UNIVERSIDADE ESTADUAL DE CAMPINAS

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REGULAÇÃO HORMONAL E INTERAÇÕES CÉLULAS-MATRIZ EXTRACELULAR NA PRÓSTATA VENTRAL DE RATOS

Tese apresentada ao Instituto de Biologia para a obtenção do título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

Orientador: Prof. Dr. Hernandes Faustino de Carvalho

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SUMÁRIO

1. Resumo

A próstata é uma glândula associada ao sistema reprodutor que, tanto para seu desenvolvimento quanto para a sua manutenção no animal adulto, é altamente dependente da ação androgênica. A manutenção e a fisiologia dessa glândula dependem de uma complexa interação entre os compartimentos epitelial e estromal. A privação androgênica, seja por castração ou por terapia anti-androgênica, leva a uma reestruturação desse órgão. Este trabalho teve por objetivos: (a) Avaliar o efeito da privação androgênica, após a castração, sobre o comportamento do estroma prostático durante o processo de remodelação glandular, com especial ênfase às fibras de colágeno, (b) Identificar as células estromais envolvidas no processo de remodelação, (c) Caracterizar o fenótipo das células musculares lisas após a castração e (d) Definir a cinética das modificações dos principais compartimentos teciduais prostáticos, assim como definir as principais alterações da matriz extracelular a elas associadas, da primeira à décima segunda semana do desenvolvimento pós-natal, levando em conta as variações dos níveis séricos de testosterona. Para tal foram utilizados ratos Wistar em diferentes idades. Animais adultos foram castrados e as próstatas foram removidas 7, 14 e 21 dias após a castração. Outro grupo de animais foi mantido em biotério e semanalmente, desde o nascimento até a idade adulta (12 semanas), e grupos de 5 animais foram sacrificados com o intuito de avaliar o desenvolvimento prostático. Após as devidas tomadas de peso corpóreo, coleta de sangue para avaliação hormonal e pesagem da próstata, fragmentos dos órgãos foram fixados e processados para microscopia de luz e eletrônica de transmissão. Testes histoquímicos para elementos do estroma, imunocitoquímicos e citoquímicos ultra-estruturais foram realizados. A avaliação estrutural, ultra-estrutural, estereológica e morfométrica, bem como os devidos testes estatísticos para averiguação da

significância dos achados foram realizados. Os resultados mostraram que após a privação androgênica pela castração, a próstata ventral de ratos sofre uma reorganização fibrilar intensa e este fenômeno é dependente da interação epitélio-estromal nesse órgão. O processo de reorganização das fibras de colágeno da matriz extracelular na próstata ventral de ratos após castração sugere um possível papel para as células musculares lisas - o de reorganizar o estroma prostático. Essas células, após a privação androgênica, apresentam uma grande plasticidade fenotípica, sendo hábeis na modificação dos fenótipos contráteis para outros mais sintéticos. Isto pode ser observado devido ao aumento no volume das organelas de síntese, tais como retículo endoplasmático e complexo de Golgi. Os resultados permitem sugerir ainda que, a aquisição de um fenótipo sintético após a castração esteja associada à síntese de elementos da matriz extracelular prostática. Durante o desenvolvimento pós-natal existe uma clara variação nos níveis de testosterona e, alternadamente aos picos deste hormônio, a próstata responde com proliferação epitelial nas segunda e sexta semanas. A formação do lúmen se dá nas três primeiras semanas e acúmulo de secreção luminal nas sexta e décima segunda semanas. Durante o desenvolvimento prostático ainda foi verificado que, para o desenvolvimento de novos brotos epiteliais, quando da proliferação epitelial, uma rede de fibras reticulares é estabelecida na base do epitélio em formação.

2. Abstract

The prostate is an accessory gland of the reproductive system, which depends on androgen stimulation to develop and to exert its function in the adult. The prostate homeostasis depends on a complex interplay between the epithelial and stromal compartments. Androgen deprivation attained by surgical or chemical castration leads to marked remodeling of the organ. This work was undertaken (a) to evaluate the effects of androgen deprivation on the stromal organization, with special attention to the collagen fibers, (b) to identify the stromal cell responsible for the main events of this remodeling, (c) to characterize the smooth muscle cell phenotypic modulation after castration, and (d) to define the kinetics of the different tissue compartments during the post-natal development with respect to the modifications in serum testosterone concentration. Wistar rats were employed throughout this work. Groups of adult rats were castrated and the prostate was analyzed 7, 14 and 21 days later. For the developmental analysis, groups of animals from the first to the 12 post-natal weeks were employed. Body weight, serum testosterone concentration, and prostatic weight were determined and prostate fragments processed for histology or transmission electron microscopy using different protocols. Histochemical tests, immunocytochemistry, stereological procedures and morphometry were amongst the analytical techniques employed. The results demonstrated that amongst the stromal alterations, there is a remarkable reorganization of the collagen fibers. There is an apparent involvement of the smooth muscle cells in this process. The smooth muscle cells of the rat ventral prostate showed high phenotypic plasticity, alternating from a contractile to a synthetic phenotype, as observed by an increase in the relative volume of synthetic organelles, such as endoplasmic reticulum and Golgi complex. There is a clear correlation between the raising testosterone concentration and the post natal development of the prostate. We detected cell proliferation peaks at the 2^{nd} and 6^{th} weeks;

the lumen was formed during the first three weeks and accumulation of secretory material in the lumen at the 6^{th} and 12^{th} weeks. Associated with epithelial budding, a reticulin-based basket is formed around the protruding epithelial cells, demonstrating an intimate association between epithelial growth and stromal reorganization.

3. Introdução

Interações célula-matriz extracelular

A matriz extracelular (MEC) é definida como uma complexa mistura de componentes protéicos, proteoglicanos e glicoproteínas adesivas que propiciam suporte mecânico para células e tecidos. Tais proteínas, estruturais e regulatórias da MEC, atuam cooperativamente para regular a ampla variedade de processos celulares (Pupa et al., 2002).

Cada tipo celular apresenta determinados receptores para o seu micro-ambiente e as interações destes receptores com a MEC influencia a forma, o desenvolvimento e a resposta celular a moléculas solúveis, incluindo citocinas e fatores de crescimento, da forma descrita no clássico modelo de reciprocidade dinâmica de Lin e Bissell (1993). É também amplamente conhecido que a MEC, dependendo de seu contexto, regula processos celulares distintos como o crescimento, morte, adesão, migração, invasão, expressão gênica e diferenciação. Tais eventos celulares regulam processos fisiológicos como desenvolvimento embrionário, morfogênese tecidual, angiogênese e também, transformação e metástases em processos patológicos (Ingber e Folkman, 1989; Juliano e Haskill, 1993; Liotta e Kohn, 2001; Bissell et al., 2002; Wang et al., 2002).

A estrutura da próstata ventral de ratos

A próstata é um órgão acessório do aparelho reprodutor masculino que contribui com uma fração importante do líquido seminal e com a capacitação e sobrevivência dos espermatozóides, contribuindo com íons, lipídios estruturais de membrana, substâncias imunossupressoras e

antiinflamatórias.

A próstata ventral de ratos é composta por um conjunto de estruturas túbulo-alveolares, nas quais as estruturas epiteliais encontram-se envolvidas por um estroma (Aümuller et al., 1979). Na próstata ventral de rato, cada lobo prostático consiste de oito conjuntos de dutos que se originam a partir da uretra como uma estrutura tubular simples que se ramifica distalmente. Esse conjunto de dutos é dividido em três regiões morfológica e funcionalmente distintas, denominadas distal, intermediária e proximal, de acordo com sua posição em relação à uretra (Lee et al., 1990; Shabsigh et al., 1999). Na região distal, são encontradas células epiteliais colunares altas com atividade proliferativa, circundada por células musculares lisas que formam uma camada esparsa e descontínua, associada a um grande número de fibroblastos (Nemeth e Lee, 1996). Na região intermediária, as células epiteliais também são colunares altas, apresentando características de células secretoras, sem atividade proliferativa. Nessa região, a camada de células musculares lisas é fina e contínua. Na região proximal, as células epiteliais são cúbicas e baixas, sendo freqüentes células apoptóticas e células musculares lisas formando uma camada espessa. Tanto na região intermediária como na proximal, o tecido fibroso está presente no espaço entre os dutos e, ocasionalmente, intercalando a camada de células musculares lisas.

As unidades secretoras e os seus dutos são constituídos por diferentes tipos celulares, as células epiteliais luminais, geralmente cilíndricas e altas e diferenciadas com capacidade secretora e as células epiteliais basais, menores e achatadas ou arredondadas, representando uma população proliferante (Ichihara *et al*, 1978, Zaviacic, 1999; Timms et al., 1999; Hudson et al, 2001; Santos et al., 2003). Um terceiro tipo celular, de localização intermediária, são as células neuroendócrinas.

A distinção entre os diferentes tipos celulares é possível utilizando-se marcadores específicos. As células epiteliais luminais, além de expressarem atividade para enzimas

específicas, como a fosfatase ácida prostática (Custódio et al., 2003) e antígeno prostático específico - PSA, podem ser identificadas pela expressão seletiva de citoqueratinas CK8 e CK18 (Hudson et al., 2001; van Leenders and Schalken, 2003), e também alguns antígenos de superfície, como os CD25, CD40 e CD147, dentre outros 25 sub-tipos (Liu & True 2002). As células basais expressam a proteína nuclear p63 (Signoretti et al., 2000) e podem ser marcadas por imunocitoquímica pela expressão diferencial das citoqueratinas CK5 e CK14 (Prins, *et al.*, 1991; Hayward *et al.*, 1996a; Hayward *et al.*, 1996b; Hudson et al., 2001).

As células neuroendócrinas do epitélio prostático têm sido diferencialmente marcadas das demais por responderem positivamente à reação histoquímica de impregnação por prata (Grimelius, 1968) e à reação imunocitoquímica a anticorpos anti-cromogranina A (Signoretti et al., 2000).

Existe ainda uma tendência atual de se classificar as células prostáticas em um quarto sub-tipo, as células intermediárias, que expressam, numa fase transitória, citoqueratinas comuns às células basais e luminais (Hudson et al., 2001). A esses tipos celulares têm sido atribuída a responsabilidade por lesões específicas na próstata, nas quais esses tipos celulares podem ser reativos a antígenos de proliferação celular, tais como o Ki67 e p27Kip1 (van Leenders et al., 2003).

A interação epitélio-estroma desempenha um papel fundamental na regulação e manutenção da atividade funcional da próstata (Lee, 1996). Assim, as diferenças fenotípicas das células epiteliais encontradas ao longo dos dutos prostáticos parecem estar relacionadas à distribuição diferenciada dos fibroblastos e das células musculares lisas (Prins et al., 1992; Lee et al., 1990; Nemeth e Lee, 1996).

Além das células musculares lisas e fibroblastos, outros tipos celulares também são encontrados no estroma, como mastócitos, células endoteliais e pericitos, juntamente com

terminações nervosas e gânglios sensitivos. Cada célula desempenha um papel importante e específico na manutenção e função secretora da próstata ventral.

As células musculares lisas (CML) representam 22% da área total da próstata humana (Shapiro et al., 1992), predominando ao redor dos dutos, onde encontram-se em íntimo contato com a membrana basal das células epiteliais. Já na próstata ventral de ratos, as CML ocupam cerca de 5% do volume total da glândula e cerca de 14% do estroma (Antonioli et al, 2004). As CML têm um papel preponderante nos mecanismos de estimulação parácrina, especialmente sobre o epitélio (Farnsworth, 1999) e, provavelmente, também sobre as demais células estromais.

Entre as células epiteliais e o estroma encontra-se a membrana basal. Essa estrutura é extremamente importante no controle das atividades celulares e, principalmente, na manutenção da fisiologia das células epiteliais (Hayward et al., 1998). Composta principalmente de colágeno tipo IV, proteoglicanos e laminina, a membrana basal é essencial para a manutenção do fenótipo diferenciado e secretor das células epiteliais glandulares (Labat-Robert et al., 1990). Os principais componentes das membranas basais foram detectados na próstata humana normal (Knox et al., 1994) e nas membranas basais dos carcinomas com diferentes graus de diferenciação tumoral, com exceção do colágeno do tipo VII, que está ausente dos dutos neoplásicos.

Carvalho e Line (1996) descreveram as modificações associadas à membrana basal das células epiteliais e das células musculares lisas ocorridas após a privação de andrógenos, demonstrando que eventos apoptóticos seguem-se à perda de adesão da célula à membrana basal e que existe um retardo na absorção das membranas basais residuais, as quais se tornam extremamente pregueadas e laminadas e contêm moléculas intactas de laminina, mesmo após 21 dias de castração. Por outro lado, Ilio e colaboradores (2000) observaram que a laminina está presente uniformemente na membrana basal ao longo do sistema de dutos prostáticos, enquanto o

colágeno tipo IV é encontrado na membrana basal da região distal e intermediária, embora esteja praticamente ausente na região proximal. Eles também demonstraram que o processo de involução prostática inicia-se com a perda ou redução do colágeno tipo IV, nas diferentes regiões dos dutos, durante os primeiros dias após a castração, enquanto a laminina permanece intacta. Após o período de pico de apoptose (3º e 4º dias após a castração), o colágeno tipo IV volta a ser encontrado na membrana basal dos dutos. Os autores sugerem uma associação direta entre o aumento da apoptose e a perda de colágeno tipo IV.

A matriz extracelular do estroma, além de apresentar os componentes de membrana basal das células musculares lisas, é formada também por fibras de colágeno tipo I e tipo III, as quais sofrem um extenso rearranjo na próstata em regressão. Esse rearranjo é caracterizado por um aspecto pregueado das fibras ao redor dos dutos e está intimamente associado às funções assumidas pelas células musculares lisas após a castração (Vilamaior, 1998).

Microfibrilas de colágeno do tipo VI e fibras do sistema elástico também são encontradas no estroma prostático e apresentam modificações durante a involução prostática. Esses componentes parecem estar envolvidos no controle de alguns aspectos do comportamento celular e desempenham um papel estrutural, mantendo a integridade do órgão (Carvalho e Line, 1996; Carvalho et al., 1997a; 1997b).

Dado o envolvimento das células musculares lisas nas funções prostáticas, sua participação na estruturação do estroma e na reorganização pós-castração e o seu provável papel nas interações epitélio-estroma (Cunha et al., 1986) e na “reação estromal” nos processos de invasão epitelial (Tuxhorn et al., 2001; 2002), o estudo do papel dessas células na produção de componentes da matriz extracelular, assim como das metaloproteinases da matriz (MMPs) e seus inibidores, parece ser fundamental para uma melhor compreensão das funções do estroma em diversos processos relacionados com a modulação do crescimento e da função prostática.

Eixo hipotálamo-hipófise-testículo-próstata e a regulação por hormônios esteróides sexuais

O controle neuroendócrino do metabolismo, do crescimento e de certos aspectos da reprodução é mediado por uma combinação de sistemas neurais e endócrinos centrados no eixo hipotalâmico-hipofisário. A secreção de gonadotrofinas (hormônios luteinizante – LH e folículo-estimulante – FSH) pela adeno-hipófise é regulada pelo hipotálamo, através do hormônio liberador de gonadotrofinas (GnRH) (Klonoff e Karam, 1995). O LH é uma glicoproteína que estimula a ovogênese nas fêmeas e a espermatogênese nos machos. Neste último, a sua principal ação ocorre através do estímulo da produção de testosterona pelas células de Leydig (Klonoff e Karam, 1995).

Os andrógenos são requeridos para o crescimento normal e para as atividades funcionais da próstata. No homem, os principais andrógenos circulantes são a diidrotestosterona (DHT) e a testosterona (T). Essa última é produzida, na sua maior parte (cerca de 95%), pelos testículos. As glândulas adrenais contribuem com menos de 5% da produção dos esteróides sexuais (Coffey, 1992; Cheng et al., 1993; Geller et al., 1984), sendo regulada pelo hormônio adrenocorticotrófico (ACTH). Nos dois tecidos existem pelo menos duas vias biossintéticas a partir da pregnenolona; a via predominante é a δ-5, que resulta na produção de androstenediona e testosterona; já a via δ-4, menos proeminente, leva à síntese de deidroepiandrosterona (DHEA) e androstenediol. Uma vez sintetizada, a maior parte da DHEA é inativada via sulfatação, enquanto uma pequena fração é convertida em androstenediona e, a seguir em T, nos tecidos periféricos e na próstata (Cheng et al., 1993). Em homens saudáveis, os andrógenos adrenais contribuem minimamente na função prostática normal, embora em estados tumorais da próstata a contribuição da adrenal pode ser

suficiente para promover o crescimento prostático, principalmente após orquiectomia (Geller et al., 1984).

Estima-se que apenas 2 a 3% da T encontra-se disponível em sua forma livre, sendo que o restante encontra-se ligado a proteínas séricas como a SHBG (*sex hormone-binding globulin*), a albumina e a globulina de ligação com corticoesteróide (Vermeulen, 1973). Entre as três proteínas, a SHBG possui a maior afinidade pela testosterona.

O receptor de andrógenos (AR) atua fundamentalmente como fator de transcrição. Ele fica localizado no citoplasma. Com a ligação da T ou da DHT, ele dissocia-se de uma proteína HSP (*heat shock protein*), dimeriza-se e é translocado para o núcleo, onde, em conjunto com uma série de co-ativadores e co-repressores, ativa ou inativa genes. Hoje são conhecidas ações não genômicas do AR, que envolveriam a ativação de várias vias de sinalização e diferentes efeitos sobre o comportamento celular (Heilein e Chang, 2002). As características principais destas ações são a sua velocidade de ocorrência, antecipando qualquer efeito de ativação e transcrição gênica (Castoria et al., 2003). Adicionalmente, existe também a possibilidade de ação do AR na ausência de ligantes (Cenni e Picard, 1999; Culig et al., 2002), além da possibilidade de modificação da atividade do AR por modificações pós-traducionais, dentre elas a fosforilação (LaFevre-Bernt e Ellerby, 2003; Hirawat et al., 2003; Thompson et al., 2003). O AR clássico tem 132kDa e possui várias características em comum com os membros da família dos receptores nucleares, como os receptores de estrógeno, de progesterona, dos hormônios da tireóide e com os PPAR (receptores para os ativadores da proliferação dos peroxissomos) (Jacobs et al., 2003). O gene que codifica o AR possui uma região regulatória com sítios de ligação para a CREB (proteína de ligação com o elemento de resposta ao AMP cíclico) e para a SP1 (Nakayama et al., 2000).

A conversão de T em 5α-diidrotestosterona (DHT) é realizada pela enzima 5α-redutase. Embora os dois hormônios atuem através do mesmo receptor de andrógeno (AR), parece que exercem funções distintas. Assim, a função da 5α-redutase é essencial para a morfogênese prostática, sendo que sua deficiência resulta na quase total inexistência do órgão. Na vida adulta, a concentração tecidual de DHT é mantida elevada, quando comparada com os níveis plasmáticos. Ambos os andrógenos são capazes de manter a atividade prostática, porém a DHT apresenta 1,6 a 1,9 maior potência que a testosterona (Rittmaster et al., 1991; Wright et al., 1999).

Dois tipos de 5α-redutases foram identificados. A 5α-redutase tipo I encontra-se na maioria dos tecidos, enquanto a 5α-redutase tipo II, que é codificada pelo gene SRD5A2, com localização cromossômica 2p23 (Labrie et al., 1992), predomina nos tecidos genitais, incluindo a próstata. Quando a finasterida, inibidor específico da 5α-redutase tipo II, foi administrada a homens para o tratamento de hiperplasia prostática benigna (BPH), os níveis séricos de DHT diminuíram cerca de 70%; enquanto os níveis prostáticos diminuíram de 85 a 90% (Bartsch et al., 2000). A quantidade remanescente de DHT na próstata pode ser atribuída à ação da isoenzima do tipo I.

A DHT intracelular é rapidamente metabolizada em uma reação reversível para 3α, 17β-androstenediol (3α-diol) ou para 3β, 17β-androstenediol (3βAdiol). A enzima responsável por esta via de inativação é a 3β-hidroxiesteróide desidrogenase tipo II (3β-HSD). O 3βAdiol é finalmente convertido irreversivelmente em esteróides inativos hidrossolúveis, através da ação da 3β adiol hidroxilase (CYP7B1) (Martin et al., 2001). Teoricamente, a inibição da 5α-redutase tipo II causaria acúmulo de testosterona aumentando os níveis de AR na próstata (Weihua et al., 2002). A aromatase poderia converter o excesso de testosterona em estrógeno que atuaria sobre os receptores ERα e ERβ. Isso poderia resultar em crescimento displásico da próstata. No entanto, não existem estudos verificando essa hipótese, nem estudos que forneçam informação

precisa sobre o padrão de expressão de algumas enzimas consideradas chave no metabolismo prostático, como a aromatase, a 5α-redutase, a CYP7B1 e a 3β-HSD na próstata e que procurem averiguar sua modulação pelos andrógenos.

Adicionalmente, está bem estabelecido que alguns dos andrógenos circulantes são convertidos a estrógenos em vários tecidos periféricos, através da enzima aromatase (Simpson et al., 1999). Interessantemente, a aromatase foi identificada na próstata humana, sugerindo um local de aromatização e uma possível fonte local de estrógeno (Tsugaya et al., 1996).

Ainda que a próstata seja dependente de andrógeno, os estrógenos influenciam as funções normais e as mudanças patológicas nesse órgão. Isso pode ser devido à presença dos dois receptores de estrógeno no órgão (Weihua et al., 2001).

As evidências de uma ação direta dos estrógenos na próstata surgem da observação da existência de receptores para estrógenos, ERα e ERβ, nas células prostáticas estromais e epiteliais, respectivamente. Um efeito direto do estrógeno, estimulando o crescimento prostático, foi demonstrando em camundongo hipogonadais (*hpg*), que não possuem andrógenos circulantes. O crescimento induzido foi bem menor que nos controles e estava associado a diferentes neoplasias (Bianco et al., 2002). Procurando definir a cinética de apoptose do epitélio prostático frente à castração e ao tratamento com estrógeno, foi sugerido que o estrógeno possui um efeito inicial direto sobre a próstata, antecipando a apoptose das células epiteliais, quando comparado com a castração (Garcia-Flórez e Carvalho, 2003).

Além disso, há também um mecanismo denominado *imprinting* estrogênico, que se caracteriza pela exposição perinatal ao estrógeno e a manifestação de efeitos na adolescência e na vida adulta. Os efeitos do estrógeno têm ação não normotônica, ou seja, efeitos opostos quando diferentes dosagens são empregadas. Dosagens baixas resultam em estímulo do crescimento,

enquanto dosagens mais elevadas resultam em redução do crescimento. Os efeitos do *imprinting* estrogênico são múltiplos e melhores conhecidos através de seus efeitos feminilizantes por ação direta no hipotálamo, além de atuar antecipando a puberdade em ratos (Putz et al., 2001b). Altas doses de estrógeno no período neonatal induzem também uma quase completa eliminação da expressão do receptor de andrógeno, como determinado por imunocitoquímica (Putz et al., 2001a). Foram observados ainda rearranjos das junções comunicantes (Habermann et al., 2001) e alterações na expressão dos receptores para o ácido retinóico (Prins et al., 2002). O estrógeno parece exercer suas ações através do receptor de estrógeno do tipo β, presente no estroma prostático. Recentemente foi publicado que o E2 causa uma redução do AR, por destinar o produto protéico ao sistema de degradação por proteassomos (Woodham et al., 2003). Apesar dessas evidências, os mecanismos moleculares envolvidos no *imprinting* estrogênico são desconhecidos.

Desenvolvimento prostático: Períodos embrionários, pós-natal e pré-púbere

A estimulação por andrógenos é absolutamente necessária para o desenvolvimento da próstata, assim como para as demais estruturas sexuais masculinas (Cunha et al., 1987). A morfogênese prostática é dependente da produção de andrógenos pelos testículos do feto (Pointis et al., 1980). O desenvolvimento da próstata não é determinado pelo sexo genético, mas sim pela exposição aos andrógenos, tendo sido demonstrado que o seio urogenital (UGS) de fêmea ou macho podem formar tecido prostático funcional, caso eles sejam estimulados por andrógenos no período adequado (Takeda et al., 1986).

O brotamento prostático é iniciado pela ação androgênica pré-natal (Timms et al., 1994). As subseqüentes morfogênese dutal, canalização e citodiferenciação epitelial também precisam de estimulação androgênica e estão associados a um aumento perinatal transitório na concentração de testosterona (Donjacour e Cunha, 1988). Embora a testosterona seja o primeiro andrógeno produzido pelos testículos fetais, a diidrotestosterona (DHT) é a responsável pela morfogênese prostática (Taplin e Ho, 2001). A DHT é produzida no seio urogenital (UGS) pela redução da testosterona pela enzima 5α-redutase. Essa enzima foi detectada no UGS e na genitália externa de ratos, coelhos e humanos (Wilson et al., 1983).

A diferenciação do epitélio prostático ocorre paralelamente à maturação do estroma prostático. Os andrógenos atuam sobre os receptores de andrógeno (AR) no mesênquima urogenital (UGM) para induzir a proliferação epitelial, a ramificação dutal e a citodiferenciação nos subtipos celulares basal e luminal (Cunha et al., 1987; 1992). Por sua vez, o epitélio próstatico em desenvolvimento direciona os padrões de diferenciação do músculo liso próstatico (Hayward et al., 1998). Nem o epitélio prostático nem o músculo liso prostático são capazes de se desenvolver na ausência do outro tecido (Hayward e Cunha, 2000). Durante a organogênese, uma das funções dos andrógenos é reduzir a camada de células musculares lisas que envolve a uretra (Thomson et al., 2002). Quando essa camada é espessa, dada a ausência de andrógenos, como acontece nas fêmeas, o brotamento inicial a partir da uretra não consegue atingir o mesênquima do seio urogenital e o desenvolvimento prostático não ocorre.

No período pós-natal, o desenvolvimento prostático é também dependente de andrógenos já que a castração de ratos neonatos inibe o crescimento e o desenvolvimento da próstata durante a puberdade, um efeito que pode ser revertido com a administração de testosterona (Cunha et al., 1987; Corbier et al., 1995).

A próstata de neonato é sensível aos andrógenos. Assim, a administração de testosterona acelera o crescimento da próstata, sendo possível atingir precocemente o crescimento máximo (Berry e Isaacs, 1984). Na puberdade apresenta-se o início do crescimento prostático que é caracterizado por um aumento do peso seco da próstata e por um pequeno incremento no número das ramificações (Sugimura et al., 1986). Isso indica que a próstata em desenvolvimento é sensível às baixas concentrações de andrógenos para a ramificação dutal, e que a sua resposta aos níveis altos de andrógenos na puberdade (incremento de peso seco) é diferente da resposta inicial (Hayward e Cunha, 2000).

Uma boa correlação entre as propriedades de invasão tumoral e o crescimento normal das estruturas epiteliais foi estabelecido para a glândula mamária (Wiseman e Werb, 2002) e para o pulmão (Kheradmand et al., 2002), pelo menos no que se refere a um estado proliferativo aumentado do epitélio, à necessidade de degradação da matriz extracelular adjacente e à colonização de espaços anteriormente ocupados pelo estroma. Esse quadro parece ser também verdadeiro na angiogênese (Feng et al., 1999), quando a membrana basal é degradada e substituída por uma matriz provisória de fibrina, na qual as células endoteliais proliferam e a partir de onde invadem o tecido adjacente. Parece então necessário uma melhor caracterização das alterações da matriz extracelular e da participação de MMPs e de seus inibidores, no processo de crescimento e de regressão epitelial prostáticos, utilizando-se o crescimento pós-natal e a castração como modelos experimentais, procurando-se identificar os mecanismos moleculares envolvidos no seu controle.

4. Objetivos

Com a finalidade de adquirir novos conhecimentos que contribuam para os estudos da regulação hormonal nas interações células-matriz extracelular na próstata ventral de ratos, este trabalho teve por objetivos:

1. Avaliar o efeito da privação androgênica, após a castração, sobre o comportamento do estroma prostático durante o processo de remodelação glandular, com especial ênfase às fibras de colágeno.

2. Tentar identificar as células envolvidas nesse processo.

3. Caracterizar os fenótipos das células musculares lisas prostáticas após a castração.

4. Definir a cinética dos principais compartimentos teciduais prostáticos, assim como definir as principais alterações da matriz extracelular a ela associadas, da primeira à décima segunda semana do desenvolvimento pós-natal.

5. Correlacionar os dados morfológicos e estereológicos obtidos para o desenvolvimento prostático com as alterações nos níveis séricos de testosterona.

5. Artigos científicos

Os resultados obtidos permitiram a elaboração de três artigos científicos, dos quais, um já se encontra publicado, um foi submetido e o outro será submetido para publicação.

5.1. Vilamaior PSL, Felisbino SL, Taboga SR, Carvalho HF. 2000. Collagen fiber reorganization in the rat ventral prostate following androgen deprivation: A possible role for smooth muscle cells. **Prostate,** 45: 253-258.

5.2. Vilamaior PSL, Taboga SR, Carvalho HF. 2003. Androgen regulation of smooth muscle cells function in the rat ventral prostate: Morphological evidences for a contractile to synthetic transition after castration. **Prostate,** *submetido.*

5.3. Vilamaior PSL, Taboga SR, Carvalho HF. 2003. Alternating proliferative and secretory activities contribute to the post-natal growth of the rat ventral prostate. **Anat Embryol.,** *a ser submetido.*

The Prostate 45:253–258 (2000)

Collagen Fiber Reorganization in the Rat Ventral Prostate Following Androgen Deprivation: A Possible Role for Smooth Muscle Cells

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BACKGROUND. Stroma plays an essential role in glandular function in different systems. In the prostate, it is responsible for the development and maintenance of the differentiated state of the epithelium. The marked reduction in the epithelial compartment of the prostate gland following castration is followed by a similarly important reorganization of the stroma. In this work, we characterized the reorganization of collagen fibers in the ventral prostate of castrated rats.
METHODS. Histochemical tests and immunohistochemistry for type I and III collagens plus confocal microscopy of triple-labeled (collagen III, actin, and DNA) tissue sections were employed.
RESULTS. We showed that collagen fibers are composed of type I and type III collagens and that they are progressively concentrated around the epithelial structures (ducts and acini) and become increasingly undulated and folded. Double-labeling of collagen fibers and F-actin demonstrated that smooth muscle cells (SMC) are intimately associated with collagen fibers.
CONCLUSIONS. The results demonstrated a marked reorganization of the collagen fibers, and suggest an active role of the SMC in the reorganization of the fibrillar components of the stroma. *Prostate 45:253–258, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **castration; histochemistry; polarizing microscope; remodelling; stroma**

INTRODUCTION

Stroma influences epithelial function by producing growth factors and by establishing a proper environment with the production of a structurally and compositionally adequate extracellular matrix. In the prostate, smooth muscle cells (SMC) and fibroblasts play important roles in producing a number of autocrine and paracrine factors that contribute to organ homeostasis. They also produce a proper environment composed of collagen [1,2], elastic fibers [3], proteoglycans [4–6], and microfibrils [7], among other macromolecules. All these components are, to different extents, responsive to androgen ablation, as attained by castration. However, the specific involvement of stromal cells on either the extracellular matrix or the reorganization of fibrillar components has not been determined. In this work we employed histochemical techniques and immunohistochemistry to define the composition and organizational aspects of collagen fibers in the rat ventral prostate following castration. Besides describing the structural aspects of collagen fibers, we also observed a clear involvement of smooth muscle cells in the reorganization of these fibrillar components.

MATERIAL AND METHODS

Animals

Three-month-old Wistar rats were employed. A group of animals was castrated and sacrificed 7, 14,

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Figs. 1–12. Figs. 1–4. Masson's trichrome stained ventral prostate sections. **Fig. 1.** Control rat. Collagen fibers (arrow) are scant and intimately associated with the SMC. ep, epithelial structures. **Fig. 2.** Effects of castration 7 days after surgery. Number of SMC around the epithelial structures (ep) is increased, but collagen is still not prominent as after 14 days (**Fig. 3**) and 21 days (**Fig. 4**). In these latter experimentation points, collagen gradually accumulates and becomes progressively wavy (arrows) (Figs. 1–4, ×1,070). **Figs. 5–8.** Ventral prostate sections subjected to the silver impregnation of Gömöri [8]. **Fig. 5.** This section corresponds to the control, in which reticulin fibers (arrow) are straight and aligned with the base of the epithelial structures (ep). Epithelial infoldings rest on a collagenous scaffold. **Fig. 6.** Prostate section 7 days after castration. Number of collagen fibers below the epithelial structures is increasing, but they are still aligned. The collagen fibers (thick arrow) are in the outer layers, while the reticulin fibers (thin arrows) are close to the base of the epithelium (ep). **Figs. 7 and 8.** On days 14 (Fig. 7) and 21 (Fig. 8) after castration, reticulin fibers are curled and/or wavy (thin arrow). The staining of the fibers more distally to the epithelial structure corresponds to that of collagen fibers (thick arrow), as opposed to the reticulin fibers more closer to the base of the epithelium (ep). These show the same aspect of collagen folding and the presence of reticulin fibers (thin arrow) and collagen fibers (thick arrow) (Figs. 5–8, ×1,070). **Figs. 9–12.** Picrosirius red/hematoxylin-stained ventral prostate sections, as seen under polarizing microscope. **Fig. 9.** This section corresponds to the control. Collagen fibers are scant and show a green interference color. Only a few fibers are reddish-orange. **Figs. 10–12.** Seventh, 14th, and 21st days after castration, respectively. An increase in density of collagen fibers, as well as a progressive transition from the green color observed in the control, towards the red interference color which predominates in the castrated animals can be observed. The arrow in Figure 10 shows a highly undulated collagen fiber at the base of the epithelial structure (ep).

and 21 days after surgery. Controls were sham-operated and examined after 21 days, and compared to nonoperated animals sacrificed at the beginning of the experiment. At least three rats were used for each experimental point.

Histological Procedures and Histochemistry

The ventral prostate was dissected out and immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. The samples were then washed, dehydrated, cleared in cedar wood oil, and embedded in Paraplast Plus embedding medium. Sections (7 μm) were dewaxed and subjected to either of the following procedures: 1) Masson's trichrome, 2) the silver impregnation for reticulin by Gömöri [8], and 3) picrosirius red [9]. The slides were mounted in Canada balsam or Entelan and examined with Zeiss Axioskop equipped for polarization microscopy and microphotography.

Immunohistochemistry

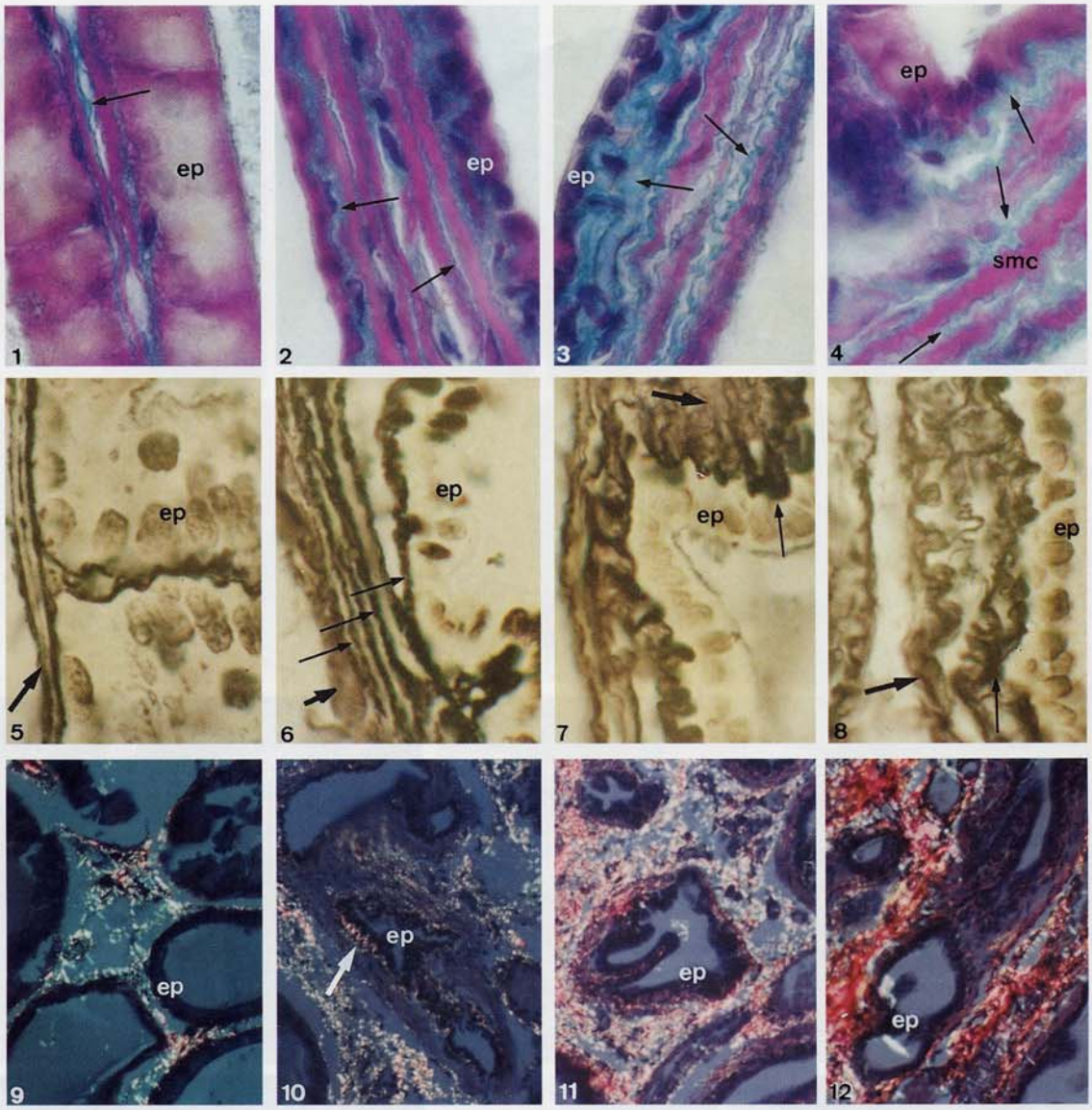
Samples were fixed as above and embedded in TissueTeck, before freezing in octanol and sectioning in a Microm cryostat. Sections (7 μm) were collected in silanized glass slides, allowed to dry, and then blocked first with 3% hydrogen peroxide in water for 30 min (to block endogenous peroxidase activity) and then with 3% bovine serum albumin (BSA) in PBS for 1 hr at room temperature. Primary rabbit anti-type I and anti-type III collagen antibodies (Chemicon International, Inc., Temecula, CA) were diluted in a solution of 3% BSA in PBS, according to the manufacturer's directions, and applied to the sections for 1 hr at room temperature. After 3 × 5-min washes with PBS, the material was incubated with a secondary peroxidase-conjugated goat anti-rabbit Ig (Sigma Chemical Co., St. Louis, MO) diluted 1:400 in 3% BSA in PBS for 1 hr at room temperature. The sections were washed again and the peroxidase activity revealed with DAB.

Alternatively, the secondary antibody was a fluorescein-conjugated goat-anti rabbit Ig (diluted 1:120) in 3% BSA in PBS. Rhodamin-conjugated phalloidin (25 μM; Sigma Chemical Co.) was added to the secondary antibody. In this situation, the anti-type III collagen antibody was used and the sections were mounted in a DABCO (Sigma Chemical Co.) solution containing 5 μM DAPI (Sigma Chemical Co.). Observations were carried out using a BioRad MRC1024UV confocal microscope.

RESULTS

Only a few thin collagen fibers were observed in the extracellular matrix of control noncastrated rats. They were closely associated with the base of the epithelium and with the smooth muscle cells (Fig. 1). Masson's trichromic revealed a progressive accumulation of collagen around the epithelial structures in the ventral prostate of castrated rats (Figs. 2–4). The number of smooth muscle cell layers had increased by 7 days after castration, and collagen accumulation was more evident after 14 and 21 days. At these latter two points, the smooth muscle cells showed an irregular outline which was intimately associated with collagen fibers.

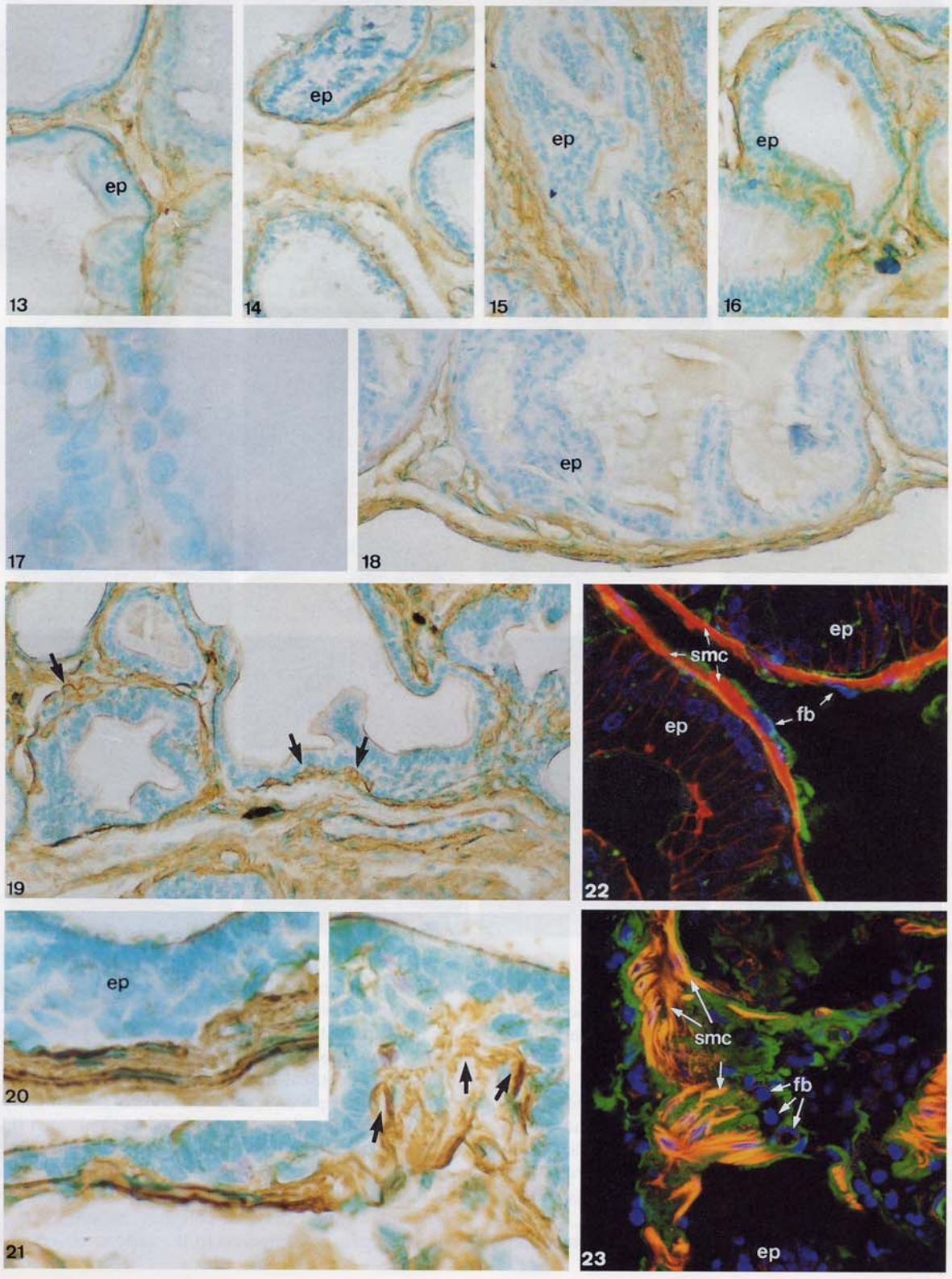
Silver impregnation according to Gömöri [8] confirmed that reticulin fibers were scarce at the base of the epithelial structures, including the epithelial infoldings (Fig. 5). A progressive increase in the number of collagen fibers around the epithelial structures occurred after castration. A progressive folding and pleating of collagen fibers was detected as well (Figs.



6–8). This procedure also showed that reticulin (mainly type III collagen) concentrated in the vicinity of the epithelial structures, while collagen fibers (mainly type I collagen) accumulated in the outer regions around the epithelium (Figs. 6–8).

Polarization microscopy of picrosirius red-stained sections revealed a progressive increase in the density of fibrillar components around the epithelial structures in the ventral prostates of castrated rats (Figs. 10–12), as compared to noncastrated controls (Fig. 9). The predominant interference color of the birefringence exhibited by collagen fibers in the control animals was green, while there was a progressive change towards red in the castrated animals (Figs. 10–12).

Immunohistochemistry revealed the presence of type I and type III collagen in the collagen fibers of the prostates of control animals (Figs. 13, 18). Only a weak diffuse staining was seen in the control reaction, especially in the secretion accumulated in the lumen (Fig. 17). The progressive increase in the area occupied by stroma in the castrated animals paralleled an increase in the amount of type I collagen (Figs. 14–16). No specific organization of type I collagen was seen in these preparations, as compared to the aspects observed in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Figs. 13–23. Figs. 13–16. Immunocytochemical localization of type I collagen in the ventral prostate. **Fig. 13.** Control. Reaction is diffuse throughout the stroma. **Figs. 14–16.** Castrated rats, 7, 14, and 21 days, respectively, after orchiectomy. A progressive increase in immunoreactive fibers around the epithelial structures is observed. Details of the fiber organization are not easily observed (Figs. 13–16, ×215). **Fig. 17.** Control for immunocytochemical reaction, from which the primary antibody was omitted. Only a weak diffuse reaction was obtained (×430). **Figs. 18, 19.** Immunocytochemical localization of type III collagen in the ventral prostate. **Fig. 18.** Control. Reaction to the anti-type III collagen antibody is localized at the base of the epithelium (ep) and aligns with the SMC. **Fig. 19.** Ventral prostate section 14 days after castration. The accumulation of type III collagen at the base of the epithelial structure is readily seen. Its wavy aspect is complementary to the surface of the stromal cells around the epithelium (arrows) (Figs. 18 and 19, ×215). **Figs. 20, 21.** Aspects of immunolocalization of type III collagen in the ventral prostate of a rat 21 days after castration. Staining is intense around the stromal cell underneath the epithelium (ep). Some aspects of the folding and undulation of collagen fibers can be observed in Figure 21 (arrows) (Figs. 20 and 21, ×430). **Fig. 22.** Confocal microscopy of a tissue section of ventral prostate from a control rat, subjected to immunocytochemistry for type III collagen (fluorescein green fluorescence) and stained for F-action with rhodamin-labeled phalloidin (red fluorescence) and DAPI (cell nuclei exhibit a blue fluorescence). Staining for collagen is compatible with the immunoperoxidase result shown in Figure 13. SMC show intense staining by phalloidin. ep, epithelium (×350). **Fig. 23.** Confocal microscopy of a tissue section of ventral prostate of a rat 21 days after castration, subjected to immunocytochemistry for type III collagen (fluorescein green fluorescence) and stained for F-action with rhodamin-labeled phalloidin (red fluorescence) and DAPI (cell nuclei exhibit a blue fluorescence). The SMC are no longer elongated but show a spinous aspect. Their surface is complementary to the foldings of the collagen fibers. fb, fibroblasts; ep, epithelium (×350).

the tissue sections processed for histochemistry. The immunostaining for type III collagen was more conspicuous than that obtained for type I collagen. A reaction was observed especially around the stromal cells in the prostate of the control animals (Fig. 18).

Type III collagen was found in the prostates of castrated animals and corresponded to fibrillar components beneath the epithelium (Figs. 19–21), and showed similar but not identical aspects to those observed in the silver-impregnated tissue sections. In Figure 19, aspects of type III collagen-containing fibers can be seen; these aspects follow the outline of stromal cells at the base of the epithelial structures and accumulate in the increasing stroma. Their presence around blood vessels and an intimate association with vascular SMC can also be noted. Immunostaining was greater by the 14th and 21st days after castration (Figs. 20, 21). Aspects of folding and pleating can be observed 21 days after castration (Fig. 21).

Double-labeling of type III collagen fibers and F-actin with the anti-type III collagen antibody and with rhodamin-conjugated phalloidin, respectively, revealed the association of SMC and collagen fibers in the regressing prostate. The ventral prostates of control rats showed that the SMC formed a single layer below the epithelium (Fig. 22). Collagen was a minor component found in intimate association with the SMC and fibroblasts (Fig. 22). Castration resulted in an altered phenotype of the SMC, which were isolated from other SMC and showed a spinous aspect (Fig. 23). The surface infolds of the SMC were closely associated with collagen fibers (Fig. 23). Fibroblasts were also seen, but no differences were seen in the castrated animals as compared to controls, except that the laminar organization in the noncastrated situation was lost.

DISCUSSION

This study presents results that demonstrated the reorganization of collagen fibers in the regressing prostate. Collagen fibers become progressively undulated and pleated in the ventral prostate of castrated rats. The double labeling protocols also demonstrated that the SMC were intimately associated with the undulating collagen fibers, suggesting an active role of these cells in the reorganization of the extracellular matrix following the involution of the epithelial structures attained by androgen depletion.

An early study by Müntzing [1] described an association between prostatic growth and collagen content in the rat ventral prostate, allowing him to hypothesize that collagen plays a growth-limiting role in the prostate gland. Later, Izumiya and Nakada [2] studied the effect of β-aminoproprionitrile, an inhibitor of lysil oxidase, the enzyme responsible for the first step in the cross-linking formation in both collagen and elastin, and showed that this drug reduced the amount of collagen in the prostate gland and increased prostate size, reinforcing the idea of Müntzing [1] that collagen restricts prostate growth, at least in young rats. Furthermore, estradiol-17β induced a decrease in prostate weight, which was accompanied by increments not only in collagen, but also in noncollagenous protein and elastin [10]. Both increased biosynthesis and decreased degradation seemed to contribute to collagen accumulation in the prostates of estradiol-treated rats [10]. The authors did not examine the effects of castration specifically on the collagen content and metabolic/catabolic changes of incorporated ^{3}H-proline in the prostate.

The results presented here show an apparent accumulation of collagen fibers around the epithelial struc-

tures, which become progressively atrophic after castration. Though this effect may not reflect an actual increase in collagen content, it demonstrates that the collagen/epithelial ratio is changed in the prostates of androgen-depleted rats. Furthermore, the specific organization of collagen fibers adopted in castrated animals reveals an altered microenvironment in which the regressing epithelium rests.

Our study also revealed that the altered phenotype of smooth muscle cells, reported by others as being synthetic instead of contractile [11], is involved not only in the synthesis and secretion of extracellular matrix components among other molecules [3], but also in the active remodeling of fibrillar components of the stroma. Fibroblasts did not present such a clear association with the fibrillar components, though they are expected to react to the new hormonal situation and to the reorganized extracellular matrix. However, this suggested role for smooth muscle cells must be tested in future experiments.

The fibrillar reorganization shown to occur after castration in the prostate gland seems relevant to the discussion of the effects of hormonal therapies on prostatic physiology, and should be taken into account in the consideration of stroma-epithelium interaction in normal physiology and diseases of the prostate gland.

In this sense, the different techniques employed in this work seem to offer a wide range of possibilities to follow structural modifications of collagen fibers. The Masson's trichromic and silver impregnation give good information about the distribution and organization of collagen fibers. Picrosirius red staining, besides offering good information on the distribution of collagen fibers, also provides information on the degree of compactness of the component macromolecules inside the fibers (as observed by the modifications in the interference colors of birefringence shown under the polarizing microscope) [12]. On the other hand, immunocytochemistry imparts molecular specificity to the structural aspects and may be used in double-labeling experiments. However, by depending on the penetration of large antibody molecules and conjugates, immunocytochemistry may not reveal complete areas occupied by a given antigen. Furthermore, steric hindrance is especially significant in collagen immunolocalization, if one considers the compactness of collagen fibers. Thus, the histochemical/immunocytochemial approach seems adequate for avoiding such drawbacks.

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Androgen regulation of the smooth muscle cell function in the rat ventral prostate: morphological evidence for a contractile to synthetic transition after castration

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ABSTRACT

Prostatic remodelling following bilateral orchiectomy is a complex phenomenon involving regressive changes in both epithelial and stromal compartments. The regressive kinetics of the different prostatic compartment differs and the stroma:epithelium ratio increases. The smooth muscle cell (SMC) is the main cell type of the prostatic stroma and is involved with the stromal remodelling. The objective of this work was to evaluate the involvement of the rat ventral prostate SMC in secretory activity and whether this function is modulated after androgen deprivation. Cell morphology was examined at both light and electron microscopy levels and the organelles involved in secretory function were labeled by the zinc-iodide-osmium (ZIO) method to ultrastructural analysis and their relative volume determined by stereology. Castration resulted in marked morphological changes of the SMC, which adopted a spinous-aspect and abandoned the layered arrangement observed in prostate of non-castrated rats. Transmission electron microscopy showed an concentration of organelles, including mitochondria in perinuclear regions. The relative volume of ZIO reactive organelles increased progressively after castration, reaching significantly higher figures after 14 days of androgen deprivation. Since previous work have demonstrated that SMC express SMC markers even 21 days after castration and are able to respond to adrenergic stimulation, we concluded that differentiated SMC are able to shift from a predominantly contractile to a more synthetic phenotype without changing its differentiantion status.

INTRODUCTION

Smooth muscle cell (SMC) is a predominant cell type in the prostate stroma. Its primary function is the organ contraction during ejaculation, allowing the accumulated secretion to be ejected to the urethra. Interest in the SMC is twofold. Firstly, this cell is the main responsible for the benign prostatic hyperplasia (BPH), the main symptom of which is urethral obstruction and intense pain, released by the use of anti-adrenergic therapy. Secondly, SMC have been implicated in paracrine regulation of the epithelial structure and function, producing key regulatory factors responsible for the organ homeostasis. Imbalance in these paracrine interactions with the epithelium would result in abnormal growth of either epithelium (in the prostatic intraepithelial neoplasias and in adenocarcinomas) or SMC (in BPH) (Cunha et al., 1996).

The SMC phenotype changes in response to physiological and pathological conditions regardless their location. In this process, the primarily contracting cell is recruited for new functions, mostly synthesizing extracellular matrix molecules and other factors. It is frequently said that a shift from the contractile phenotype to the synthetic phenotype takes place. This transition has been reported for the ventral prostate of rats treated with estradiol (E2) (Zhao et al. 1992). As demonstrated for vascular SMC, the process also involves downregulation of smooth muscle-specific markers, such as smoothelin, SM22, caldesmon and calponin (Yuan-jie et al., 2003, Dovendans and van Eys, 2002).

The effects of androgen deprivation on the rat prostate and coagulating glands have been investigated. Besides epithelial regression, which is mainly due to synthetic arrest and epithelial apoptosis (Brandes, 1966; Kerr and Searle, 1976), the stroma is also submitted to extensive remodelling, with changes in collagen (Müntzing, 1981, Vilamaior et al., 2000), elastic fibers (Carvalho et al., 1996, Vilamaior et al. 2003), proteoglycans (Kofoed et al., 1990, Terry and

Clark, 1996a,b), microfibrils (Carvalho et al., 1997), tenascin (Vollmer et al., 1999 and Tuxhorn et al., 2002) and basement membrane (Carvalho and Line, 1996).

The SMC express functional androgen receptors and are affected by androgen deprivation. After surgical castration, their absolute volume remains constant, but they occupy progressively greater proportion of the stroma and of the gland as a whole (Antonioli et al., 2003a). Despite the morphological alteration, adopting a spinous aspect, the SMC in the prostate of castrate rats possess SMC-specific myosin heavy chain molecules and α-actin plus smoothelin mRNA, as shown by immunocytochemistry and RT-PCR, respectively (Antonioli et al., 2003a), suggesting that, at least after 21 days of androgen-deprivation, the SMC maintain the SMC differentiated phenotype.

Researches from our laboratory have suggested that the SMC would be at least in part responsible for the reorganization of collagen fibers in the remodelling prostatic stroma (Vilamaior et al. 2000), and their ability to reorganize a collagen gel was confirmed *in vitro* (Antonioli et al., 2003b).

The objective of this paper was to evaluate the function of the SMC a little further, trying to quantify modifications in synthetic activity under androgen deprivation. To accomplish this task we have employed the zinc-iodide-osmium procedure to label synthetic organelles and secretory vesicles at the ultrastructural level in the ventral prostate of rats subjected to bilateral orchiectomy.

MATERIAL AND METHODS

Experimental Design

Three-month-old Wistar rats were employed in this study. Animals were obtained from the Central Stock-breeder of Universidade Estadual de Campinas, UNICAMP, (Campinas, SP, Brazil), maintained under controlled temperature and lighting conditions, and with free access to rodent food (Labina® ration, Purina, Agribrands do Brasil Ltda, Paulínia, SP) and water. Bilateral orchiectomy was achieved via scrotal incision under phenobarbital anesthesia. Ventral prostates were dissected out and weighed 7, 14, and 21 days after surgical castration. Age-matched rats were employed as controls. Five animals were used for each group.

Histology and Histochemistry

For light microscopy, the removed ventral prostates were immediately fixed by immersion, during 24 hours, in Karnovsky fixative, (0.1M phosphate buffer, pH 7.2, containing 5% paraformaldehyde and 5% glutaraldehyde). Fixed tissues were dehydrated in a grade series of ethanol and embedded in paraffin (Histosec, Merck) or glycol methacrylate resin (Leica historesin embedding kit). Sections were cut at 3 μm and stained with hematoxylin-eosin (Behmer et al., 1976) for general studies. Analysis were carried out using either a Zeiss Jenaval or an Olympus light microscope.

Transmission Electron Microscopy

Tissue fragments were fixed by immersion with 3% glutaraldehyde plus 0.25% tannic acid solution in Millonig´s buffer, pH 7.3, containing 0.54% glucose, during 24 hours (Cotta-Pereira et al., 1976). After washing with the same buffer, the material was post- fixed with 1% osmium tetroxide, for 1 hour, washed again, dehydrated in graded acetone series and embedded in Araldite. Ultrathin silver sections were cut using a diamond knife and contrasted with 2% alcoholic uranyl acetate (Watson, 1958) and then with 2% lead citrate in sodium hydroxide solution for 10 minutes (Venable and Coggeshaal, 1965). Grids were examined in a Leo-906 transmission electron microscope operating at 80 kV.

Zinc Iodide-Osmium (ZIO) method

Ventral prostate fragments were fixed for 12 hours with 3% glutaraldehyde solution in 0.1M phosphate buffer, pH 7.3, and incubated for 22 hours at 10^{o} C in a solution containing zinc, iode, TRIS-aminomethane, and osmium tetroxide (Reinecke and Walter, 1978). Samples were then dehydrated using a graded acetone series and embedded in Epon-812 resin at room temperature. Polymerization was performed at 60^{o} C for 48 hours. Ultra thin silver sections were observed in a Leo-906 transmission electron microscope operating at 80 kV.

Stereological evaluation

A computer assisted stereological method has been elaborated to quantify the zinc iodide-osmium tetroxide (ZIO) reactive sites in the prostatic SMC. SMC were observed and documented on negative films (SO-163 Kodak electron microscopic film). Only cells sectioned along the

equatorial plane, as determined by the presence and format of the cell nucleus were considered for further analysis. Ten these cells were documented and analyzed for each experimental time point. Negative films obtained at the transmission electron microscope were scanned and images stored with 400DPI in TIFF format. The images were then accessed using the Image Pro-Plus (Media Cybernetics) and superimposed with the Weibel-multipurpose test system M168 (Weibel, 1978). Counting the coincident points of the test system and dividing them by the total number of points within the SMC determined the relative volume of the ZIO reactive organelles.

Statistical analysis

The stereological data were evaluated by analyses of their mean ± standard error (SE). The statistical analysis was performed using the Statistica 6.0 software (StatSoft, Inc. 1984-1996). The hypothesis test Anova, Tukey HSD test and, Median test were employed and, $p \leq 0.05$ was considered statistically significant.

RESULTS

Castration resulted in marked weight loss of the VP. After 21 days of castration, the organ weight was reduced to about 10% of the control. There were marked reductions in the luminal and epithelial relative and absolute volumes. The stroma, in its turn, showed an increase in the relative volume, i.e. the stroma:epithelium ratio increased, but it also showed a reduction in absolute volume.

The SMC of the control VP are long and found as one to three cell layers in the intermediate region of the gland ductal system (Figure 1a). Androgen deprivation for 21 days resulted in marked alterations of the SMC. The cells became shorter and exhibited a spinous aspect (Figure 1b). They also showed stronger staining by eosin. At the ultrastructural level, the SMC were typical (Figure 2a), with a prominent external lamina. The cytoplasm was filled with myofilaments and punctuated by electron dense plaques and attaching to marginal dense plaques. The cells had a number of caveolae close to the plasma membrane. A few mitochondria were scattered throughout the cytoplasm. After androgen withdrawal, the SMC (Figure 2b) showed an increased concentration of organelles close to the cell nucleus, an apparent reduction in the myofilaments. Most characteristically, they presented an irregular outline, giving the cells the spinous aspect observed at the light microscope. Though the external lamina was not as well defined as in the control, it was easily recognizable in the micrographs. The irregular outline of the SMC was complimentary to collagen fiber profiles in the extracellular matrix. The SMC also showed close proximity to fibroblast processes.

After the ZIO procedure, intensely dark staining of synthetic organelles and secretory vesicles was observed. In the SMC of non-castrated rats, these organelles showed restricted distribution along the cell, with few organelles close to the cell nucleus and some secretory

vesicles at the cell periphery (Figure 3a). In the VP of castrated rats, there was an apparent increase in the number of reactive organelles. Furthermore, the perinuclear space is also reactive, contributing to the pool of ZIO reactive organelles (Figure 3b). Stereology demonstrated confirmed the apparent increase in the concentration of ZIO reactive organelles in castrated animals (Figure 4 and Table I). There was a progressive increment in the relative volume of ZIO reactive organelles, following castration. The relative volume of these organelles has duplicated after 21 days of androgen deprivation. Statistical analyses demonstrated that the increase observed after 14 days was statistically significant ($p \leq 0.05$), as compared to the 7 days period and the control.

DISCUSSION

The present results demonstrate a progressive increase in the organelles involved with secretory processes. Even though the reaction mechanism is not completely known, the ZIO technique labels rough endoplasmic reticulum cysternae, Golgi complex, secretory vesicles and, in some instances, the perinuclear space. We believe that the relative volume of these organelles is a good measure of the cellular investment in the secretory process.

In this sense, our results clearly demonstrate a progressive involvement of the SMC in secretion, following castration. After 14 days of androgen-deprivation, the relative volume of ZIO-reactive is significantly greater than in control.

These data confirm previous findings of Zhao et al. (1992) with respect to the morphological changes, but contrast with their stereology to determine the relative volume of individual organelles on ordinary transmission electron micrographs, and found only slight increase in the rough endoplasmic reticulum relative volume after either estradiol treatment or castration. The authors conclude that, in spite of the drastic morphological changes associated with SMC atrophy, there was no activation of these cells to a synthetic phenotype, even though they suggest an involvement of SMC on the synthesis of extracellular matrix components.

Horsfall et al. (1994) have studied the aging lateral prostate of the Guinea pig and human prostate bearing hyperplasic lesions and reported an increase of synthetic apparatus of the SMC. The authors also suggest that this increase is associated with the accumulation of extracellular matrix observed in aged individuals.

In a previous work on the modifications of the collagen fibers of the rat ventral prostate in response to bilateral orchiectomy, we have observed an intimate contact between these fibers and the SMC (Vilamaior et al. 2000). We have then suggested that SMC are actively involved in the

reorganization of the fibrillar components of the prostatic stroma in the absence of androgens. Later, we have demonstrated the ability of prostatic SMC to contract type I collagen gels and this was modulated by insulin and testosterone (Antonioli et al. 2003b), demonstrating that SMC can take part in the stromal remodelling and that this function is androgen-regulated.

It has been postulated that in adenocarcinomas, the SMC take part in the phenomenon of "reactive stroma" (Tuxhorn et al. 2001). This term refers to the stromal modifications in response to abnormal epithelial proliferation and invasion. The authors describe that the SMC undergo marked morphological changes, including a decrease in the cytoskeletal components besides an increase in the fibroblasts in the area, suggestive that SMC encompass a dedifferentiation process. However, no study using the expression of currently accepted SMC differentiation markers (Dovendans and van Eys, 2002) has been performed in this system.

We have previously employed immunocytochemistry and RT-PCR and defined that the ventral prostate SMC express SMC specific myosin heavy chain and α-actin and the mRNA for smoothelin, even after 21 of androgen deprivation by bilateral orchiectomy. This allowed us to suggest that the SMC maintain the differentiated phenotype in the absence of androgen stimulation, within the experimental time course (Antonioli et al., 2004). These data are in apparent contrast with a previous report by Hayward et al. (1997), who showed the disappearance of SMC markers after long-term androgen deprivation and concluded that SMC dedifferentiate in the regressing prostate. However, they used different markers.

To conclude we suggest that the SMC in the rat ventral prostate is able to shift from a predominantly contractile to a more synthetic phenotype without changing its differentiation state, with respect to specific cytoskeletal markers. This assumption is reinforced by the fact that ventral prostate SMC from control and castrated animals are able to contract under adrenergic

stimulation as well as respond to adrenergic antagonists (Homma et al., 2000). Finally, it is probable that at least part of the secretory material produced by the SMC corresponds to extracellular matrix components.

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Legend of Figures

Figure 1: Hematoxylin-eosin stained semi-thin section of rat ventral prostate. **(a)** Morphological aspect of the SMC in the control prostate (arrow at left). Arrow at right= fibroblast. **(b)** Spinous-like aspect of prostatic SMC in the 21-castred animal (arrow). 1250x.

Figure 2: Ultrastructural aspect of the SMC at conventional transmission electron microscopy. Partial view of SMC of the control **(a)** and 21-castrated group **(b)**. The androgen withdraw promotes the increase of the endoplasmic reticulum (rer) Golgi complex (G) and mitochondria (mi). n = nucleus; arrows= basal membrane; co = collagen. 12000x.

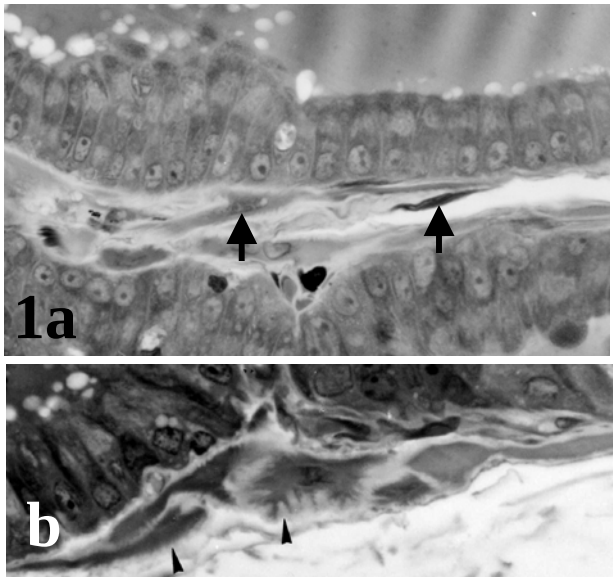
Figure 3: Ultrastructural aspects of the SMC after ZIO procedure. **(a)** Few organelles ZIO positive are observed in the control prostatic SMC (arrows). **(b)** Detail in the Golgi enlarged region (G). **(c)** An increase of synthesis organelles is observed in the prostatic SMC of 21-castrated animals. The inset shows the positive marcation in the Golgi apparatus. n = nucleus. a,c = 12000x; b = 20000x.

Figure 4: Frequency histogram of quantification of the ZIO reactive organelles relative volume in the prostatic SMC at the control and castrated rats.

Table I: Relative volume values of ZIO reactive organelles in the smooth muscle cells of the ventral prostate in the control and castrated groups. Standard Deviation (SD); Standard Error (SE).

	Control group	*7 days castrated group*	*14 days castrated group*	*21 days castrated group*
Mean	13.0[a]	19.4[a]	23.6[b]	26.8[b]
SD	5.6	4.3	7.8	7.6
SE	2.1	1.9	3.5	3.4
p		*0.35178*	*0.0503**	*0.0088*

[a,b] = different indeces indicate statistical differences inter-group. *Decision based on additional analysis using Median test at $p<0,05$.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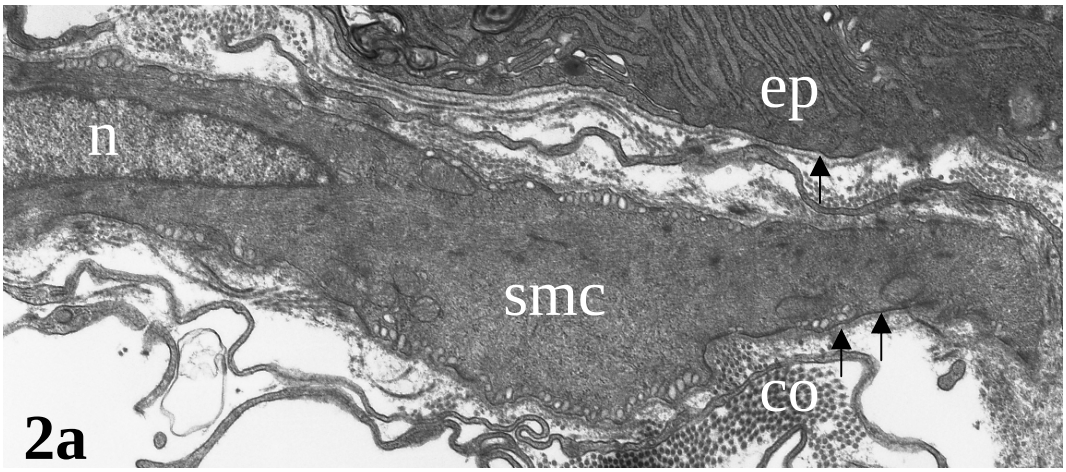
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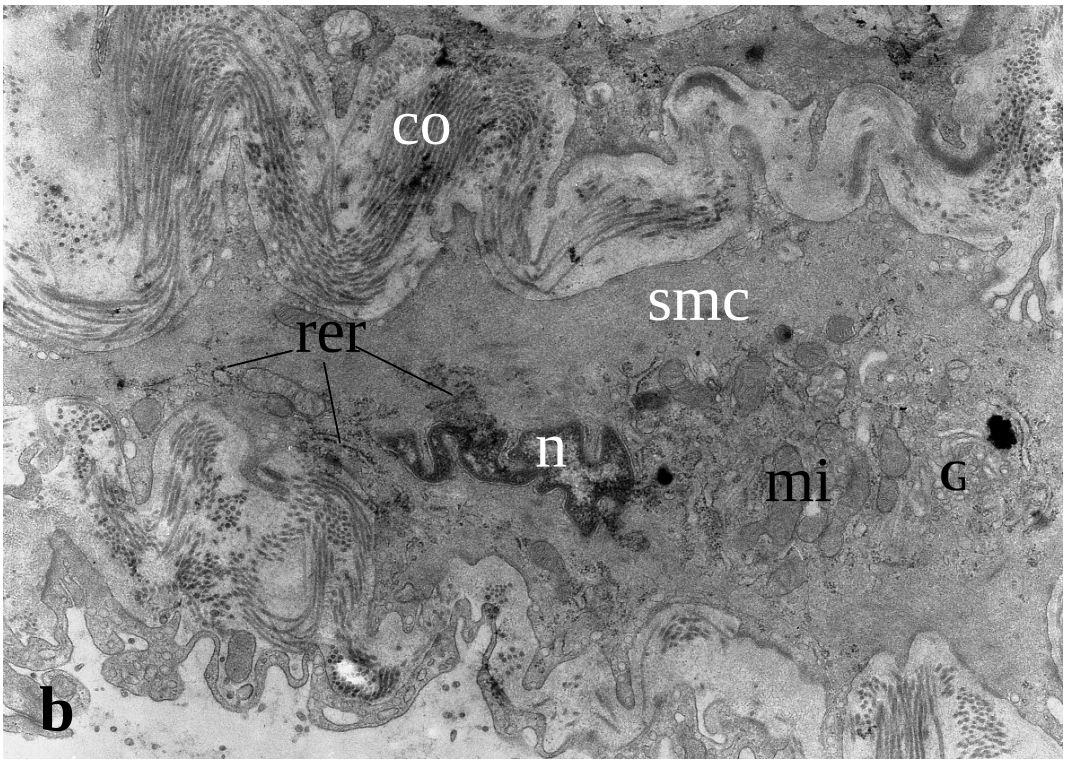
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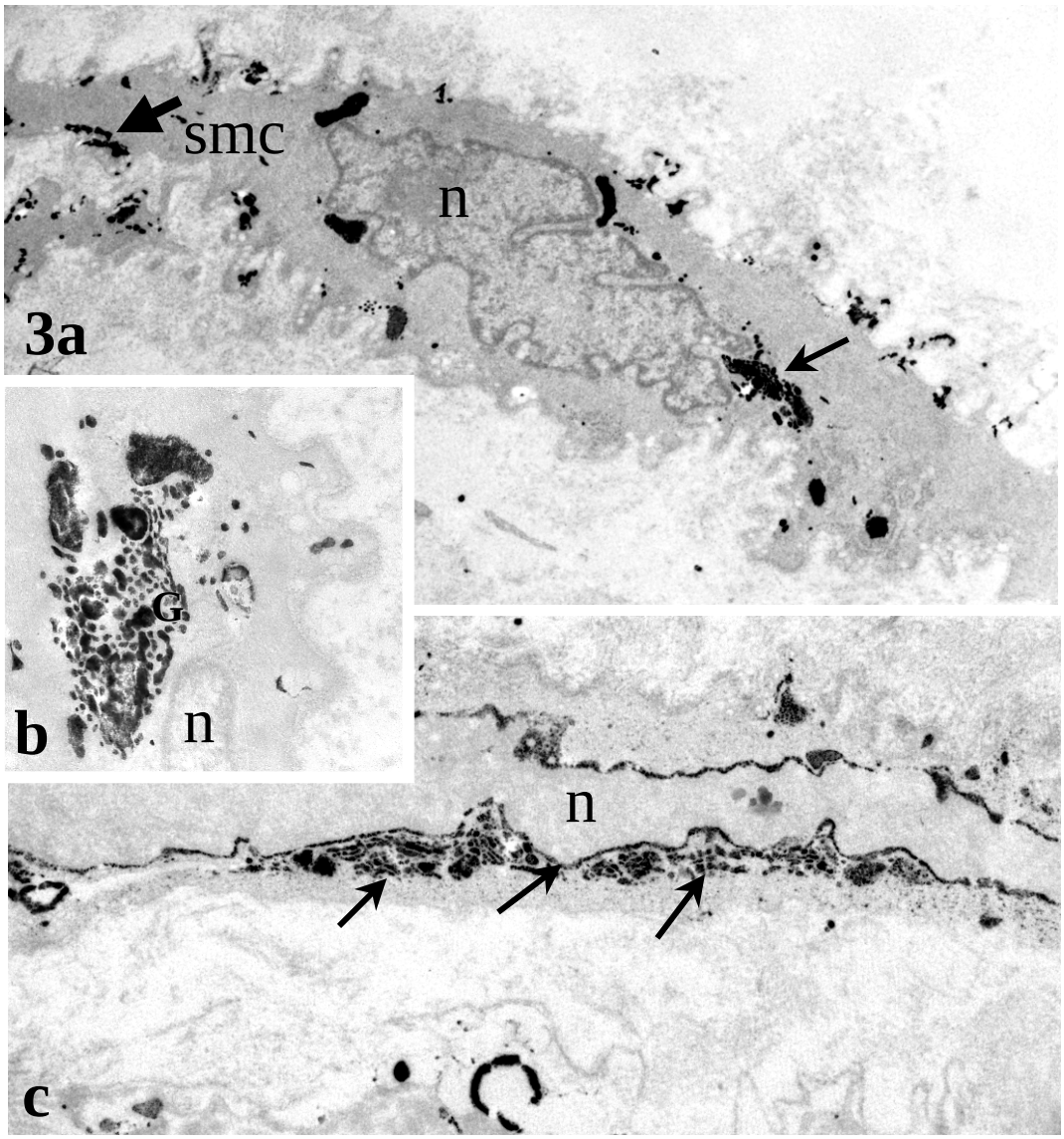
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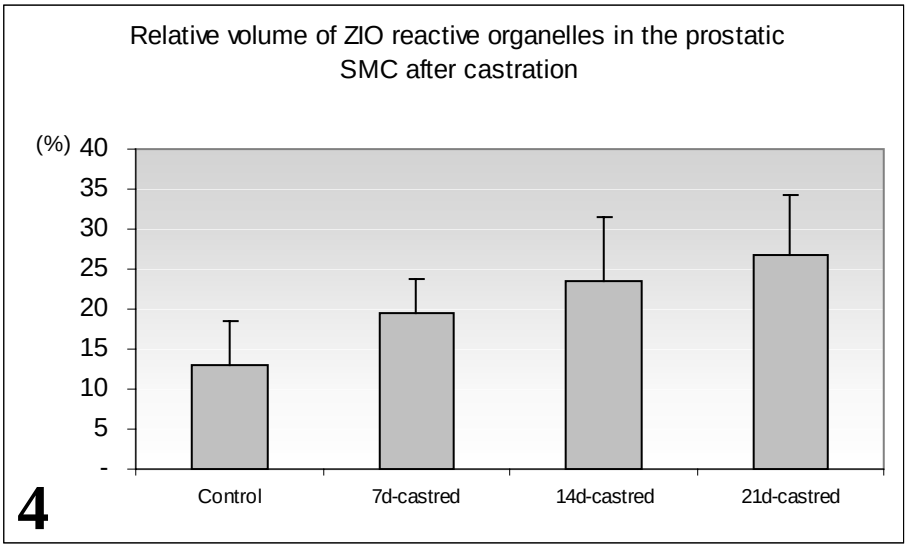
Relative volume of ZIO reactive organelles in the prostatic SMC after castration
(%)
40
35
30
25
20
15
10
5
-
Control
7d-castred
14d-castred
21d-castred
4

Alternating proliferative and secretory activities contribute to the post-natal growth of the rat ventral prostate

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Running Title: Proliferation and secretion during the rat ventral prostate post-natal growth.

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Abstract

Using morphological and stereological analyses we have analysed the post natal development of the rat ventral prostate, trying characterize the behavior of the different tissue compartments. Animal and prostate growth as measured by their weight was paralleled to the variations in the serum testosterone levels along the first 12 post natal weeks. The relative and absolute volume of the epithelium, glandular lumen, smooth muscle cells (SMC) and non-muscular stroma were determined by stereology. Epithelial height was measured using image analysis. The rat ventral prostate (VP) shows a progressive growth after birth. The relative weight of the organ demonstrate an initial growth within the first three weeks, a resting pre-pubertal period, in which growth only reflects overal body weight increase. At the seventh week the VP starts to grow steadly up to the twelfh week. This second growth phase coincides with begining of the testosterone concentration in the serum. The relative volume analysis clearly identified the luminal compartment formation within the three post natal weeks, an increase during puberty and a final raise at the 12^{th} week. It also showed a decrease in the relative volume of the non-muscular stroma and of the SMC with growth. Absolute volume of the luminal compartiment showed three phases of growth (1-3; 6-9 and $11\text{-}12^{th}$ weeks). On the other hand, the increase in the absolute volume of the epithelium was steady up to the 8^{th} week and then showed a marked increase up the 10^{th} week, being observed morphologically as an epithelial infolding and budding, and decreased slightly thereafter. The epithelial reorganization is secondary to an increase in the mitotic index initiated at the 6^{th} week. The epithelium showed a two week delay as compared to the lumen, after the pubertal testosterone accumulation, increasing only up to the 10^{th} week. The epithelial height was variable along the phases of epithelial growth, being intimately related to the synthetic activity of the epithelium. The results suggest the post-natal

growth of the prostatic gland alternate between phases of epithelial proliferation and synthesis/accumulation of the secretory products in the lumen. Additionally, there is a characteristic array of the reticulin fibers in association with the epithelial buds, indicating an important role of the stromal cells in adapting the estroma to the epithelial growth.

Key words: rat ventral prostate; stereology; epithelial growth; epithelium-stroma interaction.

Introduction

Prostatic development is not determined by the genetic sex, but by exposure to androgens. It has been demonstrated that the urogenital sinus of female and males may form functional prostatic tissue if it is properly stimulated by androgen at the correct embryonic stage (Takeda et al., 1986). So, prostatic morphogenesis is dependent on the androgen production by the testis in the fetus (Pointis et al., 1980; Cunha et al., 1987).

Epithelial budding is initiated by the pre-natal androgen action at about the 17.5 day *post coitum* (Timms et al. 1994). The subsequent ductal morphogenesis, canalization and epithelial differentiation also depends on androgens and take place in association with a trasient increase in the serum testosterone concentration (Donjacour et al., 1988). Even though testosterone is the androgen produced by the fetal testis, diidrotestosterone is responsible for the prostatic morphogenesis (Taplin & Ho, 2001). Diidrotestosterone is produced by the urogenital sinus by the enzyme 5- reductase. This enzyme has been detected in the urogenital sinus and external genitalia of rats, rabbits and humans (Wilson et al. 1983) and its deficiency results in the anomalous growth of the external genitalia and complete absence of the prostate.

The epithelial differentiation in the prostate takes place in parallel to the development of the stroma. Androgens act upon androgen receptors in the urogenital mesenchyme to induce epithelial proliferation, ductal branching and differentiation of the epithelial cells (Cunha et al., 1987; 1992). In its turn, the developing epithelium directs the differentiation of the smooth muscle (Hayaward et al., 1998). Neither epithelium nor smooth muscle are capable to develop in the absence of each other (Hayward & Cunha, 2000). During organogenesis, one of the functions of the androgens

is to reduce the smooth muscle that surrounds the urethra (Thomson et al., 2002). If the smooth muscle layer is thick, given the absence of androgen stimulation, as it occurs in the females, the initial budding from the urethra cannot reach the urogenital sinus mesenchyme and there is no development of the prostate gland (Thomson et al., 2002).

The neonatal prostate is sensible to androgens. Testosterone administration accelerates prostatic growth, and adult size may be reached much earlier (Berry & Issacs, 1984), whereas neonatal castration inhibits pubertal prostatic growth and development, and this effect may be reversed by testosterone administration (Cunha et al., 1987; Corbier et al., 1995). During puberty, there is an additional increase in the prostatic weight and a slight increase in the number of ductal branching (Sugimura et al., 1986). This suggests that the prostate in sensitive to the low levels of androgens for the ductal branching and that the response to the increasing levels of testosterone during puberty is different from the initial response (Hayward & Cunha, 2000).

A good correlation between the invasive properties of tumor cells and the normal growth of epithelial structures was established for the mammary gland (Wiseman & Werb, 2002) e for lung (Kheradmand et al., 2002), at least with regard to the increased proliferation of the epithelium, the need of extracellular matrix remodelling and colonization by the epithelium of spaces previously occupied by the stroma. This picture seems to be true also during angiogenesis (Feng et al., 1999), which involves dissolution of the basement membrane and its substitution by a provisory fibrin matrix, used by the endothelial cells to invade the adjacent areas.

As mentioned earlier, epithelium-stroma interactions are important factors in prostatic development. However, a detailed study of the post natal growth considering

the different tissue compartments is still lacking in rat ventral prostate, while it has been extensively studied in the mouse (Singh et al., 1999).

Furthermore, the prostate has been demonstrated to be adequate for the testing of environmental risk assessement, given its extreme dependency on androgen stimulation and high susceptibility to estrogenic stimulation, via an imprinting mechanism, with an yet unkown molecular players and strain specific behavior (Prins et al. 2001; Putz et al. 2001a, 2001b).

Given the usefulness of rat ventral prostate in enviromental risk assessment, the demonstration of strain specific variations, the sensibility of prostatic development and function to androgen stimulation and disturbance by steroid compounds and the importance of epithelial-stromal interaction in this organ, we have found reasonable to undertake a detailed analysis of the prostatic development in Wistar rats, trying to define morphological and stereological parameters for the main tissue compartments, as well as defining the immediate changes in the extracellular matrix, from the first post-natal week to adulthood. Pursuing this task we have employed a series of analyses, which demonstrated the differential kinetics of the prostatic compartments in post-natal life.

Material and methods

Animal protocol

Sixty male Wistar rats obtained from CEMIB/UNICAMP were used. Five animals were used for each time point (Weeks 1-12). They were maintained in a controlled environment with free access to food and water. Experiments were performed

according to the *Guide for Care and Use of Laboratory Animals*. Rats were weighed before sacrifice and sacrificed by cervical dislocation within 15 s after being removed from their cages. Ventral prostate was carefully dissected out and weighed.

Serum testosterone quantification

Blood samples were collected of all animals either after decaptation or cardiac punction. Serum testosterone was quantified using a modular chemiluminescence immunoassay analyzer ECi (Johnson & Johnson), accordingly with Weeks and Woodhead (1984). Triplicate measurements were performed for each sample and serum samples from three animals were used for each time point.

Histology and cytochemistry

The ventral prostate was dissected out and immediately fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 hr. The samples were then washed, partially dehydrated, and embedded in Leica historesin. Two-micrometer sections were obtained and stained with hematoxylin and eosin or subjected to the Gömöri`s silver impregnation technique for the identification of collagen and reticular fibers (Behmer et al. 1976; Taboga and Vidal, 2003). After silver impregnation, reticulin fibers (mostly type III collagen) appear black, while collagen fibers (mostly type I collagen) appear gold (Taboga and Vidal, 2003).

Stereological analyses and morphometry

Six microscope fields from the hematoxylin and eosin stained sections, from three animals for each group were photographed and stereology carried out using Weibel's system and a 168-point grid (Weibel, 1963), using the same parameters as Huttunen et al. (1981). Microscopical fields representing predominantly proximal or distal ductal regions were avoided, given the predominance of muscular and epithelial compartments in each of these regions (Lee et al., 1990). The relative volumes of the epithelium, lumen, smooth muscle and non-muscular stroma were determined. The total stroma was the sum of the smooth muscle and non-muscular stroma. For the calculation of the total volume of each of these compartments, we considered the mean prostatic weight measured for each group.

The epithelium height was measured from hematoxylin and eosin stained sections using the Image Pro Plus software ©Media Cybernetics, after digitalization of the microscopical images. Calibration was based on measurements taken from an Olympus graded microscopical slide. Seventy-five measurements were done for each experimental point, avoiding to measure the epithelial cells of the ductal distal region.

Mitotic cell frequency

Historesin sections were subjected to Feulgen's reaction. They were hydrolyzed with 4N HCl for 1h15 and then reacted with Schiff's reagent for 40 min. After extensive washing, the sections were dehydrated and mounted in Canada's balsam (Márquez et al.,

2001). The number of mitotic cells was determined by counting mitotic figures per microscopical field. Forty fields were taken at random and the mitotic figures were counted (15 fields per animal; three animals).

Statistical Analyses

The stereological data were evaluated by analyses of their mean ± standard error (SE). The statistical analysis was performed using the Statistica 6.0 software (StatSoft, Inc. 1984-1996). The hypothesis test Anova and Tukey HSD test were employed and $p \leq 0.05$ was considered statistically significant.

Results

Body weight and testosterone levels

There was a continuous body weight growth in the rats employed in this work (Fig. 1). This growth continued from the first to the 9th week and reached a plateau at about 250g up to the 12th week. The serum testosterone concentration was low (~0.75ng/mL) until the fifth week, raised to 1.5-2.0 ng/mL from the 6th to the 8th week and then reached the adult level (~3.5ng/mL) at the 9th week (Fig. 2). It was observed a greater variation in the serum testosterone level in the early adult life, as demonstrated by oscillation in the mean concentration, as well as by the greater standard error of the mean (SEM).

Ventral prostate growth and histological modifications

The ventral prostate showed an initial post-natal growth, a resting period from the 4th to the 6th week and a pubertal growth up to the adult size (Fig. 3). The resting period observed in the relative weight was not so evident when the absolute weight of the gland was taken into account (Fig. 3, inset).

The histological analysis showed a marked changes in histological organization, from the first to the second week and from the 7th to the 8th weeks (Fig. 4). The first transition in histological organization was characterized by the formation of the lumen, which is filled with secretory material and a reduction in the stromal area and cellular density (Figs. 4a, b), while the second transition is characterized by the enlargement of lumen (Figs. 4g, h). There was also a noticed increase in epithelial budding at the 7th, 8th and 9th weeks (Figs. 4g-i). Besides budding, one could also observe and increase in the epithelial folding in this period. Both epithelial budding and folding disappeared in later stages (Figs. 4 k,l).

The epithelial changes involve a differentiation step from the first to second week and slight variations thereafter (Fig. 5). The Golgi complex area (faintly stained supranuclear area) is clearly observed at the third week (Fig. 5c). Variations of the epithelial height were noticed and quantified. The measurements of epithelial height are shown in Figure 8. After a progressive increase up to the 6th week, there was a marked drop in the mean epithelial height and then a return to the maximum epithelial height.

The determination of the relative volumes of the different prostatic compartments (Fig. 6) confirmed some of the histological observations. The formation

of the lumen takes place within the first three week and the lumen becomes the predominant tissue compartment in the prostate, thereafter. The proportion of the lumen to the other compartments is maintained up to the 8th week and then shows a further increase, reaching as much as 70% of the prostatic volume. The stromal volume decreases from the first to the second week, remains constant up to the 8th week and then decreases again, from the 8th to the 9th week. The smooth muscle cells behave similarly. The second fall in the relative volume of the stroma balances and increase in the epithelial volume, which takes place after the 8th week.

The analysis of the absolute volume variation of the different compartment clearly shows the contribution of both epithelium and lumen to the growth of the prostatic gland (Fig. 7 and Table I). After the formation of the lumen within the first three weeks, there was a prominent growth of this compartment after the 6th week. This is then followed by an increase in the epithelial compartment, which is observed after the 8th week. Both compartments plateau at the 9th and 10th weeks, respectively, but the lumen showed a further increase at the 12th week. The smooth muscle cells also showed a contribution to the overall increase in prostatic volume, with a pattern very similar to the epithelium, but anticipating it, showing a pubertal phase beginning at the 6th week.

Cell proliferation

The frequency of mitotic cells was also evaluated. The mean of mitotic cells per microscopical field showed an initial peak at the second post-natal week and second one at the 6th week. This second peak showed a slow decline up to the 10th week. Mitotic cells were almost absent at the 5th, 11th and 12th (Figure 9).

Collagen fiber reorganization

The epithelial growth at the first post-natal week results from budding of the epithelial structures. The silver impregnation technique allowed the observation of a well organized basket of reticulin fibers in association with the protruding epithelial cells (Fig. 10a-d). Since silver impregnation also stains the cell nucleus, the cells inside this "reticulin basket" could be simultaneously observed.

From the 3rd to the 6th week, the reticulin fibers established a thin, continuous layer in intimate contact with the base of the epithelial structures. Collagen fibers were concentrated at the interlobular spaces (Fig. 10 e, f). At the seventh week, when epithelial budding is again occurring, reticulin fibers showed the same basket-like association with the protruding epithelial cells (Figs. 10g-h). Reticulin fibers were also delimitating the small epithelial structures, developing from the development of these buds. At the 12th week, such structures were rare, but still found (Fig. 10i).

Discussion

This paper presents a detailed analysis of the post-natal growth of the rat ventral prostate. It has been demonstrated that the VP grows progressively after birth. Most of the prostatic growth in rodents occurs after birth so they are adequate for studies of occupational exposition to a series of steroidal compounds. In this sense, the adequate characterization of prostatic development seems necessary, specially to avoid strain-specific variations (Putz et al. 2001a). Furthermore, the post-natal growth is complex,

and the characterization of growth kinetics for the different compartments is believed to contribute to the knowledge on prostatic physiology.

After the early post-natal mass increase, a resting period is observed between the 4^{th} and 6^{th}, in which the growth follows the general body growth, and precedes the pubertal growth initiated at the 7^{th} week. A marked phenomenon, taking place within the first three weeks, is the formation of the lumen, which then become the predominant tissue compartment of the organ. This aspect, as well as the proliferative response and branching of the epithelium, are thought to be induced by the serum testosterone peak, taking place immediately after birth (Corbier et al., 1995). It is interesting to note that the peak of prostate growth at the third week occurs one week after the observed peak of cell proliferation, demonstrating that cell growth and lumen enlargement contribute to the increase in weight. The epithelial proliferation within the first three weeks has been reported before (Weihua et al. 2002).

The epithelium and non-muscular stroma occupies approximately the same relative volume in the organ up to the 8^{th} week. Thereafter, there is a decrease in the stroma, which is compensated by an equivalent increase in the epithelium. These two episodes correspond to two steps in glandular morphogenesis and a progressive involvement with secretory activity. The variation in the absolute volume confirms these findings. It also illustrates that the VP shows a two-phase response to the increasingly testosterone levels during puberty. First, there is a clear activation of the secretory activity, manifested by the increase in the absolute volume of the lumen, and then a delayed increase in the epithelial compartment, which begins two weeks later. This may reflect either a sequence of events associated with progressive synthetic activity or two independent responses, triggered at different serum testosterone levels. As a third

possibility, it may be considered that the increase in epithelial volume is initiated at the 6th by the proliferative response of the epithelial cells, and only observed two weeks later, after the proper growth and differentiation of the epithelial cells. This is suggested by a peak of mitotic activity in the epithelial at the 6th week, coincident with the raise in testosterone concentration. This peak is secondary to a higher peak observed at the second week. The pattern of mitotic activity demonstrates that the two main phases of prostatic growth results, at least in part, from epithelial cell proliferation. The occurrence of mitotic cells correlates well with previous findings on the distribution of ^{3}H-thymidine incorporation (Banerjee et al., 1991), which detected an increase in proliferating cells at about the 6th week (45 days) in the intermediate region, but not in the distal region of Sprague-Dawley rats, even though we have not distinguished among the ductal regions.

It seems that the accumulation of secretion in the lumen, results in a marked predominance of this compartment at the 12th week, which in turn results in a decrease of the epithelial compartment, perhaps by a negative effect on the synthetic activity. However, there was not any variation in the epithelial height, from the 9th to the 12th week. This increase in the lumen volume is associated with the disappearance of the epithelial infoldings as well as the growth of the buds observed at the seventh week.

It is apparent from the present results, that prostatic growth is characterized by a two- phase phenomenon. First, there is a proliferative response with the formation of epithelial infolding and/or budding. Second, there is a marked increase in the accumulation of secretion in the lumen, due to the stimulation of the synthetic activity. This biphasic growth pattern was also observed for the female Mongolian gerbil prostate after experimental testosterone administration (Santos et al., 2003).

However, the present results suggest that the pubertal growth of the ventral prostate of male rats is characterized by a secretory-proliferative-secretory response of the epithelium to the raising serum testosterone concentration.

Testosterone is then setting the growth response of the organ. Exogenous testosterone is able to anticipate prostatic growth, however, this responsiveness is lost with aging (Banerjee et al. 1994).

It is worth mentioning that the absolute volume of the smooth muscle cells also increases during puberty and decreases again after the 8^{th} week. We cannot ascertain at the moment whether this variation reflects a variation in the number of the smooth muscle cells, the hypertrophy of preexisting cell or a combination of both. The definition of the factors involved in this increase in the smooth muscle cell absolute volume is certainly important for the characterization of the VP physiology.

One important observation was the budding of the epithelial structures. This is important because it reflects the sites of epithelial growth and occupation of the stroma, notably after the 7^{th} week, and contributes to the decrease in the stromal volume, whereas the epithelial volume is increasing. Silver impregnation allowed us to observe the modifications of the reticulin fibers in association with these epithelial buds. Contrary to our expectations, there seems not to exist an ongoing process of reticulin fiber degradation. Instead, we observed the formation of a reticulin fiber basket around the protruding epithelium. However, signs of collagen degradation could be observed a little deeper in the extracellular matrix adjacent to the budding sites, and mostly associated with collagen fibers. These extracellular matrix alterations are expected to result from stromal cell activities, then pointing to the existence of specific epithelial-stromal with the possible involvement of matrix metalloproteinases, as observed in the

mammary gland (Wiseman & Werb, 2002) and usual in the initial morphogenesis of the prostate (Marker et al. 2002).

Figure 11 summarizes the main events of the post-natal development of the rat ventral prostate.

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Legends of figures

Figure 1: Weekly weights of body in normal rats, from 1 to 12 weeks of age. Values are represented as grams (means±SEM values).

Figure 2: Means±SEM values of serum testosterone concentration (ng/mL) distribution during post natal weeks of development.

Figure 3: Weekly absolute and relative weights (means±SEM values) of rat ventral prostate from 1 to 12 week of post natal development. Organ absolute weights are represented as grams (inset) and, relative weights are represented as grams per gram of body weight.

Figure 4: Ligth microscopical aspects of hematoxylin-eosin stained sections of rat ventral prostate from 1 to 12 week of post natal development (**a-l** respectively). (*) new little alveoli; (arrow) epithelial budding. Scale bar represents 50 μm.

Figure 5: Ligth microscopical aspects of hematoxylin-eosin stained sections of rat ventral prostate from 1 to 12 week of post natal development (**a-l** respectively). Detailed wiew of the epithelial region. (*) apical Golgi region; (arrow) mitosis. Scale bar represents 5 μm.

Figure 6: Graphic plot of means±SEM values of rat ventral prostate compartments (epithelium, lumen, stroma and, smooth muscle cells) relative volume (%) obtained from 1 to 12 week of post natal development.

Figure 7: Graphic plot of means±SEM values of rat ventral prostate compartments (epithelium, lumen, stroma and, smooth muscle cells) absolute volume (x100 ml) obtained from 1 to 12 week of post natal development (**a**). Figures **b** and **c** show expended views of the values obtained for epithelium and smooth muscle cells volumes.

Figure 8: Weekly morphometric epithelium height (means±SEM values) of rat ventral prostate from 1 to 12 week of post natal development. Morphometrical data are represented as micrometer.

Figure 9: Mitotic figures per microscopical field of ventral prostate from 1 to 12 week of post natal development (means±SEM values).

Figure 10: Ligth microscopical aspects of Gömöri's method stained sections of rat ventral prostate. The collagen and reticular fibers distribution are observed during the post natal development. **a-d**: Cross section of the prostatic alveolus at differents foccal planes during 1st week. The images show the lateral buddig with reticulin basket associated (arrows). Similar figures are observed from the 7th and 12th weeks (**h-i**) of rat prostatic post natal development (arrows). In the 3th and 6th weeks this images are observed in minor frequence (**e-f**). Other morphological indicative of epithelial growth are the presence of little alveoli observed associated with the major secretory structures (**g)**. Detailed view of the epithelial region. (*) new alveoli in formation. Figs. a-d, h,i = scale bar represents 5 μm; Figs. e and g = scale bar represents 50μm; Fig. f = scale bar represents 25μm.

Figure 11: Schematic diagram of the selected events detected during the 12 weeks of rat ventral prostate development.

Table I: Absolute volume of the different compartments of the rat ventral prostate during the post natal development.

Post natal age (weeks)	*Epithelium*	*Lumen*	*Stroma*	*Smooth Muscle Cell*
1	0.13±0.01	0.02±0.003	0.18±0.01	0.07±0.004
2	0.74±0.07	0.59±0.07	0.44±0.05	0.13±0.01
3	1.16±0.14	1.89±0.21	0.89±0.13	0.26±0.03
4	0.98±0.01	1.57±0.19	0.67±0.15	0.29±0.03
5	1.42±0.14	1.72±0.20	1.27±0.15	0.29±0.03
6	1.75±0.14	2.58±0.30	1.33±0.22	0.43±0.06
7	2.25±0.21	4.41±0.36[a]	1.96±0.30[a]	0.68±0.07[a]
8	2.77±0.34[a]	6.51±0.85[b]	3.47±0.67[a,e]	0.85±0.12[a,e]
9	5.15±0.74[b]	11.16±0.96[d,e]	1.86±0.51[b,d]	0.63±0.11[d]
10	8.45±1.15[b,c]	11.03±1.44[d,e]	1.73±0.43[b]	0.65±0.11[c]
11	6.70±0.52[b]	9.11±0.84[c,e]	2.40±0.37[b,c]	0.49±0.08[b]
12	6.16±0.83[b]	18.57±1.18[*]	1.87±0.14[b,d]	0.70±0.14[a]

[a,b,c,d,e,] = different indices idicate statistically differences to $p \leq 0.05$;

* = significative differences in respect all ages ($p \leq 0.05$)

Figure 1

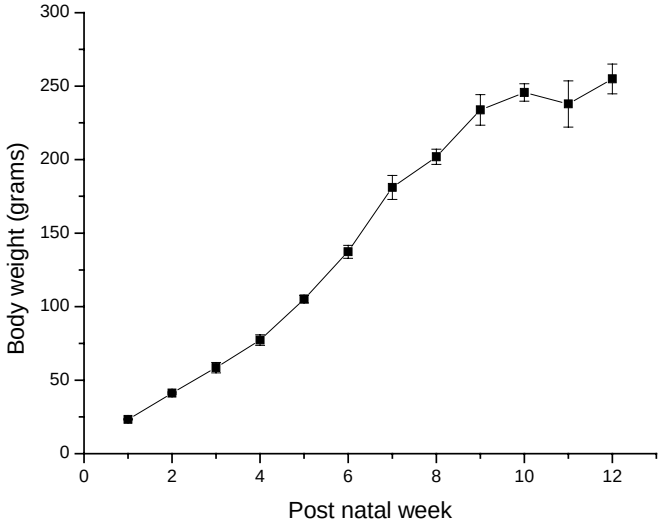


Figure 2

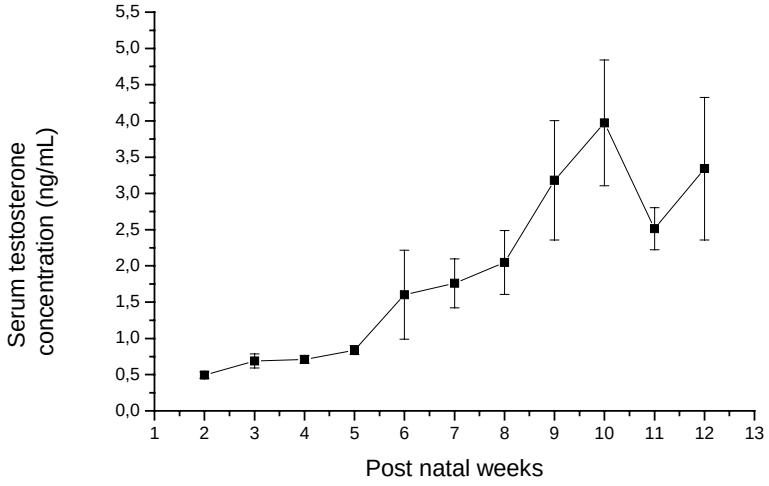
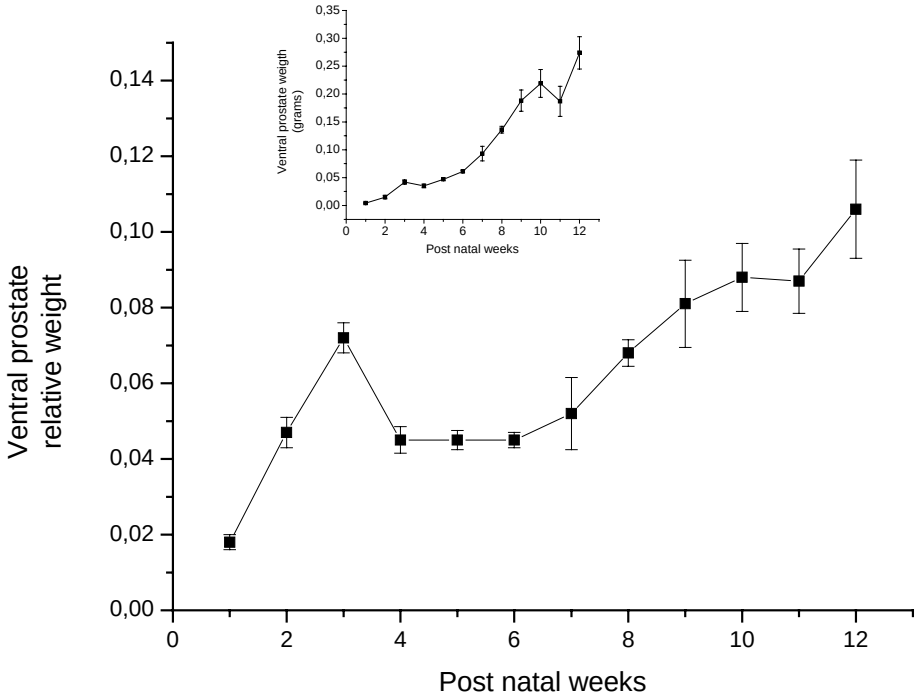
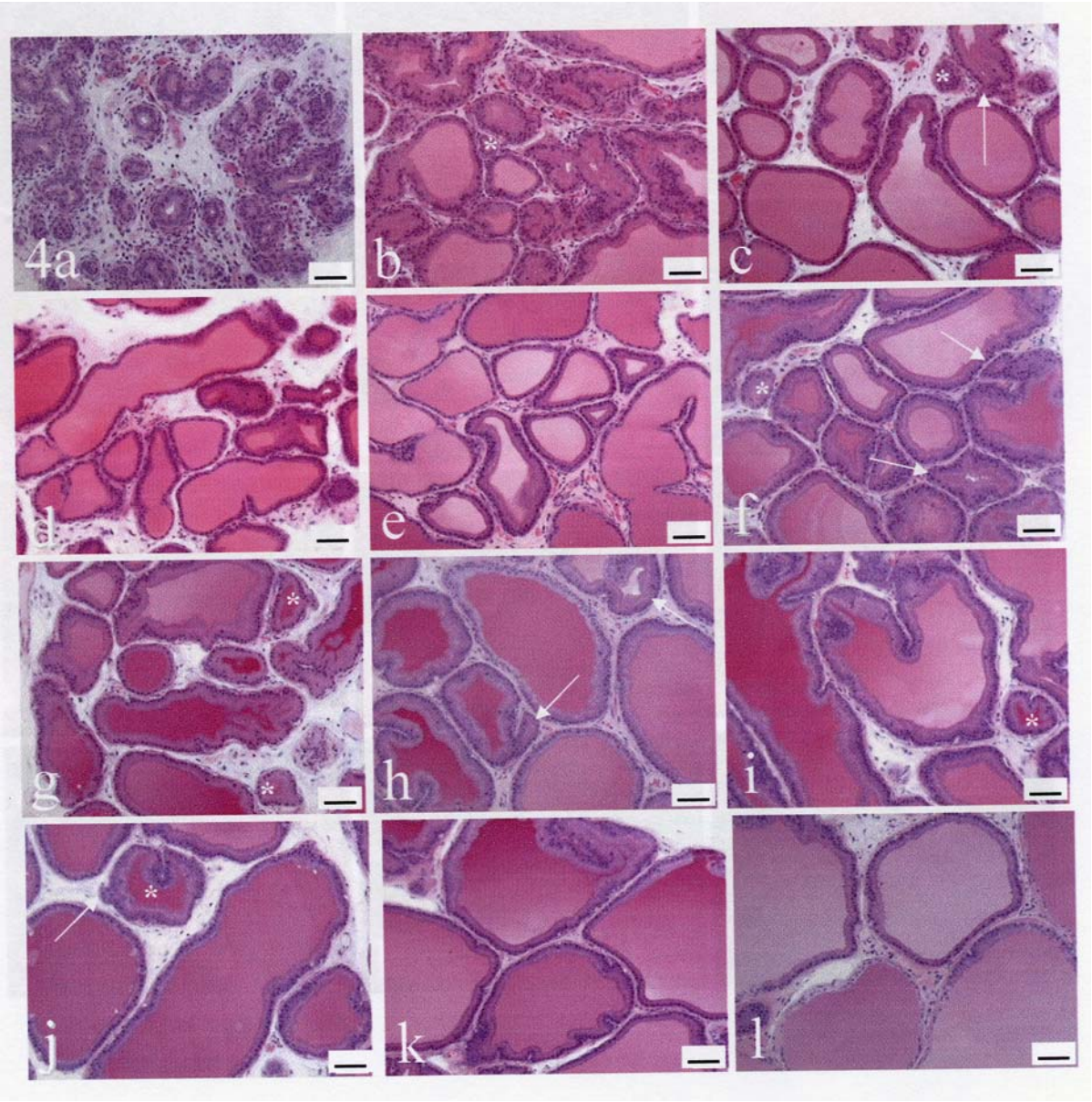


Figure 3


Ventral prostate relative weight
0,00
0,02
0,04
0,06
0,08
0,10
0,12
0,14
0
2
4
6
8
10
12
Post natal weeks
Ventral prostate weigth (grams)
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0,35
0
2
4
6
8
10
12
Post natal weeks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b
c
d
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l

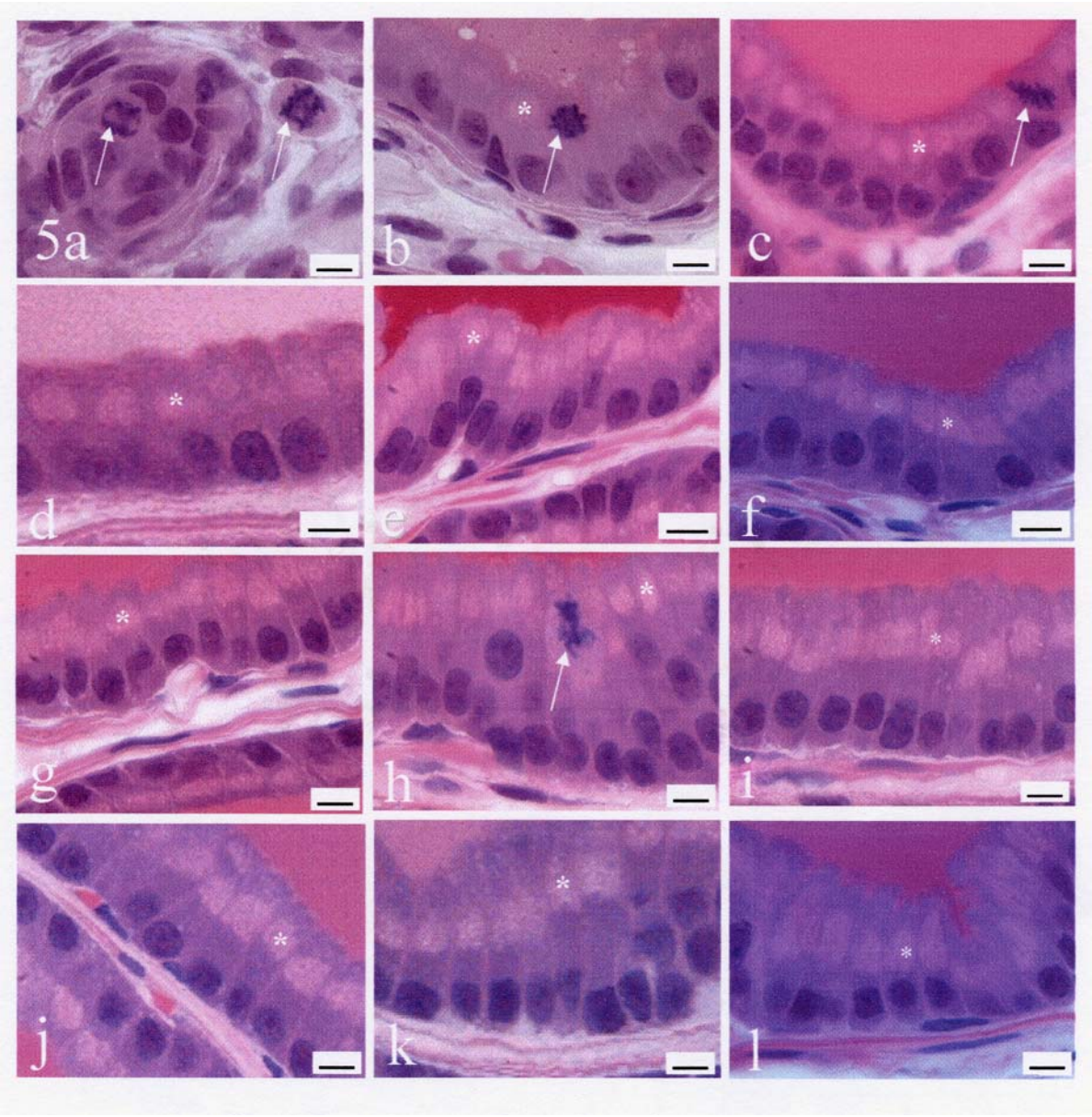
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Figure 6

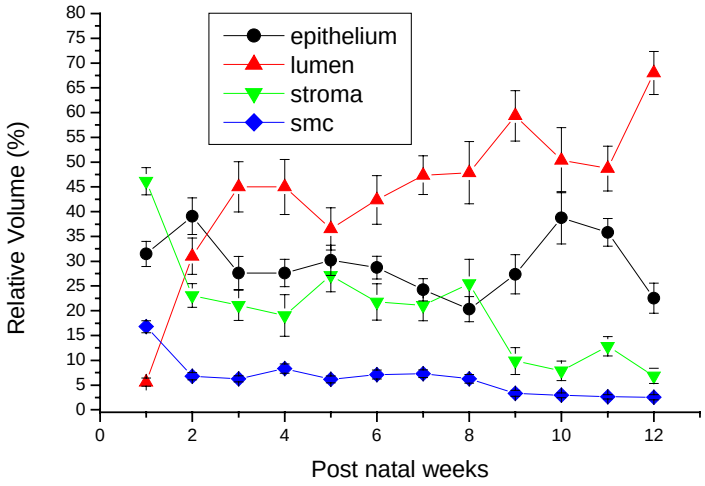
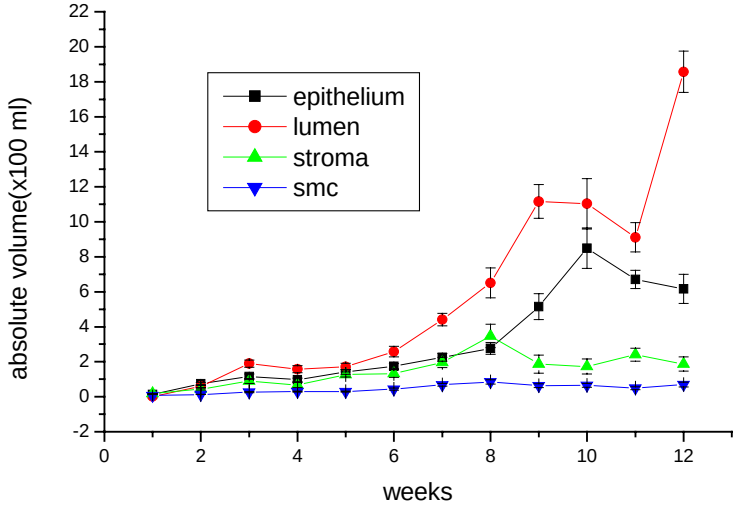
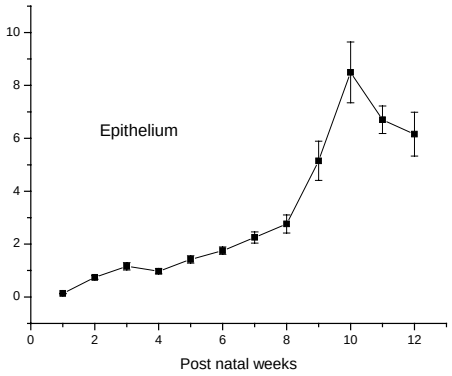

epithelium
lumen
stroma
smc
Relative Volume (%)
Post natal weeks
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
0
2
4
6
8
10
12

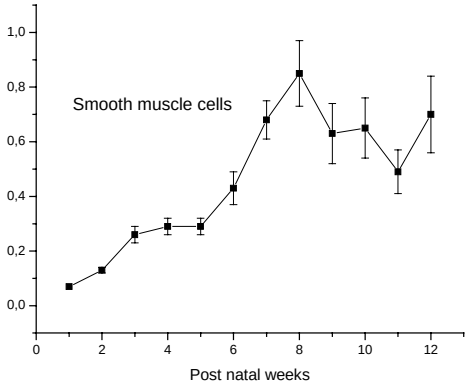
Figure 7


absolute volume(x100 ml)
weeks
epithelium
lumen
stroma
smc

a


Epithelium
Post natal weeks

b


Smooth muscle cells
Post natal weeks

c

Figure 8

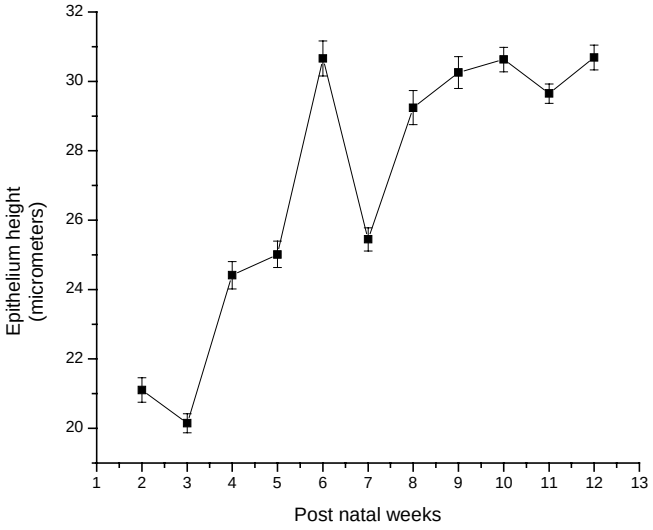
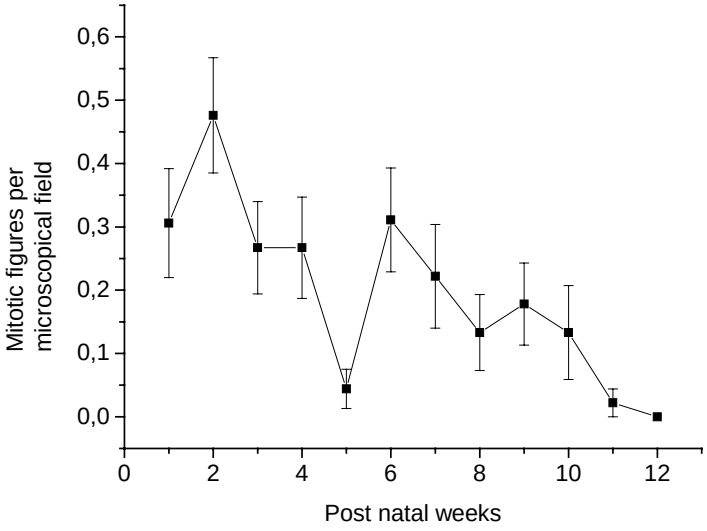


Figure 9



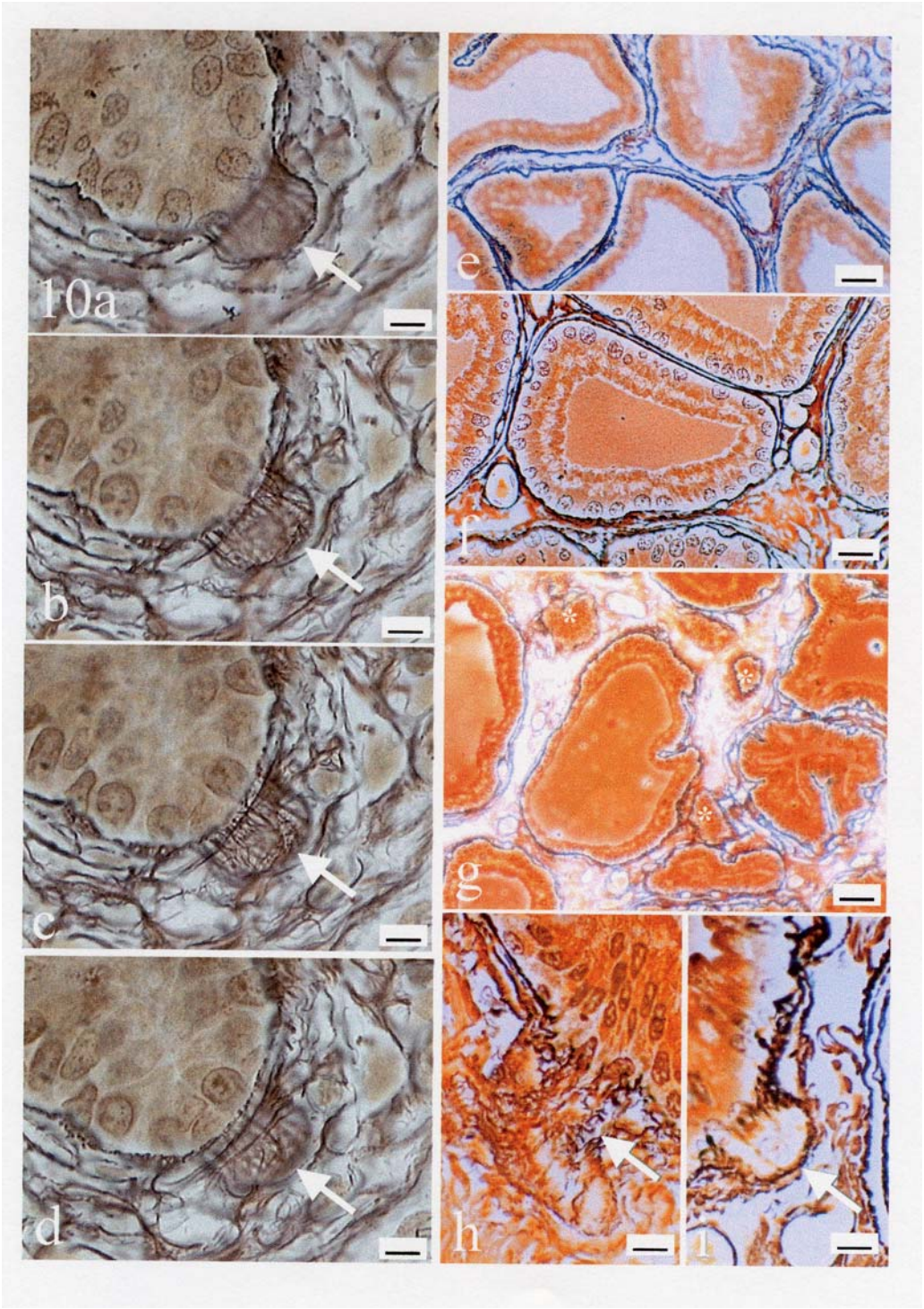

10a
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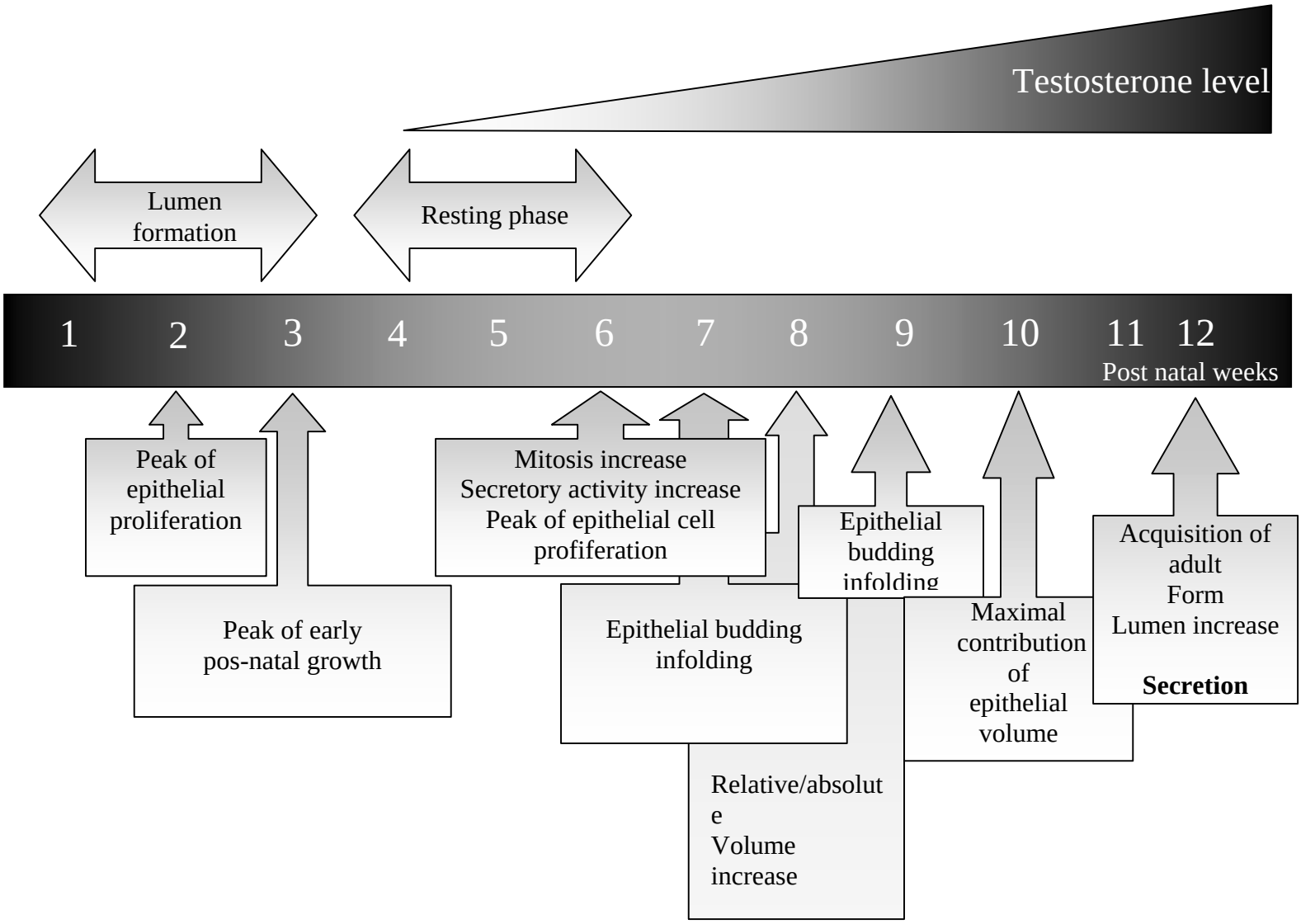
Figure 11


Testosterone level
Lumen formation
Resting phase
1 2 3 4 5 6 7 8 9 10 11 12
Post natal weeks
Peak of epithelial proliferation
Peak of early pos-natal growth
Mitosis increase
Secretory activity increase
Peak of epithelial cell profiferation
Epithelial budding infolding
Epithelial budding infolding
Relative/absolute Volume increase
Maximal contribution of epithelial volume
Acquisition of adult Form
Lumen increase
Secretion

6. Conclusões

1. Após a privação androgênica pela castração, a próstata ventral de ratos sofre uma intensa reorganização das fibras de colágeno;
2. Há um aparente envolvimento das células musculares lisas na reorganização das fibras de colágeno;
3. As células musculares lisas da próstata ventral de ratos, após a privação androgênica, apresentam uma grande plasticiade fenotípica, assumindo um fenótipo mais voltado à síntese e secreção, como pode ser observado devido ao aumento no volume das organelas de síntese, tais como retículo endoplasmático e complexo de Golgi, sem perder sua capacidade contrátil;
4. Durante o desenvolvimento pós-natal da próstata ventral existe uma clara correlação entre o aumento nos níveis de testosterona sérica e os eventos relacionados com o desenvolvimento pós-natal;
5. Há dois momentos principais de proliferação epitelial, que ocorrem na segunda e sexta semanas. Esses pontos de proliferação estão associados à formação da luz, que ocorre nas três primeiras semanas e a episódios de aumento na atividade secretora que são mais marcantes nas 6ª. e 12ª. semanas, no crescimento geral do órgão e
6. Foi observada a formação de uma estrutura em forma de cesta das fibras reticulares em associação aos brotamentos epiteliais, nas regiões em que essa estrutura se projeta em direção ao estroma, o que consiste em mais um exemplo das interações epitélio-estroma existentes na próstata.

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