Induction of Inducible Nitric Oxide Synthase, Argininosuccinate Synthase, and GTP Cyclohydrolase I in Arthritic Joints of Human Tumor Necrosis Factor-α Transgenic Mice

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ABSTRACT. Objective. Transgenic mice that express human tumor necrosis factor-α (Tg197 h-TNF-α) develop polyarthritis at 3 to 4 weeks of age leading to severe joint destruction at 8 to 10 weeks of age. Studies have suggested that inducible nitric oxide synthase (iNOS) activity can modulate the progression of arthritis. We investigated the induction of iNOS together with argininosuccinate synthase (AS) and GTP cyclohydrolase I (GTPCH), 2 of the rate-limiting enzymes for high output NO generation, in the Tg197 h-TNF-α transgenic model of arthritis.

Methods. We used 4 and 8-week-old Tg197 h-TNF-α transgenic mice and wild-type CBA × C57B1/6 control mice to investigate the expression of iNOS with respect to that of AS, GTPCH, and 3-nitrotyrosine by quantitative RT-PCR and immunocytochemistry. Urinary NO metabolites were analyzed using a chemiluminescence assay.

Results. Inducible NOS, AS, and GTPCH mRNA was found in all study groups; however, only iNOS mRNA showed a clear increase in 4-week-old Tg197 h-TNF-α transgenics in comparison to age matched wild-type controls. Abundant iNOS protein expression was found in macrophages and vascular smooth muscle cells in hyperplastic synovium and pannus. AS expression was found in vascular endothelium and fibroblasts of the inflammatory synovium and pannus. GTPCH immunoreactivity was mostly restricted to macrophages in inflammatory synovium. Localization of 3-nitrotyrosine overlapped with that of iNOS, indicating formation of reactive nitrogen species. Consistent with the high output NO generation, there was a 5-fold increase in urinary NO metabolites in 8-week-old Tg197 h-TNF-α transgenic mice.

Conclusion. We characterized the Tg197 h-TNF-α transgenic model of inflammatory arthritis in terms of high output NO-generating pathway, and showed that both AS and GTPCH are intimately associated with inflammatory arthritis. The concomitant induction of AS and GTPCH with that of iNOS suggests that they may be important modulators of arthritis, and that they may represent novel targets for modulation of disease activity. (J Rheumatol 2003;30:652–9)

Key Indexing Terms:
ARGININOSUCCINATE SYNTHASE
3-NITROTYROSINE
GTP CYCLOHYDROLASE
NITRIC OXIDE
TUMOR NECROSIS FACTOR-α

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Persistent nitric oxide (NO) production by the cytokine inducible NO synthase (iNOS) has been suggested as one of the contributing factors for the development of inflammatory lesions and joint destruction/deformations seen in rheumatoid arthritis (RA)1,2 and several animal models of
the disease. In most model systems, induction of iNOS synthesis and high output NO production precede the onset of the clinical hallmarks of the disease such as erythema and joint swelling. NO production has been shown to persist through the chronic phase of the disease, suggesting that NO may be involved not only in the initiation but also in the maintenance of inflammatory arthritides. The animal models that have been characterized in terms of iNOS-driven high output NO production include the streptococcal cell-wall fragment model, MRL/lpr mice, adjuvant-induced arthritis, and collagen II induced arthritis. Many reports agree that inhibition of NOS activity can be pharmaco-lytic against polyarthritis. However, the use of various animal models of RA and the availability of an array of pharmacological compounds that inhibit NOS enzyme activity have raised much controversy. In these rodent models, the increase in NO typically precedes the onset of the symptoms of inflammatory arthritis. Prophylactic administration of L-NMA or L-NAME (nonselective inhibitors of NOS activity) have been shown to suppress the disease onset, whereas aminoguanidine (which exhibits selectivity towards iNOS activity) has been shown to have little effect in preventing the development of arthritis. L-NIL (a selective inhibitor of iNOS activity) has indeed been shown to aggravate the inflammation associated with the streptococcal cell-wall fragment model. Further, a recent report shows that in homozygous iNOS gene-deficient mice with antigen induced arthritis, the clinical scores of joint inflammation were higher in iNOS mutants compared with their wild-type counterparts. Despite these conflicting reports on the various inhibitors of NOS activity and models of arthritis, NO metabolite concentrations in serum and synovial fluid have been shown to correlate well with the acute flares of the disease. Prednisolone, glucosamine, methotrexate, and anti-tumor necrosis factor (TNF-α) therapy have all been reported to reduce endogenous NO synthesis in correlation with amelioration of the disease. These studies together with those of Sakurai, et al and McInnes, et al have suggested that intraarticular NO production may be a contributory factor for the induction and maintenance of RA. NO reacts with superoxide at near diffusion-limited rate to form peroxynitrite, a potent oxidant capable of damaging the activity of 2 urea cycle enzymes, namely, argininosuccinate synthase (AS) and argininosuccinate lyase (AL). The synthesis of iNOS and AS mRNA follow a similar time course after immunostimulation with lipopolysaccharide or cytokines such as TNF-α, interleukin 1 (IL-1), and/or interferon-γ (IFN-γ). Additionally, NOS enzyme activity is dependent on the synthesis and availability of BH4 that involves GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in the GTP–biotin pathway. GTPCH has been shown to be induced in several cell types in parallel with iNOS and AS mRNA as a result of TNF-α, IL-1, and/or IFN-γ stimulation. We have hypothesized that spontaneous overexpression of TNF-α, as seen in Tg197 h-TNF-α transgenic mice, who characteristically develop chronic polyarthritis with 100% phenotypic penetrance of the disease, leads to increased synthesis and coinduction of iNOS, AS, and GTPCH, together with peroxynitrite induced oxidative damage evidenced by 3-nitrotyrosine (3-NT). For the study, we used 4 and 8-week-old Tg197 h-TNF-α transgenic mice and age-matched nontransgenic CBA × C57B1/6 wild-type controls.

MATERIALS AND METHODS

Tg197 human-TNF-α transgenic mice. Transgenic mice carrying and expressing human-TNF-α gene constructs with modified 3’ region were developed. The 3’ region of the human TNF-α gene containing its 3’ untranslated and 3’ flanking sequences was replaced with that of the human β-globin gene. Microinjection of the construct into mouse zygotes resulted in founder transgenic mice, some of which (designated Tg197) developed swelling of the ankle joints and exhibited impaired movement. A male Tg197 (treated with an anti-h-TNF-α antibody) was mated with an F1 female and the litter was grown in a specific pathogen-free environment. The transgenic line of arthritic Tg197 h-TNF-α mice was maintained by breeding into F1 female (CBA × C57B1/6) mice. These Tg197 h-TNF-α mice develop 100% phenotypic penetrance of disease progeny with progressive polyarthritis and impairment of hind leg movement. Human TNF-α expression has been described in the arthritic joints of Tg197 mice. For this study, Tg197 h-TNF-α transgenics were bred and killed at 4 and 8 weeks of age to represent the acute and advanced/chronic stages of inflammatory arthritis.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA was isolated using TRIzol reagent (GibcoBRL, Life Technologies, Paisley, Scotland) from frozen ankle joints of 4 and 8-week-old CBA × C57B1/6 wild-type controls and Tg197 h-TNF-α mice (n = 6 for all 4 groups). The amount of the RNA was measured spectrophotometrically and the quality was determined with ethidium bromide stained 1% agarose gel under ultraviolet light. Five micrograms of the RNA was DNase treated (Promega, Madison, WI, USA), and 2.5 μg of that was transferred to cDNA with SuperScript Preamplification System using oligo(dT)12-18 for priming and RNase H for removal of mRNA according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis without enzyme and without sample were used for negative controls.

Quantitative PCR amplification was performed from 0.25 μg cDNA in LightCycler™ SYBR Green I PCR mix by LightCycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). PCR amplification was performed using 0.25 μM of target-specific primers for β-actin (accession X03672): sense 5’-CTTCTTTTGCAGCTCTTTGGT-3’ and antisense 5’-GTGCCAGATCTCTCCATGT-3’, producing a 310 bp band; for iNOS (accession U43428): sense 5’-TCCAAAGTACGAGTGGTCC-3’
and antisense 5′-TGGTCACATTGCTTCTGG-3′, producing a 299 bp band; for AS (accession XM-123777); sense 5′-TGGAATGAAGTCCC-GAGGTA-3′ and antisense 5′-ACGGTCATGCTCACCAGCTC-3′, producing a 315 bp band; and for GTPCH (accession L09737); sense 5′-GGCTGCTTACTGTCCTCATT-3′ and antisense 5′-AGCCCAATATG-GACCCCTTCCT-3′, producing a 251 bp band. For primers, corresponding sequences were searched from NCBI Entrez search system and sequence-similarity search was done using the NCBI blastn program. The identity of the PCR product was verified by a melting curve analysis. Serial dilutions of cloned PCR fragments in plasmid DNA were used to determine the copy number of the amplicon per 1000 β-actin mRNA copies. The amplified PCR fragments were cloned with a TOPO TA cloning kit (Invitrogen) into a pCRII-TOPO vector. Plasmid DNA was isolated with a High Pure Plasmid Isolation Kit (Roche). The concentration of the plasmids was analyzed spectrophotometrically. The plasmid used for quantitative PCR was sequenced using fluorescein labeled dye terminator kits supplied by ABI (Applied Biosystems, Foster City, CA, USA) and analyzed on an automatic 373A sequencer (Applied Biosystems). The acquired sequence was verified with NCBI blastn program. Each individual sample was amplified at least 2 times in all genes. Statistical significance was evaluated by 2 tailed unpaired Student t test between the age matched wild-type and Tg 197 h-TNF-α transgenic groups. A p value < 0.05 was regarded as statistically significant and the data are expressed as mean ± SEM.

RESULTS
Nontransgenic CBA × C57B1/6 wild-type control mice showed intact synovial intima and normal articular structure with no evidence of inflammation (Figure 1A). In contrast,
4 and 8-week-old Tg197 h-TNF-α transgenics had a progressive arthritis characterized by synovial hyperplasia, granulomatous lesions, and extensive erosions of cartilage and subchondral bone (Figures 1B, 1C). In 4-week-old Tg197 h-TNF-α transgenics, the distal tibial epiphysis and associated calcaneous bone were affected with discrete erosions of cartilage and underlying bone, even in the absence of pannus tissue overlying the cartilage (Figure 1B). In contrast, 8-week-old Tg197 h-TNF-α transgenics had massive joint destruction. Synovium was intensely hyperplastic and infiltrated by numerous macrophages and polymorphonuclear cells with lymphocytes dispersed among areas of granulomatous lesions. An extensive pannus formation was evident, with destruction of the underlying cartilage and subchondral bone (Figure 1C).

The copy numbers of iNOS, AS, and GTPCH mRNA from 4 and 8-week-old Tg197 h-TNF-α transgenics are shown in Table 1. Although the copy numbers of all the 3 enzyme mRNA studied seemed to increase in accord with disease progression, only iNOS mRNA showed a statistically significant difference between 4-week-old Tg197 h-TNF-α transgenics and age matched CBA × C57B1/6 wild-type controls (2.00 ± 0.894 vs 18.00 ± 3.759 iNOS mRNA copies/1000 β-actin mRNA copies; p = 0.0020).

However, it was of interest that both AS and GTPCH mRNA copy numbers were substantially higher than those of iNOS in all wild-type and transgenic groups studied (Table 1).

The cellular distribution of the molecules investigated was similar in 4 and 8-week-old Tg197 h-TNF-α transgenics, despite clear differences in the degree of tissue damage. Therefore, the results are discussed as Tg197 h-TNF-α transgenics compared with CBA × C57B1/6 wild-type controls. In wild-type controls, iNOS expression was limited to a few bone marrow cells. In Tg197 h-TNF-α transgenics, many of the infiltrating macrophage-like and some of the polymorphonuclear cells in hyperplastic synovium and pannus were heavily immunoreactive for iNOS (Figures 2A, 2B). Most of these cells were identified as macrophages and granulocytes using mouse-specific antibodies. Many of the macrophages, granulocytes, and lymphocytes infiltrated into the synovial fluid were immunoreactive for iNOS (Figure 2A). Inducible NOS was also found in vascular smooth muscle of the subintimal synovial stroma, together with 3-NT immunoreactivity. Many of the articular chondrocytes and macrophages within both subchondral and deep bone erosions showed intense immunoreactivity for iNOS (Figure 3A). Localization of 3-NT mostly overlapped with that of iNOS, but was also observed in the extracellular matrix, especially in granulomatous lesions surrounded and sometimes infiltrated by macrophages expressing iNOS (Figures 2B, 2C). No immunoreactivity for any of the antigens investigated was seen in control sections, where the primary antibody had been omitted or replaced with an irrelevant antibody of the same IgG class (Figure 3B).

In the CBA × C57B1/6 wild-type controls, AS immunoreactivity was observed in the vascular endothelium, but not in the synovial intima/subintima or cells in the deeper stroma. There was widespread induction of AS in Tg197 h-TNF-α transgenics with arthritis, most notably in fibroblast-like cells in hyperplastic synovium and pannus and macrophage-like cells at sites of subchondral bone erosions (Figures 4A, 4B), with only a partial overlapping with that of iNOS. Intense GTPCH immunoreactivity was evident in macrophages in the intima/subintima and deeper stroma in Tg197 h-TNF-α transgenics, but could not be found in the synovium from the CBA × C57B1/6 wild-type controls (Figures 5A, 5B, 5C).

As the severity of arthritis was seen to progress with age, NO metabolite NO_2−/NO_3− levels were analyzed (as a confirmation of iNOS enzyme activity and NO generation) from overnight urine samples. A significant increase in urinary NO_2−/NO_3− was found in 8-week-old Tg197 h-TNF-α transgenics compared with CBA × C57B1/6 wild-type controls (588.0 ± 194.9 and 114.2 ± 12.6 µM, respectively; p = 0.0415).

**DISCUSSION**

The development of the Tg197 h-TNF-α transgenic mouse line, which constitutively overexpress intact human-TNF-α protein, has provided a predictive genetic animal model of spontaneous RA. Thus the Tg197 h-TNF-α transgenic line provides a model for investigations of modulators or copromoters of RA. In the Tg197 h-TNF-α transgenic model, TNF-α upregulates IL-1 synthesis with subsequent exacerbation of synovial inflammation, cartilage, and bone erosion, together with IL-1 mediated maintenance of inflammation. Administration of neutralizing antibodies to either TNF-α or IL-1 receptors has been shown to have

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**Table 1. Results of quantitative reverse transcripese-polymerase chain reaction analyses.**

<table>
<thead>
<tr>
<th>Group</th>
<th>iNOS</th>
<th>AS</th>
<th>GTPCH</th>
</tr>
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<tbody>
<tr>
<td>Wild-type, 4 weeks</td>
<td>2.00 (0.894)</td>
<td>128.5 (43.37)</td>
<td>518.8 (148.0)</td>
</tr>
<tr>
<td>Tg 197, 4 weeks</td>
<td>18.00 (3.759)*</td>
<td>190.3 (27.51)</td>
<td>514.8 (103.1)</td>
</tr>
<tr>
<td>Wild-type, 8 weeks</td>
<td>2.17 (0.401)</td>
<td>59.0 (8.521)</td>
<td>578.8 (194.1)</td>
</tr>
<tr>
<td>Tg 197, 8 weeks</td>
<td>25.33 (15.21)</td>
<td>309.2 (135.5)</td>
<td>899.3 (283.0)</td>
</tr>
</tbody>
</table>

Results are normalized and given per 1000 β-actin copies (mean ± SEM). * p = 0.002.
prophylactic effects on the development of the clinical and histopathological hallmarks of RA.\textsuperscript{29,34,35}

Our study provides evidence that the L-arginine/NO pathway, including AS and GTPCH, the 2 rate-limiting enzymes for high output NO generation, is induced in the inflamed joints of Tg197 h-TNF-\(\alpha\) transgenics. We observed that iNOS is found most abundantly \textit{in situ} in macrophages that are involved in destruction of the articular architecture. These cells produced such quantities of NO that peroxynitrite was formed, as shown by the widespread localization of 3-NT. The high urinary NO\(_2^-\)/NO\(_3^-\) concentrations provided further evidence of activation of the L-arginine/NO pathway. In 8-week-old Tg197 h-TNF-\(\alpha\) transgenics urinary NO metabolites NO\(_2^-\)/NO\(_3^-\) were increased 5-fold compared with the nontransgenic wild-type controls. This was further substantiated by the fact that iNOS mRNA increased significantly during the acute phase of the disease. We did not detect clear differences in iNOS, AS, or GTPCH expression and/or their distribution between 4 and 8-week-old Tg197 h-TNF-\(\alpha\) transgenics, despite the progressive nature of the disease. Although iNOS is often only transiently induced during the acute flares of inflammation, the constitutive overexpression of h-TNF-\(\alpha\) in Tg197 h-TNF-\(\alpha\) transgenic joints seems to be sufficient to maintain the activation of iNOS protein for prolonged periods of time and throughout the pathogenesis of joint disease.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Localization of iNOS and 3-NT in 8-week-old Tg197 h-TNF-\(\alpha\) transgenic mice. (A) Many infiltrating macrophages in hyperplastic synovium (arrows) and macrophages, granulocytes, and lymphocytes within the synovial fluid/exudate (arrowheads) were heavily immunoreactive for iNOS (A and B). (B) Fibrin deposition was evident within granulomatosus synovial stroma infiltrated predominantly with macrophages that were also immunoreactive for iNOS (arrows). (C) Peroxynitrite induced oxidative damage and nitrosylation of tyrosine groups was detected with antibodies to 3-NT. 3-NT immunoreactivity was extensively localized to the granulomatosus extracellular matrix surrounded and infiltrated by iNOS-expressing macrophage-like cells. 3-NT was also localized to some inflammatory cells that appeared to have a similar distribution to those that were characterized by iNOS immunoreactivity (arrows). Original magnification \(\times100\).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Inducible NOS localization in 8-week-old Tg197 h-TNF-\(\alpha\) transgenic bone erosions. (A) Immunoreactivity for iNOS was localized to macrophage-like cells (arrows) within the calcaneous bone erosions. (B) The immunoreactivity was absent when antiserum for iNOS had been replaced with an irrelevant antibody with the same IgG class. Original magnification \(\times100\).}
\end{figure}
destruction. Perhaps more important, coinduction of AS and GTPCH protein was also maintained from acute (4 weeks) to chronic phase (8 weeks) of the disease. This implies that the increased synthesis of L-arginine and BH4 were both maintained and able to provide the essential substrate (L-arginine) and cofactor (BH4) for prolonged activity of iNOS with subsequent high output NO generation, as evidenced by 3-NT and urinary NO metabolites.

We observed widespread iNOS expression in the inflamed joints of Tg197 h-TNF-α transgenics with spontaneous arthritis. Immunoreactivity for 3-NT was shown to overlap and in part exceed that of iNOS. Previous studies have described 3-NT in serum and synovial fluid in RA, whereas none was detected in healthy controls or osteoarthritis19. In a recent study by Mapp and co-workers, 3-NT was immunolocalized to vascular smooth muscle cells and CD 68+ macrophages in human RA synovium21. Detection of 3-NT has commonly been taken as evidence of NO mediated peroxynitrite generation18-20,33. Peroxynitrite is a potent and highly reactive oxidant species that can suppress cellular respiration and cause damage to cell membrane lipids and DNA bases, contributing to cell injury and death. Peroxynitrite forms in a reaction between NO and superoxide and its decomposition produces the hydroxyl and nitronium ions that contribute to nitration of aromatic amino acids including tyrosine18. Peroxynitrite may have other proinflammatory actions as well. One of the proinflammatory actions of TNF-α is through NO mediated induction of cyclooxygenase-2 transcription and transla-

Figure 4. AS immunoreactivity in 4 and 8-week-old Tg197 h-TNF-α transgenics. (A) In 4-week-old Tg197 h-TNF-α transgenics, AS-immunoreactive fibroblast-like cells were evident in the inflammatory synovium and macrophage-like cells in pannus and at sites of subchondral bone erosions (arrows). (B) In 8-week-old Tg197 h-TNF-α transgenics, AS expression was evident in numerous stromal fibroblast-like cells in synovium and pannus. Original magnification (A) ×100 and (B) ×75.

Figure 5. GTP cyclohydrolase immunoreactivity in 4-week-old CBA × C57B1/6 wild-type control and 4 and 8-week-old Tg197 h-TNF-α transgenic synovium. (A) Immunoreactivity for GTP cyclohydrolase was not found in the wild-type control synovium. In contrast, GTP cyclohydrolase immunoreactivity was widespread within the macrophage-like cells (arrows) in the subintimal and deeper stroma and pannus from 4-week-old (B) and 8-week-old (C) Tg197 h-TNF-α transgenic mice. Original magnification ×100.
tion. Peroxynitrite can mediate induction of cyclooxygenase activity by serving as a substrate for the peroxidase activity of the enzyme. Taken together, the colocalization of 3-NT in the inflammatory joints of Tg197 h-TNF-α transgenics with iNOS positive macrophages provides a strong indication that peroxynitrite mediated oxidation and nitration occurs as a result of h-TNF-α mediated iNOS synthesis and NO generation.

In the arthritic joints of Tg197 h-TNF-α transgenics, AS and GTPCH protein were coinduced together with iNOS. AS and GTPCH mRNA copy numbers seemed to increase in accord with disease progression, although they did not reach significance concentrations due to high intraassay variability. This may have been due to the severity of inflammation with subsequent mRNA cleavage and degradation. However, the results of the quantitative RT-PCR assay showed that both AS and GTPCH mRNA were much higher than iNOS mRNA copy numbers in all groups studied. This may imply that the synthesis of AS and GTPCH, 2 of the rate-limiting enzymes for high output NO generation, is more strictly regulated at the posttranscriptional than the transcriptional level in vivo. AS and GTPCH have not been previously studied in inflammatory arthritides. AS and argininosuccinate lyase (AL) together confer the ability of cells to regenerate the NO substrate L-arginine from L-citrulline (a coproduct of NO activity). In inflammation and during periods of high output NO generation, the availability of free L-arginine is rate-limiting to NO synthesis. While AL mRNA and protein synthesis are constitutive or only weakly regulated, AS mRNA and protein synthesis, at least in vitro, are thought to be highly regulated and induced by immunostimulants such as TNF-α, IL-1, and IFN-γ.

In this study, AS was localized in CBA × C57Bl/6 wild-type controls in the vascular endothelium. In contrast, in Tg197 h-TNF-α transgenic arthritic joints AS was induced in fibroblasts in hyperplastic synovium and pannus and some macrophage-like cells at sites of subchondral bone erosions. An interesting finding was that iNOS was mostly localized to macrophage-like cells, while AS expression was most common in fibroblasts, with only discrete macrophage-like cells being positive. Therefore, there was only partial overlapping of iNOS and AS, implying possible paracrine interactions between macrophages that synthesize iNOS and their neighboring fibroblasts that synthesize AS. Nevertheless, these findings imply that the arginase-citrulline cycle is strongly induced in the Tg197 h-TNF-α transgenics with spontaneous arthritis.

We characterized the Tg197 h-TNF-α transgenic mouse model of RA in terms of the high output NO-generating pathway including AS and GTPCH, the 2 rate-limiting enzymes necessary for maximal NO production. The concomitant induction of GTPCH with iNOS and AS is essential for high output NO generation and that the GTP-bioterin pathway is induced in arthritis, at least in the Tg197 h-TNF-α transgenic model of RA.

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