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Interactions between retinoids and TGF βs in mouse morphogenesis

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Summary

Using immunocytochemical methods we describe the distribution of different TGF β isoforms and the effects of excess retinoic acid on their expression during early mouse embryogenesis (8½ - 10½ days of development). In normal embryos at 9 days, intracellular TGF β1 is expressed most intensely in neuroepithelium and cardiac myocardium whereas extracellular TGF β1 is expressed in mesenchymal cells and in the endocardium of the heart. At later stages, intracellular TGF β1 becomes very restricted to the myocardium and to a limited number of head mesenchymal cells; extracellular TGF β1 continues to be expressed widely in cells of mesenchymal origin, particularly in head and trunk mesenchyme, and also in endocardium. TGF β2 is widely expressed at all stages investigated while TGF β3 is not expressed strongly in any tissue at the stages examined.

Exposure of early neural plate stage embryos to retinoic acid caused reduced expression of TGF β1 and TGF β2 proteins but had no effect on TGF β3. Intracellular TGF β1 expression was reduced in all tissues except in the myocardium, while extracellular TGF β1 was specifically reduced in neuroepithelium and cranial neural crest cells at early stages. TGF β2 was reduced in all embryonic tissues. The down-regulation of intracellular TGF β1 was observed up to 48 hours after initial exposure to retinoic acid while some down-regulation of TGF β2 was still seen up to 60 hours after initial exposure.

TGF βs are known to modulate the expression of various extracellular matrix molecules involved in cell growth, differentiation and morphogenesis. The interaction between retinoic acid and TGF β is discussed in relation to morphogenesis.

Key words. TGF β, retinoic acid, mouse embryogenesis, immunocytochemistry.

Introduction

Peptide growth factors play key roles in the co-ordination and control of cell division, differentiation and morphogenesis during embryonic development. The most functionally diverse family of growth factors is transforming growth factor β (TGF β). In mammals, three TGF β isoforms (as well as the closely related bone morphogenetic proteins) are present in embryonic and adult tissues and most cells express several distinct receptors for TGF β (Massagué et al., 1991). The multifunctional nature of this family of growth factors is complex in that the actions of TGF β are cell type-specific, including both promotion and inhibition of cell proliferation and differentiation (Sporn and Roberts, 1988; Roberts et al., 1990; Moses and Yang, 1990; Barnard et al., 1990), as well as modulation of the extracellular matrix and influencing cell migration (Ignotz and Massagué, 1986, 1987).

Expression of TGF β during murine embryogenesis has been studied immunohistochemically (Heine et al., 1987; Pelton et al., 1991), by northern blot analysis (Thompson et al., 1989; Miller et al., 1989a, b) and by in situ hybridisation (Lehnert and Akhurst, 1988; Wilcox and Derynck, 1988; Pelton et al., 1989, 1990b; Akhurst et al., 1990; Fitzpatrick et al., 1990; Schmid et al., 1991; Millan et al., 1991). These studies suggest that TGF β1 has a role in haematopoiesis, angiogenesis and osteogenesis as well as in epithelial-mesenchymal interactions. TGF β2 is widely expressed and may also play a role in epithelial-mesenchymal interactions (Pelton et al., 1989). TGF β2 may also have a role in the differentiation of neuronal tissue (Millan et al., 1991; Flanders et al., 1991) as well as in the process of chondro-ossification (Millan et al., 1991; Schmid et al., 1991). TGF β3 has been less extensively characterized but patterns of expression suggest that it interacts with TGF β1 and TGF β2 in various morphogenetic processes such as during cardiovascular and skeletal development (Pelton et al., 1990b; Millan et al., 1991). These studies show that expression of TGF β isoforms is tissue-specific and stage-specific during embryogenesis.

In vitro, TGF βs have mitogenic effects for bone, cartilage and connective tissue fibroblasts, but inhibit epithelial cell proliferation (reviewed by Sporn and Roberts, 1988). They stimulate the expression of extracellular matrix proteins and the integrin class of matrix protein receptors (Ignotz and Massague, 1987; Heino et al., 1989). TGF βs may therefore control cell-matrix interactions by regulating the composition of the

extracellular matrix as well as by modulating the ability of cells to adhere to different extracellular matrix components, thereby affecting growth, differentiation and cell function.

Retinoids are another family of regulatory molecules that undoubtedly play a fundamental role in controlling cellular proliferation and differentiation (see Sporn and Roberts, 1991, for review). Maternal retinoid excess or deficiency during pregnancy both cause abnormalities in human infants (Lammer et al., 1985; Sarma, 1959) as well as in rodents (Wilson et al., 1953; Morriss, 1972; Webster et al., 1986), indicating that retinoid levels must be within a specific range for normal development. The mode of action of retinoids involves effects on gene expression through binding to at least two families of nuclear receptors. These are the all-*trans*-retinoic acid-specific RARs, which are members of the steroid/thyroid hormone family of nuclear receptors (see Leroy et al., 1991, for review), and the RXRs, whose natural ligand is 9-*cis*-retinoic acid (Levin et al., 1992). Specific spatiotemporal patterns of expression of each of the receptors has been described during embryogenesis, and for the retinoid-specific cytoplasmic binding proteins, providing insight into the mechanisms underlying the diversity of retinoid-related effects in embryos (Dollé et al., 1989, 1990; Ruberte et al., 1990, 1991; Rowe et al., 1991).

There is good evidence that interactions between retinoids and TGF β play important roles in cellular proliferation and differentiation, including modulation of the balance between these two processes (see Roberts and Sporn, 1991, for review). In order to investigate the interaction between retinoic acid and TGF β in embryos undergoing morphogenesis, we have studied the distribution of TGF β1, TGF β2 and TGF β3 peptides in early mouse embryos with both normal and raised retinoic acid levels. Our observations suggest that retinoic acid is capable of differentially modulating TGF β expression within the embryo.

Materials and methods

Maternal retinoic acid treatment and isolation of embryos

A solution of all-*trans*-retinoic acid (RA) was made as 5 mg crystalline RA in 0.8 ml absolute alcohol in 9.2 ml arachis oil and stored under argon at 4°C Female mice were mated by exposure to males at 9am; plugs were observed at 11am Pregnant C57Bl/6 female mice of mean weight 25 g received 12 mg/kg RA by oral gavage at 7¾ days of pregnancy (late presomite stage); control pregnant females received an equivalent dose of alcohol in arachis oil alone at the same stage of pregnancy. Mice were killed at 8½ days (7-9 somite stage), 9 days (16-18 somite stage), 9½ days (22-24 somite stage), 10 days (early limb bud stage) and 10½ days of development. At each embryonic stage, at least 30 normal and 40 RA-treated embryos were examined morphologically before fixation The characteristic RA-induced craniofacial abnormalities appropriate to each stage were consistently observed in embryos from RA-treated dams (for details see Morriss-Kay et al., 1991). Embryos were explanted into Tyrode's saline, examined for malformations and fixed for 4 to 6 hours in cold 4% paraformaldehyde. After fixation, embryos were processed for wax embedding. Sections were cut at 7 μm One row of sections from a control (normal) embryo, and one row from a RA-treated embryo were mounted in parallel on each slide, matching embryonic regions in the two rows as closely as possible

Immunohistochemistry

Polyclonal antibodies to TGF β isoforms included (a) anti-LC(1-30-1) raised to a peptide corresponding to amino acids 1-30 of TGF β1 and which stains principally intracellular TGF β1 (Flanders et al., 1988); anti-CC(1-30-1) raised to the same peptide as in (a) but which stains principally extracellular TGF β1 (Ellingsworth et al., 1986); anti-LC(50-75-2) raised to amino acids 50-75 of mature TGF β2 (Flanders et al., 1990); anti-LC(50-60-3) raised to amino acids 50-60 of mature TGF β3 (Flanders et al., 1991), anti-LC-pre(81-100-3) raised to TGF β3 precursor (Flanders et al., 1991). The TGF β1 antibodies were total IgG fractions prepared by elution of serum from Protein A-Sepharose, while the other antibodies were affinity purified by elution from columns of the immunizing peptide coupled to agarose.

Embryonic TGF β was localized in tissue sections using the avidin-biotin-peroxidase kit (Vector Laboratories) as described by Heine et al. (1987) Sections were dewaxed in xylene, treated with 0.3% hydrogen peroxide in methanol for 30 minutes, to block endogenous peroxidase, permeabilized with 1 mg/ml hyaluronidase for 30 minutes at 37 C, blocked with 1.5% normal goat serum in 0.5% BSA for 30 minutes and incubated overnight at 4°C with TGF β antibodies: 15 μg/ml anti-LC(1-30-1), 10 μg/ml anti-CC(1-30-1), 2.5 μg/ml anti-LC(50-75-2), 3 μg/ml anti-LC(50-60-3), 1 4 μg/ml anti-LC-pre(81-100-3). Sections were then incubated with biotinylated goat anti-rabbit IgG for 60 minutes, followed by avidin-peroxidase for 60 minutes and treated with 0.5 mg/ml diaminobenzidine with 0.1% hydrogen peroxide until the brown reaction product was obtained. Some sections were counterstained with Light Green. Experimental controls included replacing TGF β primary antibodies with normal rabbit serum and using primary antibodies which had been blocked by previously incubating with an excess of the corresponding peptide. Antibody staining patterns that did not show any variation after RA treatment included those of the TGF β3 peptides.

In the experimental design, groups of slides were taken through the immunohistochemical procedure so as to (a) compare the staining reaction of a single antibody at each of the 5 stages, and (b) to compare the staining reaction of all 5 antibodies at a single developmental stage. (a) was carried out 5 times (i e. 50 embryos per antibody); (b) was carried out 3 times (i.e. 30 embryos per stage). The total number of embryos examined was 400.

The avidin-biotin-peroxidase technique does not allow proper quantitative analysis of data so we made computer-aided optical density measurements and performed statistical analyses, allowing some comparisons to be made between normal and abnormal staining patterns. Using the Kontron IPS image processing system, microscope images from tissue sections on slides were taken into the image memory and stored via video camera. Optical density readings of antibody staining in specific regions of each normal and RA-treated embryo section from the same slide were measured; in a single tissue (normal or RA-treated) 10 optical density measurements were made and from the 2 sets of data, 2 means were obtained. The Student's *t-test* was applied to the pairs of means and used to determine whether there was a significant change in antibody staining in RA-treated embryos as

compared with normal embryos. We considered probability (P) > 0.05 to represent a non-significant difference Measurements were taken from 25 individual experiments comparing the staining reaction of a single antibody through different stages. The total number of readings taken for each antibody was 500.

Results

Appearance of RA-treated embryos

The general development of RA-treated embryos was retarded compared with normal embryos. The morphological features of normal and RA-treated embryos have been described in more detail elsewhere (Morriss-Kay et al., 1991). Briefly, treated embryos were characterised by reduced somite number, reduced number and size of pharyngeal arches, rostrally shifted otocyst, late closure of anterior neuropore and retardation of heart development. These effects of RA on morphogenesis confirm that the embryos have been exposed to raised RA levels.

Immunohistochemical localization of TGF β

TGF β was mostly associated with mesenchyme and mesenchyme-derived structures but was also detected in some epithelial tissues. In general, retinoid treatment resulted in a reduction in TGF β polypeptide as detected by the immunohistochemical technique. The results are summarized in Table 1. Limb buds were not examined for TGF β staining at any stage.

Anti-TGF β1 LC(1-30-1) detects principally the intracellular form of TGF β1 (intracellular TGF β1). Its staining pattern between 9 and 10½ days is illustrated in Fig. 1. At early somite stages, there was generally widespread staining in normal embryos, but as development proceeded, staining became progressively restricted to specific structures such as the heart.

At 8½ days, (not illustrated) low levels of intracellular TGF β1 were associated with both primary mesenchyme and neural crest-derived mesenchyme in the head, mesoderm of the primitive streak region, and the myocardium of the heart. Little staining was present in head neuroectoderm. In RA-treated embryos staining of the cranial mesenchyme was reduced.

At 9 days of development in normal embryos (Fig. 1A), intracellular TGF β1 was uniformly distributed in head and trunk mesenchyme of both mesodermal and neural crest origins. Intense staining was seen in the myocardium but not the endocardium or reticulum of the heart. Epithelial structures such as the neural tube, foregut and otic pit were also positive for intracellular TGF β1. In RA-treated embryos (Fig. 1B), all mesenchymal staining was greatly reduced. However, epithelial and myocardial staining was not significantly affected by RA excess, even though the heart is morphologically abnormal. Staining was increased in the dorsal part of the neuroepithelium and in lateral surface ectoderm (which was thickened). The apparently increased staining in the lateral edges of the neural epithelium matches that of migrating neural crest cells (NCC) in control sections and may represent pre-

Table 1. *Localization of TGF β1, β2 and β3 during early mouse embryogenesis. The table indicates the levels of respective antibody staining in different tissues and the effects of excess RA on TGF β expression*

TGF β	Age of embryo (days)	Intense stain	Some stain	No stain	Effects of RA
Anti-TGFβ1 LC(1-30)	9	myocard	mes, NE, pharyngeal mes, NC	–	decrease in mes
	10	myocard	mes, NE, pharyngeal mes	–	decrease in mes, NE
	10½	myocard	facial mes, gut	all NE, trunk mes	no effect
Anti-TGFβ1 CC(1-30)	9	endocardium, cardiac jelly, cranial mes, BM, NC	NE, otic pit, ep, gut ep	myocard	decrease in NE, NC
	9½	endocardium, cardiac jelly	cranial mes, BM	epithelia	general increase
	10½	endocardium, cardiac jelly, ectoderm, frontonasal mes, eye, somite, BM	cranial and trunk mes	all NE	slight increase
Anti-TGFβ2 LC(50-75)	9	–	mes, ep, heart	–	decrease
	9½	–	mes, ep, most NE, heart, cranial mes, trunk mesoderm	otic ep, gut ep	decrease
	10½	–	all NE, heart, mes, somite, cranial mes	–	some decrease
Anti-TGFβ3 LC pre(81-100)	9	–	myocard, ectoderm	mes	no effect
Anti-TGFβ3 LC(50-60)	10	pericardium	mes, NE, ectoderm, somite	–	no effect

mes, mesenchyme, *ep*, epithelium, *myocard*, myocardium, *NE*, neuroepithelium, *BM*, basement membrane, *NC*, neural crest cells

migratory NCC whose emigration has been retarded by the effects of RA.

At 10 days in normal embryos (Fig. 1C), the acoustico-facial ganglia, trigeminal ganglia and the cranial neural tube were stained. The most intensely stained structure was the heart, particularly the myocardial wall of the outflow tract and ventricles (not illustrated). In RA-treated embryos (Fig. 1D), intracellular TGF β1 staining was greatly reduced in all tissues except the myocardium. Optical density measurements were made on sections of normal and RA-treated embryos on the same slide to compare the differences in antibody staining in the myocardium and cranial region separately. Application of the *t-test* to the measurements obtained indicated that there was no significant difference between normal and RA-altered TGF β1 expression in the myocardium ($P>0.05$), but intensities of reactions in other parts of the RA-treated embryo were significantly lower than in normal embryo tissues ($P<0.01$).

In 10½ day normal embryos (Figs. 1E and 1G), intracellular TGF β1 staining was restricted to the head and the heart except for some light staining in the epithelial component of the lung buds. Staining of the early lung buds was also observed by Heine et al. (1990). Only very weak staining was present in the neuronal tissues at this stage. A limited number of cranial mesenchymal cells located rostral to the first pharyngeal arch were stained but no staining was seen in the trunk region; the limit is indicated by the open arrow in Fig 1E. There were no differences in either intensity or distribution of staining for intracellular TGF β1 between control and RA-treated embryos at 10½ days (compare Figs. 1E and 1F).

Hence, RA treatment resulted in a decrease in the expression of intracellular TGF β1 protein in most tissues but never in the myocytes of the heart. These effects were evident within 12 hours of exposure to RA (8½ days), and continued to early 10 day embryos but not beyond.

Anti-TGF β1 CC(1-30-1) detects mostly extracellular TGF β1, i.e. TGF β1 associated with the extracellular matrix (ECM) (Flanders et al., 1989). Its staining pattern between 9 days 10½ days is illustrated in Fig. 2. In normal embryos, at 9 days (Fig. 2A), cranial and trunk mesenchyme, the basement membrane of the neural tube and pharyngeal arch mesenchyme were very strongly positive for extracellular TGF β1. Migrating cranial neural crest cells were particularly strongly stained. Cranial and trunk neuroepithelial cells of the neural tube, otic pit and foregut epithelia were also positive although they were less intensely stained than mesenchymal cells. In RA-treated embryos (Fig. 2B), staining of primary cranial mesenchyme and epithelial basement membrane was unaltered whereas neuroepithelial staining was completely absent; expression in NCC was also down-regulated. In normal embryos, intense staining was also associated with the endocardium and reticulum, but not with the myocardium. This localized expression of TGF β1 in the heart was unaltered by excess RA treatment (data not shown).

At 9½ days in normal embryos, extracellular TGF β1 was less abundant (Fig. 2D); all epithelial structures were absolutely negative for TGF β1 while general mesenchymal staining had become localized (i.e. staining was present in mesenchyme from the dorsal side of the neural tube to the endocardial wall of the heart). In RA-treated embryos (Fig. 2E), mesenchymal staining was increased and staining was also present in epithelial tissues such as the neural tube. However, specific endocardial expression in the heart remained normal. Optical density measurements were made on antibody staining in the endocardial walls of normal and RA-treated embryos mounted on the same slide. There was no significant difference in antibody reactivity between normal and RA-treated embryos ($P>0.05$, *t-test*).

At 10½ days in normal embryos (Fig. 2F), extracellular TGF β1 was still widely expressed, most abundantly in the endocardium and reticulum of the heart, in surface ectoderm, frontonasal mesenchyme and notochord (Fig. 2F); compared with previous stages, staining was less intense in the general cranial and trunk mesenchyme, in the pharyngeal arches and in the mesenchymal region of the lung primordia. No staining was observed in the neural tube. Very localized and intense staining for extracellular TGF β1 was also seen in somites (data not shown) including mesenchymal sclerotome cells but most abundantly in the epithelial dermamyotome of caudal somites. In RA-treated embryos (Fig. 2G), levels of extracellular TGF β1 were higher than in normal embryos, most notably in cranial and trunk mesenchyme and the neuroepithelium. An interesting distribution of TGF β1 was observed in the developing eye of both normal (Fig. 2H) and RA-treated embryos: the apposed basement membranes of the lens pit and presumptive neural retina were strongly stained for extracellullar TGF β1. This basement membrane staining extended to the nearby non-lens surface ectoderm, (presumptive cornea) and also to the peripheral part of the pigmented retina.

Therefore, extracellular TGF β1 was closely associated with mesenchymal cells and ECM-rich tissues, with staining in epithelial tissues present in some sites undergoing epithelial morphogenesis, eg. the open neural folds and in the basement membranes of the developing eye. The effects of excess RA on extracellular TGF β1 were less generalized than those on intracellular TGF β1; there were site-specific and stage-specific decreases as well as increases of expression in RA-exposed embryos. Loss of staining from neural crest-derived but not from primary mesenchyme in 9 day embryos is a particularly interesting example of the specificity of RA-related effects.

Anti-TGF β2 LC(50-75-2) detects mature TGF β2. TGF β2 was the most widely expressed TGF β isoform in mouse embryos of the stages examined here, and the effects of retinoid treatment on its distribution were the most pronounced. Staining patterns in embryos aged between 9½ and 10½ days are illustrated in Fig. 3.

At 8½ and 9 days (not illustrated), normal TGF β2

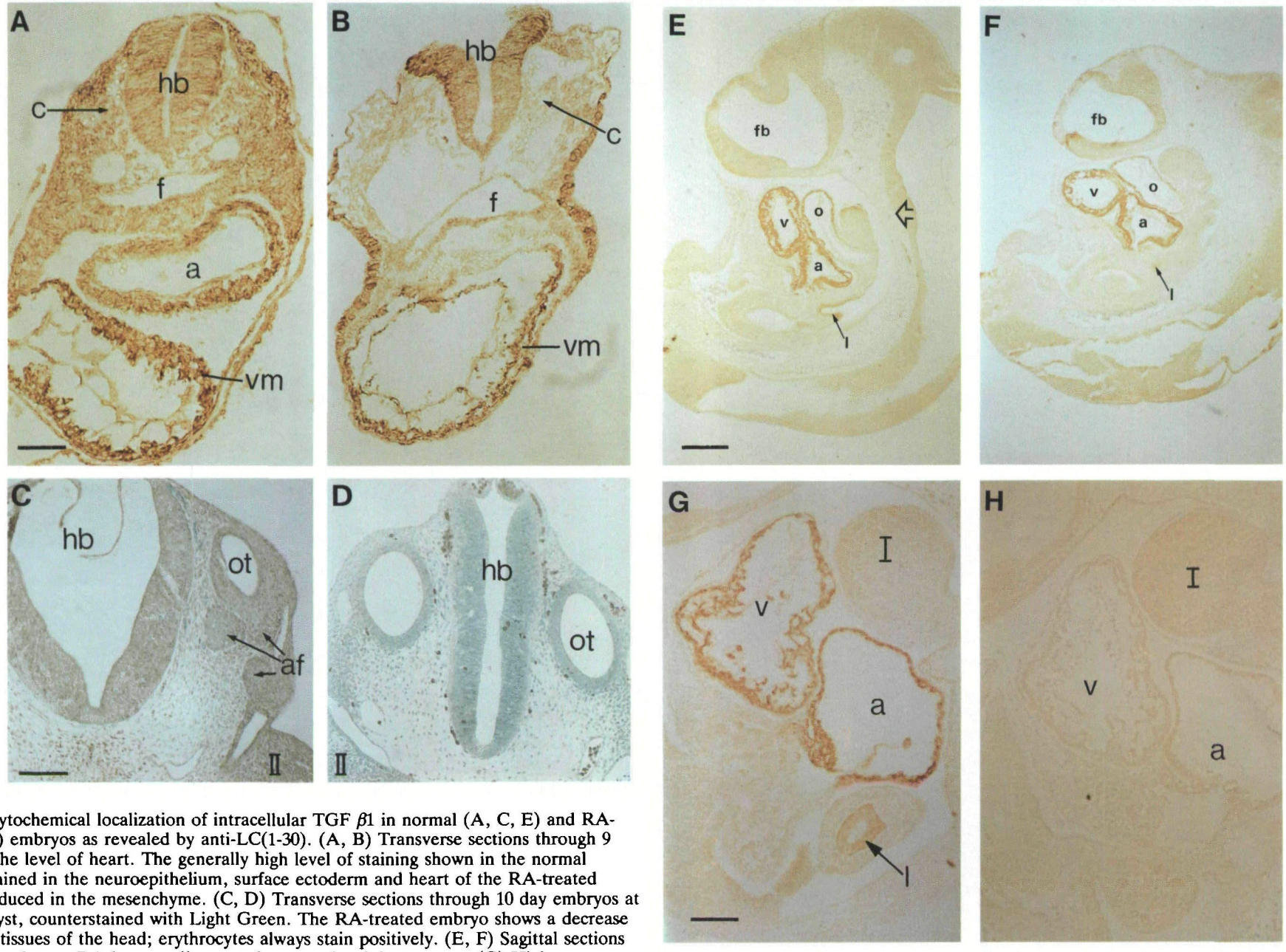
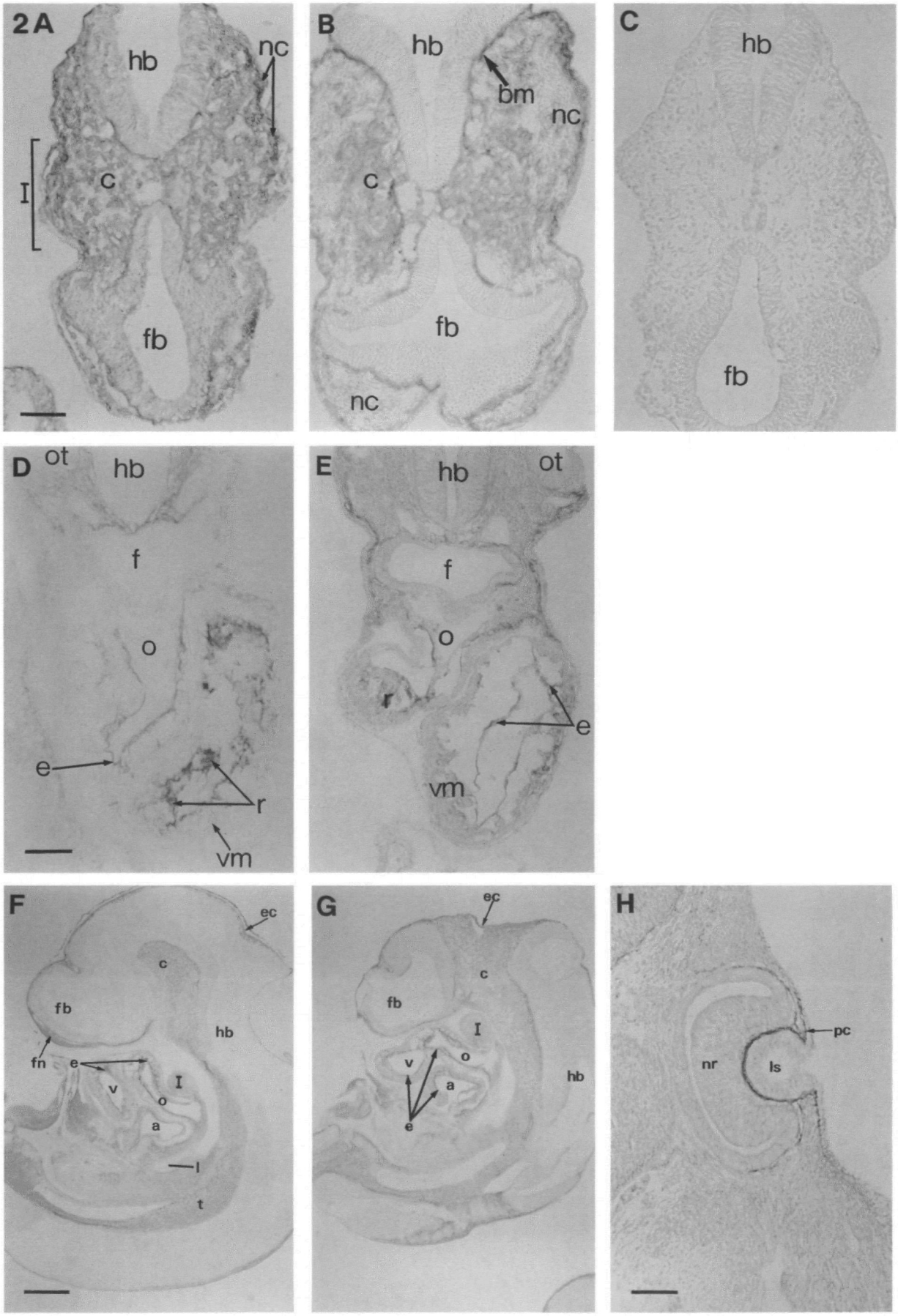


Fig. 1. Immunocytochemical localization of intracellular TGF β1 in normal (A, C, E) and RA-treated (B, D, F) embryos as revealed by anti-LC(1-30). (A, B) Transverse sections through 9 day embryos at the level of heart. The generally high level of staining shown in the normal embryo is maintained in the neuroepithelium, surface ectoderm and heart of the RA-treated embryo but is reduced in the mesenchyme. (C, D) Transverse sections through 10 day embryos at the level of otocyst, counterstained with Light Green. The RA-treated embryo shows a decrease in staining in all tissues of the head; erythrocytes always stain positively. (E, F) Sagittal sections through 10½ day embryos. RA has no effects on the normal staining pattern. (G) High power view of sagittal section from 10½ day embryo showing intracellular TGF β1 staining in the heart and lung. (H) Negative control; adjacent section to (G) stained with normal rabbit serum IgG showing background level of antibody reactivity. a, atrium; c, cranial mesenchyme; f, foregut; af, acoustico-facial ganglion complex; ot, otocyst; l, lung bud; v, ventricle; vm, ventricular myocardium; o, cardiac outflow tract; fb, forebrain; hb, hindbrain; I, first pharyngeal arch; II, second pharyngeal arch. Open arrow in E indicates limit of cranial mesenchymal staining. Scale bar represents 50 μm (A, B); 100 μm (C, D, G, H) or 250 μm (E,F).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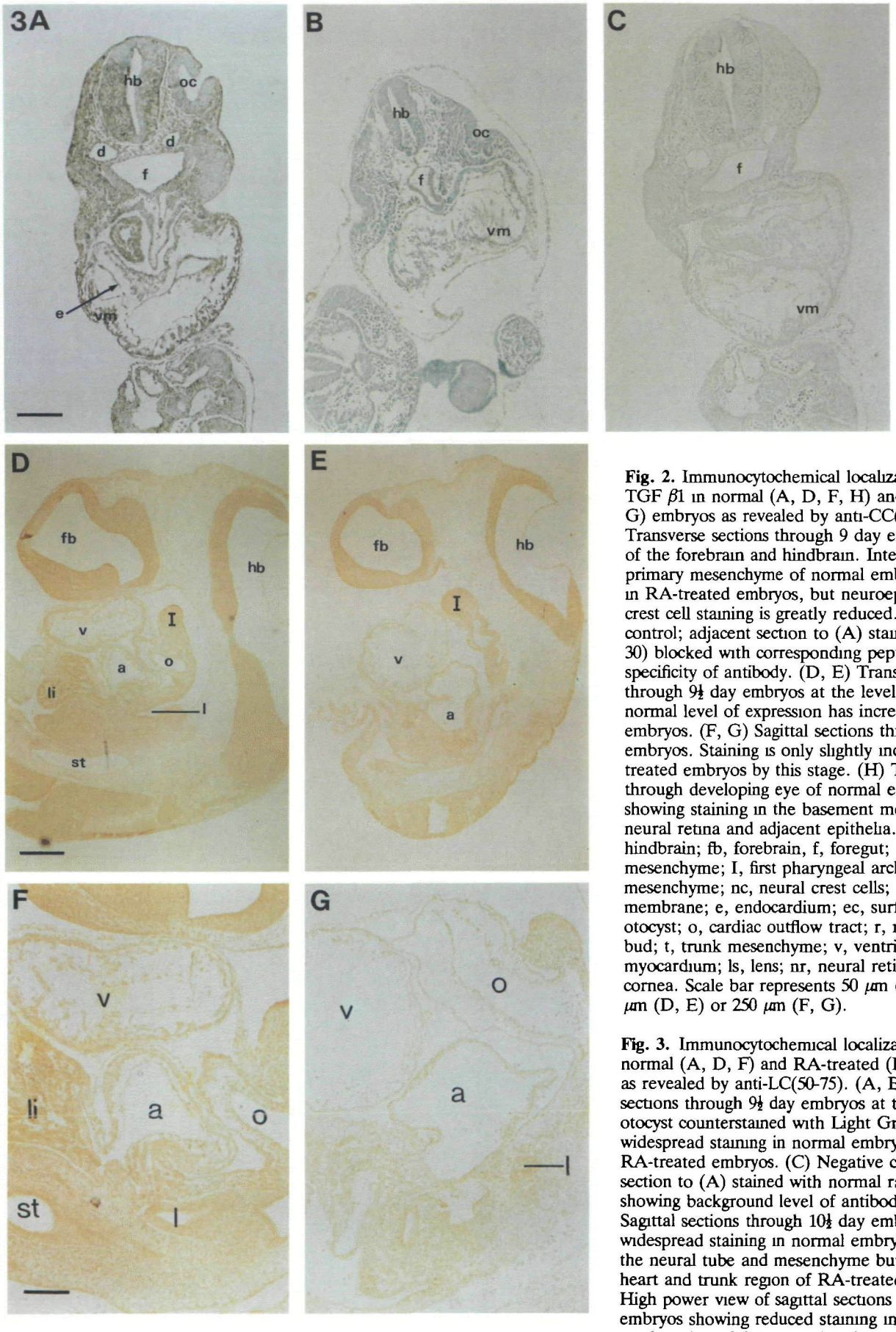

Fig. 2. Immunocytochemical localization of extracellular TGF β1 in normal (A, D, F, H) and RA-treated (B, E, G) embryos as revealed by anti-CC(1-30). (A, B) Transverse sections through 9 day embryos at the level of the forebrain and hindbrain. Intense staining in the primary mesenchyme of normal embryos is maintained in RA-treated embryos, but neuroepithelial and neural crest cell staining is greatly reduced. (C) Negative control; adjacent section to (A) stained with anti-CC(1-30) blocked with corresponding peptide showing specificity of antibody. (D, E) Transverse sections through 9½ day embryos at the level of the heart. The normal level of expression has increased in RA-treated embryos. (F, G) Sagittal sections through 10½ day embryos. Staining is only slightly increased in RA-treated embryos by this stage. (H) Transverse section through developing eye of normal embryo at 10½ days showing staining in the basement membranes of lens, neural retina and adjacent epithelia. a, atrium; hb, hindbrain; fb, forebrain, f, foregut; fn, frontonasal mesenchyme; I, first pharyngeal arch; c, primary mesenchyme; nc, neural crest cells; bm, basement membrane; e, endocardium; ec, surface ectoderm; ot, otocyst; o, cardiac outflow tract; r, reticulum; l, lung bud; t, trunk mesenchyme; v, ventricle; vm, ventricular myocardium; ls, lens; nr, neural retina; pc, presumptive cornea. Scale bar represents 50 μm (A, B, C, H); 100 μm (D, E) or 250 μm (F, G).

Fig. 3. Immunocytochemical localization of TGF β2 in normal (A, D, F) and RA-treated (B, E, G) embryos as revealed by anti-LC(50-75). (A, B) Transverse sections through 9½ day embryos at the level of the otocyst counterstained with Light Green. The widespread staining in normal embryos is abolished in RA-treated embryos. (C) Negative control; adjacent section to (A) stained with normal rabbit serum IgG showing background level of antibody reactivity. (D, E) Sagittal sections through 10½ day embryos. The widespread staining in normal embryos is maintained in the neural tube and mesenchyme but is reduced in the heart and trunk region of RA-treated embryos (F, G) High power view of sagittal sections from 10½ day embryos showing reduced staining in the heart and trunk region of RA-treated embryos. a, atrium; o, cardiac outflow tract; oc, otic pit; d, dorsal aorta; f, foregut; v, ventricle; vm, ventricular myocardium; e, endocardium; fb, forebrain; hb, hindbrain; I, first pharyngeal arch; li, liver; st, stomach; l, lung bud. Scale bar represents 100 μm (A, B, C, F, G) or 250 μm (D, E).

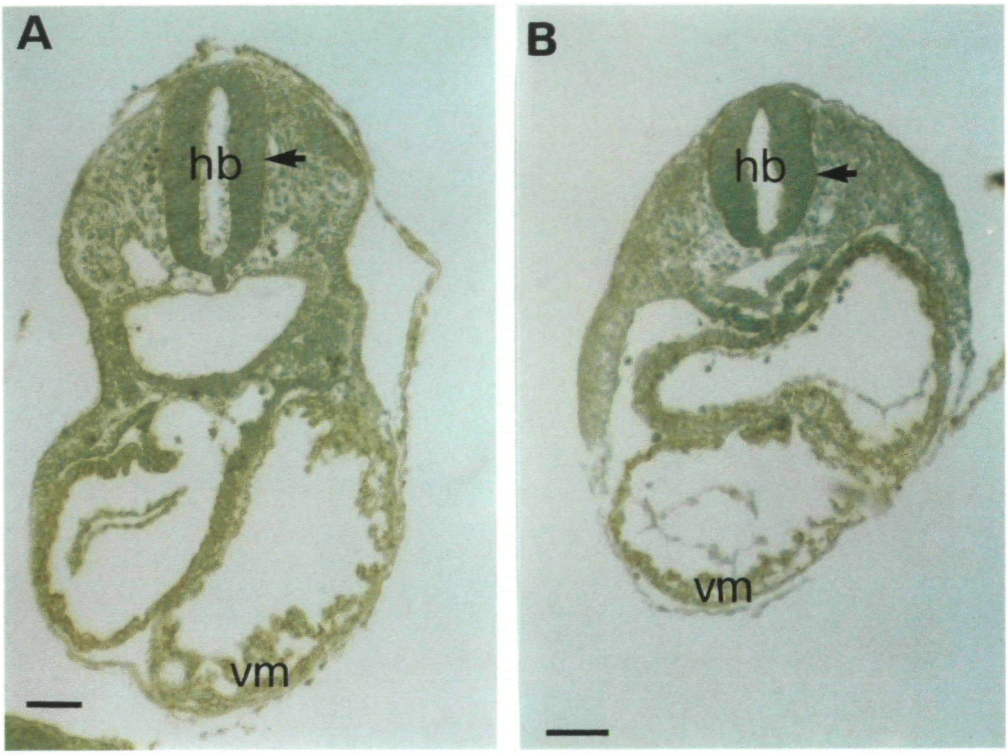

Fig. 4. Immunocytochemical localization of intracellular TGF β3 in normal (A) and RA-treated (B) embryos as revealed by anti-LC-pre(81-100-3). (A, B) Transverse sections through 9 day embryos at the level of the heart, counterstained with Light Green. Normal staining in the myocardium and on the basal surface of the hindbrain neural tube (indicated by arrows) is unchanged by RA excess. vm, ventricular myocardium; hb, hindbrain. Scale bar represents 50 μm.

staining was widespread in head and trunk mesenchyme and in all regions of the heart. Little staining was seen in the neural epithelium. RA-treated embryos showed a uniform reduction in the level of protein expression in all structures.

At 9½ days in normal embryos (Fig. 3A), both mesenchymal and epithelial tissues were stained, but epithelial structures were not uniformly stained. The basal plate (ventral half) of the neural tube was positive for TGF β2 but the alar plate (dorsal half) was only partially stained; the otic and gut epithelia were also negative at this stage. Endothelial cells of the dorsal aortae were positively stained (Fig. 3A) and differential staining was present in the mesoderm layers which are most distinct in the caudal region of the embryo (not illustrated): the paraxial (somitic) mesoderm, intermediate mesoderm, and splanchnic component of the lateral plate mesoderm were all positive for TGF β2, whereas the somatic component of the lateral plate mesoderm was only partially positive. All regions of the heart were positive for TGF β2. In RA-treated embryos (Fig. 3B), there was a very marked decrease in mesenchyme-associated staining, and an even more striking decrease in epithelial staining. Optical density measurements of antibody staining in all tissues of RA-treated embryos were compared with measurements made in corresponding tissues of normal embryos mounted on the same slide. There was a highly significant difference between normal and RA-treated TGF β2 expression patterns ($P<0.0001$, *t-test*).

In normal embryos at 10½ days, TGF β2 continued to be widely expressed (Figs. 3D and 3F), most notably in all three somitic derivatives (sclerotome, myotome and dermatome), as well as in the pharyngeal arch mesenchyme and in the mesenchyme of the midgut, stomach, lung primordia and liver. There was also clear staining in the neural tube. However, undifferentiated head and trunk mesenchyme and the heart were not intensely stained. Following RA treatment (Figs. 3E and 3G), reduced levels of TGF β2 peptide were particularly evident in the developing organs of the trunk region, but staining in the neuroepithelium was unaltered at this stage.

Generally, TGF β2 was extensively expressed but unlike TGF β1, no single tissue-type showed a particularly high level of staining. Its response to RA treatment was more generalized than that of TGF β1.

Anti-TGF β3 LC-pre(81-100-3) detects intracellular TGF β3 precursor. At 9 days, little peptide was associated with mesenchymal areas; the most prominently stained structures include the myocardium of the heart, apical and basal surfaces of the neural tube, and the apical surface of the otic pit. These expression patterns were unaltered by excess RA treatment (compare Fig. 4A and B).

Anti-TGF β3 LC(50-60-3) detects mature forms of intracellular TGF β3. General staining was associated with mesenchyme, epithelial structures and the pericardium of the heart at 10 days. RA had no effects on the distribution of this peptide (data not shown).

Discussion

In this study we describe in detail the normal protein localizations of TGF β1, TGF β2 and TGF β3 in early postimplantation stage mouse embryos undergoing neurulation, heart formation, somitogenesis and the organizational phase of craniofacial development. Embryos exposed to exogenous RA at the start of neurulation showed tissue-specific alterations in TGF β protein levels. These observations suggest that interactions between endogenous RA and TGF βs are involved in mechanisms of morphogenesis as well as in cell proliferation and differentiation.

Normal distribution of TGF β1, TGF β2 and TGF β3

Anti-LC(1-30) detecting intracellular TGF β1, recognized both epithelial and mesenchymal TGF β1; anti-CC(1-30), detecting extracellular TGF β1, recognized TGF β1 associated with the extracellular matrix as previously reported for later stages of development (Ellingsworth et al., 1986; Flanders et al., 1988; Flanders et al., 1989). The immunocytochemical staining patterns we observed showed that the protein distributions of these two TGF β1 forms were at first very similar, except in the heart, but they gradually became mutually exclusive as development advanced. In the heart, we consistently observed clear association of extracellular TGF β1 with the ECM-rich cardiac jelly and in the endocardium. The cardiac jelly is initially acellular, and is composed predominantly of fibronectin, heparan sulphate proteoglycan, chondroitin sulphate proteoglycan, but also tenascin, laminin and collagen type IV (Markwald et al., 1978; Tuckett and Morriss-Kay, 1986; Armstrong and Armstrong, 1990). Stimulation of proteoglycan synthesis by TGF β1 has been observed in vitro (Choy et al., 1990). Cardiac valves and septae develop after endothelial cells transform into mesenchymal cells and migrate into the cardiac jelly to populate it (Manasek, 1976); the epithelomesenchymal transformation is thought to be initiated by a signal emanating from ventricular myocardium (Potts and Runyan, 1989). Such processes of cell transformation, migration and proliferation may be mediated by TGF β1. Akhurst et al. (1990) noted restricted TGF β1 RNA expression in endothelial cells contributing to the endocardial cushion tissue. This is interesting since in the present study the extracellular form of TGF β1 peptide was localized to the endocardium.

The possible function of intracellular TGF β1 seen in the myocardial wall of the heart is less clear. The staining was always intense and was present at all stages examined; the intensity increased as heart development advanced. Since the anti-LC(1-30) is thought to indicate sites of TGF β1 synthesis, it is worth noting that Akhurst et al. (1990) did not detect any mRNA within the myocardium. Thompson et al. (1989) also detected uniform staining of intracellular TGF β1 in cardiac myocytes in neonatal (and adult) mice and most recently, Heine et al. (1991) have localized this staining specifically to myocardial mitochondria in adult mouse

heart. The importance of TGF β1 for normal cardiac function is indicated by the observation that it responds rapidly to cardiac ischaemia; intracellular TGF β1 staining is lost in cardiac myocytes within one hour of ligation of a coronary artery, after which there is a marked increase in staining of myocytes at the border of the infarcted area (Thompson et al., 1988). Also, exogenous TGF β1 can protect the myocardium from ischaemic injury (Lefer et al., 1990).

We have observed that migrating cranial neural crest cells are positive for extracellular TGF β1. Craniofacial development relies on correct and extensive neural crest cell migration from midbrain and hindbrain neuroepithelium to specific destinations in the embryonic head; in addition to neural derivatives, they differentiate to form the connective and skeletal tissues of the face (Noden, 1983, 1988; Tan and Morriss-Kay, 1985, 1986). Previous reports involving embryos older than those studied here indicate that many cranial neural crest cell derivatives, including developing palate, facial mesenchyme and teeth express TGF β mRNAs (Schmid et al., 1991; Pelton et al., 1990a; Fitzpatrick et al., 1990) and TGF β proteins (Heine et al., 1987; Pelton et al., 1991). These studies and our own results suggest that TGF β1 is important for normal cranial neural crest cell function at all stages of facial development. TGF β1 may control cell migration by modulating the ability of cells to recognise and adhere to components of the ECM (Heino et al., 1989), or through the upregulation of tenascin gene transcription (Pearson et al., 1988), since tenascin promotes the mobility of neural crest cells (Halfter et al., 1989).

Extracellular TGF β1 appears to have a role in early eye development. The distribution of the CC(1-30) antibody staining observed here suggests that it may be involved in the cellular proliferation of the neural retina, in the formation of the primary vitreous body, in invagination of the lens and/or formation of the cornea. Recently Wood et al. (1991) localized collagen II to the basal side of the neural retina and lens in a pattern identical to that observed here for the TGF β1 CC antibody.

One important difference between the TGF β1 and TGF β2 staining patterns in normal embryos at 10½ days was the high level of TGF β2 staining in the neuroepithelium of the neural tube and brain whereas TGF β1 was negative. Some TGF β3 staining was also observed. Flanders et al. (1991) have suggested that both TGF β2 and TGF β3 may have unique roles in nervous system development, although Millan et al. (1991) and Schmid et al. (1991) did not observe detectable levels of TGF β2 and TGF β3 mRNA within the neural tube at this stage.

Comparisons of mRNA localization patterns of TGF βs (Schmid et al., 1991; Millan et al., 1991) with our protein localizations in early mouse embryos show that some tissues co-express mRNA and peptide, suggesting autocrine mechanisms of TGF β action, but in other tissues mRNA and peptide are expressed in adjacent cell types suggesting paracrine methods of action. Examples in which mRNA and peptide are coexpressed include TGF β2 mRNA (Millan et al., 1991) and peptide in the cardiac myocardium at 9½ days. TGF β2 peptide was also localized to frontonasal mesenchyme at 10½ days and transcripts are similarly localized at the same stage (Millan et al., 1991). In general, TGF β2 patterns of peptide localizations correspond better with mRNA patterns of expression compared with those of TGF β1. This may be because unlike TGF β1 and TGF β3, TGF β2 has no cell-adhesion receptor recognition sequence (RGD residues); this sequence may be involved in tissue targeting of the growth factor (Roberts and Sporn, 1990). Hence, intracellular localization of both transcripts and peptide suggests an autocrine role for TGF β2 in cell regulation as suggested by Millan et al. (1991) for epithelially derived TGF β2.

TGF β1 may play a role in paracrine regulation of morphogenetic interactions within individual tissues (Lehnert and Akhurst, 1988). In the embryo stages examined here, most tissues consist of either epithelia or mesenchyme and are not highly differentiated, making it difficult to investigate the functions of TGF β1 as a paracrine regulator of morphogenesis. However, at 10½ days intracellular TGF β1 was localized to the epithelial component of the lung bud only and extracellular TGF β1 was observed in the surrounding mesenchyme only. This suggests various roles for epithelial TGF β1 (which may have been secreted from the epithelium into the surrounding mesenchyme), during early stages of lung morphogenesis, as well as in branching, cleft formation and matrix deposition at later stages, which involve careful regulation of cell growth and differentiation (Heine et al., 1990; Pelton and Moses, 1990). In some tissues, peptide is present where there is apparently no expression of mRNA. A prominent example is the strong and uniform expression of intracellular TGF β1 in the cardiac myocardium. No detectable levels of transcript are correspondingly expressed (Schmid et al., 1991; Millan et al., 1991). The reasons for this are unclear and we can only speculate that perhaps a novel TGF β1 message (that is not detected by existing probes) is present in the myocytes; this possibility is supported by the observation that TGF β1 mRNA in the porcine heart is alternatively spliced (Kondaiah et al., 1988).

Distribution of TGF β1, TGF β2 and TGF β3 in RA-treated embryos

Surprisingly, retinoic acid treatment resulted in a substantial decrease of intracellular TGF β1 and TGF β2 protein production at most stages examined - indeed the effects continued up to two days after maternal administration. In the case of extracellular TGF β1, raised levels of peptide at 9½ days and to a lesser extent, 10½ days, may indicate that excess RA can induce the cellular secretion of TGF β1. In contrast to TGF β1 and TGF β2, excess RA had no significant effects on the expression of TGF β3 further indicating the different mechanisms of regulation of different TGF β isoforms.

The loss of extracellular TGF β1 staining in neuroepithelium and in migrating cranial neural crest cells after RA treatment may be biologically significant with respect to retinoid-induced craniofacial defects, which

involve abnormal neural crest cell migration and an altered pattern of gene expression within the neuroepithelium (Webster et al., 1986; Morriss-Kay et al., 1991).

Our observations indicating tissue-dependent down-regulation of TGF β1 and total down-regulation of TGF β2 in all embryonic tissues are in contrast to previous observations from one of our laboratories in which it was clearly demonstrated that retinoic acid is a powerful inducer of TGF β2 but not of TGF β1. Increase in TGF β2 RNA was observed at the posttranscriptional level in cultured keratinocytes and in vivo after application of retinoic acid to mouse epidermis (Glick et al., 1989). The differences between these results on a single tissue and those of whole, early embryos illustrates the complex and varied functional interactions of TGF β. These include other regulatory molecules interacting with TGF β, the effects of normal levels of retinoic acid on these factors, and the interaction between the TGF β isoforms themselves. The prolonged nature of the effect of RA on TGF β expression is interesting since pharmacokinetic studies have shown that orally administered RA reaches a peak in 1 hour and falls to near normal levels within 8 hours (Creech-Kraft et al., 1989). Therefore, the effects of RA on TGF β expression are maximal well after RA levels have peaked in the embryo. Careful comparisons between our results and the spatiotemporal distribution patterns of the different RARs and retinoid binding proteins (Dollé et al., 1990; Ruberte et al., 1991) show no obvious correlations.

Retinoic acid and TGF β appear to be closely linked key regulators of growth and differentiation. Assigning specific functions to the different TGF β isoforms in the embryo has been difficult even though they are differentially expressed. In the heart we may speculate that TGF β1 has a unique role in myocardial development; this role is not affected by retinoic acid excess, but is affected by retinoid deficiency (our unpublished observations). Our observations suggest that interactions between TGF β and retinoic acid during normal and abnormal development are complex, and differ from those observed at later stages in more differentiated tissues.

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