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Inhibition of Bacterial Cell Wall-Induced Leukocyte Recruitment and Hepatic Granuloma Formation by TGF-β Gene Transfer

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Intraperitoneal injection of streptococcal cell walls (SCW) into Lewis rats results in dissemination of SCW to the liver, spleen, bone marrow, and peripheral joints. The uptake of SCW by Kupffer cells in the liver initiates a chain of events largely mediated by T lymphocytes and macrophages. Local synthesis and secretion of cytokines and growth factors in response to the persistent SCW lead to the evolution and maintenance of a chronic T cell-dependent granulomatous response and result in granuloma formation and irreversible hepatic fibrosis. In an attempt to impede the development of the chronic granulomatous lesions in the liver, we injected a plasmid DNA encoding TGF-β1 i.m. to the SCW animals to determine the effect of TGF-β1 gene transfer on the course of liver inflammation and fibrosis. A single injection of plasmid DNA encoding TGF-β1 resulted in virtual abolition of the development of the SCW-induced hepatic granuloma formation and matrix expansion. TGF-β1 DNA not only reduced key proinflammatory cytokines including TNF-α, IL-1β, IFN-γ, and IL-18, but also inhibited both CXC and CC chemokine production, thereby blocking inflammatory cell recruitment and accumulation in the liver. Moreover, TGF-β1 gene delivery inhibited its own expression in the liver tissue, which is otherwise up-regulated in SCW-injected animals. Our study suggests that TGF-β1 gene transfer suppresses hepatic granuloma formation by blocking the recruitment of inflammatory cells to the liver, and thus may provide a new approach to the control of hepatic granulomatous and fibrotic diseases. The Journal of Immunology, 1999, 163: 4020–4026.

Fibrosis, a pathologic response to tissue injury, involves excessive accumulation of extracellular matrix proteins and disruption of tissue function. Hepatic fibrosis has been associated with a variety of cytokines and growth factors, including TGF-β, which is increased in chronic liver diseases, including chronic hepatitis, alcoholic cirrhosis, and parasitic diseases such as schistosomiasis (1–5). The ability of TGF-β to promote synthesis of both extracellular matrix and protease inhibitors, while inhibiting matrix-degrading enzymes, favors matrix accumulation essential to tissue repair. Loss of control between fibrogenesis and fibrolysis eventually results in fibrosis characterized by inappropriate proliferation of mesenchymal cells and deposition of matrix molecules, impacting on both function and architecture of an afflicted tissue (6–8). Recent studies utilizing TGF-β transgenic mice have strengthened the link between TGF-β1 and liver fibrosis (1, 9, 10).

Due to the potential life-threatening consequences of liver failure as a result of hepatic fibrosis, efforts have centered on defining targets for therapeutic manipulation. Animal models are indispensable for understanding the cellular and molecular events in liver fibrosis and for assessing interventional approaches. One model of liver fibrosis is initiated by a systemic injection of bacterial cell walls from Group A streptococci (SCW).2 When these peptidoglycan-polysaccharide complexes are taken up by liver Kupffer cells, TGF-β and other proinflammatory cytokines are produced (11, 12), which then induce a chain of events largely mediated by T lymphocytes and macrophages (13). Neutrophil accumulation during the first several days after SCW injection is then replaced by predominantly mononuclear cells. Members of the CXC chemokine family, mediating neutrophil recruitment, and mononuclear cell chemotactic CC chemokines, including MCP-1, are expressed in a temporal manner in SCW-inflamed livers.3 Local synthesis and secretion of cytokines and growth factors in response to the persistent SCW are responsible for the evolution and maintenance of a chronic T cell-dependent granulomatous response (11, 13). The granulomas, composed of large central cores of mononuclear cells, become surrounded by fibroblastic cells, the latter contributing to development of the collagenous capsules (14). After several weeks, severely granulomatous livers may constitute up to 12% of a rat’s body weight compared with ~4% in normal rats. The chronic nature of this response and inability to clear the poorly biodegradable SCW leads to unrelenting attempts at tissue repair by hepatic stellate cells (13, 15). It is these continued attempts to wall off and repair hepatic injury that lead to development of oftentimes irreversible fibrosis and hepatic failure.

Based on recent evidence that TGF-β gene transfer through direct i.m. injection of plasmid DNA-encoding TGF-β1 can ameliorate the signs and symptoms of arthritis (16), we utilized this procedure to determine whether a similar regimen would influence the course of liver inflammation and fibrosis. A single injection of plasmid DNA-encoding TGF-β1 resulted in virtual abolition of the development of the SCW-induced hepatic granuloma formation and matrix expansion. Although counterintuitive that a profibrotic

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2 Abbreviations used in this paper: SCW, streptococcal cell walls; MCP-1, monocyte chemoattractant protein-1; CINC, cytokine-induced neutrophil chemoattractant; MIP, macrophage inflammatory protein; IP-10, inflammatory protein-10; MIF, migration inhibitory factor.

molecule would inhibit fibrosis, we found that TGF-β1 DNA reduced key cytokine and chemokine production, thereby blocking inflammatory cell accumulation in the liver to contribute to the reduced granulomatous pathology and fibrogenesis.

**Materials and Methods**

**Induction and treatment of liver granulomas**

Female Lewis rats (80–100 g; Charles River Breeding Laboratories, Wilmington, DE) were injected i.p. with group A SCW-peptidoglycan-polysaccharide complexes (30 µg rhemannose/g body weight) (Lee Laboratories, Grayson, GA) (13, 17). At indicated intervals following SCW-injection, livers were obtained, weighed, and processed for analysis.

Human TGF-β1 cDNA isolated from pHRTHTGF-β (59954; American Type Culture Collection, Manassas, VA) was cloned as a 1.6-kb XhoI fragment into pCMV (American Type Culture Collection, Manassas, VA), and injected i.m. into three sites (300 µg total) in the femoris muscle 5 days after SCW-injection, unless otherwise stated for each group.

**Histopathology and immunohistochemistry**

Liver tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained by the Masson Trichrome method for histopathologic analysis (18). For cytokine detection, the prepared tissues were stained with monoclonal anti-TGF-β1, -2, and -3 Ab (Genzyme) using the ABC Elite Kit (Vector Laboratories). Briefly, deparaffinized sections were incubated with biotinylated secondary Ab, washed, and treated with peroxidase substrate solution for 5 min and counterstained with methyl green for microscopic examination.

**Cytokine ELISA**

Plasma samples were collected either from tail bleeding at times specified for each experiment or from intracardiac puncture at the termination of the experiments. Plasma levels of monocyte chemotactic protein-1 (MCP-1), TNF-α, and IFN-γ were determined by ELISA according to the manufacturer’s instructions (BioSource International, Camarillo, CA).

**RT-PCR**

For RT-PCR, total liver RNA (~50 µg) was digested for 1 h with 1 µg RQ1 DNase and fractionated through RNase-free spin columns as recommended by the supplier (Qiagen) to remove trace genomic DNA. cDNAs were synthesized from 2 µg RNA in 40 µl of 1× RT buffer (Promega) containing 400 units Moloney murine leukemia virus RT, 80 µM oligo(dT), and 0.2 mM each dNTP for 15 min at 42°C. Subsequent PCR reactions were performed with 0.25 µM chemokine primer pairs using the profile: 0.5 min at 94°C, 0.5 min at 55°C, 0.5 min at 72°C. Chemokine PCR primers included the CXC chemokine cytokine-induced neutrophil chemoattractant (CINC; GEN/EMBL D11444), CINC2 (D21095), macrophage inflammatory protein 2 (MIP2; X65647), and inflammatory protein 10 (IP-10; U22520) as well as CC chemokine MCP-1 (M57441). GAPDH cDNA (UB200) was used as an internal control.

**Statistical analysis**

Data are expressed as mean ± SEM. A one-way ANOVA followed by Scheffe’s posthoc test was used to detect differences in liver weight after SCW-injection and/or TGF-β treatment. Plasma levels of different cytokines/chemokines were compared among groups by the nonparametric Mann-Whitney U test. In all analyses, differences were considered statistically significant at p < 0.05.
FIGURE 1. TGF-β1 gene delivery inhibits SCW-induced liver granuloma formation. Livers were excised from SCW-injected (A), and TGF-β1 DNA-treated SCW animals (B) 4 wk after SCW-injection. C, Liver weight expressed as percent of body weight was determined upon liver removal from control, SCW-injected, TGF-β1 DNA-, and vector-treated SCW animals (n = 6 per group). *, p < 0.05.

TGF-β1 within the first 3 days, which then declined over the next 2–3 wk (16). Following a single i.m. injection of TGF-β1 DNA 5 days after SCW administration, granuloma formation in the liver was virtually abolished (Fig. 1, A and B). As a quantifiable measure of liver pathology, liver weights were compared between treated and untreated animals (Fig. 1C). A >2-fold increase in liver weight above controls, evident following SCW dissemination to the liver and development of granuloma, was reduced to baseline levels in animals receiving TGF-β1 DNA treatment on day 5 (9.7 ± 1.0 vs 5.23 ± 0.37, n = 6, p < 0.05). Administration of TGF-β1 DNA nearly 2 wk after SCW induction of liver inflammation still effectively diminished liver inflammation, granuloma formation, and fibrosis as monitored 2 wk later (Fig. 1C). Control plasmid did not significantly reduce liver weight in the SCW-granulomatous animals (Fig. 1C), and neither plasmid DNA encoding TGF-β1 nor vector alone had any effect on control (no SCW) animal liver weights (data not shown).

Effects of TGF-β1 on liver histopathology

At the microscopic level, cellular recruitment was dramatically reduced, particularly during the chronic T cell-dependent, macrophage-mediated phase of the response (Fig. 2). Whereas multiple encapsulated granulomas are evident in the untreated animals (Fig. 2, A and C, day 28), no intact granulomas were observed in TGF-β1-treated animals upon gross examination. Decreased numbers of mononuclear cells and multinucleated giant cells, normally abundant in SCW-induced evolving granulomas (Fig. 2, A and C), were apparent following treatment (Fig. 2, B and D).

Effect of TGF-β1 DNA on cytokine mRNA in SCW-inflamed livers

As a measure of inflammation in the livers, mRNAs for several proinflammatory cytokines were monitored over a 3–4 wk interval with or without treatment with TGF-β1 DNA 5 days after SCW injection. As evident in Fig. 3A, quantification of RNase protection assays (ratio of cytokine mRNA to GAPDH) revealed that, in livers experiencing SCW-induced inflammation, TNF-α mRNA modestly increased in the acute response, but was most pronounced in the chronic granulomatous phase (Fig. 3A, day 14–25). In contrast, the control livers expressed extremely high levels of mRNA for the cytokine, macrophage migration inhibitory factor (MIF), which was decreased >2-fold throughout the inflammatory process, from day 4 through 3–4 wk of surveillance (Fig. 3B). IL-1β, IL-1ra, and IL-18 were all increased compared with control liver tissue obtained from animals mock-treated with PBS (Ctrl, Fig. 3C, 4 wk shown). Following treatment with TGF-β1 DNA, expression of the proinflammatory cytokines TNF-α, IL-1β, IL-18 (Fig. 3), and lymphotoxin β (data not shown), as well as IL-1ra (Fig. 3C) was reduced markedly. MIF was, conversely, restored to its control mRNA levels after treatment with plasmid DNA-encoding TGF-β1, whereas, the control plasmid did not effect this reversal of MIF mRNA expression. Interestingly, T cell cytokines commonly associated with immune suppression were not induced by the TGF-β1 gene transfer. Neither IL-4 nor IL-10 mRNA appeared to be increased in livers from SCW-treated animals receiving therapy (data not shown).

Protein analysis of two representative cytokines in the circulation revealed that both TNF-α and IFN-γ, which are elevated 20-fold or 2- to 3-fold, respectively, in SCW-injected animals, were significantly decreased by treatment of the animals with TGF-β1 DNA 5 days after SCW injection (Fig. 4). Vector-treated animals maintained high levels of both cytokines. These data are consistent with the mRNA expression patterns in the inflamed and treated livers.

Effect of TGF-β1 DNA on leukocyte recruitment

The dramatic reduction in cellular infiltrate (Fig. 2) suggested that chemotaxis of circulating cells was inhibited either directly or indirectly upon treatment with TGF-β1 DNA. Although our prior studies provided evidence that circulating TGF-β levels were elevated in animals receiving TGF-β1 DNA, effectively eliminating
a chemotactic concentration gradient emanating from the inflamed locus (16), we also examined whether TGF-β1 DNA might influence chemokine levels. Chemokine expression during the progression of SCW-induced inflammation was analyzed by RT-PCR analysis. Total RNAs from liver tissues of animals at days 17 and 26 of the SCW-induced inflammation or following TGF-β1 gene delivery were examined (Fig. 5A). CINC, CINC2, MIP2, and MCP-1 were progressively up-regulated with time in SCW-injected animals. The increase in CINC mRNA level during both early and late chronic granulomatous diseases (days 17 and 26) was significantly suppressed by TGF-β1 DNA treatment. Additionally, CINC2, MIP2, and MCP-1 mRNA levels were also markedly suppressed by TGF-β1 DNA injection when examined on day 26. Whereas no significant changes in IP-10 mRNA were observed as the disease progressed, a decrease in IP-10 late in chronic disease was detected with TGF-β1 DNA treatment (day 26 in Fig. 5A). Control vector DNA did not inhibit any of the chemokines evaluated in this study. We next evaluated the impact of TGF-β1 DNA on circulating MCP-1 levels. MCP-1 levels in the plasma of animals with liver inflammation followed a biphasic course (Fig. 5B). Following treatment of animals with TGF-β1 DNA 5 days after the fibrogenic dose of SCW, the levels of circulating MCP-1 were reduced to control levels and did not rebound, even during the interval otherwise associated with the onset of chronic inflammation (day 17 in Fig. 5B).

Suppression of matrix production

Although TGF-β up-regulates its own production (22–24) and is present throughout granuloma evolution (11, 12), the i.m. administration of TGF-β1 DNA 5 days after SCW-injection did not notably augment TGF-β protein or RNA levels in the granulomatous liver, as determined by immunohistochemical analysis and RNase protection assay (Fig. 6, A–C). Perhaps surprisingly, TGF-β mRNA levels decreased in the livers from animals treated with TGF-β1 DNA compared with untreated animals (Fig. 6C), likely reflecting the decreased inflammatory infiltrate. Since TGF-β is a mediator of matrix synthesis, the reduction in TGF-β was consistent with the minimal evidence of collagen and other matrix protein deposition. Since the fibril-forming collagen type I and III represent >80% of total liver collagen in SCW fibrosis (21), we evaluated mRNA levels for these two matrix proteins. Both type I and III collagen mRNAs were elevated in the fibrotic livers of SCW-injected animals, but following TGF-β1 DNA therapy, collagen levels did not appear significantly elevated above control liver tissues (Fig. 6D).

Discussion

Intramuscular injection of plasmid DNA-encoding TGF-β1 dramatically reduces liver inflammation, granuloma formation, and fibrosis triggered by bacterial cell walls. Whereas clearance of the bacterial peptidoglycan-polysaccharide complexes by Kupffer cells typically initiates a cascade of inflammatory cell recruitment, activation and release of fibroplasia-inducing molecules (11–14,
prolonged interstitial and pleural fibrosis (26). Altogether, these mediated gene transfer localized in rat lungs was associated with group). PBS, PBS-injected control animals; p

21), the single injection of TGF-β1 DNA interrupts this pathologic sequence of events. Administration of TGF-β1 DNA before the acute response had no significant benefit (data not shown), but when given during the acute response or even in early chronic phase, the evolution of granuloma and hepatic fibrosis was markedly attenuated.

One apparent advantage and disadvantage of TGF-β delivery via plasmid DNA-encoding TGF-β1 is the apparent transient nature of its delivery. The disadvantage reflects the necessity to administer TGF-β1 DNA at the optimal time in the disease process for efficacy. Giving TGF-β1 DNA before initiation of liver inflammation or early in the process is less efficacious than providing it at the time when mononuclear cells become key players, also implicating this population as primary targets. As an advantage, the controlled secretion of chemotactic factors at inflammatory sites. Cell-specific leukocyte recruitment is dependent on chemokine superfamily of chemoattractants with at least partial specificity for individual classes of leukocytes, notably the chemokine superfamily of chemoattractants (27, 28). In general, CXC chemokines are chemotactic for neutrophils and/or T cells, while CC chemokines usually attract mononuclear cells. In this regard, the marked reduction in inflammatory cell infiltrate is consistent with the ability of TGF-β1 gene transfer to suppress the production of MCP-1, a potent mononuclear cell chemoattractant, as well as other members of the chemokine family. The critical role for MCP-1 in mononuclear cell recruitment is documented in the recently generated MCP-1 and MCP-1 receptor knockout mice (29–31) in which migration is data suggest that by generating latent TGF-β, serious localized fibrotic consequences can be minimized.

Because TGF-β is a key fibrogenic mediator, typically enhancing extracellular matrix deposition and inhibiting proteolytic activity (1, 6–8), it might appear surprising that TGF-β1 treatment could inhibit rather than enhance liver fibrosis. However, the interruption of the fibrotic sequelae in this SCW model may, in fact, be secondary to the ability of TGF-β1 DNA to inhibit the inflammatory response to SCW in the liver. The increase in TGF-β appears to influence the nature and scope of the localized tissue inflammatory response and appears to be dependent on the circulating, rather than local, levels of TGF-β.

From a histopathologic vantage point, the reduction in cellular infiltrate in the liver following TGF-β1 DNA treatment, even when delivered during acute inflammation or nearly 2 wk after the initial deposition of SCW in the liver, was most striking. Perhaps the key step regulating the extravasation of peripheral blood leukocytes is the controlled secretion of chemotactic factors at inflammatory sites. Cell-specific leukocyte recruitment is dependent on chemoattractants with at least partial specificity for individual classes of leukocytes, notably the chemokine superfamily of chemoattractants (27, 28). In general, CXC chemokines are chemotactic for neutrophils and/or T cells, while CC chemokines usually attract mononuclear cells. In this regard, the marked reduction in inflammatory cell infiltrate is consistent with the ability of TGF-β1 gene transfer to suppress the production of MCP-1, a potent mononuclear cell chemoattractant, as well as other members of the chemokine family. The critical role for MCP-1 in mononuclear cell recruitment is documented in the recently generated MCP-1 and MCP-1 receptor knockout mice (29–31) in which migration is
compromised. In the liver, MCP-1 is not only produced by inflammatory cells, but is also a product of hepatic stellate cells (32). Which cellular source(s) of MCP-1 is targeted by TGF-β is yet unclear. Recent in vitro evidence also suggests that mesangial cell-derived TGF-β has an antagonist activity on MCP-1 (33, 34). The importance of this target is underscored by the ability of TGF-β1 DNA to block liver pathology, even when administered nearly 2 wk after the initiation of the inflammatory response. This is the interval during which MCP-1-mediated recruitment is likely most critical as it marks the onset of monocyte and lymphocyte dominance in the liver lesions. By inhibiting mononuclear cell recruitment, which typically persists for the 4 wk evaluated, the chronic T cell-dependent lesions responsible for mediating stellate cell recruitment, proliferation, and matrix generation are blunted. In this respect, the CXC neutrophil and T cell attractant, CINC, which in addition to inflammatory cells, is also produced by hepatic stellate cells upon activation in vitro or injury in vivo (35), was inhibited by TGF-β1 DNA treatment. Additionally, suppression of CINC2 and MP2 mRNA during late chronic disease likely contributes to the blockade of inflammatory cell recruitment to the liver. Some chemokines, such as RANTES and IP-10, are constitutively expressed and may be less amenable to regulatory controls, even though a suppression in IP-10 mRNA level was observed during late chronic disease in TGF-β1 DNA-treated animals.

Beyond the inhibition of chemokine production, the elevated levels of circulating TGF-β may also impair emigration of leukocytes from the vessels into the liver due to neutralization of the TGF-β chemotactic concentration gradient otherwise radiating from the injured tissues (16, 22). Since we know that elevated circulating levels of endogenous TGF-β are associated with remission of SCW-induced inflammation (X.-y. Song, W. Chen, R. B. Sartor, J. B. Allen, and S. M. Wahl, manuscript in preparation) and that exogenous delivery of TGF-β (36) or the induction of oral tolerance with increased endogenous levels of TGF-β (37) all have a similar antiinflammatory profile, there must be a commonality in its molecular mechanisms. As a reflection of the reduced inflammatory response, we demonstrate that cytokines typically associated with liver inflammation, TNF-α and IFN-γ, are suppressed in animals given TGF-β1 DNA. This is evident both in the liver and as measured by protein levels in the circulation. Other inflammatory mediators, IL-1, IL-1ra, and IL-18 are also expressed at reduced levels, with the exception of MIF, which exhibits a unique regulatory profile (Fig. 3B; X.-y. Song, L. Zeng, M. Frank, and S. M. Wahl, manuscript in preparation).

Of significance is the local reduction in TGF-β levels found in the livers of animals treated with TGF-β1 DNA. While the reduction in liver TGF-β expression is likely secondary to the suppressed inflammatory and granulomatous response, this reduction in a key fibrogenic molecule most probably contributes to the amelioration of hepatic fibrosis. In liver, hepatic stellate cells are the primary mesenchymal cells responsible for organ fibrosis, and these cells are TGF-β responsive (38). The bacterial cell wall tissue response induces the hepatic stellate cells to generate abundant type III and type I collagens and other matrix components (21). As a potent autoinducer of itself, inhibiting TGF-β production could have multiple downstream ramifications, both direct and indirect, to decrease matrix synthesis by hepatic stellate cells and/or increase its degradation (39–42). Numerous locally synthesized growth-regulatory and matrix-inducing factors required in the differentiation of hepatic stellate cells may be compromised under these circumstances. Moreover, since SCW induces monocyte production of vascular endothelial growth factor, which regulates liver endothelial and hepatic stellate cell function (43), and basic fibroblast growth factor (bFGF; T. Greenwell-Wild and S. M. Wahl, manuscript in preparation), reduced macrophage accumulation would reduce the molecules responsible for angiogenesis and fibrosis. Moreover, bFGF reportedly enhances MCP-1 (45), and interruption of this cytokine cycle likely contributes to diminished recruitment.

Thus, administration of SCW initiates a persistent insult to which the host responds by marshalling leukocyte recruitment and activation that progresses to granuloma formation and, ultimately, fibrosis. The systemic delivery of TGF-β1 consequent to gene transfer can profoundly influence the course and outcome of this otherwise pathologic response. TGF-β-dependent inhibition of the dysregulated and uncontrolled wound-healing response to bacterial cell wall-mediated liver injury enables restoration of homeostasis. While the suppression of chemokines may provide a partial explanation for the reduction in inflammation and fibrosis, it is likely that multiple additional pathways are influenced pursuant to this treatment. As these pathways continue to be revealed, new insights into the potential utility of this approach in chronic inflammatory and fibrotic diseases may emerge.

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