UNIVERSIDADE ESTADUAL DE CAMPINAS

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Desenvolvimento do tendão elástico de aves: Características estruturais e aspectos ultra-estruturais relacionados à região elástica e à fibrocartilagem

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Biologia Celular

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Dedico este trabalho

À minha família
Meu pai Edgar (in memorian)
minha mãe Ivone, minha avó Antonina e
minhas irmãs Lidiane e Luciane,
Pelo imenso carinho.
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dedicado e por estar sempre
ao meu lado, tornando cada dia melhor.

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"Nossos fracassos são,

às vezes,

mais frutíferos

que os êxitos"

(Henry Ford)

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Resumo

Embora os tendões realizem o papel mecânico de transmitir forças de tensão geradas pela contração muscular ao osso no qual ele se insere, muitas vezes há funções ou propriedades adicionais frente a uma demanda biomecânica diferenciada.

A região elástica corresponde à maior parte do tendão elástico da asa de frangos e é responsável pelas propriedades mecânicas principais do tendão. Entretanto, a presença de um sesamóide fibrocartilaginoso torna o tendão elástico num excelente modelo de diferenciação de regiões distintas ao longo de um tendão, principalmente quanto à investigação dos fatores que levam à diferenciação destas duas regiões modificadas: a região elástica e a fibrocartilaginosa. Este projeto teve por objetivo a caracterização da capacidade do envolvimento de células, a nível estrutural e ultraestrutural, quanto aos aspectos relacionados à elastogênese. Estes aspectos seriam importantes pois auxiliariam no entendimento de interrelações existentes entre elastina-colágeno-proteoglicano. Esta análise é de grande relevância para a biologia dos tecidos elásticos, pois o tipo de interações observados entre as células e as fibras elásticas, não é evidente para outros tecidos como aorta e ligamento nucal. Para isto análises em microscopia de luz usando várias técnicas de coloração para identificar a matriz extracelular em geral, e algumas particularidades, assim como análises ultraestruturais desde a fase embrionária, e técnicas imunocitoquímicas foram usadas. Neste trabalho, pudemos demonstrar que as células da região elástica tem um ativo papel não somente na síntese dos componentes da matriz extracelular mas também estão envolvidas com o estabelecimento de domínios distintos e específicos para a formação de fibras de colágeno e fibras elásticas, bem como a organização de elementos fibrilares na região elástica. Dois tipos celulares predominantes são encontrados na fibrocartilagem. Células parecidas com fibrocondrócitos são encontradas junto aos fibroblastos ou ocupando diferentes domínios onde, aparentemente, estão envolvidas

com a síntese de componentes específicos da matriz extracelular. Com o desenvolvimento ocorre um progressivo acúmulo de proteoglicanos na matriz extracelular, que são inicialmente difusos, mas depois ficam restritos à matriz pericelular dos fibrocondrócitos. Há um progressivo espessamento das fibras e feixes de colágeno, que se arranjam com um padrão de rede de basquete. Associados à superfície destas fibras e feixes de colágeno existem fibras elásticas. Embora estas diferentes regiões pareçam se formar diante de diferentes estímulos/necessidades mecânicas, a natureza específica destes fatores não pode ser ainda identificada.

Abstract

Tendons are usually suited for the transmission of mechanical forces from the muscle of origin the the bone of insertion. However, in many instances there are additional functions and properties differing from the tendon ordinary array, facing different biomechanical demands. The elastic tendon of the chicken wing has an elastic region that corresponds to a large extent of its length and is responsible for the main mechanical properties of this tendon. However, the presence of a fibrocartilaginous sesamoid turns this tendon in an excelent model of the lengthwise variation in tendon morphology and, specially, with to the investigation of factors leading to the development of the elastic and fibrocartilaginous regions. This work has as objective the characterization of the developmental steps of the elastic region and fibrocartilage, with emphasis in the participation of cell in the collagen fibrillogenesis and elastogenesis, at the structural and ultrastructural levels. These aspects are important because they could help understanding the relationships between elastin-collagen-proteoglycans in these complex systems. This work was also considered important, because the type of cell-matrix interactions observed before, are not found in the tissues like the aorta or nuchae ligament. The methodology involved light microscopy of tissue sections after different cytochemical stainings, immunocytochemistry and transmission electron microscopy applied to samples taken from embryos and post-hatched individuals.

In this work we could show that: 1) the cells of the elastic region has an active role not only in the synthesis of the extracellular components but also are involved with the establishment of specific and distinct domains for the formation of collagen and elastic fibers, as well as are involved with the isolation and organization of the fibrillar elements in the elastic region. 2) Fibrochondrocyte-like cells are found in the fibrocartilage besides fibroblasts, either occupying different domains and apparently being involved with the synthesis of different extracellular

matrix components. 3) There is a progressive accumulation of proteoglycans in the extracellular matrix and they are initially diffuse but then restricted to the fibrochondrocyte pericellular matrix. 4) Collagen fibers/bundles are progressively thicker, arranged in as basket weave-like pattern and show thin elastic fibers found at their surfaces and in the middle-substance. 5) Though these different regions seem to derive from different mechanical demands, the specific differentiation factors could not be delineated at this point.

1. Introdução

1.1. Propriedades dos tendões

Os tendões realizam o papel mecânico de transmitir forças de tensão geradas pela contração muscular ao osso no qual ele se insere.

Os tendões são constituídos de tecido conjuntivo denso, apresentando um predomínio de matriz extracelular em relação aos componentes celulares. Os tendões possuem colágeno tipo I (65-80%) e elastina (1-2%). Os proteoglicanos de baixo peso molecular correspondem a 90% do total de proteoglicanos encontrados em tendões típicos, onde perfazem menos de 1% do peso total (Vogel *et al.* 1984). O colágeno do tipo VI e outras glicoproteínas não colagênicas também estão presentes (Felisbino e Carvalho, 1999). Os tendões podem variar sua forma e tamanho, eles são achatados ou cilíndricos, e encontrados na origem, na inserção ou formando inserções tendinosas dentro de músculos (O'Brien, 1997).

O principal responsável pelas características biomecânicas dos tendões e ligamentos é o arranjo fibrilar resultante da associação de moléculas de colágeno com outros componentes da matriz extracelular. O desenvolvimento dos tendões envolve uma seqüência de deposição dos componentes da matriz extracelular organizados em um complexo esquema hierárquico, onde as moléculas de colágeno agregam-se formando fibrilas que são agrupadas em fibras e estas em feixes. A agregação é modulada por outros componentes da matriz como os pequenos proteoglicanos decorim e fibromodulim (Vogel e Trotter, 1987; Hedbom e Heinegård, 1989) e por um ativo papel das células que formam domínios pericelulares (Trelstad e Hayashi 1979; Birk e Zycband 1994).

Em um tendão os feixes de fibras de colágeno não estão agrupados ao acaso, mas organizados em feixes envolvidos por uma membrana externa de tecido conjuntivo, chamada epitendíneo. Do epitendíneo partem septos

muito finos de tecido conjuntivo que se dirigem para o interior do tendão, dividindo-se em feixes, chamado de peritendíneo, e este envolve cada feixe de fibras de colágeno. Cada fibra de colágeno, por sua vez, é envolvida por uma camada muito fina, chamada endotendíneo (Junqueira e Carneiro, 1999).

Segundo Kannus (2000) o arranjo tridimensional das fibras e os feixes de fibras do tendão é complexa. As fibrilas de colágeno são orientadas longitudinalmente, transversalmente e horizontalmente, alguns grupos de fibrilas que correm longitudinal apresentam-se em forma de espiral (trança), esta estrutura tridimensional é importante pois durante os movimentos, os tendões são expostos a forças vindas de várias direções, portanto este tipo de estrutura previne danos e dissociação das fibras.

Vogel *et al.* (1986) demonstraram que tendões sujeitos a diferentes forças mecânicas são remodelados pelas células. Um aspecto importante dos tendões é a capacidade de se repararem após um trauma (Vogel e Koob, 1989).

Ao contrário da maioria, alguns tendões podem apresentar grandes quantidades de elastina, dando a estas estruturas propriedades elásticas como aquelas observadas no ligamento nucal.

A integridade mecânica dos tecidos é proporcionada por redes compostas de colágeno fibroso, onde colágeno em combinação com proteoglicanos, podem aumentar o aspecto viscoso e melhorar assim a lubrificação de sítios que estejam sujeitos a forças de compressão (Silver *et al.*, 2000; Okuda *et al.*, 1987). Os proteoglicanos são importantes elementos estruturais da matriz e contribuem para a arquitetura dinâmica de alguns tecidos e órgãos.

Os proteoglicanos que estão presentes em muitos tecidos elásticos podem participar da regulação da síntese de elastina e sua deposição durante novos períodos de crescimento (McGowan, 1993).

Diversas linhas de estudo têm sugerido que pequenos proteoglicanos ricos em leucina (SLRPs) são importantes na fase de crescimento das fibrilas de colágeno (Graham *et al.*, 2000). As interações específicas que ocorrem

entre as fibrilas de colágeno e os proteoglicanos provavelmente auxiliam na organização da matriz extracelular, e podem estar envolvidas no comportamento do tendão quando este está sob tensão, sendo que estes proteoglicanos podem formar pontes entre as fibrilas de colágeno contribuindo para a resistência do tecido (Scott, 1988; Cribb e Scott, 1995). A matriz extracelular muitas vezes contém agrecam o que permite ao tendão embeber água e resistir à compressão (Vogel e Koob, 1989).

Além disso, resultados obtidos por Reimboth *et al.* (2000) sugerem que o ácido hialurônico pode ter um papel no agrupamento das microfibrilas e que a forma glicosilada do biglicam pode estar associada à formação da fibra elástica.

Experimentos *in vitro* têm demonstrado que a estimulação mecânica, bem como fatores de crescimento modulam o padrão de expressão dos proteoglicanos por células do tendão (Vogel e Hernandez, 1992; Vogel, 1996).

1.2. Fibrocartilagem

Alexander *et al.* (1983) definem regiões de tendões "wrap around" como aqueles que contornam os ossos em uma articulação, antes da inserção, em uma direção que difere daquela do músculo. A fibrocartilagem é encontrada ligando tendões e ligamentos ao osso e é provida de uma gradual transição de propriedades mecânicas entre eles (Woo *et al.* 1988). Isto também ocorre quando tendões passam ao redor dos ossos, resistindo à compressão e provendo-se de uma superfície plana e lisa. Durante a contração muscular, forças de compressão atuam nessa região do tendão induzindo a formação de um tecido fibrocartilaginoso, que aparentemente adapta o tendão à transmissão de forças induzindo a formação desta superfície curva (Vogel e Koob, 1989; Ralphs *et al.* 1991; 1992; Rufai *et al*, 1992). Segundo Benjamin *et al.* (1986) a fibrocartilagem pode ter um papel funcional e biomecânico.

A fibrocartilagem é um tecido transicional com propriedades estruturais e funcionais intermediárias entre tendão e cartilagem. A fibrocartilagem é geralmente uma região pobremente vascularizada. As células encontradas na fibrocartilagem podem estar arranjadas irregularmente ou em fileiras e são, na sua maioria, fibrocondrócitos (células semelhantes à condrócitos) e fibroblastos (Benjamin e Evans, 1990).

No tendão as fibrocartilagens desenvolvem-se em dois sítios principais. Elas são encontradas na inserção no osso e em sítios sujeitos a forças de compressão e fricção contra superfícies, além da força de tensão normal exercida pelo músculo (Vogel e Koob 1989). O desenvolvimento da fibrocartilagem pode ocorrer por metaplasia da cartilagem ou do tecido fibroso (Benjamin e Evans, 1990).

As fibras elásticas ou do sistema elástico são encontradas nas fibrocartilagens dos tendões, variando em conteúdo, tipos e organização (Benjamin e Evans 1990; Carvalho e Vidal, 1994b; Covizi *et al.*, 2001).

Segundo Perez-Castro e Vogel (1999) as células da fibrocartilagem do tendão flexor digital do bovino adulto exibem altos níveis de expressão para colágenos dos tipos I e II, biglicam, decorim e agrecam sugerindo que a fibrocartilagem no tendão adulto é um tecido dinâmico. O acúmulo de colágeno tipo VI ao redor de fibrocondrócitos na região de compressão parece ser um bom marcador para distintas forças mecânicas sobre os tendões (Felisbino e Carvalho, 1999).

Um tecido semelhante à fibrocartilagem de mamíferos mas com alguns aspectos distintos de estrutura celular e fibrilar, é encontrado em tendões de *Rana catesbeiana* (Carvalho e Vidal, 1994a; Carvalho e Felisbino, 1999).

Rufai *et al.* (1992) propôs que o acúmulo de condroitim sulfato na matriz extracelular e a expressão de vimentina são marcadores moleculares para a resposta de células do tendão que está sujeito à forças de compressão (Ralphs *et al.*, 1991; Benjamin *et al.*, 1991).

A região de compressão do tendão de *Rana catesbeiana* estudada por Carvalho e Felisbino (1999) foi classificada como fibrocartilaginosa em três aspectos: primeiro fibras de colágeno são dispostas com padrão de rede de basquete, segundo, há um grande acúmulo de proteoglicanos e, terceiro, células arredondadas estão dispostas em lacuna, reforçando que estas características desenvolvem-se frente à forças de compressão e fricção envolvidas no desenvolvimento e manutenção de fenótipos fibrocartilaginosos na região de compressão em diferentes sistemas.

Ratos *Wistar* com 12 meses ou ainda mais velhos apresentaram um aumento na composição do colágeno tipo II na fibrocartilagem sujeita a compressão. Relacionada à idade ocorre uma diminuição de condroitim sulfato na suprapatela (Ghosh e Taylor 1987) e no disco intervertebral (McDevitt, 1988). A partir da observação de que o condroitim sulfato diminui em alguns tecidos com a idade, foi sugerido que as células estejam sintetizando poucos glicosaminoglicanos (Benjamin *et al.*, 1991).

Fatores que levam à diferenciação dos tendões durante o desenvolvimento são ainda pouco conhecidos. Um trabalho isolado demonstrou que membros da família TGF-β são capazes de levar à diferenciação de estruturas semelhantes a tendões, quando implantados subcutaneamente em ratos (Wolfman *et al.*, 1997). Com respeito à diferenciação de fibrocartilagens em regiões sujeitas a forças de compressão, foi sugerido que a própria força mecânica leva à diferenciação dos fibroblastos em fibrocondrócitos, ao rearranjo dos componentes fibrilares e ao acúmulo de proteoglicanos (Evanko e Vogel, 1990; Vogel, 1996). Experimentos *in vitro* demonstraram que o TGF-β foi capaz de estimular tendões fetais a sintetizarem macromoléculas típicas das fibrocartilagens (Vogel e Hernandez, 1992).

1.3. O tendão elástico nas aves

Nas aves, o tendão elástico origina-se no músculo deltoídeo localizado na extremidade anterior do húmero e insere-se em vários pontos na articulação distal do rádio e da ulna, sendo responsável pelo posicionamento das asas no estado de repouso. A complexidade do tendão elástico e suas propriedades elásticas são notáveis e dependentes de um grande conteúdo em elastina e de interações interfibrilares com o colágeno (Oakes e Bialkower, 1977; Brown *et al*, 1994a). Este tendão possui propriedades mecânicas e interações complexas entre colágeno-elastina-proteoglicanos (Brown *et al* 1994b). Como descrito por Carvalho *et al*. (2000), este tendão apresenta uma grande variação morfológica ao longo do seu comprimento, compreendendo três destas regiões tipicamente fibrosas, mas com um aumentado número de fibras elásticas. Elas conectam o músculo deltoídeo à região elástica propriamente dita (que ocupa cerca de 60% do comprimento do tendão e cerca de 80% de sua massa), a região elástica é conectada a um sesamóide fibrocartilaginoso e este sesamóide ao sítio de inserção óssea.

Esta complexidade estrutural faz com que este tendão seja um excelente modelo para o entendimento à diferenciação de regiões distintas ao longo de tendões.

1.4. As fibras elásticas: composição, organização e formação

A movimentação dos organismos depende da articulação entre suas partes que são unidas por tecidos flexíveis capazes de resistir a estiramentos. Muitas proteínas estão envolvidas neste processo, a resilina em artrópodes, abductina em moluscos e a elastina em vertebrados (Sage e Gray 1979).

Em vertebrados, a elasticidade é provida de uma proteína chamada elastina, que constituem um polímero insolúvel composto de várias moléculas ligadas covalentemente umas às outras por ligações cruzadas, e que

resultam nas fibras elásticas, responsáveis pelas propriedades elásticas dos tecidos (Debelle *et al.*, 1998; Mecham e Heuser 1991, Rosenbloom *et al.* 1993). Essa proteína é encontrada em tecidos que resistem ao estiramento, o que, é importante para o funcionamento adequado de órgãos como aorta onde pressões diferentes são geradas por movimentos sistólicos e diastólicos (Wolfe *et al.*, 1993), contribuindo então para propriedades biomecânicas de vários outros órgãos incluindo vasos sanguíneos e pulmão. No estágio inicial de desenvolvimento dos membros, a distribuição da elastina *in vivo* é similar à observada em cultura de explantes.

A tropoelastina é o precursor solúvel da elastina amorfa (Hsiao *et al.*, 1999; Wolfe *et al.*, 1993; Franzblau *et al.*, 1989). Resultados obtidos por James *et al.* (1998) demonstraram um controle transcricional da expressão da elastina, que aumenta durante o desenvolvimento do pulmão.

Resultados obtidos por Ichiro *et al.* (1990) demonstraram que o fator de crescimento epidermal (EGF) diminui a produção de elastina e a proliferação da célula muscular lisa. Este fator está envolvido com processos ateroscleróticos na inibição da elastogênese. Outros fatores mitogênicos como TGF-β (transforming growth factor β) e o IGF-I (insulin-like growth factor) são estimuladores da síntese de elastina pelas células musculares lisas (Foster *et al.*, 1989).

A organização do sistema elástico compreende três tipos de fibras: as oxitalânicas, elaunínicas e elásticas.

A fibra elástica é uma estrutura complexa encontrada na matriz extracelular que contém, além da elastina, outras glicoproteínas microfibrilares, enzimas como a lisil-oxidase e proteoglicanos. Algumas proteínas do sistema elástico encontram-se associadas às microfibrilas (Monte *et al.* 1996). As fibras elásticas podem ser degradadas e subsequentemente reparadas, como mostra o estudo feito *in vitro* por Morris *et al.* (1998).

Em tecidos elásticos, feixes de microfibrilas conectam as fibras elásticas entre si e a outros componentes estruturais e celulares (Ramirez e Pereira, 1999).

Componentes elásticos identificados no tendão que suporta pressão são importantes para a organização supramolecular, especialmente na manutenção do arranjo das fibras de colágeno na região de compressão, e sua morfologia de "crimp" na região de tensão (Carvalho e Vidal 1995).

O processo no qual se dá a formação das fibras elásticas é denominado elastogênese, no tendão elástico de aves assim como nos outros tecidos esse processo ocorre da mesma maneira sendo que a elastina amorfa é depositada sobre um esqueleto de microfibrilas.

Esse processo é complexo e consiste de vários eventos principais para a construção da fibra elástica, que se inicia dentro da célula (Debelle *et al.*, 1998). A formação da fibra elástica (elastogênese) foi observada em experimentos realizados com culturas de células musculares lisas da aorta de coelho (Toselli *et al.*, 1981). O desenvolvimento da fibra elástica começa com a deposição de microfibrilas que variam entre 10 e 12 nm de diâmetro formadas por fibrilinas (Schuwartz e Fleischmajer 1986) e que são denominadas fibras oxitalânicas. Estas desenvolvem-se em fibras elaunínicas pela deposição de uma pequena quantidade de elastina amorfa, como sugerido por Gawlik (1965). A deposição de elastina ocorre sobre um molde composto por microfibrilas

Feixes de microfibrilas aparecem como uma primeira estrutura na elastogênese. Em estudos de micrografias eletrônicas é possível visualizar estes feixes de microfibrilas que formam o esqueleto/molde para a deposição de elastina. Isso ocorre na elastogênese normal tanto em modelos *in vivo* , quanto *in vitro* (Fukuda *et al.*, 1993). Segundo Cleary e Gibson (1983) durante a formação da fibra elástica uma rede microfibrilar aparece na matriz extracelular, seguindo-se a deposição de elastina amorfa sobre as microfibrilas. Esta relação sugere que as

microfibrilas contribuem para a fibrilogênese direcionando a deposição da tropoelastina.

As fibrilinas que compõem as microfibrilas do sistema elástico estão distribuídas em uma ampla variedade de tecidos e órgãos e encontram-se associadas à elastina nas fibras elásticas ou em feixes livres de elastina. Na trama de fibras elásticas as fibrilinas conectam as fibras elásticas entre si, e a outros componentes estruturais (Mariencheck *et al.* 1995).

A expressão das proteínas microfibrilares ocorre durante o desenvolvimento do tecido elástico, consistente com uma função associada a elastogênese (Mariencheck *et al.* 1995). Acredita-se que a deposição de moléculas de fibrilina antecede a elastogênese, uma vez que as microfibrilas aparecem antes da deposição da elastina (Ramirez e Pereira, 1999). Segundo Zhang *et al.* (1995) a fibrilina 1 está presente geralmente em estruturas que sofrem estresse devido a forças mecânicas, sugerindo que esta glicoproteína pode ser responsável pela função estrutural das microfibrilas enquanto a fibrilina 2 regularia o processo de formação da fibra elástica.

Wolfe *et al.* (1993) sugeriram que o IGF-I (Insulin-like growth factor-I) é um importante modulador da elastogênese na aorta. O IGF-I mostrou-se potente indutor de elastogênese, pois quando administrado a animais, induziu um alto nível de mRNA de tropoelastina coincidindo com o aumento de IGF-I no soro. Este efeito não foi observado em órgãos como pulmão e coração (Foster *et al.*, 1989).

Algumas alterações no arranjo e distribuição das fibras elásticas, durante sua formação podem ocasionar sérias disfunções para indivíduos. Na doença de Hurler ocorre acúmulo de dermatam sulfato e heparam sulfato. O acúmulo de dermatam sulfato causa inativação da proteína de 67kDa que facilita a secreção e associação da tropoelastina às fibras elásticas (Hinek *et al.* 2000).

O enfisema pulmonar tem como principal causa a desorganização das fibras elásticas (Morris *et al.*, 1998). Na pele, a desorganização

das fibras elásticas pode causar o escleroderma, caracterizado pelo acúmulo em excesso de colágeno (Davis *et al.*, 1999).

Mutações no gene da elastina causam doenças como síndrome de William, cutis laxa e estenose da valva aórtica. Outras síndromes que afetam drasticamente a integridade da fibra elástica incluem a síndrome de Marfan, doença de Menkes e pseudoxantoma elasticum (Hinek *et al.*, 2000). A síndrome de Marfan é uma desordem do tecido conjuntivo que afeta o sistema esquelético, ocular e vascular (Pyeritz, 1993), causada principalmente por mutações no gene da fibrilina 1. Sendo a fibrilina o principal constituinte extracelular das microfibrilas, na sua ausência podem ocorrer falhas na formação da fibra elástica durante o estágio de desenvolvimento fetal (Bunton *et al.*, 2001).

1.5. Papel celular na formação de fibras elásticas

Enquanto o colágeno organiza-se em fibrilas na ausência de moléculas adicionais, em função das características físico-químicas de suas moléculas (Linsenmayer, 1991, Prockop e Hulmes, 1994), a elastina não forma estruturas fibrilares espontaneamente. Existe uma sugestão da participação das células na formação da fibra elástica, envolvendo principalmente a proteína p67 presente na membrana das células (Davis *et al.*, 1999). No tendão elástico esta interação célula-fibra elástica parece ser mais evidente, conforme sugerido por Carvalho *et al.* (2000). A possível razão para isto talvez seja a necessidade de aumentar o número de fibras (sua densidade) e sua espessura (com constante deposição de elastina) garantindo sua individualidade e impedindo o crescimento desorganizado (resultando em fibras com espessura variável ao longo de seu comprimento) e impedindo a fusão entre elas.

Segundo Birk *et al.* (1991) as células têm um papel fundamental na organização das fibrilas de colágeno em fibras e feixes, porém estes conceitos não estão tão claros para o desenvolvimento das fibras elásticas.

O ligamento nucal bovino contém um tipo celular que tem como principal função a produção de elastina (fibroblasto) (Mecham *et al.*, 1981). Resultados obtidos por Monte *et al.* (1996), sugerem que fibroblastos e miofibroblastos podem estar envolvidos na síntese de microfibrilas e formação da fibra elástica no fígado.

Em contraste com a simplicidade do ligamento nucal, a aorta é um tecido mais complexo, no qual as fibras elásticas são organizadas em folhas concêntricas ou lamelares entre camadas das células musculares lisas que produzem um grande volume de fibras elásticas. Esta complexa organização garante à aorta a capacidade de acomodar o fluxo sangüíneo.

Como foi sugerido por Carvalho *et al.* (2000), as células, presentes na matriz extracelular dos tendões elásticos de aves deveriam ter um papel muito importante para a integridade deste tecido não somente na formação das fibras elásticas como também na organização destas fibras dentro do tecido. As primeiras fibras elásticas aparecem durante o desenvolvimento no embrião com 14 dias, como pequenas fibras de 1μm de diâmetro alinhadas ao longo eixo do tendão entre as células fusiformes. Estas fibras aumentam em diâmetro entre os 11° e 19° dias pós-eclosão atingindo 30 μm de espessura (Kirkaldy-Willis *et al.*, 1967), estes autores sugeriram que este aumento ocorre por fusão entre as fibras elásticas.

Estas interações entre elastina e células podem ter um papel importante na morfogênese e na manutenção da integridade dos tecidos elásticos como parede arterial, pulmão, pele (Groult *et al.* 1998).

Dadas todas estas características que envolvem a deposição da matriz extracelular e o comportamento celular durante a formação dos tendões e as diferenciações morfofisiológicas que se desenvolvem frente a demandas biomecânicas, pareceu-nos importante levar adiante este trabalho para um melhor entendimento do desenvolvimento deste tendão.

2. Objetivo

Este trabalho teve por objetivo aprofundar conhecimentos sobre a estrutura e composição na formação do tendão elástico de aves e contribuir para o entendimento da biologia dos tecidos elásticos e dos tendões em geral. Ênfase foi dada à seqüência de eventos que levam à diferenciação das regiões elástica e da fibrocartilagem, em especial às características celulares relacionadas à formação dos componentes fibrilares da matriz.

3. Artigos

3.1. **Pimentel SB**, Carvalho HF (2002). Development of the elastic tendon of the chicken wing: The role of cells in elastogenesis

3.2. **Pimentel SB**, Carvalho HF (2002). The development of the fibrocartilage in the elastic tendon of the chicken wing

3.1. Development of the elastic tendon of the chicken wing: The role of cells in elastogenesis

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Short title: Elastogenesis in chicken wing tendon

Abstract

Morphological, immunocytochemical and ultrastructural methods were used to investigate the role of cells during elastogenesis of in the elastic tendon of the chicken wing. Intimate contact of the cell processes with elastic fibers was observed in adult birds. During development, there was a sequential appearance of microfibril bundles that became progressively impregnated with amorphous elastin which eventually predominated in fully developed elastic fibers. The growing elastic fibers were usually enveloped by recesses in the cell surface. The tendon cells were polarized and were involved in secreting and directing elastic and collagen fibers to different compartments of the plasma membrane. These results show that cells are intimately involved in the organization of the fibrillar components during tendon differentiation.

Introduction

The elastic tendon of the chicken wing is a complex structure responsible for maintenance of the wing in the resting position. This tendon has five

morphologically distinct regions (Carvalho *et al.*, 2000) and its biomechanical properties are provided by the elastic region which extends along three-quarters of the tendon length. This elastic region is rich in elastic fibers, which are arranged longitudinally, and has thin collagen fibers that run perpendicular or oblique to the elastic fibers (Oakes and Bialkower, 1977; Carvalho *et al.*, 2000). Proteoglycans also occur in the elastic region, and can be identified by ruthenium red staining (Oakes and Bialkower, 1977). The proteoglycans of the elastic region present in the D1 ultracentrifugation fraction have been partially characterized and are represented by a large chondroitin sulfate-bearing proteoglycan and a biglycan-like molecule (Carvalho *et al.*, 2000).

Elastin, collagen fibers and proteoglycans act in concert to provide the tissue with remarkable elastic properties (Oakes and Bialkower, 1977; Brown *et al.*, 1994).

Electron and polarization microscopy of relaxed or tensioned elastic tendon have shown that loading the elastic tendon results in stretching of the elastic fibers and alignment of the collagen fibers. Removal of the mechanical load relaxes the elastic fibers and allows the collagen fibers to resume the wavy or convoluted array seen in the resting state. This alignment of collagen fibers results in a long toe region in stress-strain experiments. Elastic fibers are also responsible for the elastic recovery that results in the folding of collagen fibers since elastase treatment led to straightening of the latter fibers and a marked increase in tendon length (Oakes and Bialkower, 1977).

In previous work, we observed that cell processes ocurred in intimate association with elastic fibers of the elastic region and suggested that this could represent either a mechanism to contribute to the control of tissue homeostasis or an indication of a role for cells in elastic fiber formation. Based in these findings, we decided to examine the development of the elastic tendon using light and electron microscopy, with particular attention to the role of cells in elastic fiber formation.

The results obtained extend the earlier work of Kirkaldy-Willis *et al.* (1967) on elastogenesis in the elastic tendon by showing that tendon cells can

simultaneously synthesize and organize elastin and collagen fibers, and this contribute actively to determine the structure and organization of elastic fibers.

Materials and Methods

Animals:. Embryos 10-, 15- and 20-days-old and 1-, 15- and 30-day-old chicks of *Gallus gallus domesticus* were used in this study. The elastic region of the wings of specimens from each age group was examined using light and transmission electron microscopy. Fifteen-day-old chicks were used for immunocytochemistry.

Light microscopy: Fragments of the elastic region were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 h, washed with distilled water, dehydrated in a graded ethanol series and embedded in historesin (Leica Microsystems, Heidelberg, Germany). The sections were stained by hematoxylin-eosin, toluidine blue for the observation of distribution of proteoglycans, Weigert's resorcin-fuchsin and picrosirius-hematoxylin for observation of the distribution of collagen fibers.

Transmission electron microscopy: Tissue fragments were fixed in 3% glutaraldehyde and then in 0.25% tannic acid in Millonig's buffer for at least 2 h at room temperature. After washing in the same buffer, the tissues were post-fixed with 1% osmium tetroxide and washed again before dehydration in an acetone series (Cotta-Pereira *et al.*, 1976). The material was then embedded in Epon 812. Thick sections (1 μm) were cut and stained with 1% toluidine blue in 1% sodium carbonate, pH 10, for examination and selection of areas of interest. Silver thin sections were contrasted with 2% uranyl acetate in methanol (Riva, 1974) and lead citrate (Reynolds, 1963) and examined and documented in a Leo 906 transmission electron microscopy.

Immunocytochemistry: Tendon fragments were embedded in Tissue Tek cryo-embedding solution and frozen in liquid nitrogen. Frozen sections (6 μm) were obtained and fixed with 4% formaldehyde in PBS for 1 h at 4°C and then washed three times with PBS. After block in with 3% bovine serum albumin (BSA) in 10 mM Tris buffer containing 0.15 M NaCl and 1% Tween 20 (TST) for 1 h at room temperature, the sections were incubated with an anti-elastin monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:100 in TST containing 3% BSA overnight at room temperature. Following these washes with TST buffer, the sections were incubated for 1 h with a FITC-conjugated anti-mouse polyvalent antibody (Sigma Chemical Co.) diluted 1:200 in TST buffer containing 1% BSA. The sections were then treated with TRITC-conjugated phalloidin and DAPI (Sigma Chemical Co.) for 30 min. The preparations were mounted in Vectashield (Vector Labs, Berlingame, CA, USA) and observated with an Olympus fluorescence microscope.

Results

Microscopy and immunocytochemistry

The development of the elastic region of the elastic tendon involved the gradual deposition of elastin as isolated fibers (Figs. 1-4, 8 and 12), with the cell density decreasing as matrix deposition increased. Toluidine blue showed no staining in the extracellular matrix, demonstrating the absence or low amount of glycosaminoglycans, even in the early stages. Collagen deposition was slow before hatching, but gradually increased with age after hatching. H&E staining (Fig. 9) demonstrated that the cell nuclei were slightly larger than in the earlier staged (10 and 20-day-old embryo), but no significant difference from the previous stages, except for an increase in the extracellular spaces. Toluidine blue staining revealed no glycosaminoglycans in the extracellular matrix (Fig. 10). However collagen

remained a minor component of the extracellular matrix (ECM). The thin collagen fibers were undulated and occurred between growing elastic fibers (Figs. 7 and 11). Figures 13-15 show the of immunolocalization of elastin and actin staining with phalloidin, and reveal the intimate association between cell processes and elastic fibers.

Electron microscopy

The ECM of the elastic region in 10-day-old embryos was dominated by microfibril bundles and a few collagen fibrils. The cells were rich in actin filaments and microtubules and a large nucleolus (Figs. 16 and 17). By the 15th day of incubation, little elastin deposition was visible in the microfibril bundles (Fig. 18). Cell processes associated with the forming extracellular fibrillar elements were rich in microtubules (Fig. 19). By the 20th day, elastic fibers were seen as irregularly distributed patches of amorphous elastin inside the microfibril bundles (Fig. 20).
One day after hatching, the chicks showed increased deposition of collagen fibrils that were undulated and followed the outline of the cells, the latter having a very irregular profile (Figs. 21 and 22). Elastic fibers increased in size and oppened to fuse together. At this stage, there was a clear association between the growing elastic fibers and recesses in the cell cytoplasm. In later stages, as the elastic fibers grew thicker, cell processes embraced individual elastic fibers, and the relationship between the undulating collagen fibers and the irregular surface of the associated cells was clearly observed (Figs. 23-25).

Discussion

In a previous study, we observed cell processes wrapping around individual elastic fibers in the elastic region of the elastic tendon (Carvalho *et al.*, 2000). We

have now investigated whether this association represented an active role for cells in the formation of elastic fibers.

Bundles of fibrillin-based microfibrils generally scaffold the deposition of elastin, and the sequence of deposition of these components (i.e., fibrillin and then elastin) has been demonstrated for a series of tissues.

Whereas collagen molecules self-assemble into fibrillar structures, elastin coacervates result from the self-aggregation of elastin molecules are formless. The assembly of elastic structures is dependent on two aspects. First there is usually a scaffold of fibrillin-based microfibrils which progressively become impregnated with amorphous elastin to a point where the elastic component predominates over microfibrils (Mecham and Heuser, 1991). Second, at sites where elastic fibers with a uniform diameter are formed instead of laminae, isolation of the growing structure into pericellular domains is necessary in order to provide proper distribution of the building blocks (Mecham and Heuser, 1991).

By isolating the growing elastic fibers, the cells may help to avoid the fusion of the elastic fibers as they grow thicker, especially in the elastic tendon where the density of such fibers is high.

The association between cells and elastic fibers seems necessary in order to correctly define the shape, thickness, and alignment of the growing elastic fibers. The present study confirmed the findings of Kirkaldy-Willis *et al.* (1967) and demonstrated that cells in the elastic tendon are active not only in the synthesis and secretion of ECM components, but also in the creation of specific domains where the supramolecular assemby of collagen and elastin can occur. The involvement of tendon cells in producing and organizing collagen fibrils into fibers and bundles has been demonstrated in a series of papers by Birk *et al.* (1995, 1994, 1990). Mecham and Heuser (1991) mentioned a possible association between growing elastic fibers and recesses in the plasma membrane, and we have shown here a clear involvement of cells in defining the limits and direction for the growth of elastic fibers. We also observed, that elastic tendon cells are functionally polarized in that they secrete

elastin and collagen to distinct domains of the plasma membrane while simultaneously helping in their assembly.

Tendon morphogenesis is based on a series of intrinsic and extrinsic factors. Kardon (1998) demonstrated that the formation of tendon primordia and initial differentiation of myogenic precursors occurred independently, and that later events, such as segregation of the tendon mass into individual tendons, required reciprocal interactions between muscle and tendon. Kirkaldy-Willis *et al.* (1967) studied elastogenesis in the elastic tendon and reinforced the proposal by Petry (1951) that the initial appearance of the elastic tendon coincides with the period of peak motility of the embryo which provides the mechanical stimulus for alignment of the mesenchymal cells. On the other hand, compressive fibrocartilage develops in tendons at the sites of mechanical compression, this providing clear evidence for the influence of mechanical stimulation in the differentiation and maintenance of the tendon functional phenotype (Gillard *et al.*, 1979; Vogel and Koob, 1989; Benjamin and Ralphs, 1998). More recently, based on a study of the plantaris longus tendon of the bullfrog, we suggested the existence of a developmental program that establishes the proper environment for the tissue to respond to mechanical forces (Carvalho and Felisbino, 1999). Intrinsic factors are thus part of a developmental program which includes a programmed expression of genes coding for different ECM components, as well as an active role for tendon cells in organizing the secreted building blocks into functional collagen and elastic fibers. Extrinsic factors include mechanical loading that occurs only after the muscle has reached a certain degree of development, and other extraneous factors such as the availability of vitamins in the diet.

Acknowledgements

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Figures legends

Figs. 1 and 2. Sections of the elastic region from a 10-day-old embryo. Figure 1 is an H&E stained section showing that the tissue has a high density of cells with a relatively uniform nuclear phenotype and that these cells are aligned with the long axis of the tendon. No metachromasy was observed after toluidine blue staining (Fig. 2), reflecting the absence or very low content of glycosaminoglycans. Slender fibrillar elements are distinguishable amongst the cells. 400x

Figs. 3 and 4. Sections of the elastic region from a 20-day-old embryo. Figure 3 is an H&E stained section showing that the cells have an enlarged cytoplasm, that is elongated and extends amongst the fibrillar elements of the extracellular matrix. The cell outlines are slightly irregular. The fibrillar elements in the matrix are thicker. The cell density is not as high as it was on the 10th day. Toluidine blue staining (Fig. 4) revealed the cell nuclei but no extracellular staining. 400x

Figs. 5-8. Sections of the elastic region from a 1-day-old chick. H&E staining (Fig. 5) showed that the cell density decreased as the deposition of extracellular matrix increased. There was little cytoplasm extending amongst the growing elastic fibers. Toluidine blue staining (Fig. 6) revealed the absence of staining in the extracellular matrix. Figure 7 is a picrosirius red stained section showing the presence of relatively thin and undulating collagen fibers (arrows) that appear as a minor component amongst the growing elastic fibers (Fig. 8). The latter fibers become progressively thicker and are aligned predominantly with the long axis of the tendon, although some groups appear to run in different directions, as shown by Weigert's fuchsin-resorcin staining. 400x

Figs. 9-12. Sections of the elastic region from a 15-day-old chick. H&E staining (Fig. 9) demonstrated that the cell nuclei were slightly larger than in the earlier staged (10 and 20-day-old embryo), but no significant difference from the previous

stages, except for an increase in the extracellular spaces. Toluidine blue staining revealed no glycosaminoglycans in the extracellular matrix (Fig. 10). There was an increase in the amount of the collagen fibers that appear as aligned dots amongst the elastic fibers. This interrupted pattern resulted from wavy pattern of collagen fibers (arrows) which were only partially sampled in this section (Fig. 11). Weigert's fuchsin-resorcin staining revealed (Fig. 12) of slightly thicker elastic fibers a large number aligned predominantly with the long axis of the tendon. 400x

Figs. 13-15. Immunocytochemical localization of elastin in the elastic region of a 15-day-old chick. Elastic fibers were labelled only on their surfaces and appeared as hollow tubes (green staining). Filamentous actin was revealed by rhodamin-labeled phalloidin and appear in red (arrows). The processes of the elongated cells were seen associated with the surface of the elastic fibers either longitudinally or enveloping the fibers. Nuclei appeared blue after DAPI staining. Figures 14 and 15 are details of figure 13. Fig. 13 550x and Figs. 14 and 15 1,350x.

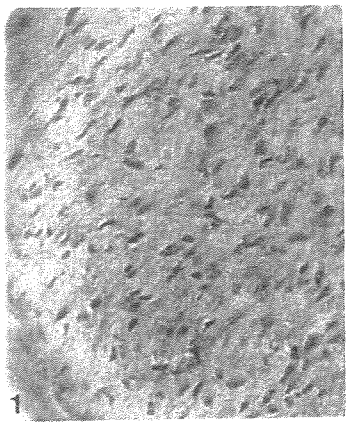
Figs. 16 and 17. Ultrastructural aspects of the elastic region from a 10-day-old embryo. Cells had large nuclei, loose chromatin. There were recesses that delimited areas of the extracellular matrix in which microfibril bundles (Fig. 16 – long arrow) or small groups of collagen fibrils (Fig. 17) were organized. The cytoplasm was rich in cytoskeletal elements, mainly actin filaments (mf) and microtubules (short arrow). M = mitochondrion. Fig. 16 28,446x and Fig. 17 47,432x.

Figs. 18 and 19. Ultrastructural aspects of cells in the elastic region of a 15-day-old embryo. The microfibril bundles showed some accumulation of elastin (white arrowhead) resembling elaunin fibers. Abundant cell processes (arrow) delimited areas of the extracellular matrix. Figure 19 is a detail of a cell in the elastic region showing the abundance of microtubules in the cytoplasm (white arrowheads). Fig. 18 22,000x and Fig. 19 61,160x

Fig. 20. Ultrastructural aspect of an immature elastic fiber in the elastic region. The fiber appears as a group of patched amorphous elastin associated with microfibrils. Although the amount of elastin was high, there was no fusion, as shown by the interrupted spaces in the interior of the fiber. 47,762x

Figs. 21-23. Ultrastructural aspects of the elastic region in a 1-day-old chick. Figure 21 is a low power view showing the cytoplasm and nucleus (arrow) of cells with an irregular profile. The undulating collagen fibers (asterisks) follow the grooves in the cell surface, whereas, the surface facing the growing elastic fibers is smoother. Fig. 22 is a cross section through the elastic region. The elastic fibers (e) are surrounded by cell processes, with a single cell having many membrane recesses and lodging many elastic fibers. Figure 23 shows the cells and extracellular matrix fibrillar components. Elastin fibers are thicker and aligned with each other. The cells retain an irregular outline and are in intimate contact with collagen fibers that are not alingned, but have an irregular path. The cells are rich in rough endoplasmic reticulum, indicating an active role in the synthesis of matrix components. Fig. 21 21,000x and Fig. 22 45,255x and Fig. 23 16,261x

Figs. 24-25. Ultrastructural aspects of the elastic region in a 15-day-old chick. Figure 24 shows a cell process with an irregular side associated with convoluted collagen fibrils (col) and a smooth surface in contact with the elastic fiber (at the botton of the figure). Figure 25 is another detail of the same region in cross-section, showing the intimate association of the cell processes with the cross-sectioned elastic fibers (e) and, in other instances, with groups of collagen fibrils (col). Figs. 24 45,255x and Fig. 25 35,000x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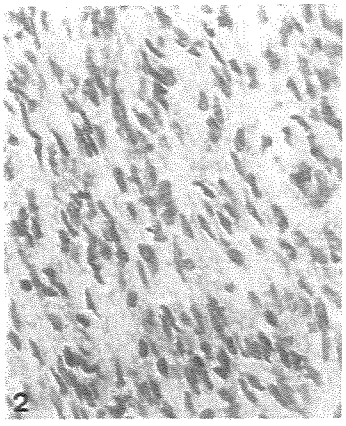
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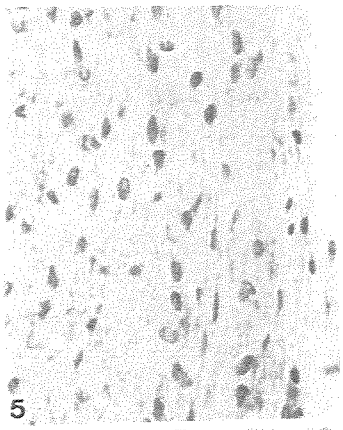
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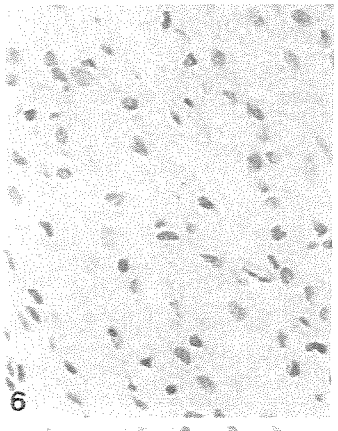
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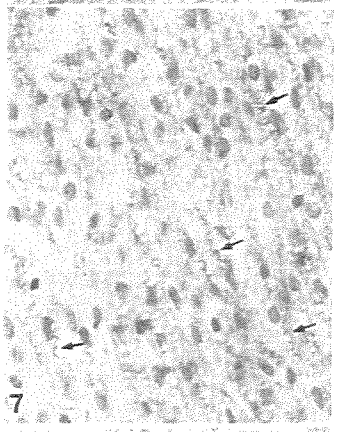
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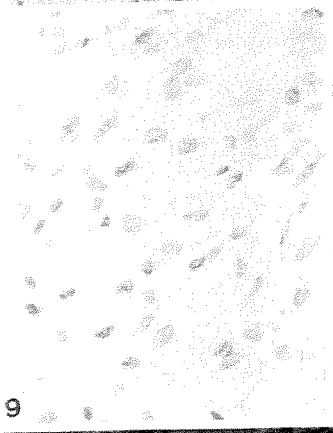
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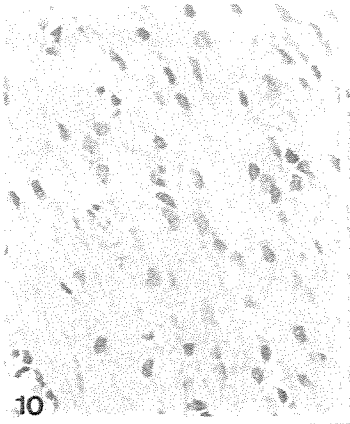
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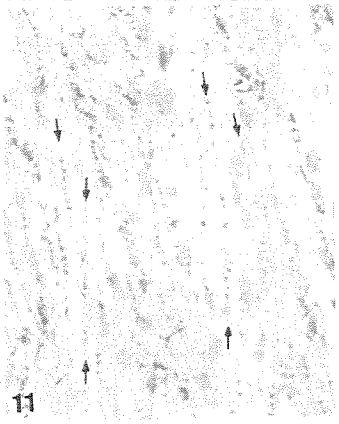
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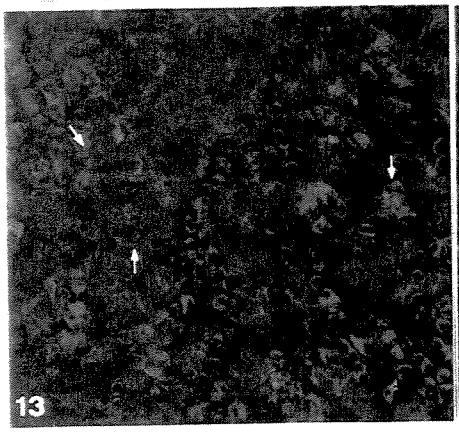
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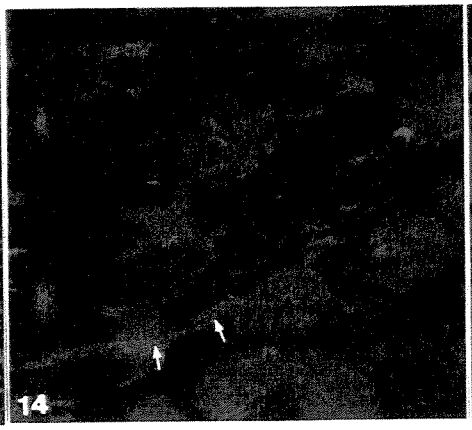
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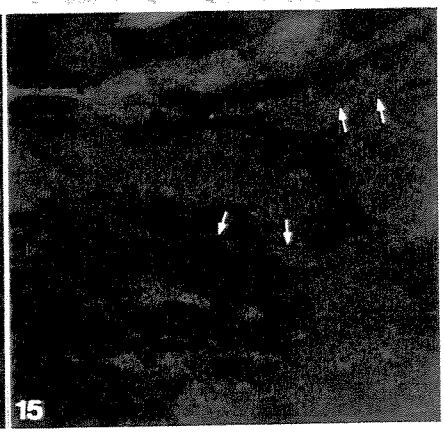
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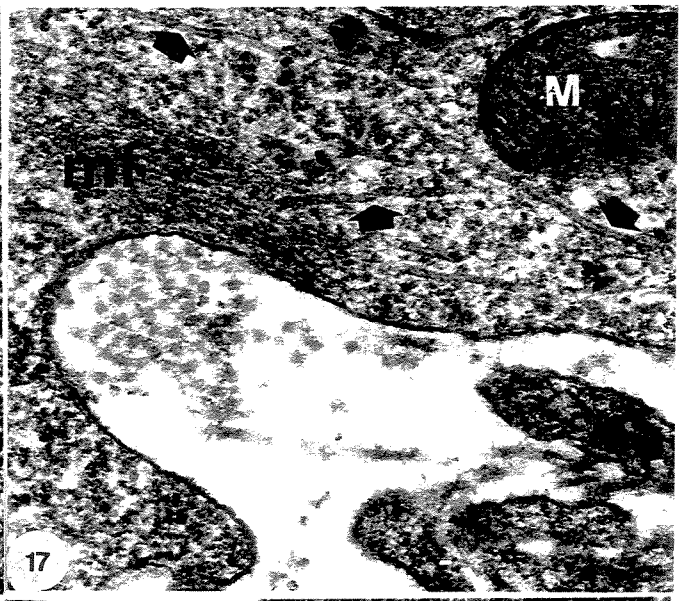

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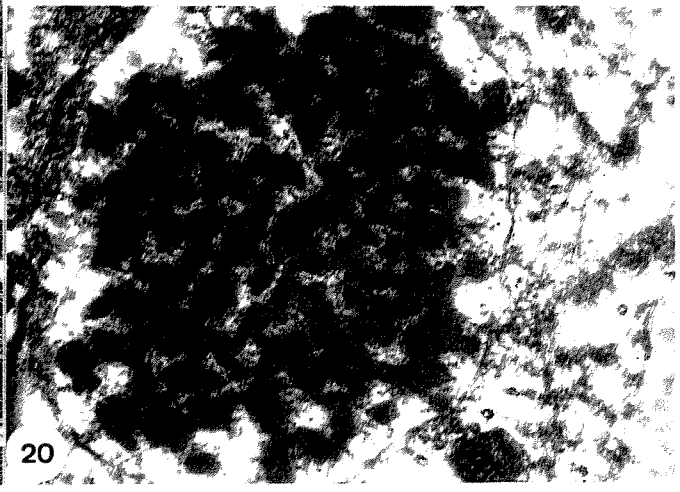

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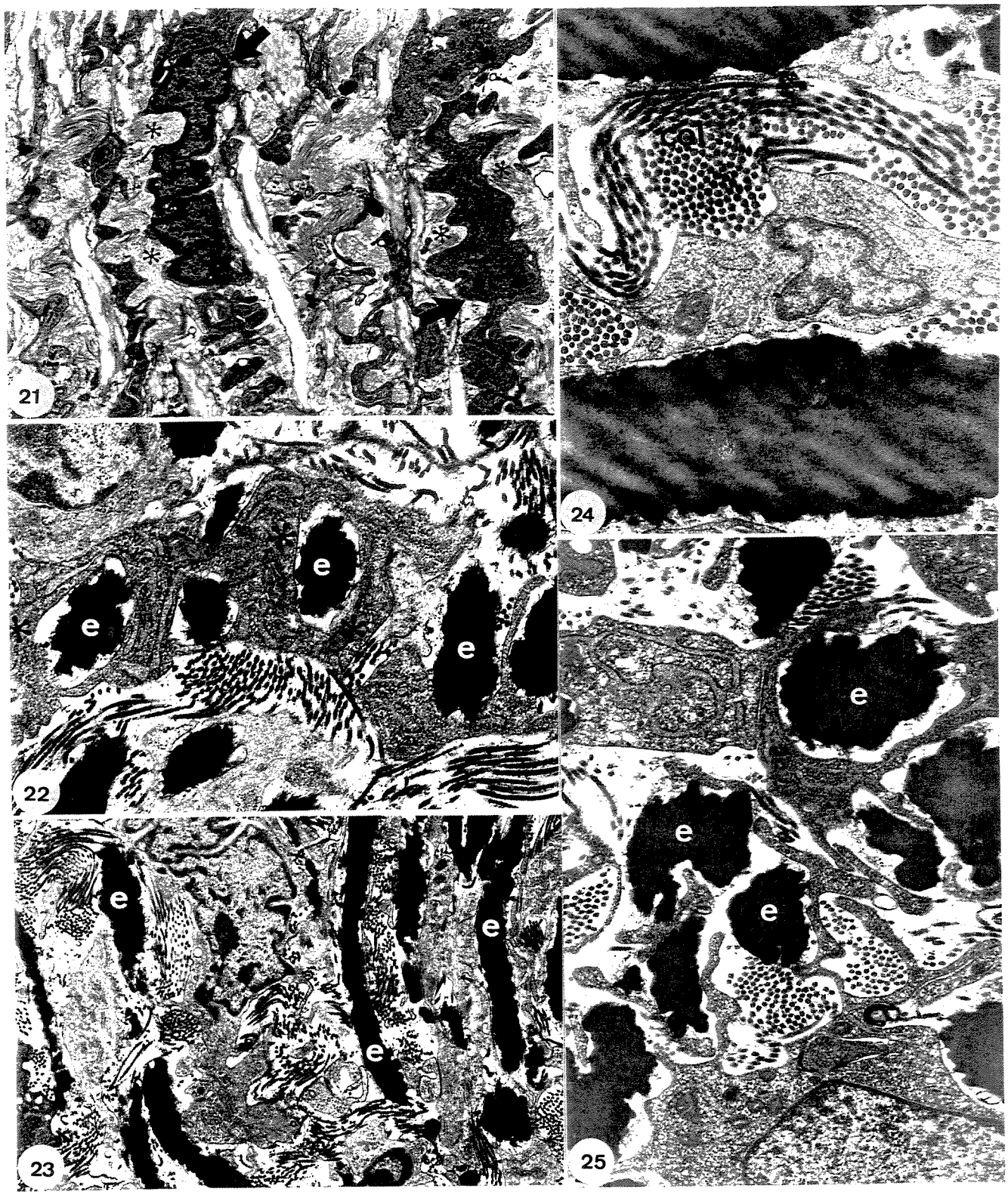
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3.2. The development of fibrocartilage in the elastic tendon of the chicken wing

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Key words: development, elastic tendon, fibrocartilage
Short title: Fibrocartilage in the elastic tendon of chicken wing

Abstract

The elastic tendon of the chicken wing has five morphologically distinct regions. One of these regions is a distally located fibrocartilage from which fibrous connections extend to the capsule of the distal radius. In adult birds this region shows the characteristics of a tendon-compressed fibrocartilage, with an accumulation of proteoglycans between thick collagen bundles arranged in a basket-weave formation. We have studied the development of this fibrocartilage in order to of compare it with other tendon fibrocartilages and have tried to identify the factors involved in fibrocartilage differentiation. This fibrocartilage initially developed by cell enlargement and the accumulation of vimentin, with the simultaneous deposition of proteoglycans in the extracellular matrix and then an increase in the amount and thickness of collagen bundles. Elastic fibers were minor components associated with the collagen bundles. Cells could be classified into two main types. One was typically fibrocartilaginous and the other was fibroblast-like, the latter occurring in close association with the collagen bundles. These results establish the steps in the development of the elastic tendon fibrocartilage and provide a basis for future studies.

Introduction

Tendons are relatively uniform structures composed of collagen fibers and bundles interspersed with a few fibrocytes. Although usually the quiescent structures, tendons can adapt to mechanical stimulation and can contribute to the repair of lesions. Though tendon structure can be altered. Following exposure to compressive forces, the differentiation of a fibrocartilage structure or, in the case of highly elastic tendons, though results the accumulation of a large amount of elastic fibers. The elastic tendon of the chicken wing is remarkable for its longitudinal morphological variation, wich consists of a long elastic region and a fibrocartilage interconnected by typically fibrous regions (Carvalho et al. 2000).

The fibrocartilage in the elastic tendon of the chicken wing is located distally, immediately before insertion of the tendon into the distal radius capsule, apparently results from compressive forces as it is pressed against the distal end of the radius. This fibrocartilage resembles typical tendon compressive fibrocartilage in possessing an increased amount of proteoglycans, thick collagen fibers and bundles disposed in a basket-weave fashing, as well as rounded fibrochondrocytes. Fibroblast-like cells are also seen in close association with the collagen bundles (Carvalho et al. 2000).

Biochemical analyses have shown that fibrocartilage possesses as much as 10-times the amount of sulfated glycosaminoglycans observed in other regions and also has chondroitin sulfate/keratan sulfate large ratio in the D1 ultracentrifugation fraction (Carvalho et al. 2000).

In the present work, we examined the developmental stages in the growth of the fibrocartilage region in order to define the steps of differentiation and to identify possible factors responsible for differentiation compared to other systems.

Materials and Methods

Animals: Embryos 10, 15 and 20 days old and 1-, 15- and 30-day-old chicks of *Gallus gallus domesticus* were used in the study. The elastic region of the wings of specimens from each age group was examined using light and transmission electron microscopy. Fifteen-day-old chicks were used for immunocytochemistry.

Light microscopy: Fragments of the fibrocatilage were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 24 h, washed with distilled water, dehydrated in a graded ethanol series and embedded in historesin (Leica Microsystems, Heidelberg, Germany). The sections were stained by hematoxylin-eosin, toluidine blue for the observation of distribution of proteoglycans, Weigert's resorcin-fuchsin and picrosirius-hematoxylin for observation of the distribution of collagen fibers.

Transmission electron microscopy: Tissue fragments were fixed in 3% glutaraldehyde and then in 0.25% tannic acid in Millonig's buffer for at least 2 h at room temperature. After washing in the same buffer, the tissues were post-fixed with 1% osmium tetroxide and washed again before dehydration in an acetone series (Cotta-Pereira et al., 1976). The material was then embedded in Epon 812. Thick sections (1 μm) were cut and stained with 1% toluidine blue in 1% sodium carbonate, pH 10, for examination and selection of areas of interest. Thin sections were contrasted with 2% uranyl acetate in methanol (Riva, 1974) and lead citrate (Reynolds, 1963) and examined and documented in a Leo 906 transmission electron microscope.

Immunocytochemistry: Fragments of fibrocartilage were embedded in Tissue Tek cryo-embedding solution and frozen in liquid nitrogen. Frozen sections (6 μm) were obtained and fixed with 4% formaldehyde in PBS for 1 h at 4°C and then washed three times with PBS. After blocking with 3% bovine serum albumin (BSA) in 10

mM Tris buffer containing 0.15 M NaCl and 1% Tween 20 (TST) for 1 h at room temperature, the sections were incubated with an anti-vimentin monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:100 in TST containing 3% BSA overnight at room temperature. Following three washes with TST buffer, the sections were and incubated for 1h with a FITC-conjugated anti-mouse polyvalent antibody (Sigma Chemical Co.) diluted 1:200 in TST buffer containing 1% BSA. The sections were then treated with TRITC-conjugated phalloidin and DAPI (Sigma Chemical Co.) for 30 min. The preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed with an Olympus fluorescence microscope.

Results

Histological organization structure and immunocytochemical identification of vimentin

The fibrocartilage appeared as a morphological entity as early as day 10 of incubation. At this stage, the structure was highly cellular and the cells were not uniform in size and/or nuclear shape (Fig. 1). Only a few thin collagen fibers were observed and a metachromatic matrix appeared around the cells (Fig. 2). After incubation for 20 days, the fibrocartilaginous region showed an increased amount of collagen fibers, and larger cells with a more uniform rounded phenotype, elipsoidal nucleus and an enlarged, clear cytoplasm, all of which resulted in a lower cell density (Fig. 3). The metachromatic non-fibrillar matrix occurred around cells and collagen fibers (Fig. 4). In one-day post-hatching chicks, the fibrocartilage showed rounded cells with a large, slightly eosinophilic cytoplasm between the collagen fibers/bundles and other cells; there was cytoplasm in close contact with or inside the collagen bundles (Fig. 5). The metachromatic matrix occupied most of the extracellular space, including the collagen fibers/bundles (Fig. 6). Collagen fibers

were thick and wavy, and were arranged in different directions (Fig. 7). Thin elastic fibers were present on the surface of the collagen fibers, and also in their interior (Fig. 8). Fig. 9 shows a hematoxylin-eosin stained section of fibrocartilage from a 15 day-old chick. Most of the aspects noted above were preserved, except that the extracellular matrix now occupied a larger area of the tissue (Fig. 9). The metachromatic matrix was excluded from areas occupied by collagen fibers and concentrated around the cells (Fig. 10). Collagen fibers were thicker and easily observed as bundles crossing the structure in different directions (Fig. 11). Elastic fibers were still thin but more frequent at this age (Fig. 12). Figure 13 shows the triple labelling of a section of tendon fibrocartilage from a 30-day-old chick for actin, vimentin and DNA. Actin occurred in rounded cells (fibrochondrocytes) and elongated cells (fibroblasts) associated with the collagen bundles. Vimentin, on the other hand, restricted to fibrochondrocytes.

Ultrastructure

Though easily recognized morphologically, the fibrocartilaginous region of the elastic tendon of embryos on the 10 th day of incubation could not be distinguished from typical mesenchymal tissues (Fig. 14). The cells were spherical and had a large nucleus with loose chromatin and a prominent nucleolus. The cytoplasm was scarce, with the rough endoplasmic reticulum as the major organelle. The cells were connected to each other by specialized junctions. The extracellular matrix was scarce, with occasional collagen fibrils and granular structures linked through filaments.

By the 20 th day of incubation, the fibrocartilage contained generally round cells with large nuclei and partially clumped chromatin, as well as heterochromatin associated with the nuclear envelope. The cytoplasm was somewhat larger because of the accumulation of intermediate type filaments, lipid droplets, mitochondria and glycogen granules (Fig. 15). Those cells in close contact with collagen fibers had no

intermediate-type filaments and showed cell processes extending into the surrounding matrix (Fig. 16). The extracellular matrix showed an increased content of collagen fibrils and a higher concentration of filaments/granules on the 10th day of incubation (Figs. 15 and 16).

The fibrochondrocytes of chicks one day after hatching showed a higher accumulation of intermediate-type filaments which occupied most of the cytoplasm. Cellular organelles were segregated to a restricted area of the cytoplasm and were mostly represented by the rough endoplasmic reticulum (Fig. 17). The extracellular matrix also occupied a larger area, with a marked accumulation of collagen fibers. The cells associated with these collagen fibers were typical fibroblasts and resembled those of immature tendons, with processes extending amongst the collagen fibrils. Each of these cells apparently organized a fiber or bundle (Fig. 18).

The fibrochondrocytes of fibrocartilage from 15-day-old chicks accumulated a large amount of intermediate-type filaments and glycogen granules. The extracellular matrix around these cells showed the increased number of filaments and granules seen in the ealier stages (Fig. 19). As the collagen fiber/bundles increased in thicknes identification of the thin elastic fibers within them was possible (Fig. 20).

Discussion

We have previously shown that the elastic tendon has five morphologically distinct regions and that the fibrocartilaginous region shows aspects that resemble those of tendon compressive fibrocartilages especially with regard to cell type and extracellular matrix content (Carvalho et al., 2000). Whereas the elastic tendon has been known for a long time (Oakes and Bialkower, 1977), the fibrocartilage region has been relatively neglected, probably because the predominant elastic region, which occupies approximatelly three-quarters of the tendon length, is unique in its

elastic properties and has attracted the attention of researchers interested in tendon diversity. Oakes and Bialkower (1977) studied the ultrastructure and biomechanical properties of the elastic tendon and referred to the existence of a sesamoid wich they used to handle the specimens during the mechanical tests. Earlier, Kirkaldy-Willis et al. (1967) had studied the development of the elastic tendon but restricted their observations to the elastic region.

Here, we have demonstrated that the fibrocartilage exists as a morphological entity as early as day 10 of incubation. However, at this stage, the histological appearance resembles that of typical mesenchymal tissue, with round cells containing a large nucleus and nucleolus. These cells are separated from each other by a fine extracellular matrix but remain connected by cell junctions. The fibrochondrocytes develop by accumulating increasing numbers of intermediate-type filaments (at least partly composed of vimentin, as identified by immunocytochemistry), glycogen granules and lipid droplets, while other organelles are restricted to a small area of the cytoplasm. This arrangement is similar to that observed for fibrochondrocytes found in other tendon fibrocartilages in rabbits (Merrilles & Flint, 1980), dogs (Okuda *et al.* 1987), rats (Ralphs *et al.* 1992) and frogs (Carvalho and Felisbino, 1999). Subsequent alterations occur progressively until adulthood.

A second cell type observed corresponded to fibroblasts and was prominently associated with collagen fibers. These cells were not as elongated during development and their nuclei were slightly rounded. Ultrastructurally level, these cells were much like fibroblasts from immature tendon (Birk et al., 1994), with extending cell processes that organized the surrounding collagen fibrils into fibers and bundles. Such cell diversity in fibrocartilage also occurs in bullfrog tendon (Carvalho and Vidal, 1994a).

The accumulation of vimentin is a marker for differenting tendon fibrocartilage (Rufai et al., 1992). Vimentin accumulates in the fibrochondrocytes of bullfrog plantaris longus tendon fibrocartilage during development (Carvalho and

Felisbino, 1999). The accumulation of intermediate-type filaments was observed in the fibrocartilage of the elastic tendon observed here by transmission electron microscopy and immunocytochemisty allowed for the identification of these filaments as vimentin.

The fibrochondrocytes produced a proteoglycan-rich extracellular matrix, as identified by the metachromatic staining with toluidine blue and ultrastructurally by the presence of granules connected by filaments (the latter likely representing hyaluronic acid). Whereas during early development the metachromatic staining was diffuse, in later stages it was excluded from the collagen fibers/bundles, being concentrated around the fibrochondrocytes. In adulthood, the content of sulfated glycosaminoglycans reach 40 mg/g of dry tissue, which corresponds to a 10-fold increase as compared to other regions of the same tendon. A large amount of these glycosaminoglycans (~85%) corresponds to a large chondroitin sulfate/keratan sulfate molecule isolated by ultracentrifugation (Carvalho et al., 2000).

Collagen accumulation was noted by the 20th day of incubation and increased steadily thereafter. The collagen fiber/bundles crossed the tissue in different directions and become progressively thicker during development. The formation of elastic fibers accompanied the development of the collagen fibers, with which they were intimately associated. The physiological reason of this association is unknown, although the presence of elastic fibers was previously associated with maintaining the collagen fibers in a undulated or wavy conformation (Carvalho and Vidal, 1994b, c).

In this study, it was not possible to identify the factors responsible for the differentiation of the elastic tendon fibrocartilage. Kirkaldy-Willis et al. (1967) mentioned that the elastic tendon first appeared on the 9th day of incubation, corresponding to the peak cell motility of the embryo. This movement would generate mechanical stimuli for alignment of the cells and would induce then to secrete elastin. Whether these mechanical forces could also be responsible for differentiation of the fibrocartilaginous region is unknown. Experiments involving

the implantation of the fibrocartilaginous in other regions of the embryo could help to distinguish between humoral and mechanical factors. As discussed elsewhere for the plantaris longus tendon (Carvalho and Felisbino, 1999), in addition to mechanical factors (Vogel and Koob, 1989; Vogel, 1996), programmed development could also coordinate the structural and compositional modifications seen in the development of tendon compressive fibrocartilages.

Acknowledgements

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Figures legends

Figs. 1 and 2. Sections of fibrocartilage from an embryo on the 10th day of incubation. Figure 1 is an H&E stained section showing that the tissue had a high density of cells with different nuclear phenotypes and non-homogenous staining after staining with toluidine blue. Metachromatic material was observed around the cells (Fig. 2). Only occasional and slender collagen fibers were observed (arrows). 400x

Figs. 3 and 4. Sections of fibrocartilage from an embryo on the 20th day of incubation. Figure 3 is an H&E stained section showing that the cell phenotypes were more uniform in terms of nuclear phenotype. The cytoplasm was enlarged and showed weak or no staining. The cell density was not as high as on the 10th day. Toluidine blue staining (Fig. 4) revealed the presence of metachromatic material around the cells and between the collagen fibers/bundles (arrows). 400x

Figs. 5-8. Sections of fibrocartilage from a 1-day-old chick. H&E staining (Fig. 5) showed that the rounded cells were even larger, with a slightly eosinophilic cytoplasm, and that a second cell type with little cytoplasm was associated with the collagen fibers (arrows). Toluidine blue staining (Fig. 6) revealed the presence of glycosaminoglycans in the extracellular matrix. Figure 7 is a picrosirius red stained section showing the presence of relatively thin and isolated collagen fibers interspersed with the rounded cells. Figure 8 is a Weigert's fuchsin-resorcin stained section demonstrating the presence of very thin elastic fibers associated with the collagen fibers. 400x

Figs. 9-12. Sections of fibrocartilage from a 15-day-old chick. H&E staining (Fig. 9) revealed two main cell types, the rounded fibrochondrocytes and fibroblasts that were associated with the collagen fibers. Toluidine blue staining (Fig. 10) showed that the glycosaminoglycans concentrated around the rounded cells and were

excluded from the collagen fibers. The latter were easily observed after picrosirius red staining (Fig. 11), wich showed them to be thicker and arranged in bundles. Their arrangement, in a basket weave pattern can also be seen. Weigert's fuchsin-resorcin staining (Fig. 12) showed an increased content of thin elastic fibers which paralleled the increase in collagen fiber content and thickness. 400x

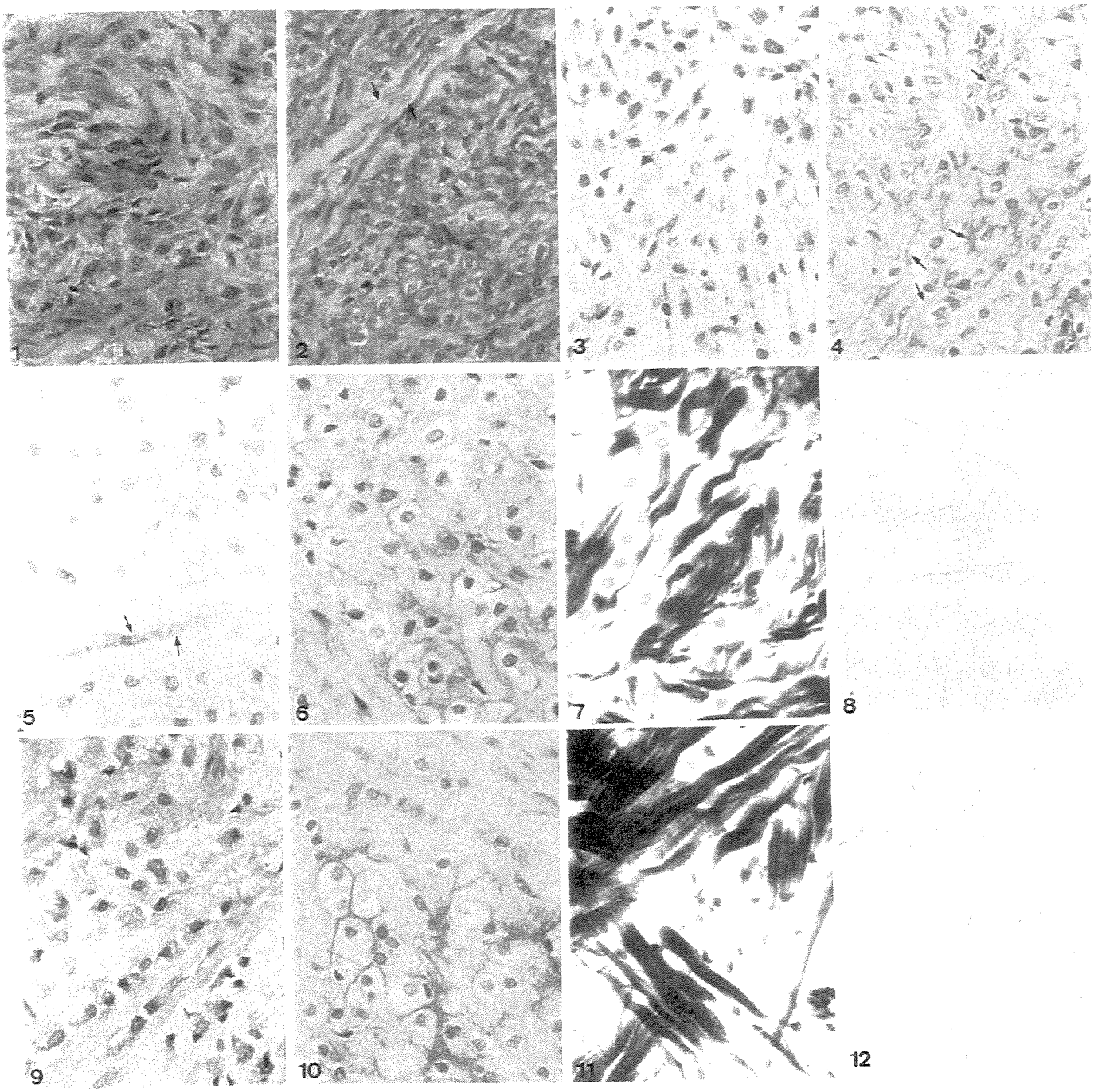
Fig. 13. Immunocytochemical localization of vimentin in the fibrocartilage of a 30-day-old chick. The fibrochondrocytes (rounded cells) showed immunolabelling for vimentin (green), which appeared in a punctuated pattern. Filamentous actin was revealed by rhodamin-labelled phalloidin and appeared in red. Note that the processes of the elongated fibroblasts associated with the collagen fibers showed staining for actin but not for vimentin (arrowheads). 1,350x

Fig. 14. Ultrastructural aspects of cells in the fibrocartilaginous region of an embryo on the 10th day of incubation. The cells were typically mesenchymal, with a large nucleus, loose chromatin and a large nucleolus. The cells contacted to each other through cell junctions (long arrow), but were separated from each other by extracellular matrix composed of a finely fibrillar component that linked granules and occasional collagen fibrils (short arrows), rough endoplasm reticulum (rer). 13,200x

Figs. 15 and 16. Ultrastructural aspects of cells in the fibrocartilage of an embryo on the 20th day of incubation. The rounded cells showed a significant accumulation of intermediate-type filaments, lipid droplets (L) and glycogen granules in the cytoplasm. There was no contact with other cells such is seen earlier (Fig. 14). Figure 16 is a detail of a fibroblast-like cell associated with collagen fibers (C). These cells resemble fibroblasts from immature tendons, with abundant cytoplasm and many cell processes (long arrow) extending amongst the collagen fibrils, and with rough endoplasmic reticulum as the predominant cytoplasmic organelle. Fig. 15 7,913x and Fig. 16 17,050x

Figs. 17 and 18. Ultrastructural aspects of fibrocartilage in a 1-day-old chick. Figure 17 is a low power view showing fibrochondrocytes with a large amount of intermediate-type filaments in the cytoplasm (asterisks) and the accumulation of glycogen at the cell periphery. The other cytoplasmic organelles are concentrated in a restricted area of the cytoplasm. These cells are usually nestel between collagen fibers (col). Figure 18 is an aspect of a fibroblast found in association with collagen fibrils. The cell processes extend amongst the collagen fibrils (asterisks) and delimit groups of fibrils in a manner resembling the development of normal collagen fibers in growing tendons. Fig. 17 7,546x and Fig. 18 12,589x

Figs. 19 and 20. Ultrastructural aspects of fibrocartilage in a 15-day-old chick. Figure 19 shows a detail of the cytoplasm of fibrochondrocytes, with the predominance of intermediate-type filaments (if) and glycogen granules (g). Figure 20 is a detail of a collagen fiber showing the presence of thin but mature elastic fibers (e) amongst the collagen fibrils (col). Collagen fibrils are not aligned as in typical tendons. Fig. 19 27,125x and Fig. 20 21,000x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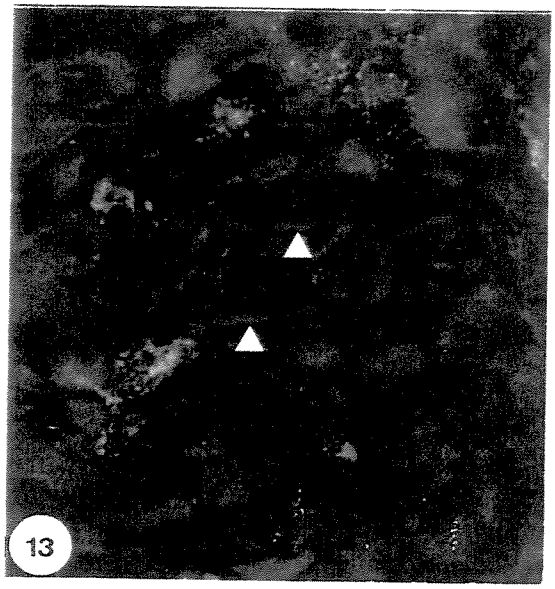
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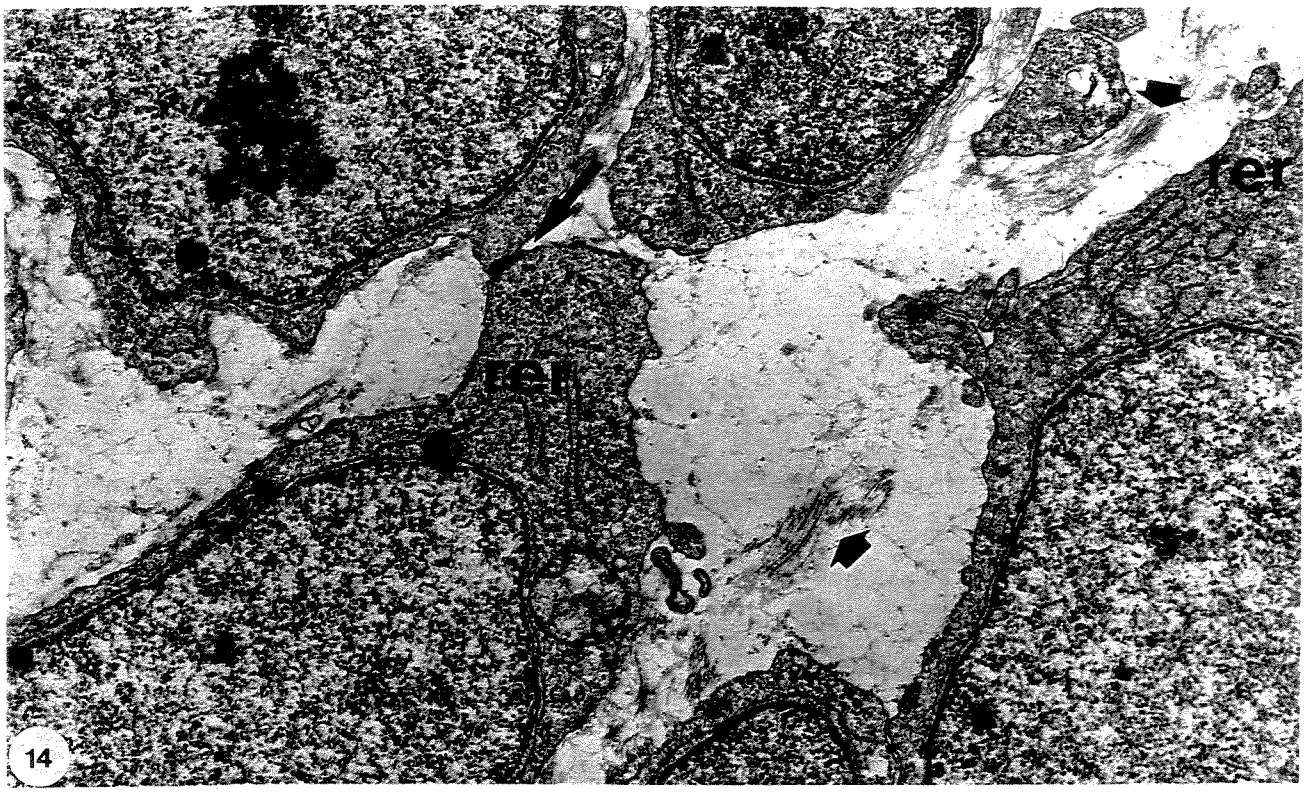
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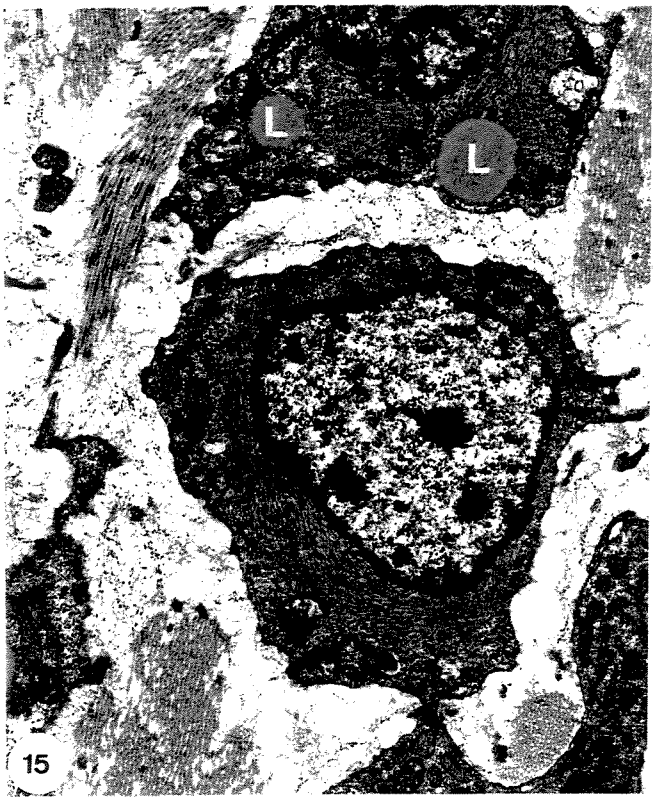
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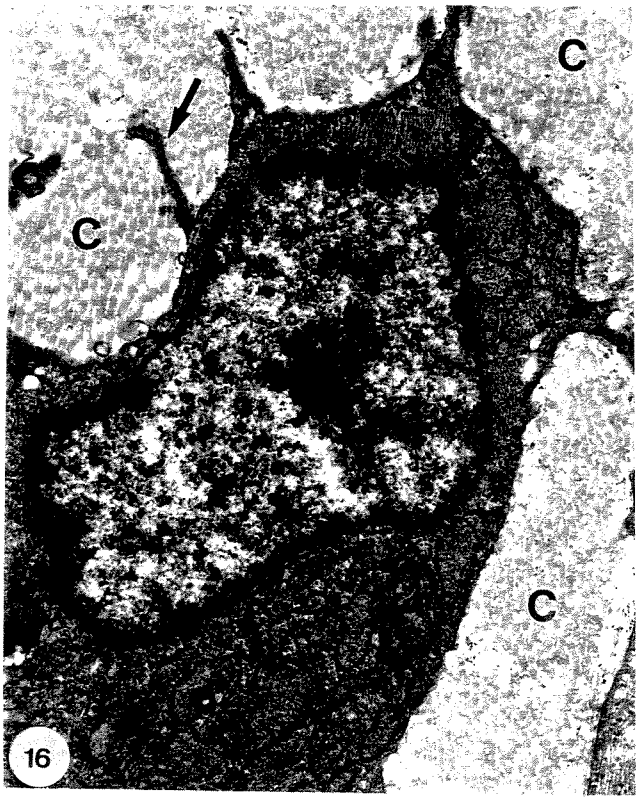
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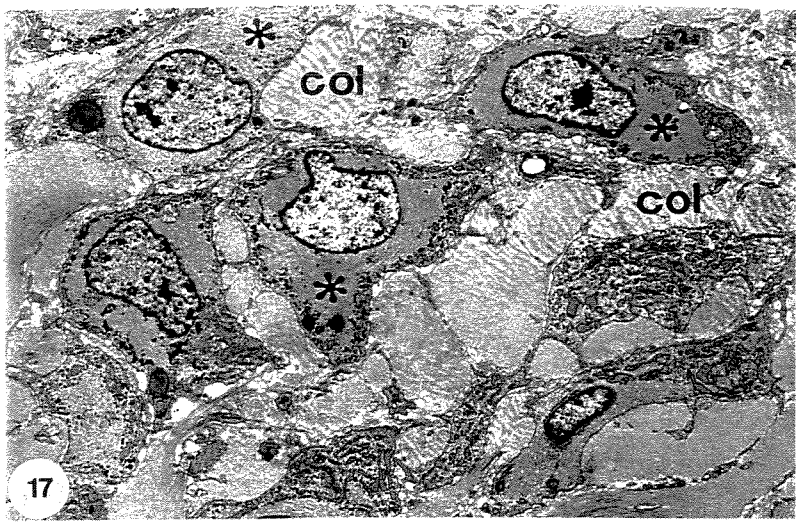

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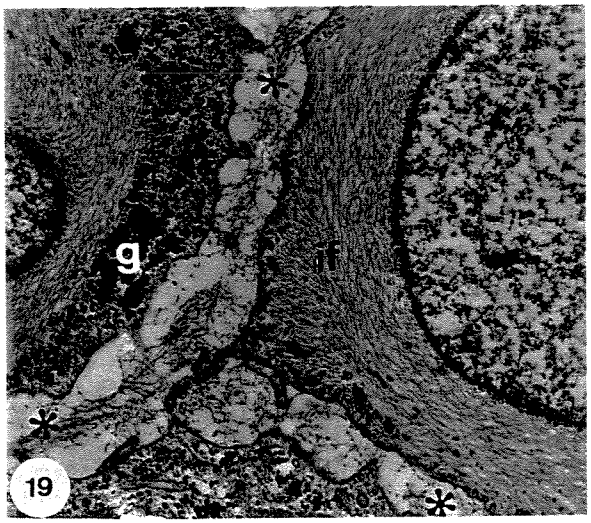

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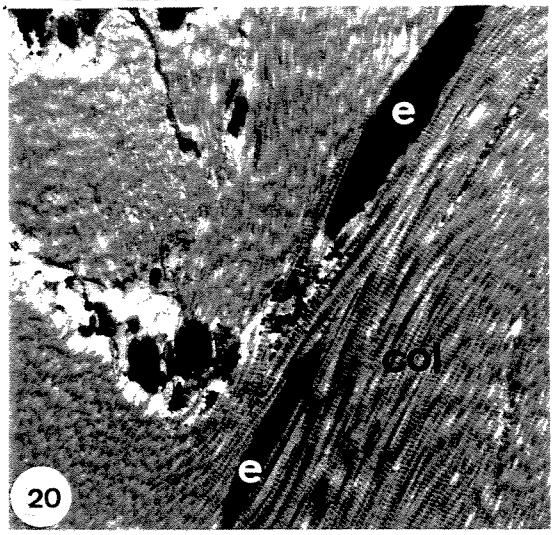

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4. Conclusões Gerais

1. As células do tendão elástico têm um papel ativo não somente na síntese e secreção de componentes da matriz extracelular, como também estão envolvidas com a formação de domínios extracelulares específicos para a formação das fibras de colágeno e de elastina.

2. As células da região elástica são polarizadas organizando elementos fibrilares distintos em domínios da membrana plasmática.

3. O desenvolvimento da fibrocartilagem assemelha-se aos de outras fibrocartilagens presentes em tendões, com a diferenciação de dois tipos celulares: os fibrocondrócitos e os fibroblastos.

4. Cada um destes dois tipos celulares parece ocupar regiões definidas e produzir componentes distintos da matriz extracelular.

5. A expressão da vimentina nos fibrocondrócitos, parece ser um fator importante pois essa expressão pode estar relacionada a uma resposta das células a existência de forças de compressão.

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