Maternal Rescue of Transforming Growth Factor–β1 Null Mice

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Maternal sources of transforming growth factor–β1 (TGF-β1) are shown here to contribute to the normal appearance and perinatal survival of TGF-β1 null newborn mice. Labeled TGF-β1 crossed the placenta and was recovered intact from various tissues after oral administration to mouse pups. TGFβ-1 protein was also detected in cells recovered from breast milk. In immunohistochemical analyses, TGF-β1 null embryos and null newborn pups born to TGF-β1 heterozygotes stained positive for TGF-β1, whereas those born to a null female were negative and had severe cardiac abnormalities. These results suggest an important role for maternal sources of TGF-β1 during development and, more generally, provide evidence for maternal rescue of targeted gene disruption in the fetus.

Targeted disruption of TGF-β1 gene expression in mice results in diffuse and lethal inflammation (*1*) and provides a valuable model of autoimmune disease. TGF-β1 belongs to a large family of proteins known to be important for normal development (*2*); this family includes the other TGF-β isoforms (TGF-β2 and TGF-β3 in mammals), the activins, inhibins, bone morphogenetic proteins, and the Müllerian inhibiting substance (*3*). Although the embryonic expression pattern of TGF-β1 mRNA and protein suggests that this protein has a vital role during development (*4, 5*), TGF-β1 null newborns from a cross of TGF-β1 heterozygotes are indistinguishable from littermates that are homozygous or heterozygous for the normal allele. Histological abnormalities in TGF-β1 null mice appear at 7 days of life, when mononuclear cells begin to adhere to vascular endothelium and then initiate a progressive inflammation involving the liver, lungs, heart, skeletal muscles, and other organs. Both class I and class II antigens of the major histocompatibility complex (MHC) are aberrantly overexpressed in the tissues of TGF-β1 null mice (6), which is analogous to the enhanced expression often observed in autoimmune disease (*7*).

The absence of a phenotype in TGF-β1 null newborns has led to the suggestion that there is redundancy in the expression of the various isoforms of TGF-β and that TGF-β1 is not essential for normal development (*8*). An alternative explanation is that TGF-β1 is supplied to the fetus or newborn from maternal sources. Transplacental transfer has been documented for other growth factors (*9*), and TGF-β isoforms have been detected in human and bovine colostrum and in bovine milk (*10*).

To determine if TGF-β1 is transferred from mother to fetus, we injected a ^{125}I-labeled mixture of active and latent TGF-β1 into the hearts of pregnant female mice that were TGF-β1 heterozygotes (*11*). Latent complex was included because it has a longer half-life than active TGF-β in vivo (*12*). Each fetus was delivered by cesarean section at specified time intervals, and labeled protein was immunoprecipitated from both maternal and fetal tissues with TGF-β1 antiserum (Fig. 1A). Identification of labeled protein as TGF-β1 was substantiated by the protein's characteristic shift in molecular mass from 25 to 12.5 kD when analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing versus reducing conditions. There was a time-dependent increase of radioactivity (an eightfold increase from 15 to 45 min) within fetal tissue (*13*).

To delineate patterns of incorporation, we processed one fetus from each experiment for autoradiography. Intracellular localization of radioactivity was apparent in fetal lung (Fig. 1B), muscle (Fig. 1D), and liver tissues. Extracellular localization was also evident in areas surrounding hypertrophic chondrocytes in bone (Fig. 1C) and in the bone matrix. Thus, TGF-β1 crosses the placenta to the fetus, where it is taken up by cells and bound by extracellular matrices.

Maternal sources of TGF-β1 may also be important during the neonatal period. We detected approximately equal concentrations of TGF-β1 (112 to 236 pM) and TGF-β2 (128 to 216 pM) in murine milk from TGF-β1 heterozygotes at the first week postpartum (*14*). In addition, immunohistochemical studies of cells present in mouse milk showed intracellular localization of TGF-β1 (Fig. 2). This observation suggests that maternal cells with a biosynthetic capacity for TGF-β1 [including lymphocytes and macrophages, which are transferred to the newborn during suckling (*15*)] could serve as a continuing source of this protein. Furthermore, gastrointestinal absorption of intact TGF-β1 was observed after the administration (by gavage) of radiolabeled TGF-β1 into the stomachs of 5-day-old pups. As early as 15 min after administration, TGF-β1 was found in neonatal lung, heart, and liver tissues (Fig. 3A). At 45 min, there was a greater accu-

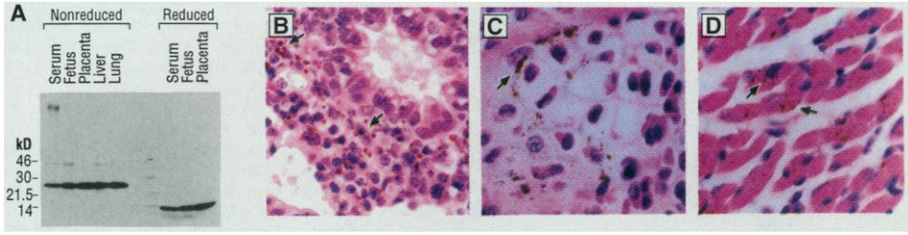

Fig. 1. Transfer of ^{125}I–TGF-β1 from mother to fetus. Pregnant female mice (days 15 to 20 of gestation, anesthetized with sodium pentobarbital) received an intracardiac injection of ^{125}I–TGF-β1 (65 ng of active TGF-β1 in physiological saline; specific activity, 1.7 μCi/pmol) (*11*). (**A**) Tissues from the mother (serum, lung, and liver), fetus, and placenta were homogenized in ice-cold immunoprecipitation (IP) buffer [1% Triton X-100, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.25% SDS]; the homogenates were clarified by centrifugation and incubated with rabbit polyclonal antibody to TGF-β1 (1% in IP buffer) overnight at 4°C (this antiserum was not affinity-purified and was used only for IP experiments). Protein A–Sepharose beads (Pharmacia) were added during the final 2 hours, after which the beads were washed three times with IP buffer. Protein was eluted by boiling in SDS loading buffer, and samples were subjected to SDS-PAGE analysis on 4 to 20% tris-glycine gels under both reducing and nonreducing conditions (each lane contains equivalent counts per minute). (**B** through **D**) Localization of maternally transferred ^{125}I–TGF-β1. After administration of labeled TGF-β1, each fetus was delivered by cesarean section, fixed in formalin, embedded in paraffin, and sectioned for autoradiography. Sections were counterstained with hematoxylin and eosin (H&E). Radioactivity was concentrated in areas surrounding blood vessels and airways in the lung (B), in matrix surrounding hypertrophic chondrocytes in bone (C), and in muscle (D), in a pattern resembling the normal immunohistochemical localization of TGF-β1 in these tissues (magnification, ×1250).

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mulation of TGF-β1 in the kidney, which suggests renal excretion (Fig. 3B). These data identify maternal milk as a source of TGF-β1 for the neonate.

The functional importance of maternally derived TGF-β1 was documented by the birth of several TGF-β1 null mice to a homozygous null female. Although these animals always die before breeding age, survival was improved by daily intraperitoneal injections of dexamethasone, which suppresses the function of lymphocytes and macrophages and reduces the expression of MHC antigens (*16*). Improved survival was accompanied by histologic evidence of diminished tissue inflammation (*17*), and this intervention allowed for successful pregnancy in a single homozygous null female, resulting in seven live-born pups. Four were homozygous for the mutant allele and died within 1 day. All four displayed severe cardiac abnormalities (Fig. 4), including poorly formed ventricular lumina and disorganized ventricular muscle and valves. Notably, these cardiac abnormalities were not seen in the three heterozygous pups, which suggests that endogenous synthesis of TGF-β1 during embryogenesis can rescue the phenotype, even when there are no maternal sources of the protein. More important, these results indicate that in the absence of endogenous synthesis, maternal sources of TGF-β1 are vital for normal cardiogenesis and embryonic development.

Immunohistochemical studies of TGF-β1 showed substantial differences between null mice born to heterozygous females and those born to the null female. TGF-β1 null mice born to heterozygous females showed strong staining of TGF-β1 in many tissues, including skeletal muscle (Fig. 4E), whereas a null mouse born to the TGF-β1 null female showed no staining in the same tissues (Fig. 4F). This finding suggests that maternally acquired TGF-β1 protein is partially responsible for the characteristic staining patterns seen during embryogenesis.

It is unclear whether active or latent TGF-β1 is the most physiologically relevant form transferred to the fetus and whether this protein is transferred in a complex with carrier proteins. Immunoglobulin G (IgG) has been shown to associate with TGF-β1 in a form that is capable of regulating lymphocyte function (*18*). Thus, IgG transferred from mother to fetus through the placenta (*19*), and to the neonate through the milk (*20*), may also be an important source of TGF-β1. In addition, there is evidence that maternal blood cells are transferred to the fetus through the placenta (*21*), which suggests that active production of TGF-β1 may actually occur within the null fetus. Similarly, maternal leukocytes (delivered to the neonate during suckling) have been shown to transit both gastric and intestinal epithelia (*15*); these cells may not only provide an important component of cellular immunity but may be another source of TGF-β1.

Although our data have not defined the precise contribution of maternal TGF-β1 to normal postpartum development, they substantiate the hypothesis that maternal TGF-β1 is critical for the null fetus. Thus, the normal appearance at birth of TGF-β1 null mice born to heterozygous females may not be solely attributable to a redundant expression pattern of the three TGF-β isoforms. Our results do not exclude a role for redundancy, as we did not detect gross abnormalities in organs other than the heart in the TGF-β1 null offspring of the null female. The embryonic lethality observed in homozygous null progeny of TGF-β1 heterozygotes (*1*) also remains unexplained but suggests the existence of other important and apparently

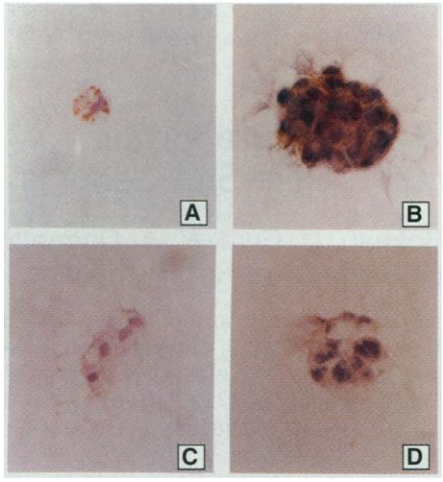

Fig. 2. TGF-β1 localized in mouse milk cells. Lactating TGF-β1 heterozygous mothers were milked during the first week after delivery of a litter; smears and cytospins were prepared from milk suspensions. Slides were stained for TGF-β1 with the antibody LC(1–30) (*4*). TGF-β1 was found intracellularly (**A** and **B**); controls stained with normal rabbit serum were negative (**C** and **D**). Slides were counterstained with H&E. (A) and (C), magnification ×810; (B) and (D), magnification ×1250.

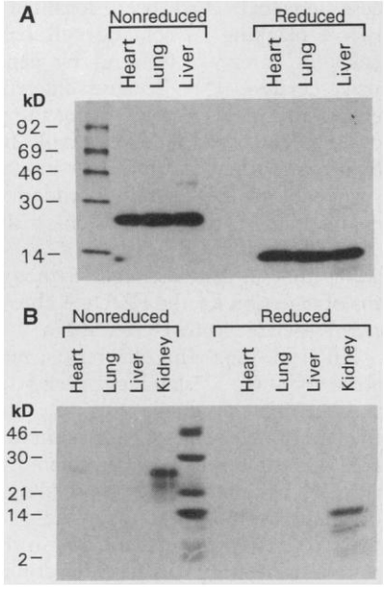

Fig. 3. Absorption of orally administered TGF-β1. Mouse pups (5 days old) received 1.5 μCi of ^{125}I–TGF-β1 in saline through a gastric tube and were killed 15 min (**A**) and 60 min (**B**) later. Tissues were homogenized and processed as described in Fig. 1A for immunoprecipitation of TGF-β1.

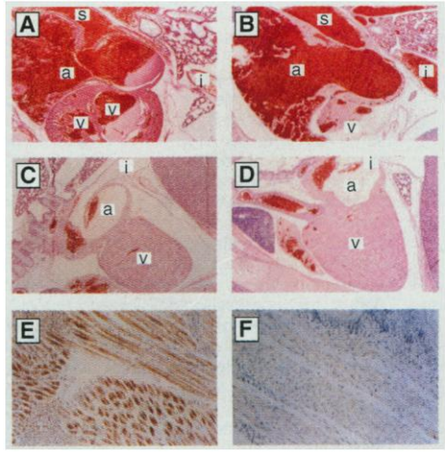

Fig. 4. Abnormal cardiac development in TGF-β1 null mice in the absence of maternal TGF-β1. A TGF-β1 null female was treated with daily intraperitoneal injections of dexamethasone (1.5 mg per kilogram of body weight), starting at day 15 of life and mated with a heterozygous male. All seven offspring (delivered at day 71 of the female's life) were killed and embedded in paraffin, and serial, sagittal step-sections of their tissues were stained with H&E. (**A**) Heart from a pup heterozygous for the TGF-β1 allele. (**B** through **D**) Hearts from TGF-β1 null littermates. Ventricular lumina were poorly formed; this was occasionally accompanied by a disordered proliferation of myocytes and unusual atrioventricular junctions. (i, inferior vena cava; s, superior vena cava; v, ventricle; a, atria). (**E**) Skeletal muscle from a TGF-β1 null mouse born to a TGF-β1 heterozygous female and (**F**) skeletal muscle from a TGF-β1 null mouse born to the TGF-β1 null female, each stained for TGF-β1. Animals were killed, embedded in paraffin, sectioned, and stained for TGF-β1 with the antibody LC(1–30) (*4*). The TGF-β1 null newborns of the null female were negative for TGF-β1 protein (F), whereas progeny of TGF-β1 heterozygotes were clearly positive (E). (A) through (D), magnification ×45; (E) and (F), magnification ×165.

uncompensated functions of TGF-β1.

Finally, although targeted gene disruption may be a valuable tool for evaluating the roles of cytokines, oncogene products, and other proteins during embryogenesis, our data show that in some instances, a gene knockout is not equivalent to a protein knockout. The potential contribution of maternal protein to the fetal phenotype should be considered in other gene disruption experiments, particularly those targeting genes that encode secreted proteins.

REFERENCES AND NOTES

1. M. M. Shull *et al.*, *Nature* **359**, 693 (1992); A. B. Kulkarni *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 770 (1993).
2. *Mol. Reprod. Dev.* **32** (June 1992).
3. J. Massagué, *Annu. Rev. Cell Biol.* **6**, 597 (1990); A. B. Roberts and M. B. Sporn, in *Peptide Growth Factors and Their Receptors*, M. B. Sporn and A. B. Roberts, Eds. (Springer-Verlag, New York, 1990), vol. 1, chap. 8; D. M. Kingsley, *Genes Dev.* **8**, 133 (1994).
4. U. I. Heine *et al.*, *J. Cell Biol.* **105**, 2861 (1987); K. C. Flanders *et al.*, *ibid.* **108**, 653 (1989).
5. F. A. Millan, F. Denhez, P. Kondaiah, R. Akhurst, *Development* **111**, 131 (1991).
6. A. G. Geiser *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9944 (1993).
7. G. F. Bottazzo *et al.*, *N. Engl. J. Med.* **313**, 353 (1985); T. Hanafusa *et al.*, *Lancet* ii, 1111 (1983).
8. H. P. Erickson, *J. Cell Biol.* **120**, 1079 (1993).
9. M. Popliker *et al.*, *Dev. Biol.* **119**, 38 (1987).
10. D. A. Cox and R. R. Bürk, *Eur. J. Biochem.* **197**, 353 (1991); H. Tokuyama and Y. Tokuyama, *Cell Biol. Int. Rep.* **13**, 251 (1989).
11. Unlabeled latent TGF-β1 was acid activated in the presence of ^{125}I-labeled TGF-β1. Upon neutralization, the latent complex reformed, incorporating the labeled protein. Incorporation was verified by incubation of the TGF-β1 preparation with 300 μM disuccinimidyl suberate cross-linking reagent (Pierce) for 15 min at 4°C and analysis of the products by SDS-PAGE. Iodination was carried out by the chloramine-T method; the labeled product was recovered by passage over a Sephadex G-25 column (Pharmacia LKB, Piscataway, NJ). Animal protocols were approved by institutional review; animals (C57 BL/6J × SVJ 129 mice) subjected to surgical procedures received sodium pentobarbital analgesia (0.05 to 0.1 mg per kilogram of body weight) by intraperitoneal injection.
12. L. M. Wakefield *et al.*, *J. Clin. Invest.* **86**, 1976 (1990).
13. Time-dependent transplacental transit by TGF-β1 was observed after injection of pregnant mice (Fig. 1). Fetal tissue processed at 15 min contained from 198 to 421 cpm per milligram of protein; those collected at 45 min contained from 2800 to 3527 cpm per milligram of protein. At 45 min, less than 4% of total counts per minute injected was present in any one fetus. Results are a range from three separate experiments, with two specimens obtained for each time point and each tissue.
14. Mice in the newborn and neonatal periods were killed by CO_2 narcosis, and stomach contents (milk) were removed for analysis by sandwich enzyme-linked immunosorbent assay for TGF-β1 and TGF-β2 (*22*). Pooled specimens were extracted in acid-ethanol and dialyzed extensively against 4 mM HCl at 4°C, with the use of 3500 MW-cutoff Spectrapor dialysis tubing (Spectrum Medical Industries, Los Angeles, CA). Samples were clarified by centrifugation, lyophilized to dryness, and redissolved for analysis.
15. L. L. Seelig Jr. and J. R. Head, *J. Reprod. Immunol.* **10**, 285 (1987); L. L. Seelig Jr. and R. E. Billingham, *Transplantation* **32**, 308 (1981).
16. A. Celada, S. McKercher, R. A. Maki, *J. Exp. Med.* **177**, 691 (1993).
17. J. J. Letterio and A. G. Geiser, unpublished data.
18. R. M. Stach and D. A. Rowley, *J. Exp. Med.* **178**, 841 (1993).
19. F. W. R. Brambell, *The Transmission of Passive Immunity from Mother to Young* (North-Holland, Amsterdam, 1970); S. C. Adeniyi-Jones and K. Ozato, *J. Immunol.* **138**, 1408 (1987).
20. D. W. Dresser, *J. Reprod. Immunol.* **18**, 293 (1990); D. R. Abrahamson and R. Rodewald, *J. Cell Biol.* **91**, 270 (1981).
21. M. Shimamura, S. Ohta, R. Suzuki, K. Yamazaki, *Blood* **83**, 926 (1994).
22. D. Danielpour, *J. Immunol. Methods* **158**, 17 (1993); *Growth Factors* **2**, 61 (1989).

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Implications of *FRA16A* Structure for the Mechanism of Chromosomal Fragile Site Genesis

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Fragile sites are chemically induced nonstaining gaps in chromosomes. Different fragile sites vary in frequency in the population and in the chemistry of their induction. DNA sequences encompassing and including the rare, autosomal, folate-sensitive fragile site, *FRA16A*, were isolated by positional cloning. The molecular basis of *FRA16A* was found to be expansion of a normally polymorphic p(CCG)$_n$ repeat. This repeat was adjacent to a CpG island that was methylated in fragile site–expressing individuals. The *FRA16A* locus in individuals who do not express the fragile site is not a site of DNA methylation (imprinting), which suggests that the methylation associated with fragile sites may be a consequence and not a cause of their genesis.

Fragile sites are points on chromosomes that tend to break nonrandomly when exposed to specific chemicals or conditions of tissue culture. A major group are the rare folate-sensitive fragile sites, which segregate in families in a non-Mendelian manner (*1*). The only fragile sites that have been cloned [*FRAXA* (*2*) and *FRAXE* (*3*)] are in this group and are both on the X chromosome. One of these (*FRAXA*) is associated with the most common form of familial mental retardation, fragile X syndrome (*2*). The two X chromosome fragile sites are unstable p(CCG)$_n$ trinucleotide repeats adjacent to CpG islands that become hypermethylated when the number of copies of the repeat exceeds certain limits. None of the autosomal, folate-sensitive fragile sites have been cloned, and nothing is known of their clinical significance, perhaps because none have been reported in homozygous form. To gain further understanding of the relations between DNA sequence, position in the genome, and chemistry of cytogenetic expression of fragile sites, we have characterized the rare folate-sensitive fragile site (*FRA16A*) in band p13.11 of human chromosome 16.

A long-range restriction map in the vicinity of *FRA16A* (Fig. 1) was generated by means of anonymous DNA sequences. These sequences had been localized by physical mapping to somatic cell hybrid breakpoint intervals (*4*) and by genetic linkage analysis (*5*). Because differential methylation has been observed for the previously characterized X-linked fragile sites, DNA from normal and *FRA16A* individuals was subjected to restriction endonuclease digestion with methylation-sensitive enzymes (Not I and Bss HII).

This analysis demonstrated distinct patterns of digestion for the *FRA16A* chromosomes associated with clustered Not I and Bss HII sites, suggesting differential methylation of a CpG island associated with expression of the fragile site (*6*). Yeast artificial chromosomes (YACs) containing DNA from this region were isolated and analyzed for the presence of these clustered Not I and Bss HII sites (*7*). One of the YACs (769H1) was found to contain these rare-cutting restriction sites adjacent to a p(CCG)$_n$ hybridizing sequence. The YAC was subcloned into lambda, and clones containing the p(CCG)$_n$ repeat were isolated (Fig. 1). One of the subclones, pf16A3, was used as a probe to demonstrate instability of this sequence in pedigrees transmitting the *FRA16A* fragile site (Fig. 2).

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