
15–180	(47.33 ± 8.17) – (38.78 ± 6.05)	0.04
90–180	(38.19 ± 7.41) – (38.78 ± 6.05)	1

* JIA = juvenile idiopathic arthritis; TNF = tumor necrosis factor.
† By Wilcoxon's signed rank test with Bonferroni corrections.

pair capacity of cells from our study groups, DNA damage was induced with 100 μM H_2O_2, which caused high DNA damage level without significant cell mortality. H_2O_2 can penetrate the cell membrane easily and initiate the generation of highly reactive species through the transition metal catalyzed Haber-Weiss reaction (5).

The DNA damage in our cells should be properly repaired before DNA replication and cell division, in order to prevent the critical mutations and susceptibility to chronic diseases and cancer. The risk of cancer introduced with the use of anti-TNF drugs is controversial. An increase in lymphoma and solid cancers has been reported in RA patients receiving anti-TNF treatment (9). However, RA itself is also associated with an increased risk of lymphoma as outlined above. Thus, we thought that studying DNA damage would reflect a certain aspect of the possible association of the disease and treatment on the risk of malignancy.

The comet assay has been used for screening mutagen sensitivity and DNA repair capacity in human peripheral lymphocytes (10). In a study performed by Andresson et al, there was a correlation between the sensitivity to the DNA damaging effect of H_2O_2 and the DNA repair capacity in human lymphocytes (11). In our study, the sensitivity of the lymphocytes to the damaging effect of H_2O_2 was higher in the JIA patients as compared with controls. It may be speculated that inflammation is effective in this impairment. In fact Ramos-Remus et al have assessed genotoxicity in their RA patients through a "micronuclei assay" (12). They have shown that with this method genotoxicity was increased in RA. They showed no difference among patients that used methotrexate and thus suggested that the increased micronuclei, reflecting genotoxicity was associated with the disease itself and not with the use of MTX (12).

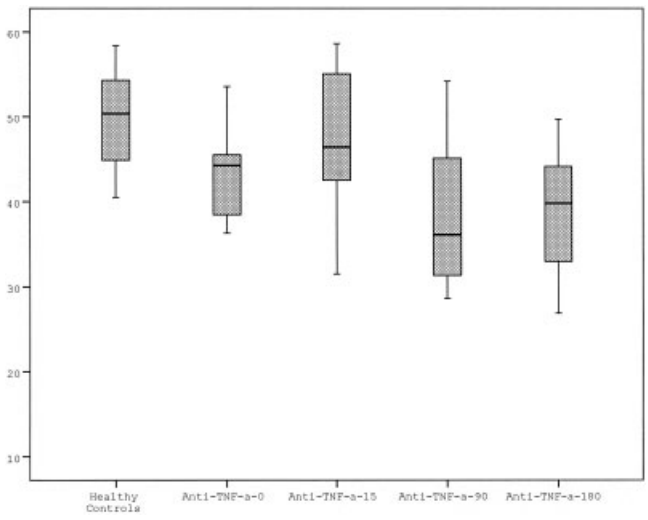

Figure 3. Median DNA repair capacity values (%) of the control patients and the patients with juvenile idiopathic arthritis during treatment. Anti-TNF = anti–tumor necrosis factor.

There is evidence for increased oxidative stress in RA (13). In turn, the reactive oxygen molecules produced by an activated oxidative stress have the potential to damage the nucleic acids and DNA. Altindag et al have shown increased DNA damage in their RA cohort and suggested that the DNA damage they observed was primarily due to this oxidative stress (14). Oxidative stress also impairs the DNA repair mechanisms. The products of oxidative stress are cytotoxic and mutagenic. In fact, somatic mutations of p53, a tumor suppressor gene, have been shown in the synovium of RA patients (15). TNFα enhances the damaging effects of reactive oxygen species. It has been suggested that increased free radicals may contribute to the cancer risk, and oxidized DNA bases have been associated with malignancy (16). In addition, increased inflammation per se may also be associated with this risk through the activation of reactive oxygen species (ROS) or directly through the inflammatory cytokines (14). These facts may explain the increase of DNA damage in JIA patients when compared with controls. Furthermore, this may explain the initial improvement of the DNA damage parameters observed with anti-TNF treatment in our cohort. Anti-TNF treatment may be expected to decrease the DNA damage through the down regulation of the ROS and blocking of a major inflammatory cytokines in these patients. In fact, the initial improvement in the DNA repair mechanism and decreased DNA damage observed at 2 weeks may be explained by these effects. However, the DNA damage slowly increased in the first 6 months along with impairment in the DNA repair mechanism. The mechanism yet remains obscure. On the other hand, we do not know if this effect fades away at later time points or whether the repair mechanism is subsequently restored because of the suppression of inflammation. However, we suggest that the increased DNA damage and impaired DNA repair as compared with the MTX treatment warrants further studies to elucidate the significance of these genotoxic effects and determine whether they are long term.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication. Dr. Demirkaya had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Demirkaya, Cok, Durmaz, Ulutas, Ayaz, Besbas, Özen.
Acquisition of data. Demirkaya, Cok, Durmaz, Ulutas, Ayaz, Besbas, Özen.
Analysis and interpretation of data. Demirkaya, Cok, Durmaz, Ulutas, Ayaz, Besbas, Özen.

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