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ORIGINAL PAPER

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Cultivation of human tenocytes in high-density culture

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Abstract Limited supplies of tendon tissue for use in reconstructive surgery require development of phenotypically stable tenocytes cultivated in vitro. Tenocytes in monolayer culture display an unstable phenotype and tend to dedifferentiate, but those in three-dimensional culture may remain phenotypically and functionally differentiated. In this study we established a three-dimensional high-density culture system for cultivation of human tenocytes for tissue engineering. Human tenocytes were expanded in monolayer culture before transfer to high-density culture. The synthesis of major extracellular matrix proteins and the ultrastructural morphology of the three-dimensional cultures were investigated for up to 2 weeks by electron microscopy, immunohistochemistry, immunoblotting and quantitative, real-time PCR. Differentiated tenocytes were able to survive over a period of 14 days in high-density culture. During the culture period tenocytes exhibited a typical tenocyte morphology embedded in an extensive extracellular matrix containing cross-striated collagen type I fibrils and proteoglycans. Moreover, expression of the tendon-specific marker scleraxis underlined the tenocytic identity of these cells. Taken together, we conclude that the three-dimensional high-density cultures may be useful as a new approach for obtaining differentiated tenocytes for autologous tenocyte transplantation to support tendon and ligament healing and to investigate the effect of tendon-affecting agents on tendon in vitro.

Keywords Tenocyte · High-density culture · Collagen type I · Quantitative PCR · Scleraxis

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Introduction

Tenocytes are highly specialised mesenchyme-derived cells responsible for the synthesis and maintenance of a mechanically unique connective tissue capable of withstanding the high tensile forces subjected to tendon in vivo (Josza et al. 1991). Tenocytes are embedded in an extensive three-dimensional network of extracellular matrix components consisting predominantly of collagen type I fibrils (>95% of tendon collagen), other types of collagen (type III and type V), proteoglycans, elastin and fibronectin (Bernard-Beaubois et al. 1997; Kannus 2000; Rees et al. 2000). These tendon-specific matrix components give tendon its resilience and biomechanical stability.

Under normal physiological conditions fully developed tendon is a poorly vascularised tissue with a low density of cells which exhibit low mitotic activities (Ahmed et al. 1998). This is considered to be one reason why tendon healing takes a considerably long time and in most cases it results in production of a mechanically inferior extracellular matrix in the tendon defect (Woo et al. 1999; Möller et al. 2000). Some attempts have already been made by tissue engineers to improve tendon rupture healing by transplantation of in vitro cultured tenocytes seeded in matrices or of in vitro differentiated autologous mesenchymal stem cells (Cao et al. 1994; Awad et al. 1999; Schulz-Torres et al. 2000; Koob et al. 2001). A critical drawback of cellular transplantation approaches using in vitro cultured tenocytes is the loss of differentiated function that occurs during prolonged monolayer culture (Schwarz et al. 1976). Extended monolayer culture of tenocytes has been reported to lead to decreased levels of

collagen type I and decorin transcripts within passages (Bernard-Beaubois et al. 1997). Previous work from our laboratory and several other groups has clearly demonstrated that three-dimensional culture models of mesenchyme-derived cells are capable of preventing dedifferentiation because they closely replicate the extensive cell–matrix interactions that occur in vivo. We have previously adopted high-density culture in vitro models to culture chondrocytes or mesenchymal cells of mouse limb buds and to redifferentiate dedifferentiated chondrocytes (Zimmermann et al. 1992; Shakibaei et al. 1993; Shakibaei and de Souza 1997; Shakibaei 1998; Schulze-Tanzil et al. 2002). However, since this approach has not yet been applied to tenocytes and it is not yet established if high-density culture favours the differentiated state of tenocytes in vivo some important questions arise: (1) Are tenocytes able to survive in three-dimensional culture? (2) Do they produce under these conditions tendon-specific extracellular matrix markers? Therefore, in order to address these issues, we have developed a high-density three-dimensional culture system for cultivation of phenotypically authentic human tenocytes in vitro. A combination of ultrastructural, biochemical and molecular tools was employed to establish the phenotypic identity of tenocytes in high-density culture. Specifically, the expression of scleraxis which has recently been reported as tendon-specific marker (Schweitzer et al. 2001; Edom-Vovard et al. 2002) was studied by quantitative, real-time PCR over a period of 14 days in high-density culture.

Materials and methods

Antibodies, growth medium and chemicals

Polyclonal anti-collagen type-I (AB749), polyclonal and monoclonal anti-fibronectin (AB1945/MAB122), monoclonal anti-β1-integrin (MAB1977), proteoglycan (MAB2005) antibodies and alkaline phosphatase-conjugated secondary sheep anti-mouse and sheep anti-rabbit antibodies for immunoblotting (AP303A, AP304A) were purchased from Chemicon International, (Temecula, CA, USA). Monoclonal anti-chondroitin sulphate (C8035) antibody was obtained from Sigma (Munich, Germany).

Goat anti-rabbit (GAR) and goat anti-mouse (GAM) FITC-linked secondary antibodies for immunofluorescence microscopy were purchased from Dianova (Hamburg, Germany). Gold-conjugated goat anti-rabbit immunoglobulin with 10-nm gold particles (GAR-10 nm) was purchased from Amersham (Braunschweig, Germany).

Growth medium [Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum, 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1% glutamine and 1% essential amino acids] was obtained from Seromed (Munich, Germany). OCT compound embedding medium and trypsin/EDTA (EG 3.4.21.4) were purchased from Sigma (Munich, Germany), and LR White and Epon were obtained from Plano (Marburg, Germany).

High-density culture

The peritendineum of a piece of human tendon (healthy finger tendon of one male middle-aged donor) obtained during tendon-rupture surgery, was carefully removed before culturing in growth medium for several days. After 1–2 weeks, tenocytes continuously migrated from this explant and adhered to Petri dishes. Three to five days later, when cell density had approached confluence, these cells were removed using 0.05% trypsin/1.0 mM EDTA and were multiplied for ten passages in monolayer culture to gain enough cells for introduction to high-density culture. For preparation of high-density cultures as described in detail previously for chondrocytes (Zimmermann et al. 1992; Shakibaei 1998), tenocytes were washed twice in growth medium and pelleted by centrifugation (1,200 rpm for 3 min). Eight microlitres of the cell sediment were pipetted onto a membrane filter with a pore diameter of 0.2 μm (Sartorius, Göttingen, Germany) on the top of a stainless steel grid at the medium–air interface in a Petri dish. Cultures were grown for 2 weeks at 37°C in a humidified atmosphere with 5% CO_2 and medium was changed every 3 days.

Light microscopy

Semithin sections of high-density cultures embedded in Epon or tenocytes monolayer on glass plates were stained for 1–2 min in 1% toluidine blue (Merck, Darmstadt, Germany), rinsed several times in aqua bidest. and examined under a light microscope (Axiophot 100; Zeiss, Jena, Germany).

Immunofluorescence microscopy

The high-density cultures were immersed in OCT embedding medium and immediately frozen in liquid nitrogen. Cryosections, 10 μm thick, were cut and fixed with methanol (10 min). Sections of cultures were overlaid with bovine serum albumin (BSA) [ambient temperature (AT), 10 min], rinsed with phosphate-buffered saline (PBS) and incubated with primary antibodies (1:30 in PBS) in a moist chamber overnight at 4°C. They were rinsed several times with PBS and incubated with secondary antibody [goat anti-rabbit or goat anti-mouse immunoglobulin conjugated with FITC (GAM- or GAR-FITC) diluted 1:50 in PBS] for 1 h at AT. The sections were washed three times with PBS, air dried and covered with Fluoromount mountant. Sections were examined under a light microscope (Axiophot 100).

Transmission electron microscopy

High-density cultures were fixed in Karnowsky for 1 h at AT followed by postfixation in 1% OsO_4 solution (in 0.1 M phosphate buffer). Cultures were rinsed and dehydrated in an ascending alcohol series before embedding in Epon. Sections were cut on a Reichert Ultracut and contrasted with 2% uranyl acetate/lead citrate. A transmission electron microscope (TEM 10; Zeiss, Jena, Germany) was used to examine the cultures.

Immunoelectron microscopy

High-density cultures were fixed in 3% paraformaldehyde and 0.25% glutaraldehyde in PBS for 1 h, rinsed in PBS/BSA and gradually dehydrated in ethanol. High-density cultures were embedded in LR White. Ultrathin sections were prepared and placed on nickel grids. Sections were incubated with 1% BSA in 0.01 M PBS, pH 7.0, and 0.5% Tween 20 at AT for 10 min. The sections were incubated with primary antibodies (anti-collagen type I) at a dilution of 1:40 in the PBS/BSA/Tween 20 solution overnight at 4°C. The sections were incubated with secondary antibody conjugated with GAR immunoglobulin with 10-nm gold particles (1:30) for 1 h at AT. Each step was followed by washing in PBS/BSA/Tween 20. Sections were contrasted with an aqueous saturated solution of 5% uranyl acetate for 20 min, osmium tetroxide for 5 min and 1% tannic acid for 30 min at AT. For inspection a Zeiss TEM 10 electron microscope was used.

Immunoblotting

Immunoblotting was performed as described previously (Shakibaei et al. 1999). Cells of high-density culture were extracted with lysis buffer [50 mM TRIS/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin and 1 mM PMSF] on ice for 30 min. Protein determination was done with the bicinchoninic acid system (Uptima; Interchim, Montlucon, France) using BSA as a standard. Samples of similar total protein concentration were separated by SDS-PAGE (5% or 7.5% gels) under reducing conditions and proteins were transferred onto nitrocellulose membranes. Membranes were preincubated in blocking buffer [5% (w/v) skimmed milk powder in PBS/0.1% Tween 20] for 30 min and incubated with primary antibodies (1:1,000 in blocking buffer) for 1 h at AT. Membranes were washed three times with blocking buffer before being incubated with alkaline phosphatase-conjugated secondary antibodies (1:5,000 in blocking buffer) for 30 min. Membranes were washed twice in blocking buffer and then three times in 0.1 M TRIS, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Specific antigen–antibody complexes were revealed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (*p*-toluidine salt; Pierce, Rockford, IL, USA) as substrates for alkaline phosphatase. Densitometric evaluation of immunoblots was performed using "Quantity one" (Biorad, Munich, Germany).

Statistical analysis

The results of immunoblotting were expressed as the means ± SD of a representative experiment performed in triplicate. The means were compared using Student's *t*-test assuming equal variances. $P<0.05$ was considered statistically significant.

Gene expression analysis

Total RNA was prepared from monolayer and high-density cultures using Tri Reagent (Sigma, UK). High-density cultures were ground up in the Tri Reagent using Molecular Grinding Resin (Geno Technology, St. Louis, USA). cDNA was synthesised from 1 μg of total RNA using M-MLV reverse transcriptase and random hexamers (both from Promega, Southampton, UK) in a 25-μl reaction. Aliquots (1 μl) were amplified by PCR in 25-μl reaction volumes on an MJ Research Opticon real-time PCR machine using a SYBR Green Core kit (Eurogentec, Seraing, Belgium) with gene-specific primers designed using ABI Primer Express software. Relative expression levels were normalised using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). Human primer sequences for real-time PCR have been described previously for GAPDH and collagen type I and type II (Martin et al. 2001). Primer sequences for scleraxis were as follows: forward 5′–3′ CCTGAACATCTGGGAAATTTAATTTTAC; reverse 5′–3′ CGCCAAGGCACCTCCTT. All primers were from Invitrogen, Paisley, UK. Primer specificity was confirmed both by sequencing of PCR products and confirmation of single product amplification using melting curve analysis. A positive control for chondrogenic cell phenotype was obtained from cDNA made from passage 2 human articular chondrocytes cultured for 2 weeks in pellet culture in chondrogenic media as described by Barbero et al. (2003).

Results

Cell culture

Explants of human tendon (about 3–5 mm) were cultured in a culture flask with growth medium. After 1–2 weeks typical tenocytes began to migrate from the tendon tissue, adhering to the culture flask and forming a monolayer (Fig. 1). This monolayer was passaged ten times to gain sufficient cells for the preparation of high-density cultures.

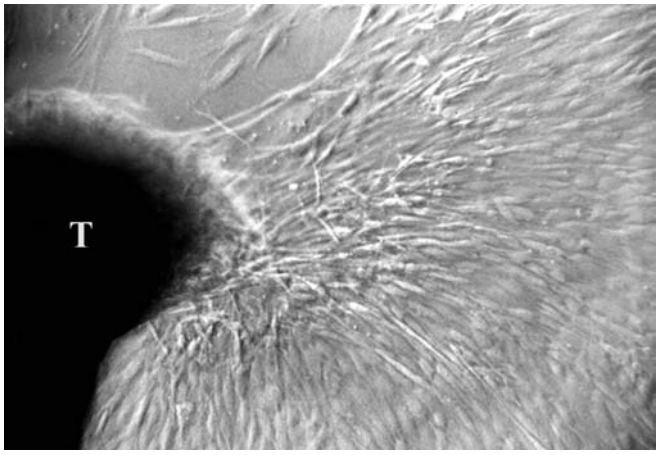

Fig. 1 The peritendineum of a piece of human tendon (*T*) obtained during tendon surgery, was carefully removed before the tendon was cultured in growth medium for several days. After a few days tenocytes migrated from this explant culture forming a monolayer. ×20

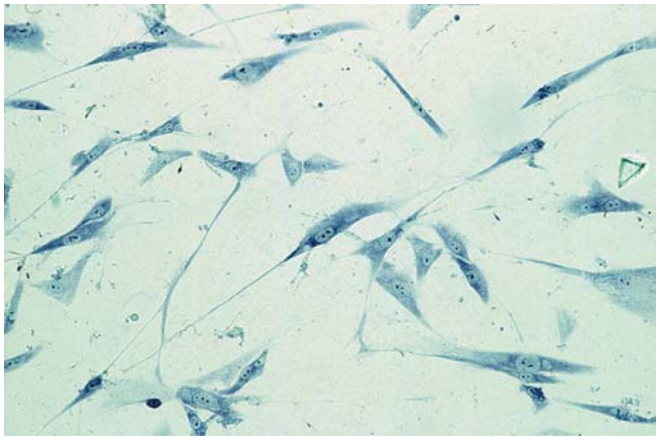

Fig. 2 Tenocytes in monolayer culture stained with toluidine blue. Tenocytes exhibited long cytoplasmic processes that formed contacts between cells. ×60

Light microscopy: monolayer and high-density cultures

In monolayer cells appeared like typical tenocytes. They exhibited long cytoplasmic processes with direct contact with neighbouring cells and a spindle-shaped nucleus (Fig. 2). On the first day of high-density culture cells made intimate contact. Over the following days intercellular spaces widened. Tenocytes formed aggregates of cells that had the same curled orientation surrounding nodules of cells (data not shown).

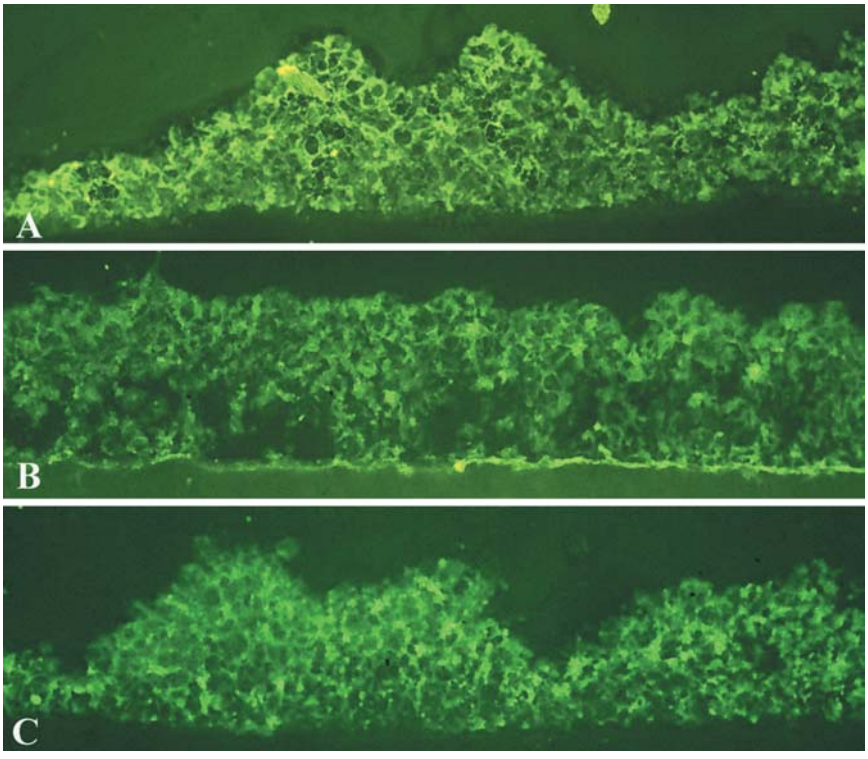

Fig. 3A–C Tenocytes cultured for 7 days in high-density culture labelled with anti-collagen type I (**A**), anti-fibronectin (**B**) and anti-proteoglycan (**C**) antibodies and examined by immunofluorescence microscopy. All the labelling observed was distributed in the extracellular matrix and at the cell surface. ×160

Immunofluorescence microscopy

Immunofluorescence microscopy of antibody-labelled cryosections of high-density cultures was performed to reveal the distribution of typical extracellular matrix components in tendon, for example collagen type I, fibronectin and proteoglycans (Fig. 3A–C). Labelling with anti-collagen type I-specific antibodies revealed the presence of collagen type I in high-density cultures of day 3 (data not shown), and increasing amounts of collagen type I on days 7 (Fig. 3A) and 14 (data not shown). Collagen labelling was localised around the tenocytes in the extracellular matrix. Fibronectin-labelling was detected at day 3 onwards (Fig. 3B). It was evenly distributed at the cell surface and in the extracellular matrix of tenocytes in high-density culture. Production of extracellular matrix proteoglycans could be demonstrated from day 3 onwards (Fig. 3C).

Transmission electron microscopy

On the first day in culture tenocytes made intimate contact; the cells had large, mostly euchromatic nuclei and exhibited a rounded shape. Cell organelles such as rER, mitochondria, granules and vesicles became visible (Fig. 4A). On day 3 cells exhibited a more spindle-like shape and increasingly detached from each other (Fig. 4B). Cells had an irregular elongated darkly stained nucleus and intercellular spaces widened. Cell organelles were clearly visible, i.e. rER, free ribosomes, Golgi apparatus, glycogen granules and vesicles. Cells developed many microvilli-like and sheet-like cytoplasmic processes. By means of these processes cells remained in contact with neighbouring cells. In these contact areas cell–cell contacts such as gap junctions were discernible (Fig. 4A, B). The first indication of an extracellular matrix was in the immediate pericellular space near the cell membrane (Fig. 4B). On day 7 many bundles of extracellular matrix fibrils were clearly visible in the newly formed extracellular matrix (Fig. 4C). Extracellular matrix fibrils formed bundles of parallel-orientated fibrils. Fibril bundles made contact with cell membranes and fibrils increased more and more on day 14 onwards (Fig. 4D). On day 14 intercellular spaces widened further and contained much more matrix. Typically cross-striated collagen fibrils that had tight contact to the cell membrane were visible, some of them arranged in bundles along the tenocytes and others orientated more transversally (Fig. 4D).

Immunoelectron microscopy

To further characterise the composition of fibrils of the tenocyte extracellular matrix, immunoelectron microscopy was performed. Matrix fibrils of 7- and 14-day-old high-density tenocyte cultures were specifically labelled with gold-conjugated secondary antibodies directed against anti-collagen type I primary antibodies. Gold particles were evenly distributed all over the fibril network in the extracellular matrix of tenocytes cultured for 7 days in high-density culture as shown in Fig. 4E. On day 14, cross-striated extracellular matrix fibrils were clearly labelled with gold particles demonstrating that these fibrils consist of collagen type I as shown in Fig. 4F.

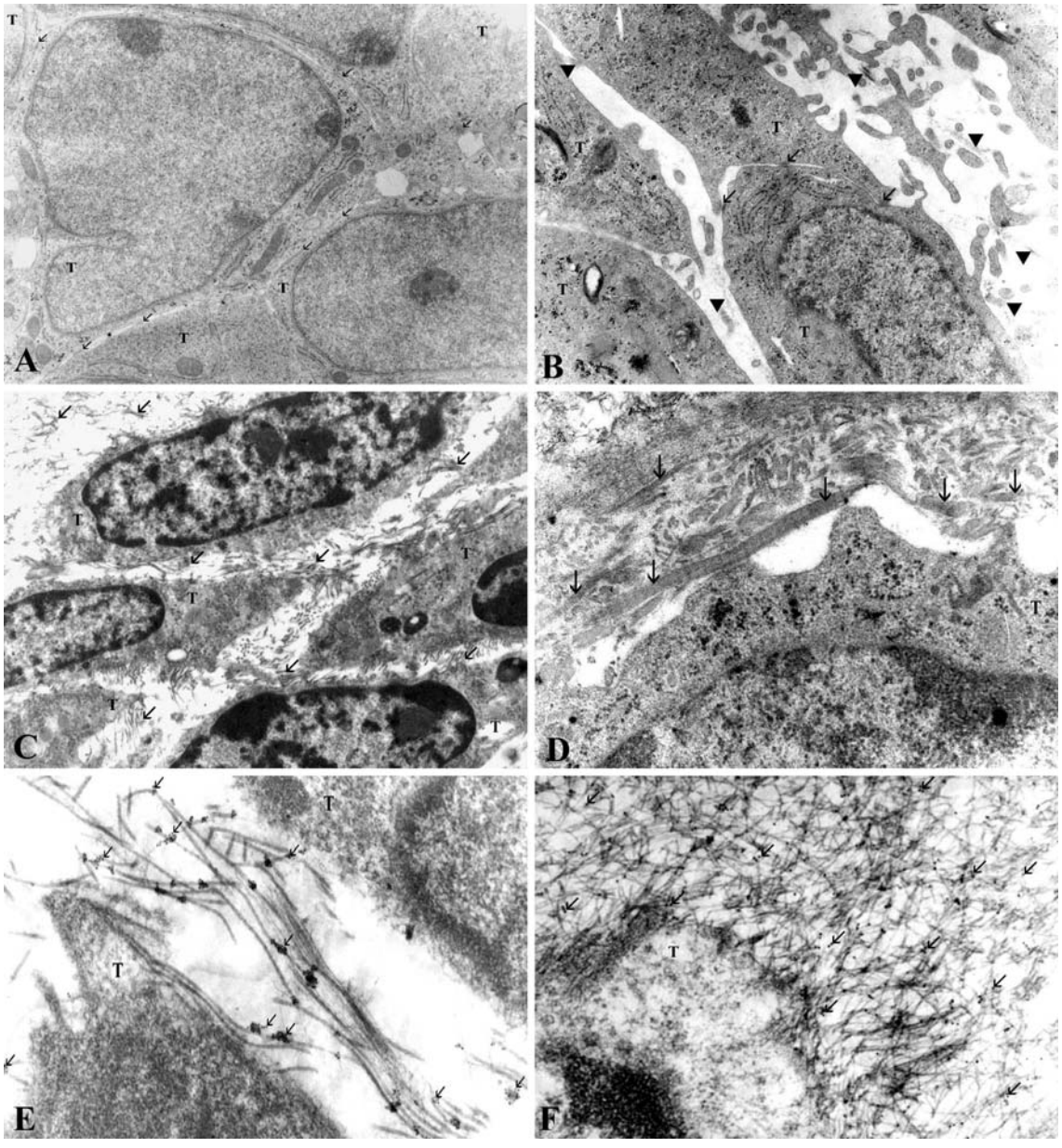

Fig. 4A–F Day 1 (**A**) tenocytes (*T*) aggregate together in intimate contact and exhibit a rounded morphology with large (mostly euchromatic) nuclei (**A**). On day 3 (**B**) cells become elongated and more detached from each other. Some cell organelles became clearly visible (for example, rER) and also cell–cell contacts such as gap junctions (**A**, **B**; *arrows*). Intercellular spaces widened further and many small microvilli-like and large sheet-like cytoplasmic processes became evident. The first signs of an extracellular matrix were visible in the pericellular space near the cell membrane (**B**; *arrowheads*). On day 7 (**C**) bundles of extracellular matrix fibrils were clearly visible (**C**; *arrows*) in the matrix. Intercellular spaces contained much more matrix on day 14 (**D**). Collagen fibrils formed bundles of parallel-orientated fibrils (*arrows*) that were typically cross-striated and had tight contacts with the cell membrane (**D**). To characterise more clearly the fibrils of the extracellular matrix, immunoelectron microscopy was performed. Matrix fibrils of 7-day-old (**E**) and 14-day-old (**F**) high-density tendon cultures were specifically labelled with immunogold-coupled secondary antibodies directed against anti-collagen type I primary antibodies. Extracellular matrix fibrils were specifically labelled with immunogold particles (*arrows*) demonstrating that fibrils consisted of collagen type I. **A–F** ×5,000

Immunoblotting

Immunoblotting was employed to reveal the composition and quantity of tenocyte extracellular matrix in high-density cultures. Investigation of 1-, 3-, 5-, 7- and 14-day old high-density cultures showed production of collagen type I (Fig. 5), fibronectin (data not shown), proteoglycans (Fig. 6) and β1-integrins (Fig. 7). The synthesis of all of these matrix proteins and β1-integrins appeared to increase continuously in this culture system until day 14. Collagen type I and proteoglycans were produced from the first day. Weak production of fibronectin could be observed for the first time on day 3 of culture. On day 5 production of fibronectin was clearly visible (data not shown). Results of densitometry revealed increasing

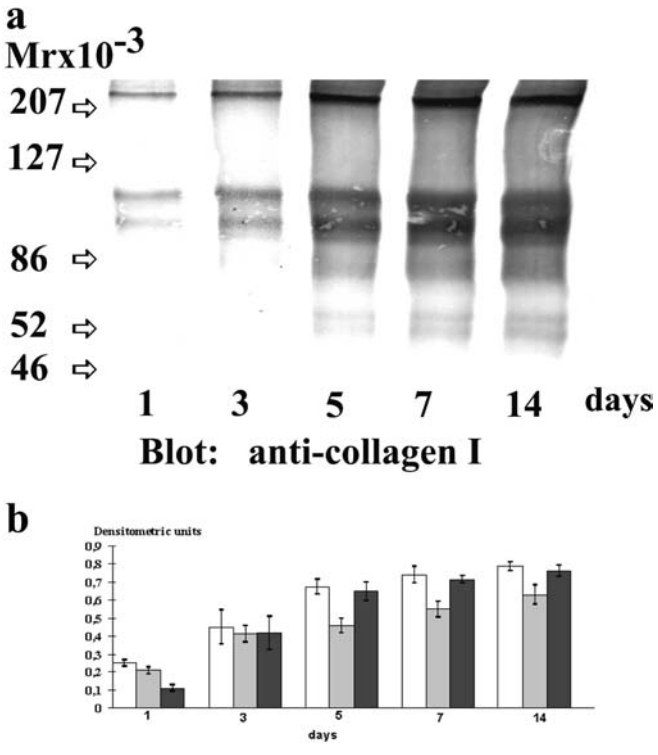

Fig. 5a, b Evidence for the presence of collagen type I in high-density culture revealed by immunoblotting. Tenocytes from high-density cultures were lysed and equal amounts (50 μg protein) of total protein were separated by 5% SDS-PAGE gels and analysed by immunoblotting with anti-collagen type I antibodies (**a**). Quantitative densitometry (**b**) demonstrates increasing deposition of collagen type I in high-density cultures up to day 14. Values are means ± SD of a representative experiment performed in triplicate. Data shown are representative of three independent experiments. *White, grey and solid bars* represent different molecular forms of the same protein

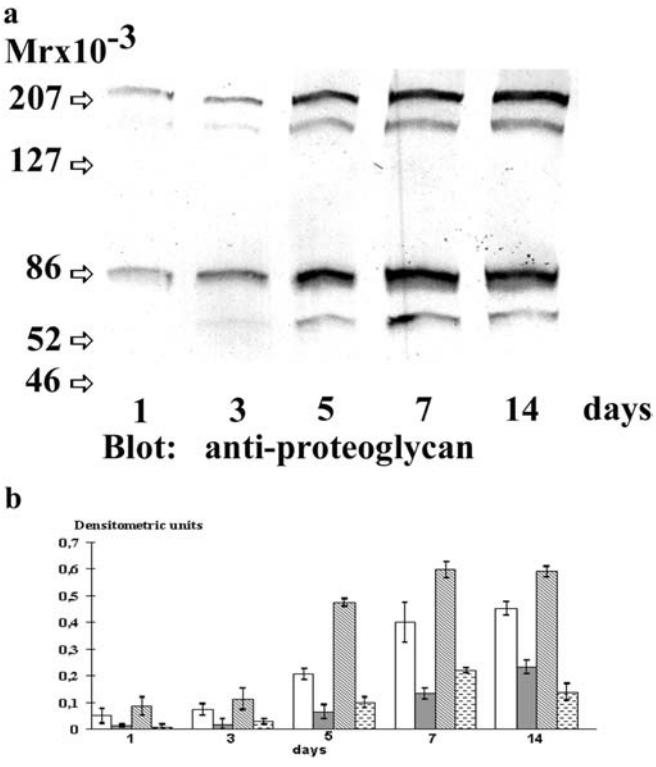

Fig. 6a, b Evidence for proteoglycan production in high-density culture revealed by immunoblotting. Fifty micrograms of total protein were separated by SDS-PAGE on 5% gels and immunoblotted with anti-proteoglycan antibodies (**a**). Quantitative densitometry (**b**) reveals increasing deposition of proteoglycans in high-density cultures until day 14. Values are means ± SD of a representative experiment repeated in triplicate. Data shown are from three independent experiments. *White, grey and solid bars* represent different molecular forms of the same protein

production of these matrix proteins until day 14 (Figs. 5b, 6b, 7b).

Quantitative, real-time PCR

Expression of transcripts of collagen type I, collagen type II and the tendon marker scleraxis relative to the housekeeping gene GAPDH was determined by real-time PCR in tenocyte high-density cultures compared to chondrocyte pellet cultures which were used as a control. Gene expression of collagen type I showed a slight decrease after day 1 until day 14 in high-density culture (Fig. 8).

Collagen type II expression was investigated to exclude transdifferentiation of tenocytes to fibrochondrocytes. Low level expression of collagen type II could also be demonstrated in tenocyte high-density cultures which was comparable to the expression in tenocyte monolayer cultures, but the expression observed was a thousand times lower than that in chondrocyte cultures (Fig. 9). The expression of collagen type II by tenocytes has already been described by Martin et al. (2003) in the distal region of tendon and in tendon fibroblasts of tendon sheath, epitenon and endotenon (Klein et al. 2001). Scleraxis expression increased during the whole culture period of tenocytes in high-density culture (Fig. 10).

Discussion

The results we have presented in this paper indicate that tenocytes are able to survive over a 2-week period in the high-density culture system. Ultrastructural investigations confirmed morphological characteristics of typical spindle-shaped tenocytes embedded in an increasing extracellular matrix containing cross-striated thick collagen type I fibrils. Tenocytes cultured in the high-density system also expressed the tendon-specific marker scleraxis.

Since tenocyte transplantation and tendon tissue engineering may have potential as future approaches to support tendon rupture healing, fully differentiated vital tenocytes with the capacity for producing a tendon-specific extracellular matrix will need to be cultivated for repair of tendon defects. Especially important is the synthesis of collagen type I fibrils by transplanted tenocytes for conferring mechanical stability (tensile strength) to

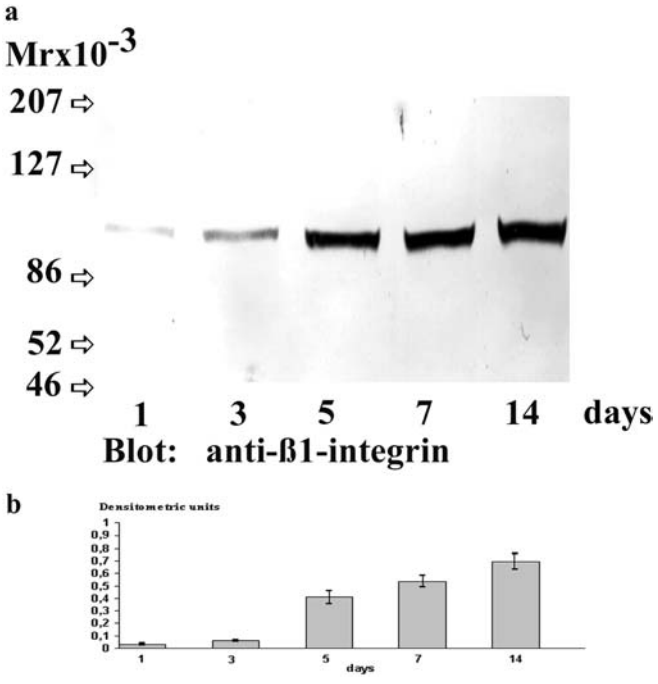

Fig. 7a, b β1-integrin synthesis in high-density cultivated tenocyte up to day 14 revealed by immunoblotting (**a**). Cell lysates (50 μg total protein) were separated by SDS-PAGE on 7.5% gels and immunoblotted with anti-β1-integrin antibodies. Quantitative densitometry (**b**) shows increasing expression of β1-integrin in high-density cultures until day 14. Values are means ± SD of a representative experiment performed in triplicate. Data shown are representative of three independent experiments

regenerated tendon. Evans and Trail (1998) observed that tendon cells naturally form three-dimensional structures during in vitro culture. Accordingly, we have tested a three-dimensional, high-density system for tenocyte cultivation. In the first few days of culture we noticed many similarities between tenocytes and chondrocytes in high-density cultures which included formation of densely packed cells with tight intercellular surface contacts (Schulze-Tanzil et al. 2002). We were able to demonstrate the presence of cell–cell contacts such as gap junctions by electron microscopy in the first days of tendon high-density cultures. Gap junctions localised to cytoplasmic processes of tenocytes are important in tendon tissue to allow cell communication throughout an extensive extracellular matrix (D'Andrea and Vittur 1997; Banes et al. 1999; Benjamin and Ralphs 2000). In the days that followed the cells became detached from one another by the produced matrix.

The extensive cell–cell interactions during the first step of connective tissue differentiation are crucial and are mediated by cell adhesion molecules and surface receptors such as cadherins and integrins (Bee and von der Mark 1990; Widelitz et al. 1993; Oberlender and Tuan 1994; Tavella et al. 1994; Shakibaei 1998; Mobasheri et al. 2002). However, it is still not clear whether integrins play an important role during the early stages of differentiation of tenocytes in this culture system as has been previously shown for chondrocytes (Shakibaei 1998). Indeed, integrins have been reported by Martin-Bermudo (2000) to be involved in tendon cell differentiation in *Drosophila* embryos. In our high-density culture model we observed a continuous increase of β1-integrins until day 14; adhesion molecules such as integrins play an essential role in interactions between matrix components and cells. Even on the first day production and expression of collagen type I was evident as shown by immunoblotting and PCR. The onset of matrix synthesis correlated with prominent cell organelles observed by electron microscopic investigation. Benjamin and Ralphs (2000) reported that rER and Golgi complexes are particularly prominent during early development, when these cells produce large numbers of collagen fibres. We found

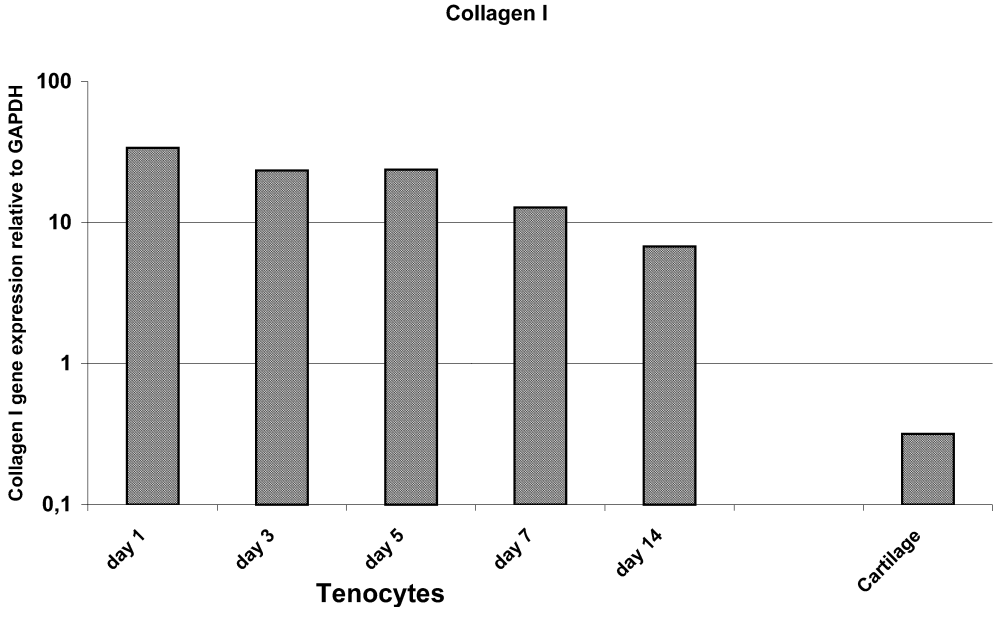

Fig. 8 Collagen type I expression relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) in tenocyte high-density cultures up to day 14 investigated by real-time PCR. Continuous expression of collagen type I could be shown in tenocytes by real-time PCR up to day 14 which was more than ten times higher than in chondrocyte pellet cultures

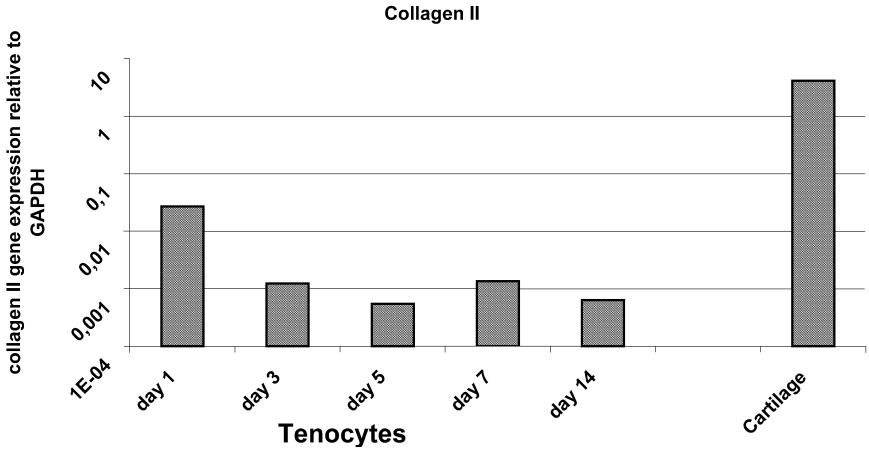

Fig. 9 Collagen type II expression relative to the housekeeping gene GAPDH in tenocyte high-density culture up to day 14 was demonstrated by real-time PCR. Expression of collagen type II in tenocytes decreased significantly after day 1 in high-density culture and was more than 100 times lower than chondrocyte pellet cultures

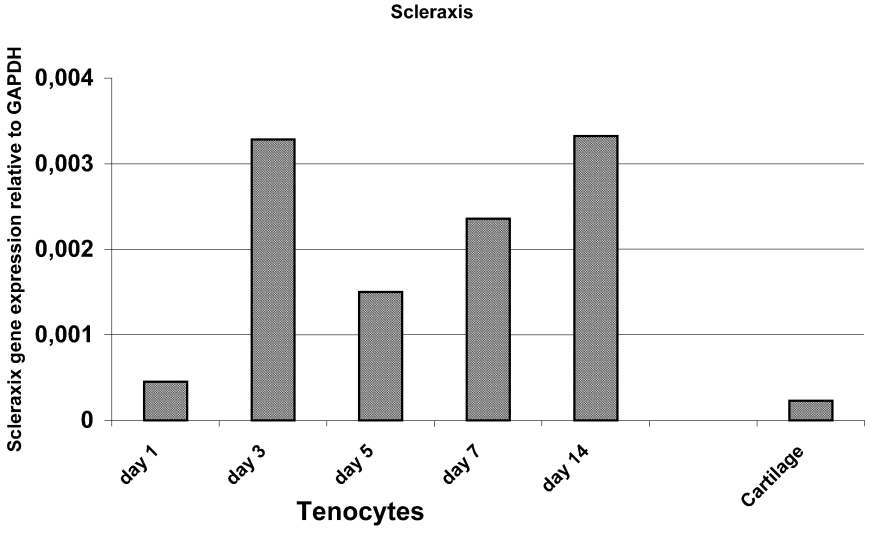

Fig. 10 Expression of the tendon marker scleraxis relative to the housekeeping gene GAPDH in tenocyte high-density culture was investigated up to day 14 by real-time PCR. Robust expression of scleraxis was evident that was lower on day 1, peaked on day 3 and continuously increased thereafter until day 14. Scleraxis expression was significantly higher than in chondrocyte pellet cultures

typical cross-striated collagen type I fibrils that were further characterised by immunoelectron microscopy with anti-collagen type I antibodies. Extracellular matrix increased during the whole culture period in high-density culture as shown by western blot analysis. Cells produced an ECM that was indistinguishable from that of primary tenocytes demonstrating the differentiated state of cells.

Tenocytes are able to transdifferentiate to fibrocartilage cell lineage (Rooney et al. 1993; Ehlers and Vogel 1998). Therefore, we tested also the expression of collagen type II, the cartilage-specific collagen type. We found some collagen type II expression that was comparable in tenocyte monolayer and high-density cultures, but low compared to chondrocyte pellet cultures. Other authors have reported collagen type II expression in tendon in vivo (Klein et al. 2001; Martin et al. 2003). Electron microscopic investigation did not reveal morphological features of fibrocartilage such as rounded cells, but typically elongated spindle-shaped tenocytes with darkly stained nuclei. Moreover to confirm tenocyte lineage, expression of the tendon-specific marker scleraxis (Schweitzer et al. 2001; Edom-Vovard et al. 2002; Salingcarnboriboon et al. 2003) was investigated and could be demonstrated by quantitative, real-time PCR. Scleraxis is a basic helix loop helix type transcription factor. This transcription factor family regulates growth and differentiation of numerous cell types. Scleraxis plays an essential role in mesoderm formation (Brown et al. 1999). It marks the tendon progenitor population that forms the fourth somitic compartment the "syndetome" and is continuously expressed through differentiation into the mature tenocyte and ligament cells (Schweitzer et al. 2001; Brent et al. 2003). Moreover, scleraxis null-mutant embryos are unable to form mesoderm (Asou et al. 2002). In this study scleraxis expression increased from day 1 to day 14 which may be indicative of tenocyte differentiation in high-density culture.

We conclude that the high-density culture is an attractive model for long-term cultivation of human tenocytes in vitro. This culture system seems to promote differentiation of tenocytes. It may serve as a new model system to test the effects of drugs that influence tendon biology in vitro and could also be useful for autologous tenocyte cultivation. We propose that growing proliferating tenocytes in monolayer culture for several passages will permit investigators to obtain sufficient quantities of

cells for subsequent introduction to high-density culture. Tenocytes cultured in this way may be used for autologous tenocyte transplantation and improve healing of tendon defects or in studies aimed at furthering our understanding of the biology of these cells.

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