UNIVERSIDADE ESTADUAL DE CAMPINAS

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IDENTIFICAÇÃO E LOCALIZAÇÃO DE PROTEOGLICANOS/ GLICOSAMINOGLICANOS E DO COLÁGENO TIPO VI NA SÍNFISE PÚBICA DO CAMUNDONGO DURANTE A PRENHEZ

ANÁLISE ULTRA-ESTRUTURAL, IMUNOHISTOQUÍMICA E BIOQUÍMICA

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Mônica de Campos Pinheiro e aprovada pela Comissão Julgadora.

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"It is true that we cannot be visionaries until we become realists. It is also true that to become realists we must make ourselves into visionaries".

Roberto Mangabeira Unger

À minha mãe, Herondina de Campos

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SUMÁRIO

RESUMO

A sínfise púbica é uma articulação não sinovial, do tipo anfiartrose, que conecta os dois ossos púbicos através de um disco fibrocartilaginoso. Durante a prenhez, a sínfise púbica de camundongos passa por numerosas modificações estruturais, facilitadas por hormônios, a fim de permitir a passagem dos fetos pelo canal de parto. Essas modificações incluem um aumento da flexibilidade dessa articulação e a transformação do disco fibrocartilaginoso em um ligamento interpúbico flexível e elástico. O desenvolvimento do ligamento interpúbico envolve o aumento da biossíntese dos componentes da matriz extracelular, principalmente colágeno, proteoglicanos e glicosaminoglicanos e a mudança na relação entre síntese e degradação dos mesmos. Levando-se em consideração que o *turnover* destes componentes na sínfise de camundongos foi caracterizado por estudos bioquímicos e de microscopia de luz, pouco se sabe a respeito dos aspectos ultra-estruturais e histoquímicos de componentes da matriz extracelular, tais como os proteoglicanos e o colágeno tipo VI, bem como das interações entre as diferentes macromoléculas extracelulares. Esses aspectos são importantes porque podem ajudar a entender as relações entre os diferentes componentes da matriz extracelular nesse modelo. Nesse sentido, o objetivo desse trabalho foi caracterizar os glicosaminoglicanos, proteoglicanos e colágeno tipo VI na articulação interpúbica de camundongos nos diferentes estágios da prenhez. Atenção especial foi dada para as interações entre proteoglicanos e colágeno VI com outras macromoléculas da matriz. Para isso, foram empregadas análises citoquímica, imunohistoquímica, uso de sonda para detecção de ácido hialurônico, além de análises bioquímica e ultra-estrutural. Os resultados obtidos demonstraram que: 1) os proteoglicanos presentes na sínfise púbica de animais virgens são tecido-específicos e refletem as funções mecânicas de cada um dos tecidos no papel biológico global da sínfise púbica. 2) existe uma variação na quantidade e distribuição dos glicosaminoglicanos e proteoglicanos, como, por exemplo, o aumento de condroitim sulfato e o ácido hialurônico, durante a prenhez. Essas observações indicam que os proteoglicanos e glicosaminoglicanos devem ter um papel importante na formação do ligamento interpúbico e no aumento da extensibilidade desse ligamento no final da prenhez de camundongos. Estas macromoléculas podem ter um efeito importante nas propriedades de tensão do tecido. 3) A distribuição do colágeno tipo VI na sínfise púbica de camundongos virgens e prenhes é diferente nos vários tecidos que compõem essa articulação. Essa diferença pode refletir exigências funcionais diferentes para

esse tipo de colágeno. A localização pericelular sugere um papel na regulação da interação célula-matriz, protegendo a célula contra estresse mecânico. Entretanto, a interação do colágeno tipo VI com outros componentes da matriz extracelular tais como colágenos fibrilares pode ter um importante papel na organização do espaço interfibrilar, provavelmente associado às propriedades elásticas do tecido.

SUMMARY

Pubic symphysis is a nonsynovial amphiarthrodial joint that connects the two pubic bones by a fibrocartilaginous disk. During pregnancy, the mouse pubic symphysis undergoes a number of hormonally facilitated structural modifications to enable the passage of the fetuses through the birth canal. These modifications include an enhanced flexibility of the joint and the transformation of the fibrocartilaginous disk into a flexible and elastic interpubic ligament. The development of the interpubic ligament involves an increase of biosynthesis of the extracellular matrix components, mainly collagen, proteoglycans and glycosaminoglycans, in addition to a change in the relation between the synthesis and degradation of such components. The turnover of these components in mouse symphysis has been well characterized by biochemical studies and light microscopy. However, relatively little is known about the ultrastructural and histochemical aspects of the extracellular matrix components, such as proteoglycans and type VI collagen, as well as the interactions between different extracellular macromolecules. These aspects are important since they can help in the understanding of the relationship between the different extracellular matrix components in this model. Hence the aim of this study was to characterize the glycosaminoglycans, proteoglycans and type VI collagen in the mouse interpubic joint through the different stages of pregnancy. Special attention was given to the interactions between proteoglycan and type VI collagen with other matrix macromolecules. The methodology used involved cytochemical analysis, immunohystochemistry, hyaluronic acid probes, biochemical and ultrastructural analysis. The results showed the following: 1) Proteoglycans present in virgin pubic symphysis were tissue specific and reflected the mechanical functions of each tissue in the overall biological role of the pubic symphysis. 2) There is a variation in the amount and distribution of the glycosaminoglycans and proteoglycans during pregnancy as, for example, a greater presence of GAGs, such as chondroitin sulphate and hyaluronic acid. These observations indicate that proteoglycans and glycosaminoglycans must play an important role in the formation of the interpubic ligament along with its increased extensibility during late pregnancy in the mouse. These macromolecules may have important effect on tensile properties of this tissue. 3) The distribution of type VI collagen in virgin and pregnant mice pubic symphysis is different in various tissues that compose this joint. This may reflect the different functional demands for this collagen. The pericellular localization suggests a role in the regulation of cell-matrix interaction, protecting the cell against mechanical stress.

However, the interaction of type VI collagen with other ECM components, such as fibrillar collagens, may also play an important role in the organization of the interfibrillar space, probably associated with elastic properties of the tissue.

1. INTRODUÇÃO

1.1. Características morfológicas da sínfise púbica

As articulações ou junturas são uniões funcionais existentes entre quaisquer partes rígidas componentes do esqueleto, sejam ossos ou cartilagens. Elas permitem, não somente a união entre essas estruturas, mas também a mobilidade entre essas partes. As articulações variam em estrutura e disposição, sendo freqüentemente especializadas para desempenhar determinadas funções. No entanto, apresentam aspectos estruturais e funcionais em comum, sendo classificadas em três grandes grupos, baseando-se no grau de movimento e na natureza do elemento que se interpõem às peças que se articulam: junturas fibrosas (sinartroses) ou imóveis, junturas cartilagíneas (anfiartroses) ou com movimentos limitados, e junturas sinoviais (diartroses) ou junturas de movimentos amplos (Gardner et al., 1988; Gray, 1988).

Nos mamíferos, a sínfise púbica é uma articulação cartilagínea, não sinovial, que possui um grau limitado de movimento resultante da deformação de um coxim de conexão existente entre os ossos púbicos (Gamble et al., 1986; Gray, 1988). Esse coxim pode ser formado por cartilagem hialina (sincondrose) ou fibrocartilagem (sínfise), de acordo com a espécie animal (Ruth, 1932; Gamble et al., 1986; Ortega et al., 2001).

O termo sínfise, de origem grega, significa "crescendo junto", sendo que tal denominação é bastante apropriada, quando se descreve a articulação que está localizada na confluência dos ossos púbicos. Nesse contexto, cada osso púbico consiste de um corpo e dois ramos; na puberdade, o ramo superior funde-se ao ílio e o ramo inferior ao ísquio. A face sinfisal do corpo de cada osso é recoberta por uma fina camada de cartilagem hialina, que se une à do lado oposto por uma massa fibrocartilaginosa, o disco interpúbico. Assim, a sínfise púbica faz a conexão dos corpos dos ossos púbicos no plano mediano (Hall, 1947; Talmage, 1947a-b; Crelin, Levin, 1955; Storey, 1957; Steinetz et al., 1965; Ham, 1972; Gamble et al., 1986; Sgambati et al., 1996).

Estudos biomecânicos da pelve humana mostraram que os ossos do quadril atuam como arcos, transferindo o peso do pilar principal da região sacral para o quadril. A sínfise púbica faz a união desses dois arcos, sendo a integridade mecânica da articulação mantida por um conjunto de ligamentos, denominados circunferenciais. Os ligamentos suprapúbico,

púbico anterior e púbico posterior pouco contribuem para a estabilidade de toda a articulação, enquanto o ligamento púbico inferior ou arqueado é o principal promotor da estabilidade (Gamble et al., 1986; Gray, 1988; Sgambati et al., 1996). Embora esses ligamentos sejam morfologicamente classificados como quatro estruturas distintas, eles agem de forma difusa e harmônica, como se fossem uma estrutura única e contínua. Juntos, eles neutralizam as forças de tensão e cisalhamento, permitindo um movimento mínimo da articulação durante a maioria das atividades do indivíduo (Gamble et al., 1986).

A pelve feminina difere da masculina por apresentar ossos mais delicados e anatomia menos robusta. Além disso, as dimensões externas da pelve são maiores em machos, enquanto que dimensões internas são maiores em fêmeas, o que torna a cavidade pélvica feminina mais rasa e larga (Gamble et al., 1986; Gray, 1988). Graus variáveis de dimorfismo sexual são classicamente descritos no homem (Crelin, 1969b) e em modelos experimentais como o camundongo (Gardner, 1936; Crelin, Levin, 1955; Crelin, 1960), o rato (Crelin, Brightman, 1957; Bernstein, Crelin, 1967; Uesugi et al., 1992), o coelho (Lovell, 1965) e o morcego (Crelin, Newton, 1969). Como a pelve, a sínfise também exibe dimorfismo sexual, apresentando o disco interpúbico mais delgado, porém alongado no sentido vertical em machos, e mais largo em fêmeas. A mobilidade da articulação interpúbica nas fêmeas é 2-3 mm maior do que em machos; além disso, as mudanças hormonais e mecânicas que ocorrem na prenhez contribuem para que essa mobilidade aumente para 8-10 mm. Essas diferenças anatômicas refletem as adaptações necessárias para o parto (Crelin, 1969b; Gamble et al., 1986; Gardner et al., 1988; Sgambati et al., 1996).

Durante a prenhez, a pelve de mamíferos passa por extensas modificações com intuito de proporcionar um parto normal. Esse processo está sob controle hormonal e requer adaptações tanto da cérvix do útero como de toda a pelve, incluindo a sínfise púbica (Sherwood, 1994).

Com relação à sínfise púbica, as alterações estruturais levam a mudanças na estabilidade apresentada pela articulação, que variam conforme a espécie. Dentre essas alterações, verifica-se um aumento da flexibilidade da sínfise que pode ou não estar associado à separação dos ossos púbicos, em decorrência da transformação do disco fibrocartilaginoso, em um ligamento interpúbico. Tanto o aumento da flexibilidade como o aumento do diâmetro do canal de parto são vitais para o sucesso do parto em várias espécies (Hall, 1947; Talmage, 1947a-b; Wahl et al.,1977; Schwabe et al., 1978; Gamble et al., 1986; Sherwood, 1994).

A transformação da articulação fibrocartilaginosa em um ligamento flexível e extensível, durante a prenhez, foi descrita em várias espécies, incluindo a cobaia (Ruth, 1937; Talmage, 1947a-b; Wahl et al., 1977), o morcego (Crelin, 1969a; Crelin, Newton, 1969), o camundongo (Gardner, 1936; Hall, 1947; Steinetz et al., 1957; Storey, 1957; Linck et al., 1975) e o homem (Crelin, 1969b; Vix, Ryu, 1971; Gamble et al., 1986). No entanto, tal processo não é observado em espécies como, por exemplo, ratos e coelhos (Ruth, 1932; Crelin, Brightman, 1957; Samuel et al., 1998; Ortega et al., 2001).

O afastamento dos ossos púbicos, em camundongos, inicia-se por volta do 12º dia de prenhez com o desenvolvimento do ligamento interpúbico, podendo este aumentar em média 1mm por dia até o parto, que geralmente ocorre na noite do 19º dia de gestação. A separação dos ossos púbicos se deve não somente à formação do ligamento, mas em parte pelo entumescimento da cartilagem hialina que recobre as superfícies articulares, reabsorção das superfícies mediais desses ossos, e posterior substituição da região de osso reabsorvido e da cartilagem articular por tecido conjuntivo fibroso que compõem o ligamento. Nas etapas finais do processo de afastamento dos ossos púbicos, inicia-se o relaxamento do ligamento interpúbico, resultante da dissociação dos componentes da matriz extracelular (MEC) do tecido conjuntivo (Hall, 1947; Talmage, 1947a-b; Hisaw, Zarrow, 1950; Perl, Catchpole, 1950; Frieden, Hisaw, 1953; Steinetz et al., 1957; Storey, 1957; Cullen, Harkness, 1960; Wahl et al., 1977; Schwabe et al., 1978).

Após o parto, a reaproximação dos ossos púbicos se faz necessária para que ocorra o restabelecimento rápido da forma e função da articulação. Esse processo requer a involução do ligamento interpúbico, permitindo que a estrutura da sínfise retorne à dimensão e aparência semelhantes àquelas de um animal virgem (Hall, 1947; Steinetz et al., 1957; Storey, 1957; Horn, 1960).

Embora as evidências disponíveis indiquem o estrógeno e a relaxina como componentes reguladores chaves das transformações da sínfise púbica durante a gestação, ainda existem aspectos desconhecidos do mecanismo preciso da influência hormonal nesse processo.

Nesse contexto, o estrógeno está associado à formação do ligamento interpúbico levando ao afastamento dos ossos púbicos e cabe à relaxina um importante papel, induzindo o relaxamento do ligamento, com conseqüente aumento da flexibilidade da articulação. Assim, as mudanças que ocorrem na sínfise púbica de modelos como a cobaia e o camundongo, durante a prenhez ou experimentalmente pela ação combinada de hormônios

estrogênicos e relaxina, podem ser divididas em dois processos: 1) separação dos ossos púbicos e 2) relaxamento do ligamento interpúbico (Talmage, 1947a-b; Perl, Catchpole, 1950; Crelin, Levin, 1955).

Entretanto, devemos levar em consideração que, em animais tratados experimentalmente com hormônios, a separação dos ossos púbicos pode ser induzida pela ação de um único hormônio, o estrógeno, enquanto que o relaxamento é causado pela ação de um segundo hormônio, a relaxina, após tratamento prévio com estrógeno. Isto certamente não é o que ocorre durante a prenhez, em que a influência contínua de ambos os hormônios leva a mudanças no tecido sinfisal (Talmage, 1947a).

1.2. Aspectos gerais sobre o tecido conjuntivo e a matriz extracelular

Os tecidos conjuntivos são responsáveis pela definição e manutenção das formas do corpo, sendo a MEC mais abundante do que as células que ela envolve. De fato, a matriz é o componente principal do tecido conjuntivo e responde pelas propriedades físicas desse tecido.

A MEC corresponde aos complexos macromoleculares relativamente estáveis, formados por moléculas de naturezas diferentes, que são produzidas pelas células modulando a estrutura, fisiologia e biomecânica dos tecidos (Hay, 1991; Carvalho, Recco-Pimentel, 2001).

O emprego de métodos específicos e sensíveis na investigação de aspectos da remodelação da MEC tem proporcionado uma rápida evolução no conhecimento dos seus componentes e interações. Ao mesmo tempo tem explicitado que, além de fornecer o suporte estrutural para os tecidos, a matriz se constitui em importante meio através do qual transitam informações entre células, podendo influenciar o comportamento celular e participando direta ou indiretamente de uma série de processos que incluem morfogênese, diferenciação e migração celulares (Kielty et al., 1992; Martínez-Hernández, Amenta, 1993; Huijing, 1999).

De modo geral, a MEC pode ser dividida em 3 componentes principais: os fibrilares, os não fibrilares e as microfibrilas. Os fibrilares são representados pelos colágenos fibrilares e pelas fibras do sistema elástico; já os componentes não fibrilares correspondem aos proteoglicanos e ao grande grupo de glicoproteínas não colagênicas. As microfibrilas da

MEC são formadas pelo colágeno VI e pelas microfibrilas associadas à elastina, sendo que as primeiras pertencem à superfamília dos colágenos e as últimas formam com a elastina, o sistema elástico (Carvalho, Recco-Pimentel, 2001).

As variações nas quantidades relativas dos diversos tipos de macromoléculas, bem como suas complexas interações moleculares e celulares definem os arranjos tridimensionais característicos das diferentes modalidades de tecido conjuntivo, resultando em uma matriz que se adapta às demandas funcionais específicas de cada órgão (Kielty et al., 1992).

O principal componente da maioria das MECs dos tecidos conjuntivos é o colágeno, sendo seus arranjos supra-moleculares responsáveis pela estrutura e função tecidual (Linsenmayer, 1991; Nimmi, 1993; Birk, Linsenmayer, 1994). Entretanto, deve ser considerado que, de fato, os arranjos fibrilares não são estruturas isoladas, mas sim superestruturas da MEC que resultam de interações entre componentes colagênicos e não colagênicos (Huijing, 1999).

Assim como os colágenos fibrilares, as diversas macromoléculas que ocupam o espaço extracelular fazem interações e associações múltiplas que garantem à matriz ser reconhecida como uma unidade funcional singular (Brewton, Maine, 1994). Nesse contexto, os proteoglicanos e o colágeno VI são moléculas essenciais.

Os proteoglicanos pertencem a um grupo distinto de macromoléculas solúveis que apresentam tanto um papel estrutural como metabólico, na MEC (Iozzo, 1998-99; Culav et al., 1999). Importantes funções dessas moléculas abrangem suporte mecânico, adesão, proliferação e migração celulares, fibrilogênese e organização tecidual (Hakkinen et al., 1993; Iozzo, 1998).

Estudos recentes têm demonstrado a interação dos proteoglicanos com constituintes da matriz como o ácido hialurônico, colágeno e elastina, com particular ênfase na interação com o colágeno (Birk, Linsenmayer, 1994). Essas macromoléculas são elementos chaves na fibrilogênese dos diferentes tipos de colágeno e na regulação do arranjo supra-molecular das fibrilas na MEC dos tecidos conjuntivos (Vogel, 1994; Iozzo, Murdock, 1996). Além de se associarem a outros componentes da MEC, os proteoglicanos são conhecidos por estarem envolvidos na interação célula-matriz (Hascall et al., 1991).

Detalhes da biossíntese, composição bioquímica, distribuição e função dos proteoglicanos e glicosaminoglicanos nos diferentes tecidos e órgãos podem ser

encontrados em algumas revisões clássicas: Montes, Junqueira, 1988; Heinegard, Oldberg, 1989; Hardingham, Fosang, 1992; Yanagishita, 1993; Vogel, 1994; Iozzo, Murdock, 1996; Iozzo, 1998-99.

Assim como os proteoglicanos, o colágeno VI faz interações heterotípicas com outros componentes da MEC, bem como com receptores celulares influenciando a organização e a fixação das células à matriz (Kielty et al., 1992; Timpl, Chu, 1994; Felisbino, Carvalho, 1999). Desta forma o colágeno VI parece atuar como uma âncora nas interações célula-matriz e matriz-matriz. Nesse contexto, Nakamura et al. (1994) sugerem que os proteoglicanos possam mediar as interações entre o colágeno tipo VI e os colágenos fibrilares. Supõe-se que proteoglicanos/glicosaminoglicanos estejam fortemente relacionados às interações: fibrila de colágeno-colágeno VI e célula-colágeno VI. (Senga et al., 1995; Wiberg et al., 2001).

Os aspectos referentes à estrutura, biossíntese, distribuição e função do colágeno VI podem ser encontradas nas seguintes referências: Bruns et al., 1986; Keene et al., 1988; Timpl, Chu, 1994; Felisbino, Carvalho, 1999; Tulla et al., 2001; Wiberg et al., 2001.

A integridade da MEC é resultante de um balanço dinâmico entre síntese e degradação bem como das interações de seus diferentes componentes. Mudanças nesse balanço podem ter efeitos profundos na composição e organização da MEC, interferindo na histoarquitetura e fisiologia dos tecidos.

1.3. Remodelação da sínfise púbica na prenhez

Os órgãos reprodutores de roedores fêmeas respondem de modo semelhante aos de outros mamíferos, no que diz respeito ao controle hormonal da prenhez, parto e pós-parto, possuindo estruturas e funções definidas pelas características do tecido conjuntivo (Kroc et al., 1958).

A cérvix do útero, pelo fato de apresentar o tecido conjuntivo denso como seu principal componente, possibilita a observação de fenômenos que ocorrem na MEC, em virtude da síntese, deposição e reabsorção de suas macromoléculas (Golichowski et al., 1980; Junqueira et al., 1980; Downing, Sherwood, 1986; Vasilenko, Mead, 1987; Winkler, Rath, 1999).

Assim como a cérvix, o ligamento interpúbico é constituído por um tecido conjuntivo homogêneo, onde a remodelação da matriz ocorre rapidamente durante a prenhez em resposta aos estímulos hormonais, favorecendo substancialmente o estudo de fenômenos que dizem respeito a MEC (Wahl et al., 1977).

Embora as características morfológicas da sínfise e do ligamento interpúbico de várias espécies de mamíferos tenham sido exploradas à microscopia de luz, são escassos os relatos que enfocam os aspectos bioquímicos e histoquímicos dos componentes macromoleculares da MEC nessa articulação.

A caracterização bioquímica de componentes da matriz da sínfise púbica mostrou que ela é fundamentalmente sintetizada e secretada pelos fibroblastos. A matriz é constituída principalmente por água (60 a 70%), sendo que o colágeno contribui com 30 % do peso seco da fibrocartilagem. Aproximadamente 85% do colágeno é do tipo I e 15% do tipo II, havendo uma pequena quantidade de colágeno do tipo V (Samuel et al., 1996). No que diz respeito aos glicosaminoglicanos foi identificado o ácido hialurônico e, dentre os sulfatados, o condroitin sulfato como principal componente, seguido de dermatam sulfato e de uma fração não identificada (Viell, Struck, 1987). Do ponto de vista morfológico, a sínfise púbica é composta por uma trama fibrilar de colágeno, densamente empacotada, que circunda os fibroblastos, na qual se encontra pouca substância fundamental (Hall, 1947; Storey, 1957; Pinheiro, 1998). Os componentes do sistema elástico restringem-se a pequenos feixes de microfibrilas localizadas, principalmente, na fibrocartilagem e na cartilagem hialina (Moraes, 2001).

Os diferentes fatores hormonais e mecânicos, que atuam sobre a sínfise púbica durante a prenhez, produzem modificações na composição e no estado de agregação nos componentes macromoleculares da MEC desse tecido.

Estudos morfológicos e bioquímicos mostraram que as alterações que ocorrem na sínfise, durante a prenhez ou experimentalmente sob estímulo combinado de hormônios estrogênicos e da relaxina, são caracterizadas por uma modificação na deposição de complexos macromoleculares da matriz, bem como na síntese e secreção de enzimas envolvidas no processo de remodelação, como as catepsinas e as colagenases (Hall, 1947; Talmage, 1947a-b; Frieden, Hisaw, 1953; Crelin, Levin, 1955; Manning et al., 1965; Steinetz, Manning, 1967; Chihal, Espey, 1973; Whal et al., 1977; Schwabe et al., 1978; Weiss et al., 1979; McDonald, Schwabe, 1982; Viell, Struck, 1987). Associada a essas modificações, há a diferenciação de populações celulares do tecido conjuntivo. Assim, os condrócitos,

fibrocondrócitos e fibroblastos da sínfise passam a sintetizar e secretar quantidades crescentes de componentes da MEC.

As alterações que ocorrem na composição da MEC da sínfise púbica variam conforme a espécie. Em ratos, ocorre uma redução no peso da sínfise púbica, atribuído a uma diminuição significante no conteúdo de colágeno. Quando os níveis de relaxina são máximos (na prenhez ou experimentalmente) tanto o colágeno total como a sua concentração (conteúdo de colágeno total em relação ao peso seco de tecido) diminuem, sem no entanto mudar a proporção dos tipos de colágeno. Analisando as frações solúveis e insolúveis, a relaxina não tem efeito significativo na solubilidade do colágeno. A proporção de colágeno solúvel e insolúvel permanece inalterada (1:10) (Samuel et al., 1996-98).

A redução no conteúdo de colágeno da sínfise púbica de ratos pode ser atribuído a degradação do colágeno pela ativação do sistema colagenolítico, sem que o espectro dos tipos de colágeno seja alterado (Samuel et al., 1996-98).

Quanto ao componente elástico, foram encontradas fibras elaunínicas na matriz interterritorial da cartilagem hialina e na periferia dos feixes de fibras de colágeno do pericôndrio; além de fibras oxitalânicas na matriz ao redor dos condrócitos. Com relação ao conteúdo total de água e de glicosaminoglicanos, não ocorrem alterações significativas na sínfise púbica de ratos, durante a prenhez (Samuel et al., 1998; Ortega et al., 2001).

Ao que parece, a sínfise púbica de ratos passa por um *turnover* rápido do tecido conjuntivo durante a prenhez e parto, com discretas modificações no conteúdo e organização dos componentes da MEC. Suspeita-se que essas alterações envolvam a dissociação e desarranjo do colágeno, porém esses dados não são conclusivos (Samuel et al., 1998).

Os achados referentes à sínfise púbica de rato são contrários àqueles descritos para o camundongo e a cobaia, que relatam um aumento na quantidade de colágeno total na sínfise de animais prenhes ou tratados com estrógeno/relaxina e uma diminuição na sua concentração. Nessas condições, a quantidade de colágeno passa a representar 70% do peso seco do tecido sinfisal. Além disso, a relaxina induz uma mudança na solubilidade do colágeno, havendo um aumento no conteúdo de colágeno solúvel em relação ao colágeno total e uma diminuição na fração insolúvel (Wahl et al., 1977; Weiss et al., 1979).

Os dados referentes aos níveis de colagenase são contraditórios. De acordo com Wahl et al. (1977), esses níveis aumentam, em cobaias, durante a prenhez, mas a sua

concentração permanece baixa, uma vez que o peso do ligamento também aumenta. No entanto, Weiss et al. (1979) encontraram uma atividade aumentada de colagenólise após a administração de relaxina em camundongos, um resultado que se assemelha àqueles encontrados recentemente em ratos. No pós parto há o aumento nos níveis de colagenólise do ligamento interpúbico, envolvidas no rápido catabolismo do colágeno (Wahl et al., 1977).

Estudos *in vitro* com fibroblastos humanos mostraram que a relaxina diminui a síntese e secreção de colágeno e aumenta a sua degradação, uma vez que esse hormônio é capaz de estimular a expressão da colagenase e diminuir os seus inibidores (TIMP) (Unemori, Amento, 1990).

Com relação às fibras do sistema elástico, há uma variação nos tipos e distribuição das fibras, bem como um aumento no diâmetro e no comprimento aparente das mesmas durante a prenhez em camundongo. Ao que parece, esse componente da matriz exerce importantes papéis, tanto impedindo o rompimento do ligamento durante a sua evolução, quanto recuperando a organização dessa estrutura após o parto (Moraes, 2001)

Outro componente tecidual que muda na prenhez é a água; seu conteúdo aumenta aproximadamente 20% na sínfise púbica de camundongos, sendo o provável responsável pelo aumento do peso total do tecido. Esse acréscimo na quantidade de água pode ser atribuído a um aumento no conteúdo de ácido hialurônico (Zhao et al., 2000). De acordo com Viel, Struck (1987), a principal mudança no metabolismo de glicosaminoglicanos da sínfise púbica de camundongos, após tratamento com relaxina, é um aumento significante no conteúdo de ácido hialurônico concomitantemente a uma redução, não significativa, das outras três frações de glicosaminoglicanos. Associado a essas modificações, esses autores observaram uma maior atividade da N-acetilglucosaminidase no soro desses animais, contraditório aos resultados dos glicosaminoglicanos, mas que pode ser interpretado como uma indicação de estímulo de *turnover* dessas macromoléculas no tecido.

Recentemente foi demonstrado que em camundongos *knockout* para o gene da relaxina, o ligamento interpúbico no final da prenhez não distende significativamente devido a um arranjo mais compacto do colágeno; além disso não apresentam diferenças bioquímicas e aumento de água significativos. Isso indica que durante a prenhez, as mudanças no colágeno (tanto bioquímicas como histológicas) induzidas pela relaxina devem ser resultantes da degradação e/ou reorganização das fibras colágenas. Dessa forma, esse hormônio parece modular a síntese e degradação através da regulação da expressão do

colágeno, colagenase e inibidores de metaloproteases tecido-específicas (TIMP) (Zhao et al., 2000).

Embora o mecanismo preciso do relaxamento do ligamento, nas etapas finais do afastamento dos ossos púbicos, seja pouco explorado, sugere-se que ocorra um rápido *turnover* da MEC. Inicialmente foram levantadas duas hipóteses para justificar esse processo, uma que envolveria principalmente a despolimerização dos componentes não-fibrilares (Perl, Catchpole, 1950; Frieden, Hisaw, 1951; Catchpole et al., 1952, Crelin, Levin, 1955; Steinetz, Manning, 1967) e outra na qual haveria a dissolução de fibras de colágeno (Hall, 1947; Talmage, 1947a-b; Storey, 1957; Chihal, Espey, 1973).

Posteriormente, o emprego de métodos bioquímicos evidenciou que o relaxamento é um processo complexo que envolve a degradação enzimática do colágeno através de aumento da expressão de colagenase e diminuição de seus inibidores, bem como mudanças na sua arquitetura (Wahl et al., 1977; Weiss et al., 1979). Da mesma forma que o colágeno, componentes da substância fundamental, a exemplo dos glicosaminoglicanos, passam por alterações tanto quantitativas como no seu estado de agregação. Tais alterações ocorrem em função da síntese e secreção de enzimas envolvidas no processo de remodelação (Viell, Struck, 1987).

Frieden, Hisaw (1951) e Golichowski et al. (1980) sugerem que as mudanças na composição de polissacarídeos da matriz podem afetar as inter-relações entre fibrilas de colágeno e desse modo mudar as propriedades físicas do tecido de órgãos reprodutores, sob estímulo hormonal. Supõe-se que a formação de fibrilas e fibras colágenas mais finas, com menor quantidade de ligações cruzadas, talvez sejam mais susceptíveis ao ataque por enzimas proteolíticas. Esse fator, juntamente com o aumento de glicosaminoglicanos na sínfise permite a retenção de moléculas de água no tecido, levando a um aumento da permeabilidade e distensibilidade do tecido, facilitando a passagem dos fetos pelo canal de parto.

Quanto aos aspectos morfológicos, a transformação da sínfise púbica, durante a prenhez ou em condições experimentais, pode ser traduzida como um processo intenso de síntese e degradação de componentes da MEC, juntamente a uma reorganização ativa desses, levando a uma mudança na histoarquitetura e no grau de relaxamento do tecido conjuntivo.

Durante a prenhez quando há a formação entre os ossos púbicos, de um ligamento, este é formado por fibras colágenas grossas que apresentam uma ondulação característica das fibras colágenas de tendões e ligamentos, descrita na literatura como *crimp* (Baer et al., 1988; Gathercole, Keller, 1991; Carvalho, 1995; Vidal, 1995; Battlehner et al., 1996; Pinheiro, 1998). Nas etapas finais da prenhez, o relaxamento do ligamento é caracterizado pelo afastamento das fibras colágenas mais finas, frouxamente empacotadas ou largamente separadas com considerável quantidade de substância fundamental entre elas. As fibras se mostram menos onduladas e parcialmente dissociadas em componentes fibrilares mais finos. A análise ultra-estrutural demonstra que as fibras estão desorganizadas, e em algumas áreas as fibrilas aparecem digeridas. Há também uma redução dos diâmetros fibrilares e aparecimento de um componente granular difuso sugestivo de um processo de colagenólise (Chihal, Espey, 1973; Pinheiro, 1998). Esse padrão morfológico é semelhante ao descrito na cérvix uterina humana por Junqueira et al. (1980), o qual permitirá a dilatação do tecido necessária para o momento do parto.

Embora as características morfológicas da transformação da sínfise púbica em um ligamento apontem para um intenso processo de remodelação da matriz, em que inicialmente há um aumento da síntese de seus componentes para a formação desse ligamento e posteriormente uma extensiva despolimerização da substância fundamental e do colágeno, para que ocorra o relaxamento da estrutura, pouco se conhece sobre a composição bioquímica e os aspectos das interações entre os componentes da matriz do ligamento interpúbico.

1.4. Considerações finais

A sínfise e o ligamento interpúbico, a exemplo de sítios anatômicos onde o tecido conjuntivo têm função predominantemente mecânica, apresentam uma combinação apropriada de dois atributos principais - a capacidade de resistir a grandes forças de tensão (resistência à tração e compressão) e a capacidade de recuperar a forma e estrutura quando essas forças cessam. Sendo assim, é importante compreender, como seus componentes macromoleculares - colágenos, glicosaminoglicanos e proteoglicanos, elastina, sais minerais e água - conferem ao tecido seus atributos mecânicos, ainda que seja difícil determinar as contribuições individuais desses diversos componentes (Parry et al., 1978; Parry, Craig, 1988).

O modelo experimental da sínfise púbica de camundongos constitui um sistema adequado para o estudo do metabolismo de componentes da MEC sob estímulo hormonal, uma vez que a formação e a degradação desses componentes ocorre em um período relativamente curto. Além disso, o ligamento interpúbico é largamente empregado para o conhecimento da atividade biológica de polipeptídeos da família da relaxina.

De acordo com Zern, Reid (1993), as influências dos componentes da MEC na fisiologia e patofisiologia são dinâmicas, mudando constantemente e agregando efeitos cooperativos com hormônios, fatores de crescimento e outros sinais. Assim, o sinergismo entre os estímulos hormonais, as respostas celulares e os componentes da matriz afetam tanto a remodelação dessa matriz como a síntese e secreção de hormônios e fatores de crescimento. Desse modo, o reconhecimento das modificações morfológicas e bioquímicas apresentadas pela MEC é um elemento chave no entendimento da ação de hormônios e citocinas.

Portanto, estudos que enfocam aspectos da deposição e interação de componentes da MEC no processo de transformação da sínfise púbica em um ligamento extensível, durante a prenhez, podem contribuir para o conhecimento da regulação metabólica no processo de remodelação da matriz.

2. OBJETIVOS

Considerando-se as características morfológicas e bioquímicas da matriz extracelular, o processo de transformação gradual da sínfise púbica do camundongo em um ligamento extensível, e o reconhecido papel biológico dos proteoglicanos, a proposta do presente trabalho tem como objetivos:

1. Descrever a distribuição dos glicosaminoglicanos e proteoglicanos na sínfise púbica durante a formação do ligamento interpúbico na prenhez.
2. Identificar a natureza química dos glicosaminoglicanos constituintes da sínfise de animais virgens e do ligamento interpúbico durante a prenhez.
3. Avaliar as possíveis funções do colágeno tipo VI, através da sua localização, nos tecidos que compõem a sínfise púbica de camundongos virgens e o ligamento interpúbico, durante a prenhez.

De acordo com os resultados obtidos, este trabalho pretende ainda fornecer subsídios para a compreensão do processo de remodelação que ocorre nos mais diversos tecidos conjuntivos; além de contribuir para trabalhos subseqüentes que visam caracterizar os componentes da matriz extracelular e suas interações.

3. ARTIGOS

3.1. Artigos relacionados à tese

3.1.1. **Pinheiro MC**, Joazeiro PP, Mora OA, Toledo OMS (2002) Ultrastructural localization and characterization of the proteoglycans in the mice pubic symphysis.

3.1.2. **Pinheiro MC**, Joazeiro PP, Toledo OMS (2002) Ultrastructural, immunohistochemical and biochemical analysis of glycosaminoglycans and proteoglycans in the mice pubic symphysis during pregnancy.

3.1.3. **Pinheiro MC**, Joazeiro PP, Carvalho HF, Toledo OMS (2002) Distribution of type VI collagen in virgin and pregnant mice pubic symphysis.

3.2. Artigo não relacionado à tese (não anexado)

3.2.1. Toledo OMS, Marquezini MV, Jia KB, **Pinheiro MC**, Mora OA (2002) Biochemical and cytochemical characterization of extracellular proteoglycans in the inner circular smooth muscle layer of dog small intestine.

Ultrastructural localization and characterization of the proteoglycans in the mice pubic symphysis. Mônica C Pinheiro, Paulo P Joazeiro, Oswaldo A Mora, Olga MS Toledo

ABSTRACT

Analysis of proteoglycans (PG) in hyaline cartilage, fibrocartilage and dense connective tissue, those which constitute the mice pubic symphysis, was studied by electron microscopy staining with cuprolinic blue dye in "critical electrolytic concentration method" together with selective glycosaminoglycans degradative enzymes.

Ultrastructurally, proteoglycans were demonstrated as electron-dense granules and filaments of several sizes. PG-granules were exclusively found in hyaline cartilage in interterritorial matrix and in a regular array around the chondrocytes. They represented supramolecular aggregates of chondroitin sulfate proteoglycans. In the fibrocartilage and connective tissues the staining with cuprolinic blue showed only proteoglycans as filaments. They were identified by size, topography and susceptibility to enzyme digestion as F1-filament (40-60nm), a collagen fibril-associated also found in hyaline cartilage; F2-filament (70-90nm), localized preferentially in interfibrillar spaces, arranged between bundles of collagen fibers; F3-filament (40-70nm), scattered throughout elastic fiber surfaces. F1 and F2-filaments containing chondroitin sulfate chains whereas, F3 filaments were heparan sulfate proteoglycans, according with their susceptibility to specific enzymes.

The proteoglycans present in virgin pubic symphysis were tissue specific and reflects the mechanical function of each tissue in the overall biological role of the pubic symphysis.

INTRODUCTION

The pubic symphysis is a nonsynovial amphiarthrodial joint formed by two pubic bones coated by articular hyaline cartilage pads. These pads are linked by a dense connective tissue with a thin central area of fibrocartilaginous tecidual transition (Gamble *et al.*, 1986). This structure is involved dorsal and ventrally by a fibrous connective tissue. The symphysis connects the two pubic bones in the midle plan of the pelvis, and circunferential ligaments provides most of the joint stability, neutralizes shear and tensile stresses, permitting minimal motion during most activities of the animal body (Gamble *et al.*, 1986; Benjamin and Evans, 1990).

This structure meets the functional demands placed on it due to the different connective tissues of which it is composed. Such tissues are tailored to furnish mechanical support. The mechanical functions provided by the connective tissues, such as the ability to resist tension, compression and extensibility are determined by the amount, type and arrangement of the extracellular matrix macromolecules.

During pregnancy, structural modifications of pelvic girdle occur in many mammalian species, including rodents and humans, to enable safe passage of fetus at birth and animal movement (Hall, 1947; Talmage, 1947a; 1947b; Storey, 1957; Crelin, 1960; Lovell, 1965; Crelin 1969; Crelin and Newton, 1969; Steinetz *et al.*, 1965; Wahl *et al.*, 1977; Uesugi *et al.*, 1992; Sherwood, 1994; Samuel *et al.*, 1996).

Early histological studies in pubic symphysis showed that during pregnancy the articular cartilage and the medial ends of pubic bones are partially reabsorbed and the interpubic fibrocartilage observed in virgin animals is replaced by a flexible and elastic ligament. This ligament occupies the interpubic gap and allows sufficient separation of the pelvic bones at parturition (Steinetz *et al.*, 1957; Storey, 1957). This process depends on the hormonal regulation.

Several reports have shown that the components of extracellular matrix of symphysial connective tissues undergoes remarkable changes in the pubic symphysis during pregnancy in rodents and humans (Steinetz *et al.*, 1965; Viell and Struck, 1987; Samuel *et al.*, 1996). However, one lacks a detailed study of the localization and characterization of the proteoglycans. Information about the pattern of proteoglycans in virgin mouse pubic symphysis should constitute a useful basis for evaluating changes in proteoglycans distribution in the symphysis during pregnancy.

This paper describes an *in situ* ultrastructural cytochemical study on the distribution of proteoglycans of the virgin mouse pubic symphysis. The cationic dye cuprolinic blue used according to the critical electrolytic concentration associated with specific enzymatic treatments allowed for an accurate localization and characterization of the biochemical nature of glycosaminoglycan chains.

MATERIALS AND METHODS

Three-month old virgin female mice of a Swiss derived strain bred in the outhyside at the University State Campinas were employed in this study. The animals were housed in a 12 hr light/12 hr darkness schedule at 22° C, with unlimited access to food and water. Animal's studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, 1996).

The animals were deeply anesthetized with ether, killed by cervical dislocation, and the pubic symphysis was removed.

Light microscopy

Specimens were fixed in 4% paraformaldehyde in 0.1M phosphate buffer for 24 hours at 4°C, decalcified in 5% EDTA with 4% paraformaldehyde in 0.1M phosphate buffer for one week at 4°C, dehydrated and embedded in paraffin. Six-micrometer sections were stained by Masson's Thricromic technique.

Cuprolinic Blue Staining

Specimens were fixed overnight (room temperature) in 2.5% glutaraldehyde in 0.025 M sodium acetate buffer (pH 5.6) containing 0.30 M $MgCl_2$ and 0.2% cuprolinic blue (BDH Chemicals LTD, Poole, England). After cuprolinic blue staining the tissue slices were washed (3 times for 10 min each wash) in the same buffer solution without cuprolinic blue, immersed in 1% aqueous sodium tungstate for 30 min, and dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% sodium tungstate. Thereafter the slices were embedded in Epon 812. Thin sections of 70-80 nm in thickness were postained with uranyl acetate and lead citrate and examined in a ZEISS EM-900 at 60 kV electron microscope.

Enzyme Treatments

For enzymatic treatment the freshly samples were prefixed in 0.1% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 1-2h at room temperature. Following fixation the slices were washed extensively with the specific buffers to be used for subsequent enzyme treatments. Specimens were then incubated for 24 hr at 37ºC with the following enzymes: 1U/ml chondroitin AC-II lyase (EC 4.2.2.5) in 0.05 M Tris-HCl acetate buffer, pH 7.2, containing 0.05 M NaCl; 1U/ml chondroitin ABC lyase (EC 4.2.2.4) in the same vehicle. The protease inhibitors 0.1M 6-aminohexanoic acid, 0.01 M EDTA, 0.01 M N-etylmaleimide and 1mM phenylmethylsulfonyl fluoride were added to the reaction mixture in order to prevent proteolytic reaction. Controls were incubated in the same buffer solution containing the protease inhibitors but without enzyme. After the enzyme digestions the pubic symphysis specimens were stained with CB, as described above.

Morphometric Analysis

Distances were measured directly in electron micrographs using a Bausch and Lomb measuring magnifier. A minimum of 100 measurements of proteoglycan filaments was made in each studied region of tissue of the pubic symphysis. The magnification of the electron microscope was calibrated with a diffraction grating.

RESULTS

Light Microscopy

The study of Masson's trichrome section of virgin mouse pubic symphysis by light microscopy disclosed various features. Basically, the pubic symphysis is formed by the following structures: a) the articular caps of hyaline cartilage that covered the pubic bones at the medial ends, b) the interpubic disk of fibrocartilage sandwiched between the laterally situated hyaline cartilage and c); a region of dense connective corresponding to the most superficial layer of pubic joint continuos to the fibrous perichondrium/periosteum.

The hyaline cartilage and fibrocartilage areas of the joint showed typical chondrocytes surrounded by abundant extracellular matrix. Cells in the hyaline cartilage were similar to the typical hyaline articular cartilage in that they were spherical, isolated or arranged in small clusters. The whole hyaline and fibrous cartilage are covered dorsally and ventrally by a typical layer of dense connective tissue were the predominant cell type is fibroblast (figure 1).

General Aspects of the Cytochemical Reaction

After treatment with cuprolinic blue the proteoglycans of the virgin pubic symphysis were observed as electron dense filaments and granules of several sizes. The PG-granules could be distinguished into two classes; one consisted of granules measuring 10 – 90 nm of diameter, while the other class consisted of granules with a maximum diameter of 180 nm. According to their sizes, localization and enzymatic susceptibility the proteoglycan-filaments were classified as F1, F2, F3. These filaments measuring 40 to 90 nm in length (table 1). The proteoglycans analyses were performed in distinct connective tissues that formed the pubic symphysis structure: caps of hyaline cartilage; fibrocartilage disk; and out layer of dense connective tissue.

Hyaline Cartilage

Micrographs of sections through the pubic symphysis showed in the extracellular matrix of the hyaline cartilage three types of proteoglycan precipitates that could be distinguished in typical localization: 1) In the interterritorial matrix where small, thin PG-filaments with a maximum length of 60 nm, termed F1, masked the delicate fibrilar collagen network. These filaments sometimes at the interterritorial matrix were collagen fibril-associated. One-second class of larger, thick and heavily staining PG precipitates, in a shape of granules, named G1, was also observed in the interterritorial matrix. They were alleatoraly distributed through the interfibrillar spaces. These granules measured up to 10nm and down to 90nm in diameter (Figure 2A, 2C); 2) In the territorial matrix F1-filaments were also observed along with another PG-precipitates in a granule-like shape. These granules appear as irregular structures, which are very large, thick and heavily cuprolinic blue stained. They were named G2 and no direct relation of these granules and collagen fibrils could be observed. Their minimal diameter was 20nm and their maximal 180nm (Figure 2A, 2B). The overall contrast of the territorial matrix compared to the interterritorial matrix were the enhances of closer spacing of the PG-granules seen in the territorial matrix; 3) In a pericellular zone around the chondrocytes, an area that appears at low magnification to be devoid of fibrilar components, a layer of matrix consisting mainly of G2 granules was observed (Figures 2A, 2B).

In cartilage, both chondroitinases AC-II or ABC removed completely the F1 filaments in the interterritorial and territorial matrices. After chondroitinase AC-II and ABC treatment, PG-granules (G1) concentration in the interterritoral matrix were reduced, whereas the G2-granules concentration (Figures 4A, 4B) present in the territorial matrix were slight affected.

Fibrocartilage

In this tissue the cytochemical reaction demonstrated two positive cuprolinic blue filaments one similar of F1-filaments observed in hyaline cartilage measuring 40-60 nm of length, and another one larger and with heavy staining cuprolinic blue, named F2-filament measuring 70-90 nm of length. The F1-filaments lying to collagen fibrils at regular distance, which corresponds to the D-period, a collagen banding repeat distance (about 64 nm). F1-filaments were arranged mainly orthogonal to the collagen fibrils. The F2-filaments, on the other hand, were localized between bundles of collagen fibrils or at places where the collagen fibrils are somewhat separated from each other (Figure 2D). Both F1 and F2 filaments were completely removed by the treatment with chondroitinases AC and ABC (Figure 4C).

Connective Tissue

The PG-filaments of dense connective tissue consists of thin, small cuprolinic blue filaments with 40-60 nm of length closely associated with collagen fibrils at regular intervals (Figure 2E), similar to those found in fibrocartilage (Figure 2D) and hyaline cartilage (Figure 2C), the F1-filaments. Almost all PGs were directed perpendicularly to the long axis of collagen fibrils. These filaments were entirely removed by chondroitinase AC-II treatment (Figure 4D, 4E).

Another type of PG-filaments (F3), almost the same size of F1-filaments measuring 40 to 70nm of length were observed at the surface of elastic fibers without a preferential direction (Figure 4F). These precipitates were also observed at elastic fibers of fibrocartilage. They are still visible after chondroitinases AC-II or ABC digestions (Figure 4F).

Effect of Glycosaminoglycan-Degrading Enzymes and Control of the Cytochemical Reaction

Proteoglycans were characterized by the predominant chemical composition of their glycosaminoglycan side chains.

Incubation of glutaraldehyde-fixed tissues in buffer solution at neutral pH did not reduce or alter the number and shape of the different PG-CB precipitates (Figures. 3A, 3B and 3C). However, when incubation was performed under the same conditions but in the presence of glycosaminoglycan degrading enzymes, partial or complete loss of the PG precipitates, depending on the specificity of the enzyme used, was observed.

DISCUSSION

It is well established that cuprolinic blue containing $MgCl_2$ at critical electrolyte concentrations preserves the proteoglycans of tissues and results in the presence of electron dense filaments (Scott, 1980). Our results showed by this method two distinct form of proteoglycan precipitates; filaments-like or granules-like shape in mouse pubic symphysis tissues.

The electron dense filaments could be distinguished into three proteoglycan classes according with their length, localization and specific susceptibility of the enzymes.

The F1-filaments measuring 40-60 nm length were distributed along the hyaline cartilage, fibrocartilage and connective tissues associated with collagen fibrils. Their specific susceptibility to chondroitinase AC-II treatment allowed identifying this collagen-associated proteoglycans as composed exclusively by chondroitin sulfate.

F1-filaments sometimes were closely associated with collagen fibrils and perpendicularly orientated with respect to the collagen axis fibrils at a periodic spacing.

Decorin, a chondroitin sulfate/dermatan sulfate substituted proteoglycans has one glycosaminoglycan chain, either chondroitin or dermatan sulfate (Fisher 1993). This leucine-rich repeat motif proteoglycan family was shown to interact with collagens and influence collagen fibrillogenesis (Vogel *et al.*, 1984). Decorin is able to interact with the collagen I and II (Tsuprun and Santi, 1996) surfaces at the "d"and "e" bands (Scott and Haigh, 1986; Scott, 1988).

Although no immunocytochemical study has been performed, the results obtained by the enzymatic treatments associated with the typical distribution among the collagen fibrils lead us to consider that the collagen fibrils in the mouse pubic symphysis are associated with the proteoglycan decorin with chondroitin sulfate chain. Similar result was observed by van Kuppevelt *et al.* (1984), using cuprolinic blue staining to study collagen-proteoglycan interactions in mouse lung.

The F2-filaments, organized as a network in the intercellular spaces and not collagen-attached appear to be also proteochondroitin sulfate. This could be concluded from the observations that the enzymatic treatment with chondroitinase AC-II completely removed F2-filaments.

According to their core protein several proteoglycans can be distinguished (Yanagishita, 1993). For example, decorin, the collagen-attached PG with only one

chondroitin sulfate or dermatan sulfate attached to a core protein. Versican, a large interstitial PG with a similar core of aggrecan but only chondroitin or dermatan sulfate side chains.

The post-translation modification of chondroitin or dermatan sulfate proteoglycans appears to be flexible in that differentially glycosylated forms of these proteoglycans may be spatially and developmentally regulated. The biological significance of this modulation still remains unknown, however, this apparent regulation of glycosylation clearly supports the idea that multiple factors determine the attachment of a glycosaminoglycan chain to the core protein, a for example the endogenous levels of xylosyl-transferase (Hocking *et al.*, 1998).

The F3-filaments with localization at the surface of elastic fibers represents a proteoglycan, which bears heparan sulfate as its glycosaminoglycan component since they were not affected by digestion with chondroitinases AC-II or ABC.

Heparan sulfate proteoglycans has been demonstrated at the surface of elastic fibers in some studies (Völker *et al.*, 1986; 1987; Pasquali-Ronchetti *et al.*, 1993; Erlinger, 1995; Hinek *et al.*, 2000). The physiological value of proteoglycan associated with elastic fibers is not entirely clear.

Proteoglycans make a major contribution to the mechanical properties of articular cartilages, where the tissue has a load bearing function. The predominant proteoglycan present in cartilage is the aggrecan, which has more than 100 chondroitin sulfate glycosaminoglycan side-chains. Other than that aggrecan self-assembles into a supramolecular structure with as many as 50 aggrecans bound to a single strand of hyaluronan forming supramolecular aggregates of very large size. Additional link proteins contribute to the cohesiveness of these aggregates.

Usually, the pericellular region and the territorial matrix have the huge aggregates of proteoglycans while in the interterritorial matrix the aggregates are smaller (Knudson, 2001). These differences could be exactness visualized in this study using cuprolinic blue method.

Our results showed proteoglycans as two granule-shaped structures. The granules were confined to the hyaline cartilage. The smallest granules, named G1 were only observed to the interterritorial matrix while the G2-granules were observed in pericellular region and in territorial matrix. The specific enzymatic treatment with chondroitinases showed that both granules were susceptible to these glicosidases indicating that chondroitin sulfate chains composed them.

On the basis of the cuprolinic blue method we showed that proteoglycans present in virgin pubic symphysis were tissue specific and reflects the mechanical function of each tissue in the overall biological role of the pubic symphysis.

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FIGURE LEGENDS

Figure 1. Photomicrographs of transverse section through the medial region of the mouse pubic symphysis stained with Masson's Trichrome. The section contains the medial ends of the pubic bones (pb), their hyaline cartilage (hc) and fibrocartilaginous (fc) caps, and the surrounding connective tissue sheaths (ct). Magnifications: 320X.

Figure 2. Electron micrographs of mouse pubic symphysis stained with Cuprolinic blue (CB) in critical electrolytic concentration (CEC). A) Low magnification of hyaline cartilage showing the territorial (tm) and interterritorial (im) matrix. Note the difference of density between the two regions. 11150X B) Territorial matrix cartilage: proteoglycans (PGs) are observed as electron dense granules (arrowheads) and a small amount of thin filaments (arrow). 15000X. C) Interterritorial matrix cartilage: observe the fewer amounts of electron dense granules (arrowheads) of proteoglycans and the network of fine filaments (arrow) markedly more developed. 19400X. D) Fibrocartilage: electron dense PG-filaments are almost arranged along collagen fibrils (arrows); a little amount of large PG-filaments is randomly distributed (large arrows). 41750X. E) Dense connective tissue: PGs are deeply stained filaments closely associated with the outside of collagen fibrils (arrows). 53900X.

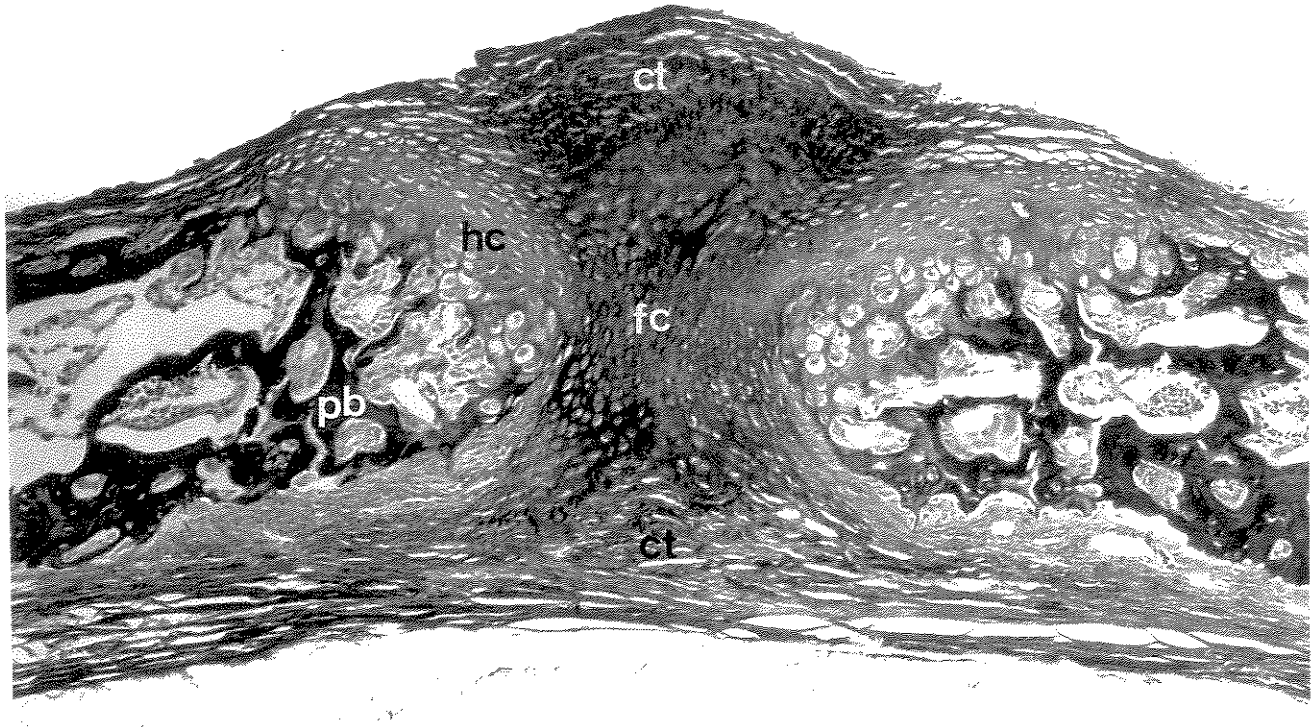
Figure 3. Electron micrographs of mouse pubic symphysis stained with CB at CEC after incubation in the same buffer solutions as used for enzyme treatment. The distribution and number of PG CB- precipitates are unaltered (compare figures 2C , 2D and 2E with 3A, 3B and 3C). A) Hyaline cartilage, 41800X; B) Fibrocartilage, 41800X and C) Connective tissue, 32300X.

Figure 4. Electron micrographs of mouse pubic symphysis stained with CB at CEC after digestion with chondroitinase AC-II or ABC. A) The chondroitin sulfate AC-II lyase treatment removes a part of the PGs precipitates at the territorial cartilage. B) In the interterritorial matrix cartilage the precipitates have been digested, only granular material remains (arrowheads). C) Fibrocartilage after digestion with chondroitinase AC. The vast majority of the precipitates are digested after enzymatic treatment. D and E) The chondroitinase AC-II treatment removes almost all PGs precipitates associated with collagen (col) and with the matrix soluble (asterisks) in the dense connective tissue. F) Elastic fiber (El) surrounded by collagen (col) after digestion with chondroitinase ABC. The collagen fibrils are almost free of precipitates. Some precipitates remain associated with the elastic fiber (arrowheads). A) 32300X; B-F) 53900X

TABLE

Table 1. Distribution of the cuprolinic blue stained proteoglycans (PG) in mouse pubic symphysis

PG-Staining structures	Size (nm)	Distribution in Tissue
G1-granules	10-90	*Hyaline Cartilage*
		Matrix Interterritorial
G2-granules	20-180	*Hyaline Cartilage*
		Matrix Territorial
F1-filaments	40-60	*Hyaline Cartilage*
		Associated or Not with Collagen Fibrils
		Fibrocartilage and Connective Tissue
		Associated with Collagen Fibrils
F2-filaments	70-90	*Fibrocartilage and Connective Tissue*
		Interfibrillar Spaces
F3-filaments	40-70	*Fibrocartilage and Connective Tissue*
		Surface of Elastic Fibers



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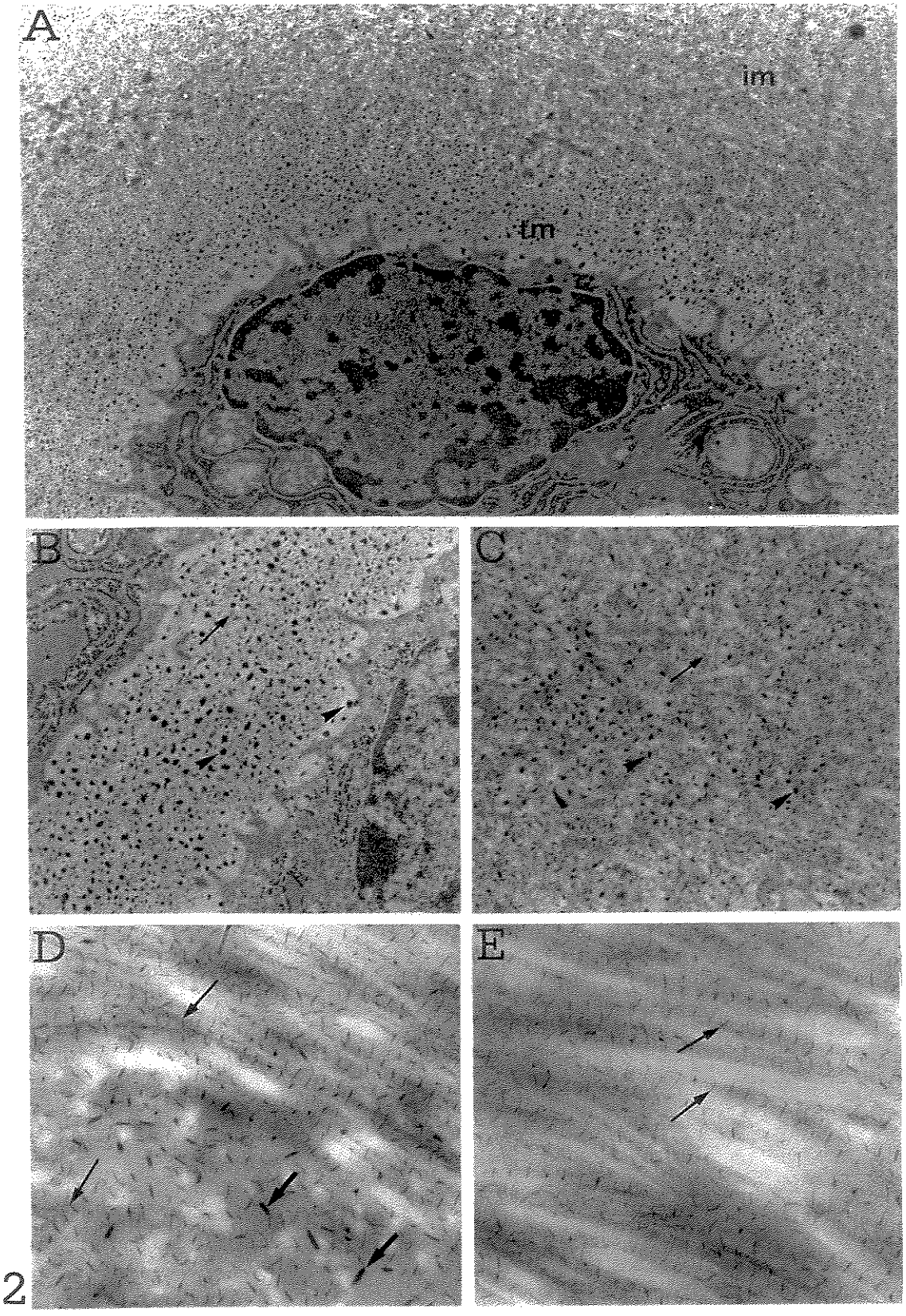

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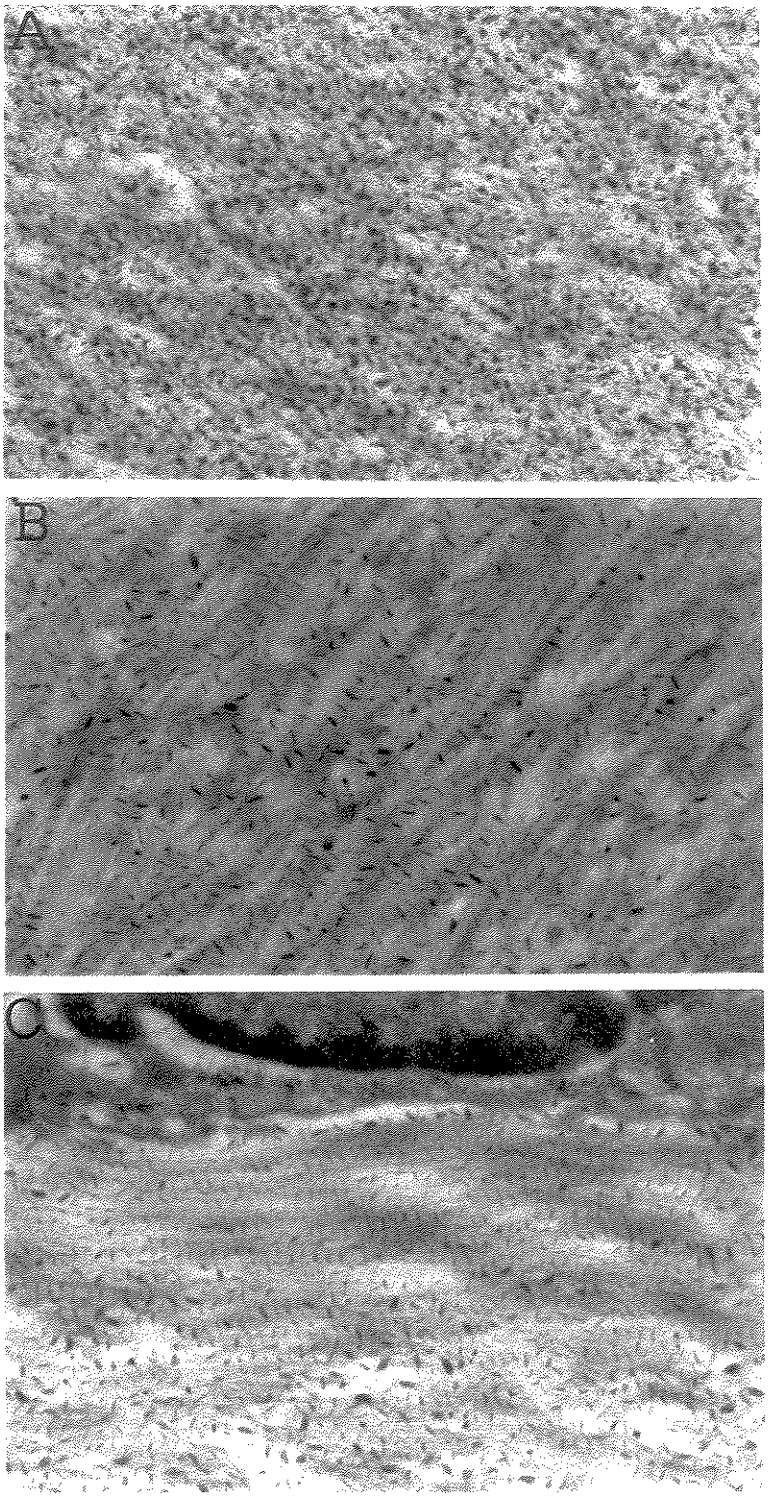

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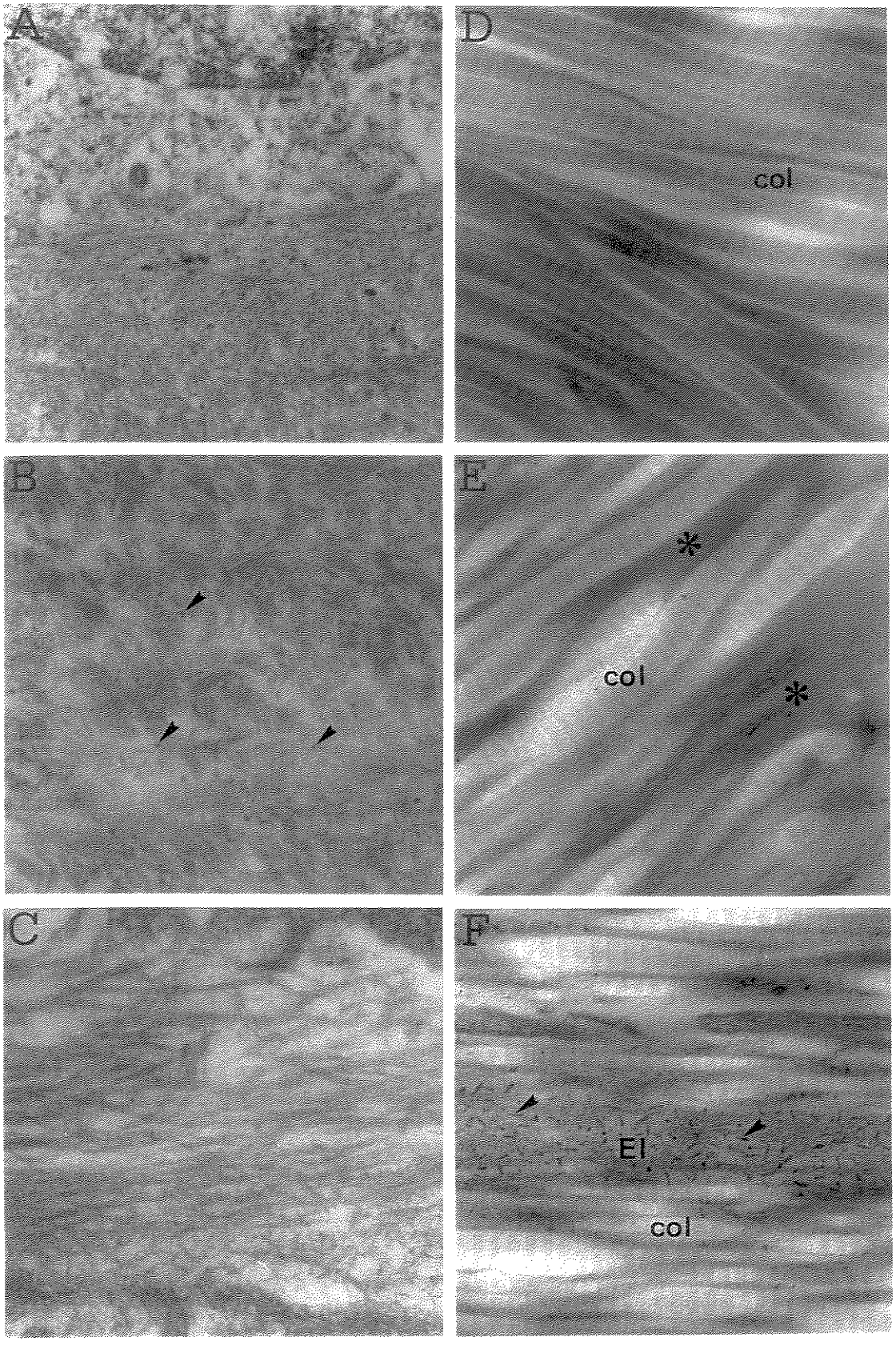

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Ultrastructural, Immunohistochemical and Biochemical Analysis of Glycosaminoglycans and Proteoglycans in the Mice Pubic Symphysis During Pregnancy. Mônica C Pinheiro, Paulo P Joazeiro, Olga MS Toledo

ABSTRACT

Ultrastructure localization of proteoglycans in the pubic symphysis of pregnant mice was studied by transmission electron microscopy staining with cuprolinic blue in a "critical electrolyte concentration" method. Proteoglycans were visualized as electron-dense filaments. The localization, appearance and its binding to collagen were similar in fibrocartilage and interpubic ligaments. The treatment with chondroitinase lyases showed chondroitin sulfate (CS) as unique sulfated glycosaminoglycan found. This sulfated glycosaminoglycan increase during pregnancy, reaching its maximal on the fifth day (118%) followed by an abrupt decrease after this day. Such decrease was of 74% on day 17. However, the decrease relative to CS contents of pubic symphysis of virgin mice to those of days 17 and 18 was of 44%.

Immunohistochemical analyses were performed using antibodies from CS, decorin, biglycan and hyaluronic acid (HA). CS, decorin and biglycan were distributed along collagen fibrils and around the cells. The changes of CS concentration during pregnancy provide further evidence that PGs are important to modifications in symphysial tissues necessaries for the formation and relaxation of the interpubic ligament during pregnancy.

The stronger binding of HA probe on day 18 may indicate a greater concentration of this GAG on one of the latest day of pregnancy. The HA, being a polyelectrolyte macromolecule, play a key role for the accumulation of the fluid in the relaxing ligament enabling successful parturition.

INTRODUCTION

During the pregnancy, the pelvic girdle in particular the pubic symphysis, to undergo numerous structural modifications. The adaptation of the pelvis is hormonally regulated and allows the passage of young through the pelvic birth canal (Sherwood, 1994).

In many mammalian species, including guinea pig, bats, mice and human, the interpubic fibrocartilage is replaced by a flexible and elastic ligament. The first changes in the mouse pubic symphysis occur at 12 days pregnancy when the biosynthesis activity of the extracellular matrix components in the center of pubic joint is altered. At the last week of pregnancy, the pubic bones begin to separate and continue to do so progressively throughout the remainder of pregnancy (Crelin, 1969; Schwabe et. al., 1978).

The development of the interpubic ligament involve the shifting of the extracellular matrix turnover and the extracellular matrix components re-arrangement (Crelin, 1969).

Proteoglycans and collagens are prominent extracellular-matrix components in the class of connective tissues.

Proteoglycans (PGs) are a class of extracellular matrix macromolecules with ability to resist compressive forces. These macromolecules are constituted of a protein core which one or more glycosaminoglycan (GAG) chains are attached. The connective tissue extracellular matrix PGs are low molecular weight and high molecular weight. Biglycan and decorin belong to the group of small PGs which has attached to the core protein one or two dermatan/chondroitin sulfate side chains (Krusius and Ruoslahti, 1986; Fisher et al., 1989). Aggrecan and versican, are examples of a large aggregating chondroitin-sulfate proteoglycans (Zimmermann and Ruoslahti, 1989). Decorin interacts with fibrilar collagens (Fleischmajer et al., 1991; Hedbom and Heinegard, 1989) and intervenes in fibrillogenesis (Neame et al., 2000). Biglycan has been mostly localized in the pericellular region (Bianco et al., 1990; Fleischmajer et al., 1991). However, evidence for an interaction with fibrillar collagen has been provided (Schönherr et al., 1995).

The common molecular feature of collagens is a triple helix region, which is necessary to provide the collagens with the ability to resist tensile forces. Distinct types of collagen with individual characteristics serve specific functions in a variety of tissues. Collagens fibrils may also be formed of more than one type of collagen for example, type I combine with type VI and type II with type IX to form heterotypic fibrils (Culav et al., 1999).

In this way, the various mechanical behaviors of the different connective tissues such as the ability to resist tension, compression, mainly the types and proportions of two classes

of macromolecules: proteoglycans and collagens, determine the properties of extensibility and torsion.

The influence of the GAGs on the symphysial relaxation process was early reported by Storey, 1957. Viell and Struck, 1987 analyzed the changes in the GAG metabolism after relaxin treatment. However, the composition and the changes of the proteoglycans/GAGs in the tissue components of the symphysial structure as the collagen-proteoglycans interactions are yet to be determined.

This study attempt to describe the changes in that occur when the pubic symphysis relaxes during pregnancy. The ultrastructural and immunohistochemical, localization of proteoglycans in mice pubic symphysis during the pregnancy was reported. The content and type of glycosaminoglycan side chains of this proteoglycans were demonstrated by biochemical analysis. The relevance of these findings to collagen-proteoglycans interactions was discussed.

MATERIALS AND METHODS

Virgin female Swiss mice 3 month old and over 25-30g of body weight (Center for Animal Care of State University of Campinas, SP, Brazil) were used. The animals were housed in a 12 hr light/12 hr darkness schedule at 22° C, with unlimited access to food and water.

To obtain pregnant specimen, virgin females were caged overnight with males and a "plug" in the vagina the following morning indicated successful mating and this day were designated the first day (D1) of pregnancy. The delivery could be expected on day 19. Pubic symphysis samples were achieved from animals during pregnancy (D12, D15, D17 and D18). Virgin mice in estrus were also used as control. Estrus was determined by vaginal smears according to Shorr (1941).

The animals were deeply anesthetized with ether, killed by cervical dislocation, and the pubic symphysis were dissected out and immediately fixed as below or stored at -70°C for the biochemical analyses. Animal's studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, 1996).

Ultrastructural Cytochemistry

Specimens were fixed overnight (room temperature) in 2.5% glutaraldehyde in 0.025 M sodium acetate buffer (pH 5.6) containing 0.30 M $MgCl_2$ and 0.2% cuprolinic blue (BDH Chemicals LTD, Poole, England). After cuprolinic blue staining the tissue slices were washed (3 times 10 min) in the same buffer solution without cuprolinic blue, immersed in 1% aqueous sodium tungstate for 30 min., and dehydrated in ascending concentrations of ethanol, the 30 and 50% concentrations containing 1% sodium tungstate. Thereafter the slices were embedded in Epon 812. Thin sections of 70-80 nm in thickness were postained with uranyl acetate or phosphotungstic acid and lead citrate, and examined in a ZEISS EM-900 at 60 kV electron microscope.

Enzyme Treatments

Pubic symphyses were prefixed in 0.1% glutaraldehyde and 1% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 1-2h at room temperature. Following fixation the symphysis were washed extensively with the specific buffers to be used for subsequent enzyme treatments. Specimens were then incubated for 24 hr at 37ºC with the following enzymes: 1U/ml chondroitin AC-II lyase (EC 4.2.2.5) in 0.05 M Tris-HCl acetate buffer, pH 7.2, containing 0.05 M NaCl; 1U/ml chondroitin ABC lyase (EC 4.2.2.4) in the same vehicle. The protease inhibitors 0.1M 6-aminohexanoic acid, 0.01 M EDTA, 0.01 M N-etylmaleimide and 1mM phenylmethylsulfonyl fluoride were added to the reaction mixture in order to prevent proteolytic reaction. Controls were incubated in the same buffer solution containing the protease inhibitors but without enzyme. After the enzyme digestions the pubic symphysis specimens were stained with CB, as described above.

Samples (50μg) of the GAGs isolated from fibrocartilage of pubic symphysis of virgin animals or from interpubic ligament from days 12, 15, 17 and 18 of pregnancy were digested with the enzymes above cited. After the enzyme digestions the pubic symphysis specimens were submitted to electrophoresis.

Morphometric Analysis

Distances were measured directly in electron micrographs using a Bausch and Lomb measuring magnifier. A minimum of 100 measurements of proteoglycans filaments was made in each studied region of tissue of the pubic symphysis. The magnification of the electron microscope was calibrated with a diffraction grating.

Histochemistry and immunohistochemistry

Specimens were fixed in Carnoy's mixture, i.e. (ethanol:chloroform:acetic acid 6:3:1 by volume) for 24 hours at 4°C, decalcified in 7% EDTA with 2% paraformaldehyde in 0,1M phosphate buffer pH 7,4 for 5 days at 4°C, dehydrated and embedded in paraffin. Serial sections (7μm in thickness) were cut in transversal plane thought the symphyseal region and mounted in silanized slides.

Deparaffinized sections were immersed in methanol containing 0,3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity, washed in PBS, and exposed to normal serum for 20 minutes. Excess serum was gently blotted off and sections were incubated with primary antibodies diluted in PBS 0,01M with 1% BSA (Sigma Chemical, St. Louis, MO, USA) overnight at 4°C in humidified chamber. The antibodies were anti-chondroitin sulfate monoclonal antibody (diluted 1:250; Clone CS-56, Sigma); anti decorin (diluted 1:500; Dr. L Fischer) and anti-biglycan policlonal antibodies (diluted 1:500; Dr. L Fischer). The sections were washed with PBS 3 times, and incubated with biotinylated universal secondary antibody solution (Novostain Super ABC kit, Novocastra) for 30 minutes at room temperature. This was followed by another rinsing with buffer and incubated with Novostain ABC kit contain avidin DH and biotinylated horseradish peroxidase H reagents for 30 minutes at room temperature. After rinsing with 0,05M Tris/HCl buffer peroxidase activity was visualized with 0,05% diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) at pH 7,4 in 0,05M Tris/HCl buffer plus 0,03% hydrogen peroxidase. The sections were rinsed in distilled water, dehydrated and mounted in Entelan. Counterstaining was performed using methyl green. As negative controls for primary antibodies, PBS was used in place of antibodies.

Histochemistry for hyaluronic acid

Sections were deparaffinized, rehydrated and rinsed in 0,1M TBS (pH 7,4). After that, the sections were blocked with 3% bovine serum albumin (BSA) in 0,1M TBS for 1 hour at room temperature and were incubated with FITC (fluorescein isothiocyanate) conjugated hyaluronic acid-binding protein (HA probe) diluted in TBS containing 1% BSA for 2 fours in humidified chambers. After rinsed in 0,1M TBS slices were mounted in Vectashield (Vector Laboratories, Berlingame, CA, USA) and observed with an Olympus fluorescence microscope. As negative controls, a digestion by 40U hyaluronidase (Streptomyces hyalurolyticus – Sigma) in 0,1M TBS for 2 hours at 37°C was performed before HA probe reaction. Control sections were counterstaining with DAPI (Sigma Chemical) for 15 minutes (Caterson et al., 1982).

Biochemical Analyses of Sulfated Glycosaminoglycans

The Sulfated Glycosaminoglycans present in pools of fibrocartilage or interpubic ligament obtained from virgin (25 females) or pregnant mice (25 females) respectively, were isolated by papain digestion (1mg/10mg of dry tissue in sodium acetate buffer 0.1M, pH 5.0, cysteine 5mM and EDTA 5mM) for 24h at 60°C (Toledo and Mourão, 1979), alkaline hydrolysis and alcoholic precipitation. The identification of GAGs was based on their electrophoretic mobility and degradation with specific glycosaminoglycan lyases (Dietrich and Dietrich, 1976). After electrophoresis, GAGs were fixed in the gel with 0.1% Cetavlon (N-acetyl-N,N,N-trimethyammonium bromide) and stained with 0.1% toluidine blue in acetic acid: ethanol: water (0.1:5, V/V). The GAG quantification was performed by densitometry of the agarose slides.

RESULTS

In the pubic symphysis during pregnancy, the first morpho-physiological changes occurred essentially on the symphysial tissues at the central region of this joint. Therefore, this study analyzed the proteoglycans of the fibrocartilage and surrounded connective tissue on the 12th day of pregnancy and the interpubic ligament on the 15, 17 and 18th days of pregnancy. For the cytochemical analyses the interpubic ligament observed after 15th of pregnancy was divided into two regions: outer layer and inner layer.

The proteoglycans observed by cytochemical treatment with Cuprolinic blue showed a predominant filaments-like shape. These filaments measuring 40-150nm in length were classified as F1 and F2 according to their sizes, spatial distribution and association with specific components of the extracellular matrix. The type of the sulfated glycosaminoglycans attached to the protein core of proteoglycans was defined by the use of specific glycosaminoglycan lyases.

12th day of pregnancy

After staining with Cuprolinic blue, the connective tissue that surrounded the fibrocartilage on the 12th day of pregnancy showed a predominant distribution of thin filaments measuring 40-60nm in length, named F1. These F1-filaments were associated to collagen fibrils at regular intervals which correspond to the D-period (Figure 1). Two distinct populations of filaments appear in the fibrocartilage on the 12th day of pregnancy (Figure 2). One population of F1-filaments and the second heavily staining filaments measuring 60-

150nm in length classified as F2. The F1-filaments were collagen fibril associated while the F2-filaments showed two typical localization: between bundles of collagen fibrils and at interfibrillar spaces (Figure 2). Both F1 and F2-filaments were completely removed by chondroitinases AC-II or ABC. No differences could be observed in its susceptibility to chondroitinases AC-II or ABC (Figures 11 and 12).

The disappearance of the PG-filaments after chondroitinases treatment permitted a better recognizing of the collagen fibril structure and arrangement. The figures 11 and 12 show the collagen arrays for the surrounding connective tissue and fibrocartilage, respectively. Although no morphometrical analysis has been done we noted that fibrocartilage-collagen fibrils were thinner than those observed on surrounding connective tissue. Furthermore, a prominent aligned in parallel of densely packed collagen fibrils was observed for the surrounded connective tissue. Differently, the fibrocartilage exhibited collagen fibrils in various orientations and loosely packed.

On the electrophoresis, a single band with migration similar to the standard chondroitin sulfate was observed. After chondroitinase AC digestion this band disappeared completely (Figure 32). The Graphic 1 showed the concentration of this glycosaminoglycan that was around 2,8 μg/mg dry tissue.

Immunohistochemistry revealed a diffuse positive staining for chondroitin sulfate in the territorial matrix fibrocartilage and a strong immunoreaction along the extracellular matrix of the surrounded connective tissue (Figure 17). The biglycan and decorin core proteins were localized preferentially between the fiber bundles of the surrounded connective tissue while in the fibrocartilage was observed a moderate staining for biglycan and decorin around fibrochondrocytes (Figures 20 and 23). A focal staining for biglycan was also observed on the extracellular matrix of the fibrocartilage (Figure 23). A positive reaction for hyaluronic acid was uniformly observed in the fibrocartilage and concentrated around the fibrochondrocytes. Hyaluronic acid was also positive in the surrounded connective tissue (Figure 28).

15th day of pregnancy

On the 15th day of pregnancy, the outer layer of the interpubic ligament showed small Cuprolinic blue filaments measuring 40-60nm of length closely associated with collagen fibrils at regular intervals, similar to the F1-filaments observed on the 12th day of pregnancy. Another class consisting of larger, thick and heavily staining PG-filaments was preferentially localized around bundles of collagen fibrils or at places where the collagen fibrils are somewhat separated from each other. This filament measured 60-150nm of length and was

similar to the F2-filaments found on the fibrocartilage on the 12th day of pregnancy (Figure 3). The PG-filaments distribution at the inner layer of the interpubic ligament was similar to the outer layer. Therefore, at this region the F2-filaments were shorter than F2-filaments found at the outer layer of this ligament, having then maximum length around 120nmm (Figures 4 and 5). The F1 and F2-filaments were digested totally by chondroitinase AC-II or ABC (Figures 13 and 14).

The glycosaminoglycans of 15th day of pregnancy displayed an electrophoretic mobility similar to that of chondroitin sulfate standard (Figure 32). Digestion with chondroitinase AC-II or ABC caused a total disappearance of the electrophoretic band. The concentration of the glycosaminoglycans on this day of pregnancy reached their maximum, which was 4,38 μg/mg dry tissue (Graphic 1).

The reaction with the anti-CS antibody was spread over the tissue but slightly concentrated on the collagen fiber surface (Figure 18). Moderated reaction for decorin was observed along the collagen fibers (Figure 21). However, only a weak diffuse staining for biglycan was seen in the interpubic ligament on this day of pregnancy (Figure 24). A strong reaction for acid hyaluronic was regularly distributed along the collagen fibers of this ligament (Figure 30).

17 and 18th days of pregnancy

In these days of pregnancy, two major classes of Cuprolinic blue positive-filaments can be distinguished; a class of small, thin, collagen fibril-associated filaments, the F1-filaments (40-60nm of length) and a class of larger, thick and heavily staining PG-filaments measuring 60-100nm in length, the F2-filaments. These filaments occurred indistinctly on the outer and inner layer of the interpubic ligament (Figures 6 to 9). On the 18th day of pregnancy, mostly on the inner layer of the interpubic ligament, was observed areas totally collagen-free. The filaments on these areas were longer and deeply Cuprolinic blue staining compared with F1 and F2-filaments (Figure 10). The staining disappeared completely when digested with one of the chondroitinases AC-II or ABC (Figures 15 and 16).

The biochemistry analysis of glycosaminoglycan found one these days presented chondroitin sulfate as the only glycosaminoglycan in the interpubic ligament (Figure 32). However, differently with the 15th day the concentration of this glycosaminoglycan decreases drastically (1,1 μg/mg dry tissue) on 18th day of pregnancy (Graphic 1).

Immunohistochemistry revealed the presence of chondroitin sulfate on the surface of the collagen fibers of the interpubic ligament on the 18th day of pregnancy (Figure 19). As

can be seen decorin and biglycan reactive positive to the staining between the collagen fibers and around the cells present in the ligament (Figures 22 and 25). An intense reaction for hyaluronic acid was observed along the collage fibers, similarly the reaction seen on the 15th day. A gradual increase in reactivity of hyaluronic acid could be observed over late days of pregnancy (Figure 31).

DISCUSSION

The connective tissues with predominantly mechanical function which presented a combination of two peculiar qualities: the ability of resisting tension and compression forces and the capacity to recover their initial state when the forces were ceased. These properties of the connective tissue depend primarily on their extracellular matrix elements: collagen and proteoglycans.

The striking feature of the fibril-forming collagen is their ability to resist to tensile loads. The proteoglycans on the other hand present the ability to resist compressive forces. Therefore, tissues with high proteoglycans content were able to resist compressive forces, an ability best exhibited by hyaline cartilage. Differently, tissues with high collagen-fiber content and low amounts of proteoglycans resist tensile forces as tendons and ligaments (Culav et al., 1999).

The pubic symphysis, a nonsynovial amphiarthrodial joint, meets the functional demands placed on it being composed by different connective tissues. Pregnancy leads to changed mechanical stress placed on the pubic symphysis. This, in turn, produces changes in the extracellular matrix of the symphysial connective tissues (Storey, 1957).

On the 12th day of pregnancy, the pubic symphysis is submitted to tension and compressive forces in an analogous situation as in virgin animals. The analysis of the proteoglycans from the central region of this joint demonstrated that F1 and F2-filaments were removed by the enzymatic treatment by chondroitinase ABC or AC-II indicating that they were composed by chondroitin sulfate. The chondroitinase AC-II cleaves GAGs at N-acetylgalactosamine linked to D-glucuronic acid, but not the N-acetylgalactosamine linked with L-iduronic acid that is found in high levels in dermatan sulfate. The chondroitinase ABC lyase, on the other hand, cleaves GAGs at both disaccharide units (Saito et al., 1968).

Considering their localization and the immunohistochemical detection, the symphysial F1-filaments were preferentially decorin. Biglycan and decorin have very similar core proteins, which are substituted with two or one glycosaminoglycan chains, respectively (Oldberg et al., 1989). The physiological role of biglycan is not fully understood, and collagen-binding

properties has not been demonstrated (Fleischmajer et al, 1991). By the results of biochemistry of sulfated glycosaminoglycans and chondroitin sulfate immunohistochemistry, the decorin core proteins are substituted with chondroitin sulfate. F2 filaments, due to its size, localization and chodroitin sulfate side-chains probably belong to the class of versicans.

The slightly increased content of CS compared with virgin animals (graphic 1) indicating that proteoglycan changes already occur on the 12th day of pregnancy. Furthermore, the extracellular matrix collagen fibrils in various orientations, loosely packed and intermingled by proteoglycans resemble a typical fibrocartilage. In fact, as a fibrocartilage this tissue reflects its intermediate nature between dense connective tissue and hyaline cartilage, where resistence to compressive and tensile strength are necessary.

On the 15th day of pregnancy, the most striking finding was the increase of sulfated glycosaminoglycan contents. An increase of 118% was observed when compared with the CS concentration from pubic symphysis of virgin animals. Besides this, a planar undulation along the length of the collagen fibers positioned in most parallel array was visualized by light microscopy (Figures 19 and 30). This collagen undulation, a "crimp" structure, has a considerable role in tissues submitted to high level of tension (Carvalho, 1995, Kaneko et al., 2001; Feitosa et al., 2002). Although the "crimp" angle and length was not measured, we can note the increase of undulation of collagen fibers in interpubic ligament on day 15th compared with day 18th, where they were loosely undulated. Tension was the main mechanical force acting upon pubic symphysis on this day (Storey, 1957).

On the 18th day of pregnancy, the sulfated GAGs contents decreased abruptly. The decrease of CS contents relative to the pubic symphysis of virgin mices was 44%. However, compared the contents of day 15 to days 17 and 18th the decrease was of 74%. Hyaluronic acid, a high molecular glycosaminoglycan, which is known to be a polyelectrolyte macromolecule, accumulates in the interpubic ligament on days 17 and 18th. Similar data, increase of hyaluronic acid and decreased of sulfated glycosaminoglycans content, after relaxin treatment in mice pubic symphysis was observed by Viell and Struck, 1987. Hyaluronic acid has been recognized as water retaining extracellular matrix component. This data associated with the work of Zhao et al., 2000 who showed on day 18,5th of pregnancy a large increase in water content (20%) in the mice pubic symphysis indicate the water retaining role of HA in the interpubic ligament.

The accumulation of water may contribute to the increased extensibility of the interpubic ligament that occurs during late pregnancy.

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FIGURE LEGENDS

Figure 1. TEM of the distribution of PGs at the surrounded connective tissue of the mouse pubic symphysis on the 12th day of pregnancy (DOP). Many CB-positive structures (F1-filaments) appear attached to collagen fibrils (arrows). X 40000

Figure 2. TEM of the fibrocartilage of mouse pubic symphysis on the 12th DOP. Thin PG-filaments (F1) occupy the ECM (arrows) and a little amount of large PG-filaments (F2) is randomly distributed (large arrows). X 47500

Figure 3. TEM of the out layer of the mouse interpubic ligament on the 15th DOP. Electron dense PG-filaments (F1) are almost arranged along collagen fibrils (arrows). Observes F2 filaments (large arrows) that form a net between the collagen fibrils. X 40000

Figure 4 and 5. TEM of the inner layer of the mouse interpubic ligament on the 15th DOP. Most of F1-filaments are preferentially connected to the collagen fibrils (arrow). A network of large electron-dense filaments (F2) are present (large arrows). X 40000

Figure 6. TEM of the outer layer of the mouse interpubic ligament on the 17th DOP. Thin F1-filaments (arrows) are present in addition to large, thick F2- filaments (large arrows). The thin PG-filaments can be seen regularly associated with collagen fibrils. X 31400

Figure 7. TEM of the inner layer of the mouse interpubic ligament on the 17th DOP. Thin PG-filaments (F1) are arranged along collagen fibrils (arrows) and thick PG-filaments (F2) are randomly distributed at interfibrillar space (large arrows). X 38400

Figure 8. TEM of the outer layer of the mouse interpubic ligament on the 18th DOP. Observes F1-filaments located regularly at the collagen fibrils (arrows) and at the interfibrillar spaces (large arrows). X 46800

Figure 9 and 10. TEM of the inner layer of the mouse interpubic ligament on the 18th DOP. The distribution of PGs is very similar to that found in outer layer (Fig. 8). Thick PG-filaments (F2) are visible (large arrows). The arrows point thin PG-filaments (F1). The interfibrillar space contains a dense meshwork of PG-precipitates (Fig. 10). X 25000; X 40000

Figure 11. TEM of the surrounded connective tissue of the mouse pubic symphysis on the 12th DOP after digestion with chondroitinase AC-II. The collagen fibrils (col) are almost free of precipitates. X 69000

Figure12. TEM of the fibrocartilage of the mouse pubic symphysis on the 12th DOP. PG-filaments were completely removed after incubation with chondroitinase AC-II. X 62000

Figure 13. TEM of the outer layer of the mouse interpubic ligament on the 15th DOP. The chondroitinase AC-II treatment removes almost all PG precipitates associated with collagen (col) and with the matrix soluble (asterisk). X 50100

Figure 14. The effects of chondroitinase AC-II treatment at the inner layer of mouse interpubic ligament on the 15th DOP is very similar to that found in outer layer interpubic ligament. Col, collagen fibril; asterisk, interfibrillar space. X 69000

Figure 15. TEM of the outer layer of the mouse interpubic ligament on the 18th DOP. Chondroitinase AC-II treatment removes all PGs filaments associated with collagen fibril (col) and in interfibrillar spaces (asterisk). Ultrathin section was stain with phosphotungstic acid and lead citrate. X 36800

Figure 16. TEM of the inner of the mouse interpubic ligament on the 18th DOP. No electron dense filaments are present after incubation with chondroitinase AC-II. Ultrathin section was stain with phosphotungstic acid and lead citrate. Asterisk, matrix soluble. X 40000

Figure 17. Immunocytochemistry for chondroitin sulfate of the mouse pubic symphysis on the 12th DOP. Reaction for chondroitin sulfate is diffuse, all over the extracellular matrix of the surrounded connective tissue and concentrated at territorial matrix fibrochondrocytes (arrowheads). In connective tissue the reaction is located between the collagen fibers (arrow). X400

Figures 18 and 19. Immunocytochemistry for chondroitin sulfate of the interpubic ligament on 15th DOP and 18th DOP, respectively. The reaction for chondroitin sulfate is located between the collagen fibers (arrows). X400; X520

Figure 20. Immunocytochemistry for decorin of the mouse pubic symphysis on the 12th DOP. Reaction for decorin was faint, and mostly located between the collagen fibers of the connective tissue (arrow) or around the fibrochondrocytes (arrowhead). The cytoplasmatic reaction for decorin is sometimes observed in fibrochondrocytes (insight). X400

Figure 21 and 22. Immunocytochemistry for decorin, in interpubic ligament on 15th DOP and 18th DOP respectively. Reaction for decorin was also diffuse throughout the extracellular matrix but more intense along the collagen fibers (arrows). The arrowheads point a positive reaction around the cells of the interpubic ligament on the 18th DOP. X520

Figure 23. Immunocytochemistry for biglycan of the mouse pubic symphysis on the 12th DOP. Biglycan is located primarily within the collagen fibers in the connective tissue (arrow). The asterisk points a diffuse reaction for biglycan in the fibrocartilage. X520

Figure 24 and 25. Immunocytochemistry for biglycan, in interpubic ligament on 15th DOP and 18th DOP respectively. Reaction for biglycan was present throughout the matrix but more intense along the collagen fibers (arrow). Moderate reaction was present in the pericellular space (arrowhead). X400

Figure 26 and 27. Control of the interpubic ligament on the 12th and 15th DOP respectively. Reaction was negative. Counterstaining with methyl green. X200

Figure 28. Reaction for HA probes of the mouse pubic symphysis on the 12th DOP. In the fibrocartilage, reaction is strong pericellular (arrowheads) and only weak interterritorial matrix (asterisk). HA probes are mostly located between the collagen fibers of the connective tissue (arrows). X400

Figure 29. Control of the mouse pubic symphysis on the 12th DOP. Reaction was negative. Counterstaining with DAPI. X520

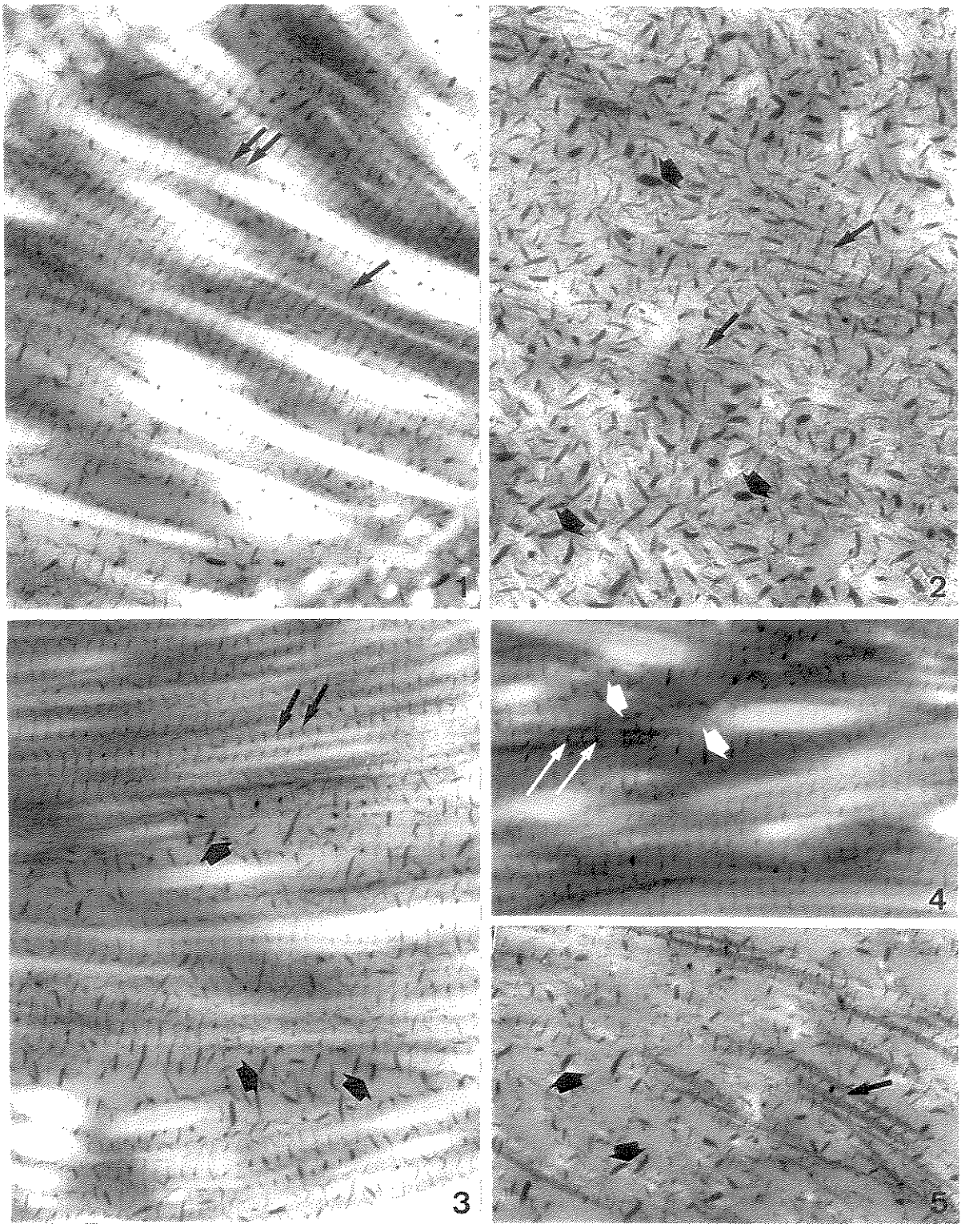
Figures 30 and 31. Reaction for HA probes of the interpubic ligament on 15th DOP and 18th DOP, respectively. The reaction for hyaluronic acid is located between the collagen fibers (arrows). Note the undulation of collagen fibers, a "crimp" structure. X400

Figure 32. Agarose gel electrophoresis of GAG extracted from fibrocartilage of virgin specimens or from interpubic ligament of pregnant mices over the later days of pregnancy before (A-B) and after (C) incubation with chondroitin AC-II lyases.

ST= standard mixture containing 5 ug of each glycosaminoglycans: chondroitin 4/6 sulfates (CS); dermatan sulfate (DS) and heparan sulfate (HS). OR= origin. Vg= Virgin and 12d, 15d, 17d and 18d = 12, 15, 17 and 18th days of pregnancy, respectively.

Graphic 1. Total amount of glycosaminoglycans of the fibrocartilage of virgin specimens or of the interpubic ligament of pregnant mices over the later days of pregnancy.

Virg = Virgin and dop = days of pregnancy, respectively.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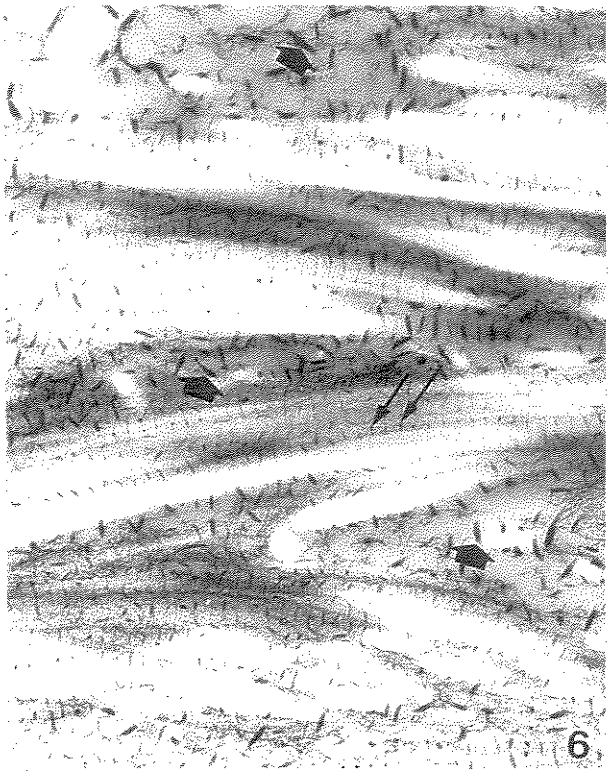
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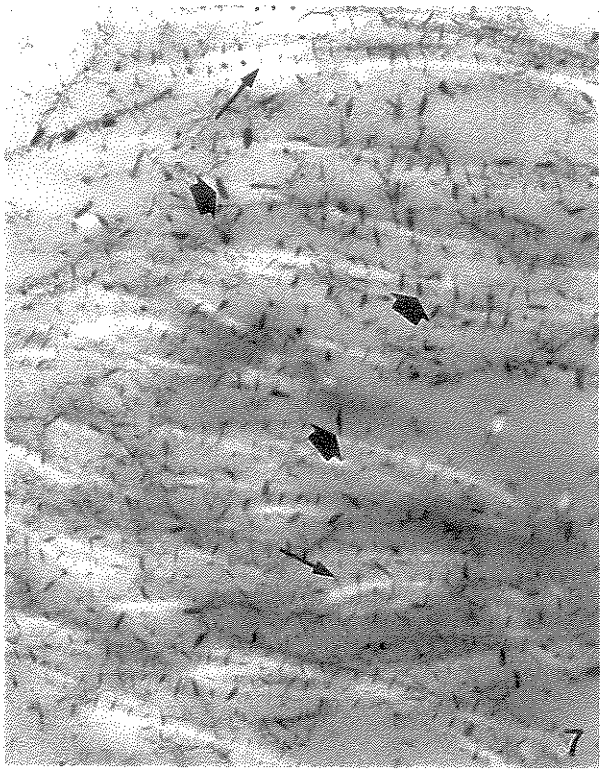
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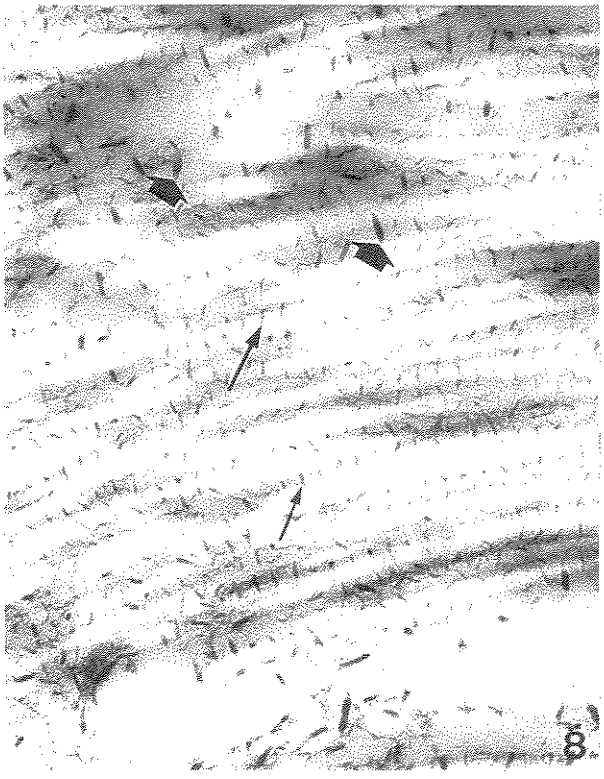
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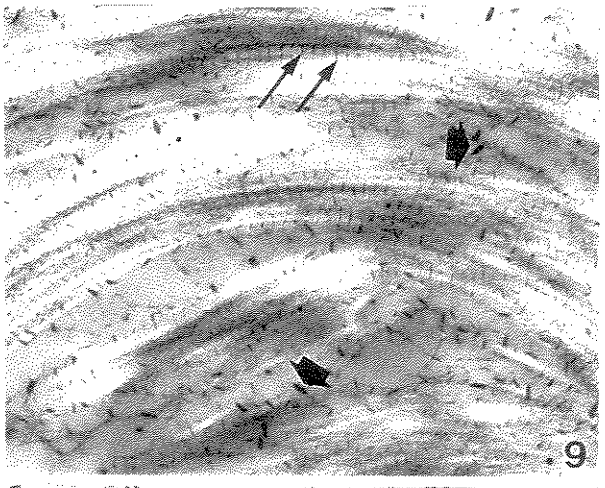
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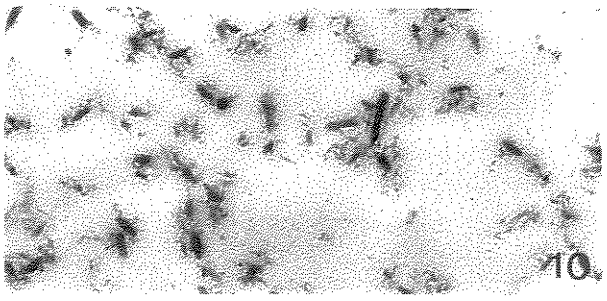
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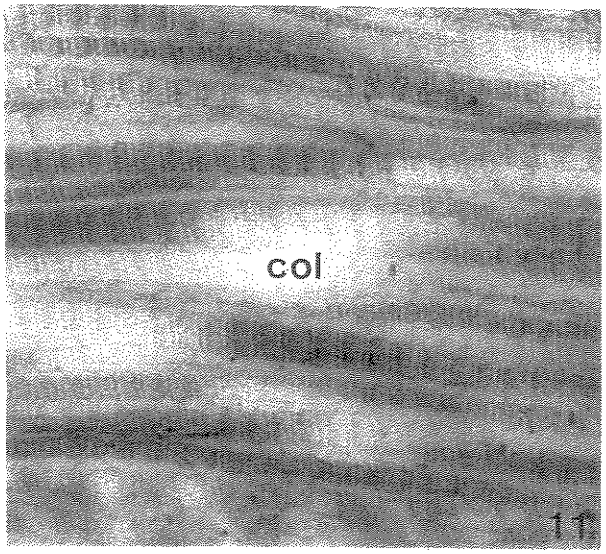
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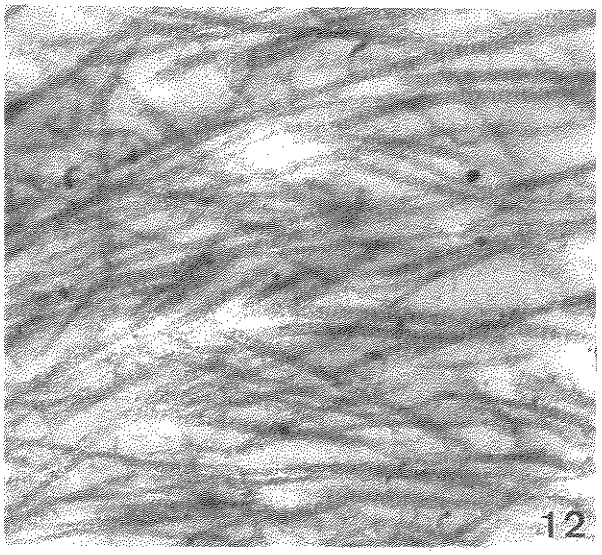
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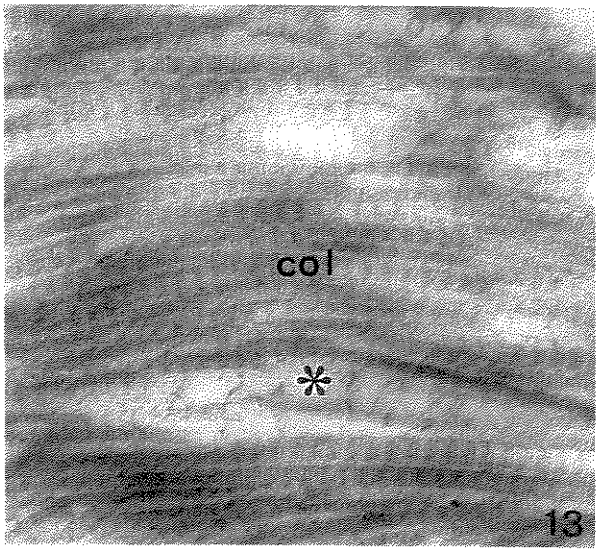
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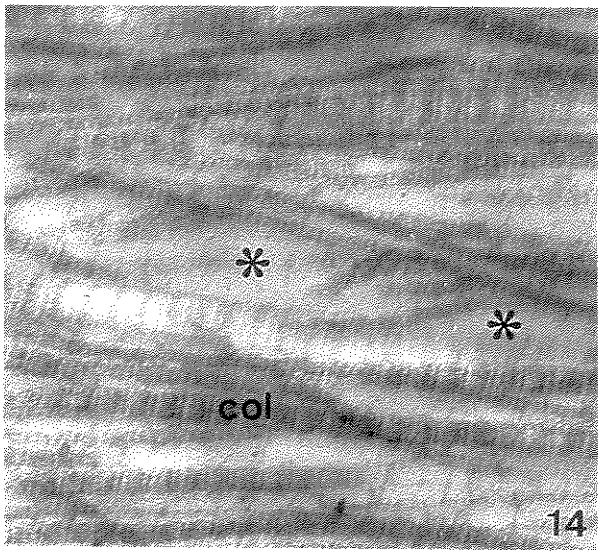
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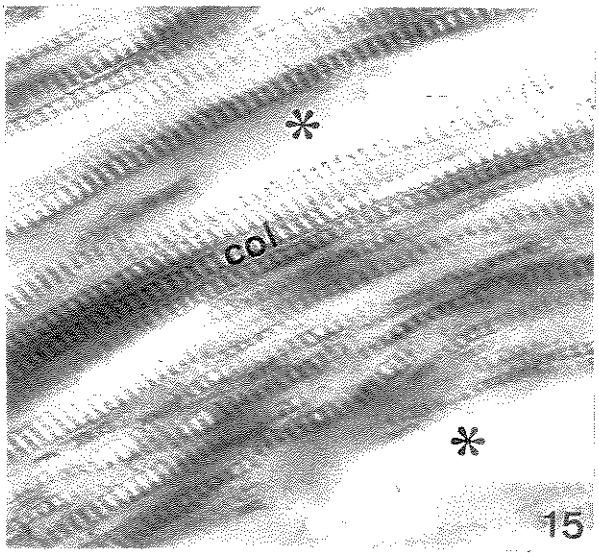
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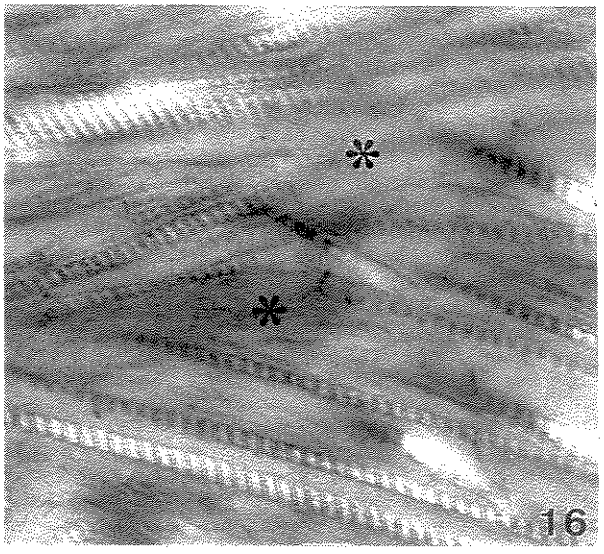
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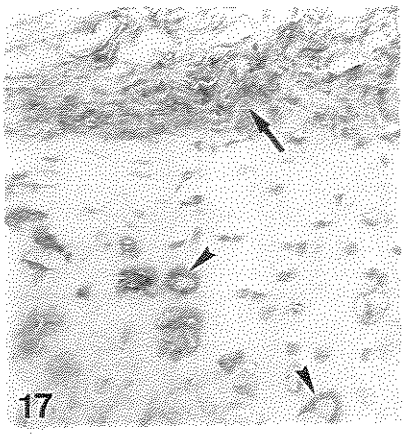
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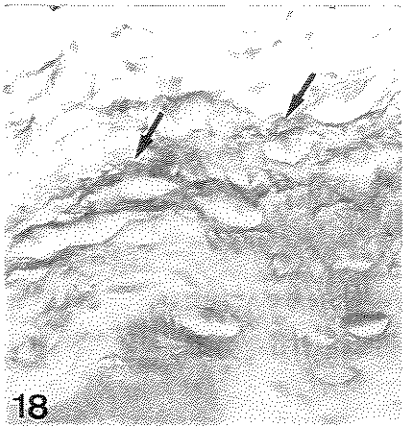
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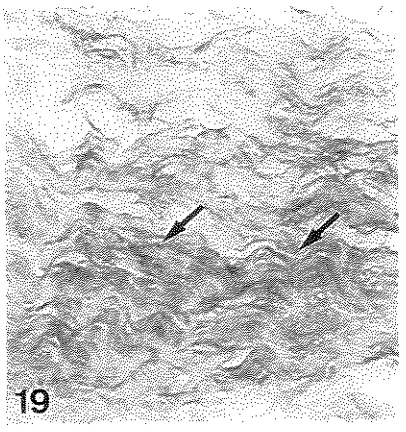
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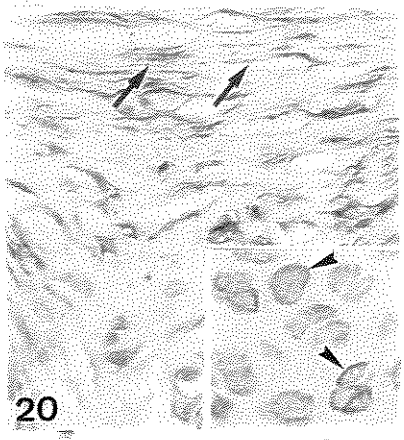
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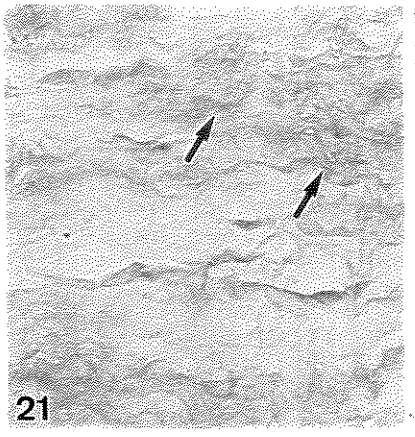
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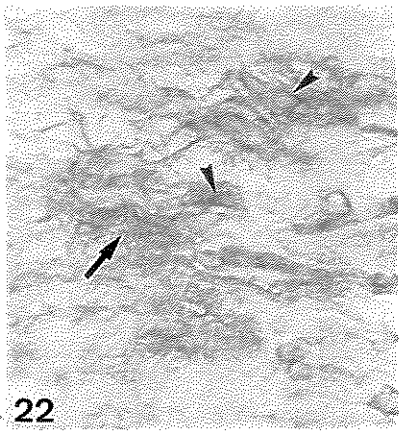
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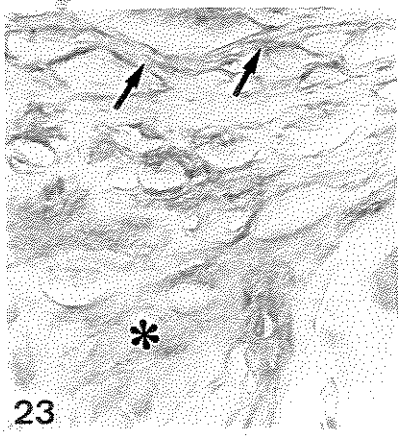
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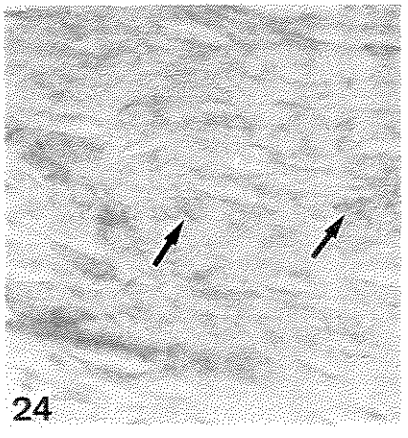
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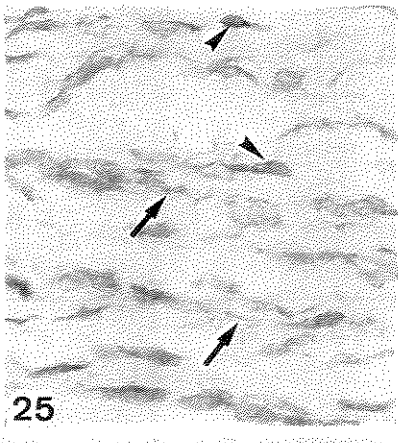
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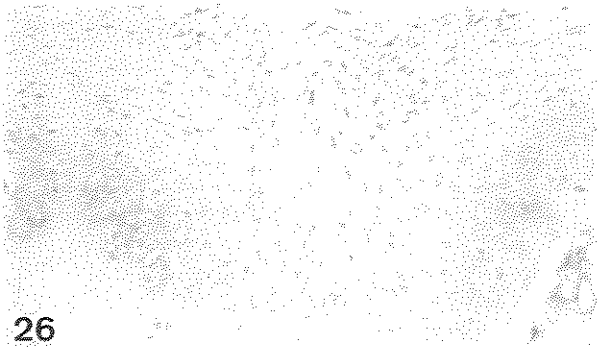
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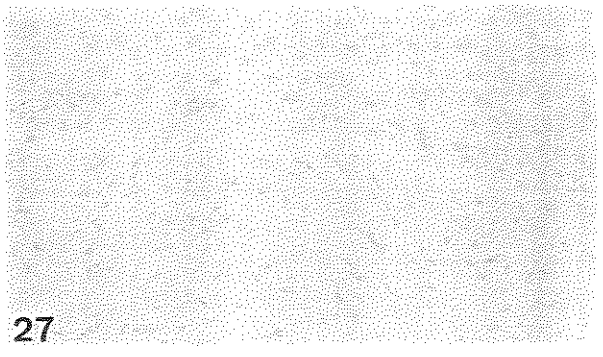
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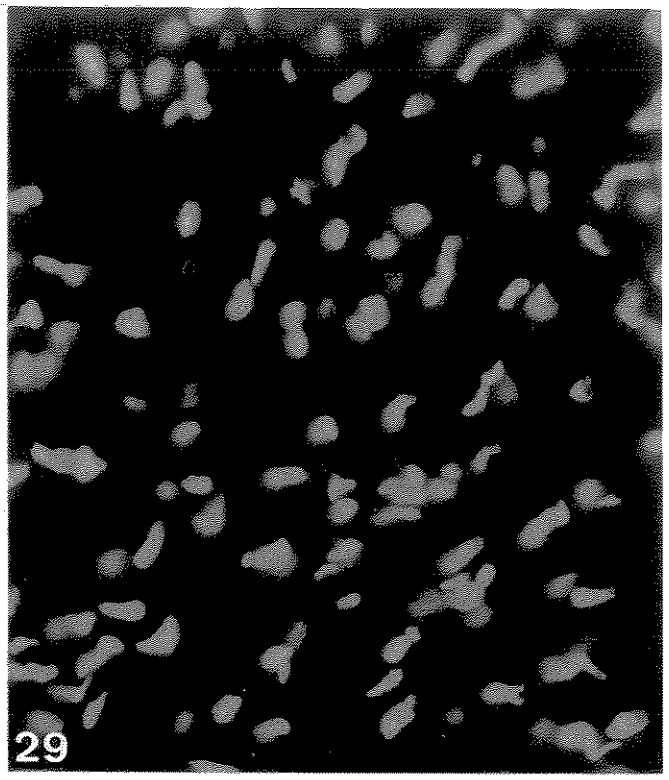
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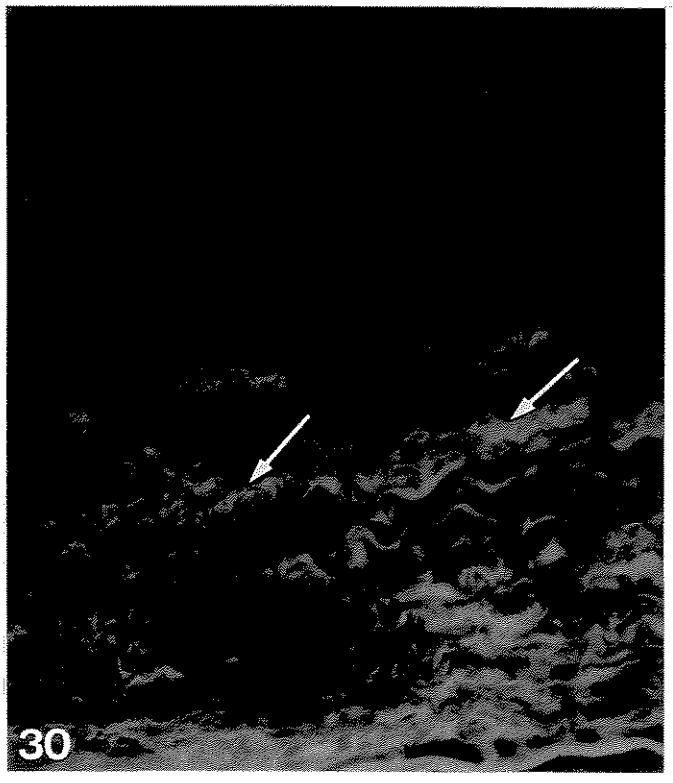
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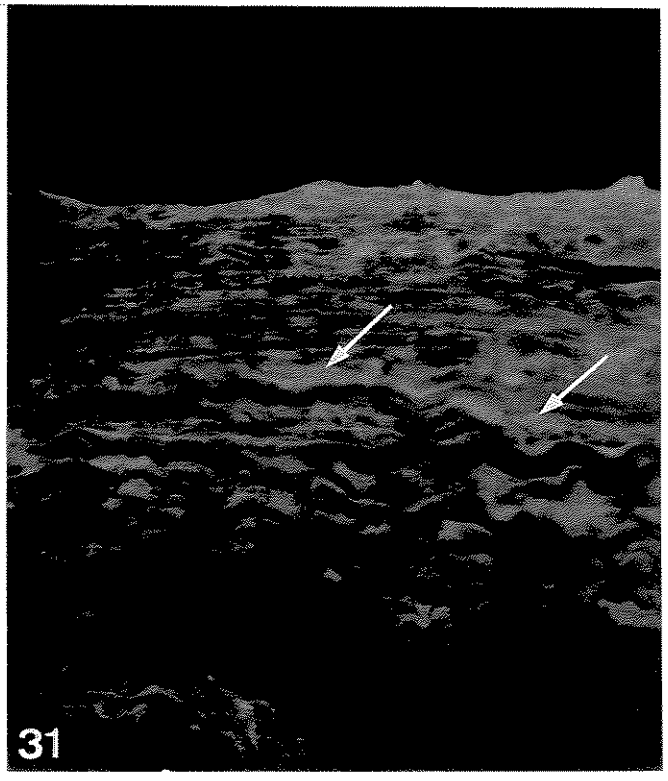
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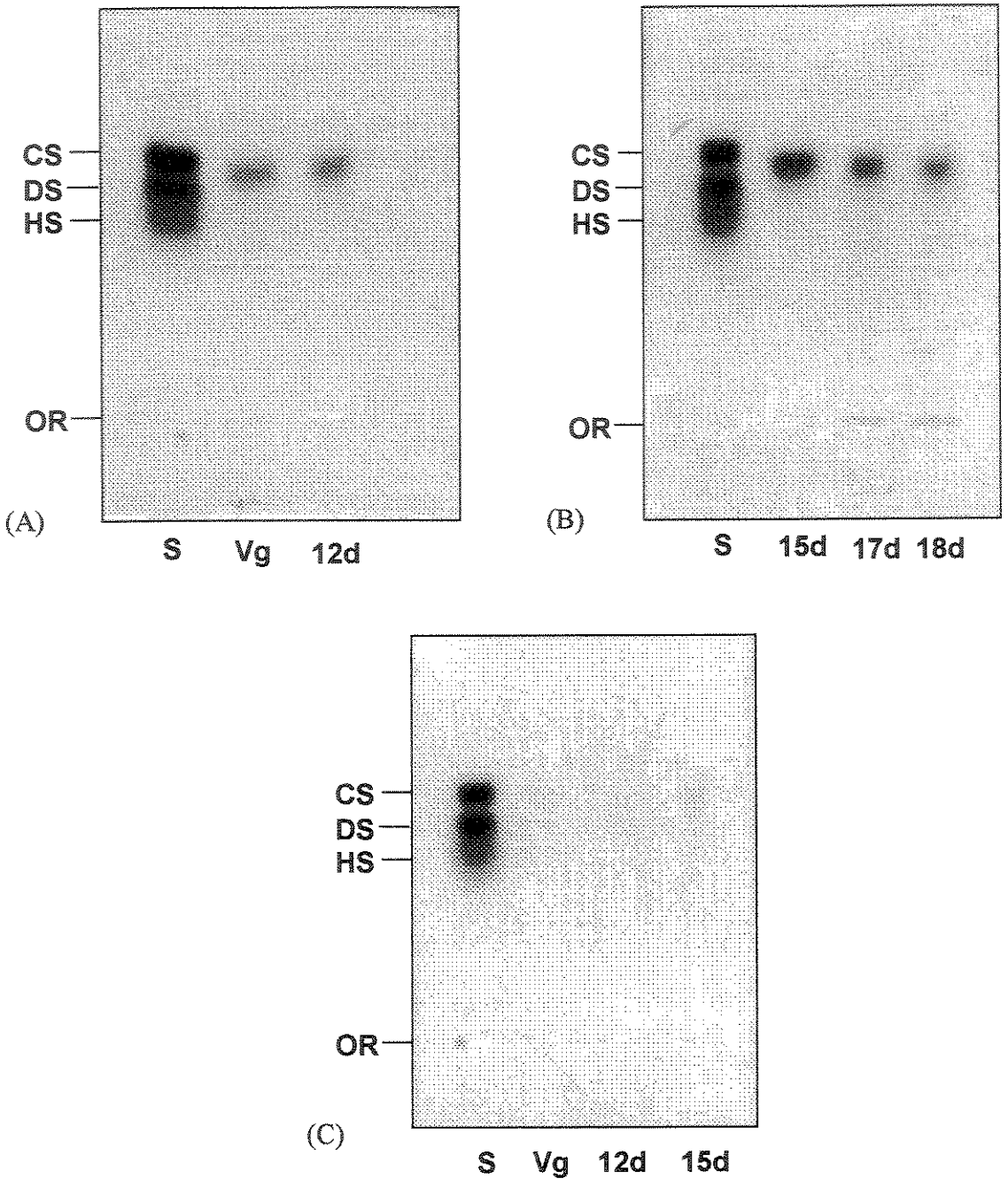
Figure 32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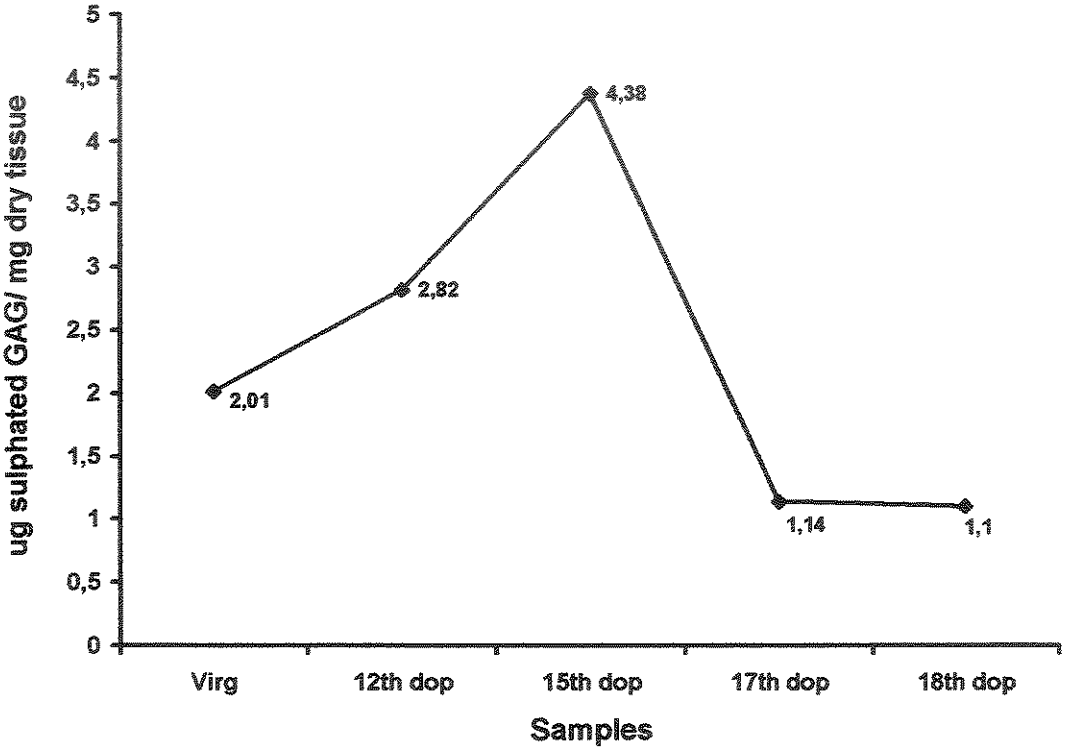
Graphic 1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Distribution of type VI collagen in virgin and pregnant mice pubic symphysis.

Mônica C Pinheiro, Paulo P Joazeiro, Hernandes F Carvalho, Olga MS Toledo

ABSTRACT

The pubic symphysis is a nonsynovial amphiarthrodial joint localized at the confluence of the pubic bones. During pregnancy, the mouse pubic symphysis undergoes a number of hormonally facilitated modifications, including the transformation from a fibrocartilage disk into a flexible and elastic interpubic ligament, to enable safe delivery of the young. The development of this ligament involves an increase in the biosynthesis of the extracellular matrix and the changes in turnover of these components. These modifications, associated with the extracellular components, are largely neglected when considering the arrangement of the individual components and interaction between different extracellular macromolecules, even though collagen was shown to have important roles on remodeling pubic symphysis during pregnancy. Type VI collagen is present in the extracellular space in a wide range of connective tissues. It appears to be an integral component of the matrix with a pivotal influence on matrix organization and on attachment of cells within the ECM, both in normal development and in tissue maintenance, and also in several major diseased states. In this study, we have applied immunocytochemistry and ATP treatment for the ultrastructural identification of type VI collagen in different connective tissues in virgin and pregnant mice pubic joint after ATP treatment by transmission electron microscopy. The distribution of type VI collagen in virgin and pregnant mice pubic symphysis is different in the various tissues that compose the joint. This may reflect the different functional demands for this collagen. The pericellular localization suggests a role in regulating cell-matrix interaction protecting the cell against mechanical stress. However, the interaction of type VI collagen with other extracellular matrix components such as collagen fibrils may play an important role in the organization of the interfibrillar space, probably associated with elastic properties of the tissue.

INTRODUCTION

The main structural component of the connective tissue matrix is the fibrillar collagens. Collagen fibrillogenesis is dependent on several other non-fibrillar collagenous and noncollagenous matrix molecules. These molecules provide sites for potential interactions with matrix components and cell interactions. In particular, collagen VI has been shown to develop specific interactions with cells (Poole et al., 1992; Aumailley et al., 1991) and fibrillar collagens (Keene et al., 1998).

Type VI collagen molecule, presented a large globular domain at each end linked by three genetically distinct α chains assembled into a triple-helical domain. These monomers aligned anti-parallel to form disulfide-bonded dimmers, and later tetramers by lateral association. The tetramers were later secreted from the cell and arranged end to end to form thin, beaded microfilaments (Furthmayer et al., 1983). The microfibrils demonstrated a unique 100 nm periodic pattern by electron microscopy (Bruns et al., 1986) and with cross-sectioned diameters of 3-5nm (Kielty et al., 1991).

In tissues, type VI collagen microfilaments are difficult to be visualized by electron microscopy. However, the treatment of the tissue with ATP solution produced type VI aggregates into characteristic bundles of ordered microfilaments (Nakamura et al. 1994, Felisbino & Carvalho, 1999). In the aggregated form, the globular domains aligned laterally producing dark bands averaging 44nm in width, while the triple-helical domain produced light bands having approximately 67nm in width.

The type VI collagen function is probably of anchoring extracellular matrix components and cell attachment to the surrounding matrix, since ultrastructurally the microfibrils are found associated with fibrillar interstitial collagen assembled as heterotypic fibrils (Keene et al., 1998), with fibrillin containing microfibrils elastic fibers (Everts et al., 1998; Finnis & Gibson, 1997), with proteoglycans such as decorin and biglycan (Bidanset et al., 1992; Wiberg et al., 2001), in contact with basal lamina (Kuo et al., 1997; Tiedemann et al., 2001, Tulla et al. 2001) and with the cell (Poole et al., 1992). Studies *in vitro* have demonstrated that collagen VI is a potent substrate for promoting cell adhesion. (Aumailley et al., 1991, Araújo et al. 2002).

The pubic symphysis is a nonsynovial amphiarthrodial joint localized at the confluence of the pubic bones. In the nonpregnant adult mouse, a fibrocartilaginous disk that is continuos with the cap hyaline cartilage of the pubic bones forms this joint (Gamble et al., 1986). This fibrocartilage disk is anterior and posteriori surrounded by layers of dense connective tissue

continuous with perichondrium/periosteum. During pregnancy, the mouse pubic symphysis undergoes a number of hormonally facilitated structural modifications, including the transformation from a fibrocartilage into a flexible and elastic interpubic ligament, to enable safe delivery of the young (Sherwood, 1994).

The compositional variety of connective tissues present in the same functional structure makes the pubic symphysis an ideal model of study of the interstitial extracellular components. In this work, we identified and localized the type VI collagen in hyaline cartilage, fibrocartilage, dense connective tissue and interpubic ligament pubic symphysis of mice by immunohistochemistry and electron microscopy.

MATERIALS AND METHODS

Virgin female mice (approximately 90 days of age) of a Swiss-derived strain bred in the countryside at the University State of Campinas were used n this study. Animals were fed hay ad libitum and given free access to water.

To obtain pregnant specimen, virgin female were mated with males of the same strain (in proportion of 2:1) and observed each morning for the presence of vaginal plugs. The day on which a plug was found was considered as the first day of pregnancy. Pregnant animals were used on 15th and 18th days of pregnancy. Virgin mice in estrus were also used. Estrus was determined by vaginal smears according to Shorr (1941).

Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, 1996)

For immunohistochemistry, four animals were used for each experimental point and two animals per group were studied in electron microscopy. The animals were sacrificed by cervical dislocation and pubic symphyses were dissected clean.

Transmission Electron Microscopy

Specimens were digested with 1U/ml chondroitin AC or ABC lyase (Sigma-EC 4.2.2.4) in 0,05 M Tris-HCl buffer, pH 7,2, at 37°C for 2 hours. Enzyme solution contained 0,02M calcium acetate, 10nm NEM, 1mM PMSF and 5mM benzamidine HCl. After incubation the specimens were washed with PBS, fixed with 2,5% glutaraldehyde in 0,01M sodium cacodylate buffer, pH 7,2, for 3 hr at room temperature. They were washed in the same buffer, stained with sodium tungstate, dehydrated in a graded series of ethanol and embedded in polydbed epon 812 resin. Non-incubated fragments were directly processed as

above for morphological identification of some tissue characteristics. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a LEO 906-Zeiss electron microscope.

Immunohistochemistry

Pubic symphysis were fixed in Carnoy's solution (ethanol, chloroform, acetic acid 6:3:1 by volume) for 24 hours at 4ºC. Afterward they were washed in 0,1M PBS, and then decalcified in 5% EDTA in 0,1M phosphate buffer for one week at 4ºC, dehydrated and embedded in paraffin. Six-micrometer sections were obtained in transverse plane through central region of the symphysis, dewashed with xylene and subjected to immunoperoxidase detection of type VI collagen using a monoclonal anti-human collagen type VI (Chemicon, Temecula, CA) as follows. Endogenous peroxidase activities were blocked by treatment tissue sections with 0,3% hydrogen peroxide (H_2O_2) in tris-HCl buffered saline (TBS) 0,05M pH 7,2 and washed in the same buffer. Sections were then treated with diluted normal blocking serum (Novocastra) in PBS 0,01M for 30 minutes and incubated with a 1:100 dilution of monoclonal anti-human collagen type VI, at room temperature, overnight in a moisture chamber. The sections were rinsed with TBS and were subsequently incubated with biotinylated universal secondary antibody solution (Novostain super ABC, Novocastra) at room temperature for 30 minutes. After washes with TBS, sections were incubated with Novostain ABC Kit (Novocastra, EUA) at room temperature for 30 minutes and rinsed in TBS. Then they were reacted with DAB solution. After immunostaining, the sections were counterstained with methyl green. Controls were done omitting primary antibody.

ATP-treatment and ultrastructural identification of collagen VI

Tissue fragments were incubated in 20 mM adenosine triphosphate (ATP) in phosphate buffered saline (PBS) for 1h at 37ºC (Nakamura et al., 1994; Carvalho et al 1997). Controls were incubated in PBS without ATP. ATP-treated samples and the control were fixed in 4% glutaraldehyde and 0,25% tannic acid in Milling's buffer for 1h (Cotta-Pereira et al., 1976). Then they were post-fixed in osmium tetroxide, dehydrated in acetone and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a LEO 906-Zeiss electron microscope.

RESULTS

Tissue distribution of the type VI collagen – Immunohystochemistry

The immunoperoxidase reaction with the antiserum against collagen type VI detected the presence of this protein in different connective tissues that comprised the mouse. The reaction was located around the cells and in the interfibrillar spaces.

Control section incubated without the first antibody proved to be negative (Figure 1A).

Staining reaction for collagen VI was positive in the matrix of hyaline cartilage around the cells after treatment with chondroitinases (Figure 1B). Positive reaction for type VI collagen was also observed close to the basal surface at the blood vessel wall (Figure 1C)

Intensive staining was seen in the fibrocartilage. In this tissue, the reaction was strongly positive around the fibrochondrocytes and in the interfibrillar spaces (Figures 1D and 1E). In the surrounding connective tissue, collagen type VI was identified dispersed throughout the extracellular matrix as fine fibrils between the collagen fibers, and sometimes concentrated at the fibroblast periphery (Figure 1F).

Intense staining was also seen in the pubic ligament of the pregnant mice. The paratenon was more intensely reactive which could be seen by the accumulation of the type VI collagen around the fibroblasts and associated with the collagen fibrils along the cell processes (Figure 2A). The immunohystochemical reaction identified some fibrillar components between the thick collagen fibers, which were more prominent in the outer layers of the ligament (Figures 2B and 2C).

The immunoreaction localized type VI collagen on the collagen fiber surface in the pubic ligament of the virgin and pregnant animals. Collagen VI appeared on pubic ligament deposited in a fine granular manner or as an extended meshwork of fine fibrils.

Ultrastructural identification of type VI collagen

Electron microscopic examination of the pubic symphysial connective tissues after chondroitinase treatments of the specimens revealed the presence of a microfibril network, except for hyaline cartilage. These microfibrils were observed uniformly distributed around the cells as individual structures. However, in the extracellular matrix, type VI collagen was seen forming a weblike network interwining with striated collagen fibrils. Some regions of the pubic symphysis appeared to delimit groups of the striated collagen fibrils forming collagen fibers.

The AC or ABC treatments indistinctly revealed the existence of a collagen type VI microfilaments in mice pubic symphysis.

The Figure 3 depicts one fibrochondrocyte of the virgin pubic symphysis after incubation with chondroitinase AC at 37°C for 2 hours. A filamentous network could be observed adjacent to the plasma membrane of the cell (Figures 3 and 4) and among the bundles of collagen fibrils in the fibrocartilaginous tissue (Figure 5).

The pericellular matrix of the surrounding connective tissue also presented a meshwork of microfibrils close to the cellular membrane of the fibroblast, which was extended throughout the extracellular matrix filling the spaces between the collagen fibers (Figure 6). The same distribution pattern was seen in the pubic ligament in pregnant mice. The microfibrils in the pubic ligament showed intimate connections with the collagen fibrils (Figures 6 and 7).

After ATP treatment of fresh pubic symphysis, periodic fibrils were observed. These structures were known to consist of lateral aggregates of the microfibrils of type VI collagen. They are constituted of electron dense bands interconnected to each other by microfilaments (light bands). With the exception of hyaline cartilage (Figures 8 and 9), the other components of the pubic symphysis - fibrocartilage, dense connective tissue and ligament - showed these characteristic aggregated structures.

The type VI collagen aggregates were observed at the same region where filamentous material occurred, when submitting the specimens to chondroitinase AC or ABC treatments. The fibrocartilaginous tissue was especially rich in type VI collagen around the cells, where they formed an extended network (Figure 10). Aggregates were also observed between the collagen fibrils in this tissue (Figures 11 and 12).

The interpubic ligament on the other hand, showed an accumulation of type VI collagen in the interfibrillar regions, between the collagen fibrils, and at the pericellular region. The inner layer of the central pubic ligament usually presented smaller amounts of the aggregates (Figure 13). However, the outer layer of the pubic ligament was especially rich in type VI aggregates. They were found in the interfibrillar spaces with intimate connections with the collagen fibrils (Figure 14), among the bundles of collagen fibrils (filling the spaces between the collagen fibers) (Figure 15) and concentrated around the cells, where they formed an extended network (Figures 16 and 17). In longitudinal section, type VI collagen periodic fibrils were observed between cells and striated collagen fibrils, but were more frequently observed among the collagen fibrils. The filaments ran close and parallel to the fibril surface (Figures 15 and 18).

DISCUSSION

Immunocytochemical and ultrastructural analyses were used for the determination of the distribution of type VI collagen in the mice pubic symphysis.

Type VI collagen was observed for all the connective tissues that composed the symphysis of virgin and pregnant mice being widely distributed throughout the ECM in two distinct locations: in the pericellular region and in close association with the striated collagen fibrils and fibers.

These observations demonstrated that collagen type VI constitutes an important part of the extracellular matrix of the connective tissues of the pubic symphysis. The high pericellular concentrations of type VI collagen reported clearly suggest that this collagen played an important role in the regulation of cell-matrix interaction as already suggested (von der Mark et al., 1984; Bruns et al., 1986; Aumailley et al., 1991, Sherwin et al., 1999; Araujo et al., 2002).

Ligament and dense connective tissue structures meet their functional role having tensile strength and elastic capacity against loading. Fibrocartilage has properties in between those of dense connective tissue and hyaline cartilage, having tensile and compression capacity. Type VI collagen, in mice symphysis, was widely distributed throughout the extracellular matrix in the ligament and connective tissue, whereas in the cartilages: fibrocartilage and hyaline, it was strongly pericellular. Collagen Type VI may have produced a basket-like structure around the chondrocytes to protect the cell from the great compressive force that the cartilage is exposed to due to its anatomical localization.

Type VI collagen may interact with proteoglycans present in the pericellular region (collagen fibril-free extracellular matrix) creating defined arrangements in this region of the tissue (Felisbino & Carvalho, 1999; Araujo et al., 2002). The accumulation of type VI collagen around the cells, with are large proteoglycan-rich regions, may suggest that type VI microfibrils are associated with these macromolecules. In fact, an interaction between a membrane-bound chondroitin sulfate proteoglycan found on several cell types, known as NG2, and collagen type VI has been described (Bidanset et al., 1992).

Fibrillar collagen have as their principal function, to provide great tensile strength. The presence of type VI collagen in the interstitial extracellular matrix has been linked to the occurrence of fibrillar collagens. There was evidence that proteoglycans were involved in the formation of collagen heterotypic fibrils closely associated with banded collagen fibrils and type VI collagen (Bray et al., 1990). This kind of association may indicate that type VI

collagen was essential to the mechanical properties of connective tissues, binding various components such as collagen fibers into a functional tissue while still allowing flexibility.

In ligament, a more distensible connective tissue than fibrocartilage, collagen type VI helps to build a more flexible collagen network interacting with fibrillar collagens and other components of the interfibrillar space thus creating a extracellular matrix resistent to tensile strength and with elastic capacity against loading. Also, type VI collagen is an essential component on the functional fibrillar units serving as inter-fibrillar gliding filaments (Neurath & Stofft, 1992).

We suggest that collagen type VI works as an adhesive molecule of mesenchymal tissues stabilizing the extracellular matrix and the cell matrix communication.

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FIGURE LEGENDS

Figure 1. Immunoperoxidase localization of type VI collagen in the virgin mouse pubic symphysis. A) Control reaction B) Reaction is seen concentrated around the cells (arrowheads) of the hyaline cartilage C) A detail of the association of collagen type VI with the blood vessel wall. Bar; 20μm. D and E) The reaction for type VI collagen was mostly observed in the pericellular matrix of the fibrochondrocytes (arrowheads) but was also seen as fibrillar components (asterisk) disposed in a meshwork at the fibrocartilage F) Aspects of the reaction for type VI collagen in the dense connective tissue. Some reaction was observed as fibrillar components (arrows) inside at the fiber surface. Bar; 20μm

Figure 2. Immunoperoxidase localization of type VI collagen in the interpubic ligament of the mouse in the 15th (A and B) and 18th days of pregnancy (C). A and B) Collagen type VI is found throughout the ligament. It was found around the cells (arrowheads) and especially between the collagen fibers as thin fibrils components (arrows). C) The reaction was mostly observed at the collagen fiber surfaces, which appear as thin fibrils (arrows). Bar; 20μm

Figure 3 to 5. Transmission electron micrographs of the virgin mouse pubic symphysis digested with chondroitinase ABC. 3) Note the amorphous materials (asterisks) associate with the cell bodies and citoplasmatic processes of the fibrocartilage. 4) Higher magnification of cell bodies of fibrocartilage. 5) Longitudinal section shows amorphous materials (arrow) between the collagen fibrils in dense connective tissue. Bar; 0,25μm

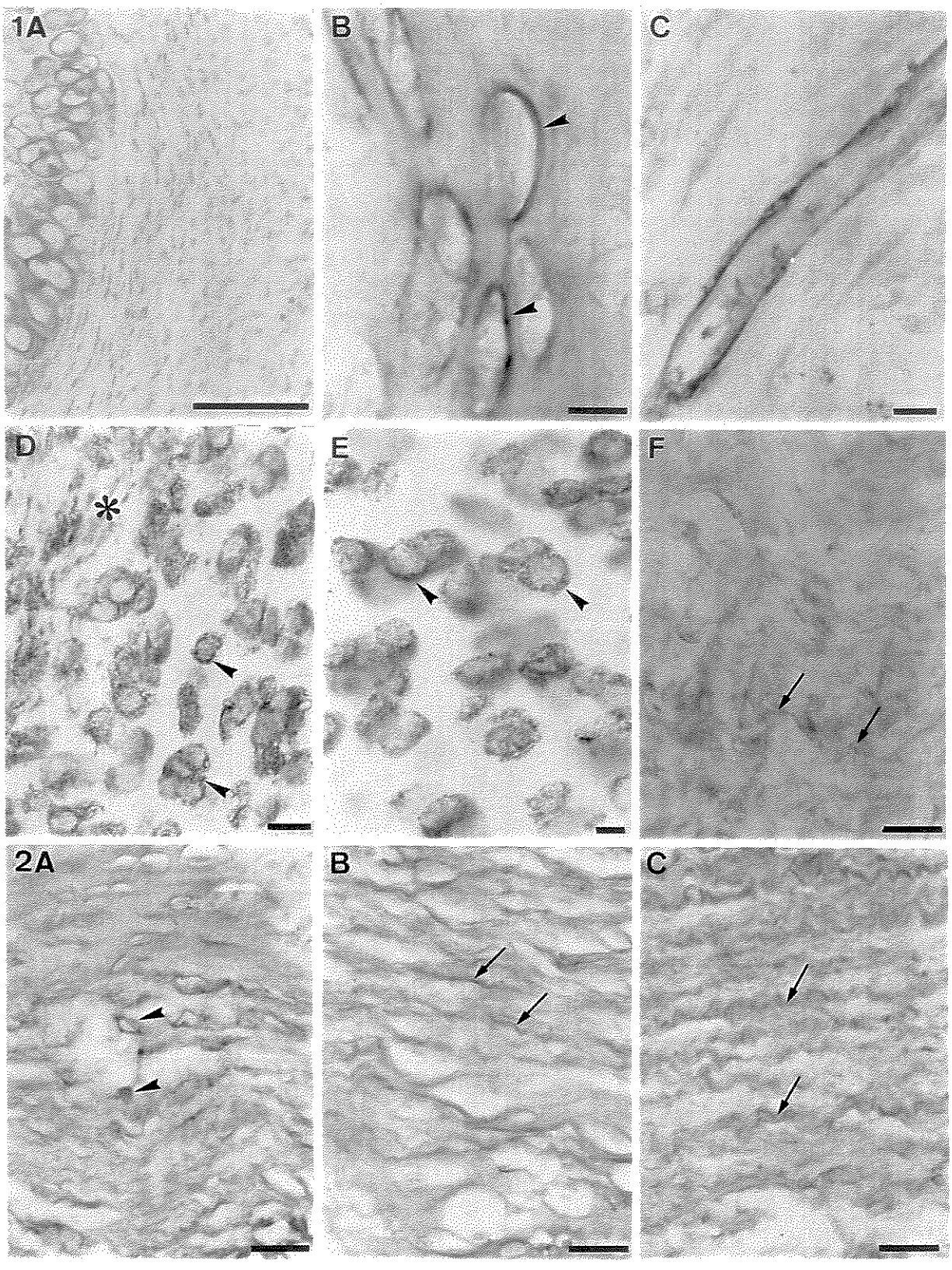
Figure 6 and 7. Transmission electron micrographs of mouse pubic symphysis during the 15th pregnancy day after chondroitinase ABC digestion. 6) High magnification shows the presence of a meshwork of microfibrils near cell bodies and amongst the collagen fibrils (asterisks). 7) A network formed by microfibrils interspersed the collagen fibrils can be observed (arrows). Bar; 0,25μm

Figure 8 to 12. Transmission electron micrographs of the virgin mouse pubic symphysis after ATP-treatment. Figure 8 is a thin section of hyaline cartilage. Inspection at higher magnification; no periodic structures were observed at pericellular matrix chondrocyte (Figure 9). Figure 10: type VI collagen aggregates form a meshwork around the fibrochondrocytes (cell) defining their pericellular matrix (asterisk). Figure 11 and 12: Aggregates of type VI

collagen in dense connective tissue. Type VI extends between the collagen fibers and fibrils (arrowheads), apparently filling part of the interfibrillar spaces. Bar; 0,6μm

Figure 13 to 15. Type VI collagen aggregates in the mouse interpubic ligament on the 15th day of pregnancy. Figure 13: The aggregates (asterisk) defined the pericellular matrix of the cell. Figure 14 is a high magnification showing the type VI collagen aggregates around the cells and occupying large areas of the interfibrillar spaces (asterisks). Figure 15: type VI collagen aggregates also appeared to be segregated from striated collagen fibrils, and they formed a network by themselves (arrows). After the ATP treatment, the microfibrils aggregated and formed the ladder-like structures (Fig.15), observed in detail in the inset as dense bands (arrowheads) connected by thin filaments. Bar; 0,3μm

Figure 16 to 18. Type VI collagen aggregates in the mouse interpubic ligament on the 18th day of pregnancy. Figure 16: Aggregates of the type VI collagen formed by ATP treatment could be observed near cell bodies (cell) and among collagen fibrils (asterisks) forming a microfilaments network in the ligament. Figure 17: The different directions of the type VI aggregates demonstrate that it forms a meshwork in the extracellular space. Figure 18: A longitudinal section of the outer layer ligament showing microfilaments (arrows) distributed among collagen fibrils. Bar; 0,3μm.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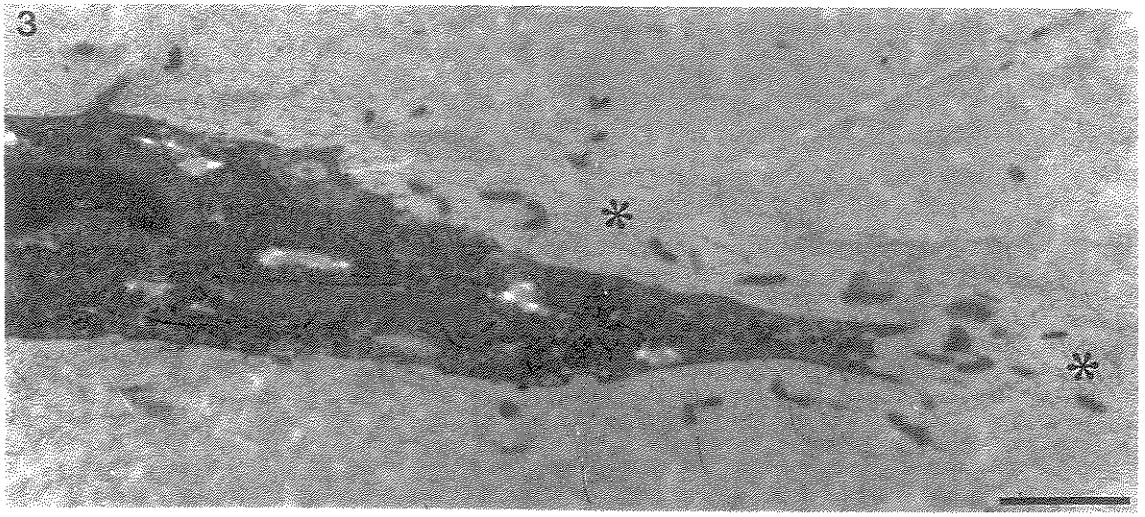
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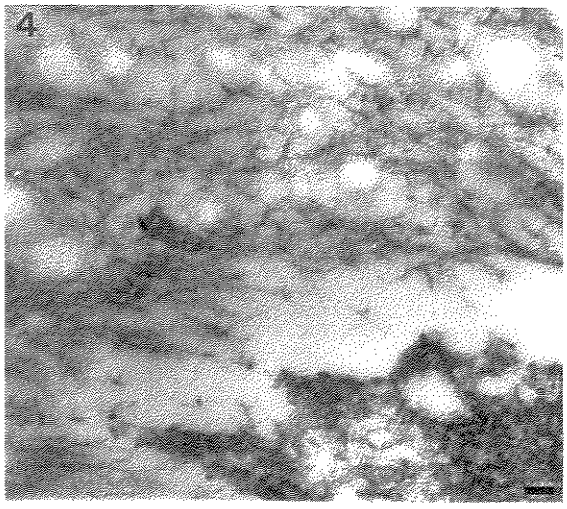
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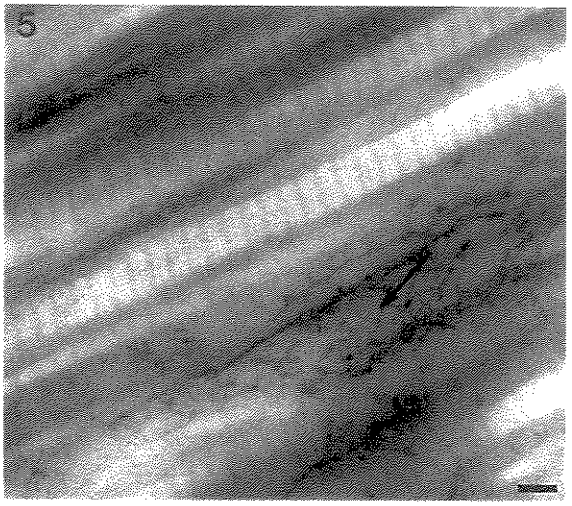
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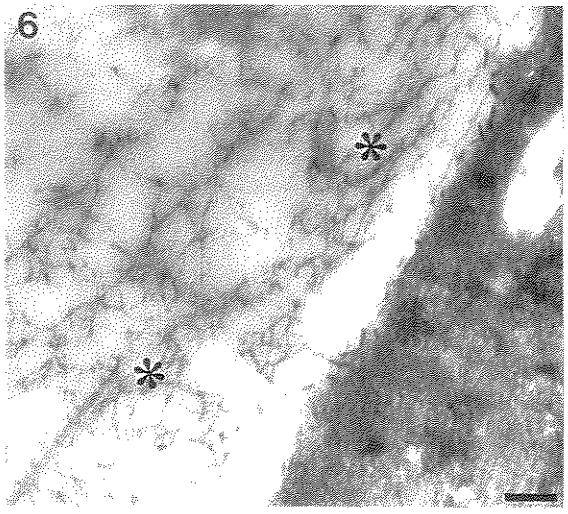
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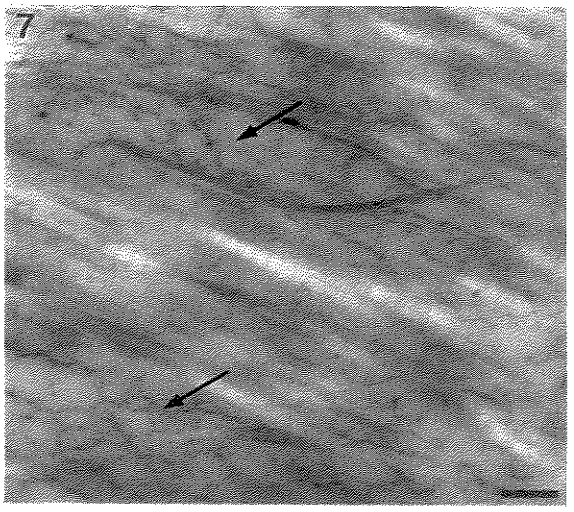
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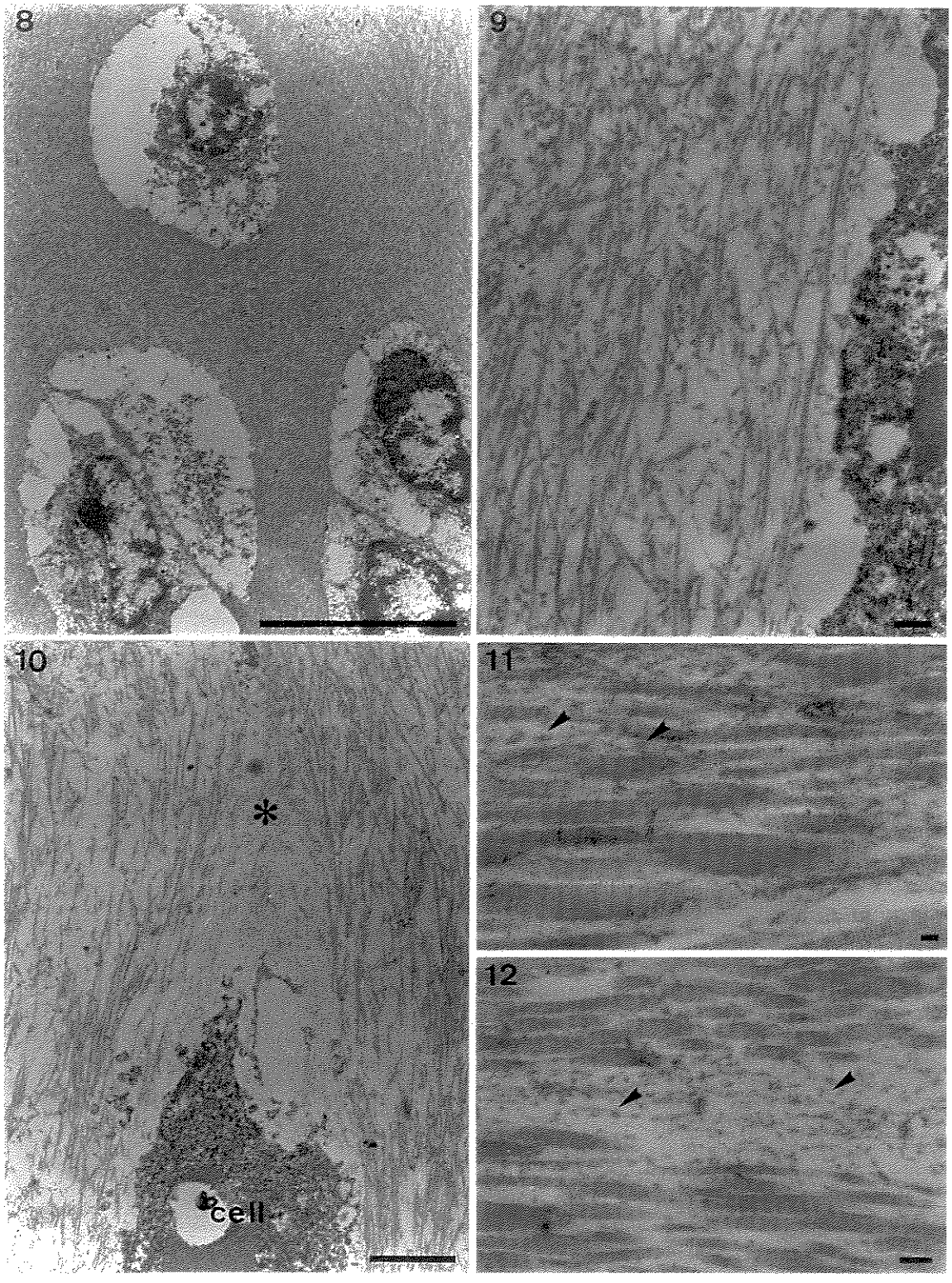
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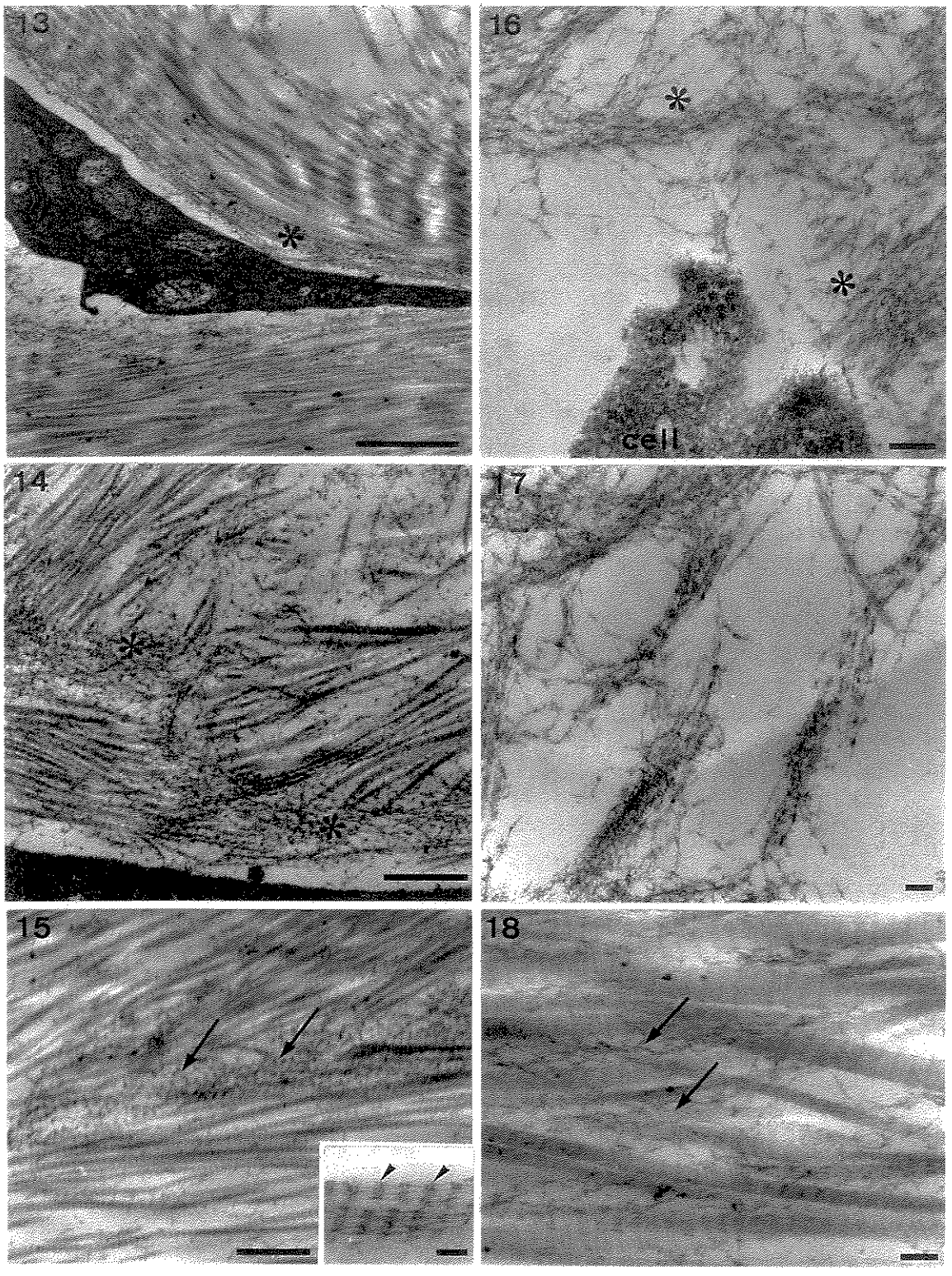
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4. CONCLUSÕES

Os resultados obtidos nessa tese permitem concluir que:

1. A sínfise púbica de camundongos virgens é formada por tecido conjuntivo denso periférico e fibrocartilagem central. A concentração, o tipo e a localização dos proteoglicanos e glicosaminoglicanos são característicos de cada um desses tecidos e refletem as suas funções mecânicas no papel biológico dessa articulação.

2. As análises bioquímica e imunohistoquímica revelam o condroitim sulfato como único glicosaminoglicano sulfatado presente na sínfise púbica de animais virgens e prenhes.

3. A concentração, o tipo e a localização dos proteoglicanos e glicosaminoglicanos variam durante a última semana de prenhez.

4. O aumento de condrotim sulfato tem início no 12º dia de prenhez, período em que se inicia a transformação da fibrocartilagem em um ligamento.

5. A localização ultra-estrutural e a análise imunohistoquímica permitem concluir que os proteoglicanos presentes no ligamento interpúbico no 15º dia de prenhez são, principalmente, decorim e biglicam. A análise qualitativa dos glicosamingoglicanos sulfatados, nesse período, mostrou que o condrotim sulfato é o glicosaminoglicano da cadeia lateral desses proteoglicanos.

6. No 15º dia de prenhez, quando a sínfise púbica está sob elevadas forças de tensão, há um aumento, em mais de 100%, na concentração de condroitim sulfato, o que sugere o papel fundamental do decorim no suporte às forças de tensão.

7. A abrupta diminuição de condroitim sulfato, observada nos últimos dias de prenhez (17º e 18º dias), associada ao aumento de ácido hialurônico e a presença de áreas desprovidas de fibrilas de colágeno, porém com grande quantidade de proteoglicanos, caracterizam o início do processo de relaxamento do ligamento, com a formação de um tecido adaptado para resistir à forças de tensão sem sofrer estiramento.

8. A partir do 15º dia de prenhez, o proteoglicano versicam é encontrado no ligamento interpúbico de camundongos, demonstrado pela análise ultra-estrutural, localização nos espaços interfibrilares, e apresentando cadeia lateral constituída de condroitim sulfato. Sua presença sugere um papel no deslizamento das fibras e fibrilas colágenas que estão sob tensão.

9. O aumento de ácido hialurônico nos últimos dias da prenhez, possivelmente, tem papel fundamental na retenção de água no ligamento interpúbico. Esse aumento de água auxiliará no fluxo de hormônios e citocinas, na manutenção da integridade estrutural do ligamento, bem como promoverá a necessária flexibilidade desse ligamento nos momentos que antecedem ao parto.

10. A distribuição do colágeno tipo VI na sínfise púbica de camundongos virgens e prenhes é diferente nos vários tecidos que compõem essa articulação e reflete as suas exigências funcionais. A sua localização pericelular sugere um papel na interação célula-matriz. A interação do colágeno tipo VI com outros componentes da matriz extracelular, tais como os colágenos fibrilares, tem um importante papel na organização do espaço extracelular, formando fibras colágenas heterotípicas capazes de resistir melhor a forças de tração.

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