Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5187-5191, May 1994
Immunology

Synthetic fibronectin peptides interrupt inflammatory cell infiltration in transforming growth factor β1 knockout mice

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Communicated by Roscoe O. Brady, December 23, 1993 (received for review November 18, 1993)

ABSTRACT **Pronounced mononuclear leukocyte (MNL) infiltration occurs in multiple organs of mice homozygous for a transforming growth factor β1 (TGF-β1) loss-of-function gene mutation [TGF-β1 (−/−)], followed by cachexia and eventually death. Consistent with the increased leukocyte adhesion and tissue infiltration, MNLs isolated from spleen, thymus, and peripheral blood of symptomatic TGF-β1 (−/−) mice, as compared to littermate controls, exhibited increased adhesion to extracellular matrix proteins and to endothelial cells *in vitro*. Incubation of TGF-β1 (−/−) MNLs with selected synthetic peptides corresponding to cell- and heparin-binding sequences of fibronectin (FN) significantly attenuated adhesion of these cells not only to FN but also to endothelial cells *in vitro*. Based on these observations, mice were treated with the FN peptides in an attempt to rescue them from tissue inflammation and cardiopulmonary failure. Daily injections of a combination of four synthetic FN peptides that interact with β1-integrins and/or cell surface proteoglycans blocked the massive infiltration of MNLs into the heart and lungs of TGF-β1 (−/−) mice. Peptide treatment initiated on day 8, coincident with the first evidence of increased leukocyte–endothelial cell interactions, not only blocked tissue infiltration but also moderated the lethal wasting syndrome.**

Transforming growth factor β (TGF-β) is a 25-kDa peptide that is produced by virtually all cells of the body (1). In mammalian species, TGF-β exists as three isoforms, TGF-β1, -β2, and -β3, but TGF-β1 has the widest tissue distribution (1, 2). To explore its role in development, growth, and other fundamental biological processes, mice deficient in a functional TGF-β1 gene [TGF-β1 (−/−)] were generated (3, 4). Although circumstantial evidence had suggested that TGF-β1 might be essential for embryogenesis (5, 6), nearly 40% of the homozygous TGF-β1 (−/−) mice were born healthy and without obvious disfigurement. However, within 10–20 days, the TGF-β1 (−/−) mice began to experience a wasting syndrome, and shortly after becoming symptomatic, the mice succumbed. Histopathological analysis of the tissues from symptomatic TGF-β1 (−/−) mice revealed dramatic numbers of mononuclear leukocytes (MNLs) in vital organs, such as heart and lung, which virtually obliterated the parenchyma, leading to organ failure and death (3, 4, 7).

Based on the potent immunosuppressive and anti-inflammatory properties of TGF-β1 (8, 9), it was suspected that the absence of TGF-β1 was responsible for uncontrolled leukocyte recruitment and activation resembling chronic inflammatory disease-associated pathology (8–10). However, the etiologic basis for the onset of this rampant "inflammation" remains unclear, although recent evidence of elevated major histocompatibility complex (MHC) expression suggests autoimmune-like mechanisms (11). The earliest detectable alteration in MHC class I and II antigens occurs in some tissues on day 6 postpartum (11), around the time that leukocyte adherence in otherwise normal-appearing tissue occurs.

Leukocyte adhesion to endothelium and to extracellular matrix (ECM) components has a complex molecular basis. The initial trafficking of circulating leukocytes to sites of inflammation is mediated by the selectin family of adhesion receptors (12–16), followed by engagement of additional cellular recognition receptors, including members of the immunoglobulin superfamily and integrins (13–15). Although less understood, there is also increasing evidence for a role of cell surface proteoglycans in mediating early steps in the inflammatory response (16–21).

Since modulation of leukocyte adhesion represents a key step in the development of inflammation, we evaluated the role of adhesion molecules in the massive tissue infiltration in TGF-β1-deficient mice. MNLs harvested from TGF-β1 (−/−) mice demonstrated increased adhesiveness to endothelial cell monolayers and to fibronectin (FN)-coated substrata, compared to MNLs from littermate controls. Inhibition of this adhesive interaction *in vitro* with integrin- and proteoglycan-binding synthetic FN peptides prompted studies directed at blocking leukocyte recruitment and pathology in tissues of the TGF-β1 knockout mice.

MATERIALS AND METHODS

Animals. TGF-β1 (−/−) mice were generated following targeted disruption of the TGF-β1 gene in mouse embryonic stem cells as described (4). Mouse genotype was verified by PCR analysis of tail DNA (11).

Spleen, Thymus, and Blood MNLs. Thymus and spleen were removed from littermates with a wild-type (+/+), heterozygous (+/−), or null (−/−) TGF-β1 genotype, and single-cell suspensions were prepared as described (22). Peripheral blood MNLs were isolated by Ficoll (Histopaque; Sigma) centrifugation (900 × *g*, 23°C, 30 min) of heparinized blood diluted in phosphate-buffered saline (PBS). The cells were resuspended in RPMI 1640 medium containing 5% (vol/vol) heat-inactivated fetal bovine serum (Sigma), 2 mM glutamine, gentamicin at 10 μg/ml, and 50 μM 2-mercaptoethanol.

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Abbreviations: TGF-β, transforming growth factor β; (+/+), wild-type TGF-β1 genotype; (−/−), TGF-β1 null genotype; (+/−), TGF-β1 heterozygous genotype; MNL, mononuclear leukocyte; ECM, extracellular matrix; VLA, very late antigen; FN, fibronectin; MHC, major histocompatibility complex; CS-1, connecting segment of FN; FN-C/H, cell- and heparin-binding fragment of FN; FITC, fluorescein isothiocyanate; VCAM-1, vascular cell adhesion molecule 1.

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Immunofluorescence Staining and Fluorocytometry. For staining, cells were suspended in PBS with 2% fetal bovine serum and 0.1% sodium azide. Cells (≈10^6 per tube) were incubated (4°C, 10 min) with rat antibody 2.4G2 to block Fc receptors (23) followed by a 30-min incubation with the primary antibody, washed, and incubated with a fluorescein isothiocyanate (FITC)-labeled secondary antibody against the host species primary antibody (Caltag, South San Francisco, CA). After washing, the cells were resuspended and analyzed using a FACScan (Becton Dickinson) (24). The monoclonal antibodies used for staining were FITC-anti-mouse CD44 (PgP-1; PharMingen), FITC-anti-rat CD49d (VLA-4; Endogen, Boston), and anti-mouse LFA-1 (KBA; Seikagaku, Tokyo).

Endothelial Cell Culture and Cell Attachment Assays. Mouse pulmonary artery endothelial cells (generously provided by Una S. Ryan, Washington University, St. Louis) were cultured in Ryan's red complete growth medium (M199 with 6.7% bovine calf serum, 3.3% fetal bovine serum, penicillin at 100 units/ml, streptomycin at 100 μg/ml, gentamicin at 10 μg/ml, and 0.2 μM thymidine) (25). For adhesion assays, eight-well glass slides (Nunc) were coated with mouse pulmonary artery cells (250 μl, 2.5 × 10^4/ml) or 8 μg of the 33-kDa cell- and heparin-binding fragment of FN (FN-C/H) or intact laminin (24, 26, 27) per well and blocked with bovine serum albumin (1 mg/ml). MNLs were added (2.0 × 10^6 cells per 0.2 ml) for 30 min at 37°C, the unattached cells were removed by two PBS washes, and the attached cells were fixed, stained, and quantitated using the Optomax image analyzer (Hollis, NH). The data are expressed as the mean of nine values ± SE.

FN Peptide Synthesis and Treatment. Human plasma FN was purified, and the tryptic/catheptic 33-kDa FN-C/H fragment of the A chain was isolated (26–28). FN polypeptides containing the arginylglycylaspartic acid (RGD) domain, the alternatively spliced connecting segment (CS-1), and two nonoverlapping sequences corresponding to the FN-C/H region (FN-C/H-I, Tyr-Glu-Lys-Pro-Gly-Ser-Pro-Pro-Arg-Glu-Val-Val-Pro-Arg-Pro-Arg-Gly-Val; FN-C/H-V, Trp-Gln-Pro-Pro-Arg-Ala-Arg-Ile) were synthesized and purified as described (26, 27). Because of the limited availability of TGF-β1 (−/−) mice, the animals received daily i.p. injections (0.4 mg/100 μl) of a pool of the four peptides for 14–19 days. To evaluate the effect of the peptides on tissue pathology, excised tissues were fixed in 10% formalin, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin/eosin for histological analysis.

RESULTS

Leukocyte Infiltration in Tissues of TGF-β1 (−/−) Mice. Although initially appearing normal, ≈1 week postpartum, mice homozygous for the TGF-β1 null mutation (−/−) began to exhibit leukocyte adherence to endothelium, followed by tissue infiltration (Fig. 1). No leukocyte adhesion was evident in tissues from 3-day-old mice (Fig. 1*A*), but significant numbers of leukocytes adhered to the vessel walls by 7–8 days (Fig. 1 *B* and *C*). By 3 weeks, the heart and lungs were typically infiltrated with massive numbers of leukocytes, with fewer cells remaining adherent on the lumenal side of the vessel (Fig. 1*D*). Not all tissues were uniformly affected, but within 2–3 weeks, nearly 100% of the null homozygotes exhibited both lung and cardiac lesions (4, 7). Whereas the lungs exhibited a multifocal mixed leukocyte infiltrate, predominantly mononuclear phagocyte attachment and infiltration were associated with lesions in myocardium (Fig. 2). The continued accumulation of macrophages in these sites nearly obliterated the parenchyma (Fig. 2 *B* and *E*), contributing to the death of the mice at 3–4 weeks of age. Other frequently infiltrated tissues include salivary glands, pancreas, stomach, and colon, whereas the kidney and brain are less often affected (4, 7).

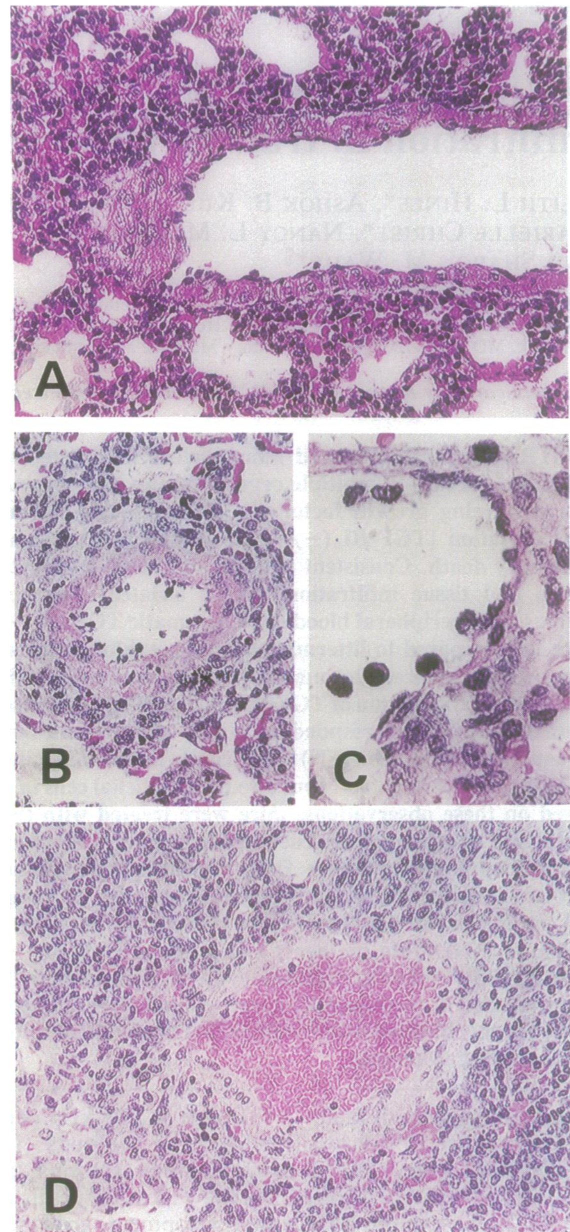

FIG. 1. Adhesion and infiltration of leukocytes in TGF-β1-deficient mice. Tissues were obtained from asymptomatic (3- and 8-day-old) and symptomatic (20-day-old) TGF-β1 (−/−) mice. (*A*) Pulmonary vein of a 3-day-old mouse showing no apparent leukocyte adhesion (n = 4). (*B* and *C*) Pulmonary vein of 8-day-old mouse with leukocyte adhesion to endothelium and perivascular accumulation (n = 6). (*D*) Pulmonary vein at 20 days with perivascular infiltration (n = 4). (Hematoxylin/eosin; *A*, ×300; *B*, ×150; *C*, ×750; *D*, ×300.)

MNL Adhesion to Endothelial Cells and ECM. To define the basis of the massive leukocyte infiltration, MNLs were isolated from TGF-β1 (+/+), (+/−), and (−/−) littermates, and their adherent properties were compared *in vitro*. MNLs from blood, thymus, or spleen of TGF-β1 (−/−) mice were more adherent to ECMs than comparable MNL populations obtained from littermate controls. As shown in Fig. 3*A*, splenocytes derived from symptomatic 21-day-old TGF-β1 (−/−) mice were more adherent (87 ± 9 cells per field) to a FN matrix than cells from either TGF-β1 (+/+) or (+/−) littermates (47 ± 12 and 45 ± 5, respectively; $P < 0.05$). Similarly, TGF-β1 (−/−) MNLs adhered more readily to

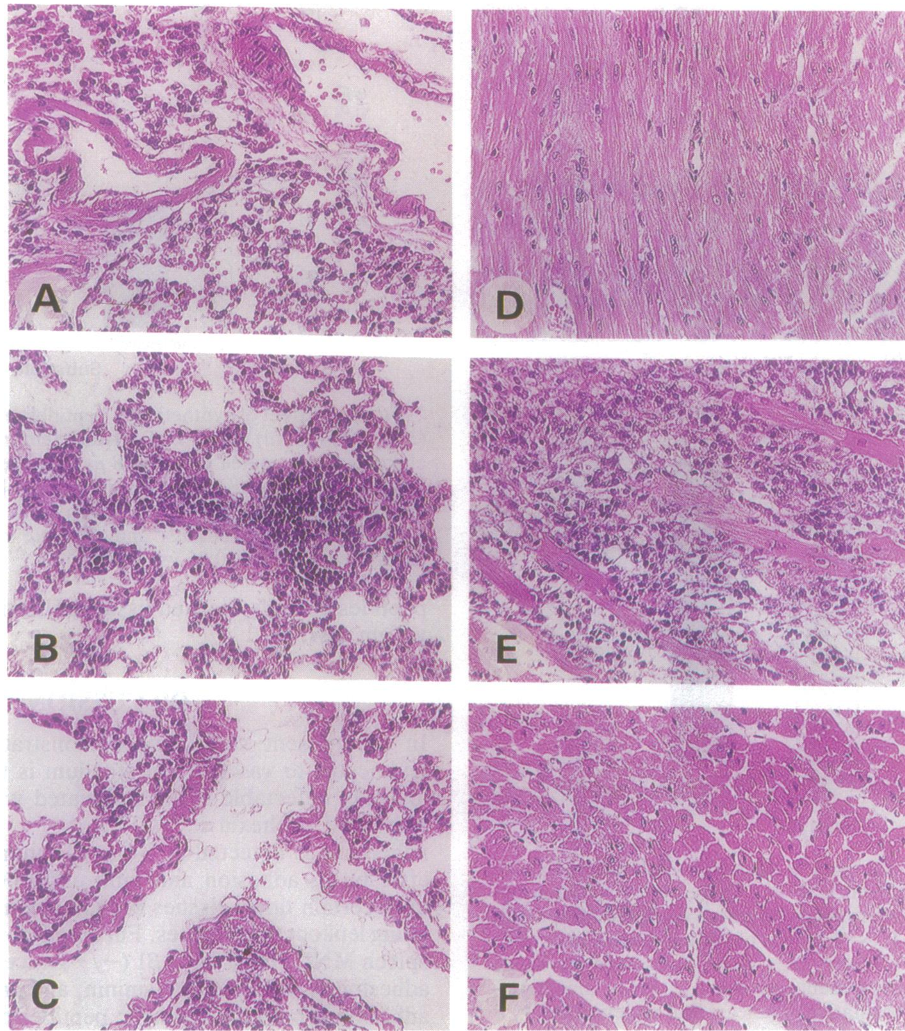

FIG. 2. Effect of synthetic FN peptides on cardiac and pulmonary histopathology. Lung (*A*) and heart (*D*) tissue representing wild-type TGF-β1 (+/+) mice and symptomatic TGF-β1 (−/−) mice (*B* and *E*, respectively) with extensive infiltration of inflammatory cells (day 19; $n = 6$) are shown. Lung (*C*) and heart (*F*) tissue from TGF-β1 (−/−) mice injected i.p. daily (days 7–22) with FN peptides (0.4 mg/100 μl), demonstrating negligible infiltrating leukocytes ($n = 2$), are shown. (Hematoxylin/eosin; ×240.)

laminin (Fig. 3*A*). The number of TGF-β1 (−/−) splenocytes binding to cultured endothelial cells was also significantly greater ($P < 0.05$) than the littermate populations (Fig. 3*B*).

Immunofluorescence Staining and Fluorocytometry. Since TGF-β1 (−/−) MNLs adhere more readily to endothelial cells and FN and laminin substrates, they were analyzed for very late antigen 4 (VLA-4), which interacts both with endothelial cells via vascular cell adhesion molecule 1 (VCAM-1) and with ECM molecules (14, 15, 28). Increased expression of VLA-4 was observed in TGF-β1 (−/−) spleen cells (Fig. 4 *Left*), suggesting a molecular basis for the greater binding capacity of these cells to the relevant substrates. Lymphocyte function-associated antigen-1 (LFA-1), the corresponding receptor for endothelial intercellular adhesion molecules 1 and 2, was also increased on the surface of TGF-β1 (−/−) MNLs (data not shown). By comparison, expression of CD44, a leukocyte cell membrane glycoprotein that interacts with several cell surface and ECM components including hyaluronate, collagen, and fibronectin (14, 17, 30), was variably elevated in the lymphoid organs (M.C., N.L.M.-F., A.B.K., J.M.W., C. L. Mackall, R. E. Gress, K.L.H., H.T., S.K., and S.M.W., unpublished results), although not significantly, as shown for the spleen (Fig. 4 *Right*).

Inhibition of Adhesion by FN Peptides. Because the increased leukocyte adhesion appears to be a precipitating factor for the lethal pathology that develops in the TGF-β1 (−/−) mice, we explored mechanisms for interrupting these events, which might rescue the animals. Based on recent evidence that specific synthetic FN peptides can modulate leukocyte adherence (27, 28), we tested these peptides for their ability to inhibit TGF-β1 (−/−) leukocyte adhesion. When the FN peptides were found to block the adherence of TGF-β1 (−/−) leukocytes to FN and endothelial cells *in vitro* (Fig. 5), experiments were initiated to administer these peptides therapeutically to the mice. Daily systemic injections of pooled CS-1, RGD, FN-C/H-I, and FN-C/H-V were initiated on day 7 or 8 postpartum, since leukocyte adhesion to the vessel wall first becomes apparent at this time, and were continued for 12–19 days. As represented by the heart and lungs, FN peptide administration resulted in a virtual block of leukocyte infiltration into the tissues of two treated animals (Fig. 2 *C* and *F*). Whereas the TGF-β1 (−/−) littermate that did not receive FN peptides exhibited characteristic inflammatory cells in the myocardium (Fig. 2*E*), the hearts from the TGF-β1 (−/−) littermates that were the recipients of daily peptide therapy contained few, if any, inflammatory cells (Fig. 2*F*) and appeared more like the wild-type TGF-β1 (+/+) tissues (Fig. 2*D*). A similar pattern of interruption of leukocyte infiltration by the peptides was evident in the lungs (Fig. 2 *A*–*C*).

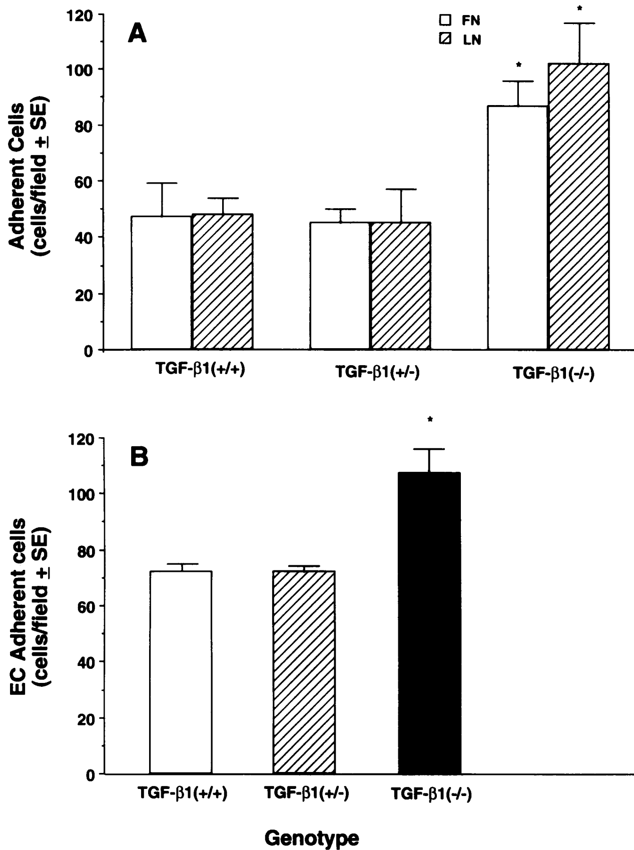

FIG. 3. Adhesion of splenocytes to matrix and cultured endothelial cells. Spleen cells from TGF-β1 (+/+), (+/−), and a symptomatic TGF-β1 (−/−) littermate were added to slides coated with FN (8 μg/ml) or laminin (LN; 8 μg/ml) (*A*) or endothelial cells (EC; ≈70% confluent) (*B*). Adherent cells were quantitated by image analysis, and each value represents the mean ± SE of nine determinations. Data are representative of at least four experiments. *, $P < 0.05$ vs. TGF-β1 (+/+) and TGF-β1 (+/−).

As a marker of symptomatology, animal weight was monitored, and as shown in Fig. 6, peptide treatment appeared to moderate the typical plateau and loss of weight evident in the untreated TGF-β1 (−/−) mouse. Although considerable variability in weight loss and other symptoms occurred, there was a correlation between peptide inhibition of tissue pathology (Fig. 2) and inhibition of weight loss (Fig. 6). In the littermates represented in Fig. 6, the TGF-β1 (−/−) mouse with the lowest weight, and perhaps most difficult to rescue, was selected for treatment, and even in this animal, the

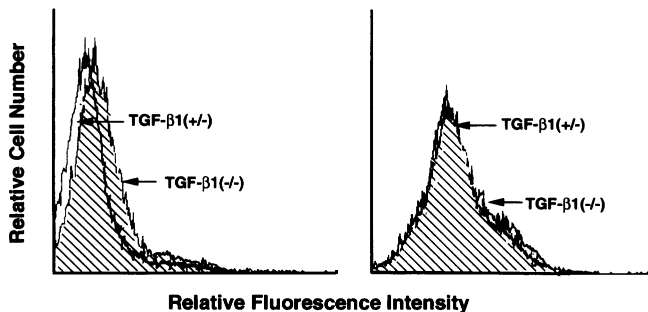

FIG. 4. Expression of VLA-4 and CD44. Spleen cells from symptomatic TGF-β1 (−/−) and control TGF-β1 (+/−) littermates were stained with FITC-conjugated anti-VLA-4 (*Left*) and anti-CD44 (*Right*) for flow cytometry. Data represent typical fluorescence histograms (n = 3).

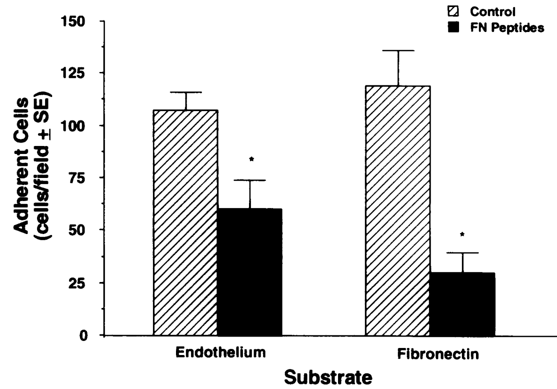

FIG. 5. Effect of synthetic FN peptides on adhesion. Spleen cells (2×10^6/200 μl) from TGF-β1-deficient mice were cultured in chamber slides coated with FN (8 μg/ml) or endothelial cells (≈70% confluent) in the presence or absence of pooled RGD, CS-1, FN-C/H-I, and FN-C/H-V (10 μg/ml). Each value represents the mean ± SE of nine determinations, and data are representative of three experiments. *, $P < 0.05$ vs. control.

peptides not only blocked tissue infiltration but also inhibited weight loss.

DISCUSSION

In the present study, we demonstrate that adhesion of leukocytes to vascular endothelium is the earliest morphologically detectable event associated with inflammatory lesions and cachexia in TGF-β1 (−/−) mice. Although no adherence is detected in 3-day-old animals, around 1 week after birth, adhesion and infiltration began and continued until certain target tissues became obstructed with accumulating leukocyte infiltrates. Furthermore, blood, thymus, and spleen MNLs from TGF-β1 (−/−) mice exhibited increased adhesion *in vitro* to FN, laminin, and endothelial cells. Cell adhesion-promoting synthetic peptides derived from FN not only inhibited leukocyte adhesion in culture but also interrupted the development of inflammatory lesions and the wasting syndrome characteristic of these TGF-β1-deficient animals.

Although the mechanism(s) that initiates the increased leukocyte adhesion in TGF-β1 (−/−) mice remains unclear, cell surface adhesion molecule expression appears to be involved. The synthetic FN peptides, used as combination

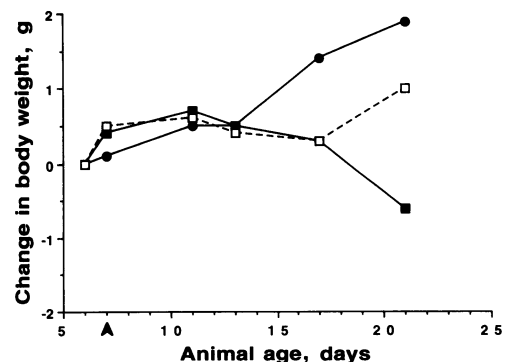

FIG. 6. Effect of synthetic FN peptides on body weight. Confirmed TGF-β1 (−/−) mice were not treated or treated i.p. with 100 μl of a FN peptide preparation (0.4 mg/100 μl) beginning on day 7 (arrowhead). Due to variability in animal weights at the onset of treatment [TGF-β1 (−/−) untreated = 6.2 g, TGF-β1 (−/−) FN treated = 4.4 g, and TGF-β1 (+/−) = 5.6 g], the day 6 weights are represented as baseline with the changes in weight determined at the indicated intervals. ●, TGF-β1 (+/−); □, FN-treated TGF-β1 (−/−); ■, TGF-β1 (−/−).

therapy in the current studies, have been shown to individually inhibit adhesion of both normal and transformed cells (19, 29) and to suppress arthritis in an experimental animal model, whereas control peptides including arginylglycylglutamic acid were inactive (27, 28). The effectiveness of these selected peptides implicates, at the minimum, β_1 integrins and likely cell surface proteoglycans, as critical mediators of the increased MNL adhesion and recruitment in these mice.

Integrin-mediated adhesion events are complex and susceptible to numerous regulatory mechanisms (13–15). In the TGF-β1 null mice, phenotypic analysis revealed a modest increase in VLA-4, the receptor for both endothelial VCAM-1 and the CS-1 domain of FN, although enhanced cell–cell and cell–matrix interactions are not dependent upon increased expression but may reflect activation of these molecules (13–15). VLA-4 ($\alpha_4\beta_1$) is expressed on T cells, B cells, and monocytes (30), all of which are represented in the knockout infiltrates. The CS-1-containing peptides may mitigate cellular recruitment by targeting both $\alpha_4\beta_1$–VCAM and $\alpha_4\beta_1$–FN interactions. The recent demonstration that $\alpha_4\beta_7$ also binds FN and VCAM (31, 32) suggests another target for the CS-1 peptides. Interference with VLA-5 ($\alpha_5\beta_1$) and other β_1 integrin-specific binding to FN by the RGD-specific peptides (27, 30) would also counteract leukocyte-binding patterns in the TGF-β1 null animals.

As cationic, hydrophilic peptides, FN-C/H-I and FN-C/H-V adhere to cell surface proteoglycans and also interact with α_4 subunits (18), presumably blocking proteoglycan and α_4-dependent adhesion. Cell surface proteoglycans, which mediate a spectrum of cell-binding activities (17–21), may represent a key target for these peptides. Moreover, if proteoglycan interactions with selectins facilitate leukocyte homing (16), these heparin-binding peptides may antagonize selectin-mediated adhesion. Since TGF-β inhibits expression of E-selectin (33), the absence of TGF-β1 in the knockout mice might enable unregulated E-selectin expression, promoting leukocyte emigration. However, if E-selectin plays a dominant role in these sequelae, it is intriguing that neutrophils are not a primary infiltrating cell type (4).

Our data indicate that the aberrant adhesive interactions in the TGF-β1 (−/−) animals are mediated at the level of the leukocytes, yet the endothelial cells must also exhibit altered adhesive properties in order to define the apparent tissue specificity for leukocyte infiltration. For example, heart and lungs in the TGF-β1 (−/−) animals are nearly always affected, whereas other tissues, such as kidney and brain, often do not have cellular infiltrates. Considerable evidence implicates TGF-β1 in normal embryogenesis and function of the heart (refs. 5, 6, and 34; reviewed in ref. 9), and consequently, in the absence of TGF-β1, formative and functional deficits may initiate an autoimmune-like phenomenon as suggested by the increased MHC expression (11). Investigations are continuing to specifically define the precipitating event(s) leading to the massive leukocyte adherence and infiltration (35) which occur in the tissues of TGF-β1 (−/−) mice.

In association with the prevention of leukocyte infiltration and tissue pathology in the TGF-β1 (−/−) mice, the FN peptide treatment reduced the weight loss that normally occurs between 2 and 3 weeks of age. These studies document the early and critical nature of aberrant leukocyte adhesive interactions in the initiation of the characteristic pathology associated with TGF-β1 deficiency. Such adhesive interactions are fundamentally important for the arrest and extravasation of leukocytes and offer potential sites for therapeutic intervention in the control of inflammatory lesions not only in this model but also in debilitating inflammatory diseases.

We thank Nandita Chopra, Neil Hardegen, and Mary Slater-Venkata for their excellent technical help, Ms. Vasiliki Angelis for preparation of this manuscript, Dr. Crystal Mackall for rat antibody 2.4G2, Dr. Una Ryan for mouse pulmonary artery cells, and Drs. Prema Mertz and John Zagorski for their critical comments. K.L.H. is a Pharmacology Research Associate Trainee (PRAT) Fellowship recipient in the National Institute of General Medical Sciences.

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