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Immunology

Transforming growth factor β_1 (TGF-β_1) controls expression of major histocompatibility genes in the postnatal mouse: Aberrant histocompatibility antigen expression in the pathogenesis of the TGF-β_1 null mouse phenotype

ANDREW G. GEISER*, JOHN J. LETTERIO*, ASHOK B. KULKARNI†, STEFAN KARLSSON†, ANITA B. ROBERTS*, AND MICHAEL B. SPORN*

*Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892; and †Developmental and Metabolic Neurology Branch, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892

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ABSTRACT **The phenotype of the transforming growth factor β_1 (TGF-β_1) null mouse has been previously described and is characterized by inflammatory infiltrates in multiple organs leading to a wasting syndrome and death as early as 3 weeks after birth. Since this phenotype occurs in the absence of any detectable pathogen, potential autoimmune disease mechanisms were investigated. We examined major histocompatibility complex (MHC) mRNA expression in tissues of the TGF-β_1 null mouse and found levels of both the class I and class II MHC mRNA elevated compared to normal or TGF-β_1 heterozygous littermates. This elevated expression was seen prior to any evidence of inflammatory infiltrates, suggesting a causal relationship between increased MHC expression and activation of immune cell populations. Cell surface expression of MHC molecules was detected by immunohistochemistry and correlated well with mRNA levels. Expression of mRNA for interferon γ and its receptor was unchanged at the ages when increased MHC expression became apparent. Down-regulation of class I MHC expression by TGF-β_1 was also demonstrated *in vitro* in fibroblasts isolated from TGF-β_1 null mice. These findings suggest that one natural function of TGF-β_1 is to control expression of both MHC classes. Altered regulation of MHC expression may be a critical step leading to the multifocal inflammation and wasting syndrome seen in the TGF-β_1 null mouse. These results suggest potential applications for TGF-β in the management of autoimmune disease, allograft rejection, and other problems associated with altered MHC expression.**

Transforming growth factor β (TGF-β) is a family of proteins (TGF-β_1, -β_2, and -β_3 in mammals) that display multiple effects on a large number of cell types (1). As a means of discerning the specific functions of TGF-β_1, we and others (2, 3) have created mouse strains that lack a functional TGF-β_1 gene. Though normal at birth, they develop a wasting syndrome due to an inflammatory response with infiltration of lymphocytes and macrophages in many organs. The TGF-β_1 null mice usually succumb by 4 weeks of age, with no identifiable pathogen responsible for the extensive immune system activation.

TGF-β has been shown to control many different aspects of the immune system, including immune cell proliferation, differentiation, and activation (4). Although TGF-β can promote cell recruitment and inflammation at sites of tissue damage, the effects of TGF-β are generally of a negative nature, suppressing immune function. The absence of TGF-β_1 (the primary isoform in activated immune cells) could release immune cells from this negative regulation, potentially contributing to autoimmune activation. Supporting this hypothesis are experiments in which TGF-β_1 administration prevents collagen-induced arthritis (model for rheumatoid arthritis) and reduces the severity of experimental allergic encephalomyelitis (model for multiple sclerosis) (5–7). In addition, neutralizing antibodies to TGF-β_1 increase the severity of experimental allergic encephalomyelitis suggesting that endogenous TGF-β_1 normally plays a role in modulating autoimmune disease (8, 9).

The inflammatory response of autoimmune disease is thought to be mediated by release of many cytokines, including interleukin 1, tumor necrosis factor α, and interferon γ (IFN-γ) (10, 11). These cytokines are normally released from activated immune cells in response to foreign antigens. In autoimmune disease, activation of immune cells is frequently elicited by the presentation of self antigens in the context of cell surface major histocompatibility complex (MHC) molecules, by cells typically uninvolved in antigen presentation. For example, class II MHC molecules are normally expressed only on a limited number of cell types involved in antigen presentation to $CD4^+$ T cells (T-helper cells). These include B lymphocytes, activated T lymphocytes and macrophages, dendritic cells, glial cells, and thymic epithelial cells (12, 13). Yet, in certain autoimmune diseases, immune activity appears to be directed against cells that inappropriately express MHC class II molecules, such as β cells in the diabetic (type I) pancreas (14, 15) and thyroid cells in Graves disease (16, 17).

There is some evidence that TGF-β can modulate both constitutive and IFN-γ-induced levels of class II MHC expression (18, 19). We have now examined the expression of MHC antigens in the TGF-β_1 null mice compared to normal or heterozygous littermates. Our data suggest that TGF-β_1 is a primary regulator of both classes of MHC molecules and implicate altered antigen presentation in the multifocal inflammatory disease of the TGF-β_1 null mouse.

MATERIALS AND METHODS

Identification of Genotypes. The distal tail (0.5–1.0 cm) was cut 1 day after birth and digested overnight at 56°C in 0.5 ml of lysis buffer [100 mM EDTA/50 mM Tris, pH 8.0/0.25% SDS/600 μg of proteinase K per ml (BRL)]. The samples were boiled for 15 min, centrifuged for 1 min, and then diluted 1:20 in 5% Chelex 100 (Bio-Rad). A 1.0-μl DNA sample was then used in a 50-μl PCR to amplify either the normal TGF-β_1 allele or the targeted (inactive) allele. The normal allele oligonucleotides were from the deleted portion of the first exon (5′-GGTCACCCGCGTGCTAATGG) and from the first

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Abbreviations: IFN-γ, interferon γ; MHC, major histocompatibility complex; TGF-β, transforming growth factor β; β_2M, β_2-microglobulin.

intron (5′-GAGAGTAAGCCCACTAGAG) to give a product of 529 bp. Targeted allele oligonucleotides were from exon 1, 5′ of the deleted portion (5′-ATGGAGCTGGTGAAACG-GAA), and from the phosphoglycerate kinase promoter (5′-TCCATCTGCACGAGACTAGT), for a product size of 375 bp. The PCR cycles were 94°C, 3 min; then 94°C, 1.5 min; 60°C, 1.5 min; and 72°C, 2.0 min × 40 cycles. The absence of a normal allele but presense of a targeted allele was indicative of a TGF-β_1 null mouse genotype.

RNA Isolation and Northern Analysis. Tissues were homogenized in GTC buffer [4.0 M guanidine thiocyanate/50 mM Tris, pH 7.0/10 mM EDTA/2% (wt/vol) sarkosyl/1% (vol/vol) antifoam A (Sigma)], CsCl purified, and then phenol/$CHCl_3$ extracted (20). Total RNA (15 μg) of each sample was separated on 1× Mops-buffered 1.2% agarose/10% (vol/vol) formalin gels and blotted to nytran filters with 10× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate) (20). Northern blot filters were hybridized with fragments of DNA labeled with a random primer kit (BRL) using 3.0×10^6 cpm/ml of hybridization buffer and the conditions of Church and Gilbert (21).

DNA Probes Used for Hybridization. The class I MHC heavy chain DNA probe (440 bp) was obtained by reverse transcription of mouse spleen RNA and PCR of the resulting cDNA product (RT-PCR) using oligonucleotides obtained from conserved sequence of the α-3 domain from clone pH-2^d-3 (22) (oligonucleotides 5′-AAAGGCACATGTGAC-CCATC and 5′-CATATCAGAGCTCTGGGAGC). The class I light chain [β_2-microglobulin (β_2M)] probe was a 1.5-kb genomic fragment from plasmid pGEM1β_2M (23). The class II MHC cDNA probes were as described (24). The Aα, Aβ, and Eβ chain class II probes each hybridized with nearly the same intensity to a 1.3-kb transcript, whereas the Eα probe did not hybridize as these mice carry the *H-2*^{b} haplotype known to be defective in expression of the Eα chain (25). A murine IFN-γ cDNA probe was also generated by RT-PCR of mouse spleen RNA using primer pairs from within the first 500 bp of sequence (oligonucleotides 5′-CGCTACACACT-GCATCTTGG and 5′-AGCTCATTGAATGCTTGGCG), providing a 397-bp product (26). Similarly, a cDNA probe to the murine IFN-γ receptor was generated (oligonucleotides 5′-AACGGCTCTGACAGTGGC and 5′-GACTTACGGC-TGGCTTTGAG) as a 650-bp product from a region near the 3′ end of the receptor sequence (27).

Immunohistochemistry. Tissue samples were mounted in OCT, frozen on a bed of dry ice, and stored at −70°C. Cryostat sections 8 μm thick were air dried at 4°C for 5 min and then incubated for 30 min at room temperature with an appropriate dilution of one of the following primary antibodies: rat anti-H-2 monoclonal antibody to the mouse *H-2* monotypic antigen, rat anti-Ia monoclonal antibody to mouse I region-associated antigens (Boehringer Mannheim), or normal rat serum control (Pierce). Sections were washed twice in Tris-buffered saline followed by a 30-min incubation at 37°C with horseradish peroxidase-coupled goat anti-rat immunoglobulin (Pierce) in 20% normal mouse serum. Sections were developed in a solution of diaminobenzidine (Sigma) and counterstained with hematoxylin.

Cell Culture. Primary cultures of mouse fibroblasts were prepared from skin of 3-day-old mouse pups, either negative (−/−) or heterozygous (−/+) for the targeted allele. Sections of skin were removed and allowed to adhere to an area of sterile vacuum grease applied to the bottom of 100-cm^2 tissue culture dishes. The surface of the plate was covered by a layer of Dulbecco's modified Eagle medium/F12 medium (GIBCO/BRL) with 20% fetal calf serum and antibiotic/antifungal agents. By 10 days in culture, fibroblasts migrate onto the plastic dish. Subconfluent cultures were passaged three times, at which point confluent monolayers were used for Northern analysis of MHC expression. Levels of MHC expression were determined by normalized densitometric analysis of autoradiographs.

Histopathology. Selected tissues from littermates either heterozygous or negative for TGF-β_1 were fixed in 10% buffered formalin and embedded in paraffin. Five-micron sections were stained with hematoxylin/eosin and analyzed for histopathology.

RESULTS

We used DNA fragments from conserved regions of the class I and class II MHC molecules as hybridization probes to examine MHC mRNA expression. Both α and β chains of the IaA and IaE class II loci as well as the α-3 domain of the heavy chain and the β_2M light chain of the class I locus were used. RNA was extracted from tissues of a 23-day-old (−/−) mouse expressing the characteristic wasting phenotype and from (+/+) and (+/−) littermates. The RNA was subjected to Northern blot analysis using the MHC probes described above (Fig. 1). Whereas the levels of MHC expression in (+/−) and (+/+) mice were low or undetectable in most nonlymphoid tissues, levels in the (−/−) mouse were increased in heart, lung, liver, kidney, and brain [data for (+/+) mouse not shown]. Levels of mRNA for IFN-γ, which is known to greatly enhance the expression of both classes of MHC molecules (28–30), were nearly identical in (−/−) and (+/−) mice as were IFN-γ receptor message levels (data not shown). Given the advanced inflammation seen in the tissues of the (−/−) mouse, it was not unexpected to find elevated MHC levels (typically induced by cytokines released from activated mononuclear cells), but the lack of change in IFN-γ expression was unexpected since Shull *et al.* (3) had reported an increase in the TGF-β_1-null mouse.

As previously described (2), all TGF-β_1 null mice older than 10 days exhibited inflammatory lesions. Most notable were those found in the heart and lung; lesions were also present in liver, kidney, salivary gland, and other organs but never in the heterozygous littermates. In an effort to discern the earliest lesions, tissues from mice of 3, 5, 6, 7, and 9 days of age were examined. The first evidence of infiltrate was typically seen within the lung at day 9 and was characterized by perivascular (venular) cuffing or adherence of neutrophils and macrophages (see Figs. 3 and 4). No lesion could be

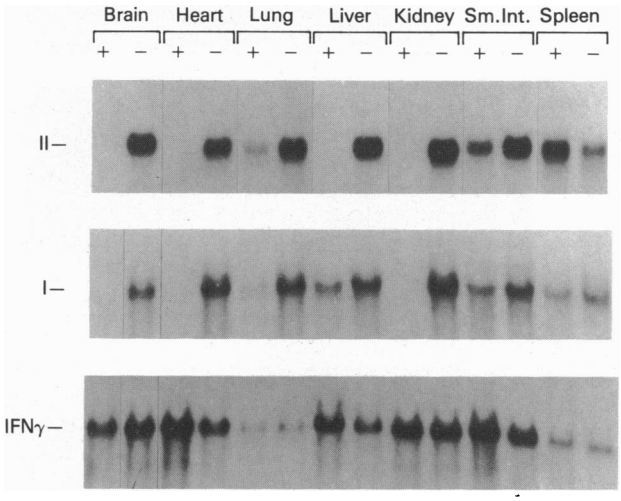

FIG. 1. Elevated expression of MHC mRNA in TGF-β_1 null mouse expressing the wasting syndrome. Northern blot analysis of RNA isolated from tissues of either heterozygous (+) or null (−) 23-day mice is shown. The hybridization probes used were as follows: top panel (II), MHC class II, Aα chain (1.3-kb transcript); middle panel (I), MHC class I heavy chain (1.8-kb transcript); bottom panel (IFN-γ), IFN-γ ligand (1.2-kb transcript). Sm.Int., small intestine.

found within the organ parenchyma at this time, and no histologic abnormalities were seen prior to this age.

To determine if the elevated MHC levels precede the tissue inflammation, we isolated RNA from both (−/−) mice and (+/−) littermates at days 3, 6, and 9. During the immediate postnatal period (day 3), no significant differences are apparent between (−/−) and (+/−) littermates (Fig. 2*A*). By day 6, elevated MHC expression is obvious in some tissues of (−/−) mice, though in others (heart and brain), the degree of elevation is much less compared to that seen in older animals (Fig. 2*B*). By day 9, MHC expression in the (−/−) mouse is similar to that of the older, wasting mice (data not shown), and all animals sacrificed on or after this age consistently exhibit similarly elevated MHC patterns. Identical Northern blots revealed no differences in IFN-γ expression (data shown for day 9, Fig. 2*C*), supporting the argument that this cytokine was not responsible for increases in MHC expression seen as early as 6 days after birth. Thus, between

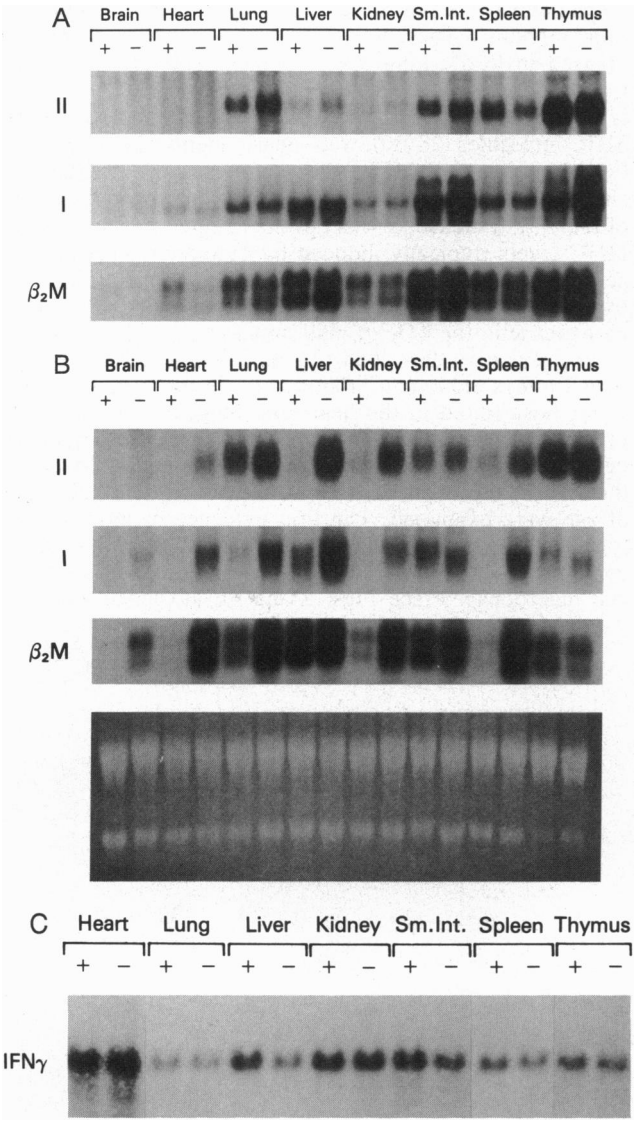

FIG. 2. Altered MHC mRNA occurs between days 3 and 6 after birth of the TGF-β_1 null mouse. Northern blot analysis (as in Fig. 1) using tissue RNA from day 3 mice (*A*), day 6 mice (*B*), and day 9 mice (*C*). Hybridization probes used included those in Fig. 1 and also the β_2M light chain of class I MHC (β_2M) (1.0- and 1.3-kb transcripts). Lanes marked + denote heterozygous, and − denotes TGF-β_1 null. RNA loading was controlled by ethidium bromide staining of ribosomal bands, as exemplified in *B*.

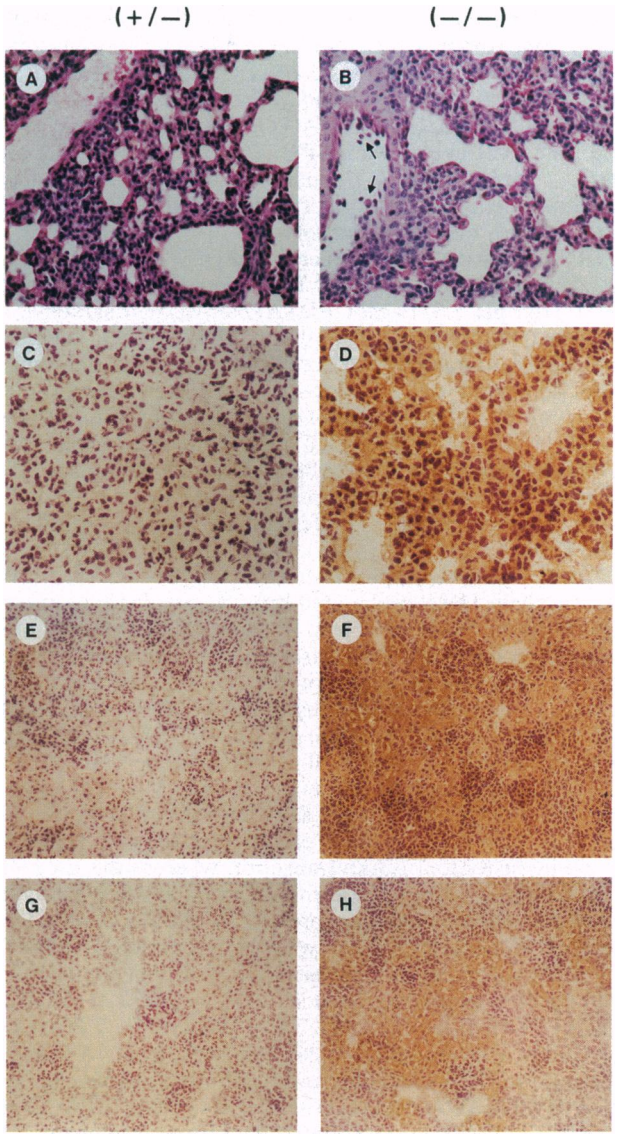

FIG. 3. Histopathology and MHC immunostaining of TGF-β_1 null mice. Hematoxylin/eosin-stained lung sections from day 9 (+/−) and (−/−) littermates are shown in *A* and *B*, respectively. Arrows (*B*) indicate the presence of marginating mononuclear cells in the (−/−) section. Immunohistochemical staining for MHC class I in the lung (*C* and *D*) and kidney (*E* and *F*) and class II in the kidney (*G* and *H*) is also shown. (×260.)

3 and 6 days after birth, a role for TGF-β_1 in regulating expression of MHC mRNA is evident.

Surface expression of MHC proteins was similarly increased in the TGF-β_1 null mice as determined by immuno-

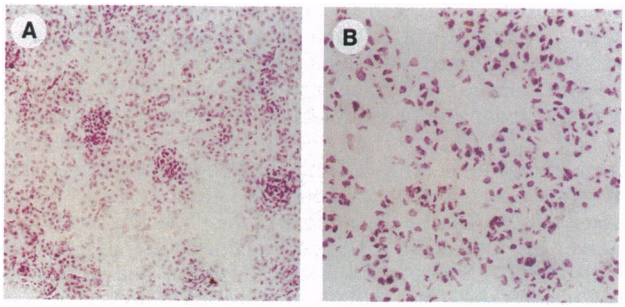

FIG. 4. Immunostaining controls. The absence of nonspecific staining within kidney (*A*) and lung (*B*) implicates the specificity of the associated MHC immunostaining. (*A*; ×68; *B*, ×134.)

histochemical analysis. Although there were distinct tissue differences in the expression of class I and class II antigens, there was a consistent increase in the expression of each antigen in (−/−) animals when compared to the same organs of (+/−) littermates (Figs. 3 and 4). Those organs with the most notable differences at the level of mRNA expression (heart, lung, and kidney) also exhibited the highest differential in surface antigen expression. Tissues such as brain, spleen, small intestine, and thymus exhibited no apparent differences by this method. The *H-2*-directed antibody provided the strongest staining, suggesting that protein expression was greater for class I than class II, although it may also reflect the relative efficacy of these antibodies for immunohistochemical staining. Within each tissue, the staining pattern for class I was more diffuse, indicating expression on several cell components within the organ parenchyma. However, the kidney showed a more distinct pattern of staining within the renal cortex, with particularly intense staining over glomeruli (class I, Fig. 3*F*). This likely represents specific induction within the vascular endothelium and possibly tubular epithelium. Analogous to the mRNA expression, these differences were present in the absence of any detectable inflammation within the organs of (−/−) mice.

To study further the relationship of TGF-β_1 to MHC expression, primary cultures of mouse fibroblasts were established. Although fibroblasts do not express class II antigens, they can express class I, allowing the evaluation of MHC regulation in the absence of other immunoregulatory factors likely to be operative *in vivo*. Confluent monolayers of (+/−) and (−/−) fibroblasts were exposed to either IFN-γ (12 ng/ml) or TGF-β_1 (5 ng/ml), or both, and compared to control (untreated) cultures. Control fibroblasts from (−/−) mice exhibited a >2-fold higher basal level of expression of both β_2M (Fig. 5) and class I heavy chain (not shown) as determined by normalized densitometric analysis. TGF-β_1 treatment resulted in a reproducible 1.5-fold reduction of IFN-γ-induced MHC expression in both (+/−) and (−/−) fibroblast cultures.

DISCUSSION

We have shown that the absence of TGF-β_1 results in a major increase in the expression of both classes of MHC antigens. This dysregulation of MHC expression was found to occur prior to the earliest detection of inflammatory cell infiltration

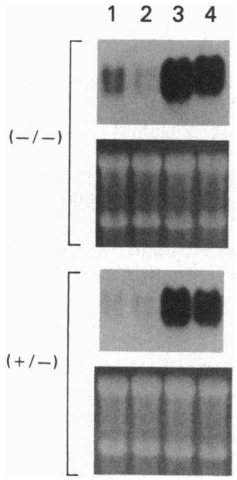

FIG. 5. *In vitro* analysis of MHC regulation. Northern blot analysis of β_2M expression in mRNA isolated from fibroblast cultures treated with either TGF-β_1 (5 ng/ml; lane 2) or IFN-γ (12 ng/ml; lane 3), or both (lane 4), and compared to vehicle control (lane 1). Ribosomal bands are shown to demonstrate equal loading of RNA for each sample. Relative levels of MHC expression were determined by densitometric analysis and normalized to ribosomal bands.

in the TGF-β_1 null mouse. Further, the increased MHC expression was seen in many different organ systems, regardless of eventual inflammatory involvement, and was not due to an increase in IFN-γ production. Thus, TGF-β_1 appears to be a natural regulator of MHC expression in the developing mouse.

The contribution of increased MHC expression to the wasting syndrome of the TGF-β_1 null mouse is not yet clear. Some theories of autoimmune disease suggest that inappropriate expression of MHC antigens may allow cells to present autoantigens, thereby initiating a circle of self-perpetuating inflammation leading to organ damage (14, 16, 31, 32). However, increased MHC expression does not necessarily lead to tissue damage (33), and in some cases, high MHC expression causes tissue damage through nonimmune mechanisms (34, 35). Other processes, such as the establishment of tolerance to organ-localized self-antigen, are potentially under the influence of TGF-β. This cytokine up-regulates CD-8 expression in immature thymocytes, and the interaction of CD-8 with MHC plays an important role in positive selection of MHC class I-restricted T cells (36, 37). Other events that control self-reactive T cells (normally present in the adult) depend, at least in part, on natural suppressor cells whose function may also be influenced by TGF-β_1 (38).

We have found that in some tissues in which high MHC levels are detected, such as the kidneys, relatively little inflammatory cell infiltration was detectable by histologic examination. This may implicate a relative difference in the ability of various cell types to present antigen to receptive T cells. Tissue-specific expression or processing of autoantigens for proper binding to MHC molecules and transport to the cell surface could make a critical difference in antigen presentation. Furthermore, local factors may affect the tissue penetration of circulating mononuclear cells (39). TGF-β has a demonstrated role in the inhibition of neutrophil adherence to endothelium (40, 41) and has also been shown to regulate integrin synthesis (42, 43), which is needed for cell–cell and cell–matrix attachment. Thus, the lack of TGF-β_1 expression may also influence the access of infiltrating cells to tissues expressing elevated MHC antigens.

The level of expression of MHC class I molecules in different tissues varies markedly, and in some tissues (such as pancreas and salivary glands) class I expression cannot be detected even with the most sensitive immunohistochemical techniques (44). There is a constitutive level of class I molecules expressed on most nucleated cells, which is important for effective presentation of foreign (e.g., viral) antigens to cytotoxic T lymphocytes in the class I-restricted immune response (45, 46). Aberrant or elevated expression of class I antigens has been associated with other processes, such as the massive induction seen in donor myocardial cells (which normally express little or no class I antigen) during allograft rejection (47).

The results of this study suggest that TGF-β_1 acts as a general regulator (repressor) of MHC expression. Within a given MHC class, the gene promoters show a high degree of conservation and are usually coordinately expressed (48). In contrast, the promoters of class I genes are quite distinct from the promoters of class II genes. Regulation by TGF-β_1 may involve presently undefined distal elements shared by both MHC classes and involved in repressing transcription. Alternatively, TGF-β may activate a negative regulatory factor, such as the recently cloned IFN consensus sequence binding protein (49), or inhibit positive regulators of IFN-γ signals, such as the IFN-γ-activating factor (50).

Finally, the delay observed in tissue infiltration and disease expression could be due to unique features of the neonatal immune system. These features include neonatal macrophages that are relatively defective in presenting antigen and an abundance of natural suppressor cells in the neonatal

spleen (51, 52). Further, there is a potential maternal contribution of TGF-β_1 (or TGF-β_1-regulated products) during gestation; determination of whether or not there is sufficient transfer to result in the observed latency period requires further investigation.

Our findings indicate that TGF-β_1 is an important component of the developmental program regulating MHC expression. As such, the TGF-β_1 null mouse will serve as a useful model to further explore the role of both TGF-β and MHC molecules in autoimmune disease. Finally, these results implicate potential clinical applications for TGF-β_1 in the setting of autoimmune disease, allograft rejection, and other disorders accompanied by altered MHC expression.

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