

Expression of Transforming Growth Factor- β 1 in Specific Cells and Tissues of Adult and Neonatal Mice

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Abstract. We have used immunohistochemical techniques to detect transforming growth factor- β 1 (TGF- β 1) in many tissues of adult and neonatal mice. Each of two antibodies raised to the amino-terminal 30 amino acids of TGF- β 1 selectively stained this molecule in either intracellular or extracellular locations. Strong intracellular staining was found in adrenal cortex, megakaryocytes and other cells of the bone marrow, cardiac myocytes, chondrocytes, renal distal tubules, ovarian glandular cells, and chorionic cells of the placenta. Marked staining of extracellular matrix

was found in cartilage, heart, pancreas, placenta, skin, and uterus. Staining was often particularly intense in specialized cells of a given tissue, suggesting unique roles for TGF- β within that tissue. Levels of expression of mRNA for TGF- β 1 and its histochemical staining did not necessarily correlate in a given tissue, as in the spleen. The present data lend further support to the concept that TGF- β has an important role in controlling interactions between epithelia and surrounding mesenchyme.

IN the preceding paper (Flanders et al., 1989), we have described the use of two antibodies, anti-CC(1-30) and anti-LC(1-30), for histochemical localization of transforming growth factor- β 1 (TGF- β 1),¹ with the former staining predominantly extracellularly and the latter intracellularly. We now report the use of these two antibodies in a histochemical survey of numerous tissues of neonatal and adult mice. Furthermore, we have performed Northern blot analysis on many of these mouse tissues to determine if the level of mRNA expression correlates with the intensity of histochemical staining, since it has been previously reported that there may be poor correlation between mRNA levels and levels of expressed protein when measurements of TGF- β are made on cultured cells (Kehrl et al., 1986; Assoian et al., 1987).

Materials and Methods

Immunohistochemistry

Rabbit polyclonal antibodies to unconjugated peptides corresponding to the

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1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF- β , transforming growth factor- β .

amino-terminal 30 amino acids of TGF- β 1 were prepared as described (Flanders et al., 1989). Tissues from 6-8-wk-old adult or 4-5-d-old neonatal NIH-Swiss mice were excised immediately after death by carbon dioxide and fixed by immersion overnight at room temperature in neutral-buffered formalin. Tissues were also obtained from adult mice anesthetized with sodium pentobarbital. Blood was cleared by brief cardiac perfusion with PBS, followed by formalin fixation in situ for 20 min. Tissue was postfixed in Bouin's solution for an additional 4-6 h, transferred to 70% ethanol, embedded in paraffin, and sectioned at 5 μ m. Rib specimens were decalcified before embedding. TGF- β 1 was localized in tissue sections using avidin-biotin-peroxidase or avidin-biotin-alkaline phosphatase as described (Flanders et al., 1989). Details of controls are given in the accompanying manuscript (Flanders et al., 1989).

RNA Extraction and Northern Hybridization

Total RNA was prepared according to Chirgwin et al. (1979) from tissues that were flash frozen in liquid nitrogen and stored at -70°C . 10 μ g of total RNA per lane was subjected to electrophoresis in formaldehyde-agarose gels and transferred to Nytran filters (Davis et al., 1986). Filters were hybridized according to Church and Gilbert (1984), using a ^{32}P -labeled, single-strand, cDNA probe for TGF- β 1 and a nick-translated cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Van Obberghen-Schilling et al., 1988).

Results

Tissue Distribution of TGF- β 1

Table I presents a summary of the tissue distribution of immunohistochemically detectable TGF- β 1 in adult mouse tissues, using both anti-LC(1-30) and anti-CC(1-30) antibodies. Relative staining intensity on a scale of 1-4+ was judged subjectively by the density of the peroxidase reaction product

Table I. Tissue Distribution of Immunohistochemically Detectable TGF- β 1 in Adult Mouse

Tissue	Localization	Antibody	
		Anti-LC (1-30)	Anti-CC (1-30)
Adrenal	Adrenocortical cells	+++	-
Bone marrow	Hematopoietic cords, megakaryocytes	++	-
Brain	ND*	-	-
Cartilage	Chondrocytes	+++	-
	Matrix	-	++
Heart	Cardiac myocytes	++	-
	Interstitial connective tissue	-	++
Intestine	Mucosal epithelium	+	-
Kidney	Distal tubules	++++	-
Liver	Biliary ductular cells, hepatocytes	+	-
Lung	Bronchial epithelial cells	+	-
Pancreas	Periductular connective tissue	-	++++
Ovary	Interstitial glandular cells	++	-
Placenta	Chorion (subpopulation)	++++	-
	Peri-chorionic connective tissue	-	++
Skin	Epidermis	+	-
	Papillary dermis	-	++
Spleen	ND	+/-	-
Testes	ND	-	-
Uterus	Periglandular connective tissue	-	+++

* None detected above background staining level of normal rabbit IgG with either anti-LC (1-30) or anti-CC (1-30).

and more quantitatively by the persistence of staining with increasing antibody dilution.

Using anti-LC(1-30), intracellular staining of specific cell types was seen in many adult tissues (Table I). With our fixation and staining protocol and a primary antibody concentration of 20 μ g/ml, the intensity of staining varied substantially between cell types. Intense cytoplasmic staining was found in costal chondrocytes, renal distal tubule cells, placental chorion cells near the site of implantation, and adrenal cortical cells. Moderate staining was seen in cardiac myocytes and cells of the bone marrow, while lighter staining occurred in such cell types as intestinal epithelial cells and biliary ductular cells. By contrast, no specific intracellular staining was seen in brain or pancreas.

Anti-CC(1-30) gives discrete extracellular tissue localization of TGF- β 1. In some cases, this extracellular staining occurs near cells which are stained intracellularly by anti-LC(1-30), as occurs in placenta, cartilage, and heart. However, in other cases, such as uterus and pancreas, staining of stroma is seen without detectable intracellular staining of any

cell type by anti-LC(1-30). Conversely, cells of the adrenal cortex, renal distal tubules, and ovarian interstitial glands display intracellular staining with anti-LC(1-30) without detectable extracellular staining by anti-CC(1-30).

Specific features of the staining patterns of TGF- β 1 in several tissues, controls to show antibody specificity, and comparisons between adult and neonatal tissue are presented below. This survey is intended as an overview of the cellular distribution of TGF- β 1 which may suggest potential tissue-specific roles, rather than as an exhaustive examination of every murine cell type.

Adrenal Tissue

Anti-LC(1-30) stains cytoplasm in the adult adrenal cortex (Fig. 1 A), primarily in the inner two cortical subdivisions, zona fasciculata and zona reticularis. A small subpopulation of pheochromocytes (neural crest-derived) in the adrenal medulla is also stained by this antibody. Specificity of staining was demonstrated by blocking of antibody with TGF- β 1 coupled to Sepharose as described (Flanders et al., 1989) (Fig. 1 B). Equivalent blocking was also obtained by preincubation of antibody in solution with peptide LC(1-30) (not shown). TGF- β 1 reactive with anti-CC(1-30) was not found in any adrenal cell type.

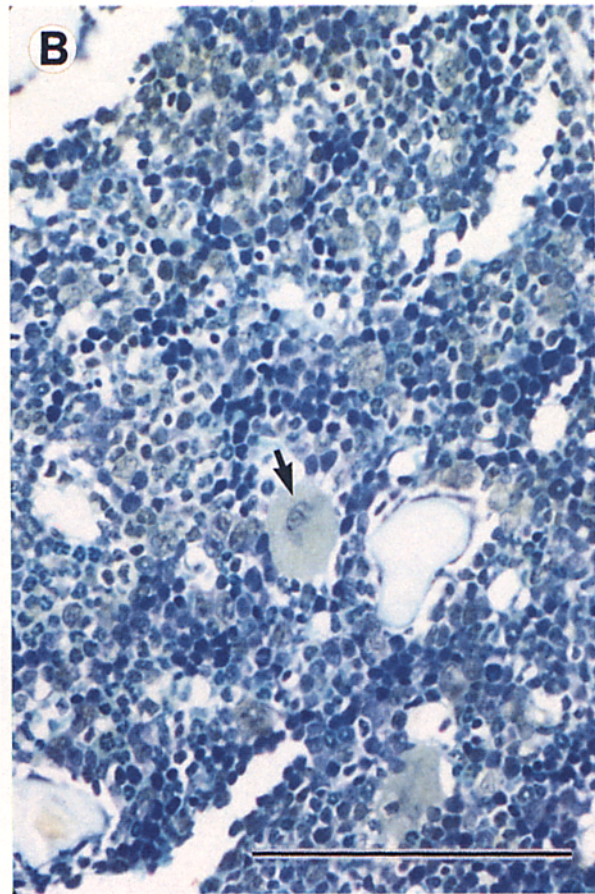
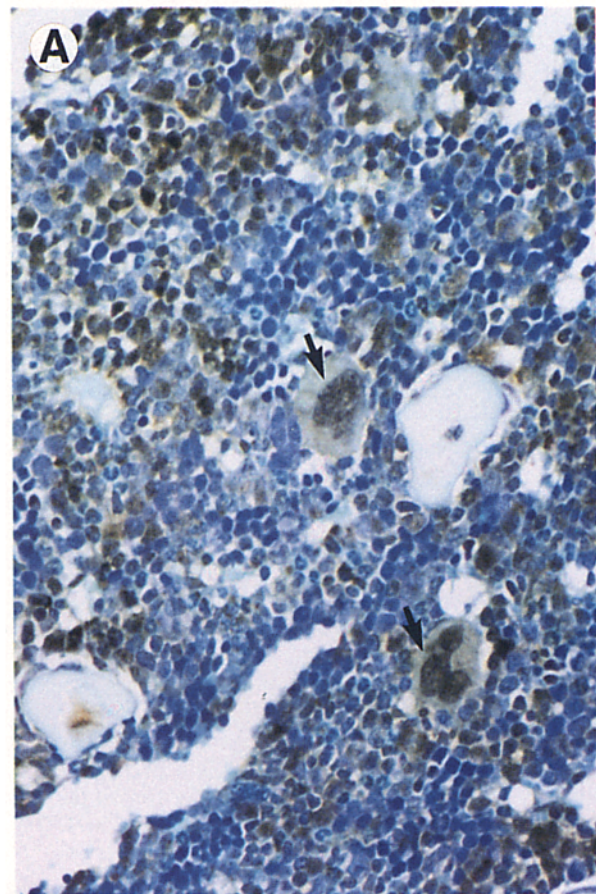
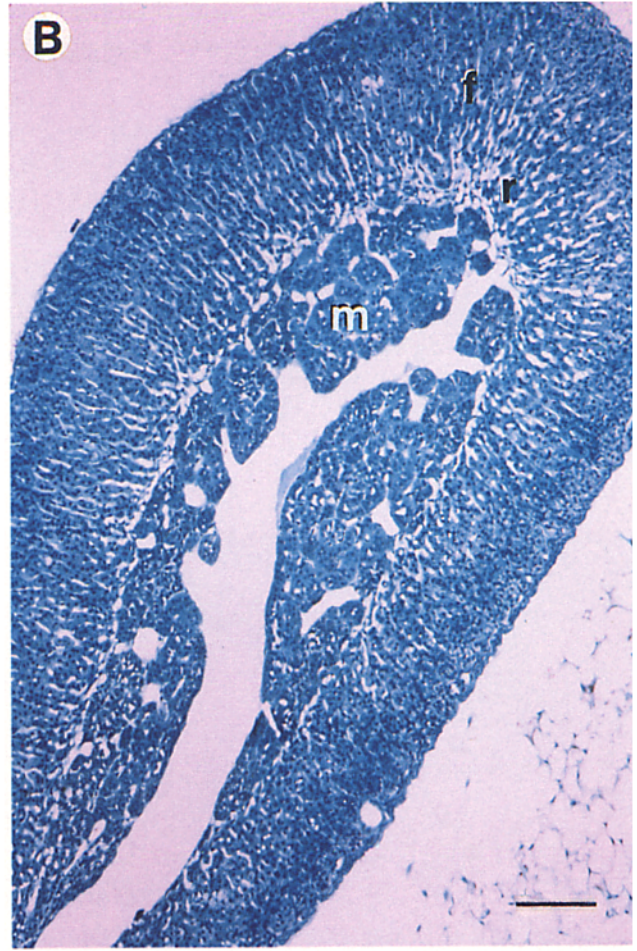
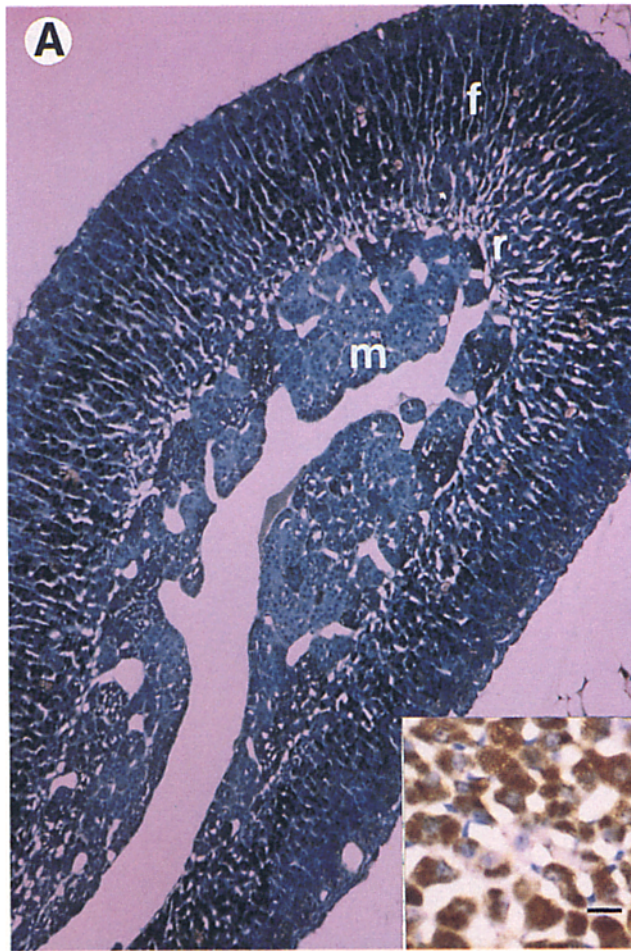
Hematopoietic Tissue

Anti-LC(1-30) stains megakaryocytes and other cells of the hematopoietic cords in red bone marrow of the adult rib (Fig. 2 A). Interestingly, this antibody stained the multilobulated megakaryocyte nucleus at a stage when platelets were not yet visible in the cytoplasm. Whether this intense staining of the megakaryocyte nucleus is related to its peculiar polyploid (up to 32 or 64 n) state remains to be determined; it is interesting to note that anti-LC(1-30) did not stain the nucleus of any other tissue or cell type. The identity of the other hematopoietic precursor cell types was not determined. Specificity of staining was demonstrated on sequential sections by blocking antibody staining with peptide LC(1-30) in solution at a 20-fold molar excess of peptide over IgG (Fig. 2 B). The same staining pattern was obtained with an alkaline phosphatase detection system (not shown), indicating that iron complexes such as hemosiderin, which are abundant in red marrow, did not give false positive staining in our experiments (Sternberger, 1986). Although Ellingsworth et al. (1986) reported a similar localization of TGF- β 1 with anti-CC(1-30) in fetal bovine bone marrow, no staining was observed in the mouse using the present staining protocol with this antibody.

In vitro studies have shown that TGF- β 1 inhibits proliferation of hematopoietic progenitor cells stimulated by interleu-

Figure 1. Immunolocalization of TGF- β in adult mouse adrenal gland. (A) Anti-LC(1-30) stains adrenal cortex, predominantly zona fasciculata (*f*) and zona reticularis (*r*). In addition, light staining is seen in a subpopulation of pheochromocytes in the medulla (*m*). Inset shows cytoplasmic localization and heterogeneity of staining in zona reticularis at higher magnification. Avidin-biotin-peroxidase detection system. Giemsa and May-Grunwald counterstains. (B) Staining is blocked in this control sequential section, stained with an equivalent aliquot of antibody preadsorbed with Sepharose to which TGF- β had been coupled. Bars: (A and B) 100 μ m; (inset) 10 μ m.

Figure 2. TGF- β in bone marrow. (A) Section through bone marrow of adult mouse rib. Anti-LC(1-30) stains megakaryocyte nuclei (arrows) and cells of the hematopoietic cords. (B) Control sequential section stained with antibody which had been blocked by preincubation with a 20-fold molar excess of peptide LC(1-30). Avidin-biotin-peroxidase, with Giemsa and May-Grunwald counterstains. Bar, 100 μ m.



kin 3 (Ohta et al, 1987; Keller et al., 1988). Even though hematopoiesis also occurs in spleen, little or no staining above background level was observed in this tissue in neonate or adult (not shown). Likewise, no staining was detected in the hematopoietic cells which are still present in neonatal liver at 4 d after birth or in gut-associated lymphoid tissue (not shown).

Heart

Cardiac myocytes are uniformly stained by anti-LC(1-30) in both the adult and neonate with somewhat greater intensity in the atria than in the ventricles (Fig. 3, *A* and *D*). Antibody to CC(1-30), by contrast, stains the fibrous cardiac skeleton (to which cardiac muscles and valves are both anchored), as well as the valves themselves (Fig. 3, *B* and *E*). In the em-

bryo, anti-CC(1-30) stained endocardial cushion tissue and developing heart valves (Heine et al., 1987).

Kidney

A subpopulation of distal tubule cells in both neonatal and adult kidney is intensely stained by anti-LC(1-30) (Fig. 4, *A* and *B*). Staining is confined to the cortex; none is seen in the collecting ducts that penetrate the medulla. This localization of TGF- β 1 is similar to that reported for EGF in the adult mouse kidney by immunohistochemical (Salido et al., 1986) and in situ hybridization (Rall et al., 1985) techniques. EGF, however, is not detectable in the kidney until \sim 2 wk after birth (Popliker et al., 1987) and is localized in the apical portions of the cells (Salido et al., 1986) in contrast to the uni-

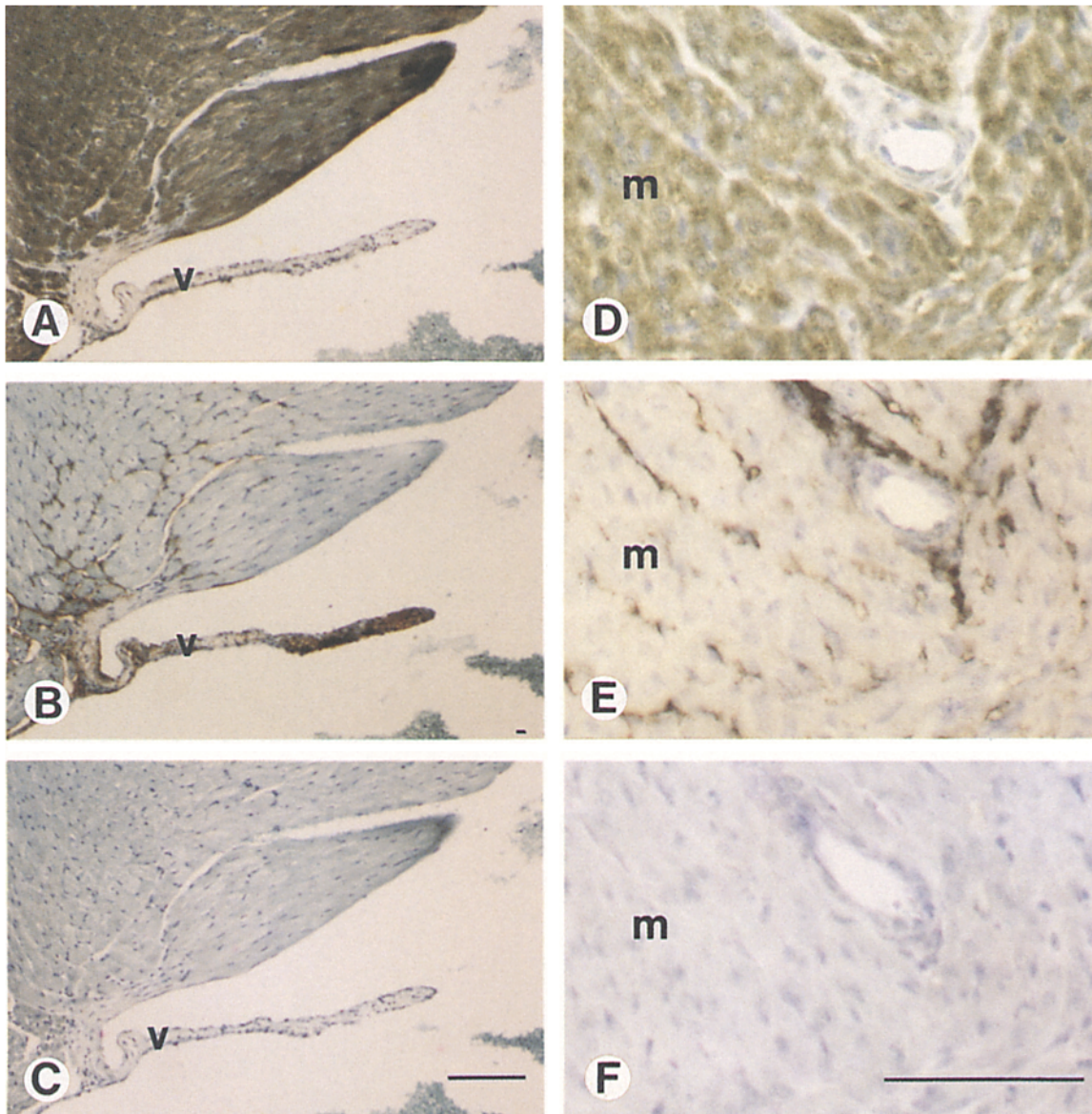


Figure 3. TGF- β in adult (*A-C*) and neonatal (*D-F*) mouse heart ventricle. (*A* and *D*) Anti-LC(1-30) stains cytoplasm of all cardiac myocytes (*m*). (*B* and *E*) Anti-CC(1-30) stains interstitial connective tissue around capillaries and connective tissue of valve leaflets (*v*). (*C* and *F*) Treatment with nonimmune rabbit IgG at the same concentration gives no staining on sequential sections. Peroxidase, with Giemsa and May-Grunwald counterstains. Bars, 100 μ m.

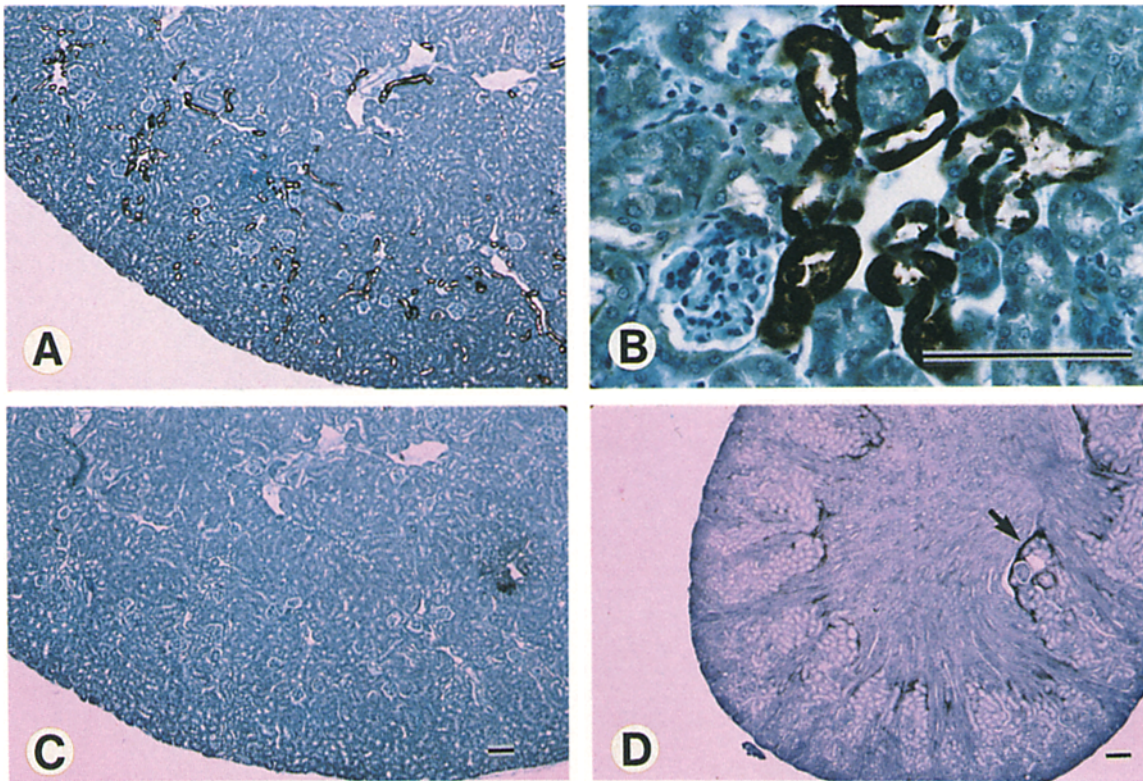


Figure 4. TGF- β in adult and neonatal kidney. (A and B) Anti-LC(1-30) stains a subpopulation of distal tubule cells in the adult mouse cortex. (B) Higher magnification of A reveals heterogeneity of staining within proximal distal tubule. (C) Anti-CC(1-30) does not stain adult mouse kidney. (D) Anti-CC(1-30) stains connective tissue of neonatal kidney in vicinity of cortical-medullary junction (arrow). Peroxidase, with Giemsa and May-Grunwald counterstains. Bars, 100 μ m.

form distribution of TGF- β 1 throughout the cytoplasm. Due to the especially intense immunoreactivity of kidney tissue with anti-LC(1-30), it was necessary to dilute primary antibody preparations to $<1 \mu\text{g/ml}$ IgG for blocking reactions. Preincubation of antibody with peptide LC(1-30) at a 400-fold molar excess greatly reduced staining, whereas preincubation with a nonhomologous peptide at the same concentration did not (not shown). Anti-CC(1-30) has little reactivity in adult kidney (Fig. 4 C), but does stain a fine network of interstitial tissue prominent at the cortical-medullary junction in the neonate (Fig. 4 D).

Placenta

We examined TGF- β 1 immunolocalization in placenta at 15 and 18 d of gestation. At both times, anti-LC(1-30) stained cells of the chorion at the interface between the implantation site and the rest of the chorion (Fig. 5 A). Placental morphology varies greatly between species, with a disc-shaped area of fusion present in rodents (Banks, 1986). The maternal portion of the chorion was not stained by anti-LC(1-30). Anti-CC(1-30) weakly stained fine connective tissue between the chorion and adjacent blood vessels (Fig. 5 B). The placenta continuously undergoes changes in size, shape, and internal structure during gestation (Bjorkman and Dantzer, 1987), and TGF- β 1 may play a regulatory role during this remodeling.

Other Organs and Tissues

As noted in Table I, immunoreactive TGF- β 1 is present in several other sites. Anti-LC(1-30) stains interstitial glandular cells of the ovary (Fig. 5, C and D), while anti-CC(1-30) stains stroma in adult uterine glands (not shown), suggesting a role in reproductive physiology (Feng et al., 1988; Skinner et al., 1987). We did not examine variations during the estrous cycle. The cytoplasm of chondrocytes in adult and neonatal cartilage is intensely stained by anti-LC(1-30) (not shown), as was also seen in embryonic cartilage (Flanders et al., 1989). In some organs such as stomach, liver, and eye, it was difficult to interpret antibody reactivity due to nonuniform staining patterns and high background with nonimmune rabbit IgG. In adult liver, light staining of hepatocytes, especially near central veins, and of biliary ductular cells with anti-LC(1-30) (not shown) may reflect hepatic uptake and processing of TGF- β 1 (Coffey et al., 1987). Other organs, such as adult brain, are clearly negative under our staining conditions.

TGF- β 1 RNA Expression

To correlate expression of TGF- β 1 protein as detected by immunohistochemical staining with steady-state levels of TGF- β 1 message, total RNA was prepared from several normal murine tissues and RNA Northern blots were hybridized to

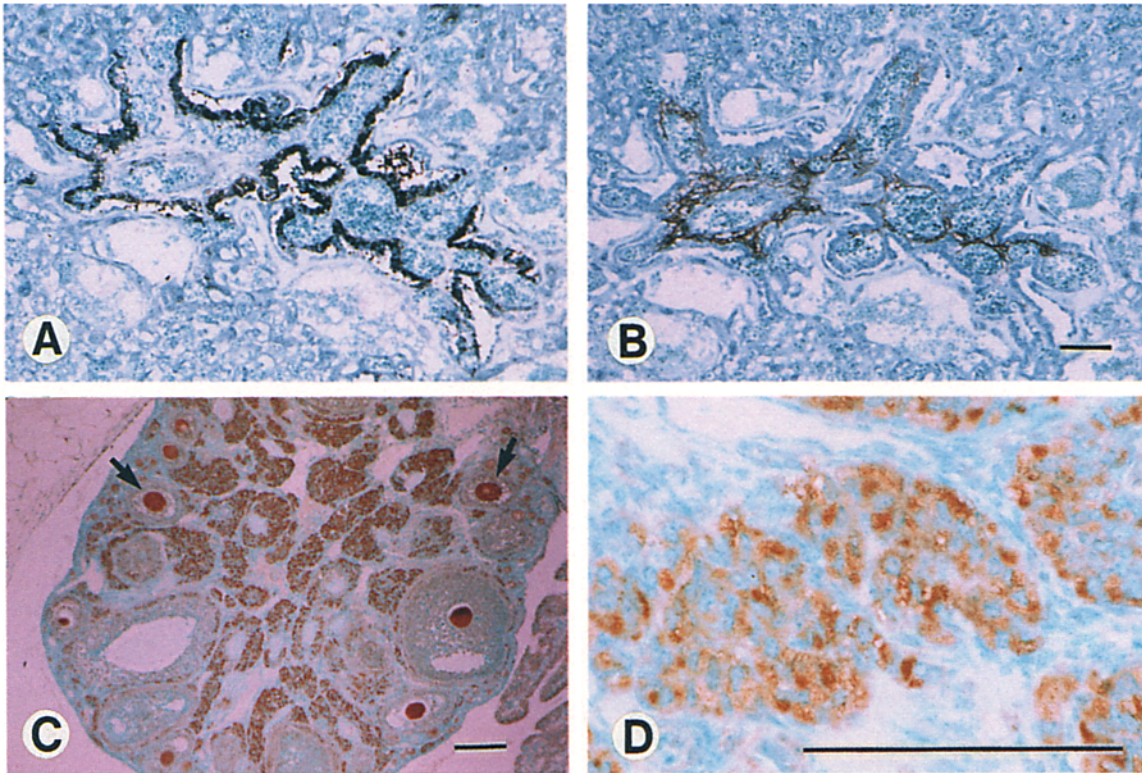


Figure 5. TGF- β in mouse placenta and ovary. (A) Placenta at 18 d of gestation stained with Anti-LC(1-30). Cytoplasm of chorion cells near site of implantation is intensely positive. (B) Anti-CC(1-30) stains stroma between chorion and blood vessels in an adjacent placental section. Giemsa and May-Grünwald counterstains. (C and D) Adult mouse ovary. (C) Anti-LC(1-30) stains interstitial glandular cells. Staining of developing ova (arrows) was artifactual and could not be competed by peptide. (D) Higher magnification of C showing specific cytoplasmic staining of interstitial glandular cells. Peroxidase, with methyl green counterstain. Bars, 100 μ m.

a single-stranded cDNA probe for TGF- β 1. The 2.4-kb TGF- β 1 transcript is detectable in all tissues examined (Fig. 6 A). The lowest levels of TGF- β 1 RNA are found in normal adult liver and skin, tissues which give light but detectable antibody staining. The highest level of TGF- β 1 RNA expression is seen in adult spleen; steady-state levels (quantitated by densitometry) are \sim 15-fold greater than those in liver. Despite the abundance of TGF- β 1 RNA however, little or no TGF- β 1 was detected in this organ with either anti-LC(1-30) or anti-CC(1-30). Since the original tissue samples for RNA analysis and immunohistochemistry were taken from different animals, it was possible that this lack of correlation was due to an unapparent infection or some other interanimal variation. Therefore, we divided the spleens of three additional adult mice for RNA and immunohistochemical preparations. High steady-state levels of TGF- β 1 RNA, comparable to those initially observed, were again found in all three spleens (Fig. 6 C), yet no staining above background levels was detected by immunohistochemistry. Intermediate levels of TGF- β 1 RNA expression were found in adult heart, kidney, small intestine, adrenal, placenta (15-d gestation), and colon (Fig. 6 A). Steady-state mRNA levels in these organs relative to adult liver are five-, four-, four-, two-, five-, and twofold, respectively. Ethidium bromide staining confirmed that RNA loading was similar in all lanes (Fig. 6 B).

TGF- β 1 RNA expression in neonatal vs. adult tissue was compared for heart, kidney, small intestine, and liver (Fig.

7 A). Steady-state message levels for the constitutive glycolytic enzyme GAPDH were used to standardize RNA loading (Fig. 7 B). When standardized to GAPDH, levels of the 2.4-kb TGF- β 1 transcript in heart and small intestine vary less than twofold between neonate and adult. Adult heart RNA however has two additional TGF- β 1 species of \sim 1.1 and 0.8 kb, which hybridize to the cDNA probe and are not seen in neonatal heart tissue. The abundance of these RNAs is 0.2- and 0.5-fold, respectively, relative to the 2.4-kb transcript. When standardized to GAPDH, neonatal liver TGF- β 1 RNA expression is twofold higher than in adult liver, while neonatal kidney RNA expression is approximately fourfold higher than in adult kidney.

Discussion

We have demonstrated the presence of immunoreactive TGF- β 1 in many neonatal and adult murine tissues, using two different antibodies raised to the amino-terminal 30 amino acids of TGF- β 1. Marked staining is found in tissues from which TGF- β 1 has been purified to homogeneity, such as kidney (Roberts et al., 1983) and placenta (Frolik et al., 1983). Staining is associated with particular cell types and is specific. Staining with anti-LC(1-30) is intracellular in epithelial (e.g., renal distal tubule), mesenchymal (e.g., costal chondrocyte), and hematopoietic (e.g., megakaryocyte) cell types, while staining with anti-CC(1-30) is extracellular and

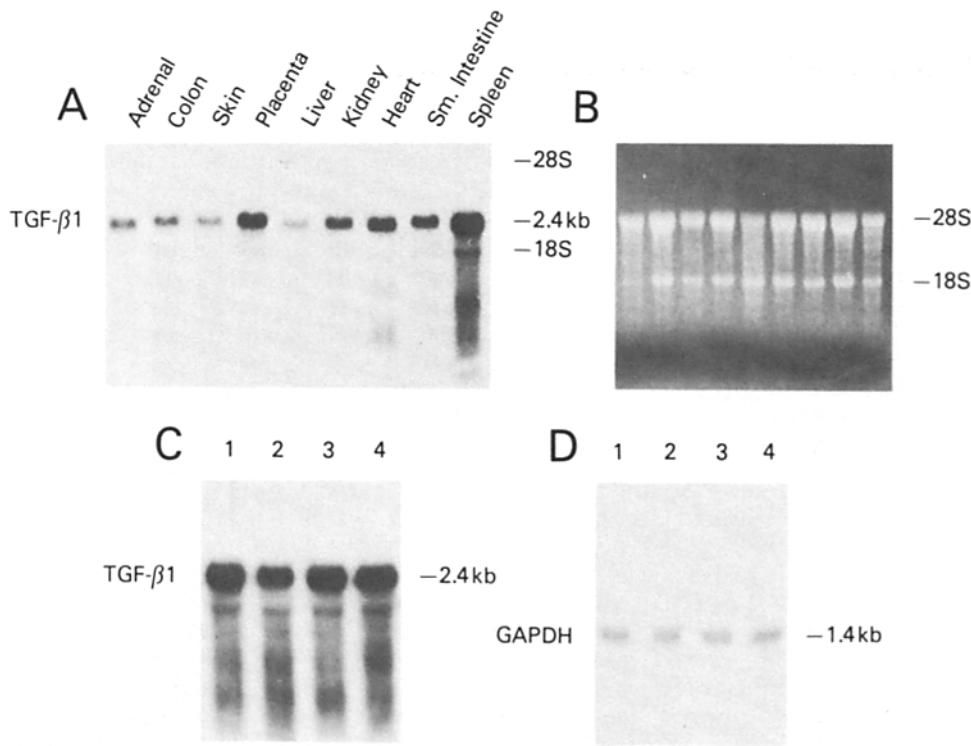


Figure 6. Steady-state levels of TGF- β 1 mRNA in adult mouse tissues. (A) Northern blot of total RNAs (10 μ g/lane) hybridized to human TGF- β 1 probe. (B) Ethidium bromide-stained Nytran blot shown in A before hybridization. (C) Northern blot of total spleen RNAs (10 μ g/lane) from three additional normal adult mice (lanes 1-3) and spleen RNA shown in A (lane 4); hybridized as in A. (D) Blot shown in C hybridized to probe for GAPDH.

associated with mesenchyme in every case (e.g., uterine stroma). Differences in primary epitope specificity appear to account for the differential staining pattern (Flanders et al., 1989), and as such, these polyclonal antibodies are functionally more analogous to monoclonal antibody preparations, recognizing a possible conformational change between intracellular and extracellular forms of TGF- β 1.

Specificity of staining has been demonstrated by blocking with TGF- β 1 or peptide and lack of reactivity with nonimmune IgG. The amino-terminal 30 amino acid sequence of

TGF- β 1 is completely conserved between human and mouse (Derynck et al., 1986). Computer search of this sequence reveals no homology with five or more adjacent amino acids of any protein in the database with the exception of TGF- β 2 (deMartin et al., 1987; Marquardt et al., 1987), TGF- β 3 (Jakowlew et al., 1988a; ten Dijke et al., 1988), or TGF- β 4 (Jakowlew et al., 1988b) making it unlikely that cross-reactivity with other proteins is responsible for the staining patterns.

The proportion of staining which represents latent, precur-

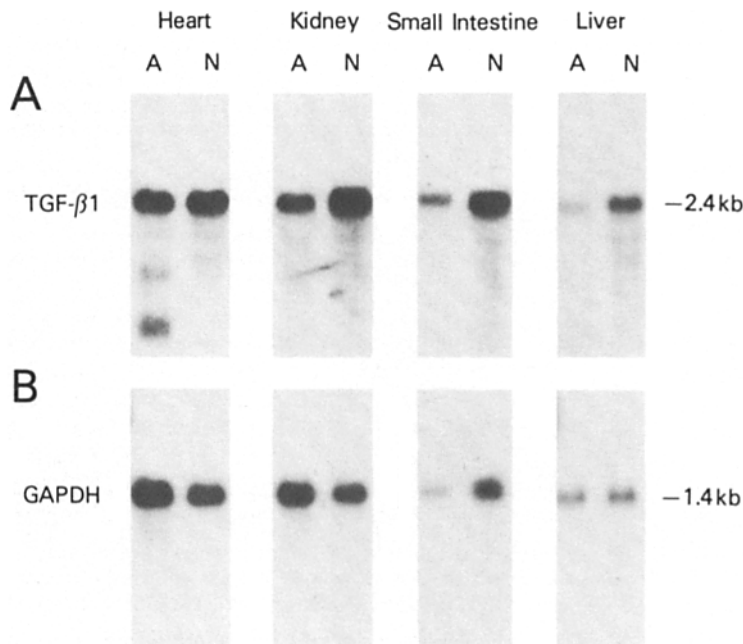


Figure 7. Comparison of steady-state levels of TGF- β 1 RNA in adult (A) and neonatal (N) mouse tissues. (A) Northern blot of total RNAs (10 μ g/lane) hybridized to human TGF- β 1 probe. (B) Blot shown in A hybridized to probe for GAPDH.

sor, or active forms of murine TGF- β is not known. In situ hybridization data are needed to correlate protein staining with sites of synthesis. Our localization of TGF- β in the epidermis of mouse skin with anti-LC(1-30) correlates well with the recently reported localization of TGF- β mRNA to the suprabasal epithelial layer in mouse skin treated with phorbol ester (Akhurst et al., 1988) and suggests that this antibody recognizes TGF- β at sites of synthesis.

Our Northern blot analysis of murine tissue RNAs demonstrates that TGF- β mRNA is detectable in every tissue examined; however, levels of this RNA do not correlate with the distribution of immunoreactive TGF- β . In particular, high levels of TGF- β mRNA are expressed in normal spleen, yet no staining above background levels was detected by either anti-LC(1-30) or anti-CC(1-30). Possible explanations for this discrepancy could include (a) protein levels below the detection limits of our assay, (b) lability or masking of epitopes in spleen (by a binding protein for example), or (c) failure of TGF- β mRNA to be translated in spleen. Studies reported by others in lymphocytes (Kehrl et al., 1986) and macrophages (Assoian et al., 1987) have shown that TGF- β mRNA may be expressed without detectable protein secretion. Conversely, positive staining in splenic macrophages can be induced by immunization of animals with bacterial cell walls with no concurrent change in splenic TGF- β mRNA levels (Wahl et al., 1988).

The cellular distribution of TGF- β in vivo may reflect important mechanisms of action in some tissues. Regulation of steroid synthesis may be a principal physiological role. In vitro studies have shown that TGF- β is a potent modulator of differentiated adrenocortical cell function; it inhibits basal steroidogenesis, as well as that induced by angiotensin II or ACTH (Feige et al., 1986; Hotta and Baird, 1986) and decreases angiotensin II-receptor numbers (Feige et al., 1987). In vitro TGF- β suppresses cortisol synthesis (Feige et al., 1987), which is confined to the zona reticularis and fasciculata. Our localization of TGF- β in these adrenal cortical zones is thus consistent with Hotta and Baird's (1986) suggestion that TGF- β plays a role in maintaining homeostasis in this organ. Placental chorion cells (Ferguson et al., 1986) and renal distal tubule cells (Sasano et al., 1988) are also known to possess steroidogenic enzyme activities and all contain immunoreactive TGF- β . Ovarian granulosa cells and testicular steroid-producing cells were negative, however.

Neonatal and adult tissue-staining patterns were compared in kidney, skin, heart, spleen, pancreas, cartilage, intestine, and liver. In all cases, neonatal staining patterns corresponded to those reported for adult tissues in Table I, although some variation in staining intensity was observed. As reported above, adult heart contains two RNA species of 0.8 and 1.1 kb that hybridize to the TGF- β probe and are not detected in the 5-d-old neonate. A novel TGF- β transcript of \sim 1.0 kb has been observed in porcine heart, which may represent an alternatively spliced messenger (Kondaiah et al., 1988). Hybrid selection and in vitro translation experiments are in progress to determine if the specific RNAs in the adult mouse heart encode unique proteins. A unique role for TGF- β in the heart is not known, although the peptide elevates actin mRNA in cell culture (Leof et al., 1986), a regulatory role which may be important in cardiac muscle.

In summary, we have found immunoreactive intracellular

and extracellular TGF- β in a wide variety of murine tissues. Staining is often particularly intense in specialized cells within a given tissue. The present results add further confirmation to previous suggestions (Heine et al., 1987; Lehnert and Akhurst, 1988) that TGF- β plays an important role in controlling interactions between epithelia and surrounding mesenchyme. Our studies also suggest that there may be new functions, as yet undetermined, for TGF- β in particular cells, such as cardiac myocytes and renal tubular cells. Further studies on the role of TGF- β in the pathogenesis of disease in specific organs may be particularly useful in elucidating the function of this peptide in normal cells and tissues.

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