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Patterns of tenascin expression during tail regeneration of the amphibian urodele *Pleurodeles waltl*

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Summary

We have determined the patterns of expression of tenascin, an extracellular matrix (ECM) glycoprotein, by indirect immunohistofluorescence and immunoblots during the post-traumatic regeneration of the tail distal part of the amphibian urodele *Pleurodeles waltl*. In normal tails of adult *Pleurodeles*, tenascin expression is mostly restricted to few connective tissues including the periosteum, the ligaments of vertebral articulation, myotendinous structures, the perimysium and the pia mater; the extracellular matrix is virtually negative. In certain areas of the adult skin the molecule is expressed around cells of the inner epidermal layer, apparently associated with the cell surface. In the first 4–6 days following tail amputation, tenascin expression increases in the stump region in areas surrounding dedifferentiating tendons; the early blastemic epithelium does not express the molecule. The local increase of tenascin in areas where cells dedifferentiate and start to migrate, precedes the increase in fibronectin that occurs later in the mesenchyme of the blastema. From the 8th day of regeneration, there is a sharp increase of the level of expression of the molecule in the extracellular matrix of the loose mesenchyme underlying the epithelium of the blastema which remains negative. The maximal expression in the matrix is reached in 4- to 6-week-old regenerates and then gradually decreased. High levels of tenascin are present in sites of muscle condensation as fibrils oriented parallel to the direction of alignment of myogenic cells and in sites of chondrogenesis particularly in regions of precartilage formation. After the second week of regeneration, tenascin is strongly expressed in the basal lamina of the regenerated skin and, after the fourth week, also at the level of epidermal–dermal junctional areas. Like in normal tail ends, in regenerates older than 8–9 weeks, tenascin expression is nearly restricted to the muscle connective tissue and myotendinous structures.

These results are discussed in view of the possible multiple morphogenetic roles of tenascin in tissue regeneration and repair.

Key words: tenascin, urodele tail regeneration, wound healing, extracellular matrix.

Introduction

Adult amphibian urodeles have the remarkable capacity to regenerate missing parts of their body after limb or tail amputation (Holtzer, 1956; Goss, 1969; Thorton, 1968; for reviews see Singer, 1952, 1974; Wallace, 1981). This epimorphic mode regeneration (Carlson, 1979) begins with a widespread histolysis of the stump region close to the amputation surface followed by wound healing and the formation of a blastema composed of undifferentiated cells thought to originate, at least in part, from differentiated stump tissues. Subsequently the blastema cells proliferate, aggregate and redifferentiate into a new appendage composed of its muscular, neural, skeletal, dermal and glandular elements (Singer, 1952; Chalkey, 1954; Hay, 1962, 1979; Iten and Bryant, 1973; Carlson, 1979; Khrestchatisky *et al.* 1988). It has been reported that amphibian limb regeneration involves profound alterations in the molecular composition of the ECM; in particular, collagens, glycosaminoglycans, fibronectin and laminin are known to be modulated during this process (Revel and Hay, 1963; Schmidt, 1970; Toole and Gross, 1971; Linsenmeyer and Smith, 1976; Mailman and Dresden, 1976; Gulati *et al.* 1983).

Recently another ECM component has been implicated in the control of the morphogenetic events that take place during embryonic development and regeneration; originally called myotendinous antigen (Chiquet and Fambrough, 1984*a*,*b*), this molecule was then

renamed tenascin (Chiquet-Ehrismann *et al.* 1986, 1988) and was shown to be very similar and possibly identical to other molecules simultaneously discovered by other laboratories: cytotactin (Grumet *et al.* 1985; Crossin *et al.* 1986; Hoffman *et al.* 1988), glioma mesenchymal extracellular matrix protein (Bourdon *et al.* 1985) and hexabrachion (Erickson and Iglesias, 1984; Erickson and Taylor, 1987); it appears furthermore that tenascin is the predominant component recognized by the J1 antibody (Kruse *et al.* 1985). As we made use of antibodies directed against tenascin, we will, for simplicity, use only this name referring to the molecule.

Electron microscopic observation of tenascin and its analogs by rotary shadowing have shown that the molecule has a six-armed structure with a central core (Erickson and Iglesias, 1984; Vaughan *et al.* 1987; Hoffman *et al.* 1988; Chiquet-Ehrismann *et al.* 1988). The complete sequence of tenascin has been determined; it contains 13.5 EGF-like sequence repeats and 11 type III fibronectin homologies, which can undergo alternative splicings and fibrinogen-like sequences (Jones *et al.* 1988, 1989; Pearson *et al.* 1988). The structural complexity of this molecule and its reported capacity to modulate differently the adhesion of different cell types (Grumet *et al.* 1985; Mackie *et al.* 1987*a*; Tan *et al.* 1987; Friedlander *et al.* 1988; Lotz *et al.* 1989) and to inhibit the attachment of cells to FN substrates (Mackie *et al.* 1988*a*; Chiquet-Ehrisman *et al.* 1988; Lotz *et al.* 1989) have led different authors to suggest that tenascin modulates cell–substrate adhesion by several different mechanisms. Recently, the use of fusion proteins containing different regions of the molecule has shown that both cell binding sites and sequences with antiadhesive effects coexist in tenascin (Spring *et al.* 1989), suggesting a possible interpretation of its versatile behavior. It has been also proposed that tenascin might act as a mediator of global cell-surface modulation capable of affecting differently the behavior of different cell types through a number of cytoskeletal and membrane responses elicited by multivalent interactions with its receptors (Friedlander *et al.* 1988).

The strikingly limited distribution of tenascin during embryonic development at specific sites of the matrix (Crossin *et al.* 1986; Riou *et al.* 1988; Tan *et al.* 1987) and the remarkable modulation of its expression where late morphogenetic rearrangements take place has suggested that this molecule is important for early embryonic development as well as for tissue differentiation. In particular, tenascin and its analogs have been implicated in the development of muscle and myotendinous structures (Chiquet and Fambrough, 1984*a*,*b*), cartilage and bone (Makie *et al.* 1987*b*), central nervous system (Grumet *et al.* 1985; Kruse *et al.* 1985; Chuong *et al.* 1987; Chiquet, 1989; Sanes, 1989), intestine (Aufderheide and Ekblom, 1988), kidney (Aufderheide *et al.* 1987), mammary glands (Inaguma *et al.* 1988) and tooth (Thesleff *et al.* 1987). In two situations, it has been shown that the expression of tenascin can be modulated in adults during regenerative processes: A very strong increase of tenascin expression occurs during the process of wound healing in the dermis and matrix underlying the migrating epithelium (Makie *et al.* 1988*a*) and strong modulations in the expression of the molecule are observed during the process of repair that follows peripheral nerve injury (Daniloff *et al.* 1989). Not much is known about the molecular factors implicated in modulating the expression of tenascin; the only substance known to affect the expression of the molecule is transforming growth factor β (TGF-β), which can induce a four-fold increase in the synthesis of tenascin by chick embryo fibroblasts (Pearson *et al.* 1988).

We report here the patterns of expression of tenascin at different stages of tail regeneration of *Pleurodeles waltl*. We have chosen to study this system as an example of morphogenesis occurring in a developmental context totally different from that present in the normal embryo, which leads, however, to the formation of a structure very similar to that obtained during normal development. If indeed tenascin has an important role in the control of the cellular events at the basis of morphogenesis, we expected its expression to be strongly modulated during regeneration. This study therefore provides the information needed to address the question of the molecular signals involved in regulating the expression of this morphogenetic molecule during an epimorphic mode of regeneration.

Materials and methods

Animals, surgical procedures

All animals used in this study were male and female adult salamanders of the species *Pleurodeles waltl* obtained from the CNRS's Amphibian Farm, centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France. Animals were reared in groups of 10–12 and maintained in circulating tap water thermostatted at 18–20 °C; the water was completely renewed twice a week. The animals were fed twice a week with beef heart or liver. Before surgery animals were anesthetized by immersion in a 0.1 % aqueous solution of tricaine methane sulfonate (Sigma) for 10–15 min; the third distal part of the tail was then amputated with a razor blade. After appropriate periods of regeneration the blastema were harvested by reamputation.

Antibodies

Polyclonal antibodies anti-chicken-fibroblast-tenascin were kindly provided by Dr Mattias Chiquet; their production and characterization have been previously described (Chiquet and Fambrough, 1984*a*,*b*). The specificity of the reactivity of these antibodies on tissues of *Pleurodeles waltl* has been recently determined by Western blots and indirect immunofluorescence (Riou *et al.* 1988). When testing the reactivity of the antibodies in immunoblots of adult organs of *Pleurodeles waltl*, we could confirm the results of Riou *et al.* (1988) finding no expression of the molecule in the liver and high levels of expression in the brain; furthermore, as reported by Chiquet and Fambrough (1984*a*), we find tenascin reactivity in adult intestine.

The preparation and characterization of anti-human-fibronectin and anti-mouse-laminin antibodies have been previously described (Rovasio *et al.* 1983; Duband and Thiery, 1987).

Immunohistochemistry

For preparation of cryostat sections, tissues were fixed overnight in 0.01 M sodium periodate, 0.75 M lysine, 2% paraformaldehyde, 37.5 mM sodium phosphate, pH 6.2 (McLean and Nakane, 1974). Samples were then rinsed in PBS and cryoprotected in 18% sucrose in PBS for several hours before being embedded in OCT compound (Tissue Tek, Miles Laboratories Inc., Naperville, IL); they were then frozen rapidly in liquid nitrogen and stored at −20°C. Serial frozen sections of 12 μm were cut in a cryostat at −22°C and collected on polylysine-coated glass slides which were then stored at −20°C until immunofluorescent staining was performed.

Sections were blocked with 5% foetal calf serum in PBS (blocking solution) for about one hour and then treated for at least three hours at room temperature with a 1:100 dilution of rabbit polyclonal antibodies against chicken fibroblast tenascin in blocking solution. After three 5 min rinses in PBS, the slides were treated for two hours at room temperature with rhodamine-conjugated sheep anti-rabbit IgG affinity-purified polyclonal antibodies (10 $\mu g\,ml^{-1}$ in blocking solution; Nordic Immunology, Tilburg, The Netherlands). After washing, the sections were mounted in Gel/Mount aqueous mounting medium (Biomeda,CA) and observed using a Leitz epifluorescence microscope. Negative controls were performed by substituting the primary antibody with a rabbit polyclonal antibody against Keyhole Limpet Hemocyanin at similar dilutions (kindly provided by Dr François Radvanyi, Ecole Normale Supérieure, Paris).

Western blots

Immediately after dissection, samples were homogenized in 7 volumes of 20 mM Tris–HCl (Sigma), 10 mM EDTA, 0.2% Triton X100, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 $\mu g\,ml^{-1}$ antipain and pepstatin, 15 $\mu g\,ml^{-1}$ benzamidine pH 8.0 (antipain, pepstatin and benzamidine were first solubilized in DMSO). The homogenization was carried out on ice in a Dounce homogenizer with a glass pestle for 4- to 15-day-old regenerates, older regenerates and normal tail tips were crushed in an ultra-turrax T25 tissue grinder (Jenke and Kunkel, IKA labortechnik, Staufen) for 15–20 s before being homogenized.

The homogenates were then briefly sonicated and centrifuged at 10000 *g* for 10 min at 4°C. Proteins present in the aqueous supernatant were resolved by electrophoresis on a 5% sodium dodecyl sulphate polyacrylamide gel (Laemmli, 1970). Proteins were then transferred onto nitrocellulose membranes (Burnette, 1981). The nitrocellulose sheets were then sequentially reacted with the primary anti-tenascin antibody, horse radish peroxidase-coupled second antibody and diaminobenzidine for color development of peroxidase activity.

Results

Tenascin expression in normal adult tail

Tenascin expression in adult *Pleurodeles* tissues was strikingly restricted to few connective tissue types so that the general level of expression of the molecule was very low. Strong tenascin immunoreactivity was present in ligaments of the vertebral articulations and in myotendinous structures involved in anchoring skeletal muscles to bony vertebrae (Fig. 1A,D), in the periosteum (Fig. 1A,B) and in certain areas of the perimysial tissue surrounding muscle fiber bundles; differentiated bones, cartilages and muscles were negative. The adult spinal cord was not labeled by anti-tenascin antibodies that strongly stained only the pia mater (Fig. 1C). The extracellular matrix of the loose mesenchyme of the tail was very weakly stained by anti-tenascin antibodies.

Certain regions of the skin displayed a strong tenascin immunoreactivity in the inner epidermal layer; the rest of the epidermis and the dermis were negative (Fig. 2A). Epidermal labeling appeared as a dotted, patchy, staining limited to areas of epidermal cell contact easily visualized in tangential sections of the skin (Fig. 2D). This staining was predominantly present in distal parts of the fin and in regions of the skin surrounding the ducts of exocrine glands, which were negative (Fig. 2C). The basal lamina of most of the adult tail skin was negative; strong lamina staining could, however, be detected in ventral portion of the adult *Pleurodeles* skin.

Fibronectin immunoreactivity was not present in the ECM of the normal *Pleurodeles* tail and was limited to most basement membranes (data not shown).

Induction of tenascin during early stages of regeneration

Within 2–3 days after tail amputation, wound healing was achieved by epidermal cell migration over the amputation surface. By the 4th day, a regeneration blastema began to form by accumulation of undifferentiated mesenchyme-like cells under the apical epidermal cap; these cells have been shown to originate in great part from dedifferentiating stump tissue cells (for review see e.g. Thornton, 1968).

When sections of early regenerates were stained with antibodies against tenascin (Fig. 3C,E), the first response that we could detect was a strong increase of immunoreactivity in the connective tissues of the stump region of 4-day-old regenerates just beneath the apical epidermal cap which was negative. A very strong staining was observed in the connective tissue surrounding the lesioned cartilage and muscles and extended towards the loose mesenchyme that was beginning to form under the apical epithelium. No corresponding increase in fibronectin immunoreactivity was detected in the stump region (Fig. 3A), on the contrary fibronectin staining became weak and patchy.

After 8 days of regeneration, the epithelium of the blastema had considerably thickened and a loose mesenchyme had accumulated under it. A very strong tenascin immunoreactivity was observed at this stage in the matrix of the outer mesenchymal zone which is very poor in cells but very rich in ECM fibrils; the thick epithelium of the blastema was negative (Fig. 3D,F). At this stage a clear fibronectin immunoreactivity was present in the ECM of the blastema mesenchyme (Fig. 3B); it kept increasing in later stages of development to reach a maximum in 4-week-old regenerates and then it decreased.

Differentiation of the regenerate

Two weeks after trauma, the regenerated blastema had considerably thickened and the ependyma of the central

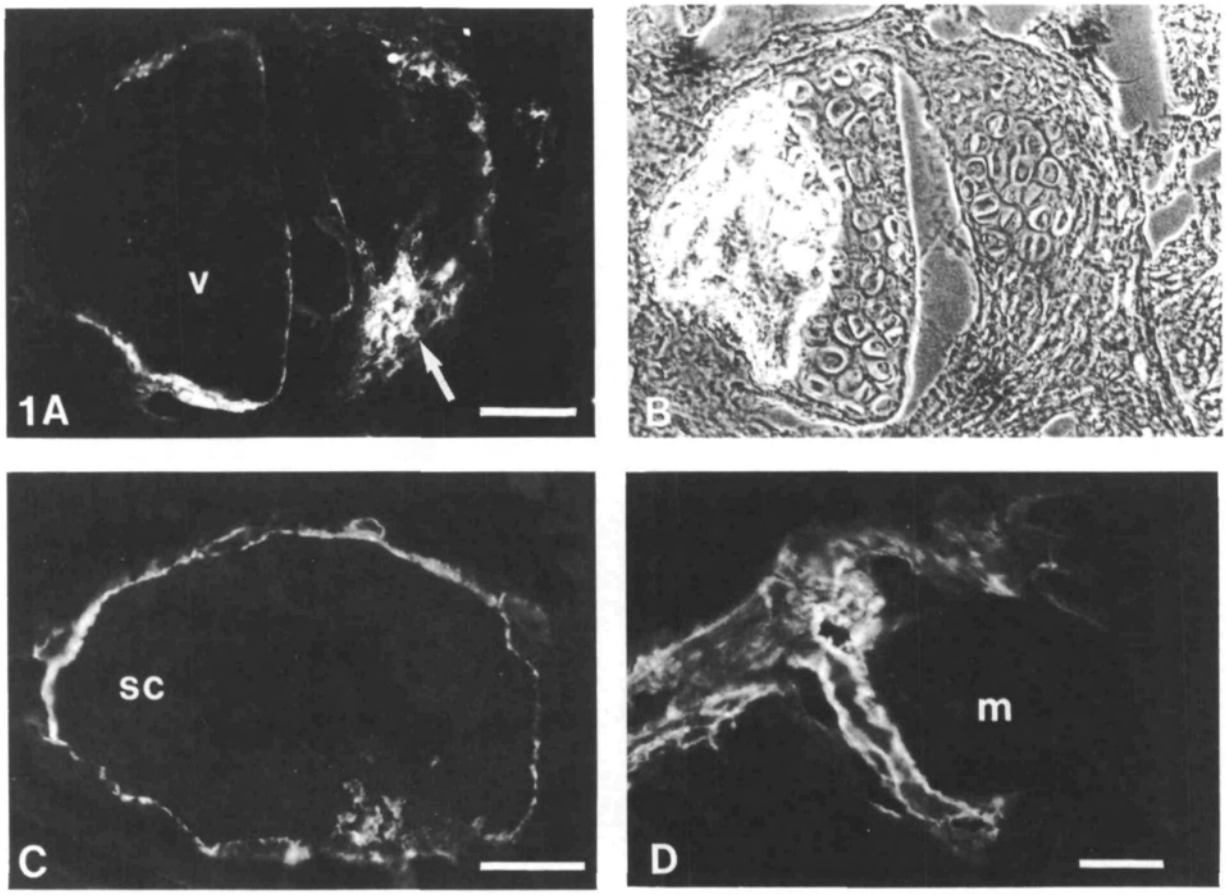


Fig. 1. Distribution of tenascin immunoreactivity in adult newt tail tissues. (A,B) Articulation between two consecutive vertebrae. Tenascin immunoreactivity (A) is present in the periosteum and in the ligaments (arrow); differentiated cartilages and bones do not express the molecule; (B) Phase-contrast image of A. (C) Section through the adult spinal cord. Tenascin immunoreactivity is almost exclusively limited to the pia mater; the central nervous system is negative. (D) Lateral tendon of the tail. Tenascin immunoreactivity is limited to the myotendinous structures and is not present in differentiated muscle. m, muscle; sc, spinal cord; v, vertebra. Bar: 50 μm.

nervous system had began to protrude in the loose mesenchyme underlying the very thick blastemic epithelium; mesenchymal cells ventral to the regenerating spinal cord began to be arranged in a circular fashion giving rise to the presumptive cartilage of the future vertebral body of the regenerate. At this stage, a very intense tenascin immunoreactivity was present in fibrous components of the matrix of the regenerate; the blastema epithelium was still negative but a tenascin-positive basal lamina at the epithelial–mesenchymal junction had developed (Fig. 4A,B). Tenascin expression was locally enhanced in the matrix surrounding the circularly arranged mesenchymal cells in areas of formation of presumptive cartilage; the regenerating nervous system was negative (Fig. 4C,D).

Four weeks after amputation, a well-differentiated tail had begin to reform over the wound region. A large vertebral body of differentiated cartilage was present in the center of the regenerate while neural and hemal vertebral arches began to condense around the regenerated spinal cord and tail blood vessel, respectively. On the lateral aspects of the regenerate, tail muscle had started to develop: a gradient of differentiation could be observed along the regenerate, the more proximal parts being the most differentiated. The cartilaginous matrix of the developing vertebra showed an intense tenascin immunoreactivity which gradually disappeared as proceeded chondrogenesis and ossification (Fig. 5A,C,D). The basal lamina underlying the skin and some regions of the inner epidermal layer were intensely stained particularly in the regenerated fin where some tenascin immunoreactivity could also be detected in the fibrillar matrix (Fig. 5B,E). This pattern of staining resembled that of fibronectin (Fig. 5F), which was, however, more uniformly distributed through the ECM; in the skin, laminin staining (Fig. 5G) was mostly limited to the epithelial basal lamina.

Initiation of myogenesis in newt tail regeneration proceeds proximally to distally and it was apparent either in the blastema proximal regions near the stump 2 weeks after trauma or in the distal part of the regenerate 4 weeks following amputation (Khreschatisky *et al.* 1988). Prominent tenascin immunoreactivity was present in premyogenic zones in longitudinal ECM fibrillar components aligned parallel to the prefusing

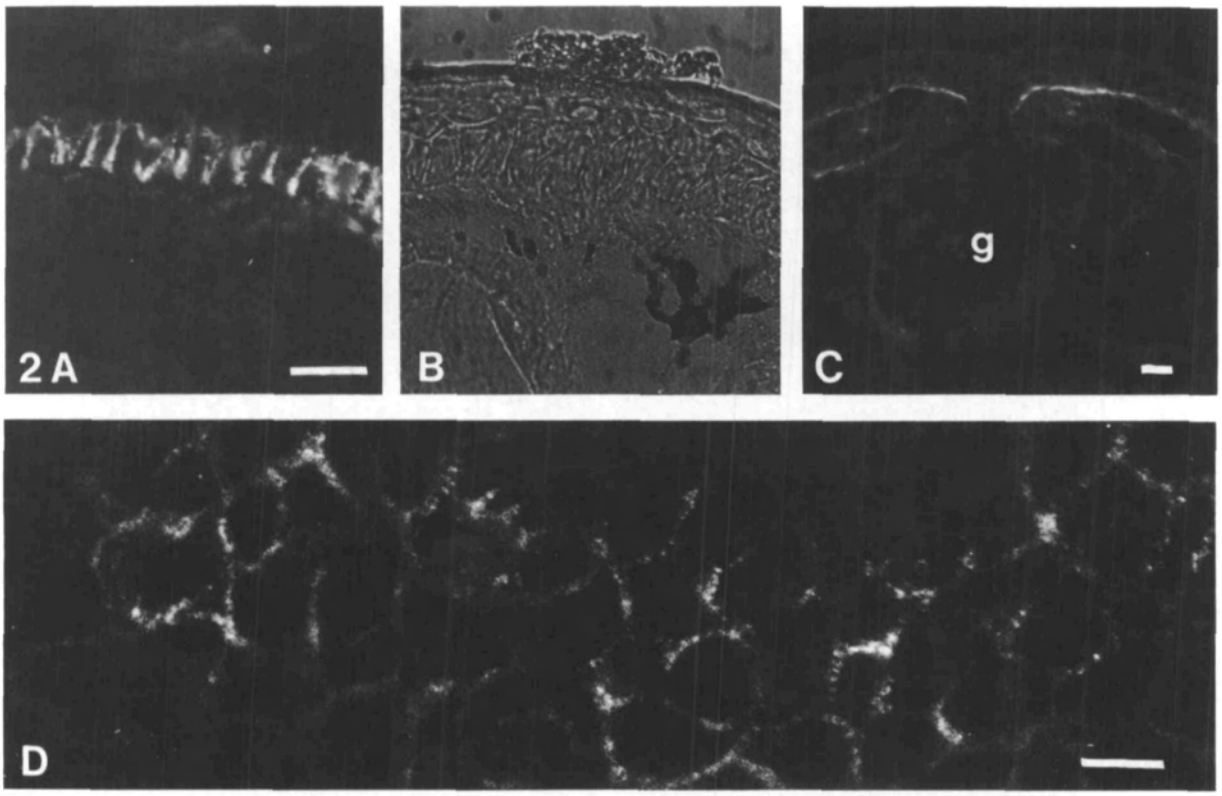


Fig. 2. Distribution of tenascin immunoreactivity in adult newt skin. (A,B) Transverse section through the fin of the tail; tenascin immunoreactivity (A) is seen on the lateral cell surfaces of the inner epidermal layer, the rest of the epidermis and the dermis are negative, (B) phase-contrast image corresponding to A. (C) Transverse section through adult ventral skin, tenascin immunoreactivity is seen in the basal lamina and inner epidermal layer of the skin in region surrounding dermal glands (g). (D) High magnification of a tangential section through adult fin skin. Tenascin staining appears as bright dotted immunoreactivity surrounding epithelial cells of the inner epidermal layer and is particularly intense in regions of cell contact. g, gland. Bar: 50 μm (A–C); 10 μm (D).

myoblast-like muscle precursor cells of the blastema (Fig. 6A,B). After myoblast fusion and during later muscle differentiation, this staining became gradually more restricted to the epimysial and perimysial sheaths (Fig. 6C,D; Fig. 5A).

Later stages of differentiation and the establishment of metamery

During later stages of differentiation of the tail, a metameric pattern gradually appeared with sequentially arranged bony vertebrae and muscle masses. Tenascin staining gradually returned to be restricted to the connective tissues where it could be detected in the normal adult. Strong staining was still present in developing cartilages of growing vertebrae and in the periosteum of differentiated bone (Fig. 7A,B). In longitudinal sections, one could clearly distinguish the metamery of the regenerated tail with striated muscle masses separated by strongly tenascin-positive connective tissue segments (Fig. 7C). Tenascin continued to be expressed at least up to the 8th week of regeneration in the matrix of perichordal regions between differentiated muscle masses and the spinal cord (Fig. 7C bottom).

Biochemical characterization of tenascin in tail regenerates

In parallel with the immunohistochemical analysis, we followed the changes in the expression of tenascin during regeneration by immunoblots. The presence of tenascin in normal entire adult tails could not be detected by immunoblot; this is most probably due to the very low level and extremely restricted pattern of expression of the molecule which was, however, detected in adult fin extracts (data not shown). 4 days after amputation, the presence of tenascin in the regenerate could be clearly revealed by immunoblots (Fig. 8, lane 1). Its expression increased gradually reaching a maximum in 6-week-old regenerates (Fig. 8, lane 4) and then it decreased. Eleven weeks after amputation, the expression of the molecule was weak, but still clearly detectable (Fig. 8, lane 6). At all stages of development tenascin appeared as two polypeptides of relative molecular mass 210 and 220×10^3.

Discussion

In this paper, we have described the patterns of expression of tenascin, an extracellular matrix glycoprotein, during the post-traumatic regeneration of the

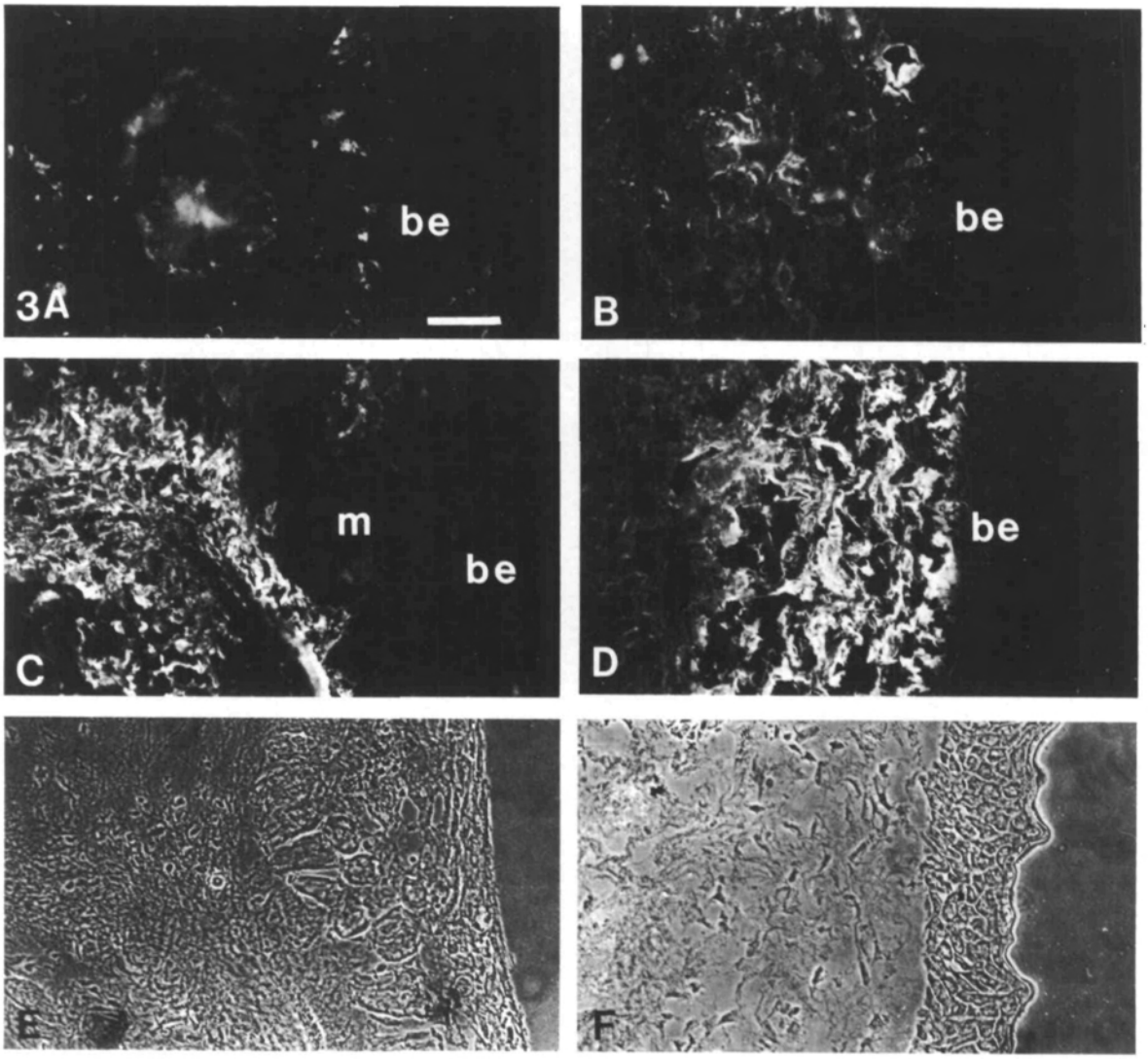


Fig. 3. Distribution of tenascin and fibronectin immunoreactivity in early stages of tail regeneration. (A,C,E) Transverse section through a 4-day-old regenerate in a plane parallel to that of the initial amputation about 200 μm under the thickened wound epithelium. Fibronectin immunoreactivity (A) is weak and limited to degenerative areas, while a very strong tenascin immunoreactivity (C) is present in all the connective tissue surrounding stump muscles and cartilage and extends towards the loose mesenchyme that is forming near the plane of amputation constituted by dedifferentiating stump cells (upper left part of A,C and E), the blastema epithelium is negative for both fibronectin and tenascin; (E) phase-contrast image corresponding to C. (B,D,F) Section through a blastema eight days after amputation. In the loose mesenchyme forming under the regenerated epithelium the immunoreactivity for both fibronectin (B) and tenascin (D) is strong, the blastemic epithelium is negative; (F) phase-contrast image corresponding to D. be, blastema epithelium; m, muscle. Bar: 50 μm.

tail distal part of the amphibian urodele *Pleurodeles waltl*. Our results are as follows. (1) In the normal adult tail of *Pleurodeles*, tenascin expression is limited to few connective tissues: the periosteum, vertebral ligaments, myotendons, the perimysium and the pia mater. (2) In certain regions of normal adult skin, tenascin is present in the inner layer of the epidermis apparently located at the cell surface. (3) Four days after tail amputation, there is a strong increase in the levels of tenascin expression in the stump tissues just beneath the regenerated blastemic epithelium which is negative. This increase in tenascin was not paralleled by an increase in fibronectin expression. (4) During further regeneration, the matrix of the loose mesenchyme underlying the blastema epithelium is very intensely stained by anti-tenascin and by anti-fibronectin antibodies. (5) Tenascin immunoreactivity is particularly prominent in areas of chondrogenesis and is absent from the regenerating nervous system. (6) Tenascin immunoreactivity is present in fibrillar matrix component oriented parallel to the direction of aligning myoblasts in myogenic areas. (7) During later differentiation, tenascin gradually disappears from differentiated cartilage and bone, but persists in myotendinous structures, in the periosteum and in regions of the matrix where chondrogenesis is still taking place.

As already mentioned, tenascin appears to modulate differently the adhesion of different cell types (Grumet

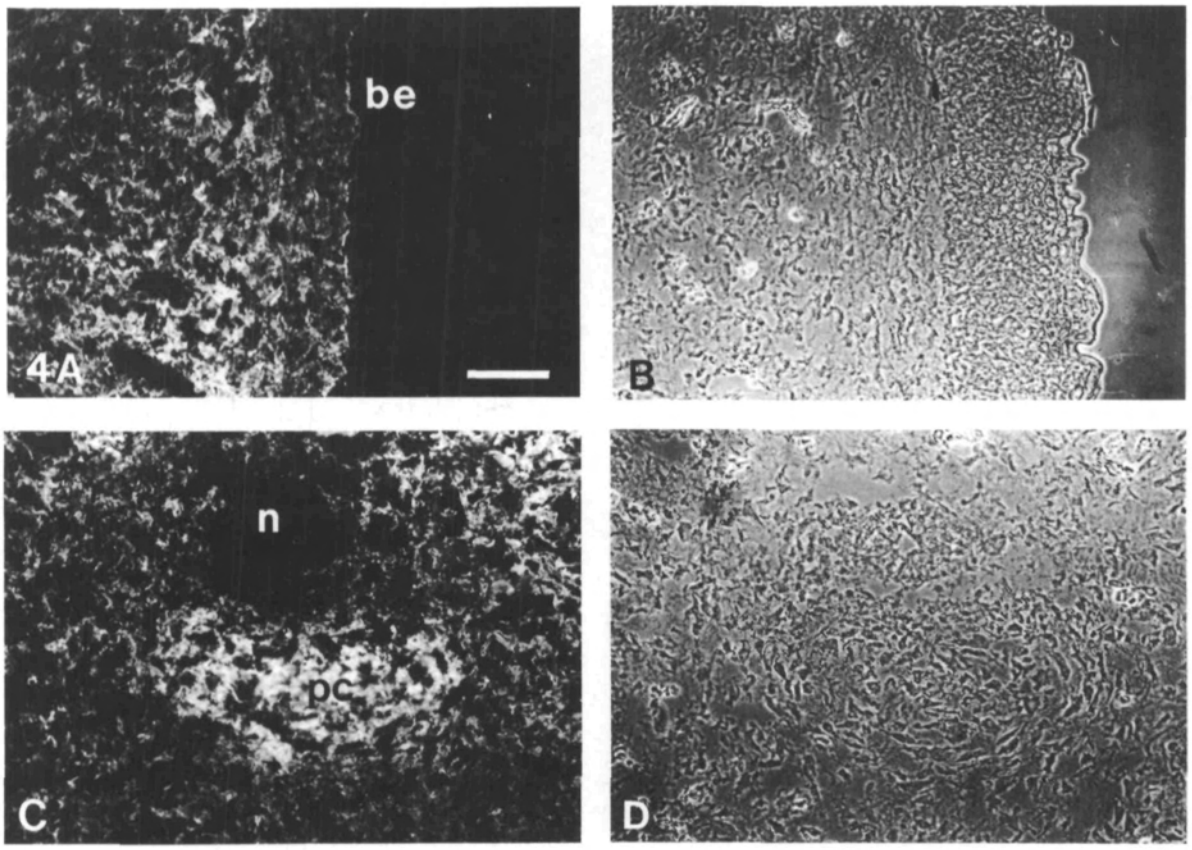
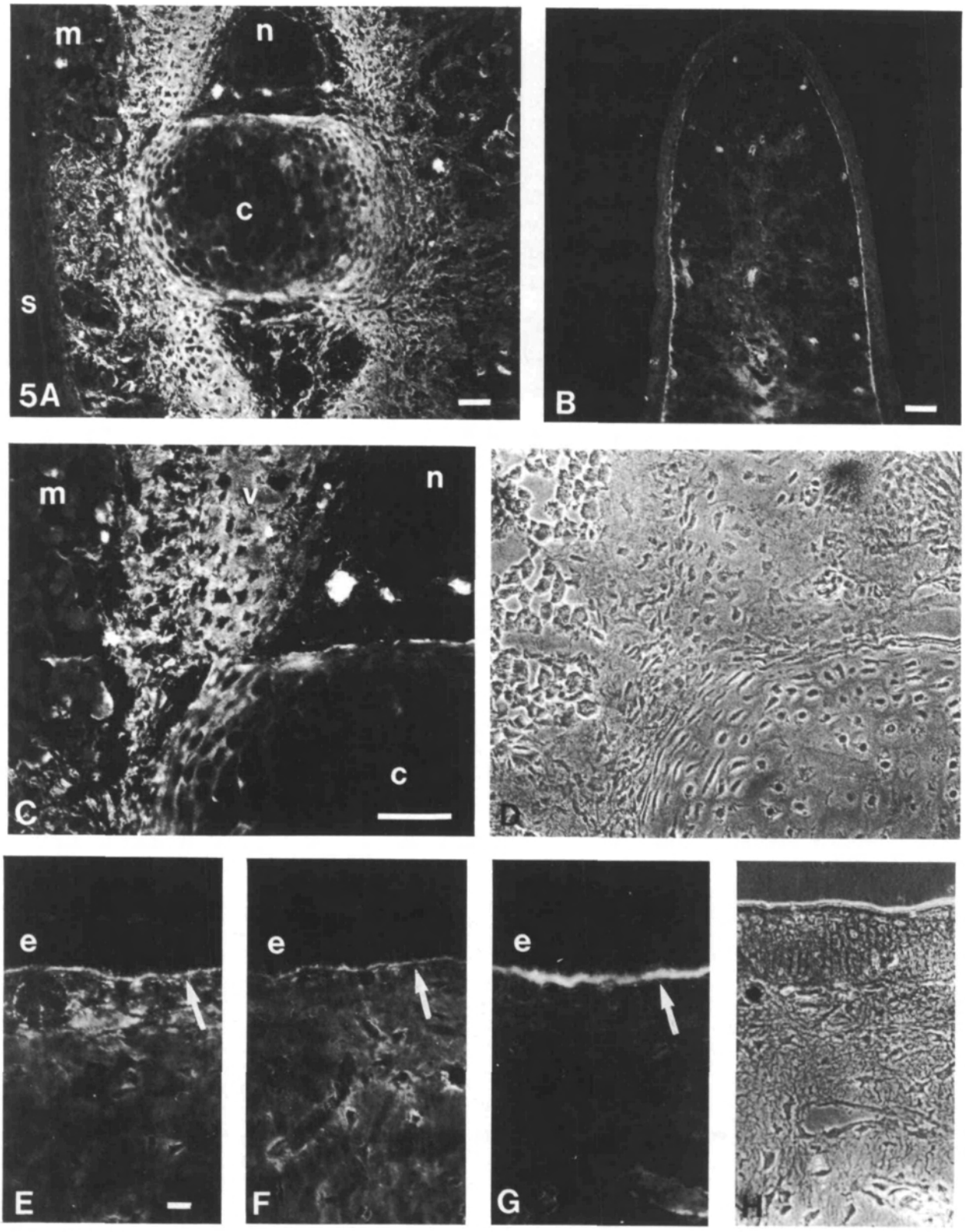


Fig. 4. Distribution of tenascin immunoreactivity after two weeks of regeneration. (A,B) Longitudinal section through a 2-week-old regenerate; (C,D) transverse section through an equivalent regenerate. All the matrix of the loose mesenchyme under the blastemic epithelium is very intensely immunoreactive to anti-tenascin antibodies (A,C). The blastema epithelium is still not labeled, but a clearly labeled basal lamina can be seen at the epithelial–mesenchymal interface (A). The ependyma of the regenerating nervous system penetrates into the mesenchyme where the first signs of cellular organization into cartilage can be detected. (C) The regenerating nervous system (n) does not express tenascin immunoreactivity that, on the contrary, is strongly induced in the procartilage of the presumptive vertebral body (pc). (B and D) Phase-contrast images corresponding to A and C respectively. be, blastema epithelium; n, regenerating nervous system; pc, procartilage. Bar: 50 μm.

et al. 1985; Mackie *et al.* 1987*a*; Tan *et al.* 1987; Friedlander *et al.* 1988; Lotz *et al.* 1989) and to inhibit the attachment of cells to FN substrates (Mackie *et al.* 1988; Chiquet-Ehrisman *et al.* 1988; Lotz *et al.* 1989); these and other *in vitro* and *in vivo* observations have led different authors to propose that tenascin might play different roles in the control of cellular behavior during development. Recent studies of the functional structure of tenascin conducted on recombinant fragments of the molecule (Spring *et al.* 1989) have provided a molecular explanation for the possible multiple functions of this molecule. It appears that tenascin contains at the same time a cellular adhesive site and an 'antiadhesive' site located in separate regions of the molecule and interacting with different receptors. In particular, the fact that the antiadhesive site is located in a fragment containing the EGF-like repeats raises the possibility that an EGF-like receptor and signaling process is involved in an active modulation of this specific function of the molecule. Tenascin could therefore influence cell movement, cell differentiation and possibly cell proliferation through complex regulatory pathways not involving simply the formation of more or less stable cell–matrix connections, but through the activation of more global cellular programs that would depend on the specific subset of receptors present at the surface of a given cell type (Spring *et al.* 1989).

The first striking modulation on tenascin expression during tail regeneration is its rapid and sharp increase in the mesenchyme underlying the regenerated blastemic epithelium, which preceeds the increase in fibronectin expression; this phenomenon can be viewed as a further example of induction of tenascin in a mesenchyme in response to the development of a new epithelium. A similar process occurs in several developing organs (Chiquet-Ehrismann *et al.* 1986; Aufderheide *et al.* 1987; Aufderheide and Ekblom, 1988; Inaguma *et al.* 1988; Thesleff *et al.* 1988; Vainio *et al.* 1989), in epithelial tumors (Mackie *et al.* 1987*a*) and in healing wounds (Mackie *et al.* 1988*a*). In the case of tail regeneration, tenascin might be elaborated by fibroblast-like blastema cells which are metabolically very



active (Bodemer and Everett, 1959; Riddiford, 1960; O'Steen, 1960; Hay and Fischman, 1961) in response to signals from the blastemic epithelium.

High levels of tenascin are generally present in the matrix of mesenchymes where the migration and proliferation of relatively undifferentiated cells has been induced either by a surgical procedures (Mackie *et al.* 1988; Daniloff *et al.* 1989) or by normal inductive signals during embryonic development (Riou *et al.* 1988). For example, high levels of tenascin are present in the

Fig. 5. Distribution of tenascin immunoreactivity after four weeks of regeneration. (A) Transverse section through the central part of a four week-old regenerate. The differentiated cartilage of the vertebral body is virtually non-reactive to anti-tenascin antibodies; strong immunoreactivity is, however, still present in the peripheral zone of young proliferating cartilage, in the perichondrial zone, in the developing cartilage of the neural arch around the spinal cord and in the matricial fibrillar components of the hemal arch surrounding the caudal blood vessel. (C,D) High magnification of a part of A, note the strong staining in the matrix surrounding cells that are going to differentiate into vertebral cartilage (v); (D) phase-contrast image corresponding to C. (B) Section through the regenerated fin; the mesenchymal fibrillar matrix is weakly stained while intense staining is present in the basal lamina under the regenerated skin. (E–H) Transverse sections through the skin of a 4-week-old regenerate; (E) pattern of tenascin immunoreactivity, (F) pattern of fibronectin immunoreactivity, (G) pattern of laminin immunoreactivity, (H) phase-contrast image corresponding to G. Tenascin and fibronectin are present in the mesenchymal matrix and basal lamina (arrow), laminin staining is mostly limited to the basal lamina. b, blood vessel; c, differentiated cartilage; e, epidermis; m, differentiated muscle; n, neural tube; s, skin. (A–D) Bar: 50 μm; (E–H) bar: 10 μm.

granulation tissue of healing wounds and in the pathways of neural crest cell migration of chick, rat, quail, *Xenopus* and *Pleurodeles* embryos (Crossin *et al.* 1986; Mackie *et al.* 1988; Riou *et al.* 1988).

During later differentiation of the regenerated tail, there is at first a strong increase of tenascin expression in areas where mesenchymal cells differentiate and get arranged in particular patterns such as in prechondrogenic regions and sites of myoblast alignment, followed then by a gradual decrease of its expression that leads to its absence from most differentiated tissues. When the regeneration process is completed, as in normal adult animals, tenascin expression is limited to a number of elastic connective tissues such as ligaments, tendons and the connective sheaths enveloping bones, nerves and muscular masses. These modulations constitute a second common feature between our results and later phases of development. Our observations of strong tenascin immunoreactivity in regions of the epidermis apparently associated with the cell surface might possibly be related to the continuous renewal of the skin that takes place during moulting of urodele amphibians.

The strong modulation of tenascin expression observed during development and regeneration suggests that tenascin plays a key role as a modulator of cell motility and differentiation. If indeed tenascin is so important for morphogenesis which are then the signals that control tenascin expression? Answer to this question might help to understand why the regenerative capacity is limited to certain species and to certain specific developmental stages (Wallace, 1981).

It has been shown that a strong increase in tenascin secretion can be induced in cultured chick embryo fibroblast by transforming growth factor β (TGF-β) (Pearson *et al.* 1988). The possible inductive role of

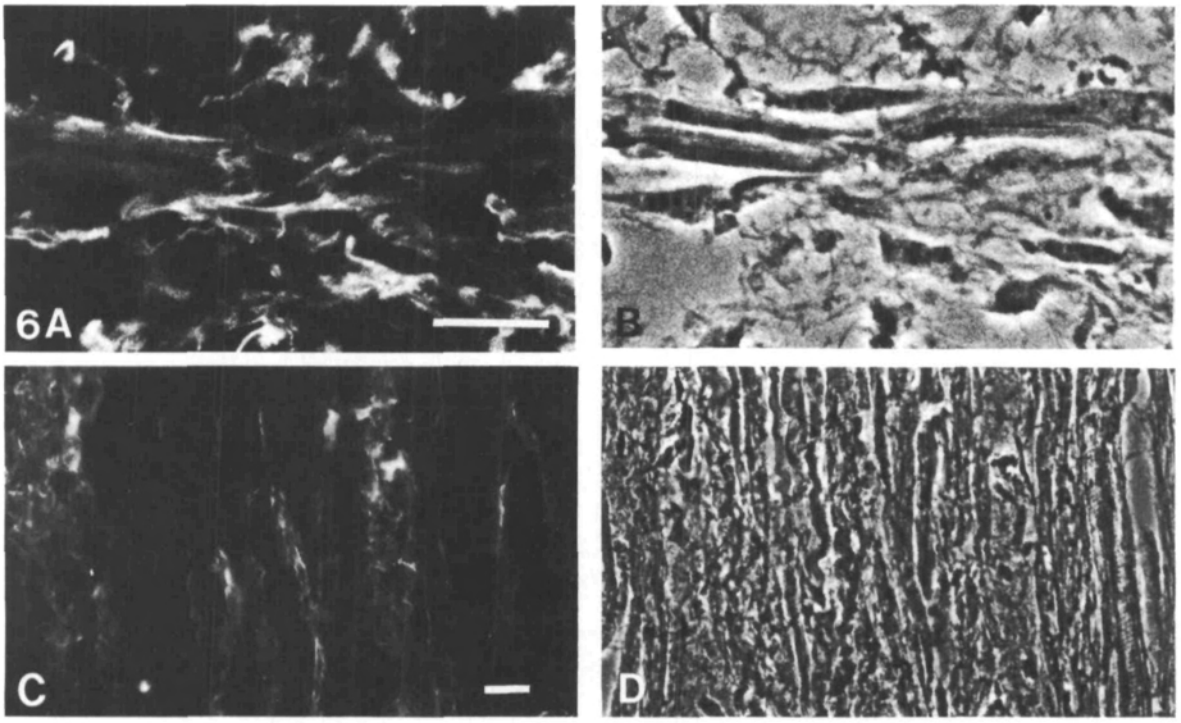


Fig. 6. Distribution of tenascin immunoreactivity in myogenic regions. (A,B) Aligning myoblasts in the dermal layer between the epidermis and the prechondrogenic zone. (A) The longitudinal ECM fibers around the muscle precursors cells are brightly stained by anti-tenascin antibodies. (C,D) After fusion and muscle differentiation, tenascin immunoreactivity persists only in the connective tissue surrounding differentiated myotubes. B and D are phase-contrast images corresponding to A and C, respectively. Bar: 50 μm.

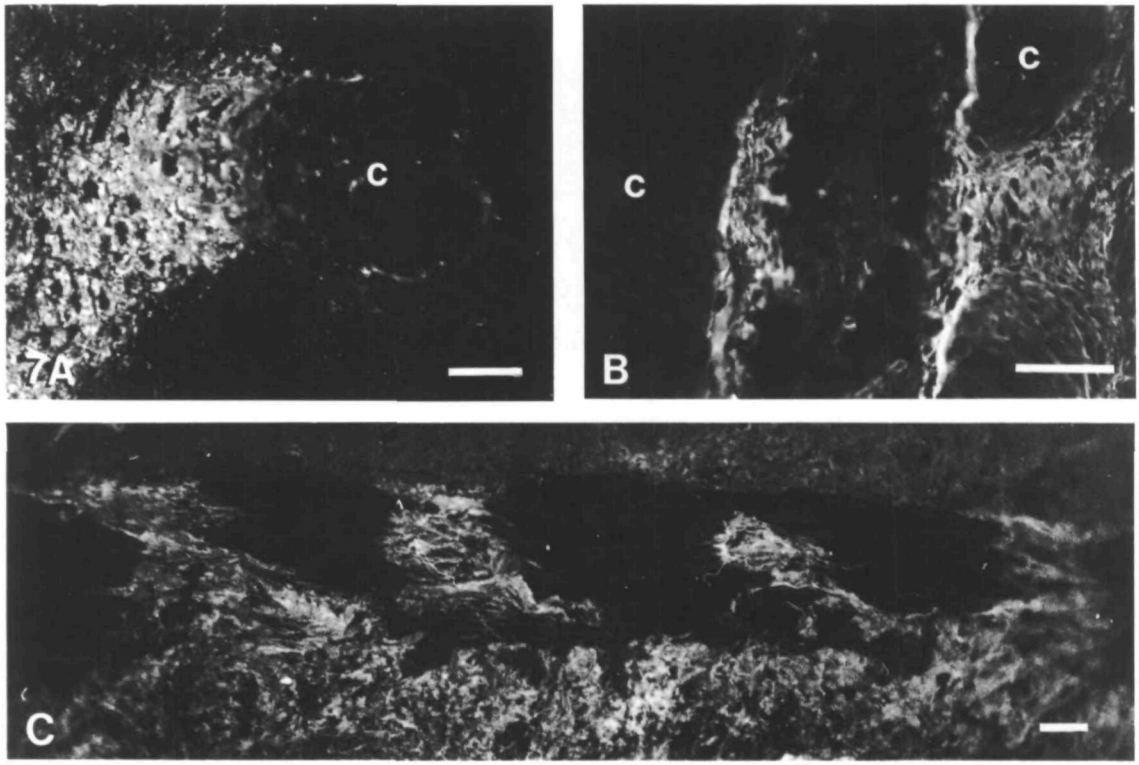
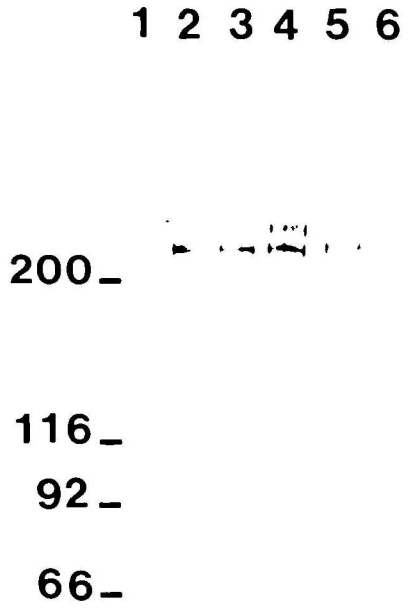


Fig. 7. Distribution of tenascin immunoreactivity in late regenerates. (A,B) Detail from transverse sections through 8 week-old regenerate. Differentiated cartilage is totally negative; tenascin immunoreactivity still persists in regions where chondrogenesis is taking place. (C) Longitudinal section through 8 week-old regenerate. Fully differentiated muscle masses do not express anti-tenascin immunoreactivity; however, the molecule is very abundant in myotendinous strands connecting the mature muscles in a metameric fashion. Tenascin immunoreactivity is still present in some regions of the perichordal matrix. c, differentiated cartilage. Bar: 50 μm.



TGF-β on tenascin is also suggested by the fact that, in the developing embryo, the pattern of expression of the two molecules is largely overlapping, being conspicuous in several mesenchymes where active cell migration is taking place and in areas of chondrogenesis (Heine *et al.* 1987). Interestingly TGF-β has been shown to accelerate the healing process of incisional skin wounds and to directly accelerate chemotaxis and synthesis of matrix molecules *in vivo* (Mustoe *et al.* 1987). One might then suggest that one of the first responses to a lesion is the local secretion of TGF-β, which activates the synthesis of tenascin (and other matrix molecules), creating the conditions for the subsequent migration of undifferentiated mesenchymal cells. Further experiments in which the presence (or the absence) of molecular modulations similar to those described in this paper in species and stages where regeneration is

Fig. 8. Immunoblots of tenascin polypeptides in regenerating adult tails. Extracts of regenerating tail at different times after trauma were resolved on SDS–polyacrylamide gels, transfered to nitrocellulose paper and immunoblotted with anti-tenascin polyclonal antibodies. Lane 1: regenerate of 4 days; lane 2: regenerate of 3 weeks; lane 3: regenerate of 5 weeks; lane 4: regenerate of 6 weeks; lane 5: regenerate of 8 weeks; lane 6: regenerate of 11 weeks. $M_r \times 10^{-3}$ of standards are indicated.

impaired, coupled with possible molecular inductions by combinations of growth factors and hormones, might then provide a key to a pharmacological approach of the regenerative process.

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