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Microsatellite Instability and Suppressed DNA Repair Enzyme Expression in Rheumatoid Arthritis

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Reactive oxygen and nitrogen are produced by rheumatoid arthritis (RA) synovial tissue and can potentially induce mutations in key genes. Normally, this process is prevented by a DNA mismatch repair (MMR) system that maintains sequence fidelity during DNA replication. Key members of the MMR system include MutSα (hMSH2 and hMSH6) and MutSβ (hMSH2 and hMSH3). To provide evidence of DNA damage in inflamed synovium, we analyzed synovial tissues for microsatellite instability (MSI). MSI was examined by PCR on genomic DNA of paired synovial tissue and peripheral blood cells of RA patients using specific primer sequences for five key microsatellites. Surprisingly, abundant MSI was observed in RA synovium compared with osteoarthritis tissue. Western blot analysis for the expression of MMR proteins demonstrated decreased hMSH6 and increased hMSH3 in RA synovium. To evaluate potential mechanisms of MMR regulation in arthritis, fibroblast-like synoviocytes (FLS) were isolated from synovial tissues and incubated with the NO donor S-nitroso-N-acetylpenicillamine. Western blot analysis demonstrated constitutive expression of hMSH2, 3, and 6 in RA and osteoarthritis FLS. When FLS were cultured with S-nitroso-N-acetylpenicillamine, the pattern of MMR expression in RA synovium was reproduced (high hMSH3, low hMSH6). Therefore, oxidative stress can relax the DNA MMR system in RA by suppressing hMSH6. Decreased hMSH6 can subsequently interfere with repair of single base mutations, which is the type observed in RA. We propose that oxidative stress not only creates DNA adducts that are potentially mutagenic, but also suppresses the mechanisms that limit the DNA damage. 


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MMR enzyme MSH6 was decreased in RA synovium. The expression of this enzyme was also suppressed in cultured FLS after exposure to reactive nitrogen species (RNS). We propose that oxidative stress not only creates DNA adducts that are mutagenic, but also relaxes the mechanisms that ordinarily repair DNA damage.

Materials and Methods

**Synovial tissue and FLS**

RA and OA synovial tissues were obtained at joint replacement surgery from patients with destructive arthritis. The diagnoses conformed to the 1987 revised ACR criteria (27). None of the patients had been treated with alkylating agents. For the MSI studies, the average ages were 75 (52–94) years and 55 (32–87) years for OA (n = 5) and RA (n = 7), respectively. The patients were female, and the synovial tissues were derived from the knees or hips, except for one RA patient who had shoulder surgery. Of the RA samples, five were rheumatoid factor positive. Four patients received methotrexate, three received leflunomide, three received low dose prednisone, and one received etanercept. OA patients were treated with analgesics and nonsteroidal anti-inflammatory drugs. Informed consent was obtained, and the protocol was approved by the University of California at San Diego Human Subjects Research Protection Committee. For microsatellite analysis, synovial tissue was snap frozen and aliquoted at the time of surgery. FLS were prepared as previously described (28). Briefly, the tissues were minced and incubated with 1 mg/ml collagenase in serum-free DMEM (Life Technologies, Grand Island, NY) for 2 h at 37 °C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% FCS (Life Technologies; endotoxin content <0.006 ng/ml), penicillin, streptomycin, gentamicin, and l-glutamine in a humidified 5% CO2 atmosphere. After overnight culture, nonadherent cells were removed and adherent cells were cultivated in DMEM plus 10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages 3 through 9, in which they comprised a homogeneous population of FLS (<1% CD11b, <1% phagocytic, and <1% FcγRII receptor positive).

**Microsatellite analysis**

MSI status was determined following DNA extraction from synovial tissue and paired autologous PBMC collected during joint replacement surgery (seven RA and five OA; see characteristics of patients above) using the genomic DNA separation kit (Qiagen, Valencia, CA). A panel of five microsatellite markers was used comprising three dinucleotide repeats (D2S123, D5S346, D17S250) and two mononucleotide repeats (BAT25 and BAT26), which serve as a National Cancer Institute MSI reference panel for colorectal cancer (18). Changes in the electrophoretic mobility of amplified PCR products are used to assess the microsatellite status. PCR amplification was performed with 100 ng of purified genomic DNA isolated from snap-frozen synovial tissue or the PBMCs in a final reaction volume of 20 μl using an MJ Research Thermocycler (PTC100; MJ Research, Watertown, MA). The primers for PCR were end labeled with [γ-32P]ATP. Products were denatured, electrophoresed on 8% denaturing polyacrylamide gels, and visualized by autoradiography. MSI was readily determined by the presence of clearly visualized altered allelic shifts in the PCR products in the synovial tissue specimens when compared with the paired control DNA derived from autologous PBMC. MSI status was independently evaluated by two blinded observers. These criteria were defined and validated by a National Cancer Institute workshop chaired by one of us (C. R. Boland) (18). Synovial tissues exhibiting allelic shifts in ≥2 of the 5 markers were considered as microsatellite high (MSI-H), as previously defined in colorectal cancer. A low level of MSI (MSI-L) was defined by the shift in only 1 of the 5 markers. Synovial tissues that did not exhibit any allelic shifts compared with the control DNA were defined as microsatellite stable (MSS).

**Western blot analysis of MMR proteins**

Tissue homogenates (100 mg) or cells (~106 cells) were suspended in cold PBS/0.1% sodium deoxycholate, frozen for 2 h at −20 °C, and thawed for 30 min on ice. After a second freeze-thaw cycle, the membranes were gently broken by mechanical pipetting (5). Protein concentrations were measured with the BSA protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples from FLS lysates (30 μg) or synovial tissue lysates (100 μg) were separated by electrophoresis using a SDS-8% polyacrylamide gel, and transferred onto a nitrocellulose membrane at 140 mA in 25 mM Tris Cl (pH 8.3), 200 mM glycine, and 20% methanol. Membranes were blocked with 5% nonfat dry milk in PBS-0.1% Tween 20 (PBS-T) for 1 h. This was followed by overnight incubation at 4 °C with mouse mono-
due to local environmental influences or variations in cellular composition rather than generalized suppression of MMR expression in RA.

**Effect of RNS and ROS on MutS MMR proteins in FLS**

Because no differences in basal MMR expression were observed between OA- and RA-cultured FLS, we studied the effect of various mediators on the components of the MMR enzyme complexes in vitro. No changes in hMSH2, 3, or 6 were observed in FLS that had been stimulated with IL-1 (1 ng/ml) or TNF-α (100 ng/ml) for up to 24 h (data not shown). We then used an in vitro model of oxidative stress to determine whether MMR expression in cultured RA FLS is regulated by RNS or ROS. FLS were cultured in the presence of the NO donor SNAP, and expression of MMR enzymes was assessed by Western blot analysis (n = 5 separate lines). As shown in Fig. 4, pretreatment of FLS with SNAP for 3 h significantly decreased hMSH6 expression 6–12 h after exposure (62% decrease at 6 h, p = 0.002). hMSH3 levels increased during the same period of time (201% increase at 6 h, p = 0.047), indicating that the effect on hMSH6 was not due to nonspecific toxicity (Fig. 4A). hMSH2 expression was not significantly changed, although there was a trend toward a modest increase after RNS exposure. Alterations in MMR protein expression were transient, and expression returned to baseline within 24–48 h. No differences

**FIGURE 2.** Expression of DNA MMR proteins in synovial tissues. Synovial tissue extracts from RA and OA were evaluated by Western blot analysis. hMSH6 protein expression was significantly less in RA than in OA, while hMSH3 expression was higher in RA in comparison with OA (*, p < 0.05). RA synovium from lanes 1–4 were MSI-H, and OA synovium from lanes 2–4 were MSS. Although there was a trend for higher hMSH2 expression in RA, the differences did not reach statistical significance.
were observed between RA and OA FLS with regard to their response to SNAP (data not shown). Hydrogen peroxide treatment also modestly decreased hMSH6 (Fig. 4B, \( p < 0.05 \)), although the effect was more prolonged. hMSH2 was not significantly changed by ROS exposure. These data indicate that the MMR pattern observed in RA synovial tissue, i.e., low hMSH6 and high hMSH3, was reproduced in cultured FLS after oxidative stress. This amount of MMR enzyme decrease is known to suppress DNA repair activity in cells, supporting the functional relevance of this phenomenon (26).

Effect of dose and duration of NO exposure on hMSH6 expression in FLS

Kinetics and dose-response experiments were then performed to characterize the effects of RNS on MMR expression. To evaluate the concentration of NO required to suppress hMSH6, RA FLS cell lines \((n = 3)\) were exposed to various concentrations of SNAP (0.01, 0.1, 1.0 mM) for 3 h. Minimal or no effect was observed for the lower concentrations, while 1 mM clearly suppressed hMSH6 \((p < 0.05)\) (Fig. 5). We then treated RA FLS cell lines with 1 mM of SNAP for 0.5–3 h, and analyzed hMSH6 protein levels. As illustrated in Fig. 6, suppression of hMSH6 correlated with the duration of exposure to SNAP \((p < 0.05)\).

Discussion

Genotoxic adducts, especially ROS and RNS, are released as a result of cell activation and can damage genomic DNA. One such product, NO, is especially prevalent in chronic inflammatory diseases such as ulcerative colitis (30) and RA (31–33). In the former, local NO production induces somatic mutations that can ultimately lead to neoplasia (34). DNA damage and mutation have also been documented in the synovial intimal lining in rheumatoid synovium, albeit without resultant tumor formation (35). To determine the mechanisms of DNA damage in inflamed tissues, we evaluated rheumatoid synovium for evidence of mutagenesis by performing...
standard microsatellite analysis. Microsatellites are tandem repeated sequences in the DNA that are primarily (but not exclusively) located in noncoding regions. Instability is defined as a microsatellite insertion or deletion mutation in genomic DNA isolated from the target tissue or tumor compared with an autologous control (usually peripheral blood) (18). Many studies demonstrate an association among MSI, DNA damage, and development of neoplasia (19, 36, 37).

Criteria for MSI-H and MSI-L phenotypes, respectively, and MSS phenotype have been carefully defined for colon tumor loci utilizing the same techniques used in our study (18). Five specific microsatellites can be evaluated for evidence of mutations and the number of mutations quantified. The phenotypes are: 1) MSI-H, with mutations in ≥40% of loci; 2) MSI-L with mutations in ≤20% of loci; and 3) MSS, with no microsatellite mutations. Our initial studies were performed on RA and OA synovial tissues and used the loci validated for colon inflammation because the mechanisms of mutagenesis are most likely similar. These experiments demonstrated clear evidence of the MSI-H genotype in RA using the same methodology and criteria used to define MSI in neoplastic disease. In contrast, OA samples were MSS. The presence of microsatellite mutations in RA is consistent with previous studies demonstrating somatic mutations and increased DNA strandbreaks in rheumatoid synovium (5, 35). It extends our hypothesis that DNA damage and mutations can occur randomly throughout the genome in RA; when nonconserved base changes occur in key genes such as p53 that provide a selective advantage, local expansion of individual clones can occur. Our recent microdissection studies on RA synovium demonstrating islands of p53 mutant cells are consistent with this observation (29).

This area, like other studies examining mutations and DNA damage in RA, is not without controversy. A previous report by Muller-Ladner and colleagues (38) suggested that microsatellites are stable in long-term cultured RA synoviocytes. Our MSI analysis focused on fresh tissue rather than cultured cells, which we believe provides a more accurate indication of the situation in situ. Their patient population also did not have evidence of p53 mutations (7), in contrast to patients from other groups (5, 6, 8). Although we do not know why our data differ, it could be related to greater severity of disease in our patients because we primarily examined destructive arthritis requiring total joint replacement. This is clearly an area that requires additional investigation, and we are currently evaluating the relationship between duration of disease, severity, and DNA damage.

The mechanisms of MSI have been evaluated in other settings, especially in hereditary nonpolyposis colon cancer (HNPCC). These patients have many mutations in the colon and frequently develop colon cancer in early adulthood. The etiology of the mutagenic propensity in HNPCC is defective DNA MMR enzymes due to inactivating germline mutations in MMR genes (22, 25, 36). This complex repair system involves many enzymes, including two key heterodimeric complexes known as MutSα and MutSβ. hMSH2 is a subunit of both dimers and is the most common defective gene in HNPCC (22). It can pair with hMSH6 to form MutSα, while hMSH3 and hMSH2 form MutSβ. MutSα mainly repairs single base mispairs, while MutSβ repairs larger insertion/deletion mispairs (39). Because MSH2 is the common component, balance between repair of single base mismatches or longer insertion/deletion loops depends on the ratio of MSH6 and MSH3. In cells that overexpress MSH3, the available MSH2 protein is sequestered into MutSβ. This leads to degradation of the partnerless MSH6 and defective repair of single base mismatches (40). Therefore, MMR deficiency can arise not only through mutation or transcriptional silencing of MMR genes, but also from an imbalance in the relative amounts of the MSH3 and MSH6 proteins.

Based on our observation that RA synovium is MSI-H, we assessed the expression of MMR proteins. hMSH2 and another MMR enzyme, hMLH1, are expressed by intimal lining cells in RA, as determined by immunohistochemistry (38). However, there is no information on the other key enzymes or the relative amounts compared with OA. Decreased hMSH6 was observed in the rheumatoid synovium compared with OA, although hMSH3 expression was significantly higher in RA. Variations in the cellular content of synovium in the two diseases might contribute, but the high expression of MMR proteins in the intimal lining suggests this region, comprising macrophage and FLS in RA and OA, is the major site. The observed pattern of MMR enzyme expression provides a potential explanation for the type of mutation observed in the intimal lining of RA, where single base pair transition substitutions account for the vast majority of abnormalities (5, 29). Also, transition mutations are characteristic of MMR defects and represent almost all of the RA mutations identified to date.

The MMR differences between OA and RA synovium led us to explore the expression and regulation of these enzymes in FLS. Basal of MMR protein expression was similar in RA and OA FLS, indicating that the abnormalities observed in the tissue were not simply due to a generalized suppression as observed in HNPCC. The pattern of MMR expression in FLS changed when they were stressed by RNS, with induction of hMSH3 and suppression of hMSH6. The decrease in hMSH6 levels was dose dependent and varied with the duration of NO exposure. The fact that other MMR proteins or housekeeping genes such as actin were unaffected indicates that hMSH6 suppression was not caused by nonspecific toxicity. NO donors such as SNAP serve as physiologic sources of NO and permit accurate assessment of reactive nitrogen effects. The results with SNAP were largely confirmed using hydrogen peroxide, which is a standard reagent that exposes cells to ROS. Although the mechanism of MMR regulation is currently unknown, the MMR protein levels in other cells correlate with mRNA expression and suggest that transcriptional regulation most likely participates (41). ROS was recently noted to decrease expression of hMSH6 in erythroleukemia cells to an extent similar to FLS (26). More important, hydrogen peroxide suppressed DNA repair activity, as determined with functional assays. This defect was reversed by the addition of rMMR enzymes to the cell extracts of stressed cells, indicating the functional relevance of the observation.

The cultured FLS studies showed that the pattern of MMR expression in RA synovium (high hMSH3/low hMSH6) was reproduced in synoviocytes that were exposed to genotoxic stimuli. This tissue culture model obviously does not completely mimic the situation in chronically inflamed synovium, and other cell types can contribute to MSI or MMR protein expression in RA synovium. Nevertheless, the levels of RNS and ROS exposure that alter MSH expression are relevant to the in vivo situation (33, 42). Suppression of hMSH6 by RNS appears paradoxical at first. However, it might represent a mechanism that protects the cell from major...
DNA damage while permitting single base pair changes. This process could provide short-term survival benefits to an organism by shifting the balance from MutSo to MutSB. It also might serve as a mechanism for establishing a mutator phenotype, which can have additional survival benefits (43).

Exposure to reactive species, especially RNS, can be directly mutagenic and can increase the likelihood of single base mismatches in RA through oxidative deamination (44). In addition, suppression of MMR mechanisms could contribute to a hypermutable state and increase the likelihood for the accumulation of somatic mutations (17). In RA, ROS production is increased by the hypoxic environment mediated by activated leukocytes, ischemia-reperfusion injury, an increased metabolic rate, and reduced capillary density (45, 46). Expression of iNOS is also markedly increased in RA, especially in the synovial intimal lining (32). Local NO production results in nitrosylation of synovial proteins and can produce mutagenic nucleotide adducts (44). Synovial expression of proinflammatory cytokine genes probably contributes to cell activation, reactive oxygen production, and iNOS induction. The cytokine networks that perpetuate disease in RA thereby provide a link between synovial inflammation, production of reactive metabolites that have mutagenic potential, and relaxation of the DNA repair system. However, a direct influence of cytokines such as IL-1 and TNF-α on MMR expression in cultured FLS was not observed.

We have not formally examined in vivo correlations between synovial cytokine production or clinical disease activity and the extent of MSI. Because the proinflammatory milieu is directly related to both processes, they probably move in tandem over time. The corollary is that suppression of cytokine networks and control of inflammation with TNF-α, IL-1, and NO production results in nitrosylation of synovial proteins and can potentially be minimized. Alternatively, replacing or augmenting targets to modify the long-term destructive potential of RA synoviocytes. By limiting local RNS or ROS production, mutagenesis can potentially be minimized. Alternatively, replacing or augmenting MMR expression could mitigate the genotoxicity in the synovium or in other chronically infected sites.

Acknowledgments

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References


CORRECTIONS


In the original article, both the concepts and language in the last paragraph of the Discussion were taken without attribution from the previous publications (References 3 and 9). We deeply regret and apologize for this lack of appropriate attribution.


In Figure 2B, the mismatch repair enzyme expression for osteoarthritis (OA) and rheumatoid arthritis (RA) was compared in a bar graph. The designation for RA and OA were reversed in the figure. The corrected figure is shown below.