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Regional differences in cell shape and gap junction expression in rat Achilles tendon: relation to fibrocartilage differentiation

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ABSTRACT

Tendon cells have complex shapes, with many cell processes and an intimate association with collagen fibre bundles in their extracellular matrix. Where cells and their processes contact one another, they form gap junctions. In the present study, we have examined the distribution of gap junction components in phenotypically different regions of rat Achilles tendon. This tendon contains a prominent enthesial fibrocartilage at its calcaneal attachment and a sesamoid fibrocartilage where it is pressed against the calcaneus just proximal to the attachment. Studies using DiI staining demonstrated typical stellate cell shape in transverse sections of pure tendon, with cells withdrawing their cell processes and rounding up in the fibrocartilaginous zones. Coincident with change in shape, cells stopped expressing the gap junction proteins connexins 32 and 43, with connexin 43 disappearing earlier in the transition than connexin 32. Thus, there are major differences in the ability of cells to communicate with one another in the phenotypically distinct regions of tendon. Individual fibrocartilage cells must sense alterations in the extracellular matrix by cell/matrix interactions, but can only coordinate their behaviour via indirect cytokine and growth factor signalling. The tendon cells have additional possibilities—in addition to the above, they have the potential to communicate direct cytoplasmic signals via gap junctions. The formation of fibrocartilage in tendons occurs because of the presence of compressive as well as tensile forces. It may be that different systems are used to sense and respond to such forces in fibrous and cartilaginous tissues.

Key words: Connexins; fibrocartilage; tendon; gap junctions

INTRODUCTION

Tendon cells are complex in shape and are arranged in short longitudinal rows within the tendon extracellular matrix (ECM). Laterally, they extend broad, sheetlike processes that make contact with similar processes from adjacent cells; longitudinally, cells at the end of rows have fine longitudinal processes that interact with the process network further along the tendon (McNeilly et al. 1996). There is therefore a 3-dimensional network of cell processes ramifying throughout tendons. The lateral cell processes wrap up collagen fibre bundles—each cell is associated with 5–8 fibre bundles and passes each bundle along to its longitudinally adjacent neighbours. This cellular organisation probably arises as a consequence of the sequence of events in the deposition of the oriented collagen matrix of the tendon in development (Birk & Zycband, 1994). It is important to appreciate this intimate association of cells and fibre bundles—the fibre bundles perform the force transmission function of the tendon, and the tendon cells maintain and modify the fibre bundles. Gap junctions, as shown by immunolabelling for their components connexin (cxn) 43 and cxn32, occur at points of contact between tendon cells, suggesting that the cell network is involved in cell–cell communication and coordination of function (McNeilly et al. 1996). There is a differential distribution of cxn43 and 32: cxn43 occurs at the points of contact of cells, whether they be between cells in the longitudinal cell rows, or between lateral cell processes from adjacent cell rows, or on the

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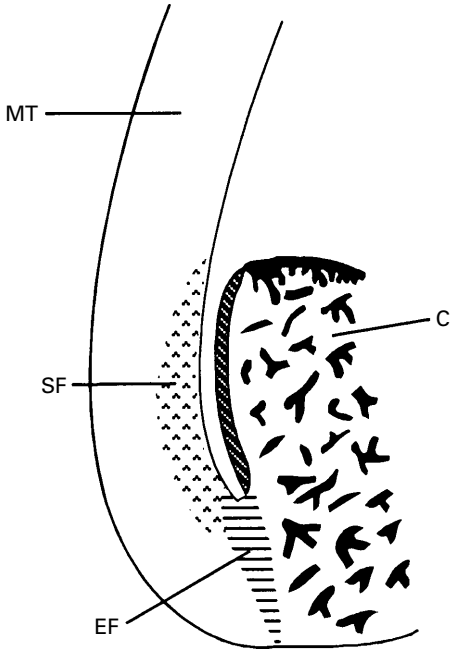


Fig. 1. Diagram to illustrate the organisation of tissues in the distal part of the rat Achilles tendon, showing the tendon midsubstance (MT), the sesamoid fibrocartilage (SF) where the tendon presses on the back of the calcaneus, and the enthesial fibrocartilage (EF) where it inserts into the calcaneus (C).

fine longitudinal cell processes. Cxn32 is usually found only between cells in the same longitudinal row. This suggests different functions for the gap junctions, as cxn32 and cxn43-containing junctions have different physiological characteristics (e.g. Veenstra et al. 1992; Elfgang et al. 1995). There is evidence that cxn43 may be important in the response of tendon cells to mechanical load, as cultured tendon cells upregulate cxn43 expression in response to tensile loading, and use gap junctions to propagate calcium fluxes from cell to cell when mechanically stimulated (Banes et al. 1995).

In the present study we have examined the regional distribution of gap junction components in tendons. In numerous sites, tendons experience compressive as well as tensile load. This occurs where they change direction by wrapping around a bony pulley, or as they approach their bony attachment. Where tendons are subject to compressive loading they become fibrocartilaginous (Vogel & Koob, 1989; Benjamin & Ralphs, 1995). Histologically, a range of fibrocartilaginous differentiation can be seen, ranging from a slight increase in matrix metachromasia with toluidine blue (i.e. increased glycosaminoglycans) in otherwise structurally normal tendon, to strongly metachromatic matrix with interwoven collagen fibres and rounded fibrocartilage cells (Benjamin et al. 1995). Biochemically, the fibrocartilage cells express cartilage type matrix macromolecules not normally seen in tendons, e.g. type II collagen and cartilage aggrecan. Such modifications in structure and matrix composition imply a profound change in the nature of the cell–cell and cell–matrix interactions.

We have chosen to study the distal part of the rat Achilles tendon. In previous studies of this and the human Achilles tendon (Rufai et al. 1992, 1995) we have shown that there are major changes in structure as the tendon approaches its attachment to the calcaneus (Fig. 1). The midsubstance of the tendon is typical regular dense fibrous connective tissue. As it approaches its attachment it passes over the back of the calcaneus, where it is pressed against the bone during dorsiflexion of the foot; the deep part of the tendon is fibrocartilaginous in this region. We have referred to this as the *sesamoid* fibrocartilage. The tendon then proceeds to its insertion, where it has a prominent *enthesial* fibrocartilage prior to its attachment to bone. Thus the pure tendon blends into sesamoid fibrocartilage and then into enthesial fibrocartilage, providing the opportunity to examine cell shape and gap junction expression in all of the variants of tendon and tendon fibrocartilage in a relatively small area. In this article we show altered cell-cell interaction and gap junction expression in fibrocartilage in comparison with pure tendon.

MATERIALS AND METHODS

Material

Achilles tendons were dissected from the hind feet of sexually mature (3–4 mo old; n = 18) male and female white Wistar rats. Specimens were briefly rinsed in phosphate buffered saline (PBS), fresh frozen onto cryostat stubs using dry ice (typical freezing time 5–10 s), and 20 μm cryosections cut in longitudinal or transverse planes. Longitudinal sections contained pure tendon, sesamoid fibrocartilage and enthesial fibrocartilage. Transverse sections were taken from each of these regions separately.

Cell staining with DiI

To examine the 3-dimensional shape of cells in the different regions of the tendons, unfixed cryosections were incubated for 10 min with the membrane label DiI (10 μM in PBS; Molecular Probes). Sections were then examined using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope (CLSM) set up for rhodamine illumination and detection (laser

wavelength 514 nm, beamsplitter 535 nm, barrier filter 535 nm). Using ×40 and ×60 objectives, optical section series were taken through the whole cryosection thickness and 3D projections prepared using Molecular Dynamics ImageSpace software running on a Silicon Graphics XS24 4000 workstation.

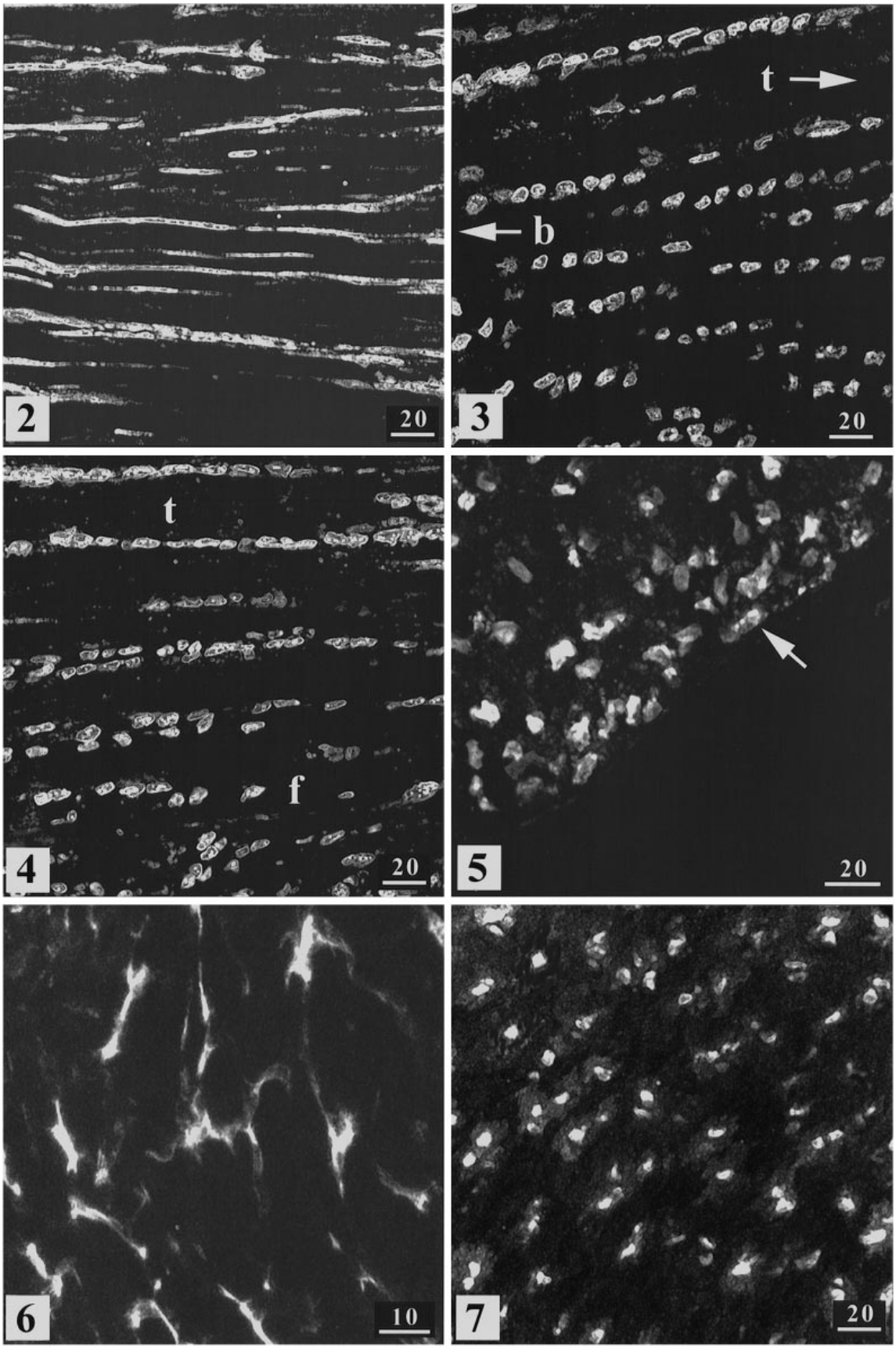
Immunolabelling and counterstaining

To investigate the distribution of gap junction proteins associated with cells in the different regions, cryosections were immunolabelled as in our previous study (McNeilly et al. 1996) by standard procedures for indirect immunofluorescence using monoclonal antibodies to cxn32 (10 μg/ml, Zymed; Goodenough et al. 1988) or cxn43 (2 μg/ml, Zymed; Beyer et al. 1988) as primary antibodies and sheep antimouse IgG Fab fragments/FITC conjugate (1:100; Sigma Chemical Co.) as the secondary antibody. Sections were counterstained with propidium iodide to provide a cellular context for the immunolabelling (0.5 μg/ml; Molecular Probes). Control sections were incubated with nonimmune mouse immunoglobulins (10 μg/ml) or the primary antibody was omitted. All sections were mounted in an aqueous mountant containing diazobicyclo 2,2,2 octane as fluorescence preservative (Johnson et al. 1982). Specimens were examined using the CLSM set up for dual channel fluorescence (laser wavelenth 488 nm, primary beamsplitter 510 nm, secondary beamsplitter 595 nm, detector filters 600 nm and 530 nm). Section series were taken and 3D modelling performed as above.

RESULTS

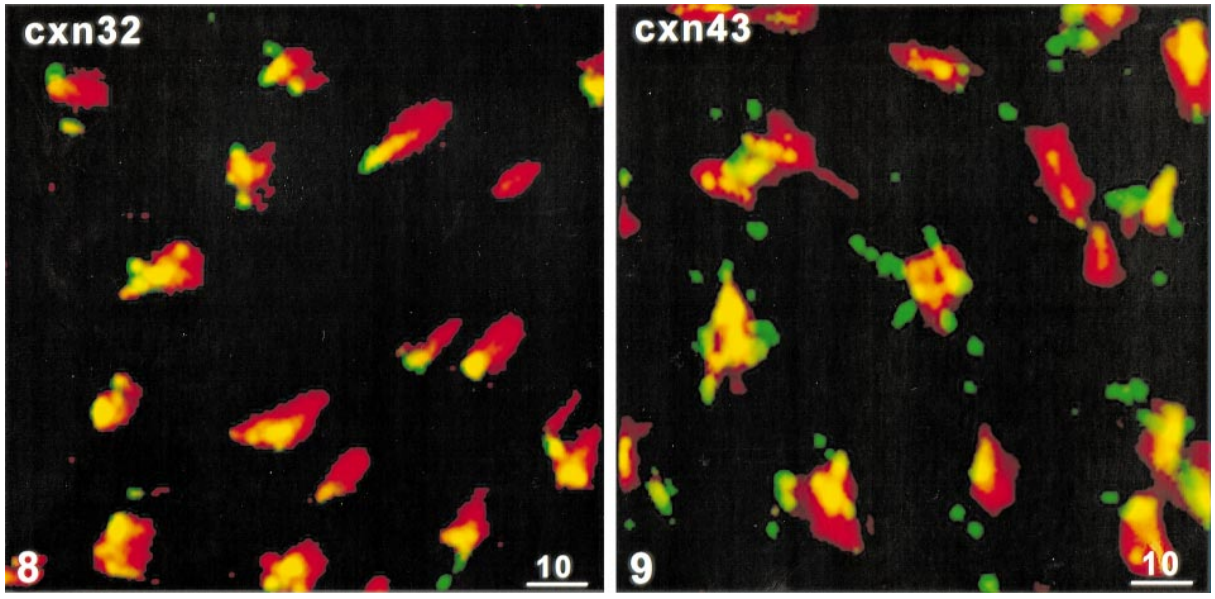
DiI staining revealed major differences in cell shape in the 3 regions studied. In longitudinal sections, midsubstance tendon cells were elongated and narrow (Fig. 2), although the precise longitudinal cell boundaries could not be seen. Lateral cell processes, being very thin, were not generally recognised in longitudinal sections (see transverse sections, below). In the enthesial fibrocartilage (Fig. 3) cells were oval in shape, but were still retained in their longitudinal rows. There was little contact between cells. In the region of the sesamoid fibrocartilage (Fig. 4) the tendon blended into the fibrocartilage. In the tendon, the part nearest the fibrocartilage proper contained oval cells with well defined boundaries, but still arranged in longitudinal rows. In the sesamoid fibrocartilage itself, the longitudinal arrangement was less obvious and eventually was lost approaching the articulating surface of the tendon. In transverse sections of the fibrocartilage at the articular surface, the fibrocartilage cells were more-or-less round, with a somewhat irregular outline (Fig. 5). There was little cell–cell contact and there were no obvious lateral cell processes. In transverse sections of midsubstance tendon (Fig. 6), cells were clearly stellate, with typical lateral cell processes enclosing collagen fibril bundles and interacting with similar processes from adjacent cells. In transverse sections of enthesial fibrocartilage (Fig. 7) cells had no lateral cell processes and were rounded.

Immunolabelling studies demonstrated variation in the occurrence of gap junctions. Junctions were particularly prominent in the midsubstance tendon, where both cxn32 and cxn43 labelling was present between cells in cell rows, and predominantly cxn43 containing junctions occurred between the lateral cell processes (Figs 8–10, 13). Cxn32 tended to form plaques of immunolabel between cells, made up of many very small foci of label (as described in McNeilly et al. 1996); cxn43 usually had a more discrete, punctate labelling pattern. High magnification views clearly showed the association of cxn32 with the cell bodies but not cell processes (Fig. 8), whereas cxn43 is clearly associated with lateral cell processes (Fig. 9). The tendon enthesis is a transitional zone containing 4 tissue types: pure tendon blends into uncalcified fibrocartilage, which in turn becomes calcified fibrocartilage, which then is attached to the bone. There was a clear transition in immunolabelling characteristics between the tendon and the enthesial fibrocartilage. As the tendon blended into the fibrocartilage, immunolabel was greatly reduced, with cxn43 label being lost before cxn32. The latter remained at points of contact between cells in cell rows until the cells had adopted their fully rounded morphology. In the main part of the uncalcified enthesial fibrocartilage, there was no label for either connexin (Figs 11, 14). Similarly, no label was observed in calcified fibrocartilage. Bone cells displayed foci of immunolabel for cxn43, presumably between cell processes in canaliculi (not shown). The gap junction label was also weak in the sesamoid fibrocartilage, with reduced cxn32 label and loss of cxn43 label; some cxn32 was present at the occasional points of contact between cells, however (Figs 12, 15). Away from the pressure bearing surface, the fibrocartilage blended into the overlying tendon; as it did so, immunolabel for gap junctions became apparent until at the farthest part from the source of compressive load (i.e. in the superficial part of the tendon),



Figs 2–7. Sections of Achilles tendon stained with the fluorescent membrane dye DiI. The images are projections of 3-D models generated by computer from confocal section series. All scale bars are labelled in μm.

Fig. 2. Longitudinal section of tendon midsubstance. Cells are narrow and elongate, with longitudinal cell boundaries not visible.



Figs 8–9. High magnification views of transverse sections of rat Achilles tendon midsubstance immunolabelled for gap junctions. Scale bars labelled in μm. Immunolabel for cxn32 (Fig. 8) consists of bright plaques of label associated with red stained cell bodies. Label for cxn43 (Fig. 9) consists of bright spots of immunolabel associated with cell bodies and cell processes (compare with Fig. 6).

the cells had the labelling characteristics of pure tendon cells.

DISCUSSION

This study clearly shows that tendon cells vary in their shape and expression of connexins according to their state of differentiation: where the tendon becomes fibrocartilaginous, cells become rounded and appear to reduce and eventually eliminate their cxn43 and cxn32 containing gap junctions. Thus, gap junction expression is altered with the change in tendon cell phenotype, and there is a fundamental change in the potential for direct cell–cell communication associated with tendon fibrocartilage differentiation. This pattern of junctional communication may be similar to that which occurs in the development of cartilage. Gap junctions are present between cells in embryonic mesenchyme (Makarenkova et al. 1997) and in the precartilaginous condensations (Dealy et al. 1994; Meyer et al. 1997), but once cartilaginous differentiation has occurred in the condensation (cells are physically separated by the ECM they are secreting to become recognisable chondrocytes) direct cell–cell communication clearly cannot occur. Gap junctions have been described in cultured chondrocytes (Donahue et al. 1995; D'Andrea & Vittur, 1996), although here cells have been removed from their matrix and plated into culture, reestablishing the possibility of direct membrane to membrane contact. They have also been reported in chondrocytes in rat calvaria (Jones et al. 1993), although as the authors point out this may be unusual for chondrocytes as the calvarial chondrocytes are generally close together and are often clustered in cell nests, giving direct membrane to membrane contact.

Fibrocartilage at the enthesis and in compressed regions of tendons and ligaments develops by meta-

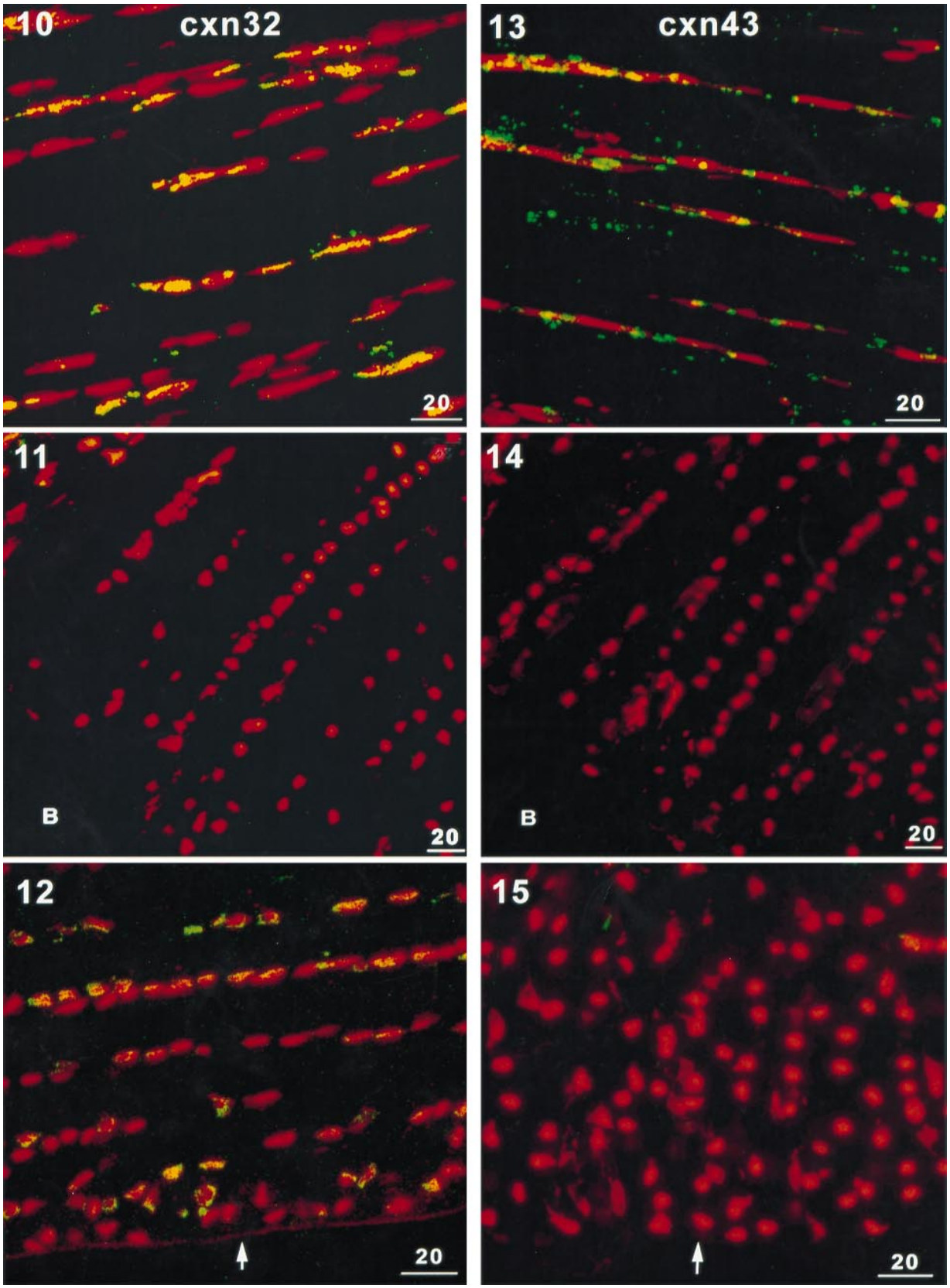
Fig. 3. Longitudinal section of enthesial fibrocartilage. Cells remain in longitudinal rows but are now rounded, with boundaries clearly visible. Direction of bony attachment indicated by 'b'; tendon by 't'.

Fig. 4. Longitudinal section of region of sesamoid fibrocartilage. The overlying tendon (t) blends into the fibrocartilage (f). The deep surface of the fibrocartilage is just beyond the bottom of the figure. Note that the fibrocartilage cells lose their organisation into longitudinal rows, and also that the fibrocartilaginous differentiation extends into the tendon—the cells in the region of the label 't' are more oval shaped and distinct than the pure tendon cells in Fig. 2.

Fig. 5. Transverse section of sesamoid fibrocartilage; arrow indicates articular surface. Cells are rounded, with few cell processes.

Fig. 6. Transverse section of tendon midsubstance. Tendon cells have many interconnecting lateral cell processes.

Fig. 7. Transverse section of enthesial fibrocartilage. Note rounded cells with few cell processes.



Figs 10–15. Sections of Achilles tendon immunolabelled for cxn32 (Figs 10–12) or cxn43 (Figs 13–15) and counterstained with propidium iodide. The images are projections of 3-D computer generated models of dual channel confocal section series. Scale bars are labelled in μm.

Fig. 10. Longitudinal section of tendon midsubstance, labelled for cxn32. Bright foci of immunolabel are associated with cells, mostly between cells within cell rows.

plasia of tendon or ligament cells (Vogel & Koob 1989; Rufai et al. 1992; Gao et al. 1996). Cells become progressively more rounded the closer they are to the highest compressive load: at the enthesis, approaching the mineralised bone/tendon interface at the attachment (Matyas et al. 1995), or in compressive regions approaching the pressure point against the back of the calcaneus. The freezing of unfixed material in the present study clearly does not cause any major cell shape changes, in comparison with conventional histological studies (Matyas et al. 1995; Benjamin & Ralphs, 1995). The DiI results suggest that cells become rounded by withdrawing their lateral cell processes, eliminating direct interaction between parallel rows of tendon cells, and then losing contact with cells in their own rows as they continue to round up. This could explain the observation that cxn32 labelling persists for longer than cxn43 when observing cells in regions of progressively greater fibrocartilaginous differentiation. Cxn43 occurs at sites of contact between lateral cell processes of adjacent cell rows, as well as between adjacent cells in the same cell row; cxn32, in contrast, usually occurs just at points of contact between cells of the same cell row (McNeilly et al. 1996). It may be that cxn43 expression ceases as the processes are withdrawn and contact is broken and cxn32 continues as contact between cells in the same row persists for rather longer.

The general function of gap junctions lies in the coordination of cell behaviour, e.g. the spread of excitation in heart or smooth muscle, coordination of ciliary beat, cell interactions in embryonic development, etc. (see Evans 1997). We suggested recently that the presence of gap junctions between the processes of tendon cells may be important in coordinating their response to mechanical load (McNeilly et al. 1996). The evidence was circumstantial, but was based on gap junction distribution on tendon cells and the intimate association of tendon cells with their extracellular matrix, coupled with the increased expression of cxn43 by tendon cells exposed to tensile loads in vitro (Banes et al. 1995). The regional variation in gap junctional expression indicates a variable ability for direct coordination of cell behaviour according to state of cell differentiation. The fibrocartilage cells, and by extrapolation cartilage cells in general, have fewer potential methods of coordination of behaviour than tendon cells. Cartilage cells must respond individually to their environment by cell–matrix interactions and with coordinating signals coming indirectly from other cells via cytokines and growth factors. Tendon cells will also have their individual interactions with their immediate extracellular matrix, and be influenced by local cytokines, but can also directly communicate via cytoplasmic electrical and chemical coupling via their gap junctions. This may also indicate a different mechanism for sensing and responding to mechanical load in tensile and compressively stressed connective tissues.

Gap junctions are also known to occur on the network of cell processes running in canaliculi between osteocytes (e.g. Jones et al. 1993; Lanyon, 1993). The present study clearly shows that there is no direct communication between bone and tendon cell networks: thus the fibrocartilage at the enthesis forms a barrier between cells in the two tissues. In development and growth, there is a close relationship between the behaviour of enthesial fibrocartilage and bone, particularly at epiphyseal attachments (Gao et al. 1996). Uncalcified and calcified fibrocartilage form part of a dynamic system which allows the growth in width of the epiphysis; essentially, endochondral ossification occurs through the calcified fibrocartilage. Clearly, direct interaction between bone cells and fibrocartilage cells is not necessary for this to occur.

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Fig. 11. Longitudinal section of enthesial fibrocartilage labelled for cxn32. There is no immunolabel in the fibrocartilage as the tendon approaches the bone (B).

Fig. 12. Longitudinal section in the region of the sesamoid fibrocartilage, labelled for cxn32. There are some labelled cells in the fibrocartilage and in the overlying tendon, although the amount of labelling is considerably less than in the tendon midsubstance. Arrow indicates the deep surface of the tendon.

Fig. 13. Longitudinal section of tendon midsubstance, labelled for cxn43. Bright foci of immunolabel occur between cells in the cell rows, and in the spaces between the cell rows.

Fig. 14. Longitudinal section of enthesial fibrocartilage labelled for cxn43. There is no immunolabel in the fibrocartilage as the tendon approaches the bone (B).

Fig. 15. Transverse section of sesamoid fibrocartilage immunolabelled for cxn43; articular surface towards bottom of figure (arrow). There is no immunolabel in the fibrocartilage.

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