Plasmid DNA Encoding Transforming Growth Factor-β1 Suppresses Chronic Disease in a Streptococcal Cell Wall–induced Arthritis Model

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Abstract

Transforming growth factor β is a potent immunomodulator with both pro- and antiinflammatory activities. Based on its immunosuppressive actions, exogenous TGF-β has been shown to inhibit autoimmune and chronic inflammatory diseases. To further explore the potential therapeutic role of TGF-β, we administered a plasmid DNA encoding human TGF-β1 intramuscularly to rats with streptococcal cell wall–induced arthritis. A single dose of 300 μg plasmid DNA encoding TGF-β1, but not vector DNA, administered at the peak of the acute phase profoundly suppressed the subsequent evolution of chronic erosive disease typified by disabling joint swelling and deformity (articular index = 8.17±0.17 vs. 1.25±0.76, $n = 6$, day 26, $P < 0.01$). Moreover, delivery of the TGF-β1 DNA even as the chronic phase commenced virtually eliminated subsequent inflammation and arthritis. Both radiologic and histopathologic as well as molecular evidence supported the marked inhibitory effect of TGF-β1 DNA on synovial pathology, with decreases in the inflammatory cell infiltration, pannus formation, cartilage and bone destruction, and the expression of proinflammatory cytokines that characterize this model. Increases in TGF-β1 protein were detected in the circulation of TGF-β1 DNA-treated animals, consistent with the observed therapeutic effects being TGF-β1 dependent. These observations provide the first evidence that gene transfer of plasmid DNA encoding TGF-β1 provides a mechanism to deliver this potent cytokine that effectively suppresses ongoing inflammatory pathology in arthritis. (*J. Clin. Invest.* 1998. 101:2615–2621.) Key words: cytokine • inflammation • immunosuppression • gene therapy • arthritis

Introduction

In addition to its role in development, differentiation, tissue repair, and tumorigenesis, transforming growth factor-β (TGF-β)[1] is a key immunomodulatory protein exhibiting both pro- and antiinflammatory activities (1–4). These profound immunoregulatory properties of TGF-β raise the potential for its therapeutic use in autoimmune and chronic inflammatory diseases. In this regard, systemic administration of TGF-β has been shown to reverse inflammation and immune-mediated pathology in vivo (5–9). The route of administration is critical, since in contrast to systemic delivery, direct local injections of TGF-β into synovium can exacerbate an arthritic response (10, 11), consistent with reversal of synovitis by intra-articularly administered neutralizing antibodies to TGF-β (12). Nonetheless, the repeated demonstration that systemically delivered TGF-β can effectively control autoimmune pathology, including arthritic lesions, is tempered by the marrow suppression, anemia, and fibrotic changes that have been associated with repetitive administration of nonphysiologic quantities of the cytokine (13, 14). Despite these limitations, TGF-β remains a potential candidate to develop for therapeutic application.

One potential route of TGF-β delivery involves the use of plasmid DNA. Upregulation of cytokine gene expression by intramuscular injection of plasmid cDNA has been used in experiments with reporter gene constructs (15–17), and to examine the effects of individual cytokines in experimental animals (18). Unlike retro- and adeno-associated viral vector–mediated gene transfer (19), direct plasmid DNA injection does not require integration of the gene into the host genome (17, 20), nor cause untoward effects associated with viral vectors (21). Moreover, the dosing regimen can be easily modified after changes in disease indices in a "druglike" manner.

This study was designed to examine the effectiveness of a plasmid DNA encoding TGF-β1 on the development of experimental arthritis induced by streptococcal cell wall (SCW) peptidoglycan-polysaccharide complexes (5, 12, 22). In this model, the acute neutrophil-dependent phase is followed by a chronic arthritis involving T-cells and macrophages that shares clinical and histopathological characteristics with human rheumatoid arthritis (22). After an i.m. injection of plasmid DNA encoding TGF-β1 into each thigh of the arthritic animals during acute inflammation or even during the early chronic phase, we demonstrate marked inhibition of disease progression. Moreover, the inhibitory effects are sustained through the 2–3-wk evaluation period and in some cases for several months after treatment, suggesting a novel approach to the amelioration of chronic erosive synovitis.

Methods

TGF-β1 construct and delivery regimen. Human TGF-β1 cDNA restricted from pBRHTGF-β (59954; American Type Culture Collection, Rockville, MD) was cloned as a 1.6-kb XhoI-EcoRI fragment into pCI, an expression vector (Promega, Madison, WI). The final expression construct of pCIHTGF-βXho contains the human TGF-β1 gene driven by a cytomegalovirus promoter. Transfection of

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1. *Abbreviations used in this paper:* AI, articular index; SCW, streptococcal cell wall; TGF-β, transforming growth factor β.

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pCIHTGF-βXho into HeLa cells was performed with lipofectamine (GIBCO BRL, Gaithersburg, MD), and production of TGF-β1 in the supernatants was determined 48 h after transfection by TGF-β1 ELISA (Promega). The plasmid DNA for animal experiments was prepared from DH5α *Escherichia coli* cells and purified with an endotoxin-free plasmid extraction kit (QIAGEN Inc., Chatsworth, CA). 37.5–200 μg of either plasmid DNA encoding TGF-β1 or plasmid vector DNA were injected i.m. into three sites in each thigh at times specified for each group of rats.

Induction and monitoring of arthritis. Arthritis was initiated in genetically susceptible female Lewis rats (Charles River Breeding Laboratories, Wilmington, MA) by intraperitoneal injection of group A SCW peptidoglycan-polysaccharide complexes (Lee Laboratories Inc., Grayson, GA) as described (22). The severity of arthritis (articular index, AI) was determined by scoring each ankle and wrist joint blinded based on the degree of swelling, erythema, and distortion on a scale of 0–4 and summing the scores for all four limbs. In parallel, hind paw swelling was measured with a plethysmometer (UGO BASILE, Varese, Italy). Statistical significance was ascertained using the Student's *t* test. Radiographs were taken with direct exposure (1:1) on X-omat film (Eastman Kodak Co., Rochester, NY) using 30 mev, 90-s exposure using a Faxitron x-ray machine (Faxitron X-Ray Corp., Buffalo Grove, IL).

PCR analysis. Femoris muscles were harvested 3 d after injection with plasmid DNA, frozen immediately, and homogenized for analysis. Total cellular DNA was isolated by proteinase K digestion

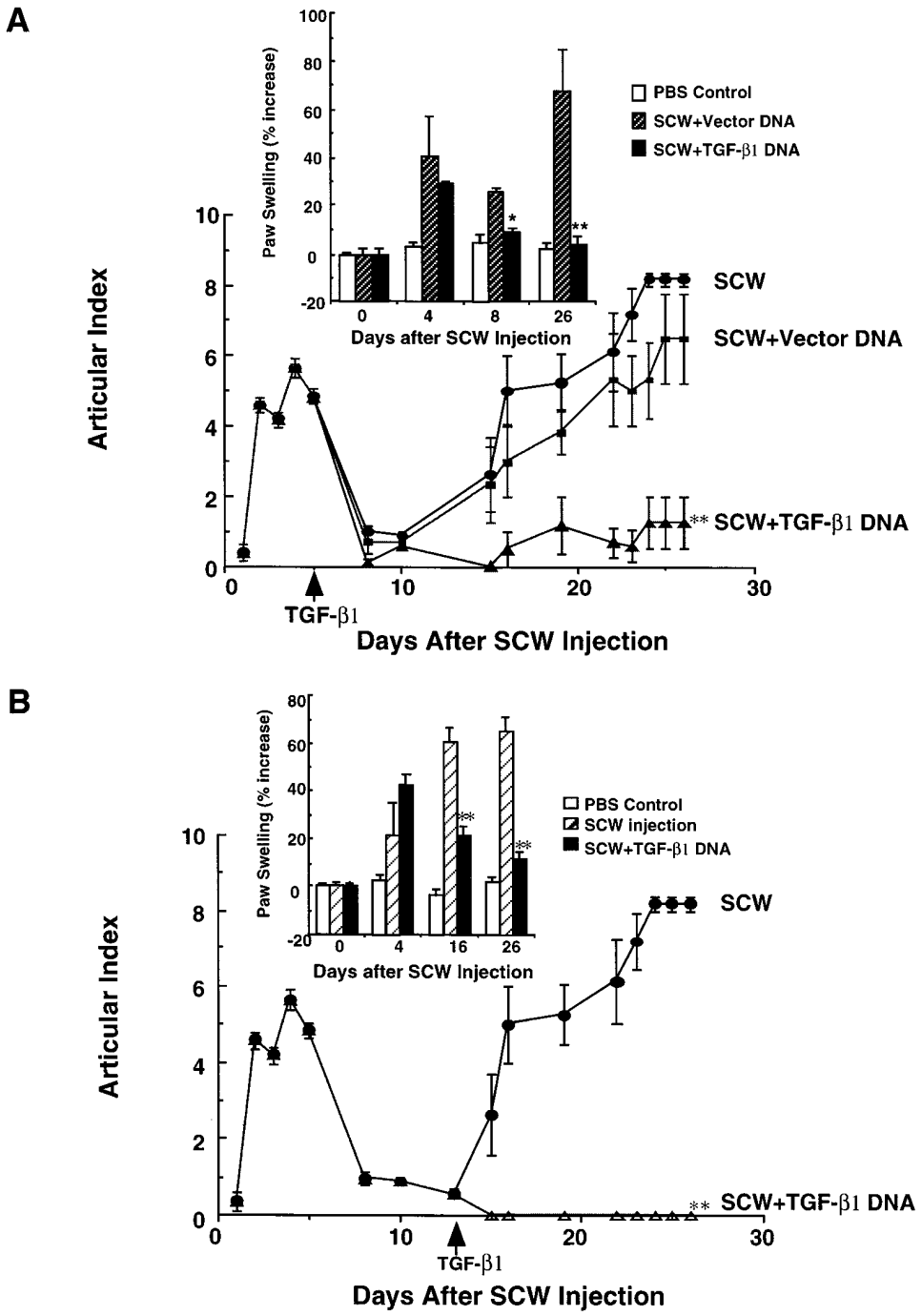

Figure 1. Plasmid DNA encoding TGF-β1 suppresses SCW-induced arthritis. (*A*) Animals were injected intraperitoneally with SCW on day 0, and on day 5 received 300 μg of plasmid DNA encoding TGF-β1 i.m. (▲, *solid bars in the inset*, $n = 6$), vector DNA (■, *hatched bars*, $n = 3$), or no treatment (●, SCW, $n = 10$ before and 6 after day 13). PBS-injected control animals had AI = 0 (data not shown), except their paw volumes are plotted in the inset (*open bars*, $n = 3$). Each point in the graph represents the mean AI±SEM for each group of animals. The paw volume is plotted as mean percent increase after SCW or PBS injection, compared with pretreatment values±SEM. (*B*) AI±SEM for animals injected with SCW (●, $n = 10$ before and 6 after day 13), or SCW followed by 300 μg TGF-β1-containing plasmid DNA on day 13 (△, $n = 3$). Paw volume is plotted as in *A* (*open bars*, PBS-injected control animals; *hatched bars*, SCW-injected animals; *solid bars*, SCW animals receiving TGF-β1 DNA on day 13). $^*P < 0.05$, $^{**}P < 0.01$.

and subsequent phenol-chloroform-isoamyl alcohol extraction method. Approximately 5 μg total cellular DNA from each tissue was subjected to PCR using primer pair 1, corresponding to sequences within the pCI plasmid (5′-TGGGCTTGTCGAGACAGAGAAG-3′) and the hTGF-β1 cDNA (5′-CGATAGTCTTGCAGGTGGAT-AGTCC-3′), generating a 379-bp product that represented transgenic TGF-β1 DNA. A second pair of primers was designed to detect only the vector pCI DNA (5′-GGTCTTACTGACATCCACTTTGCC-3′, and 5′-CATCTCCCCCTGAACCTGAAAC-3′), giving rise to a 337-bp product when pCI vector DNA was used as template. PCR conditions were 45 cycles (94°C for 1 min, 54°C for 1 min, and 72°C for 1 min), followed by extension at 72°C for 10 min. The resulting PCR products were fractionated on a 2% agarose gel and visualized with ethidium bromide. SalI and XhoI restriction enzymes were purchased from Life Technologies Inc. (GIBCO BRL).

Histology and immunohistochemistry. Excised ankle joints were fixed in 10% neutral formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections were stained with hematoxylin and eosin for histopathology (22). For immunohistochemical analysis of TGF-β expression in muscle tissue, femoris muscles were collected, cut into several small pieces, embedded in ornithine carbamyl transferase (OCT), and frozen in liquid nitrogen–cooled isopentane. 6 μm serial cryostat sections were then placed on glass slides and air dried. The sections were then stained with anti–TGF-β1, -β2, and -β3 (Genzyme Diagnostics, Cambridge, MA) at 50 μg/ml using the alkaline phosphatase staining kit and counterstained with methyl green (Vector Laboratories, Inc., Burlingame, CA).

Leukocyte counts. Peripheral blood was obtained by intracardiac puncture on day 26 and total white blood cell counts were determined by Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) analysis.

ELISA analysis. Serum samples were collected either from tail bleeding at times specified for each experiment or from intracardiac puncture when animals were killed on day 26. Serum levels of TGF-β1 and TNFα were determined by ELISA according to the manufacturer's instructions (TGF-β1 ELISA kit; Promega; TNFα kit; Biosource International, Camarillo, CA).

Results and Discussion

Therapeutic effects of TGF-β1 gene delivery on established arthritis. In initial studies, plasmid DNA encoding TGF-β1 (300 μg) was injected i.m. 5 or 2 d before the i.p. injection of SCW. In neither case did this treatment significantly diminish the acute inflammatory response (data not shown). However, additional injections of plasmid DNA 5–13 d after the onset of arthritis were associated with diminution of clinical symptoms. Since the treatment of established arthritis is more relevant than prophylaxis, we focused on gene transfer in rats already experiencing synovial pathology. Rats given an arthritogenic dose of SCW experience an acute inflammatory response within 24 h, reflecting endothelial cell swelling, platelet and fibrin thrombus formation, increased expression of class II MHC and adhesion molecules, and infiltration of phagocytic cells into the synovium (22). When 300 μg of plasmid DNA encoding TGF-β1 was delivered i.m. 5 d after the initiation of this acute response, evolution of the ensuing chronic disease was markedly suppressed. Whether measured by clinical AI (Fig. 1 *A*) or by plethysmometer, which determines paw volume (Fig. 1 *A, inset*), disease severity was reduced in TGF-β1 DNA-treated animals as compared with untreated SCW arthritic rats (AI = 8.7±0.17 vs. 1.25±0.76, $n = 6$, day 26, $P < 0.01$; and paw volume = 67.6 vs. 4% increase).

In subsequent studies, the plasmid DNA encoding TGF-β1 was injected as late as 13 d after the initiation of arthritis. This treatment essentially eliminated signs and symptoms of chronic disease as determined by both AI and paw volume (Fig. 1 *B*). In contrast, rats injected with vector DNA, whether on day 5 or 13 after onset of arthritis, developed the same extent of joint swelling and pathology as the untreated SCW-injected animals (Fig. 1 *A*).

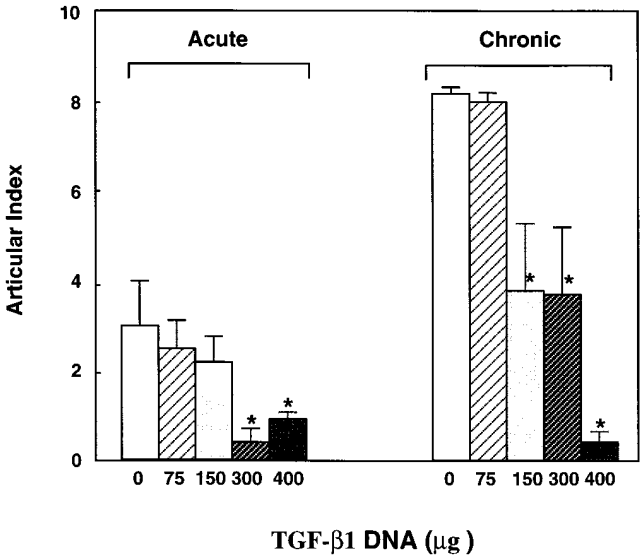

Figure 2. Dose–response of plasmid DNA encoding TGF-β1. Age-matched female Lewis rats were randomly divided into five groups. Plasmid DNA encoding TGF-β1 from 75 to 400 μg/animal was injected i.m. 5 d after SCW injection, and the effects on SCW-induced arthritis were assessed by articular index on the late acute response (day 8 after SCW) and the chronic disease (day 26). Data represent the mean AI±SEM for each group of animals ($n = 3$–6). *$P < 0.01$.

To assess whether TGF-β1 plasmid DNA injection affects arthritis in a dose-dependent fashion, 75–400 μg of plasmid DNA encoding TGF-β1 were injected i.m. 5 d after an arthritogenic dose of SCW. At the late acute phase (day 8), which was only 3 d after TGF-β1 plasmid DNA injection, only doses ≥ 300 μg were significantly inhibitory (Fig. 2). However, during the ensuing chronic disease (26 d after SCW), a dose as low as 150 μg was seen to be efficacious.

Effects of plasmid DNA encoding TGF-β1 on joint histopathology. Radiologic examination of the SCW-injected rats treated with 300 μg TGF-β1 DNA revealed profound suppression of the soft tissue swelling and disfiguring cartilage and bone erosion typical of the chronic synovial lesions (Fig. 3 *A*). Clearly impacting on tissue destruction, we observed at the cellular level that TGF-β1 DNA-treated animals exhibited greatly diminished mononuclear cell infiltration into the synovium, minimal pannus formation, and essentially no cartilage and bone destruction (Fig. 3, *C* and *D*), paralleling the radiologic features (Fig. 3 *B*). Except for modest thickening and cellularity in the synovium, the joints of TGF-β1 DNA-treated animals resembled disease-free control synovium (Fig. 3 *E*). Vector-only treated SCW arthritic animals exhibited a similar profile of histopathological features to those observed in untreated SCW-injected animals (data not shown). These dramatic results suggest that sufficient TGF-β1 is produced in the muscle and released to influence the course of inflammation and pathology in the synovium.

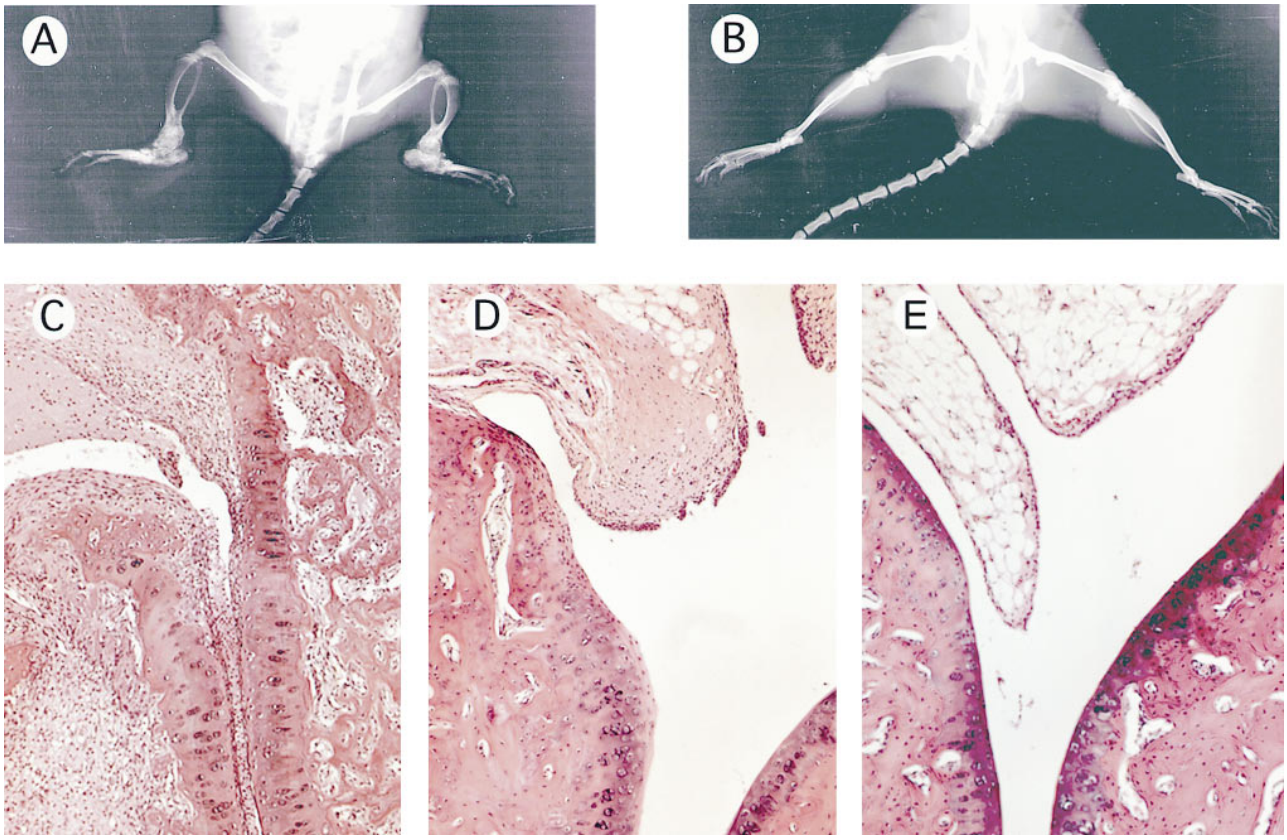

Figure 3. TGF-β1 DNA treatment inhibits pathogenesis in SCW-injected animals. Radiographs of representative animals 26 d after SCW (*A*) or after SCW and 300 μg of plasmid DNA encoding TGF-β1 on day 5 (*B*). Histopathology of synovial tissue from animals 26 d after receiving an arthritogenic dose of SCW (*C*), SCW with 300 μg of plasmid DNA encoding TGF-β1 on day 5 (*D*), or no SCW or plasmid (*E*). Original magnification, 40×.

Local and circulating levels of TGF-β1. Muscle tissue at the site of plasmid injection was excised and assessed for TGF-β1 DNA by PCR. The transgenic TGF-β1 DNA amplified with primer pair 1 (Fig. 4 *A*, lane *1*) was detected in the femoris muscles of rats given pCIHTGF-βXho, but not in vector-treated rats (Fig. 4 *A*, lane *2*). Conversely, the 337-bp vector DNA PCR product (primer pair 2) was only detected in vector-treated animals (Fig. 4 *A*, lane *3*), whereas neither PCR-product was evident in muscle tissues from PBS control animals (Fig. 4 *A*, lanes *4* and *5*). In addition, the PCR products obtained were proven to be amplified from exogenously delivered plasmid DNA encoding TGF-β1 or vector DNA, respectively, through restriction analysis of the PCR products (data not shown). Furthermore, by immunohistochemical analysis, the expression of TGF-β protein was present at the local injection site of TGF-β1 DNA and absent at the site of vector DNA injection (Fig. 4, *B* and *C*). We observed little evidence of fibrosis at the injection site, which may reflect the overexpression of latent, rather than active, TGF-β1.

Circulating TGF-β1 protein was measured in serum samples obtained from SCW- and PBS-injected animals with or without DNA treatment. 3 d after TGF-β1 DNA injection, an $\sim$ 30–40% increase in total TGF-β1 protein was detected in DNA-treated vs. untreated animals whether the animals were arthritic (SCW injected) or nonarthritic (PBS injected), which then declined over the subsequent 2–3 wk (Fig. 4 *D*). When active TGF-β1 protein (without acid activation) was measured in the circulation of DNA-treated PBS-injected nonarthritic animals, peak levels were also observed 3 d after DNA injection (Fig. 4 *D*), whereas active TGF-β1 protein was not detected in control animals that did not receive plasmid DNA encoding TGF-β1. Based on these data, the observed therapeutic effect of TGF-β1 DNA injection most likely involves increased levels of TGF-β1 protein produced by transfected muscle cells and released into the bloodstream.

Since injection of plasmid DNA encoding TGF-β1 5 or even 2 d before SCW injection failed to affect arthritis progression (our unpublished results), timing of the DNA delivery appears critical. The maintenance of elevated systemic levels of TGF-β between the peak of the acute (day 5) and the beginning of the chronic (day 13) phase of the disease may be essential for the effective dampening of the T cell–dependent immune response. These findings are compatible with our recent observations that natural killer cell–derived TGF-β released during this interval, but not sustained, contributes to the transient remission of acute arthritis (Fig. 1), which precedes the chronic destructive phase (manuscript in preparation). Although TGF-β1 levels decline in the DNA-injected animals over time, irrespective of different delivery doses, we were surprised that, in a preliminary experiment, no disease flare-ups

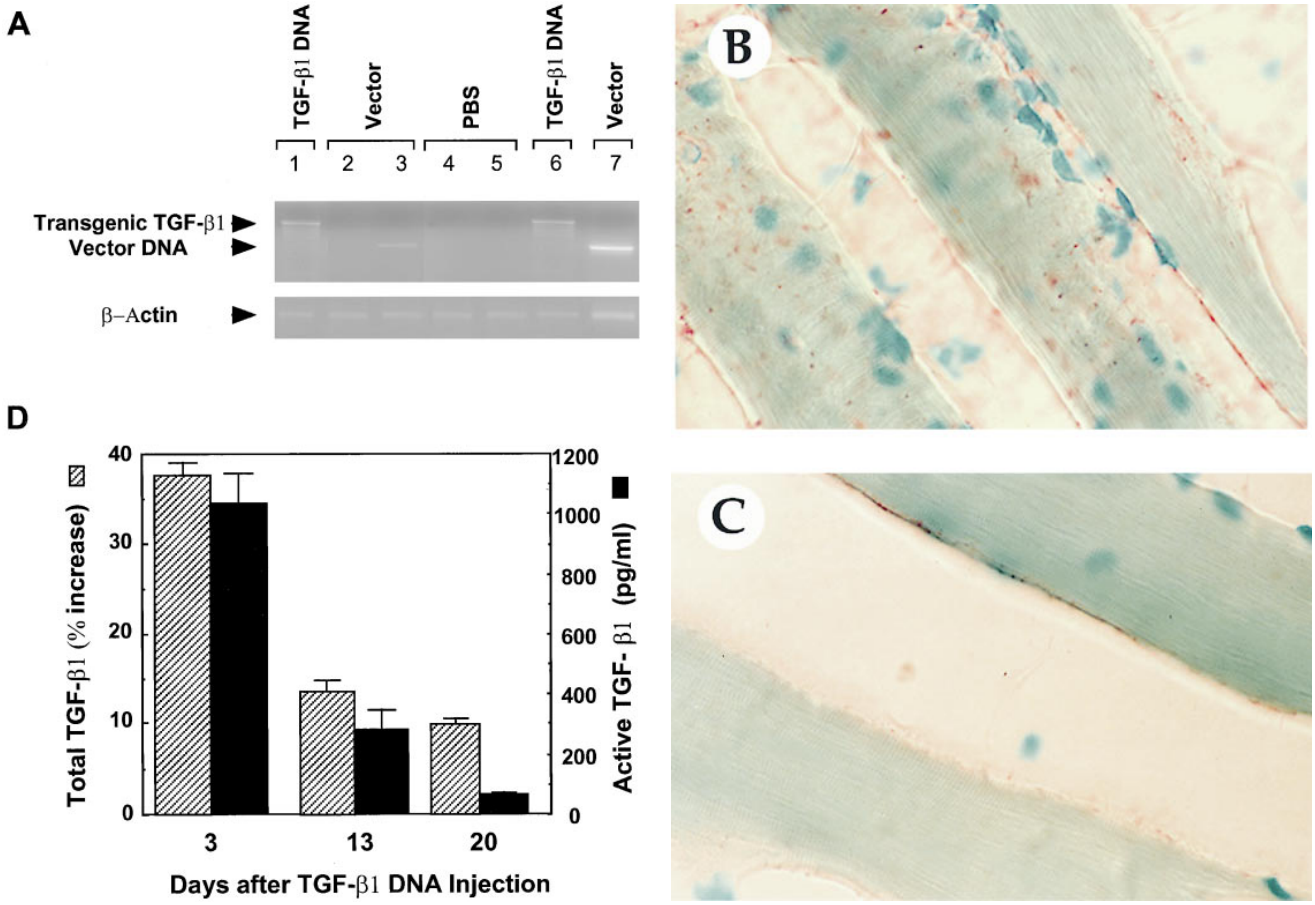

Figure 4. (*A*) Localization of TGF-β1 DNA at the injection site. Total cellular DNA from muscle tissues of treated and control animals was analyzed for the presence of either plasmid DNA encoding TGF-β1 (lanes *1, 2, 4,* and *6* using primer pair 1) or pCI vector DNA (lanes *3, 5,* and *7* using primer pair 2). Lane *1*, muscle 3 d after 300 μg TGF-β1-encoding plasmid DNA injection; lanes *2* and *3*, muscle 3 d after vector injection; lanes *4* and *5*, muscle from PBS-injected control animals. Lanes *6* and *7*, positive controls with either pCIHTGF-βXho-or pCI vector-transfected Hela cell DNA. Shown are representatives of six samples. (*B* and *C*) Detection of TGF-β1 protein expression at the local injection site. Femoris muscle tissue was obtained 3 d after TGF-β1 or vector DNA injection in SCW animals, embedded in ornithine carbamyl transferase and stained for TGF-β. Positive staining (*red*) was detected in TGF-β1 DNA-injected muscle tissue (*B*), but not in vector DNA-injected muscle (*C*). Original magnification, 100×. (*D*) Determination of circulating TGF-β1 levels. Sera were collected from SCW- or PBS-injected animals at indicated times after 300 μg TGF-β1 DNA injection. Sera were diluted 1:50 in PBS, acid-activated (total), and analyzed by TGF-β1 ELISA. Data represent percent increase±SEM above similarly treated animals without DNA. Serum levels of active TGF-β1 protein measured by ELISA after 1:10 dilution, but without acid activation from PBS-injected nonarthritic animals after 300 μg TGF-β1 DNA injection. Data shown are from three representative experiments.

were observed in DNA-injected animals that were followed for up to 3 mo after arthritogenic SCW (AI = 0.63±0.37; $n = 2$).

Systemic effects of TGF-β1 DNA delivery. To determine whether the impact of TGF-β1 was localized to the synovium or manifested systemically, we monitored the peripheral white blood cell (WBC) count in treated animals. As frequently occurs in chronic inflammatory diseases, the total number of circulating WBCs was significantly increased in SCW-injected animals (Table I). Interestingly, this leukocytosis was drastically curtailed after injection of plasmid DNA encoding TGF-β1, reflective of reduced inflammatory disease. Anemia, a common manifestation of SCW-induced arthritis (23) and a complication of chronic TGF-β administration (13), was in fact reversed by TGF-β1 DNA treatment as measured by hematocrit levels 26 d after SCW-injection (59±5% in TGF-β1 DNA-treated vs. 42±8% in untreated arthritic animals). Moreover, when circulating levels of TNFα, a key cytokine in arthritic disease (24), were measured before and after TGF-β1 DNA treatment, a significant reduction was detected (254.2 pg/ml in SCW vs. 123.5 pg/ml in DNA treated, day 26, $P < 0.05$). These and other data support a more generalized effect of TGF-β1, rather than a tissue-specific response. The failure to detect increased TGF-β protein in the already TGF-β-laden synovium (data not shown) coupled with the altered profile of tissue and systemic pathology, is reminiscent of the impact of systemically delivered TGF-β in this model (5). Since sustained TGF-β1 overproduction has been associated with increased matrix production and deposition in the kidneys (14, 25), kidney sections from animals in different treatment groups were examined by periodic acid Schiff and Masson trichrome staining. No aberrant morphological changes in glomeruli, renal tubules, renal interstitium, or vessels were observed in animals receiving ≤ 300 μg TGF-β1 DNA, whereas mild glomerulosclerosis, interstitial fibrosis, and tubular casts could be detected by 2 wk

Table I. Effect of Plasmid DNA Encoding TGF-β1 on Circulating WBC Levels

Treatment	Control	Vector control*	TGF-β1 plasmid DNA*
	WBC ($\times 10^3/mm^3 \pm$SEM)		
PBS control	6.8±0.04	6.7±4.0	8.7±7
SCW	57.8±10.6	27.9±2.8	14.7±3.9
			(12.3±0.9)‡

*Plasmid DNA encoding TGF-β1 or vector DNA was administered intramuscularly to rats 5 d after intraperitoneal injections of SCW or PBS. WBC counts were determined on day 26. ‡Animals receiving an arthritogenic dose of SCW on day 0 were given plasmid DNA encoding TGF-β1 on day 13. WBC counts were determined on day 26.

after doses ≥ 400 μg (J.B. Kopp, personal communication). Since a significant therapeutic effect on SCW-induced arthritis can be achieved with as low as 150 μg TGF-β1 plasmid DNA, this potential side effect need not deter the use of plasmid DNA encoding TGF-β1.

Although gene transfer is becoming a more attractive approach to the treatment of human diseases, vectors commonly employed in gene transfer, including retroviruses and adenoviruses (26), continue to pose problems. Adenovirus is the most widely used gene therapy vector due to its ability to enter nondividing cells (27, 28), as well as its ability to propagate without integrating into the host genome (26), yet the occurrence of vector-mediated inflammation continues to plague this approach (21). In contrast to viral-mediated gene transfer, direct plasmid DNA injection is not associated with adverse viral effects and also provides an easier means to construct and deliver more homogenous DNA. Long-term high-level expression of an erythropoietin (Epo)-encoding plasmid DNA in mice confirmed that a single intramuscular injection of plasmid resulted in physiologically relevant levels of circulating Epo sufficient to treat anemia (29). In addition, direct plasmid DNA transfer has recently been employed to effectively deliver TGF-β to rodents with lupus and colitis (30, 31).

Our study demonstrates that a single TGF-β1 DNA injection not only exerts a therapeutic effect in SCW-induced arthritis, but that this single intramuscular DNA injection overcomes the limitations of the short half life of TGF-β and the difficulties associated with daily systemic inoculation of active TGF-β protein (5). Moreover, these gene transfer studies suggest that muscle-based therapy with TGF-β1 DNA results in effective systemic delivery of TGF-β1 characterized by reduced leukocytosis, blockade of the chemotactic gradient, alterations in cytokine profiles, and reduced inflammation, all contributing to arthritis suppression (2, 5). Although TGF-β1 gene transfer into splenocytes ex vivo has recently shown efficacy in collagen-induced arthritis (32), we provide initial documentation that TGF-β1 gene therapy via naked plasmid DNA can effectively suppress the development and progression of chronic arthritis even after the onset of disease.

Acknowledgments

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