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EFEITOS DA FINASTERIDA SOBRE CULTURAS DE CÉLULAS
EPITELIAIS PROSTÁTICAS NORMAIS E TUMORAIS EM
DIFERENTES SISTEMAS IN VITRO

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Epígrafe

“If a man empties his purse into his head
no one can take it away from him.
An investment in knowledge
always pays the best interest.”

Benjamin Franklin

Lista de Abreviaturas

5-ARIs: 5 alpha reductase inhibitors
AR: androgen receptor
CaP: câncer de próstata
CD: cluster of differentiation
CM: conditioned medium
CPCR: câncer de próstata resistente à castração
CRPC: castration resistant prostate cancer
DHT: dihidrotestosterona
DMSO: dimethylsulfoxide
ECM: extracellular matrix
EGF: epidermal growth factor
ELISA: enzyme linked immunoabsorbant assay
ER: estrogen receptor
ERK: extracellular signal-regulated protein kinases
EUA: Estados Unidos da América
FAK: focal adhesion kinase
FBS: fetal bovine serum
FDA: Food and Drugs Administration
Fin: finasterida
Fin: finasteride
HPB: hiperplasia prostática benigna
IGFBP: insulin growth factor binding protein
INCA: Instituto Nacional do Câncer
IOD: integrated optical density
MC: meio de cultura
MEC: matriz extracelular
MMP: matrix metalloproteinase
MMP: metaloproteinase de matriz
MTT: dimethylthiazol-diphenyltetrazolium bromide
MWCO: molecular weight Cutoff
PAP: prostate acid phosphatase
PBS: phosphate buffered saline
PCa: prostate cancer
PCPT: Prostate Cancer Prevention Trial
PI-3K: phosphatidylinositol 3-kinase
PIA: proliferative inflammatory atrophy
PIN: prostatic intraepithelial neoplasia
PSA: prostatic specific antigen
RNA: ribonucleic acid
RPM: rotations per minute
SD: standard deviation
SFB: soro fetal bovino
SU: sino urogenital
SUS: Sistema Único de Saúde
T: testosterona
TIMPs: tissue inhibitors of matrix metalloproteinase
uPA: urokinase-type plasminogen activator
uPAR: urokinase-type plasminogen activator
USA: United States of America

Resumo



O câncer de próstata (CaP) é importante causa de morte no mundo. Além do óbvio impacto na vida pessoal do paciente e na sua família, no Brasil esta doença gera custos altíssimos para o Sistema Único de Saúde (SUS), desde o diagnóstico até o óbito. As terapias disponíveis, além de causarem complicações e efeitos colaterais indesejáveis, não proporcionam sobrevida alta ao paciente. Além disso, os casos de cura são restritos aos diagnosticados precocemente, e com intervenção rápida, antes que o tumor se torne resistente à castração química. Neste sentido, estratégias preventivas são desejáveis para a diminuição da incidência e óbitos e, dentre elas, o uso da finasterida, um fármaco inibitório da enzima 5- α -redutase, foi proposto como potencial agente quimiopreventivo após estudo conduzido pelo Prostate Cancer Prevention Trial, que demonstrou significativa diminuição na incidência de CaP no grupo de pacientes tratados, em comparação ao grupo controle. No entanto, mesmo com resultados promissores, foi detectado aumento, também significativo, do número de casos de cânceres mais agressivos (alto grau) no grupo de pacientes que recebeu finasterida, em comparação aos pacientes do grupo controle. Após intenso debate entre urologistas, biólogos e cancerologistas, ainda não há consenso sobre a natureza artefactual ou de real indução de cânceres mais agressivos pela finasterida. O órgão regulatório americano Food and Drugs Administration (FDA) declarou que o aumento dos casos agressivos pela finasterida não deve ser negligenciado, e recentemente proibiu o uso deste fármaco como quimiopreventivo para o CaP. Uma vez que casos agressivos de câncer estão comumente relacionados à superexpressão de enzimas denominadas metaloproteinases de matriz extracelular (MMPs), e consequente aumento do potencial invasivo e migratório das células tumorais, o objetivo do presente trabalho foi verificar os efeitos modulatórios sobre a atividade destas enzimas e a expressão de seus reguladores naturais TIMPs (*tissue inhibitors of MMPs*), além dos efeitos sobre a viabilidade, migração e invasividade celulares, após diferentes doses e períodos de exposição à finasterida, empregando-se dois sistemas *in vitro*. Para tanto, as linhagens de células humanas prostáticas RWPE-1, LNCaP e PC3 foram cultivadas, expostas à finasterida, e métodos de cultura celular, atividade de MMPs, expressão proteica, testes de viabilidade, migração e invasividade celular foram empregados, com ou sem a interação de proteínas de matriz extracelular, dissolvidas no meio de cultura. A exposição à finasterida não foi capaz de modular a proliferação e viabilidade das células, no entanto, significativa diminuição da atividade de MMPs e aumento de TIMPs foram observados. Uma diminuição direta na capacidade migratória e invasiva das células tumorais também foi observada. Nossos resultados comprovam a importância de se melhorarem os sistemas de cultura celulares, com a adição de proteínas para se estabelecerem interações células-matriz extracelular no ambiente *in vitro*. Embora a finasterida tenha sido contraindicada pelo FDA para a prevenção do CaP, nossos resultados apontam para efeitos inibitórios de importantes fatores complicadores, como a invasividade e habilidade de formação de metástases no CaP, em células expostas à finasterida.

Palavras chave: Câncer de próstata, finasterida, invasividade, migração, cultura celular

Abstract



Prostate cancer (PCa) is an important death cause in Brazil and other countries. Besides the obvious impact at patient's life, and their relatives, this disease consumes exorbitant resources from the Sistema Único de Saúde (SUS), from diagnosis to death. The available therapies not only cause undesirable complications and side effects, but also are inefficient at providing good survival expectancies for those affected. Moreover, the cure is only possible when the tumors are readily found and when the intervention is fast enough to prevent that the tumor become castration-resistant. In this sense, preventive strategies are desirable in order to lower incidence and death, and amongst them, finasteride (Fin) treatment, an inhibitor of the 5-alpha reductase enzyme, was proposed as a potential chemopreventive agent after the study conducted by the Prostate Cancer Prevention Trial reported a significant lower incidence of PCa cases on Fin-treated patients, when compared to control patients. However, even though these results were promising, this study also reported a significant increase on more aggressive, high-grade PCa, amongst Fin-treated patients, compared to those not exposed to Fin. After an intense debate about factual or artifactual Fin-induced high-grade PCa cases, between urologists, biologists and oncologists, there are still no decisive conclusions on this matter. The regulatory USA organ, Food and Drugs Administration (FDA), has recently declared that the higher incidence of a more serious form of PCa at the Fin-treated patients must not be neglected, and prohibited its use as a chemopreventive agent. Given that the aggressive cancer cases are commonly associated with the super-expression of matrix metalloproteinases (MMPs) enzymes, with consequently higher invasion and migration potential of tumor cells, this research was conducted to verify the modulatory effects upon the activity of these enzymes and expression of their naturally occurring inhibitors TIMPs (tissue inhibitors of MMPs), the effects on cell viability, migration and invasion potential, after different Fin doses for different periods of exposure, employing two in vitro systems. Therefore, the human prostatic cell lines RWPE-1, LNCaP and PC3 were cultivated, exposed to Fin, and cell culture, MMP activity, protein expression, cell viability, cell migration, and cell invasion assays were employed, with or without the interaction of ECM dissolved at the culture medium. Fin exposure did not modulate the proliferation and viability of cells, however, a significant downregulation of MMPs activities and upregulation of TIMPs expression were observed. A direct inhibition of the migratory and invasive potential of tumor cells was also observed, after Fin exposure. Moreover, our results confirm the importance of upgrading the cell culture systems, with the addition of proteins to establish cell-to-extracellular matrix interactions at the in vitro environment. Nevertheless the FDA has prohibited Fin use as a chemopreventive agent for PCa, our results highlights that Fin may inhibit deleterious factors, such as tumor invasion and the ability of cells to metastasize, at Fin-exposed cells.

Keywords: Prostate cancer, finasteride, invasion, migration, cell culture.

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1.1 A Glândula Prostática

A próstata é uma glândula exócrina constituinte de todos os mamíferos. É a maior glândula acessória do aparelho reprodutor masculino, responsável pela produção e secreção de enzimas, aminas, lipídios e íons metálicos, essenciais para a função correta dos espermatozoides (Costello et al., 1989), e de proteínas como o antígeno específico prostático (PSA – *prostate specific antigen*) e a fosfatase ácida prostática (PAP – *prostate acid phosphatase*) (Long et al., 2005). Além de sua importante função para a biologia da reprodução, é o sítio de origem de duas importantes doenças em indivíduos do sexo masculino: a hiperplasia prostática benigna e o câncer de próstata (CaP) (Lee et al., 2011). Comparativamente, a próstata é reconhecida por diferenças anatômicas e bioquímicas nas diferentes espécies de mamíferos, porém, apresenta arquitetura tecidual uniforme. A próstata de mamífero adulto é constituída de dois componentes principais, o epitelial e o estromal, ambos regulados por hormônios (Marker et al., 2003; Kindblom, 2003).

O epitélio consiste de uma única camada de células epiteliais colunares polarizadas, que possuem função secretora, de células basais, envolvidas no *turnover* do epitélio prostático, e de células neuroendócrinas (Figura 1). O componente epitelial luminal secreta um grande constituinte do plasma seminal do ejaculado, na uretra (Cunha et al., 1987; Kindblom, 2003). As células epiteliais colunares são caracterizadas, morfológicamente, pela sua polaridade, com o núcleo deslocado para o polo basal e as organelas secretoras deslocadas para o polo apical. Marcadores específicos como as citoqueratinas 8 e 18, além do marcador CD57, podem diferenciá-las das demais células prostáticas (Marker et al., 2003; Long et al., 2005). O compartimento estromal, ou estroma, circunda o tecido epitelial e é composto, principalmente, de fibroblastos, células musculares lisas, vasos sanguíneos e linfáticos, além de conter células nervosas, mastócitos e macrófagos (Cunha et al., 1986; Marker et al., 2003). O estroma tem função crucial na manutenção do estado diferenciado da próstata, pois produz

fatores de crescimentos e mantém um microambiente adequado para as células epiteliais mediante sinalização cruzada com o epitélio. Além disso, é responsável pela produção dos componentes de matriz extracelular (MEC) da glândula prostática, entre eles colágeno, fibras elásticas, proteoglicanos e microfibrilas, principalmente pelos fibroblastos e pelas células musculares lisas (Villamaior et al., 2000).

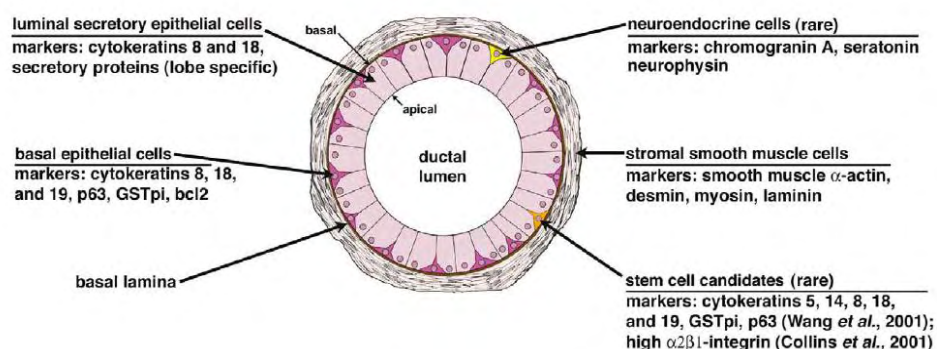


Figura 1: Representação esquemática de um corte histológico da próstata, em que se identificam células epiteliais secretoras, basais, neuroendócrinas e células do estroma. Adaptado de Marker et al., 2003.

O crescimento e desenvolvimento da glândula prostática começam na vida fetal e se completam na maturidade sexual. A próstata se desenvolve do sino urogenital (SU), uma estrutura com uma camada de epitélio derivado da endoderme, circundado por uma camada de mesênquima derivado da mesoderme (Marker et al., 2003; Cunha et al., 2004). O SU se encontra na porção caudal da bexiga em desenvolvimento e aparece em camundongos aproximadamente 13 dias após a concepção, e em humanos após cerca de sete semanas de gestação. A morfogênese da próstata se inicia, em um processo dependente dos andrógenos circulantes produzidos pelos testículos fetais (Marker et al., 2003). Na diferenciação das células epiteliais, ocorre uma divisão entre duas linhagens: a população epitelial basal, que se orienta seguindo a membrana basal (formada de colágeno IV e fibronectina), formando uma linha de células contínua no homem e descontínua nos roedores; e as células luminares

colunares, que se alinham circulando os ductos, formando a porção secretora da glândula. A porção mesenquimal (estroma) também passa por um processo de diferenciação, com o aparecimento de camadas de músculo liso circundando os ductos (Marker et al., 2003).

Após sua maturação, a próstata humana possui uma compactação considerável e sem lobos distintos, ainda assim, podem ser reconhecidas as seguintes regiões anatômicas: base, ápice, lobos esquerdos, lobos direitos e istmo (Wendell-Smith, 2000). Uma nomenclatura clinicamente mais relevante foi proposta dividindo-se a próstata em três zonas distintas, com base na morfologia destas, e nomeadas usando a uretra como ponto central anatômico de referência: zona central, zona periférica e zona de transição (McNeal, 1968 e 1981). Estas designações foram gradualmente conquistando os pesquisadores da próstata e atualmente são aceitas universalmente (Kindblom, 2003). A zona de transição é composta de lóbulos de glândulas com ductos mais curtos, em comparação aos da zona periférica, e é normalmente separada da zona periférica por tecido colágeno, que se torna mais pronunciado quando a zona de transição se expande em casos de hiperplasia prostática benigna. Ela é, também, o local mais comumente acometido por esta doença (McNeal, 1988; Lee et al., 2011). A zona periférica contém a maior parte do tecido glandular na próstata normal e representa o local mais frequente de origem de carcinomas prostáticos (McNeal, 1988; Lee et al., 2011). Esta zona é, também, o local de origem mais frequente de lesões precursoras de CaP ou neoplasias intraepiteliais prostáticas.

1.2 O Câncer de Próstata

O CaP é o câncer mais diagnosticado e a segunda causa de morte por câncer entre os homens na América e nos países da Europa Ocidental (Jemal et al., 2009; Rittmaster et al., 2009), totalizando mais de 200.000 óbitos anualmente no mundo inteiro (Wang et al., 2011). A incidência desta doença vem aumentando em razão de fatores demográficos – como o

aumento da proporção dos idosos nas populações – do aumento do número de casos detectados decorrentes da implementação dos testes sorológicos de PSA em larga escala (Mora et al., 2001), e de fatores ambientais e componentes genéticos (Chiaverotti et al., 2008). Neste sentido, projeções alarmantes de, aproximadamente, 1,7 milhão de novos casos e 0,5 milhão de mortes são estimadas para o ano de 2030 (Center et al., 2012), gerando gastos excessivos aos sistemas de saúde pública, com números igualmente alarmantes de custos de 8 bilhões de dólares anualmente (11% dos custos para o tratamento de todos os cânceres) e 81.658 dólares por paciente, desde o diagnóstico até o óbito, nos EUA (Racioppi et al., 2012). No Brasil, o número de novos casos de CaP estimado para o ano de 2012 é de 60.180 (INCA, Estimativa 2012). O custo total decorrentes de cirurgias oncológicas, quimioterapia e radioterapia ultrapassou o valor de 271.500.000,00 reais gastos pelo Sistema Único de Saúde (SUS), durante os anos de 2001 a 2005, para todos os tipos de câncer (DATASUS/SIA).

Alguns fatores aumentam as chances de um homem desenvolver o CaP, tais como: idade (acima de 50 anos); níveis do PSA (é considerado um marcador para o CaP e homens com níveis elevados de PSA apresentam maiores riscos); etnia (os descendentes africanos são os indivíduos que mais desenvolvem o CaP); histórico familiar (homens com casos de CaP na família têm o risco de desenvolver a doença aumentado em 2 a 3 vezes); dieta (homens obesos, com dieta rica em gordura animal apresentam maior predisposição); influência dos componentes genéticos e de fatores epigenéticos (Rittmaster et al., 2009). Embora a faixa de idade considerada como risco para o desenvolvimento de CaP seja a partir dos 50 anos, dados de autopsias feitas em homens jovens vítimas de traumas, com idade aproximada de 30 anos, revelaram que 1 em cada 3 próstatas analisadas apresentaram alterações histológicas compatíveis com os estágios iniciais do CaP (Sakr et al., 1994). A progressão entre o CaP pré-clínico e a doença clínica é bastante lenta e silenciosa, e neste sentido, métodos preventivos e

o acompanhamento clínico são indicados principalmente aos homens com predisposição de desenvolverem a doença (Rittmaster et al., 2009).

O CaP se manifesta como variados achados clínicos, desde formas latentes e relativamente assintomáticas até cânceres ativos, altamente agressivos e metastáticos (Mora et al., 2001). Um fator ainda não esclarecido refere-se à predileção da área onde se forma o tumor. Os tumores predominantemente se originam de células epiteliais da zona periférica da glândula, raramente na zona de transição e praticamente não ocorrem na zona central (De Marzo et al., 2007; Lee et al., 2011). Os tumores que progridem, caso não sejam tratados, podem se estender à cápsula prostática e vesículas seminais, atingir vasos sanguíneos e originar metástases em locais distantes, como nódulos linfáticos e ossos. Outra complicação, importante causa de morbidade, é a doença metastática refratária a hormônios (Mora et al., 2001). Assim como o epitélio da próstata adulta depende de androgênios para a manutenção do estado diferenciado e proliferação, as células tumorais prostáticas também dependem desses hormônios para se multiplicarem e sobreviverem em um estágio inicial. A modulação androgênica da próstata é influenciada por uma importante reação química, quando a testosterona (T) entra na célula e é convertida em diidrotestosterona (DHT), seu metabólito mais ativo. A enzima responsável por esta reação é a 5- α -redutase. A DHT se liga aos receptores androgênicos (AR – *androgen receptor*) no citoplasma, é translocada ao núcleo e ativa uma série de genes, induzindo a síntese de proteínas que estimulam o crescimento celular (Miyamoto et. al., 2005; Pienta & Smith, 2005; Brattoef et al., 2008), inibem a apoptose e estimulam a produção das proteínas da secreção, como o PSA (Rittmaster, 2008). Entretanto, invariavelmente, as células do CaP desenvolvem diferentes estratégias que as permitem se multiplicar mediante um mecanismo independente do estímulo da DHT, ou mesmo clones resistentes a androgenoterapia podem ser selecionados entre as células sensíveis. (Miyamoto et. al., 2005). Vários processos já foram relatados, como a

hipersensibilidade consequente ao aumento da expressão do AR, as mutações que modificam a especificidade do receptor, o desencadeamento da cascata sem a ligação dos androgênios e o desenvolvimento de receptores promíscuos (Miyamoto et. al., 2005; Pienta & Smith, 2005).

Atualmente é aceito que o desenvolvimento do CaP se dá em razão de agressões contínuas ao epitélio prostático acumuladas durante décadas (Rittmaster, 2008). Desta forma, a progressão do CaP envolve diferentes estágios da doença, que geralmente incluem a atrofia inflamatória proliferativa (PIA – *proliferative inflammatory atrophy*), a neoplasia intraepitelial prostática (PIN – *prostatic intraepithelial neoplasia*), o adenocarcinoma de próstata *in situ* e o adenocarcinoma invasivo (De Marzo et al., 2007; Rittmaster, 2008) (Figura 2).

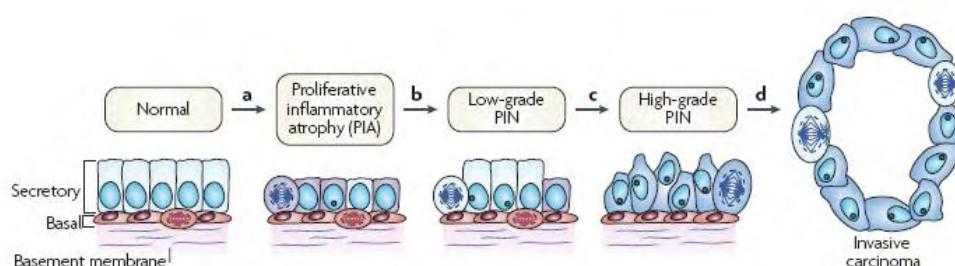


Figura 2: Estágios de desenvolvimento do CaP (De Marzo et al., 2007).

Atualmente, a PIA pode ser considerada lesão precursora do CaP, causada pela exposição contínua do epitélio prostático a agentes infecciosos, processos inflamatórios crônicos e carcinogênicos (Rittmaster, 2008). Subsequentes alterações somáticas no genoma podem levar ao desenvolvimento da PIN, uma proliferação neoplásica descontrolada, que ocasiona uma estratificação do epitélio, com núcleos aumentados e nucléolos evidentes, além do padrão de hiper cromasia (Ayala & Ro, 2007). Muitos estudos identificaram áreas de transição entre PIA e CaP, bem como entre PIA e PIN, reforçando a relação entre as três alterações da próstata (De Marzo et al., 2007). Muitas das alterações moleculares induzem ao aparecimento do CaP foram identificados na PIA. O epitélio normal da próstata apresenta altos níveis dos produtos proteicos de três genes supressores de tumores (NKX3.1, CDKN1B

e PTEN), os quais se encontram diminuídos em PIA, NIP e CaP (De Marzo et al., 2007). A correlação entre PIN e CaP também está bem fundamentada. A presença de PIN de alto grau sugere presença de carcinoma invasivo em cerca de 33% dos casos, e quando uma biópsia de múltiplos pontos é realizada, caso seja detectada presença de PIN em 4 pontos, há 75% de chances de se encontrar um carcinoma invasivo em uma nova biópsia (Ayala & Ro, 2007).

Os tumores mais agressivos na próstata podem ser definidos como os que possuem fenótipo indiferenciado, com alta velocidade de divisões mitóticas e invasividade no tecido conjuntivo fibromuscular (estroma) localizado ao redor dos ácinos (Bostwick, 2004). À medida que a massa tumoral maligna invade o tecido adjacente, as células malignas atingem os vasos linfáticos e/ou sanguíneos, causando metástases em outros órgãos. A segunda característica dos tumores agressivos é sua capacidade de induzir a formação de vasos sanguíneos para a nutrição da massa de células em expansão, processo denominado de angiogênese. Isso é necessário, pois com o crescimento do tumor, este se distancia dos vasos sanguíneos preexistentes, e a velocidade de difusão de nutrientes célula a célula não é suficiente para manter o metabolismo das áreas centrais do tumor (Bostwick, 2004).

1.3 O Tratamento do Câncer de Próstata

As estratégias terapêuticas clássicas do CaP incluem terapia de supressão androgênica, prostatectomia radical, radioterapia e quimioterapia (Allen et al., 2007; Michaelson et al., 2008; Lughezzani et al., 2012). Pacientes com CaP pouco diferenciados e baixo volume tumoral podem ser elencados para a observação monitorada, por avaliação trimestral dos níveis de PSA e biópsias de repetição, visando à intervenção rápida nos casos que progredirem (Duchesne 2011), ainda com tempo suficiente para a cura (Klotz, 2012; Bangma et al., 2012).

A prostatectomia radical é indicada para os casos em que o CaP é localizado (Lughezzani et al., 2012), mas controversa em casos avançados (Marchioro et al., 2012). Em até 30% dos pacientes operados, metástases podem-se desenvolver futuramente (Afar et al., 2004). Além disso, como se trata de um procedimento cirúrgico, existem riscos e complicações pós-operatórias, por exemplo: lesão do músculo retouretral, do ureter, da bexiga; complicações trombóticas, embólicas; e disfunção urinária e sexual (Michaelson et al., 2008; Duchesne 2011).

A radioterapia é um procedimento opcional ou concomitante à prostatectomia radical nos casos de CaP de alto grau (Allen et al., 2007; Prada et al., 2012). Ela pode ser efetuada por meio de um laser externo ou pela implantação de uma fonte radioativa dentro da próstata, técnica esta denominada braquiterapia (Michaelson et al., 2008; Duchesne 2011). Embora as inovações em medicina contribuam para a diminuição dos efeitos colaterais, estes ainda são severos e frequentes, muitas vezes fazendo com que o paciente abandone o tratamento (McNeel, 2007). As complicações mais revelantes são disfunção urinária, sequelas gastrintestinais, diminuição da potência sexual em comparação a grupos não irradiados (Michaelson et al., 2008; Duchesne 2011; Prada et al., 2012), mal-estar gerado pelos efeitos da radiação em células proliferativas (Duchesne 2011) e necessidade de pequenas intervenções invasivas (Prada et al., 2012).

A quimioterapia se baseia na administração de drogas comprovadamente citotóxicas, capazes de interferir na biologia do tumor, visando à destruição deste ou dos seus elementos de alimentação (Omlin & de Bono, 2012). Este procedimento, todavia, não distingue células sadias das doentes, e pode provocar graves efeitos colaterais que também podem levar o paciente a abandonar o tratamento. Para se atenuar tais efeitos colaterais, são necessários monitoramento acurado dos sintomas tóxicos, ajustes de dose e tratamentos profiláticos (Omlin & de Bono, 2012). Mesmo com estes cuidados, efeitos colaterais como neutropenia,

anemia, trombocitopenia, neutropenia febril e diarreia podem ser observados quando algumas drogas quimioterápicas são empregadas (Omlin & de Bono, 2012). Óbitos causados por sepse neutropênica, doenças cardíacas e falha renal podem ocorrer (Omlin & de Bono, 2012). Outro efeito indesejado da quimioterapia e da radioterapia é a possível liberação do fator nuclear $\kappa\beta$, que ativa genes intrinsecamente ligados a processos tumorais, como invasão, angiogênese e inibição da apoptose (Sweeney et al., 2004). Além das complicações acima citadas, uma droga considerada eficaz no tratamento de CaP metastático resistente à castração, o docetaxel, fornece mediana de sobrevida de apenas 19 meses (Fleming et al., 2012).

A terapia hormonal, também conhecida como terapia de supressão androgênica ou terapia de ablação androgênica, descrita inicialmente por Huggins e Hodges em 1941 (Hill et al., 2003; Pienta & Smith, 2005; Gomella et al., 2010), promove diminuição dos níveis de testosterona sérica na tentativa de diminuir a velocidade de crescimento do CaP (Gomella et al., 2010; Cannata et al., 2012) e é o padrão-ouro de tratamento de pacientes com CaP recorrente ou metastático (Pienta & Smith, 2005; Michaelson et al., 2008; Gomella et al., 2010). Estima-se que aproximadamente 660.000 homens fazem uso desta modalidade terapêutica, atualmente, nos EUA (Michaelson et al., 2008). Como complicações decorrentes da terapia hormonal podem ser citadas diminuição da densidade dos ossos, impotência, libido diminuída, aumento da massa corpórea (Sharifi et al., 2005) ginecomastia, fadiga e anemia (Michaelson et al., 2008).

1.4 A Finasterida

A finasterida é uma droga capaz de modular a quantidade de andrógenos disponíveis para o epitélio prostático e foi a primeira desenvolvida para o tratamento da hiperplasia benigna da próstata (Stoner, 1990). Ela atua sem competir com o receptor androgênico ou

antagonizar a ação da T nos tecidos alvos, mas sim inibindo a atividade enzimática da 5- α -redutase do tipo 2. Esta enzima é responsável pela conversão da T em um andrógeno mais potente, a DHT (Prahalada et al., 1994; Griffiths et al., 1997; Prahalada et al., 1998). A 5- α -redutase desempenha papel fundamental, visto que a DHT possui afinidade pelo AR até quatro vezes maior do que a T (Cai & Balk, 2011). Como a finasterida é capaz de inibir de 80% a 90% a enzima 5- α -redutase do tipo 2, e consequentemente reduzir o volume prostático para 30%-40% do original (Prahalada et al., 1994), elevar os níveis séricos de T e diminuir os níveis de DHT em 80% a 90% em relação aos níveis normais em homens e animais após a sua administração (Rittmaster, 1994), e que a próstata é uma glândula andrógeno-dependente, sua ação é capaz de interferir consideravelmente com a morfologia, histofisiologia e biologia da próstata, em geral (Cai & Balk, 2011).

Na próstata, alguns efeitos do tratamento com a finasterida já estão bem definidos e incluem inibição da proliferação celular, inibição da atividade secretória epitelial e aumento da indução de apoptose das células epiteliais (Rittmaster et al., 1996; Prahadala et al., 1998; Tindall & Rittmaster, 2007; Golbano et al., 2008). Estudos em roedores tratados com finasterida demonstraram que a inibição da enzima 5- α -redutase na próstata diminui o volume da glândula prostática, a altura das células epiteliais, a área ocupada pelo lúmen, além de promover a reorganização do estroma, principalmente pela reorganização de fibras colágenas (Prahalada et al., 1998; Rittmaster, 2008; Delella et al., 2010).

Em outubro de 1993, foi criado o Prostate Cancer Prevention Trial (PCPT), apoiado por vários institutos americanos de estudos de prevenção e tratamento do CaP, para avaliar os efeitos preventivos da finasterida no desenvolvimento do CaP. O estudo teve início com a triagem de homens a partir de 55 anos de idade e com diagnóstico negativo para CaP. Após a triagem, homens com o PSA menor ou igual a 3.0 ng/ml foram randomizados para receber 5 mg de finasterida/dia ou placebo. Os participantes foram convocados a cada 6 meses para

relatar possíveis efeitos colaterais, realizar a dosagem do PSA e o exame de toque retal, anualmente. Após 7 anos de estudo, foi recomendado a todos os participantes realizar biópsia de próstata (Thompson et al., 2009). Dentre os 4.368 homens que receberam finasterida durante 7 anos, apenas 803 (18,4%) desenvolveram câncer, contra 1.147 (24,4%) entre 4.692 pacientes que receberam placebo. Entretanto, uma maior incidência de tumores com graduação de Gleason 7, 8, 9 e 10 foi encontrada nos pacientes que receberam finasterida (37%), contra 22,2% nos pacientes que receberam placebo (Thompson et al., 2003). O PCPT concluiu que, embora a finasterida previna ou atrase o aparecimento do câncer, ela aumenta o risco de CaP de alto grau.

Após a reavaliação dos resultados encontrados pelo PCPT, foi comprovado que a finasterida aumenta as chances de detecção do CaP de alto grau em biópsias por punção, por diminuir o volume da glândula prostática, e que a droga não aumentava os riscos de desenvolver CaP de alto grau, pois este aspecto era artefactual, devido a diminuição da glândula. Assim, o uso da finasterida como quimiopreventivo do CaP foi aprovado e indicado para pacientes com predisposição ao desenvolvimento desse tipo de tumor (Thompson et al., 2009). Entretanto, nem todos os urologistas ficaram convencidos do uso seguro da finasterida na prevenção do CaP, e os principais argumentos contra foram o curto período do estudo, pois estes pacientes deveriam ter sido acompanhados por um maior período de tempo, e que o tratamento com finasterida poderia selecionar, ao longo do tempo, as células tumorais andrógeno-independentes, mais agressivas e metastáticas (Koltz & Clarke, 2008). No ano de 2012, o órgão americano Food and Drugs Administration (FDA) posicionou-se acerca do uso da finasterida para a quimioprevenção do CaP. Embora esta agência tenha confirmado o benefício encontrado no PCPT, ela conclui que 1 a cada 150 homens tratados com a finasterida (para prevenção do CaP) irá desenvolver uma forma agressiva desta doença. Com

base nessas conclusões, o FDA decidiu proibir seu uso para a prevenção do CaP (Violette & Saad, 2012).

1.5 Invasividade e Metaloproteínases de Matriz Extracelular

Para as células epiteliais tumorais atingirem vasos linfáticos/sanguíneos, têm que degradar os componentes da MEC composta de proteínas da família colágeno, proteínas do sistema elástico, glicosaminoglicanos, proteoglicanos e glicoproteínas adesivas (Visse & Nagase, 2003). Deste modo, as células tumorais malignas secretam enzimas que digerem a MEC ou induzem sua secreção por células vizinhas, sendo as mais importantes as metaloproteínases de matriz (MMPs), hialuronidases, heparanases, condroitinases, serina-proteases e cisteína-proteases (Visse & Nagase, 2003; Yip et al., 2006). As MMPs são endopeptidases dependentes de íons divalentes (especialmente Ca^{2+} e Zn^{2+}) (Visse & Nagase, 2003), que em situações normais atuam ativamente nos processos de remodelação tecidual, especialmente durante a morfogênese dos tecidos, na renovação constante dos componentes da MEC e nos processos de angiogênese (Visse & Nagase, 2003).

Vários estudos demonstraram que quando o epitélio prostático se transforma, tende a produzir MMPs em excesso, principalmente a MMP2 e a MMP9 e as direcionar para a área de invasão tumoral (Wood et al., 1997; Trudel et al., 2003; Nagle, 2004; Sauer et al., 2004). O primeiro trabalho a fazer um mapeamento das MMPs na próstata em humanos, comparando CaP *versus* epitélio normal, foi publicado em 1991. Nesse trabalho foram utilizadas 18 amostras de CaP primário, duas metástases de nódulos linfáticos, 11 amostras de próstatas saudáveis e três linhagens de CaP (LNCaP, PC3 e DU-145) para se estudar expressão do gene codificador da MMP7 (matrilisina). Por meio de estudos de *northern blotting* determinou-se que 77% dos CaPs, 50% dos nódulos linfáticos e apenas 27% das próstatas saudáveis expressavam o RNA mensageiro (RNAm) da MMP7. As três linhagens celulares estudadas

não demonstraram expressão do RNAm da MMP7. Análises adicionais foram conduzidas para o mapeamento da expressão da MMP1, MMP3 e MMP18, nas amostras de CaP e próstata sadia. Os resultados indicaram que 60% das amostras de CaP expressavam RNAm para a MMP18, enquanto nenhuma amostra de próstata sadia o fez (Pajouh et al., 1991).

Após a constatação inicial do aumento da expressão das MMPs no CaP e sua possível correlação com o processo de invasividade e potencial para metástases, uma série de estudos veio a confirmar os resultados preliminares de Pajouh e seu grupo: em 1993, pesquisadores constataram que quando a linhagem DU-145 era transfectada com um vetor contendo o gene codificador da MMP7, seu potencial invasivo aumentava em relação ao controle de transfecção (Powell et al., 1993). Posteriormente, outros estudos confirmaram a importância da MMP7, de outras MMPs como a MMP2, MMP9 e MMP14 e dos inibidores teciduais das MMPs (TIMP – *tissue inhibitor of matrix metalloproteinases*) na progressão para o fenótipo do CaP invasivo (Knox et al., 1996; Matrisian, 1999; Daja et al., 2003; Jung et al., 2003; Morgia et al., 2005).

Como diversas substâncias e compostos parecem interferir com o potencial de invasividade, mediante modulação da atividade e expressão das MMPs, quando em contato com células de CaP e células saudáveis (Delella et al., 2010; Justulin Jr. et al., 2010; Xu et al., 2009; Zhou et al., 2009), nosso grupo se interessou nos efeitos da finasterida sobre as MMPs, TIMPs e outros componentes diretamente ligados a invasividade tumoral, como a fibronectina. Experiências prévias (Felisbino et al., 2007) mostraram que, durante a orquiectomia em ratos, as células epiteliais prostáticas passavam a secretar MMP7 para o estroma logo abaixo do epitélio, próximo à membrana basal. Nesse estudo, após a restrição androgênica, a MMP7 estava co-localizada na região da membrana basal, que se encontrava descontínua e fragmentada. Sugeriu-se, desta forma, que esta enzima poderia estar envolvida

na degeneração desta membrana. A MMP7 poderia também atuar de forma parácrina ao solubilizar uma série de fatores de crescimento (Felisbino et al., 2007).

Estudo de Dellela et al. (2009) demonstrou que o tratamento com a finasterida altera o padrão de expressão da MMP2 e MMP9 na próstata ventral de ratos. Estas duas enzimas aparecem frequentemente alteradas (superexpressas) no soro e em amostras de tecidos prostáticos de pacientes com CaP agressivos. Nesse estudo, após o tratamento com a finasterida, constatou-se que os níveis de MMP2 estavam diminuídos, enquanto a MMP9 estava aumentada. Outros autores vieram a confirmar o efeito da finasterida sobre a modulação da MEC na próstata, após investigarem o efeito da terapia combinada de finasterida e doxazosina na próstata ventral de ratos Wistar que foram sacrificados após 3 e 30 dias de exposição aos fármacos. Quanto à expressão e atividade das MMPs, os autores observaram que a atividade gelatinolítica da MMP9 havia aumentado após 30 dias. Outros achados importantes foram a diminuição da expressão do RNAm para TIMP-1 e 2, além do aumento da reatividade do fator de crescimento transformador beta ($TGF-\beta$ – *transforming growth factor- β*) por imuno-histoquímica, tanto no epitélio quanto no estroma da glândula prostática (Justulin Jr. et al., 2010).

1.6 Linhagens Celulares no Câncer de Próstata Humano

Para o estudo do CaP foram desenvolvidas, no decorrer dos anos, diversos modelos experimentais *in vivo*. Entre os modelos animais citam-se: os roedores, os murinos transgênicos, os camundongos com imunodeficiência severa combinada, os modelos xeno e o cão. Cada um deles apresenta vantagens, destacando-se o baixo custo e similaridade entre os modelos de roedores e o homem, a especificidade da investigação genética associada ao CaP com o modelo de murinos transgênicos, a utilidade de estudos terapêuticos nos modelos de

camundongos imunodeficientes e a interação entre as células de CaP do paciente e o microambiente fornecido pelo modelo xeno.

Além dos modelos *in vivo*, muitos modelos de cultura celular já foram desenvolvidos, murinos e humanos, a partir de culturas primárias (finitas) ou linhagens celulares (permanentes) de CaP nestas espécies (Miki & Rhim, 2008). A possibilidade de se trabalhar com células de CaP humanas em cultura é uma grande vantagem, principalmente pela maturidade adquirida das quatro principais linhagens: a não tumoral, RWPE-1, e as três tumorais, LNCaP, PC3 e DU-145. Estas linhagens estão bem descritas, seus métodos de cultivo e expansão bem padronizados, gerando economia de tempo e agilidade ao pesquisador, além do rígido controle do microambiente no qual as células se multiplicam (meios de cultura, aditivos, saturação de CO₂, pH, arcabouços tridimensionais, superfícies dos plásticos dos frascos) (Freshney, 2005).

A linhagem não tumoral RWPE-1 foi obtida a partir da imortalização de uma cultura primária de epitélio prostático sadio, não tumoral, de indivíduo adulto de 54 anos, por pesquisadores da Universidade Estadual do Michigan, do Instituto Nacional do Câncer dos Estados Unidos da America. Após a obtenção desta cultura primária, foi realizado procedimento de engenharia celular para a imortalização e obtenção da linhagem, com o uso de cópia simples do vírus do papiloma humano (HPV – *human papilloma vírus*). A linhagem obtida, RWPE-1, não induz formação de tumores quando injetada em camundongos imunodeficientes e possui inibição de proliferação por contato, quando cultivada como monocamada em plásticos de cultura. Esta linhagem expressa os marcadores epiteliais luminais citoqueratina 8 e 18, além do AR, que se torna mais detectável quando estimulada por andrógenos. Ela também expressa o gene supressor tumoral p53 (Bello et al., 1997) e baixa quantidade do receptor de estrógeno β (ER- β) (Mimeault et al., 2006).

A linhagem LNCaP foi isolada por Schuurmans e Mulder, em 1977, e foi derivada de fragmentos de biópsias de uma metástase situada nos nódulos linfáticos de homem de 50 anos de idade (Webber et al., 1997). O CaP que gerou a metástase havia sido diagnosticado como moderadamente diferenciado um ano antes. Foi determinado que o tempo médio de duplicação celular é de aproximadamente 60–72 horas, dependendo da quantidade de soro fetal adicionado ao meio de cultura. Estas células apresentam baixo potencial de ancoragem e não formam uma monocamada uniforme em cultura celular, preferindo crescer em *clusters*, uma vez que não sofrem inibição da proliferação por contato. Contêm aproximadamente 76 cromossomos, com receptores citoplasmáticos e nucleares para andrógenos, os quais estimulam seu crescimento (Webber et al., 1997). Estas células expressam a proteína do receptor de estrógeno ER- β (Mimeault et al., 2006).

A linhagem PC3 foi isolada de metástase de uma vértebra lombar (Kaighn et al., 1979). O diagnóstico patológico para o câncer primário era adenocarcinoma de próstata pouco diferenciado. As células não possuem cromossomo Y e têm, aproximadamente, 62 cromossomos durante o 5º repique. As células crescem muito aderidas aos frascos de cultura, bem como em arcabouços tridimensionais. Estas células são classicamente conhecidas por serem andrógeno-independentes, sem expressão de AR, entretanto, em estudo prévio, elas exibiram uma fraca marcação nuclear para o AR (Webber et al., 1997). Recentemente, outro estudo demonstrou a presença do RNAm e proteína em extratos celulares desta linhagem e propôs que as células PC3 sejam agora reconhecidas como AR⁺ (Alimirah et al., 2006). Outra importante característica desta linhagem é a expressão de ER- β , sendo detectável em altos níveis pela técnica de *western blotting* (Mimeault et al., 2006), bem como a expressão da enzima aromatase (Lou et al., 2005), capaz de transformar a testosterona em estrógeno. Dessa forma, quando em um microambiente rico em testosterona, como o produzido pela ação da finasterida, a linhagem PC3 tem a capacidade de responder pelo eixo estrogênico.

Quanto à expressão de MMPs nas linhagens LNCaP e PC3, os dados existentes são controversos, principalmente em função das diferenças na sensibilidade de detecção dos métodos de estudos utilizados. Daja et al. (2003) relataram que a linhagem PC3 expressa as MMPs -1, -3, -7 -10, -13, -14, -15 e -16. Todavia, mencionam não terem encontrado RNAm de MMP2 e MMP9 na linhagem PC3. Em contrapartida, Miyamoto et al. (2005) relataram que a linhagem PC3 expressa altos níveis da MMP9, o que contribui para a alta agressividade da linhagem. Já Zhang et al. (2002) relataram terem encontrado baixa expressão de RNAm de MMP2 nas linhagens LNCaP e PC3. Miyamoto et. al. (2005) também demonstraram que a linhagem LNCaP expressa baixa quantidade de MMP9. No estudo de Daja et al. (2003) demonstrou-se que as células LNCaP expressam as MMPs -1, -7, 10, -13, -15 e -16, mas não MMP2 e MMP9. Liao et al. (2003) demonstraram que as células LNCaP expressam altas quantidades de MMP2, mas não MMP9, quando estimuladas por andrógenos diluídos no meio de cultura.

No entanto, outros estudos demonstraram a presença de RNAm e proteínas ativas de MMP2 e MMP9 no meio de cultura das células LNCaP e PC3 por método de ensaio enzimático cromogênico (Singh et al., 2004 A; Singh et al., 2004 B; Wilson et al., 2004; Singh et al., 2009). Desta forma, mais estudos são necessários para uma melhor caracterização da expressão de MMPs por estas células em cultura, principalmente por meio de métodos com maior nível de sensibilidade.

1.7 Interações Célula-Matriz Extracelular no Cultivo de Células Prostáticas

Os tecidos biológicos apresentam-se organizados em estrutura tridimensional, em que uma sofisticada interação entre diferentes tipos celulares, e entre estas diferentes células e uma variada gama de proteínas da MEC, mantém o estado diferenciado e a homeostase fisiológica e funcional do tecido. Isso é observado tanto em condições teciduais normais

(Moroz et al., 2012) como em condições patológicas (Coulson-Thomas et al., 2010). Os modelos de cultura em monocamada, como os utilizados nas pesquisas que empregam as linhagens celulares descritas acima, são importantes pois fornecem respostas rápidas, podem ser realizados em triplicatas ou mesmo repetidos até seis vezes, dispensam o uso de animais de biotério, gerando agilidade de pesquisa. No entanto, estes modelos recriam apenas uma fração de um cenário maior e mais complexo, como é o caso dos tecidos *in vivo* (O'Connor, 1999). Neste sentido, modelos de cultura que adicionam a interação das células cultivadas com proteínas da MEC são desejáveis, uma vez que reproduzem o microambiente tecidual com mais fidedignidade do que os modelos de monocamada, especialmente no estudo do CaP, no qual a interação epitélio-estroma é significativamente importante (Coulson-Thomas et al., 2010).

Técnicas simples podem fazer um modelo de cultura em monocamada mais atrativo, com interações célula-matriz. De fato, proteínas e enzimas que outrora não eram detectadas no meio de cultura no qual as células em monocamada foram cultivadas, denominado meio condicionado, passam a ser detectadas pelas mesmas técnicas quando uma única proteína da MEC é dissolvida no meio de cultura (Moroz et al., 2013). Isso foi demonstrado em estudos nos quais diferentes proteínas foram utilizadas, como fibronectina, laminina, colágeno I, colágeno IV (Kuratomi et al., 2002; Das et al., 2008; Malik et al., 2010, Moroz et al., 2013). Além de alterações na expressão de proteínas e enzimas pelas células tumorais, outras mudanças no comportamento biológico destas células foram observadas quando um modelo de cultura com proteínas da MEC foi empregado, por exemplo, aumento da aderência a substratos protéicos (Docheva et al., 2010), aumento da resistência contra agentes quimioterápicos (Thomas et al., 2010) e resistência à apoptose (Fornaro et al., 2003).

Uma das proteínas da MEC que pode ser facilmente diluída no meio de cultura é a fibronectina (Das et al., 2008). A fibronectina é uma glicoproteína multifuncional da MEC,

considerada altamente relevante para os processos de metástase e sobrevivência celular (Malik et al., 2010). Ela também é substrato para a MMP2 e MMP9 (Stanton et al., 1998; Maron et al., 2007). Além de a fibronectina ser a proteína de adesão mais abundante no sangue, ela também consegue interagir com as células metastáticas em circulação periférica (Malik et al., 2010), sendo, portanto, uma das proteínas de escolha para um modelo de cultura com interações célula-matriz.

2.1 Objetivo Geral

Determinar os efeitos da finasterida sobre a viabilidade celular, expressão e atividade de metaloproteinases de matriz extracelular e inibidores de metaloproteinases de matriz extracelular (TIMPs), além da migração e invasividade de células epiteliais prostáticas humanas em diferentes sistemas *in vitro*.

2.2 Objetivos Específicos

- Determinar a viabilidade celular das linhagens RWPE-1, LNCaP e PC3 após 24, 48 e 72 horas de exposição a duas doses diferentes de finasterida (10 μ M e 50 μ M).
- Determinar se a finasterida é capaz de modular a atividade da MMP2 e da MMP9 nas linhagens RWPE-1, LNCaP e PC3 após 24, 48 e 72 horas de exposição a duas doses diferentes de finasterida (10 μ M e 50 μ M).
- Determinar se a finasterida é capaz de modular a expressão de TIMP-1 e TIMP-2 nas linhagens RWPE-1, LNCaP e PC3 após 24, 48 e 72 horas de exposição a duas doses diferentes de finasterida (10 μ M e 50 μ M).
- Determinar se a finasterida é capaz de interferir no potencial migratório das linhagens RWPE-1, LNCaP e PC3 após 72 horas de exposição à finasterida na dose de 50 μ M.
- Determinar se a finasterida é capaz de interferir com o potencial invasivo das linhagens RWPE-1, LNCaP e PC-3 após 72 horas de exposição à finasterida na dose de 50 μ M.
- Determinar se a fibronectina é capaz de modular a atividade da MMP2 e da MMP9.

Resultados



Os resultados desta tese apresentados em artigos científicos que foram ou serão submetidos à publicação em periódicos nas áreas de biologia celular, matriz extracelular e pesquisas em câncer de próstata. Estes artigos foram denominados Artigo 1 e Artigo 2.

Artigo 1 – Submetido para publicação no periódico *PLOS ONE*

Cover Letter



25/01/ 2013

Bandana Chatterjee, Ph.D.
University of Texas Health Science Center, San Antonio, TX.
UNITED STATES OF AMERICA

Dear **Dr.Chatterjee**,

Please accept the manuscript "***Finasteride inhibits human prostate cancer cell invasion through MMP2 and MMP9 downregulation***" for publication, by consideration of the Editorial Board of ***PLOS ONE***.

In the intention to ascertain if finasteride could modulate the migration and invasion of human prostatic cells, we investigated the MMP2 and MMP9 activity, TIMP-1 and TIMP-2 expression, and the direct in vitro effects of finasteride at the migration and invasion potential, using the tumoral cell lines LNCaP and PC3, and the non-tumoral cell line RWPE-1.

This research article paper demonstrates that while finasteride did not induce cell death, its exposure downregulated MMP2 and MMP9 activity, upregulated the expression of TIMP-2, and significantly inhibited the cells migration and invasion potential, at in vitro assays.

Since finasteride is clinically prescribed for benign prostatic hyperplasia therapy, and was recently contraindicated for prostate cancer prevention by the Food and Drugs Administration, our results have important relevance as it offers additional insights on the effects of this drug on tumor aggressiveness and invasion, critically involved in high-grade prostate cancer. Moreover, given that the *PLOS ONE* has previously accepted papers on this theme, we believe our paper could contribute to this subject.

I certify that it is original and has not been published elsewhere or is being considered for publication by any other journal. All authors have contributed, read, and approved the paper. I thank you in advance for your attention to this manuscript.

Sincerely yours,

Sergio Luis Felisbino, Ph.D.

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Research Article

**Finasteride inhibits human prostate cancer cell invasion through MMP2 and MMP9
downregulation**

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Disclosure Statement: All authors report no conflicts of interest.

Short Title: Finasteride inhibits tumor cell invasion

ABSTRACT

Introduction: The use of 5- α reductase inhibitors (5-ARIs) finasteride and dutasteride for prostate cancer prevention is still under debate. The FDA recently concluded that the increased prevalence of high-grade tumors among 5-ARIs-treated patients must not be neglected, deciding to not allow its use for prostate cancer prevention. This study was conducted to verify the effects of finasteride on prostate cell migration and invasion, and its related enzymes/proteins, by human normal and tumoral prostatic cell lines. **Materials and Methods:** RWPE-1, LNCaP and PC3 cells were cultivated until 60% confluence and further exposed for different periods to either 10 μ M or 50 μ M finasteride, diluted at culture medium. Cells viability, migration and invasion were analyzed. The conditioned media was collected and MMP2 and MMP9 activities, and TIMP-1 and TIMP-2 protein expression were determined. Experiments were carried out in triplicates. **Results:** Cell viability was not affected after finasteride exposure. Finasteride significantly downregulated MMP2 and MMP9 activities in the conditioned media from RWPE-1 and PC3 cells, and upregulated TIMP-2 expression from RWPE-1 cells. Cell invasion was inhibited by finasteride 50 μ M exposure at all tested cell lines, and migration only in RWPE-1 and LNCaP. **Conclusions:** Although the debate around higher incidence of high-grade prostate cancer among 5-ARIs-treated patients remains, our findings supports that finasteride may exert attenuation effects on tumor aggressiveness and invasion, which could vary depending on the phenotype of patients' prostate cells, regarding to androgen responsiveness status.

Keywords: Prostate cancer, Finasteride, Migration, Invasion, MMP2, MMP9.

INTRODUCTION

Prostate cancer is the most common malignancy in men and accounts for \$8 billion and an average cost of \$81658 per-patient, from diagnosis to death in the USA [1]. In this sense, there are currently a number of agents for the prevention of prostate cancer under investigation [2]. Finasteride, a type 2 5- α reductase inhibitor (5-ARI) that blocks the conversion of testosterone (T) to dihydrotestosterone (DHT) [3], is a well known drug used for benign prostatic hyperplasia treatment [4], and had been also suggested to act as a prostate cancer chemopreventive agent. Prostate Cancer Prevention Trial (PCPT) demonstrated a 24.8% reduction in overall and low-grade prostate cancers risk with the administration of finasteride. However, high-grade cancers were noted in 6.4% of finasteride treated patients compared to 5.1% of men receiving placebo [5,6]. This finding led to an important question, Did finasteride induce high-grade cancer or increase its detection? This question was followed by an intense debate about factual or artifactual overestimation of high-grade cases in the finasteride-treated patients [3,7] which divided the urologists and prostate researchers. More recently, the REDUCE trial reported similar outcomes after 5-ARI dutasteride treatment. Recognizing the importance of this issue, the Food and Drug Administration (FDA) has recently revised the data from the PCPT and REDUCE trials and concluded that finasteride and dutasteride treatments may increase the risk of a more serious form of prostate cancer, deciding to not allow their use for prostate cancer prevention [8].

The high-grade prostate cancer cases, such as those observed in the 5-ARIs-treated patients, are commonly associated with an increased expression of matrix metalloproteinases (MMPs), a family of zinc and calcium dependant endopeptidases responsible for extracellular matrix (ECM) remodeling which also contributes to invasive

and metastatic phenotypes of the prostate cancer cells [9-12], and decreased expression of tissue inhibitor of matrix metalloproteinases (TIMPs) [12], a class of naturally occurring inhibitors of MMPs, that tightly regulates their activity, and are expressed in a variety of cell types [10].

Since ECM degradation is known to be a major step during cancer progression [9,11,12], our group has been investigating the finasteride effects upon MMPs and TIMPs modulation, that could explain why finasteride-treated patients had higher grade prostate cancer cases. We have previously demonstrated that finasteride treatment increased the expression of MMP9 and decreased the expression of MMP2 in the rat ventral prostate [13,14], and that it downregulated the mRNA levels of TIMP-1 and -2 in the rat ventral prostate [14]. Moreover, we have recently demonstrated that finasteride also reduce the MMP2 gelatinolytic activity in a variety of human prostate cell lines [15]. In this sense, we conducted the present study to ascertain whether finasteride treatment interferes with the migration and invasive potential of the human prostate normal RWPE-1 and tumoral LNCaP and PC3 epithelial cells that bear different androgen receptor (AR) profiles.

MATERIALS AND METHODS

Cell Cultures. RWPE-1 (non-tumoral, AR⁺), LNCaP (tumoral, AR⁺), and PC3 (tumoral, AR⁻) cell lines were acquired from American Type Culture Collection (ATCCTM), one vial of each cell line. The experiments were performed two months after the acquisition of the refereed cell lines. The RWPE-1 cells were cultivated using the Keratinocyte Serum Free Medium (InvitrogenTM), which is supplemented with 0.05 mg/ml bovine pituitary extract, 5 ng/ml recombinant human epidermal growth factor (EGF), and 1% antibiotic/antimycotic solution (InvitrogenTM). The LNCaP and PC3 cells were cultivated using RPMI medium

(Invitrogen™) supplemented with 10% fetal bovine serum (SFB) (Invitrogen™) and 1% antibiotic/antimycotic solution (Invitrogen™). All cell culture procedures were performed under strict sterile conditions and kept inside a 5% CO₂ incubator (Thermo Scientific™). The initial vial of each cell line was expanded up to 10 vials and individually cryopreserved in the respective cell culture medium, supplemented with 20% FBS and 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich™), to constitute the cell bank.

Finasteride Exposure and Cell Viability. All cell lines were then thawed and individually seeded at 2×10^4 cells/cm² in 6-well culture plates (TPP™). Experiments were carried out as triplicates. Upon reaching 60% confluence, cells were washed three times with sterile D-PBS (GIBCO/Invitrogen™), and the recommended culture medium (FBS free) was added as follows: 1) control treatment, supplemented with 0.1% DMSO; 2) low dose finasteride treatment, supplemented with 0.1% DMSO plus 10μM finasteride (Sigma™); and 3) high dose finasteride treatment, supplemented with 0.1% DMSO plus 50μM finasteride (Sigma™). Finasteride was diluted at DMSO, and the DMSO/Finasteride solution was diluted at the culture medium. During all the finasteride exposure treatments, cells were observed under a phase-contrast inverted microscope (Zeiss™) for general morphology and bacterial and fungal contamination inspections. After 24, 48, and 72 hours of exposure, the conditioned medium (CM) was collected and individually stored at -80°C. The remaining attached cells were individually retrieved by trypsin digestion and an aliquot was viability accessed by Trypan Blue staining. The remaining cells were individually centrifuged at 1200 rpm for 10 minutes to obtain a cell pellet to produce protein extracts. FBS is removed during finasteride exposure since its known to content high levels of MMPs, which would interfere with the following gelatinolytic assays.

Conditioned Medium and Cell Extracts Processing. CM was concentrated 10X using the Centriprep[®] 10,000 MWCO (Millipore[™]) tubes by centrifugation at 4900 RPM during 30 minutes. The respective cell pellets were submitted to protein extraction using a in house produced 50mM Tris-HCl, 0.2M NaCl, 10mM CaCl₂, 0.1% Triton X-100 protein extraction buffer supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich[™]) to obtain cell extracts. The protein extraction buffer was chosen after a comparison with a commercially available buffer (RIPA Buffer, Thermo Scientific[™]) which revealed better preserved MMP gelatinolytic activity by zymography (data not shown). Protein extraction was conducted by repetitive pipetting and vortexing, followed by 1 hour of rest period on ice and a final centrifugation at 5000 RPM during 20 minutes at 4°C. Finally, CM and protein extracts were submitted to protein quantification at a NanoDrop 2000 (Thermo Cientific[™]) spectrophotometer using the Protein A280/260 protocol. It is important to emphasize that the CM was FBS free; therefore, all protein on the CM was produced by the cells.

MMP2 and MMP9 Activity Assays. CM and cell extracts were submitted to measurement of total (pro-form + active form) of MMP2 and MMP9 activity using the respective Biotrak[®] Activity Assays (GE Healthcare LifeSciences[™]) according to manufacturer guidelines. Briefly, equal amounts (100µg) of pooled CM or cell extract (per triplicate treatment) was pipetted at a 96 microplate coated with anti-human-MMP2 or anti-human-MMP9 antibodies. Standards of human recombinant pro-MM2 or pro-MMP9 were pipetted in the respective plate wells, with values ranging from 0.19 to 3ng/ml (for MMP2) and 0.125 to 4ng/ml (for MMP9). These sensitivity range values were chosen after testing the less sensible ranges of 0.75 to 12ng/mL (for MMP2) and 0.5 to 16ng/mL (for MMP9). After an overnight incubation at 4°C and washing, 50µL of a 0.5mM p-aminophenylmercuric acetate

(APMA) solution was pipetted to all wells to activate the pro-forms of MMP2 and MMP9. Subsequently, 50 μ L of a detection reagent (modified urokinase + S-2444TM peptide substrate) was added to all wells. The plates were incubated at 37°C during 6 hours (for MMP2) or 2 hours (for MMP9) and the IOD was measured at a 405nm capable spectrophotometric plate reader. Finally, a fold-change graphic was made by dividing the obtained IODs from treated CM/extract by their respective control values.

TIMP-1 and TIMP-2 Protein Quantification. CM were submitted to measurement of TIMP-1 and TIMP-2 proteins using the respective solid-phase ELISA Biotrak[®] Human Assays (GE Healthcare LifeSciencesTM) according to manufacturer guidelines. Briefly, equal amounts (100 μ g) of pooled CM (per triplicate treatment) was pipetted at a 96 microplate coated with anti-human-TIMP-1 or anti-human-TIMP-2 antibodies. Standards of human recombinant TIMP-1 or TIMP-2 were pipetted in the respective plate wells, with values ranging from 3.13 to 50ng/ml (for TIMP-1) and 8 to 128ng/ml (for TIMP-2). After 2 hour incubation at 25°C and washing, 100 μ L of a peroxidase conjugate solution was pipetted to all wells and the plates were further incubated for 2 hours at 25°C. Subsequently, 100 μ L of tetramethylbenzidine (TMB)/hydrogen peroxide solution was added to all wells. The plates were incubated at room temperature during 30 minutes and the IOD was measured at a 630nm capable spectrophotometric plate reader. Finally, a fold-change graphic was made by dividing the obtained IODs from treated treatment by their respective control values.

Migration Assay. The migration potential of all cell lines was investigated using the BD BioCoatTM Migration Insert (BD BiosciencesTM). The porous membrane (8 μ m pore size) was hydrated with 500 μ l of serum-free bicarbonate based medium for 2 hours. After

hydration, 500µl of RPMI1640 media with 5% FBS (for LNCaP and PC3 cells) or 500µl of keratinocyte medium with 5% FBS (for RWPE-1 cells) was added to the lower chamber of the 24 well-plate. The prostate cell lines (RWPE-1, LNCaP or PC3) were previously cultivated under 50µM finasteride medium or control medium for 72 hours, harvested, and individually cultivated in 200µl serum free medium (with or without 50µM finasteride) in the insert at a density of 1×10^5 total cells. The plates were incubated at an incubator with 5% CO₂ at 37°C for 22h, according to manufacturer guidelines. The cells that did not migrate, on the top of the insert membrane, were scraped off with a cotton swab. The cells which migrated through the membrane pores, attracted by the FBS, were fixed in 100% methanol then stained with 0.1% toluidine blue solution for 2min. The migrated cells were digitally photographed under 200X magnification using a light microscope (Nikon™). The experiments were performed in triplicate and 5 random fields were photographed and subjected to cell counting at the ImageJ® free software. Migration index was calculated as the mean $\pm$ SD of cells counted per-field and control cells were compared to finasteride treated cells. Human skin fibroblasts (WS1 – ATCC) were employed as positive controls, under the same assay conditions as prostate cells.

Wound Healing Assay. PC3 cells were further investigated with a different migration assay. These cells were cultivated in 6-well plates at 4×10^4 cells/cm² until 100% confluence was achieved. The monolayers were wounded (scratched) in a straight line across the well with a 200µl pipette tip, using a sterile 6-well culture plate lid as a ruler, to obtain a regular scratch. The wounded monolayers were then washed twice with D-PBS (GIBCO/Invitrogen™) to remove cell debris. Culture medium with or without 50µM finasteride was added to control and treatment wells. The wound area was subsequently

inspected after 24, 48, 72h, and 96h using an inverted phase contrast microscope with digital camera. The wound healing speed was calculated as the percentage of the initial wound until total wound closure, at the different time-points, with the ImageJ[®] free software.

Matrigel Invasion Assay. The invasive potential of all cell lines was investigated using the BD BioCoat[™] Matrigel[™] Invasion Inserts (BD Biosciences[™]). The matrigel-coated porous membrane (8µm pore size) was hydrated with 500µl of serum-free bicarbonate based medium for 2 hours. After hydration, 500µl of RPMI1640 media with 5% FBS (for LNCaP and PC3 cells) or 500µl of keratinocyte medium with 5% FBS (for RWPE-1 cells) was added to the lower chamber of the 24 well-plate. The prostate cell lines (RWPE-1, LNCaP or PC3) were previously cultivated under 50µM finasteride medium or control medium for 72 hours, harvested, and individually cultivated in 200µl serum free medium (with or without 50µM finasteride) in the insert at a density of 1×10^5 total cells. The plates were incubated at an incubator with 5% CO₂ at 37°C for 22h, according to manufacturer guidelines. The uninvaded cells on the top of the insert membrane were scraped off with a cotton swab. The cells which invaded the Matrigel matrix and migrated to the opposite side of the membrane, attracted by the FBS, were fixed in 100% methanol then stained with 0.1% toluidine blue solution for 2min. The invaded cells were digitally photographed under 200X magnification using a light microscope (Nikon[™]). The experiments were performed in triplicate and 5 random fields were photographed and subjected to cell counting at the ImageJ[®] free software. Invasion index was calculated as the mean $\pm$ SD of cells counted per-field and control cells were compared to finasteride treated cells.

Statistical Analysis. The obtained data was analyzed with INSTATTM software using the two tail Student's T test ($P < 0.05$) to compare the different treatments with their respective controls.

RESULTS

Finasteride Exposure and Cell Viability. Cell viability was not affected by either finasteride doses employed at the present investigation. Cells did not present aspects of cell death and loss of viability, even after 72h of 50 μ M finasteride exposure (Figure 1). Values obtained from the trypan blue assessment are summarized at Table 1.

MMP2 and MMP9 Activity Assays. On control conditions, it was observed that PC3 cells secrete twice MMP2 and MMP9 activities in their CM, in comparison with RWPE-1 cells (IOD values – data not shown). The LNCaP cells did not secrete any detectable levels of MMP2 and 9 activities on their CM (data not shown). For cell extracts, neither cell lines presented detectable MMP2 and 9 activities, demonstrating that these cell lines do not hold MMPs at their membrane (data not shown).

Upon finasteride exposure, it is possible to observe that for RWPE-1 cells, at the low-dose employed (10 μ M), only 72 hours of exposure are able to induce downregulation of the both MMPs activity at the CM (Figure 2a), reduced by 25% and 30%, for MMP2 and MMP9, respectively, compared to control values. On the other hand, for the employed finasteride high-dose (50 μ M), MMP2 and 9 activities were downregulated at all time points tested, up to 90% and 55% after 72 hours of exposure, respectively (Figure 2a).

For the PC3 cell line, low dose finasteride exposure induced downregulation of MMP2 and 9 activities only at 24h time point (Figure 3a). On the other hand, the high dose

finasteride induced downregulation of MMP2 at the 48h and 72h time points, and MMP9 at all time points assessed, up to 70% of reduction (Figure 3a).

TIMP-1 and TIMP-2 Protein Quantification. When the cell lines were cultivated without finasteride, PC3 cells produced two times more TIMP-1 than RWPE-1 cells, while the amount of TIMP-2 was the same (IOD values). LNCaP cells produced low levels of TIMPs 1 and -2, nearing the lower detection limit of the kit assay (data not shown).

Upon finasteride exposure, for the tested RWPE-1 cell line, both doses induced upregulation of TIMP-2 expression at the 72h time point, up to 150% more expression than control treatment (Figure 2b). As for PC3 cells, TIMP-1 and -2 expressions showed no significant modulation, except for the 10 μ M finasteride dose at 24h of exposure (Figure 3b). Finasteride did not induce any significant changes in the expression of TIMP-1 and -2 in the LNCaP cell line (data not shown).

Migration Assay. Under regular culture medium, the cell lines tested exhibited different migration potential. RWPE-1 cells migrated more (mean of 32 cells/field) than PC3 cells (mean of 24 cells/field), which in turn migrated more than LNCaP cells (mean of 8 cells/field) (Figure 4). After 72h of finasteride exposure (50 μ M), migration was inhibited significantly in RWPE-1 cells ($p < 0.01$) and LNCaP cells ($p < 0.05$). PC3 cell migration was slightly inhibited, however not significantly ($p > 0.05$) (Figure 4). Human WS1 fibroblasts (positive control) migrated promptly through the membranes (Figure 4).

Wound Healing Assay. To confirm that PC3 cells migration was not influenced by finasteride exposure, a different migration assay was performed for this cell line. After the wound area was inflicted at the monolayers, it was possible to observe that finasteride

slightly inhibited cell migration (Figure 5a), however, this difference was not significant (Figure 5b), confirming the results obtained at the previous migration insert assay (Figure 4).

Matrigel Invasion Assay. Under regular culture medium, the cell lines exhibited different invasion potential. PC3 was the most invasive cell line, with a mean of 75 cells/field (Figure 6). The other tumoral cell line, LNCaP, curiously exhibited very low invasive potential, with a mean of 5 cells/field (Figure 6). The non-tumoral cell line RWPE-1 also exhibited low invasive potential, as expected, with a mean of 9 cells/field (Figure 6).

Finasteride inhibited cell invasion in all cell lines tested. PC3 cell invasion was significantly reduced ($p < 0.001$), followed by RWPE-1 ($p < 0.01$) and LNCaP ($p < 0.05$) (Figure 6).

DISCUSSION

In this study, we evaluated the effects of finasteride at the prostate cells lines RWPE-1, LNCaP and PC3, on different doses and for different periods of exposure. Initially, we demonstrated that finasteride did not induced substantial cell death, at all tested lines, under the experimental conditions and techniques employed. The maximum significant differences in cell viability, between control and treated cells, were not higher than 10%, considered normal at short time cell culture experiments. Although it was previously reported that finasteride induces apoptosis in LNCaP cells [16,17] the MTT technique employed on both papers is merely a proliferation assay, that however suitable for anti-proliferative drug screening [18], can lead to misleading of data when employed as a viability assay, without proper backup assays. This is highlighted in some reports, in

which the authors [4] first claim that finasteride reduces the viability of LNCaP cells (using the MTT assay), and immediately claim it reduces RWPE-1 cell growth (using the same MTT assay), an entirely different parameter. Therefore, although being a very basic technique, the Trypan blue staining is more suitable for viability assessment, since its membrane integrity characteristic makes only non-viable cells permissive to the dye [19]. Moreover, there are published reports that finasteride does not alter the expression profile of important proteins, commonly used as apoptotic markers, such as caspase-3, insulin growth factor-1, Bcl-2, p53 and p21; and that its exposure leads to downregulation of other apoptotic factors, such as caspase-7 and IGFBP-3, in prostate cancer samples from patients undergoing radical prostatectomy [20]. More evidence that finasteride may not always induce cell death was provided by Sawaya *et al.*, (2002) who showed that its exposure decreased the levels of caspase detected at alopecia treated patients, compared to non-affected individuals [21].

In the following experiments, we confirmed a recently published report by Moroz *et al.*, (2013) that when these cell lines are cultivated with regular culture medium, all of them displays low activities of MMP2 and 9 in their CM [15], even with more sensible methods, such as these employed on the present report, compared to regular gelatin zymography. We also demonstrated that this activity is restricted at the CM, since neither tested cell lines exhibited MMP2 and 9 activities at their cell extracts. Interestingly, the obtained IODs from the control CMs did not respect the tumoral grading of these cell lines: although the most aggressive cell line PC3 had more MMPs activities, the non-tumoral cell line RWPE-1 had more MMPs activities than the tumoral cell line LNCaP.

However, when the cell cultures were performed with diluted finasteride, PC3 and RWPE-1 exhibited significantly lower MMP2 and 9 activities on their CM. Furthermore,

our results demonstrated that only the high dose finasteride (50 μ M) was able to downregulate MMP2 and 9 activities on these cell lines, as previously reported [15]. After these observations, it was expected that the expression of TIMPs -1 and -2 could be modulated by finasteride, since the activities of MMPs were. We then proceeded to quantify the amount of TIMP-1 and -2 on the CM of these cells, under control and finasteride treated conditions. Again, the LNCaP cell line did not express significant TIMP-1 and -2 in their CM. The expression of TIMP-1 was higher in PC3 cells than in RWPE-1 cells, while the expression of TIMP-2 was similar at both cell lines, at control treated CMs. Although finasteride (50 μ M) induced downregulation of MMPs activity at PC3 and RWPE-1 cells, this modulation may occur via different mechanisms among the cells, such as upregulation of TIMP-1 and -2 at RWPE-1 cells or reduced expression of MMPs by PC3 cells. While these findings are relevant, one question was still unsolved: could finasteride induce high-grade prostate cancer? Therefore, the next experiment aimed to verify if it could directly modulate cell migration and invasion, important features for tumor metastasis to occur, and consequently, high-grade prostate cancer cases [22,23].

In this sense, finasteride, when employed at a high-dose, inhibits cell migration at the RWPE-1 and LNCaP cell lines, but not for PC3 cell line at both migration assays. Even though cell migration is an important feature for the success of cells to metastasize, cell invasion is mandatory for tumor cells to migrate through the ECM [9,11,15] in order to gain access to blood or lymph vessels, and ultimately reach circulation. In this sense, we have exposed all cell lines to 50 μ M finasteride, during 72 hours, and then subjected them to another 22 hours of finasteride exposure during the transwell-MatrigelTM invasion assay. Initially, we could observe that untreated LNCaP cells have about one-tenth of the invasion potential of untreated PC3 cells, as reported previously [23,24], and more importantly, our

results clearly demonstrate that finasteride significantly inhibits prostate cancer cell invasion at all tested cell lines. Others studies using vascular smooth muscle cells (AR⁺) and fibroblasts (AR⁺) have reported consistent inhibition of migration speed by flutamide and bicalutamide, respectively [25, 26]. Even though in the present study the cells were exposed to finasteride in a FBS-free medium, these cell lines are able to synthesize T even in a FBS-starved medium environment, acquiring complete steroidogenic ability to produce androgens [27]. In this sense, our findings confirm these previous reports [25-26], once it should be expected that the DHT would stimulate cells to migrate and invade more than T does, which does not occur on a finasteride-rich microenvironment.

Intriguingly, it is well known and reported that these three cell lines present different integrity of the androgenic signaling machinery, mainly in terms of AR mRNA and protein expression [28-32] and finasteride-treatment induced a similar result of reduced Matrigel™ invasion ability by these cell lines. Even though it is accepted that there may be a role for AR in the regulation of cell migration and invasiveness [26,33,34], the similar results obtained at the present work suggests an effect via a non-canonical mechanism of action of finasteride over PC3 cells, which remains to be confirmed by future investigations.

CONCLUSION

Although the debate around higher incidence of high-grade prostate cancer among 5-ARIs-treated patients remains, our findings supports that finasteride may exert attenuation effects on tumor aggressiveness and invasion, which could vary depending on the phenotype of patients' prostate cells, mainly regarding to their cell's androgen responsiveness status. Although these results may seem beneficial for finasteride-treated

patients, future studies are necessary to elucidate the mechanism by which finasteride exerts its effects upon prostate androgen-independent cells.

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FIGURES

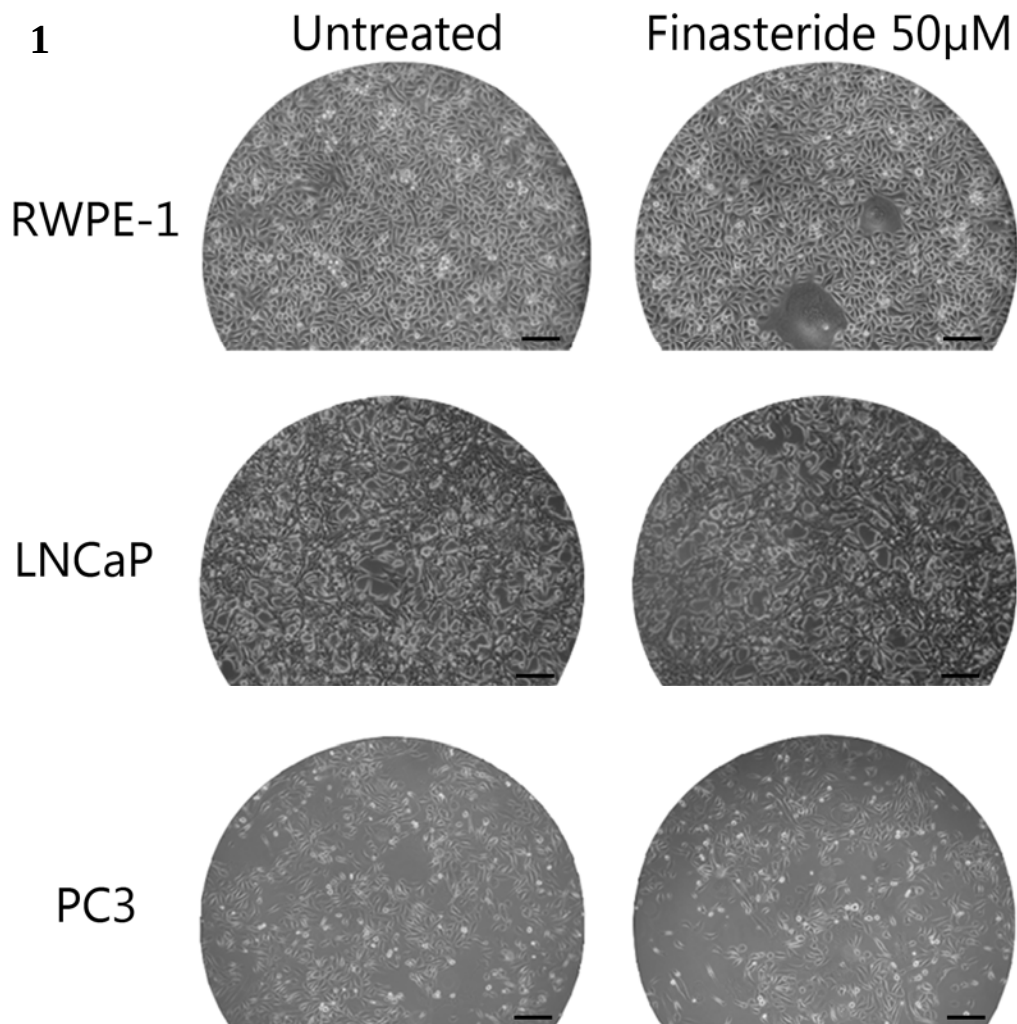


Figure 1. Representative images of human prostate cells morphology and cell density, after 72h of finasteride exposure (50 μ M). Prostate cell lines RWPE-1, LNCaP and PC3 were plated at 2×10^4 cells/cm², cultivated until reaching 60% confluence, and exposed to different finasteride exposures. General morphology was similar between untreated and finasteride-treated cells, as well the number of cells/cm². No significant apoptotic or necrotic morphological characteristics were observed. Scale bar = 100 μ m.

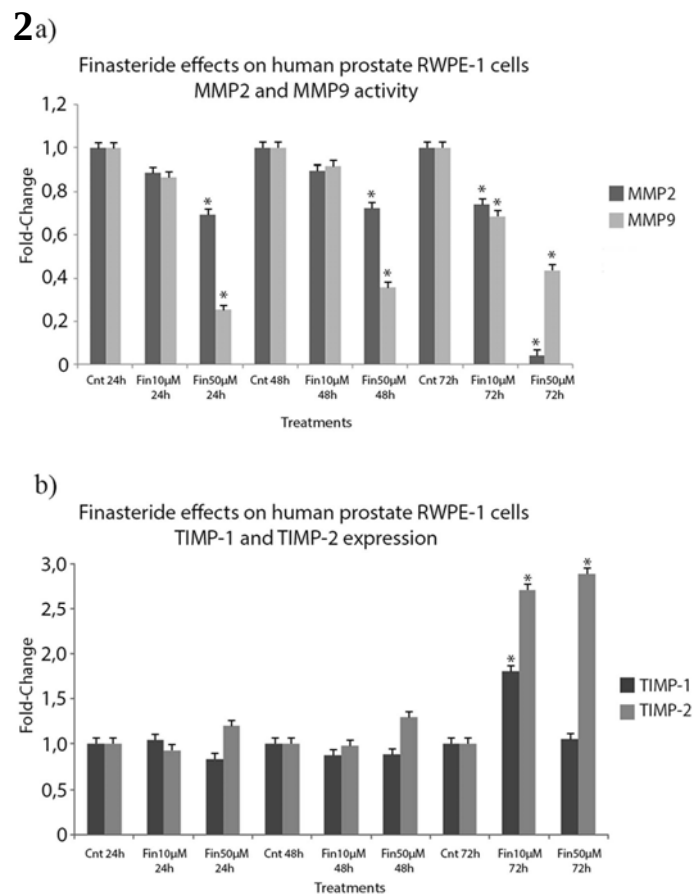


Figure 2. Finasteride effects on MMP2 and MMP9 activity and TIMP-1 and TIMP-2 expression of RWPE-1 cells. **a)** Conditioned medium of untreated (Control) and finasteride treated RWPE-1 cells were collected, concentrated and analyzed for MMP2 and 9 activities using the respective Biotrak[®] Activity Assays. Low-dose finasteride (10μM) during 72 hours of exposure downregulated MMP2 and MMP9 activity by 25% and 30%, respectively, compared to control values. Finasteride high-dose (50μM) downregulated MMP2 and 9 activities at all time points tested, up to 90% and 55% after 72 hours of exposure, respectively. **b)** Conditioned medium of untreated (Control) and finasteride treated RWPE-1 cells were collected, concentrated and analyzed for TIMP-1 and TIMP-2 protein expression using the respective Biotrak[®] Assays. Finasteride exposure, on both

doses, induced upregulation of TIMP-2 expression at the 72h time point, up to 150% more expression than control levels. Data are expressed as a fold-change graphic of the IOD values obtained for finasteride treated cells over control cells. (*) Statistically significant values with $p < 0.05$

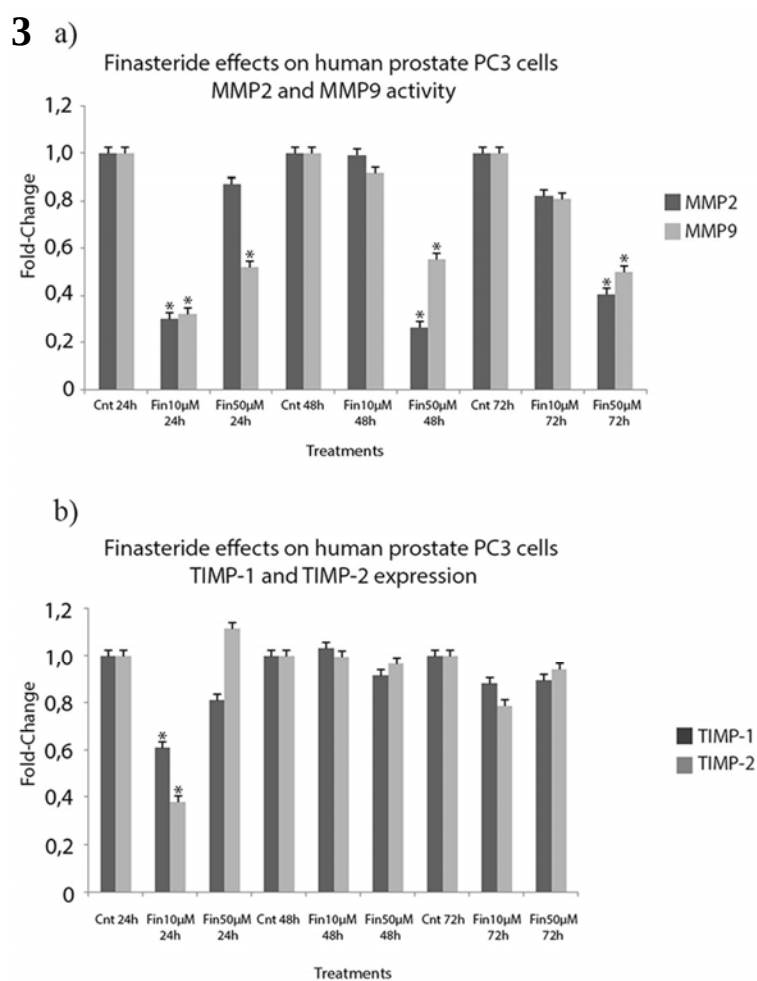


Figure 3. Finasteride effects on MMP2 and MMP9 activity and TIMP-1 and TIMP-2 expression of PC3 cells. **a)** Conditioned medium of untreated and finasteride treated PC3 cells were collected, concentrated and analyzed for MMP2 and 9 activities using the respective Biotrak[®] Activity Assays. Low dose finasteride exposure (10µM) did not

induced downregulation of MMP2 and 9 activities, except for the 24h time point. High dose finasteride induced downregulation of MMP2 at the 48h and 72h time points, and MMP9 at all time points assessed, up to 70% of reduction. **b)** Conditioned medium of untreated (Control) and finasteride treated PC3 cells were collected, concentrated and analyzed for TIMP-1 and TIMP-2 protein expression using the respective Biotrak[®] Assays. Finasteride exposure did not induce any significant modulation upon TIMP-1 and -2 expressions, except for the 10 μ M finasteride dose at 24h of exposure. Data are expressed as a fold-change graphic of the IOD values obtained for finasteride treated cells over control cells. (*) Statistically significant values with $p < 0.05$

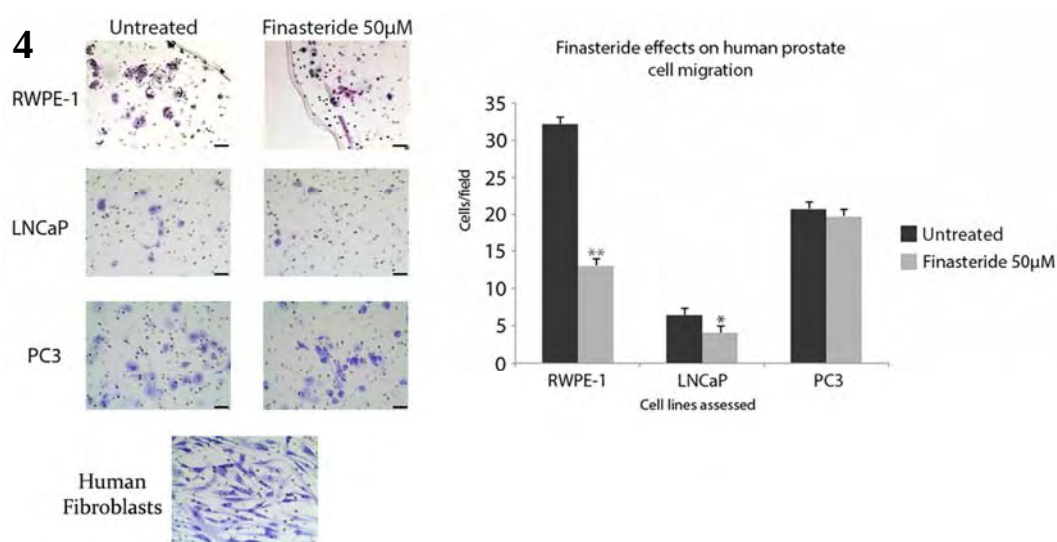


Figure 4. Finasteride effects on the migration of human prostate cell lines RWPE-1, LNCaP and PC3. These prostate cell lines were previously cultivated under 50 μ M finasteride medium or control medium for 72 hours and individually cultivated in 200 μ l serum free medium (with or without 50 μ M finasteride) in the migration insert at a density of 1×10^5 total cells. After 22h, cells that migrated through the 8 μ porous membrane were

fixed, stained and counted at five random fields on a light microscope. Experiments were performed as triplicates. Representative photomicrographs of untreated and finasteride treated cells are shown in the left side. Human WS-1 fibroblasts were employed as migration-positive control cells, and a representative image is shown in the bottom-left side. Scale bar = 40 μ m. Finasteride significantly inhibited the cell migration of RWPE-1 and LNCaP cell lines, but not PC3 cell line. Data are expressed as the mean $\pm$ SD of the migrating cells. (*) Statistically significant values with $p < 0.05$; (**) statistically significant values with $p < 0.01$

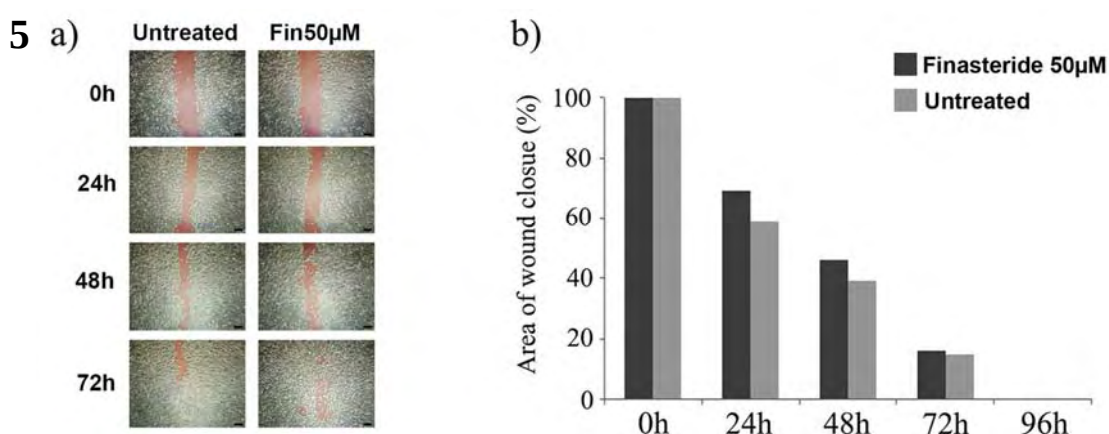


Figure 5. Finasteride effects on the migration of PC3 cell line on the wound closure migration assay. Cells were cultivated in 6-well plates at 4×10^4 cells/cm² until 100% confluence was achieved and the monolayers were scratched in a straight line. The wounded monolayers were then given culture medium with or without 50 μ M finasteride. The wound area was inspected after 24, 48, 72h, and 96h using an inverted phase contrast microscope with digital camera. **a)** Representative images showing the wound closure of finasteride-treated and control cells, at 0h to 72h. Red highlighted areas represent the open

wound area. Scale bar = 50µm. **b)** The initial red highlighted areas were measured with the ImageJ™ software and the remaining areas were calculated as a percentage of the initial wound area. A wound closure graphic was made by dividing the area values obtained at the indicated time points, for control and finasteride-treated cells. Initial wound areas (0h) were given the value of 100%. While finasteride slightly inhibited cell migration, this was not significant ($p > 0.05$).

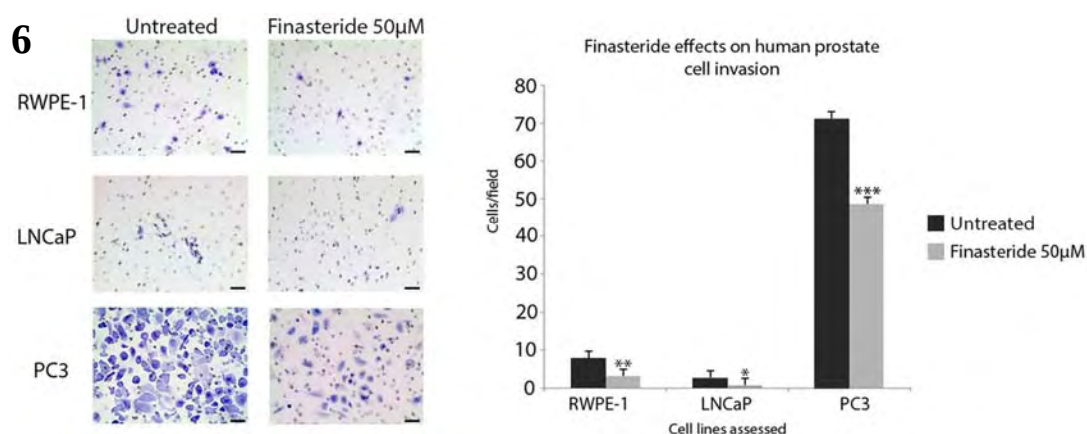


Figure 6. Finasteride effects on the invasion of human prostate cell lines RWPE-1, LNCaP and PC3. These prostate cell lines were previously cultivated under 50µM finasteride medium or control medium for 72 hours and individually cultivated in 200µl serum free medium (with or without 50µM finasteride) in the Matrigel® invasion insert at a density of 1×10^5 total cells. After 22h, cells that invaded through the matrigel matrix and porous membrane were fixed, stained and counted at five random fields on a light microscope. Experiments were performed as triplicates. Representative images of untreated and finasteride treated cells are shown in the left side. Scale bar = 40µm. Finasteride significantly inhibited the invasion capability of RWPE-1, LNCaP and PC3 cell lines. Data are expressed as the mean $\pm$ SD of the invading cells. (*) Statistically significant values

with $p < 0.05$; (**) statistically significant values with $p < 0.01$; (***) statistically significant values with $p < 0.001$

TABLES

Table 1
Finasteride effects on viability (%) of human prostate cancer cell lines RWPE-1, LNCaP and PC3.

Cell Lines	Treatments			
	Control	Finasteride 10 μ M	Control	Finasteride 50 μ M
RWPE-1				
24h	94.83 $\pm$ 2.07	94.82 $\pm$ 1.48	93.25 $\pm$ 2.04	93.25 $\pm$ 0.53
48h	85.3 $\pm$ 15.87	77.86 $\pm$ 14.65	95.20 $\pm$ 2.58	94.56 $\pm$ 1.91
72h	89.6 $\pm$ 7.66	92.13 $\pm$ 2.15	88.64 $\pm$ 1.68	80.22 $\pm$ 3.81
LNCaP				
24h	97.82 $\pm$ 1.36	92.71 $\pm$ 5.59	95.20 $\pm$ 2.55	86.23 $\pm$ 0.94
48h	97.41 $\pm$ 0.74	91.5 $\pm$ 4.33	97.68 $\pm$ 1.79	97.21 $\pm$ 1.37
72h	91.53 $\pm$ 0.13	87.91 $\pm$ 2.76	93.51 $\pm$ 3.57	66.09 $\pm$ 30.11
PC3				
24h	91.98 $\pm$ 4.71	94.87 $\pm$ 0.86	93.39 $\pm$ 1.45	95.2 $\pm$ 0.82
48h	96.32 $\pm$ 3.21	95.51 $\pm$ 2.67	92.07 $\pm$ 2.73	90.75 $\pm$ 3.58
72h	94.77 $\pm$ 5.81	94.15 $\pm$ 0.82	96.09 $\pm$ 2.21	92.66 $\pm$ 2.70

Means $\pm$ SD.

Artigo 2 – Publicado no periódico *Biochemical and Biophysical Research Communications*

Short communication

Fibronectin induces MMP2 expression in human prostate cancer cells

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ABSTRACT

High-grade prostate cancers express high levels of matrix metalloproteinases (MMPs), major enzymes involved in tumor invasion and metastasis. However, the commonly employed tumoral cell lines on prostate cancer research express small amounts of MMPs when cultivated in monolayer cultures, with common culture media. The present study was conducted to verify if culture conditions with fibronectin alters MMP2 and MMP9 expression by human prostatic epithelial cell lines RWPE-1, LNCaP and PC-3. These cells were individually seeded at 2×10^4 cels/cm², cultivated until 80% confluence, and further exposed for 4 hours to fibronectin, after which the conditioned medium was analyzed by gelatin zymography. Untreated cells were given common medium. Only RWPE-1 express detectable amounts of MMP9 when cultivated in common medium, while addition of fibronectin induced high expression of pro and active forms of MMP2 in all tested cell lines. Our findings demonstrate that normal and tumoral prostate cell lines express MMP2 activity when in contact with extracellular matrix components or blood plasma proteins such as fibronectin. Future studies about transcriptomes and proteomes in prostate cancer research using these cell lines should not neglect these important conclusions.

Key words: Fibronectin; MMP2; MMP9, LNCaP, PC-3, RWPE-1.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of male cancer-related deaths in the Western world [1,2]. Castration-resistant prostate cancer (CRPC) is the most advanced prostate cancer exhibiting reduced survival, with reported values varying between 9 and 30 months [2]. In this sense, in vitro culture systems

are widely employed in prostate cancer research and, more recently, for transcriptome and proteomic analysis, since they are fast and easy to reproduce [3]. Although these monolayer cultures provides fast and important results, they recreate only a fraction from a more complex scenario [4], in which the absence of cell-to-extracellular matrix (ECM) interactions can lead to underestimation of results, especially in prostate cancer, where the cross-signaling between epithelium and stroma is so important [5].

Matrix metalloproteinase's (MMPs) are a family of zinc and calcium dependant endopeptidases responsible for the first steps in the ECM degradation in both normal development and diseases [6,7]. A high invasive and metastatic behavior is a major characteristic of CRPC, and ECM degradation is critical to these processes [2,7]. However, the commonly employed tumoral cell lines on prostate cancer research express small amounts of MMPs when cultivated in monolayer cultures with common culture media [8,9], without ECM components.

Considering that cell-ECM cross-talk, when present at culture systems, leads to behavioral changes in tumor cell biology, such as increased adherence to substrates [10], increased survival against chemotherapeutic agents [11], and resistance to apoptosis [12], we hypothesized that it could also modulate the expression of MMPs by human prostate cells. Therefore, we conducted the present investigation exposing the classical human prostate RWPE-1, LNCaP, and PC-3 cell lines to fibronectin, a multifunctional extracellular glycoprotein, which is considered of critical relevance for the process of metastasis and cell survival [13]. Not only fibronectin is the most abundant adhesion protein in the blood, but it also interacts with circulating tumor cells [13], and therefore the ECM protein chosen for this investigation.

Furthermore, employing this culture model, we exposed these cells to finasteride, an inhibitor of the enzyme 5 alpha-reductase widely used in benign prostatic hyperplasia treatment and potentially chemopreventive agent for prostate cancer [14], to verify if in these conditions finasteride would also modulate the expression of MMP2, as previously reported in the rat ventral prostate [15], where epithelial-stroma interactions does occur.

MATERIAL AND METHODS

RWPE-1, LNCaP, and PC-3 cell lines were acquired from American Type Culture Collection (ATCC). The experiments were performed two months after the acquisition of the refereed cell lines, which were cultivated according to ATCC guidelines. These cells were then individually seeded at 2×10^4 cells/cm² in 6-well culture plates (TPP™) and the experiments were carried out as triplicates. Upon reaching 80% confluence, cells were washed three times with sterile D-PBS (GIBCO/Invitrogen™), and the recommended culture medium (FBS free) was added as follows: 1) control treatment - 0.1% dimethylsulfoxide (DMSO); 2) fibronectin treatment - 0.1% DMSO plus 25µg/mL fibronectin (Sigma™); and 3) combined fibronectin/finasteride treatment - 0.1% DMSO plus 25µg/mL fibronectin (Sigma™) plus 50µM finasteride (Sigma™). All reagents were dissolved at the culture medium. After 4 hours of exposure, the conditioned media (CM) were collected and individually stored at -80°C.

CM were concentrated using the Centriprep® 10,000 MWCO (Millipore™) tubes and submitted to protein quantification at a NanoDrop 2000 (Thermo Cientific™) spectrophotometer. Equal amounts of protein from CM (15µg) were analyzed by gelatin zymography on 8% polyacrylamide gels co-polymerized with 0.1% gelatin (Merck™) as

substrate. Purified human MMP2 (20 ng) and MMP9 (30 pg) (Calbiochem™) were also loaded as positive control. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated 24 hours in an activation buffer (50 mM Tris-HCl, 5 mM CaCl₂ and 0.02% ZnCl₂). Gels were stained with Coomassie brilliant blue R-250 and de-stained with 20% methanol and 10% acetic acid in distilled water until the clear bands had been visualized. The activities of the MMP2 and MMP9 bands were quantified using the ImageJ™ software on each sample (as triplicates). The integrated optical density (IOD) was measured and data analyzed with INSTAT™ software using the two tail Student's T test ($P < 0.05$) to compare the different treatments. Values were calculated as mean $\pm$ SD of the totality of IODs for the pro- and active forms of the MMP2 and MMP9 enzymes. Finally, a fold-change graphic was made by dividing the mean of treated by the mean of untreated values.

RESULTS

When cultivated in common culture media, all cell lines produced very low detectable levels of MMP2 activity (Fig. 1a). Fibronectin exposure significantly upregulated MMP2 activity in all cell lines, with a 25.5 fold-change in RWPE-1 cells, a 22.6 fold-change in the LNCaP cells and a 24.0 fold-change in PC-3 cells, when compared with controls (Fig. 1b). However, when these cell lines were co-exposed to fibronectin and finasteride, the activity of MMP2 was significantly lower than those treated only with fibronectin; reduced at least by 33% (Fig. 1b).

Curiously, the LNCaP cells expressed primarily pro-MMP2 while PC-3 active-MMP2 (Fig. 1a), probably related with the PC-3 higher invasive behavior. In contrast, only RWPE-1 cells expressed low but detectable amounts of MMP9, mostly in its pro-form (Fig.

1a). Fibronectin treatment induced partial activation of MMP9, while finasteride reduced this activation (Fig. 1a) ($P>0.05$).

DISCUSSION

In this study, we evaluated the expression of gelatinases A and B in the prostate cells lines RWPE-1, LNCaP and PC-3, which are widely employed in prostate cancer research, using two different culture conditions. In our initial experiments, we demonstrated that when these cells were cultivated as monolayers, using regular culture medium, all of them secreted very low amounts of MMPs in their CM, in accordance to previous results [8,9]. Interestingly, only the non-tumoral cell line RWPE-1 expressed detectable levels of MMP9 under these culture conditions. However, when the cell cultures were performed with fibronectin, which was diluted on culture medium, all tested cell lines exhibited significantly higher MMP2 levels on their CM. While this effect was observed for MMP2 in all tested cell lines, this was not observed for MMP9. Furthermore, our results demonstrated that the co-treatment of these cells, with fibronectin and finasteride, results in the same pattern of downregulation of MMP2 activity after finasteride treatment, as observed in the rat ventral prostate [15], and now reproduced in an in vitro model of human prostate cancer.

Although Das *et al.* [16] reported higher expression of MMPs -2 and 9 upon exposure of human breast cancer cells to fibronectin, and Meng *et al.* [17] reported increased invasion of lung cancer cells through MMP9 activity after fibronectin treatment, this is the first report of enhanced activity of MMPs in prostate cancer cell lines after fibronectin exposure. Our findings highlight the importance of cell-ECM interactions in the modulation of these endopeptidases. Similar results were obtained by Penttala *et al.* [18],

which showed that the interaction of LNCaP cells with the extracellular matrix plays a dominant role in uPA and uPAR gene expression, another protease system involved in ECM degradation [19].

Fibronectin has been shown to support cell proliferation and invasion, which are major events for metastasis, and to protect tumoral cells from the cytotoxic action of natural killer cells [13]. Now we have demonstrated that fibronectin can also upregulate the expression of MMPs that are directly involved in prostate cancer aggressiveness. Interestingly, our findings demonstrated that it could only induce MMP2 activity, but not MMP9, in the tested prostate cell lines. Although MMP2 has been historically considered a constitutive gene due to the absence of well-characterized regulatory elements in the MMP2 promoter region [20], recent reports on the MMP2 promoter analyses have demonstrated numerous regulatory elements that could modulate MMP2 expression [21]. The mechanism by which fibronectin induces MMP2 expression has been described in MCF-7 human breast carcinoma cells and A549 human lung cancer cells, and involves fibronectin–integrin $\alpha 5\beta 1$ interaction and/or FAK/ PI-3K/ERK signaling pathways activation [16, 17], but this mechanism remains to be confirmed for prostate cancer cells, in future studies.

Taken together, all these reports highlight the importance of the cell-ECM interactions for studding transcriptomes and proteomes from both normal and tumoral cells, at in vitro systems [3-5, 8-10, 13, 16-18]. In this sense, future investigators should be aware that RWPE-1, LNCaP and PC-3 cells, and probably other tumor cells, require cell-ECM interaction in order to express detectable levels of MMPs on their CM.

In conclusion, our results confirm that: 1) fibronectin exposure upregulates the activity of MMP2 in all tested human prostate cell lines up to 25 fold-increase and 2) by

employing a human cell culture model with cell-ECM interaction, it was possible to reproduce previous results of an *in vivo* study where finasteride downregulated the activity of MMP2 in the rat ventral prostate.

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FIGURES

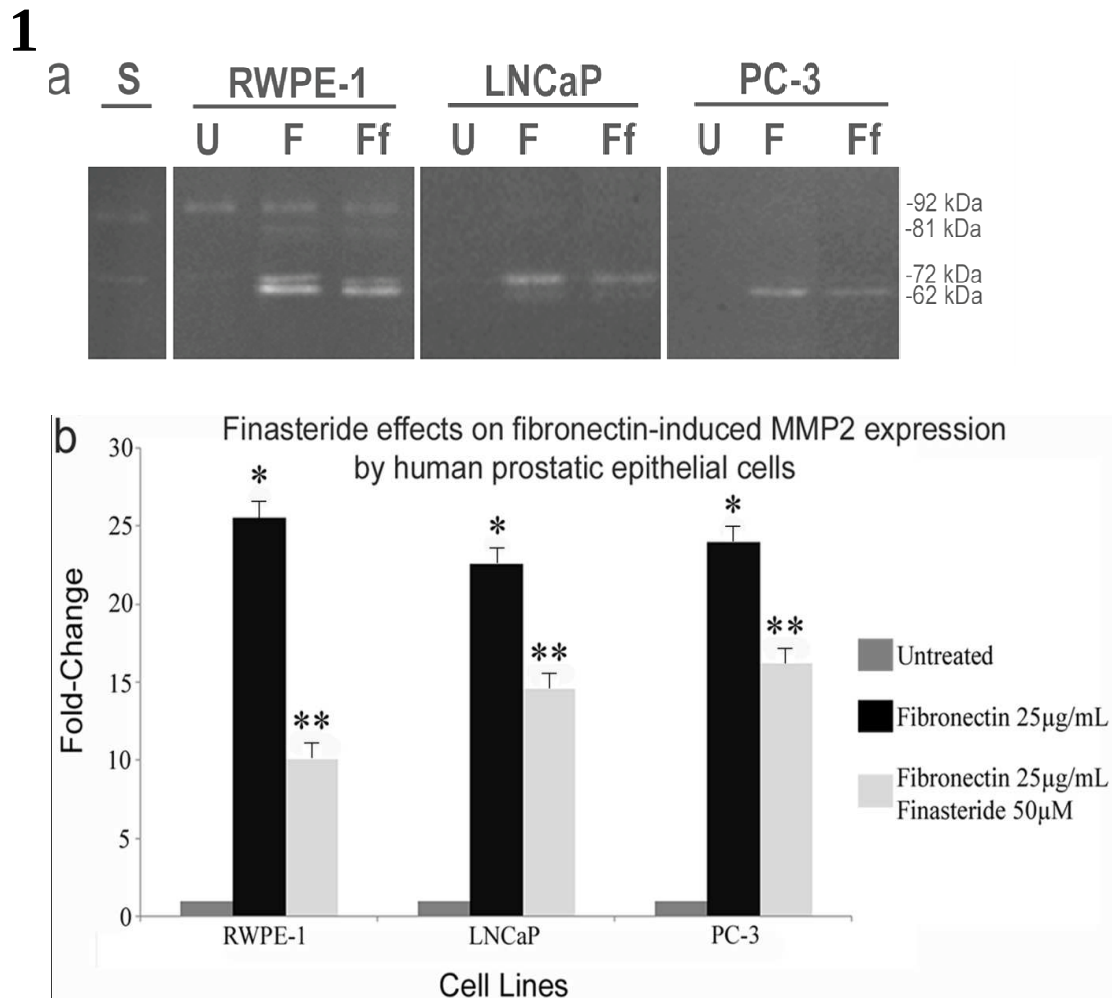


Figure 1. (a) Representative zymography of the gelatinolytic activity observed in the conditioned medium from untreated cells (U), fibronectin-treated cells (F), and fibronectin plus finasteride treated cells (Ff). The lane S corresponds to the reference standard of human active-MMP9 and pro-MMP2 enzymes. Gelatinolytic bands of 92, 81, 72, and 62 kDa were observed, which correspond to pro-MMP9, active-MMP9, pro-MMP2, and active-MMP2, respectively. In normal culture conditions only RWPE-1 cells express MMP9 activity. Fibronectin-treatment induced high MMP2 activity in all tested cell lines.

Finasteride-treatment significantly downregulated this induction. Note that LNCaP expresses mainly pro-MMP2, while PC-3 active-MMP2 after fibronectin treatment. **(b)** Densitometry analysis of the gelatinolytic bands present at the conditioned media from untreated, fibronectin treated and fibronectin/finasteride treated cells (RWPE-1, LNCaP and PC-3). The data are expressed as a fold-change of the means $\pm$ SD of MMP2 total band IOD between the fibronectin and combined fibronectin/finasteride treatments over the untreated cells, which were given the value of 1. Fibronectin treatment significantly increased MMP2 activity in the three cell lines while finasteride treatment significantly reduced total MMP2 gelatinolytic activity. *Statistically significant difference from untreated cells with $p < 0.001$. **Statistically significant difference from fibronectin treated cells with $p < 0.05$.

Conclusão

Os resultados obtidos neste estudo mostram que a exposição à finasterida foi capaz de diminuir a atividade das endopeptidases MMP2 e MMP9, e de aumentar a expressão de seus inibidores – TIMPs –; inibindo diretamente a capacidade de migração e de invasividade das células prostáticas. Desse modo, conclui-se que a finasterida pode ter um efeito benéfico ou de atenuação em cânceres de próstata de alto grau. Embora estes dados sejam promissores, é necessário elucidar os mecanismos de modulação desta droga, andrógeno-dependente ou independente, em estudos futuros.

Comprovou-se, ainda, que modelos de cultura celular podem e devem ser aprimorados, mediante simples técnicas como a diluição de fibronectina no meio de cultura, para representarem mais caracteristicamente os ambientes *in vivo* e suas interações células-matriz extracelular.

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Fibronectin induces MMP2 expression in human prostate cancer cells

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ABSTRACT

High-grade prostate cancers express high levels of matrix metalloproteinases (MMPs), major enzymes involved in tumor invasion and metastasis. However, the tumor cell lines commonly employed for prostate cancer research express only small amounts of MMPs when cultivated as monolayer cultures, in common culture media. The present study was conducted to ascertain whether culture conditions that include fibronectin can alter MMP2 and MMP9 expression by the human prostatic epithelial cell lines RWPE-1, LNCaP and PC-3. These cells were individually seeded at 2×10^4 cells/cm², cultivated until they reached 80% confluence, and then exposed for 4 h to fibronectin, after which the conditioned medium was analyzed by gelatin zymography. Untreated cells were given common medium. Only RWPE-1 cells express detectable amounts of MMP9 when cultivated in common medium, whereas the addition of fibronectin induced high expression levels of pro and active forms of MMP2 in all tested cell lines. Our findings demonstrate that normal and tumor prostate cell lines express MMP2 activity when in contact with extracellular matrix components or blood plasma proteins such as fibronectin. Future studies of transcriptomes and proteomes in prostate cancer research using these cell lines should not neglect these important conclusions.

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1. Introduction

Prostate cancer is the most common malignancy in men and the second leading cause of male cancer-related deaths in the Western world [1,2]. Castration-resistant prostate cancer (CRPC) is the most advanced form of prostate cancer exhibiting reduced survival, with reported values varying between 9 and 30 months [2]. In this sense, in vitro culture systems are widely employed in prostate cancer research and, more recently, for transcriptome and proteomic analysis because the cultures reproduce rapidly and readily [3]. Although these monolayer cultures provide rapid and important results, they recreate only a fraction of a complex scenario [4]. The absence of cell-to-extracellular matrix (ECM) interactions can lead to misleading results, especially for prostate cancer, where the cross-signaling between epithelium and stroma is so important [5].

Matrix metalloproteinase's (MMPs) belong to a family of zinc and calcium dependant endopeptidases responsible for the first steps in ECM degradation in both normal development and disease [6,7]. A very invasive and metastatic behavior is a major

characteristic of CRPC, and ECM degradation is critical to these processes [2,7]. However, the commonly employed tumoral cell lines on prostate cancer research express small amounts of MMPs when cultivated in monolayer cultures with common culture media [8,9], without ECM components.

Considering that cell-ECM cross-talk, when present in culture systems, leads to behavioral changes in tumor cell biology, such as increased adherence to substrates [10], increased resistance to chemotherapeutic agents [11], and resistance to apoptosis [12], we hypothesized that it could also modulate the expression of MMPs by human prostate cells. Therefore, we conducted the present investigation exposing the classical human prostate cell lines RWPE-1, LNCaP, and PC-3 to fibronectin, a multifunctional extracellular glycoprotein, which is considered of critical relevance for the process of metastasis and cell survival [13]. Not only is fibronectin the most abundant adhesion protein in the blood, but it also interacts with circulating tumor cells [13], and therefore the ECM protein chosen for this investigation.

Furthermore, employing this culture model, we exposed these cells to finasteride, an inhibitor of the enzyme five alpha-reductase, which is widely used in benign prostatic hyperplasia treatment and potentially a chemopreventive agent for prostate cancer [14], to ascertain whether in these conditions finasteride would also modulate the expression of MMP2, as previously reported for the rat ventral prostate [15], where epithelial-stroma interactions occur.

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2. Material and methods

RWPE-1, LNCaP, and PC-3 cell lines were acquired from the American Type Culture Collection (ATCC). The experiments were performed two months after the acquisition of the cell lines, which were cultivated according to ATCC guidelines. These cells were then individually seeded at 2×10^4 cells/cm² in 6-well culture plates (TPP™) and the experiments were carried out in triplicate. Upon reaching 80% confluence, cells were washed three times with sterile D-PBS (GIBCO/Invitrogen™), and the recommended culture medium (FBS free) was added as follows: (1) control treatment – 0.1% dimethylsulfoxide (DMSO); (2) fibronectin treatment – 0.1% DMSO plus 25 µg/mL fibronectin (Sigma™); and (3) combined fibronectin/finasteride treatment – 0.1% DMSO plus 25 µg/mL fibronectin (Sigma™) plus 50 µM finasteride (Sigma™). All reagents were dissolved in the culture medium. After 4 h of exposure, the conditioned media (CM) were collected and individually stored at -80°C .

The CM were concentrated using Centrprep® 10,000 MWCO (Millipore™) tubes and submitted to protein quantification at a NanoDrop 2000 (Thermo Scientific™) spectrophotometer. Equal amounts of protein from CM (15 µg) were analyzed by gelatin zymography on 8% polyacrylamide gels co-polymerized with 0.1% gelatin (Merck™) as substrate. Purified human MMP2 (20 ng) and MMP9 (30 pg) (Calbiochem™) were also loaded as positive controls. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated 24 h in an activation buffer (50 mM Tris–HCl, 5 mM CaCl₂ and 0.02% ZnCl₂). Gels were stained with Coomassie brilliant blue R-250 and de-stained with 20% methanol and 10% acetic acid in distilled water until the clear bands had been visualized. The activities of the MMP2 and MMP9 bands were quantified using the ImageJ™ software for each sample (as triplicates). The integrated optical density (IOD) was

measured and the data were analyzed with INSTAT™ software using the two tail Student's *t* test ($p < 0.05$) to compare the different treatments. Values were calculated as the mean $\pm$ SD of the totality of IODs for the pro- and active forms of the MMP2 and MMP9 enzymes. Finally, a fold-change graphic was made by dividing the means of the values for the treated cells by the mean of the values for the untreated cells.

3. Results

When cultivated in common culture media, all cell lines produced very low detectable levels of MMP2 activity (Fig. 1A). Fibronectin exposure significantly upregulated MMP2 activity in all three cell lines, with a 25.5-fold-change in RWPE-1 cells, a 22.6-fold-change in the LNCaP cells and a 24.0-fold-change in PC-3 cells compared with controls (Fig. 1B). However, when these cell lines were co-exposed to fibronectin and finasteride, the activity of MMP2 was significantly lower than those treated only with fibronectin; reduced at least by 33% (Fig. 1B).

Curiously, the LNCaP cells primarily expressed pro-MMP2 whereas PC-3 expressed active-MMP2 (Fig. 1A), a phenomenon most likely related to the greater invasive behavior of PC-3. In contrast, only RWPE-1 cells expressed low but detectable amounts of MMP9, mostly in its pro-form (Fig. 1A). Fibronectin treatment induced partial activation of MMP9, whereas finasteride reduced this activation (Fig. 1A) ($p > 0.05$).

4. Discussion

In this study, we evaluated the expression of gelatinases A and B in the prostate cells lines RWPE-1, LNCaP and PC-3, which are widely employed in prostate cancer research, using two different culture conditions. In our initial experiments, we demonstrated

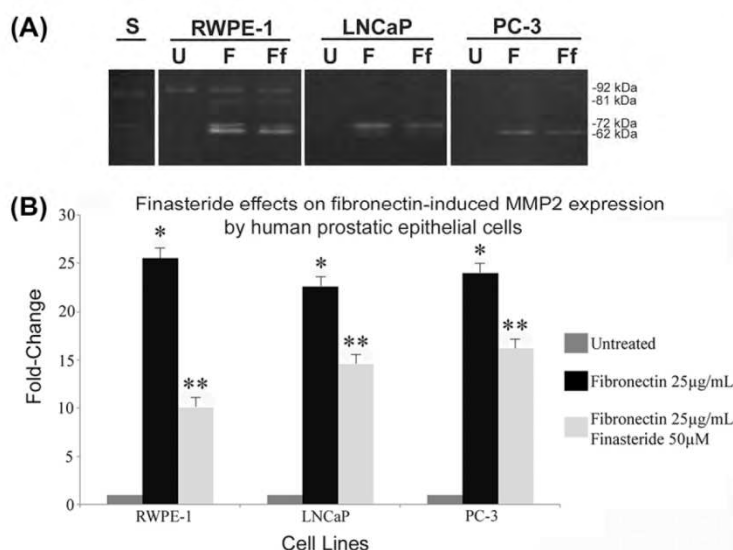


Fig. 1. (A) Representative zymography of the gelatinolytic activity observed in the conditioned medium from untreated cells (U), fibronectin-treated cells (F), and fibronectin plus finasteride treated cells (Ff). Lane S corresponds to the reference standard of human active-MMP9 and pro-MMP2 enzymes. Gelatinolytic bands of 92, 81, 72, and 62 kDa were observed, which correspond to pro-MMP9, active-MMP9, pro-MMP2, and active-MMP2, respectively. In normal culture conditions only RWPE-1 cells express MMP9 activity. Fibronectin treatment induced high MMP2 activity in all tested cell lines. Finasteride-treatment significantly downregulated this induction. Note that LNCaP expresses mainly pro-MMP2, whereas PC-3 expresses active-MMP2 after fibronectin treatment. (B) Densitometry analysis of the gelatinolytic bands from the conditioned media from untreated, fibronectin treated and fibronectin/finasteride treated cells (RWPE-1, LNCaP and PC-3). The data are expressed as the fold-change of the means $\pm$ SD of MMP2 total band IOD between the fibronectin and combined fibronectin/finasteride treatments over the untreated cells, which were given the value of 1. Fibronectin treatment significantly increased MMP2 activity in the three cell lines, whereas finasteride treatment significantly reduced total MMP2 gelatinolytic activity. *Statistically significant difference from untreated cells with $p < 0.001$. **Statistically significant difference from fibronectin treated cells with $p < 0.05$.

that when these cells were cultivated as monolayers, using regular culture medium, all of them secreted very low amounts of MMPs in their CM, in accordance to previous results [8,9]. Interestingly, only the non-tumoral cell line RWPE-1 expressed detectable levels of MMP9 under these culture conditions. However, when the cell cultures were grown with fibronectin in the culture medium, all the cell lines tested exhibited significantly higher MMP2 levels in their CM. Although this effect was observed for MMP2 in all tested cell lines, this was not observed for MMP9. Furthermore, our results demonstrated that the co-treatment of these cells, with fibronectin and finasteride, resulted in the same pattern of downregulation of MMP2 activity after finasteride treatment, as observed in the rat ventral prostate [15], and now reproduced in an in vitro model of human prostate cancer.

Although Das et al. [16] reported higher expression of MMPs-2 and 9 upon exposure of human breast cancer cells to fibronectin, and Meng et al. [17] reported increased invasion of lung cancer cells through MMP9 activity after fibronectin treatment, this is the first report of enhanced activity of MMPs in prostate cancer cell lines after fibronectin exposure. Our findings highlight the importance of cell-ECM interactions in the modulation of these endopeptidases. Similar results were obtained by Pentyla et al. [18], who showed that the interaction of LNCaP cells with the extracellular matrix plays a dominant role in uPA and uPAR gene expression, another protease system involved in ECM degradation [19].

Fibronectin has been shown to support cell proliferation and invasion, which are major events for metastasis, and to protect tumoral cells from the cytotoxic action of natural killer cells [13]. Now we have demonstrated that fibronectin can also upregulate the expression of MMPs that are directly involved in prostate cancer aggressiveness. Interestingly, our findings demonstrated that fibronectin could only induce MMP2 activity, but not MMP9, in the tested prostate cell lines. Although MMP2 has been historically considered a constitutive gene due to the absence of well-characterized regulatory elements in the MMP2 promoter region [20], recent reports on MMP2 promoter analyses have demonstrated numerous regulatory elements that could modulate MMP2 expression [21]. The mechanism by which fibronectin induces MMP2 expression has been described in MCF-7 human breast carcinoma cells and A549 human lung cancer cells, and involves fibronectin–integrin $\alpha 5 \beta 1$ interaction and/or FAK/PI-3K/ERK signaling pathways activation [16,17], but this mechanism remains to be confirmed for prostate cancer cells, in future studies.

Taken together, all these reports highlight the importance of the cell-ECM interactions for studying transcriptomes and proteomes from both normal and tumoral cells, at in vitro systems [3–5,8–10,13,16–18]. In this sense, future investigators should be aware that RWPE-1, LNCaP and PC-3 cells, and most likely other tumor cells, require cell-ECM interaction in order to express detectable levels of MMPs on their CM.

In conclusion, our results confirm that: (1) fibronectin exposure upregulates the activity of MMP2 in all tested human prostate cell lines up to a 25-fold-increase and (2) by employing a human cell culture model with cell-ECM interaction, it was possible to reproduce previous results of an in vivo study where finasteride down-regulated the activity of MMP2 in the rat ventral prostate.

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