

Tenascin/Hexabrachion in Human Skin: Biochemical Identification and Localization by Light and Electron Microscopy

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Abstract. Tenascin/hexabrachion is a large glycoprotein of the extracellular matrix. Previous reports have demonstrated that tenascin is associated with epithelial-mesenchymal interfaces during embryogenesis and is prominent in the matrix of many tumors. However, the distribution of tenascin is more restricted in adult tissues.

We have found tenascin to be present in normal human skin in a distribution distinct from other matrix proteins. Immunohistochemical studies showed staining of the papillary dermis immediately beneath the basal lamina. Examination of skin that had been split within the lamina lucida of the basement membrane suggested a localization of tenascin beneath the lamina lucida. In addition, there was finely localized staining within the walls of blood vessels and in the smooth muscle bundles of the arrectori pilorum. Very prominent staining was seen around the cuboidal cells that formed the

basal layer of sweat gland ducts. The sweat glands themselves did not stain.

The distribution of tenascin in the papillary dermis was studied at high resolution by immunoelectron microscopy. Staining was concentrated in small amorphous patches scattered amongst the collagen fibers beneath the basal lamina. These patches were not associated with cell structures, collagen, or elastic fibers.

Tenascin could be partially extracted from the papillary dermis by urea, guanidine hydrochloride, or high pH solution. The extracted protein showed a 320-kD subunit similar to that purified from fibroblast or glioma cell cultures. We have developed a sensitive ELISA assay that can quantitate tenascin at concentrations as low as 5 ng/ml. Tests on extracts of the papillary dermis showed tenascin constituted about 0.02–0.05% of the protein extracted.

TENASCIN/hexabrachion is a large oligomeric glycoprotein of the extracellular matrix. The name hexabrachion refers to the unique disulfide bonded six-armed structure that is the most common form of the glycoprotein (15, 16). The protein has been independently discovered in a number of laboratories and given several names including: hexabrachion (15, 16), myotendinous antigen (8, 9), GP250 protein (6, 7), glial mesenchymal extracellular matrix protein (3, 4), tenascin (2, 10), cytotactin (13, 22), J1 protein (30, 42), and brachionectin (17, 47; for review, see reference 16). Studies characterizing this protein indicate tenascin is a collagenase resistant protein that is distinct from types I, III, IV, VI, and VII collagen, fibrillin, laminin, entactin, fibronectin, chondronectin, and thrombospondin.

Several laboratories have described the temporal and spatial localization of tenascin during embryogenesis. This distribution is much more restricted than that of other ECM proteins including laminin and fibronectin (1, 2, 8, 10, 13, 17, 18, 22, 24, 30, 35, 37, 42, 47). Individual laboratories have focused on different tissues and functional associations, including dense connective tissues, developing central nervous system, epithelial-mesenchymal interfaces of embryos, and tumor matrix.

Chiquet and Fambrough (8) described the localization of tenascin in a subclass of dense connective tissue including perichondrium/periosteum, ligaments, and tendons as well as in developing smooth muscle tissues. Vaughan et al., (47) also noted the prominence of this protein in cartilage, from which it could be extracted using high salt. Mackie et al., (35) have reported in vitro studies that suggest tenascin plays an active role in the initiation of chondrogenesis. They proposed that tenascin may act by modulating the inhibitory effects of fibronectin on chondrocyte differentiation.

Edelman and colleagues (13, 22) have studied the appearance of tenascin in the developing central nervous system, noting specific temporal and spatial patterns of expression. They proposed that tenascin, specifically synthesized by glial cells, plays a role in neuron-glial interactions (22, 24). Other studies suggest tenascin plays a role in migration of neural crest cells, myelination, and formation of neuromuscular junctions (12, 40, 42, 44).

Tenascin expression during embryonic morphogenesis is also linked to developing epithelial-mesenchymal interfaces. Aufderheide et al., (2) reported on the induction of tenascin in developing kidney only after the differentiation of an epithelial surface from the underlying mesenchyme. Similar

findings were reported by this group in developing embryonic gut (1). In embryonic skin, several reports have noted prominent tenascin staining in the mesenchyme beneath developing skin appendages, including chicken feather papillae and rat hair follicle, tooth bud and mammary gland (8, 10, 13).

In contrast to embryonic tissue, tenascin expression is reportedly very restricted in adult tissues. However, prominent expression of tenascin in the stroma of several types of human tumors was first noted by Bourdon et al., (3, 4). Similarly, Chiquet-Ehrismann et al. (10) found the protein expressed in developing embryonic rat mammary tissue but absent in normal adult tissue. They observed prominent induction of tenascin in the matrix surrounding chemically induced mammary carcinomas (10).

Because of the association of tenascin with dense connective tissue, including developing skin, and the matrix surrounding some epidermally derived tumors, we have examined adult skin to determine if tenascin expression persists in adult human skin, to determine its ultrastructural localization, and to characterize the protein isolated from skin relative to that produced by fibroblast and tumor cells *in vitro*.

Materials and Methods

Antibodies/Antigens

Monoclonal antibody 81C6, an IgG_{2b} immunoglobulin to human tenascin (3, 4), was used for affinity purification of tenascin from culture supernatant of a human glioma cell line, U-251 MG clone 3, as previously described (18). Tenascin was further purified by gradient sedimentation on a 15–40% (vol/vol) glycerol gradient (15, 18) before immunization of rabbits. Antiserum obtained after the second and subsequent boosts was affinity-purified on a column prepared by coupling 1 mg of the purified tenascin to cyanogen bromide activated sepharose 2-B (45). The affinity-purified antisera had no detectable reactivity with collagen types I, III, IV, and V, gelatin, keratin, laminin, or fibronectin on ELISA (14) and showed no reactivity to fibrillin, collagen types I, III, or VII, laminin, fibronectin, or keratin on Western blotting (46). Antibody from preimmune serum was purified on protein A-agarose (Sigma P1406) and used as a negative control in immunolocalization and immunoblotting. A mouse myeloma IgG_{2b} immunoglobulin of no known specificity, 45.6 (3, 4), was used as a control for monoclonal studies.

A mouse monoclonal antibody to fibrillin (41) was the generous gift of Dr. L. Sakai (Oregon Health Sciences University, Portland, OR). Mouse monoclonal antibodies to laminin and type VII collagen were provided by Dr. R. Briggaman (University of North Carolina, Chapel Hill, NC). Mouse monoclonal antibody 3E3 to human fibronectin was from Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA). Fluorescein and horse radish peroxidase-conjugated goat anti-mouse and anti-rabbit were from Tago Inc. (Burlingame, CA). Peroxidase avidin-biotinylated horseradish peroxidase complex (ABC)¹ kit was from Vector Laboratories, Inc. (Burlingame, CA).

Light Microscopy

Tissue obtained from surgical specimens of autopsy material was used for immunolocalization. Fresh tissue was embedded in Tissue-Tek O. C. T. compound (Miles Scientific, Naperville, IL) and 4–6- μ m sections were cut using a microtome (E. Leitz, Rockleigh, NJ) at -21°C . Sections were fixed in cold acetone (-20°C) for 10 min and air dried. Monoclonal antibody was used at 6 $\mu\text{g}/\text{ml}$ diluted in 0.25% gelatin in PBS. Affinity-purified rabbit polyclonal antibody was used at 0.5–1 $\mu\text{g}/\text{ml}$. Sections were incubated in a humidified chamber at room temperature for 30 min. After washing in PBS, sections were incubated with fluorescein-conjugated second antibody previously adsorbed against human IgG (Tago Inc.) for 30 min at room temperature. The sections were washed with PBS, covered with 50% glycerol in PBS, and visualized (Laborlux 12; E. Leitz). For saline-split skin, tissue was incubated for 24 h at 4°C in 1 M NaCl, 10 mM Tris HCl, 0.02% sodium azide, 1 mM EDTA, 1 mM PMSF, pH 7.4 (Scaletta buffer), before embedding and sectioning. Immunolocalization with affinity-purified polyclonal

anti-tenascin was the same as that seen with 81C6 although, as expected, the signal was more intense with the polyclonal antibody.

Formalin-fixed paraffin-embedded sections were deparaffinized by incubation overnight in xylene followed by serial rehydration through 100%, 95%, and 80% ethanol. After rinsing with water, the sections were blocked for 20 min with 10% normal goat serum in PBS. Sections were incubated overnight at room temperature in a humidified chamber with affinity-purified polyclonal antibody at 1 $\mu\text{g}/\text{ml}$ in blocking buffer, which gave optimum specific staining. Preimmune control antibody was used at the same concentration. Sections stained with preimmune antibody at 30 $\mu\text{g}/\text{ml}$ gave diffuse background but no specific staining. After washing with PBS, the bound antibody was visualized by the peroxidase ABC (27) method using diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the developer. These sections afforded better preservation of the tissue morphology than frozen sections. However, the monoclonal antibody 81C6 was not reactive to formalin-fixed sections, so these were stained with the affinity-purified polyclonal antibody. Several comparisons of these sections with cryosections stained with monoclonal antibodies showed identical localization.

Electron Microscopic Immunolocalization

Freshly obtained tissue samples were cut into 2-mm blocks and washed for 4 h at 4°C with PBS. They were then incubated with affinity-purified antibody or preimmune control antibody at 2.5 $\mu\text{g}/\text{ml}$ in 20 mM Tris HCl, 0.1% BSA, 0.15 M NaCl, 0.1% sodium azide, pH 8.0 (Tris buffer) at 4°C for 43 h. After washing with PBS for 6 h at 4°C , the blocks were incubated with 10-nm gold-conjugated goat anti-rabbit antibody (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:2 with Tris buffer at 4°C for 15 h. The blocks were washed for 6 h at 4°C in PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 3 h. The blocks were extensively washed with 0.1 M sodium cacodylate, stained for 1 h at 4°C in 1% osmium tetroxide in 0.1 M sodium cacodylate, washed twice with 0.1 M sodium cacodylate, washed twice with 0.9% NaCl, and stained with 0.5% uranyl acetate in 0.9% NaCl for 2 h at room temperature. After rinsing with 0.9% NaCl, the blocks were dehydrated through 70%, 95%, and 100% ethanol followed by propylene oxide. The blocks were embedded in Epon Resin and cut in 60-nm sections (Ultracut E, Reichert Scientific Instruments, Buffalo, NY). The sections were stained for 30 min in 7.5% uranyl acetate, rinsed with water, and counterstained 10 min with Sato lead stain (43). Sections were examined using an electron microscope (model 300; Philips Electronic Instruments, Inc., Mahwah, NJ).

Tissue Extraction

Tissue obtained from autopsy or surgical specimens was incubated for 72–96 h at 4°C in Scaletta buffer. This incubation causes the skin to split within the lamina lucida, separating the epidermis from the dermis (21). After separation, the basal lamina face of each section was extracted with one of several buffers: 0.1 M 3-[cyclohexylamino]-1-propane sulfonic acid (CAPS), 0.15 M NaCl, pH 11.0; 4 M urea, 0.1 M sodium borate, 0.5 M NaCl, pH 8.4; 4 M guanidine hydrochloride; PBS; or Scaletta buffer at 4°C . Frozen sections were prepared following 2 and 7 d of extraction and stained for tenascin. The extracts were dialyzed against PBS and total protein was estimated by the method of Bradford (5) using reagents from Bio-Rad Laboratories (Richmond, CA). Purified human fibronectin was used as protein standard. Tenascin content in the extracts was estimated by ELISA (14, see below) using affinity-purified tenascin as a standard.

ELISA Assay for Tenascin

96-well plastic plates (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) were coated overnight at 4°C with monoclonal antibody 81C6 at 0.25 $\mu\text{g}/\text{ml}$ in sodium carbonate buffer (0.05 M sodium carbonate, 0.02% sodium azide, pH 9.6). After washing with PBS-Tween (0.05% vol/vol Tween 20), 100 $\mu\text{l}/\text{well}$ of purified tenascin diluted in 0.25% gelatin in PBS-Tween was loaded in a range of 0.25–16 ng/well for a standard curve. All samples were assayed in triplicate. Extracts to be tested were diluted in 0.25% gelatin in PBS-Tween. Following incubation for 2 h at 37°C , the plates were washed and incubated with affinity-purified rabbit antibody at 0.15 $\mu\text{g}/\text{ml}$ in 0.25% gelatin in PBS-Tween for 2 h at 37°C . The plates were washed and incubated with horse radish peroxidase-conjugated goat anti-rabbit IgG (Tago Inc.) at 37°C for 2 h. Bound antibody was assayed colorimetrically using orthophenylenediamine (Sigma Chemical Co.). No detectable reactivity of affinity-purified antitenascin with mouse antibody was seen. Control plates using antibody purified from preimmune serum were also negative.

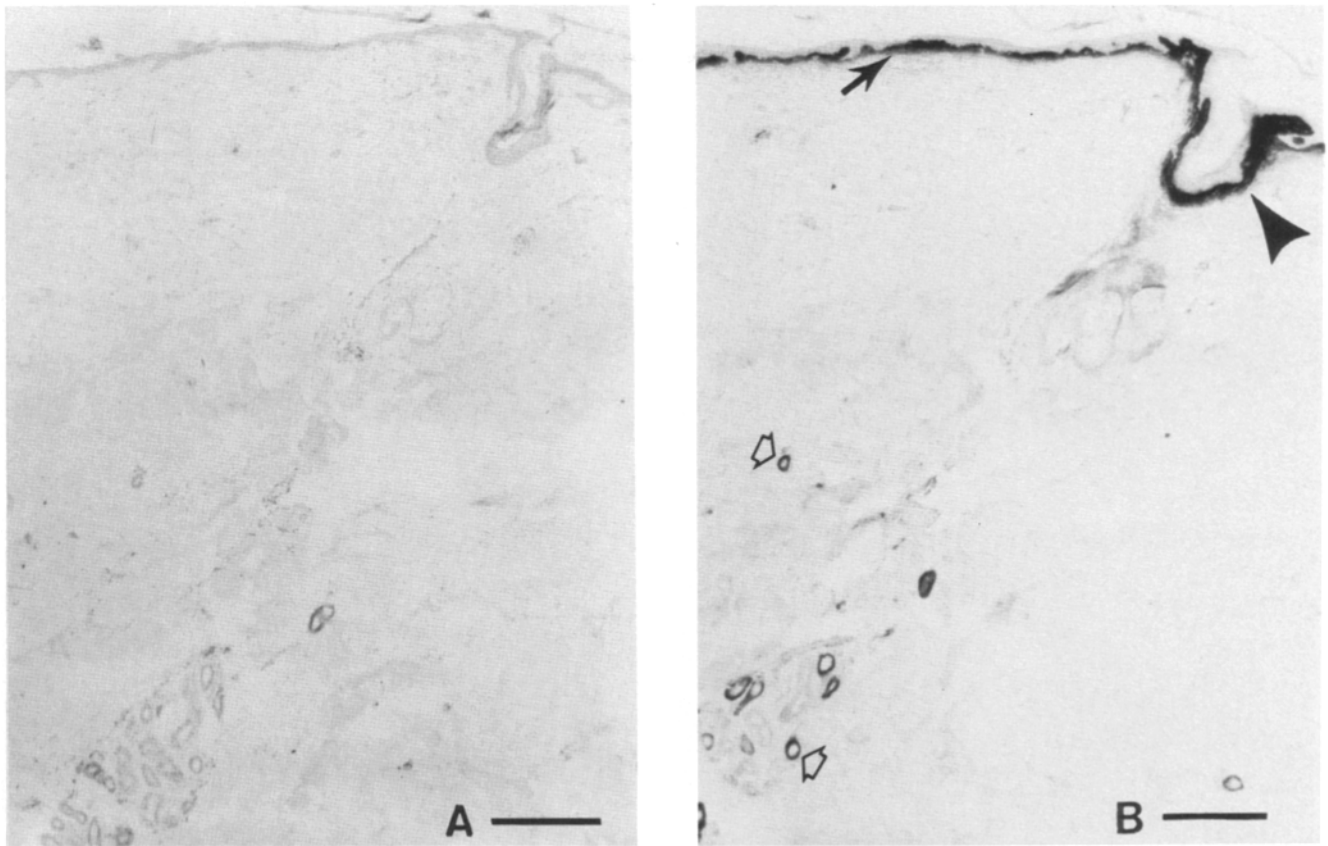


Figure 1. Indirect immunoperoxidase staining of 5- μm sections of formalin-fixed paraffin-embedded human skin using affinity-purified polyclonal antibody to human tenascin as described in Materials and Methods. Bound antibody was detected using the ABC peroxidase method. Shown are sections stained with preimmune antibody (A) and affinity-purified antibody (B). Prominent staining of the papillary dermis (arrow) is seen with enhancement around the infundibulum of the hair follicle (arrowhead). In addition, there is staining of the ductal portion of the eccrine sweat gland (open arrows). Bar, 200 μm .

Immunoblotting

Proteins were separated by PAGE by the method of Laemmli (32) on 5% gels or 4–15% gradient gels with 3% stacking gel. Proteins were transferred to nitrocellulose using a semi-dry blotting apparatus (LKB Instruments, Gaithersburg, MD) as described by Kyhse-Andersen (31). After transfer, the nitrocellulose was blocked in 3% BSA in PBS for 1 h at room temperature, incubated with monoclonal antibody at 2.5 $\mu\text{g}/\text{ml}$ or affinity-purified polyclonal antibody at 0.5 $\mu\text{g}/\text{ml}$ in 1% BSA in PBS-Tween for 2 h at room temperature, and washed with PBS-Tween. After incubation with horse radish peroxidase-conjugated antisera for 2 h at room temperature and washing with PBS-Tween, bound antibody was visualized using 4-chloronaphthol (Bio-Rad Laboratories) or diaminobenzidine (Sigma Chemical Co.).

Results

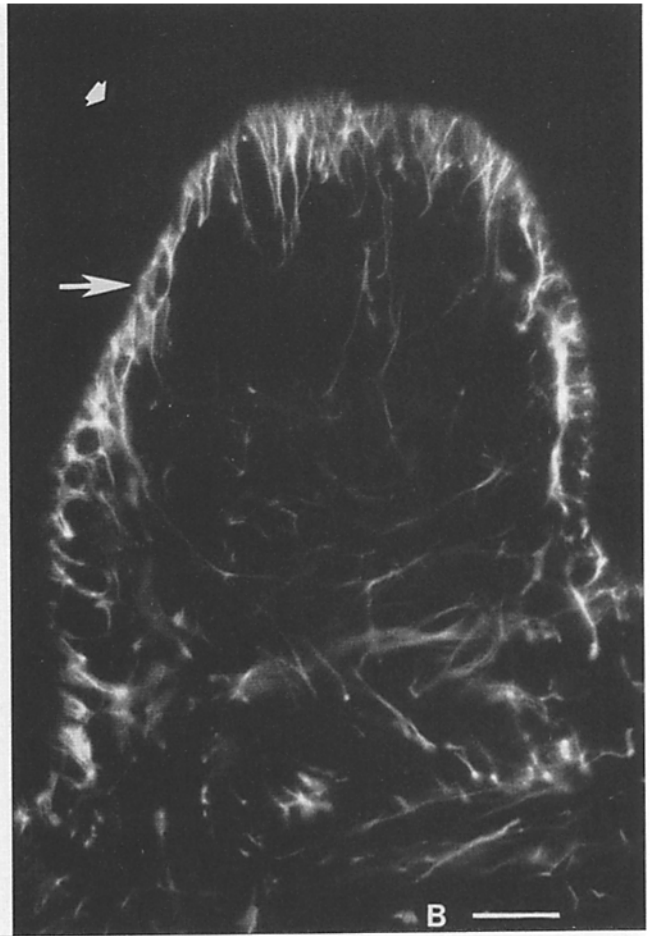
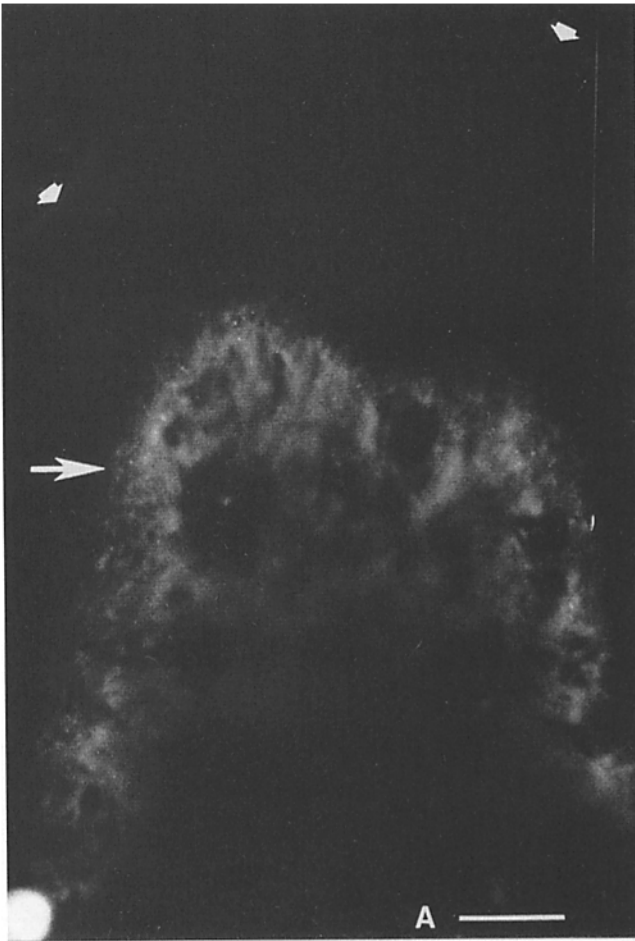
Distribution of Tenascin in Normal Adult Skin

Adult human skin from normal and pathological specimens was examined for presence of tenascin using immunofluorescent and peroxidase techniques. In normal adult skin, tenascin is localized primarily in the papillary dermis (Figs. 1 and 2). The staining was more pronounced within the papillary tips and was frequently sparse at the base of the rete ridges. The coarse patchy staining seen (Figs. 1 B, and 2 A) was distinct from the fine fibrillar pattern seen with antifibrillin staining (Fig. 2 B), and the more punctate staining seen with antifibronectin (Fig. 2 C). In contrast to staining seen with antibodies to the basal lamina (type IV collagen and laminin)

or with antibodies to the immediate sublamina space (type VII collagen, Fig. 2 D), the band of tenascin staining extended 2–10- μm into the dermis. Skin was incubated in Scaletta buffer to induce splitting within the lamina lucida (21). Immunostaining of saline split skin showed tenascin in the separated dermis, but no staining of the epidermal layer above the lamina lucida (Fig. 3).

In addition, antibodies to tenascin gave pronounced stippled staining of arrector pilorum (Fig. 4 A) and nerves as well as some staining of arteries (Fig. 4 B), capillaries, and veins. In arteries, the staining appears to be within the smooth muscle of the arterial wall and not limited to the basement membrane (Fig. 4 B).

There is striking staining of the eccrine sweat ducts but no staining of the sweat glands (Fig. 4 C). The sweat ducts extending towards the epidermis stained intensely for tenascin. The coiled portion of the gland, which is primarily sweat gland but can include up to one third duct, showed no staining in many of the deeper coils. However, there was intense staining of up to one third of the tubules in more superficial coils (Fig. 1). This pattern suggests that the tenascin staining is limited to the ductal portion of sweat glands. The eccrine sweat duct is lined by two layers of cuboidal epithelial cells: an outer cell layer that rests on a basement membrane and an inner cell layer that has a cuticular border. The tenascin staining appears to completely surround the duct and extend between the outer cell layer to the inner cell layer, but does



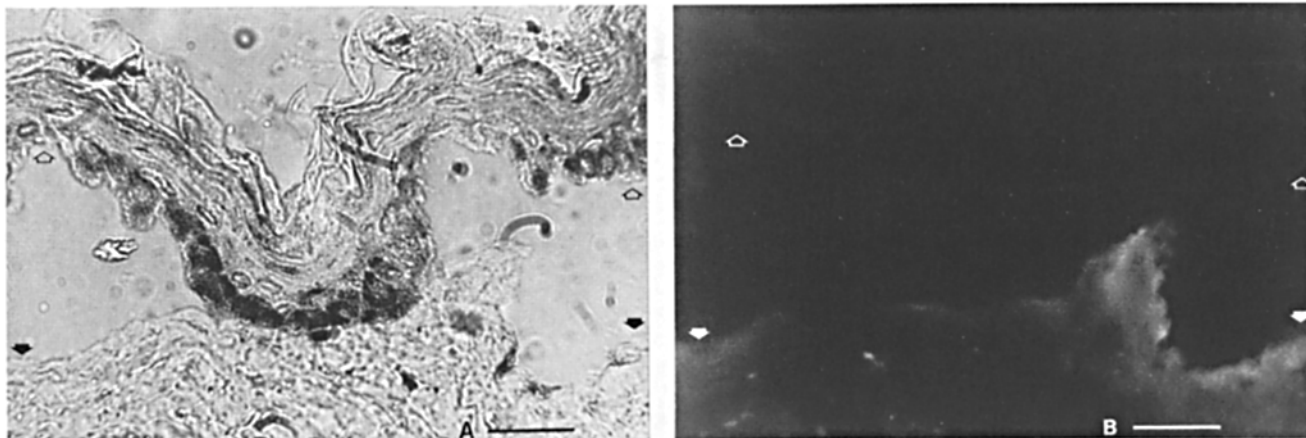


Figure 3. Indirect immunofluorescent staining of 5- μ m frozen section of saline split human skin using affinity-purified polyclonal antibody to tenascin. Human skin was incubated for 24-h in Scaletta buffer before embedding to induce splitting within the lamina lucida of the basement membrane as described in Materials and Methods. (A) phase contrast, (B) immunofluorescent staining. Roof of the split (epidermis) (open arrows), and base of the split (dermis) (solid arrows). Bar, 10 μ m.

not appear to extend to the lumen (Fig. 4 C). The staining is lost when the ducts enter the epidermis (Fig. 4 D).

Pretreatment of sections with elastase, collagenase, or hyaluronidase did not eliminate nor enhance the tenascin staining observed (not shown). Incubation of sections with PBS, Scaletta buffer, 4 M urea, or CAPS buffers was also unable to remove tenascin staining, although there was some decrease in the sections treated with the latter two (not shown). Incubation of sections with guanidine hydrochloride removed the sections from the slide.

In contrast to normal adult skin, dermis that was involved with primary malignancy (dermatofibrosarcoma protuberans, Fig. 5 A) or secondary invasion (squamous cell carcinoma, Fig. 5 B) showed marked increase in the tenascin staining in the matrix of the tumor. However, the nests of invasive squamous cells did not stain. This is in agreement with observations reported by Bourdon et al., (3) as well as Chiquet-Ehrismann et al., (10) on the distribution of this protein in tumors. In addition, examination of granulation tissue in rat skin in the late stages of healing after third degree burn (sections provided by Dr. A. Banes, University of North Carolina, NC) revealed markedly enhanced staining for tenascin (Fig. 5 C). The tenascin was prominent throughout the granulation tissue as well as laterally beneath the wound edge. The time course of induction of tenascin in wound healing is currently under study in our laboratories.

Electron Microscopic Immunolocalization

Studies on human skin demonstrated tenascin was present in small amorphous patches within the papillary dermis (Fig. 6). These patches were not labeled in control blocks stained with preimmune antibody. However, in the absence of labeling, the patches are indistinguishable from the many micro-

fibrillar components and other amorphous patches in the dermis. No labeling was seen in the basement membrane or in the zone immediately beneath the lamina densa where characteristic anchoring fibrils are seen. The tenascin patches were not specifically associated with collagen or elastic fibers or cell surfaces. The labeled patches were often smaller in size than a well-spread hexabrachion molecule (see rotary shadowed molecule in inset, Fig. 6). Examination of multiple sections from several blocks failed to reveal any long strands of labeling, although a few extended patches of label were seen (Fig. 6 B). It thus appears unlikely that tenascin associates in an extended fiber like collagen or elastic microfibrils. These findings suggest the smaller patches may represent single or small clusters of hexabrachion molecules. Enbloc staining with monoclonal antibody to tenascin gave essentially the same distribution as the affinity purified polyclonal antibody but with less intense gold labeling (not shown).

Extraction of Tenascin from Normal Skin

Extraction of tenascin from the dermal-epidermal junction of saline split skin was attempted using five buffers: (a) PBS; (b) 1 M NaCl, 10 mM Tris HCl, 1 mM EDTA, 0.02% sodium azide, pH 7.4 (Scaletta buffer); (c) 0.1 M CAPS, 0.15 M NaCl, pH 11; (d) 4 M urea in borate buffer; and (e) 4 M guanidine hydrochloride. In spite of prolonged incubation, no buffer completely removed the tenascin as assessed by immunohistochemistry. The staining observed after CAPS extraction was reduced \sim 50% and was more diffuse when compared to the other extraction buffers. Because of this, the CAPS extracts were chosen for further analysis.

The CAPS extracts were examined for tenascin by Western blotting using affinity-purified polyclonal antibodies and the monoclonal antibody 81C6. As shown in Fig. 7 A, the extract

Figure 2. Indirect immunofluorescent staining of 5- μ m frozen sections of normal human skin using monoclonal antibodies to (A) tenascin, (B) fibrillin, (C) fibronectin, and (D) type VII collagen. DEJ (long arrows), and the outer border of the epidermis (short arrows). Bar, 10 μ m.

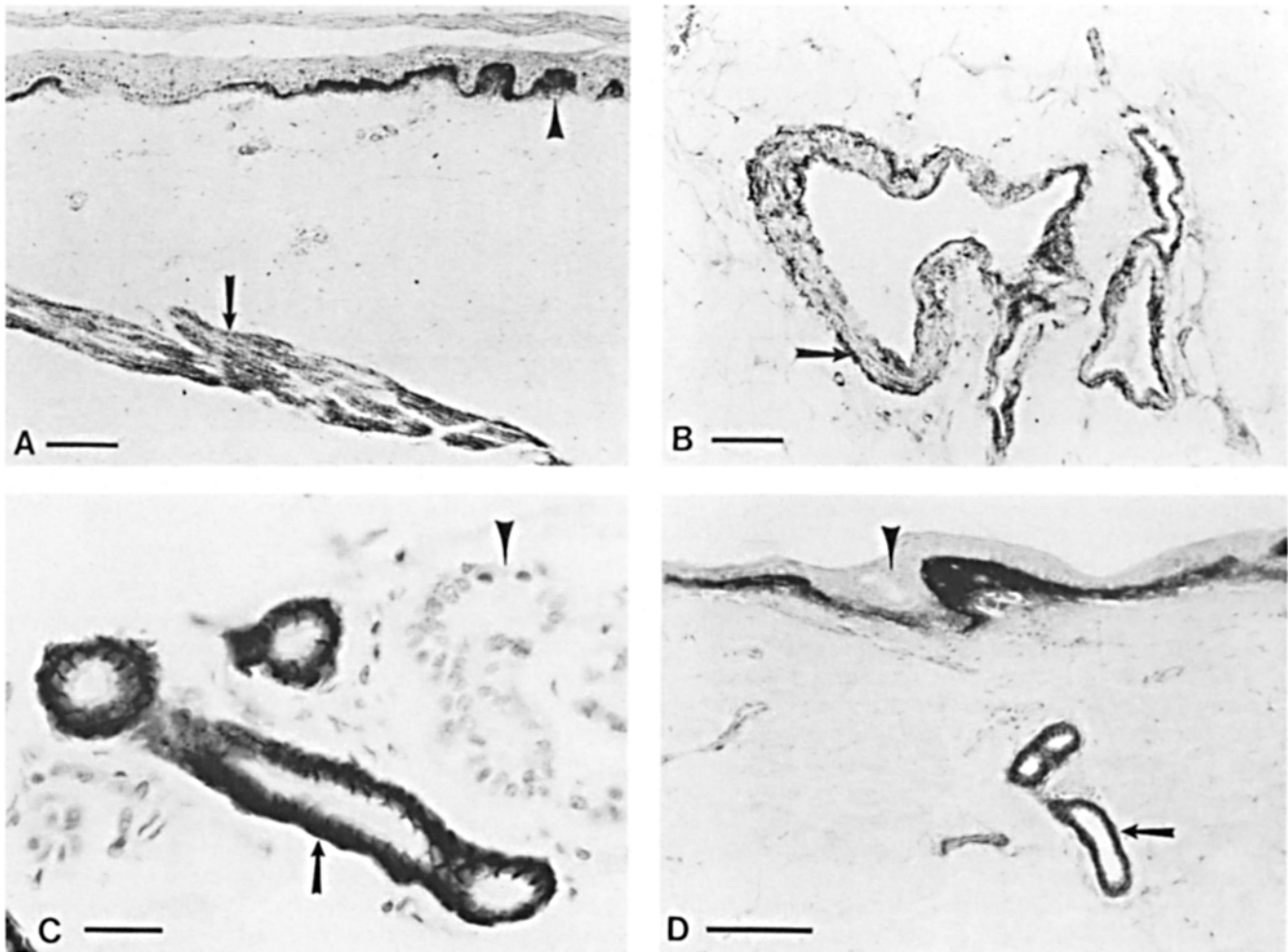


Figure 4. Indirect immunoperoxidase staining of 5- μ m sections of formalin-fixed paraffin-embedded human skin using affinity-purified polyclonal antibody to tenascin. (A) Intense staining of the papillary dermis (arrowhead) as well as a network staining of an arrector pilorum muscle (arrow). (B) Staining within the wall of an artery in the hypodermis (arrow). (C) Intense staining of the ductal portion of an eccrine sweat gland (arrow) with no staining of the adjacent gland (arrowhead). The staining appears to extend between the cells of the outer cuboidal cell layer. (D) Staining of the sweat duct (arrow) is lost upon entering the epidermis (arrowhead). Bars, (A), (B), (D) 100 μ m; (C) 20 μ m.

of the dermal face of the dermal-epidermal junction contained a 320-kD band that was recognized by both the polyclonal (lane 3) and monoclonal (lane 4) antibodies. This band had the same electrophoretic mobility as the tenascin purified from the glioblastoma cell line (lane 1). The mass of 320-kD was estimated relative to a set of very high molecular mass standards (Fig. 7 B and C) including reduced laminin, nonreduced fibronectin, reduced fibronectin, and reduced pig thyroglobulin. As noted in Fig. 7 C, these very high molecular mass standards deviate significantly from the curve estimated using high molecular mass markers (Bio-Rad Laboratories; Fig. 7 B, lane 3). This new curve is more accurate for estimating the size of similarly very large proteins, including the tenascin monomer (Fig. 7 B, lane 4 on gel, and C, arrow). Additional bands at \sim 640 kD (arrow) and at the top of the gel are noted in Fig. 7 A, lane 3. The \sim 640 kD band is thought to represent covalently linked dimers of tenascin (17). The band at the top of the gel represents complexes larger than 820 kD (the size of unreduced laminin). Whether these are trimers or complexes of tenascin

with other molecules is not known. Both bands also blotted faintly with monoclonal antibodies to tenascin. The \sim 640-kD band is also seen in silver stained SDS-PAGE gels of tenascin affinity-purified using 81C6 (Fig. 7 B, lane 4, arrow). The additional faint bands in Fig. 7 A, lane 3, may reflect breakdown products in the extract or minor alternatively spliced forms of tenascin. They are recognized by both the affinity-purified polyclonal antibody and the monoclonal antibodies to tenascin but are uniformly more intense with the polyclonal antibody. No tenascin was detected in the epidermal extracts stained with 81C6 (not shown). A very faint amount of staining was seen with the polyclonal antibody (Fig. 7 A, lane 2). This probably represents trace contamination with dermis during the mechanical separation of epidermis from dermis as neither light nor electron microscopic localization show any tenascin above the lamina lucida. However, we cannot rule out very low levels of tenascin in the epidermis.

The tenascin content of these extracts was determined using an ELISA assay. As shown in Table I, no detectable

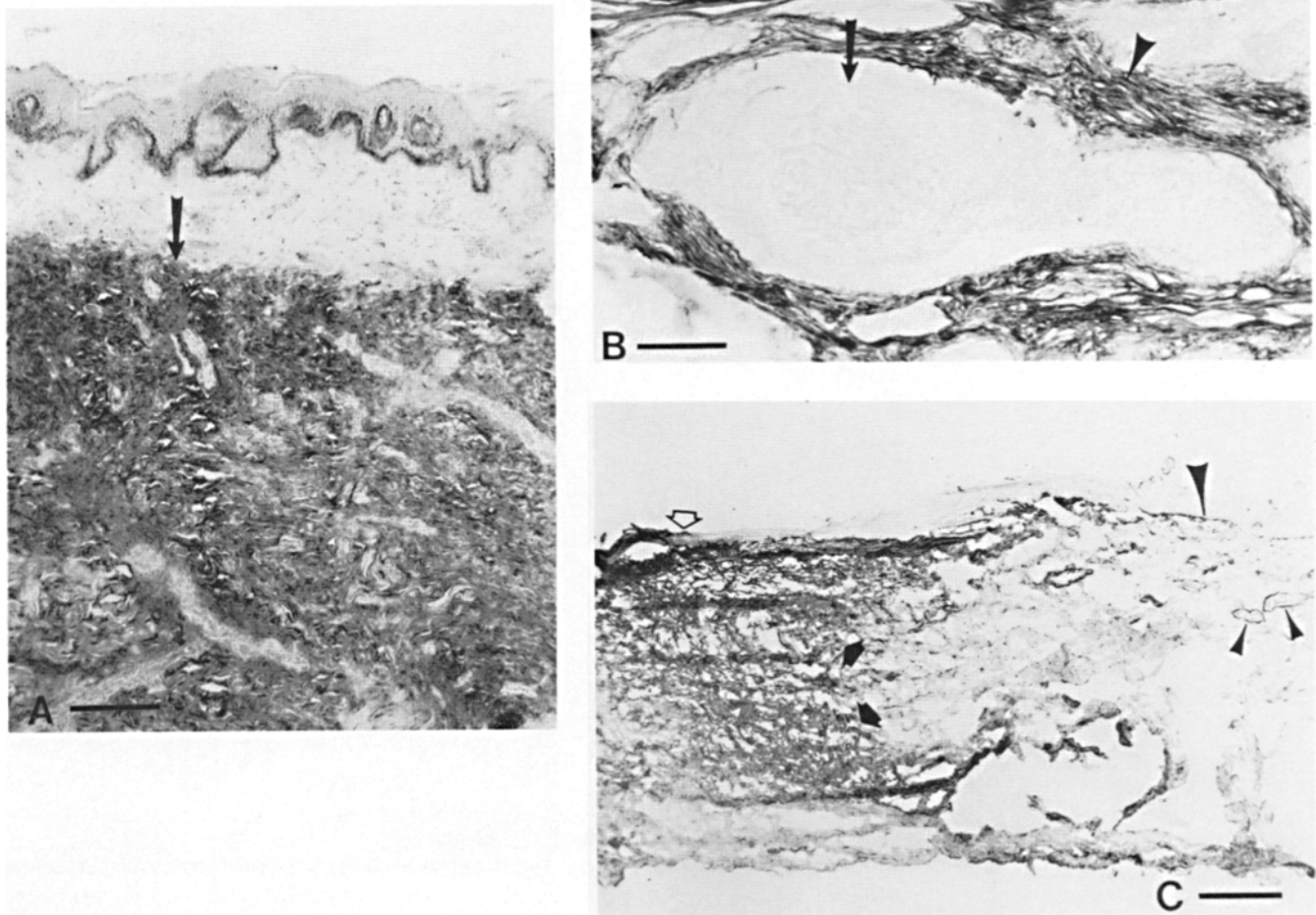


Figure 5. Indirect immunoperoxidase staining of 5- μ m sections of tumor matrix and granulation tissue in skin. (A) Dermatofibrosarcoma protuberans stained with affinity-purified antibody to tenascin. There is intense staining of the area involved with tumor (arrow) as well as the thin band of staining normally seen in the papillary dermis. (B) Invasive squamous cell carcinoma stained with monoclonal antibody 81C6 to tenascin. Note the prominent staining of the matrix surrounding the tumor islands (arrowhead) but no staining of the carcinoma cells (arrow). (C) Rat granulation tissue, 30 d after third degree burn, stained with affinity-purified antibody to tenascin. There is intense diffuse staining of the granulation tissue (solid arrows) that extends beneath the wound edge (open arrow). This is in marked contrast to the normal dermis at the right edge of the specimen that shows staining restricted to hair follicles (small arrowheads) and the DEJ (large arrowhead). Bars, (A and B) 200 μ m; (C) 400 μ m.

tenascin was extracted from the epidermal face of the dermal-epidermal junction (DEJ). While detectable amounts of tenascin were found in the extracts of the dermal face of the DEJ, these represented a small percentage of the total protein extracted (average 0.026%). However, because only part of the tenascin could be extracted from the dermis, we cannot quantitate the total tenascin found in skin. Whole dermis homogenates had less tenascin than extracts of the DEJ, in agreement with the histological studies which localized the protein to the zone adjacent to the DEJ. In contrast, an extract of a dermal tumor had significantly higher amounts of extractable tenascin than the whole skin or the DEJ extracts.

Discussion

The extracellular matrix proteins in the dermis of skin form the foundation necessary for maintenance of the structure and function of the skin. We have demonstrated that tenascin, a newly described 320-kD glycoprotein, is a structural component of normal adult dermis with a highly specific and unique distribution. Previous studies by Bourdon et al. (3) and Chiquet-Ehrismann et al. (10) failed to detect tenascin in adult mammalian skin, although studies by Crossin et al. (13) showed tenascin persisted at the dermal-epidermal interface in adult chicken skin. The failure of human skin ex-

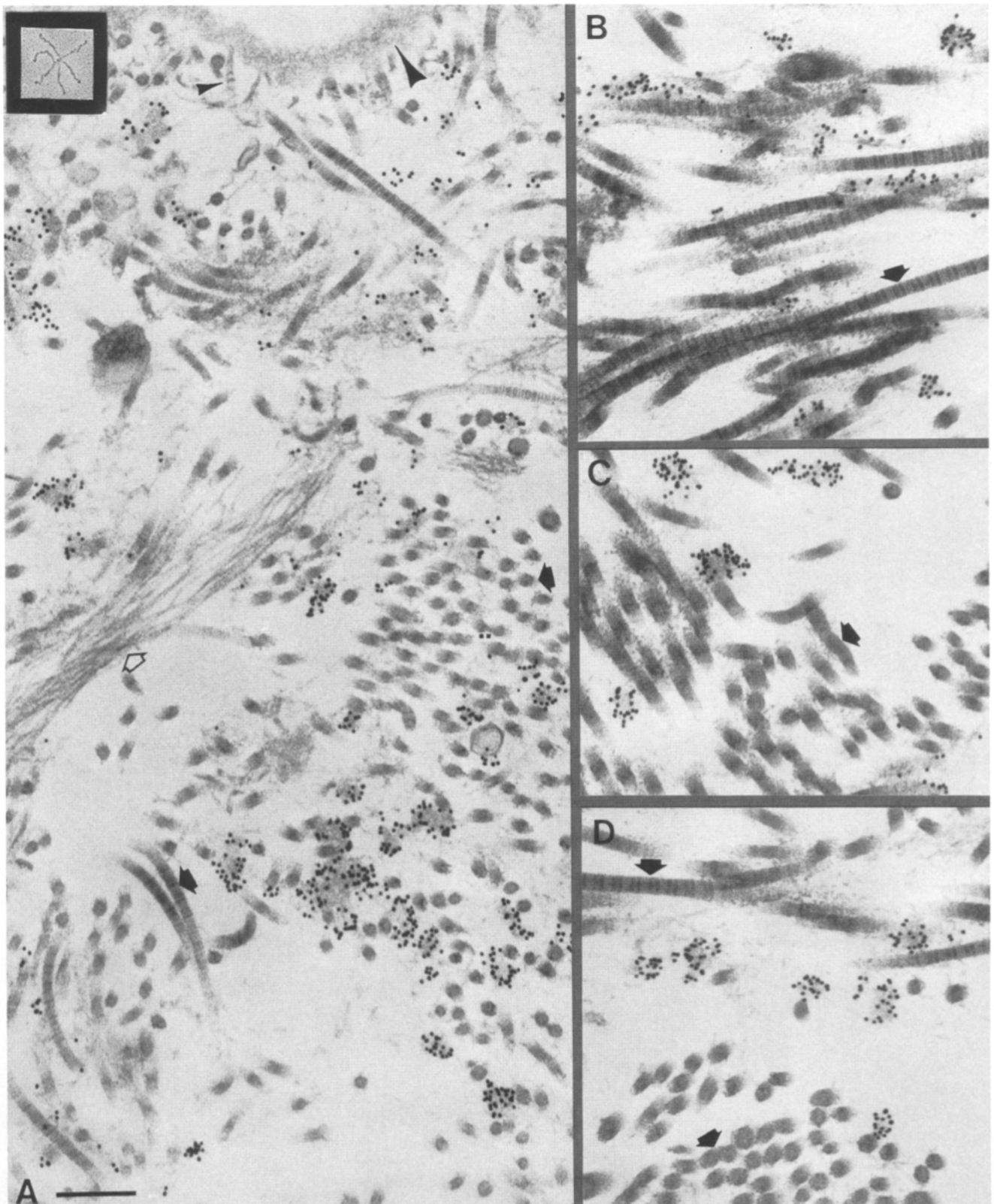


Figure 6. Electron microscopic immunolocalization of tenascin in the dermis of adult human skin. (A) Beneath the DEJ, small amorphous patches are labeled with 10-nm gold. These patches are not specifically associated with collagen (*solid arrows*) or elastic fibers (*open arrow*). No labeling of the basement membrane or immediate sublamina zone is seen (lamina densa, *large arrowhead*; anchoring fibril, *small arrowhead*). Insert shows a rotary shadowed hexabrachion molecule at the same magnification. (B), (C), (D) Deeper in the papillary dermis there are fewer patches, but they still show no obvious association with the collagen fibers (*solid arrows*). Bar, 0.2 μm .

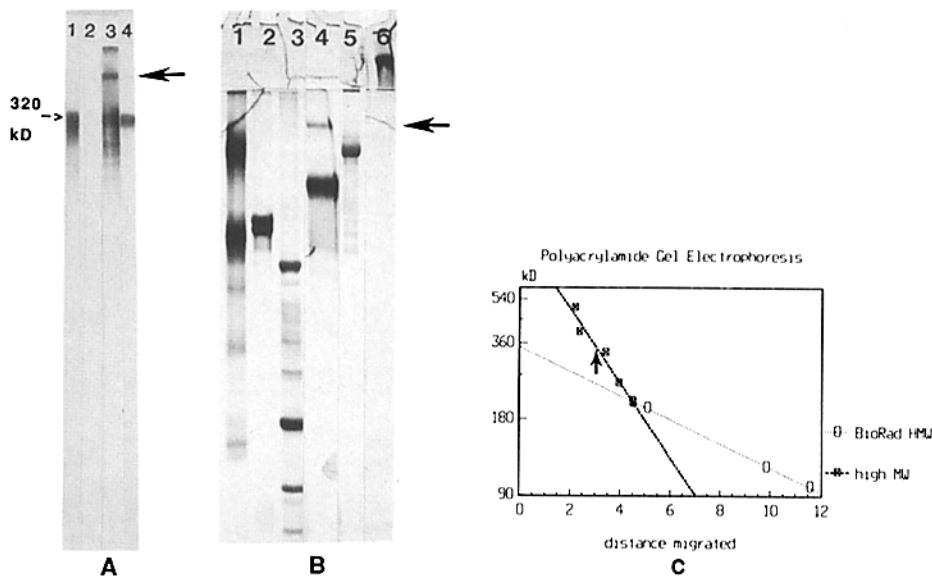


Figure 7. (A) Western blot analysis of CAPS extracts of the epidermal and dermal faces of saline split skin. Human skin was incubated in Scaletta buffer to separate the epidermis from the dermis within the lamina lucida as described in Materials and Methods. The basal lamina face was extracted with CAPS buffer and run on 5% SDS-PAGE before Western blotting. Lane 1 was loaded with purified tenascin from cultured glioblastoma cell line U-251 MG, lane 2 with the extract of the epidermal aspect of the DEJ, and lanes 3 and 4 were loaded with the extract of the dermal aspect of the DEJ. Lanes 1, 2, and 3 were blotted with affinity-purified polyclonal antibody. Lane 4 was blotted with the monoclonal antibody 81C6. (B) Silver-stained 5% SDS-PAGE on tenascin for molecular mass determination. Lane 1, reduced Engelbreth-Holm-Swarm tumor laminin (400 kD); 215 and 205 kD not individually resolved); lane 2, reduced human plasma fibronectin (250 kD); lane 3, Bio-Rad high molecular mass marker (200, 116.25, and 97.4 kD); lane 4, reduced tenascin that had been affinity-purified using monoclonal antibody 81C6; lane 5, nonreduced human plasma fibronectin (500 kD); and lane 6, nonreduced tenascin (does not enter main gel, band in stacker). The relative mobilities of these proteins were used to generate the graph in C for estimating the size of the tenascin monomer. (C) The relative mobility of tenascin (arrow). The gel estimates for tenascin range from 320 to 340 kD on this type of curve, which correlates with its comigration with reduced pig thyroglobulin (330 kD, not shown).

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tracts to immunoadsorb the monoclonal antibody 81C6 (3) was most likely because of the low tenascin content of skin relative to other tissues. In contrast to previous reports by Chiquet-Ehrismann et al. (10), we have found staining of adult rat skin very prominently around the hair follicles as well as less intensely along the DEJ. This discrepancy may reflect the lower level of staining of normal skin relative to that of the tumor matrix examined by Chiquet-Ehrismann et al., (10) as well as a poorer cross-reactivity of the rat tenascin with the antibody to chicken tenascin.

The function of tenascin in skin is not known. The prominence of this protein near the basal lamina in light microscopic studies suggests a possible role in maintenance of the DEJ. However, immunoelectron microscopy shows that tenascin is not an integral component of the basal lamina and lies beneath the anchoring fibrils. In addition, the tenascin staining reveals only discrete small patches and no evidence of organization into long fibers or fibrils. A complex network of tenascin that might play a structural role in maintaining the integrity of the subbasal lamina space cannot be ruled out. The inability to extract tenascin completely from the dermis might suggest some fraction of the hexabrachion molecules are cross-linked, possibly to other dermal components.

The pattern of tenascin staining does not reveal any obvious association with collagen fibers or elastic microfibrils to suggest a role in cross-linking these structures. A previous report on J1 protein had suggested tenascin staining of large collagen fibers as well as small collagen-associated fibers (42). Similar results were reported recently by Mackie et al. (35) for tenascin. However, these studies utilized a peroxidase reaction product to immunolocalize the tenascin. This product can diffuse small distances from the site of production and be adsorbed nonspecifically by adjacent structures

(38) so is not as specific as immunogold for electron microscopic localization. In our study, we found no immunogold labeling of collagen fibers or elastic microfibrils.

Recently, Fine et al. (19, 20, 25, 26) have reported on two new proteoglycan epitopes in the upper papillary dermis of skin, similar to what we have seen with tenascin. One of these epitopes is a heparan sulfate proteoglycan core protein (25, 26) and the second a complex proteoglycan bearing both heparan sulfate and chondroitin-6 sulfate side chains (19, 20). Several studies have suggested tenascin binds to chondroitin sulfate proteoglycans (9, 23, 47). It is possible that the tenascin in dermis is interacting with the proteoglycan matrix that is not stained in these immunoelectron micro-

Table I. ELISA Assay on CAPS Extracts of Skin

Extract	Total protein mg/ml	Tenascin μ g/ml	Percent of total protein that is tenascin
E1	0.603	<0.02	<0.003
D1	0.921	0.440	0.048
E2	0.53	<0.02	<0.004
D2	2.04	0.177	0.009
E3	0.584	<0.02	<0.003
D3	1.55	0.313	0.02
skin	16.6	<0.065	<0.0004
DFSP	26.5	17.20	0.065

Three separate skin samples were split at the DEJ, and the dermal and epidermal faces were extracted as described in Materials and Methods. In addition, homogenates of a sample of whole skin as well as a sample of a dermatofibrosarcoma protuberans were made in the same buffer. The extracts were analyzed for tenascin content and total protein as described in Materials and Methods. E1, 2, and 3 represent extracts of the epidermal face of the DEJ. D1, 2, and 3 represent extracts of the dermal face of the DEJ.

scopic sections. Double labeling studies with these new antibodies to the proteoglycan matrix and antitenascin antibodies should help define if there is colocalization of these antigens.

The tenascin staining of the ductal portion of the sweat glands suggests a tighter association with the basement membrane of these structures than with the DEJ. Because of poor preservation of these structures in the en bloc stained immunoelectron microscopy sections, we have not yet been able to visualize the precise localization of the tenascin in these ducts. At the light microscope level, the striking staining of the ductal portion of sweat glands but virtual absence of staining of the glandular or intraepidermal portions of the gland suggests a significant role for tenascin in the sweat ducts.

Several recent studies suggest tenascin may play a more significant role in the maintenance of epidermal histogenesis than as a structural component. E. Mackie et al. (35) have shown tenascin can promote chondrogenic and osteogenic differentiation *in vivo* and chondrogenesis *in vitro* in wing bud cultures from chick embryos. This differentiation is normally inhibited by fibronectin, suggesting tenascin modulates the effects of this protein. Other workers have also suggested a role for tenascin in modulating cell-fibronectin interactions (11). While some studies on purified tenascin have suggested tenascin binding to fibronectin (11, 23, 24), other laboratories find no evidence of direct tenascin-fibronectin binding (9, 34). Even though tenascin does not completely colocalize with fibronectin in skin, it is interesting to speculate that the presence of both of these proteins in the papillary dermis adjacent to the basal lamina is not coincidental.

Bourdon et al. (3) first noted the prominence of tenascin in the matrix of several carcinomas but absence of tenascin production by established carcinoma cell lines in culture. We have also observed prominent staining of the matrix of several carcinomas of epidermal origin (Fig. 6, and our manuscript in preparation). These observations, along with the unique localization of tenascin in normal dermis, suggest that fibroblast production of tenascin may be regulated by factors produced by the epidermis or carcinoma (16), a view also proposed recently by Inaguma et al. (28) in their work on rodent mammary tumors, Aufderheide et al. (1 and 2) in developing embryos, and Mackie et al. (36) in healing wounds.

Recently, studies reported by Jones et al. (29) and Pearson et al. (39) have shown tenascin contains a string of 13 repeats homologous to epidermal growth factor (EGF). These repeats have a high homology with the EGF-like repeats of the notch protein of *Drosophila* that may play a role in promoting differentiation of ectodermal cells into epidermal structures (48). It is also of note that many human glioblastomas, which show high levels of expression of tenascin (3), frequently exhibit amplification of the EGF receptor gene as well as a high degree of expression of the receptor (33, 49). The interaction of tenascin with these receptors and the effects of tenascin on keratinocyte growth and differentiation are currently under investigation in our laboratory.

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