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**ESTUDOS COM A PROTEÍNA ANEXINA A1 NO TRATAMENTO
DE PROCESSOS TUMORIGÊNICOS OU INFLAMATÓRIOS**

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mergulhei. Não se preocupe em entender, viver ultrapassa
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Clarice Lispector

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RESUMO

Anexina A1 (ANXA1) é uma proteína que em condições normais é expressa no citoplasma de diferentes tipos celulares, mas particularmente abundante nas células diretamente relacionadas com o processo inflamatório, como monócitos, neutrófilos e macrófagos. A ANXA1 e seu peptídeo Ac2-26 atuam por meio dos receptores FPR1 e FPR2/ALX, mediando respostas biológicas dessas moléculas. O objetivo desta tese foi apresentar os resultados de estudos realizados com a proteína anti-inflamatória ANXA1 no tratamento de processos tumorigênicos ou inflamatórios e compará-los com o grupo controle (sem tratamento). Na realização desses estudos, as células foram tratadas *in vitro* com o peptídeo mimético da ANXA1 e então foram avaliadas algumas características morfológicas (proliferação, migração, invasão, citotoxicidade e viabilidade celular, co-localização) e expressão gênica e proteica, com o intuito de buscarmos por quais possíveis vias a ANXA1 pode modular a expressão gênica. Além da ação antiproliferativa da ANXA1, também relatamos nos nossos trabalhos a ação dessa proteína na modulação da expressão gênica. No capítulo 3.1 observamos que a ANXA1 exógena modifica a expressão gênica, em cultura de células de carcinoma de laringe (Hep-2). Neste trabalho, relatamos os efeitos da ANXA1 na expressão gênica, utilizando análise genômica em linhagens de carcinoma e de células foliculoesteladas de adenoma pituitário (PDFS). Também foram utilizadas análises *in vivo* da pituitária de camundongos selvagens e *knockouts* para o gene da ANXA1. Pela técnica de RaSH (Rapid Subtractive Hybridization) realizada em células tratadas e não tratadas com a proteína ANXA1 ou seu peptídeo foi observada a regulação positiva da expressão do gene CCR10. No capítulo 3.2 observamos a resposta antiproliferativa nas células Hep-2, sugerindo que o peptídeo da ANXA1 pode desencadear a regulação do crescimento por interação direta com o receptor FPR/ANXA1. O peptídeo também pode ter ativado a expressão ANXA1 endógena, resultando em regulação do crescimento por um mecanismo autocrino/parácrino. Também foi avaliada a expressão da ANXA1 endógena em células tumorais e em mastócitos, no qual foi observado a imunoreatividade da ANXA1 mostrando baixa regulação nos tumores de laringe e células peritumorais, comparadas com o tecido controle. No capítulo 3.3 observamos que também houve diminuição na proliferação de células de câncer cervical após tratamento com o

peptídeo da ANXA1, agindo como um supressor tumoral, possivelmente por meio da cascata MAPK/ERK. Além disso, os resultados mostraram que o tratamento resultou em 82 genes diferencialmente expressos (pela técnica de RaSH). A expressão da proteína ANXA1 foi diminuída nas células SiHa tratadas com o peptídeo pelas análises imunocitoquímicas ultraestruturais. O gene *ID1* foi validado por RT-qPCR, confirmando a sua baixa expressão após o tratamento com o peptídeo. Também foram interligadas, pelo *Ingenuity Systems*, as vias de sinalização celular, das quais a ANXA1 participa, importantes para o desenvolvimento tumoral. No capítulo 3.4, analisando as células cultivadas da linhagem celular ARPE-19, observamos que a proliferação celular diminuiu após indução pelo LPS e após o tratamento com o peptídeo da ANXA1, a proliferação e a migração celular aumentaram. E pela tecnologia de RaSH, 80 genes diferencialmente expressos após o tratamento foram encontrados, dos quais cinco desses genes (*LRAT*, *CTGF*, *MAP1B*, *ALDH1A3* e *SETD7*), devido às suas funções estarem relacionadas com doenças oculares e/ou inflamatórias, foram validados por RT-qPCR, mas apenas dois deles (*LRAT*, *CTGF*) confirmaram a baixa expressão. Também foram interligadas, pelo *Ingenuity Systems*, as vias de sinalização celular, das quais a ANXA1 participa, importantes para o processo inflamatório. As análises *in vitro*, dos quatro artigos científicos, mostraram que o ANXA1 pode ter um importante papel nas cascatas gênicas e um papel regulatório no desenvolvimento tumoral e na inflamação ocular. Dessa maneira, os nossos dados apontam a importância das células Hep-2, SiHa e ARPE-19 como um alvo potencial de atividades gênicas envolvendo a ação da ANXA1, tendo em vista as aplicações dessa proteína como uma possível alternativa terapêutica no carcinoma de laringe, de colo uterino e nas doenças inflamatórias oculares.

Palavras-chave: cultura de células, expressão gênica diferencial, efeito antiproliferativo.

ABSTRACT

Annexin A1 (ANXA1) is a protein that under normal conditions is expressed in the cytoplasm of different cell types, but particularly abundant in cells directly related to the inflammatory process, such as monocytes, neutrophils and macrophages. ANXA1 and its peptide Ac2-26 act through the receptors FPR1 and FPR2/ALX, mediating biological responses of these molecules. The aim of this thesis was to present the results of studies carried out with the anti-inflammatory protein ANXA1 in the treatment of tumorigenic or inflammatory processes and to compare them with the control group (without treatment). In the accomplishment of these studies, the cells were treated *in vitro* with the ANXA1 mimetic peptide and then some morphological characteristics (proliferation, migration, invasion, cytotoxicity and cell viability, co-localization) and gene and protein expression were evaluated in order to search for possible pathways for ANXA1 to modulate gene expression. In addition to the antiproliferative action of ANXA1, we also report in our work the action of this protein in the modulation of gene expression. In Chapter 3.1, we observed that exogenous ANXA1 modifies gene expression in culture of laryngeal carcinoma cells (Hep-2). In this work, we report the effects of ANXA1 on gene expression, using genomic analysis in carcinoma and follicular cells of pituitary adenoma (PDFS). *In vivo* analyzes of the pituitary of wild mice and knockouts for the ANXA1 gene were also used. By Rapid Subtractive Hybridization (RASH) technique performed on cells treated and not treated with the ANXA1 protein or its peptide, the positive regulation of *CCR10* gene expression was observed. In chapter 3.2 we observed the antiproliferative response in Hep-2 cells, suggesting that the ANXA1 peptide can trigger the regulation of growth by direct interaction with the FPR/ANXA1 receptor. The peptide may also have activated the endogenous ANXA1 expression, resulting in regulation of growth by an autocrine/paracrine mechanism. The expression of endogenous ANXA1 was also evaluated in tumor cells and in mast cells, in which ANXA1 immunoreactivity was observed showing low regulation in tumors of the larynx and peritumoral cells, compared to the control tissue. In chapter 3.3 we observed that there was also a decrease in the proliferation of cervical cancer cells after treatment with the ANXA1 peptide, acting as a tumor suppressor, possibly through the MAPK/ERK cascade. In addition, the results showed that the treatment resulted in 82 differentially expressed genes (by

RaSH). Expression of the ANXA1 protein was decreased in SiHa cells treated with the peptide by ultrastructural immunocytochemical analyzes. The *ID1* gene was validated by RT-qPCR, confirming its low expression after treatment with the peptide. Also linked by Ingenuity Systems were the cell signaling pathways, of which ANXA1 participates, important for tumor development. In chapter 3.4, analyzing the cultured cells of the ARPE-19 cell line, we observed that cell proliferation decreased after induction by LPS and after treatment with the ANXA1 peptide, cell proliferation and migration increased. And by RaSH, 80 genes differentially expressed after treatment were found, of which five genes (*LRAT*, *CTGF*, *MAP1B*, *ALDH1A3* and *SETD7*), due to their functions related to ocular and/or inflammatory diseases, were validated by RT-qPCR, but only two of them (*LRAT*, *CTGF*) confirmed the low expression. Ingenuity Systems also connected the cell signaling pathways, of which ANXA1 participates, important for the inflammatory process. *In vitro* analyzes of the four scientific papers showed that ANXA1 may play an important role in gene cascades and a regulatory role in tumor development and ocular inflammation. Thus, our data point to the importance of the Hep-2, SiHa and ARPE-19 cells as a potential target of the genetic activities involving the action of ANXA1, considering the applications of this protein as a possible therapeutic alternative in laryngeal carcinoma, and in inflammatory eye diseases.

Key words: cell culture, differential gene expression, antiproliferative effect

LISTA DE ABREVIACÕES

ABC: Cassete ligador de ATP

Ac2-26: Peptídeo da ANXA1

ANXA1: Proteína Anexina A1

ALDH1A3: Família Aldeído Desidrogenase 1, membro 3

ARPE-19: Linhagem celular de Células Epiteliais Pigmentadas da Retina Humana

BRB: Barreira Hematoretiniana

CCL27: citocina de motivo CC com ligante 27

CCR10: receptor para a citocina CCL27

CECP: Carcinoma Epidermóide de Cabeça e pescoço

COX: ciclo oxigenase -2

CTGF: Fator de Crescimento de Tecido Conjuntivo

CXCR4: citocina de motivo CXC e receptor 4

EGF: Fator de crescimento epidérmico

EPR: Epitélio Pigmentado da Retina

ERK: Quinase regulada por sinais extracelulares

FPR: Receptor para peptídeos formilados

Hep-2: Linhagem de celular de carcinoma de laringe

HPV: Papilomavirus Humano

ID1: Inibidor de ligante de DNA-1, proteína dominante negativo helix-loop-helix

IL – Interleucina

INF- γ : Interferon gama

JNK - Via quinase N-terminal da c-Jun

LPS: Lipopolisacárideo bacteriano

LRAT: Lectina Retinol Acetiltransferase (fosfatidilcolina-retinol O-aciltransferase)

MAP1B: Proteína associada ao microtúbulo 1B

MAPK: Proteína quinase ativada por mitógeno

MCP-1: Proteína quimiotática para monócitos

NF- κ B: Fator de crescimento nuclear kappa B

PCR: Reação em cadeia da polimerase

PDFS: linhagem de células foliculoesteladas de adenoma de pituitária

Rantes: Expressão e secreção de Células T reguladas e normais

RaSH: Hibridização Subtrativa Rápida

RT-qPCR: transcriptase reversa – PCR quantitativo

SETD7: Domínio SET (lisina metiltransferase) 7

SiHa: Linhagem Celular de Carcinoma Cervical

siRNA: Ácido Ribonucleico de silenciamento

SKCO-15: linhagem de células epiteliais derivadas de adenocarcinoma colorretal

TGF: Fator de crescimento transformante

TNF- α : Fator de necrose tumoral alfa

VEGF: Fator de crescimento vascular endotelial

UBC: Ubiquitina C

1.INTRODUÇÃO

Anexina A1 (ANXA1) pertencente a uma superfamília bem conservada de proteínas classificadas em cinco grupos (A-E), entre esses os vertebrados (mamíferos) possuem as proteínas do grupo A (GUO; LIU; SUN, 2013). Estruturalmente essa superfamília possui duas porções, a região C-terminal da proteína, representa 80% de sua constituição, é comum para todos os membros e possui um domínio de ligação ao cálcio, composto de quatro sequências homologas repetidas (BIAOXUE; XIGUANG; SHUANYING, 2014), e a região variável N-terminal, que é única em comprimento e sequência para cada membro da família e inclui sítios potenciais de fosforilação, glicosilação e ação de peptidase (GERKE; MOSS, 2002; PERRETTI; DALLI, 2009).

ANXA1 é uma proteína que em condições normais é expressa no citoplasma de diferentes tipos celulares, mas particularmente abundante nas células diretamente relacionadas com o processo inflamatório, como monócitos, neutrófilos e macrófagos (GOULDING et al., 1990). Existem várias propostas de mecanismos moleculares, célula específica, que são responsáveis pela secreção da ANXA1, entre esses, um deles seria após ativação celular, a ANXA1 intracelular se transporta para a membrana plasmática e é então secretada pela ativação do transportador ABC (*ATP-binding cassette*). Outros mecanismos seriam pela fosforilação do resíduo de serina na porção N-terminal, ou pela fusão de grânulos de gelatinase contendo ANXA1 com a membrana plasmática, visto que há a ligação à membrana de maneira dependente de cálcio. Na presença de cálcio, a ANXA1 extracelular sofre uma mudança conformacional que leva a exposição da região N-terminal e ligação ao seu receptor FPR/ALX (PERRETTI; D'ACQUISTO, 2009). Outro mecanismo de comunicação intercelular, estudado mais recentemente, seria pelas microvesículas que transferem componentes de lipídios e proteínas celulares para células-alvo (HEADLAND et al., 2015).

A ANXA1 e seu peptídeo Ac2-26 atuam por meio dos receptores FPR1 e FPR2/ALX, mediando respostas biológicas dessas moléculas (LEONI et al., 2015; PERRETTI, 2003). A proteína ANXA1 no organismo foi demonstrada ativar a cascata p38 ativada por mitógeno e o peptídeo Ac2-26 promove a ativação da via quinase N-terminal da c-Jun (JNK) (COORAY et al., 2013). No processo inflamatório foi visto que a ANXA1 modificou o macrófago de perfil M1 para tipo M2 anti-inflamatório, atenuando a expressão de IL-6, IL-1 β e TNF- α (LI et al., 2011). A defesa do organismo contra patógenos e a resolução da inflamação são importantes ações que a ANXA1 e

seu peptídeo Ac2-26 desempenham (BUCKLEY; GILROY; SERHAN, 2014; VAGO et al., 2016).

A expressão e localização da ANXA1 têm sido investigadas, pelo nosso grupo de pesquisa, em modelos experimentais de inflamação aguda no mesentério de ratos, por meio da análise dos leucócitos extravasados e endotélio vascular (GIL et al., 2006; OLIANI et al., 2001), em inflamação crônica granulomatosa na pele de camundongos (OLIANI et al., 2008), inflamações agudas (GIROL et al., 2013) e em estudo *in vitro*, utilizando linhagens celulares de diferentes tipos de câncer (GASTARDELO et al., 2014; PRATES et al., 2015; RODRIGUES-LISONI et al., 2006; SILISTINO-SOUZA et al., 2007), e recentemente foi analisada em modelo de enxerto (TEIXEIRA et al., 2016) e implante (MIMURA et al., 2016) de pele. O grupo de pesquisa trabalha também com o estudo de tumores, destacando dois tipos: o carcinoma de cabeça e pescoço e o de colo de útero. Na realização desses estudos, as células tumorais são tratadas *in vitro* com o peptídeo mimético da ANXA1 e então são avaliadas algumas características morfológicas (proliferação, migração, invasão, citotoxicidade e viabilidade celular, co-localização) e expressão gênica e proteica, com o intuito de buscarmos quais as possíveis vias a ANXA1 pode modular a expressão gênica.

A atuação da ANXA1 é específica para cada tipo de câncer e há evidências que indicam que sua regulação e localização subcelular estão relacionadas com desenvolvimento, invasão, metástase, progressão e resistência a tratamentos (GUO; LIU; SUN, 2013; JORGE et al., 2013; POETER et al., 2013; POLLI-LOPES et al., 2013). Mundialmente, o carcinoma epidermóide de cabeça e pescoço (CECP) é o quinto tipo mais comum, surgindo aproximadamente 500.000 casos novos e cerca de 271.000 óbitos por ano (MARCUS; YEOH, 2009). No Brasil, estimam-se 11.140 casos novos em homens e 4.350 em mulheres, para o ano de 2016/2017, considerando apenas os tumores de cavidade oral (MINISTÉRIO DA SAÚDE, 2017). Os principais fatores de risco para o CECP, incluindo o câncer de laringe e o de cavidade oral (língua), são o tabagismo e o consumo de bebidas alcoólicas (HASHIBE et al., 2009). Infelizmente, a maioria dos casos é diagnosticada em estágios avançados da doença (60% nos estágios III e IV) e são células carcinogênicas escamosas, podendo variar consideravelmente em agressividade, metástase potencial e sensibilidade a radioterapia e quimioterapia (HERCHENHORN; DIAS, 2004).

Exemplos da ação da proteína são encontrados no CECP, que inclui neoplasias que surgem na cavidade oral, faringe e laringe, como foi observada a alta expressão de

ANXA1 no núcleo de células epiteliais de carcinoma oral, analisadas em 115 pacientes (LIN et al., 2008). Também foi observado que a ANXA1 pode ser usada como biomarcador de prognóstico em células de carcinoma epidermóide de esôfago, pois pacientes com baixa expressão de ANXA1 nuclear tem melhor prognóstico do que aqueles com alta expressão (HAN et al., 2014). Xiao e colaboradores (2017) observaram que a ANXA1 pode inibir *in vitro* a habilidade invasiva em células de carcinoma de nasofaringe pela interação ANXA1/S100A9/Vimentina. Enquanto a interação ANXA1/FPR2, em estudos com câncer de laringe, também pode fornecer um ponto de início para pesquisas, pois foi observado em experimentos *in vitro* e *in vivo* que a ANXA1 pode contribuir para a regulação do crescimento tumoral e metástase por mecanismos parácrinos que são mediados pelo receptor FPR2 (GASTARDELO et al., 2014).

Outro tipo de câncer estudado pelo nosso grupo é o câncer cervical, uma das principais neoplasias ginecológicas em todo o mundo, essa doença do colo do útero é associada ao papilomavírus humano (HPV) e/ou a infecção com agentes bacterianos, tais como *Chlamydia trachomatis* e *Neisseria gonorrhoea* (MOREIRA, 2014). Ele possui uma estimativa de 16.340 novos casos para o Brasil em 2016, e o risco estimado dessa doença é de 15,85 casos a cada 100 mil mulheres (MINISTÉRIO DA SAÚDE, 2017). Além dessas infecções, o microambiente tumoral também influencia no processo tumorigênico, por ser muito diferente daquele observado em tecidos normais correspondentes, sendo caracterizado por composição alterada da matriz extracelular, densidade de microvasos elevada, abundância de células inflamatórias e fibroblastos com fenótipo ativado (DE WEVER; MAREEL, 2003; TLSTY; HEIN, 2001).

A tumorigênese cervical também pode estar relacionada com a expressão da ANXA1, por exemplo em amostras de displasia e câncer cervical nos estágios I, II e III, foi visto que houve regulação aumentada da proteína fosforilada conforme progressão da doença (Robinson-Bennett et al., 2008). Enquanto outro trabalho mostra que em amostras normais e de estágio I, II e III, e câncer invasivo, foi evidenciado que a expressão da proteína reduzia conforme progressão da doença, sendo mais forte nas células normais (WANG et al., 2008). Já quando administrada a ANXA1 exógena em células de carcinoma de colo de útero *in vitro*, foi observado que houve inibição da proliferação celular e modulação da expressão gênica, pela via do gene *ID1* (PRATES et al., 2015).

O tratamento para essas neoplasias é definido em função do tipo celular, grau de diferenciação, local, extensão, presença de metástases linfonodais, características macroscópicas e envolvimento ósseo e muscular do tumor. É importante levar em consideração condições físicas, sociais e ocupacionais do paciente (HASHIBE et al., 2007). Os principais tratamentos incluem, cirurgia, terapia por irradiação e quimioterapia. A cirurgia é o método mais antigo utilizado como tratamento do câncer, mas é altamente invasivo, por isso alguns fármacos têm despertado interesse científico, entre eles a ANXA1 poderia ser uma alternativa, devido não apenas ao seu potencial anti-inflamatório, mas também antitumorigênico.

Outro processo inflamatório estudado pelo nosso grupo é a uveíte, inflamação ocular que acomete o trato uveal, que é composto por: íris (estrutura que dá cor aos olhos), corpo ciliar e coróide (composto basicamente por vasos sanguíneos). Quando ocorre o acometimento inflamatório de uma destas estruturas ou o conjunto das mesmas, denomina-se uveíte. O olho é um órgão imunologicamente privilegiado onde as respostas inflamatórias são suprimidas e muitos fatores são responsáveis por essa supressão, incluindo a presença da barreira hematoretiniana (BRB) (CAI; BRANDT, 2008). O epitélio pigmentado da retina (RPE) é um dos principais componentes da BRB que controla o fluxo de nutrientes para os fotorreceptores (LEUNG et al., 2009) e contribui de forma essencial para o privilégio imunológico ocular (STREILEIN, 2003). A inflamação é o principal fator contribuinte para o comprometimento funcional do RPE (LEUNG et al., 2009). O epitélio está envolvido em várias funções metabólicas e suporta a retina sensorial; assim, a inflamação desta área pode levar à aparência de doenças oftalmológicas (HAMILTON; LEACH, 2011; STRAUSS, 2005). A primeira linha de defesa contra patógenos é mediada por receptores, que reconhecem os invasores. Esses receptores podem iniciar a via inflamatória de NF- κ B e proteína quinase ativada por mitogênio (MAPK) para desencadear a expressão de citocinas pró-inflamatórias e quimiocinas (CORN; VUCIC, 2014).

A inflamação é um dos principais fatores que contribuem para muitos distúrbios de cegueira (LEUNG et al., 2009). Os corticosteróides são freqüentemente administrados para tratar esse processo intraocular, no entanto, existe o risco de efeitos colaterais (JONAS et al., 2003). Os corticosteróides induzem a expressão da proteína anexina A1 (ANXA1), mediadores anti-inflamatórios endógenos (FLOWER, 1988). Durante décadas, os cientistas desenvolveram conhecimentos para prevenir a perda de visão ou restaurar a visão em pacientes afetados pela degeneração da retina por meio de

terapia medicamentosa, aumento de genes ou transplante de células (WILEY et al., 2015). Foi então, observado que a ANXA1 diminui a proliferação, reestabelece a migração, diminui a expressão dos genes *CTGF* e *LRAT* e de citocinas pró-inflamatórias nas células epiteliais pigmentadas da retina, determinando que as vias de sinalização NF- κ B e Ubiquitina C (UBC) são moduladas pela ANXA1 (CARDIN et al., 2017).

Desse modo, os trabalhos relacionados com a ANXA1 têm contribuído para um maior entendimento do papel dessa proteína em processos tumorigênicos e inflamatórios. No entanto, os mecanismos pelos quais a ANXA1 atua na tumorigênese não são bem conhecidos e, considerando seu papel na inflamação, compreendem um alvo importante para estudo. Os dados disponíveis também evidenciam que há controvérsia na expressão dessa proteína na carcinogênese de cabeça e pescoço e também na de colo de útero, podendo evidenciar possíveis vias de atuação para estudos, pois ela pode ser alvo de novas intervenções terapêuticas e/ou utilizada como potencial biomarcador (BIAOXUE; XIGUANG; SHUANYING, 2014; LEONI; NUSRAT, 2016).

2. PROPOSIÇÃO

O objetivo desta tese é apresentar os resultados de estudos realizados com a proteína anti-inflamatória ANXA1 no tratamento de processos tumorigênicos ou inflamatórios e compará-los com o grupo controle (sem tratamento). Sua estrutura está dividida em capítulos que correspondem a artigos publicados, seguindo a sequência cronológica abaixo:

- *In vitro* and *in vivo* studies on CCR10 regulations by Annexin A1. (Artigo publicado no periódico FEBS Lett 2006 Feb 20; 580(5): 1431-8, doi: 10.1016/j.febslet.2006.01.072, JCR: 3,623)
- Annexin 1: differential expression in tumor and mast cells in human larynx cancer. (Artigo publicado no periódico International Journal of Cancer, 2007 Jun 15; 120(12):2582-9. doi: 10.1002/ijc.22639, JCR: 6,513)
- ANXA1Ac2-26 peptide reduces ID1 expression in cervical carcinoma cultures. (Artigo publicado no periódico Gene. 2015 Oct 10; 570(2): 248-54. doi: 10.1016/j.gene.2015.06.021, JCR: 2,415)
- ANXA1Ac2-26 peptide: as a possible therapeutic approach in inflammatory ocular diseases? (Artigo publicado no periódico Gene. 2017 May 30; 614:26-36. doi: 10.1016/j.gene.2017.02.032, JCR: 2,415)

3. ARTIGOS

3.1 *In vitro* and *in vivo* studies on CCR10 regulations by Annexin A1*

ABSTRACT

The mode of action of annexin A1 (ANXA1) is poorly understood. By using rapid subtraction hybridization we studied the effects of human recombinant ANXA1 and the N-terminal ANXA1 peptide on gene expression in a human larynx cell line. Three genes showed strong downregulation after treatment with ANXA1. In contrast, expression of CCR10, a seven transmembrane G-protein coupled receptor for chemokine CCL27 involved in mucosal immunity, was increased. Moreover the reduction in CCR10 expression induced by ANXA1 gene deletion was rescued by intravenous treatment with low doses of ANXA1. These findings provide new evidence that ANXA1 modulates gene expression.

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In vitro and in vivo studies on CCR10 regulation by Annexin A1

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Abstract The mode of action of annexin A1 (ANXA1) is poorly understood. By using rapid subtraction hybridization we studied the effects of human recombinant ANXA1 and the N-terminal ANXA1 peptide on gene expression in a human larynx cell line. Three genes showed strong downregulation after treatment with ANXA1. In contrast, expression of *CCR10*, a seven transmembrane G-protein coupled receptor for chemokine CCL27 involved in mucosal immunity, was increased. Moreover the reduction in *CCR10* expression induced by ANXA1 gene deletion was rescued by intravenous treatment with low doses of ANXA1. These findings provide new evidence that ANXA1 modulates gene expression.

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Keywords: Gene expression; Host defence; Annexin A1-null mice; Epithelial cells; Annexin A1

1. Introduction

Annexin A1 (ANXA1) is a 37 kDa member of a family of Ca^{2+} -regulated lipid-binding proteins [1]. Functional studies have identified roles for ANXA1 and its N-terminal domain [2,3] in the regulation of inflammation [4], the cell cycle [5] and as a mediator of glucocorticoid action on the release of corticotrophin (ACTH) from the anterior pituitary gland [6,7]. Many of the regulatory actions of ANXA1 in these systems have been shown to emerge promptly, i.e., within minutes to hours of contact with the protein [6]. Whilst some have been ascribed to the ability of ANXA1 to act intracellularly to disrupt signalling cascades [8], others appear to reflect interactions with cell surface receptors [9–11]. Recent studies suggest that members of the formyl peptide receptor (FPR) family of G-protein coupled receptors are particularly important in this regard [12,13]. Three members of this receptor family have been identified in human, termed FPR, FPRL1 and FPRL2. Receptor-specific ligands are not yet available, but FPR is highly sensitive to the bacterial peptide formyl-Met-Leu-Phe (fMLP), while FPRL1 shows some selectivity for lipoxin A4, a lipid metabolite, in addition to responding to high concentrations of fMLP [14].

Despite the evidence that ANXA1 might influence cellular responsiveness, the possibility that ANXA1 regulates gene expression has received scant attention. Interestingly however, the recently generated ANXA1 knockout mouse shows significant alterations in the expression of a number of gene products associated with inflammation [15,16], a finding that concurs with earlier reports that ANXA1 reduces the expression of inducible nitric oxide synthase [17].

We have used rapid subtraction hybridisation (RaSH) [18] to investigate the effects of ANXA1_{Ac2–26} on gene expression in a human cell line of epithelial origin. In addition, we have compared the effects of hrANXA1 and ANXA1_{Ac2–26} with those of fMLP (a bacterial peptide) and the stable analogue of lipoxin A4 [15-epi-16-(para-fluoro)-phenoxy-LXA4-methyl ester, ATLa-ME], an arachidonic acid metabolite, in order to explore the role of the FPR family in effecting the response to ANXA1. The data obtained from the cell line in vitro have been evaluated in vivo by studies on *Anxa1* null mice. Our findings strongly support a role for ANXA1 in the regulation of gene expression, particularly those genes concerned with the inflammatory response.

2. Materials and methods

2.1. Drugs

Human recombinant annexin A1, [19] was produced, purified and tested by SDS-PAGE for the presence of cleaved forms which were not detected, by Scientific Proteins, Witterswil, Switzerland. Furthermore, the recombinant protein did not show any phosphorylation at the N-terminal as reported previously [19]. Human ANXA1_{Ac2–26} (acetylated peptide at the N-terminal) was synthesized in the Advanced Biotechnology Centre, Imperial College London, UK. The peptide fMLP was purchased from Sigma (Sigma-Aldrich Corp., Poole, Dorset, UK) and 15-epi-16-(para-fluoro)-phenoxy-ATLa-methyl ester (ATLa, the methyl ester of lipoxin A4) was a generous gift from Professor C. Serhan (Harvard Medical School, Boston, USA). The doses of acetylated peptide (ANXA1_{Ac2–26}; 2 μM) were chosen on the basis of previous studies, performed both in vitro and in vivo, in which we showed that comparable biological activity were obtained when the ratio between fMLP or ATLa and ANXA1_{Ac2–26} was 1:20 [13,20] furthermore such molar ratio was of 1:15 between the entire recombinant ANXA1 and the peptide Ac2–26 as described previously [21].

2.2. Cell culture

The Hep-2 cell lines were derived from a human larynx epidermoid carcinoma cells (ATCC Rockville, MD, USA) and used for RaSH analysis. The PDFS cell line derived from a clinically non-functioning pituitary macroadenoma was used to confirm and compare the analysis performed on Hep-2 cells (see supplement Table 2) [22].

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2.3. Animals

Male *Anxa1*-null mice aged approximately 4 months old [15] and weighing 30 ± 4 g were used together with age- and sex-matched wild type controls. All animals were fed on a standard chow pellet diet. All procedures were carried out under licence in accordance with the UK Animals (Scientific Procedures) Act, 1986. For tissue collection the animals were killed by cervical dislocation and their lower back region was shaved. Longitudinal fragments of the dorsal skin (after shaving), trachea and ileum were obtained from each mouse (6 animals per tissue) and frozen immediately in liquid nitrogen for subsequent extraction of RNA and protein.

Anxa1 null mice were injected intravenously via the lateral tail vein with hrANXA1 (10 ng/mouse), ANXA1_{Ac2-26} (14 ng/mouse) or a corresponding volume of the saline vehicle (100 μ l). Mice (three animals per treatment) were killed with CO₂, 24 h after drug administration, and tissues collected exactly as described above.

2.4. Rapid subtraction hybridization (RaSH) and RT-PCR analysis

Hep-2 cells were plated at a concentration of 10^6 cells/in six well plates (Corning Incorporated, NY, USA) and 24 h later in culture, stimulated with ANXA1_{Ac2-26} (2 μ M; 72 h). Total RNA isolated by RNeasy (Qiagen, West Sussex, England) from control and treated cells were used for double-stranded cDNA synthesis using a standard protocol [23]. The DNA was then digested and mixed with the adaptors XPDN-14/XPDN-12 (Sigma Genosys; final concentration 20 μ M) as described by Jiang et al. [18]. 100 ng *tester* cDNA (e.g., untreated cells) was mixed with 5 μ g *driver* cDNA (e.g., ANXA1_{Ac2-26} treated cells, see also Table 1) in hybridization solution [18,24]. After extraction and precipitation the hybridization mixtures (1 μ g) were ligated to an *Xho*I digested pCDNA3 plasmid and transformed in competent bacteria. Bacterial colonies were selected randomly and amplified by PCR. The sequences of these clones were determined using the automated cycle sequencer in the Advanced Biotechnology Centre, Imperial College London. Sequences were compared with the NCBI nucleotide bank (GenBank) allowing identification of any genes up or down regulated. Experiments were then set up ($n = 3$) in the same conditions described above and RT-PCR with specific oligonucleotides (supplementary data Table 1) for the genes identified by RaSH was performed.

2.5. Semi quantitative RNA analysis

We first determined the linear range of amplification (25, 20, 35, 40 cycles) of cDNA using each of the primer sets. Amplification was within the linear range for all genes studies when 35 PCR cycles were used and this level of amplification was therefore used in all further studies. For each sample 5 μ l of the PCR amplification products was analysed on 2% agarose gels and stained with ethidium bromide. The intensities of the bands were compared to GAPDH in each sample and evaluated using the Image Master Software (SYDR-1990, SYNGENE, USA).

2.6. Western blot analysis

Expression of CCR10 (GPR2) protein (15 μ g) was examined in the cell lines and murine tissue extracts by SDS-PAGE, then transferred electrophoretically to nitrocellulose paper [25]. The blots were incubated overnight at 4 °C in incubation buffer in presence of an anti-CCR10 antiserum (diluted 1:1000, goat anti-mouse polyclonal, Abcam; or anti human CCR10 diluted 1:1000, Imgenex San Diego, CA, USA). Immunoreactive protein bands were detected by chemiluminescence (GE Healthcare, UK) after incubation with secondary

antibody (rabbit anti-goat peroxidase; 1: 100000, Biomeda; or goat anti-rabbit peroxidase conjugated antiserum, 1:25000, Sigma-Aldrich Corp.). The blots were then scanned and analyzed (HP Scanjet 5200 with Adobe Photodeluxe Business Edition, version 1.1; Cupertino, CA). The intensities of the bands were compared to β -actin (mouse monoclonal 1:5000, Abcam).

2.7. FACS analysis

For detection of FPR and FPRL1 on the cell surface of Hep-2 and PDFS cells, aliquots of suspensions of the cells (10^5 cells in 100 μ l) were added to a 96-flat well plate in triplicate and incubated at 4 °C for 1 h in the presence or absence (controls) of either: (a) a monoclonal anti-human FPR antibody (1:100, BD Pharmingen) or mouse IgG1 clone NOPC-21 (isotype-matched control antibody 1:100, BD Pharmingen, UK) or (b) a rabbit anti-human FPRL1 polyclonal antiserum raised against the peptide TVWLKEMLFYGYKYKIIDILVNP from the 3rd extracellular domain of the receptor (1:100, Neosystem Strasbourg, France). Non-specific binding was minimized by the addition of human IgG (1.6 mg/ml, Roche Diagnostics, Lewes, UK). After incubation with the primary antiserum, the cells were incubated on ice for 30 min with FITC-conjugated rabbit anti-mouse or sheep anti-rabbit IgG (1:300, Serotec, Kidlington, Oxford, UK). The cell surface fluorescence was analyzed immediately by flow cytometry using a Becton Dickinson FACScan II analyzer. At least 10000 cells per sample were counted. The data were analyzed as units of fluorescence measured in the FL1 channel (mean fluorescence intensity). A similar method was used to detect and quantify expression of the cell surface receptor CCR10 (GPR2) using a goat anti-rabbit polyclonal antibody to CCR10 (GPR2, 1:100, Imgenex San Diego, CA, USA) as a probe and FITC-conjugated sheep anti-rabbit IgG (1:300, Serotec) for detection.

2.8. Statistical analysis

Statistical significance was assessed by ANOVA followed by Bonferroni's *t* test, and $P < 0.05$ was taken as statistically significant.

3. Results and discussion

3.1. Annexin A1 modulates gene expression in cell line

Subtractive hybridisation performed on the human cell line Hep-2, identified three genes, which were downregulated by hrANXA1 (100 nM) and ANXA1_{Ac2-26} (2 μ M) treatment. The first was *ITGB1BP1* (previously named *ICAP-1A*, Table 1 and Fig. 1) a protein specifically associated with the β 1 cytoplasmic domain of integrins [26] containing putative phosphorylation sites. The phosphorylated form probably impairs cell adhesion and spreading [27], but over-expression of *ITGB1BP1* stimulates cell migration [28]. Thus, the ability of ANXA1 to impair monocytes on the endothelium in vitro [21] may be mediated, at least in part, through *ITGB1BP1* downregulation.

Since ANXA1 and ANXA1-derived peptides are potential ligands for members of the formyl peptide receptor (FPR) family, we explored the potential role of these receptors in

Table 1
Genes isolated by RaSH

	Symbol	Locus ID	Function
Upregulated 100 ng of <i>tester</i> (ANXA1 _{Ac2-26} treated cells) versus 5 μ g of <i>driver</i> (untreated cells)	<i>CCR10</i> (NM_016602)	2826	A G protein coupled receptor for chemokine CCL27 mostly involved in T cell mediated skin inflammation
Downregulated 100 ng of <i>tester</i> (ANXA1 _{Ac2-26} treated cells) versus 5 μ g of <i>driver</i> (untreated cells)	<i>TIFA</i> (NM_052864)	92610	A TRAF2 binding protein involved in the TNF-mediated signalling
	<i>FARSLA</i> (NM_004461)	2193	A tRNA synthetase family expressed in a cell cycle stage and differentiation-dependent manner
	<i>ITGB1BP1</i> (NM_004763)	9270	Accessory protein for the integrin beta 1

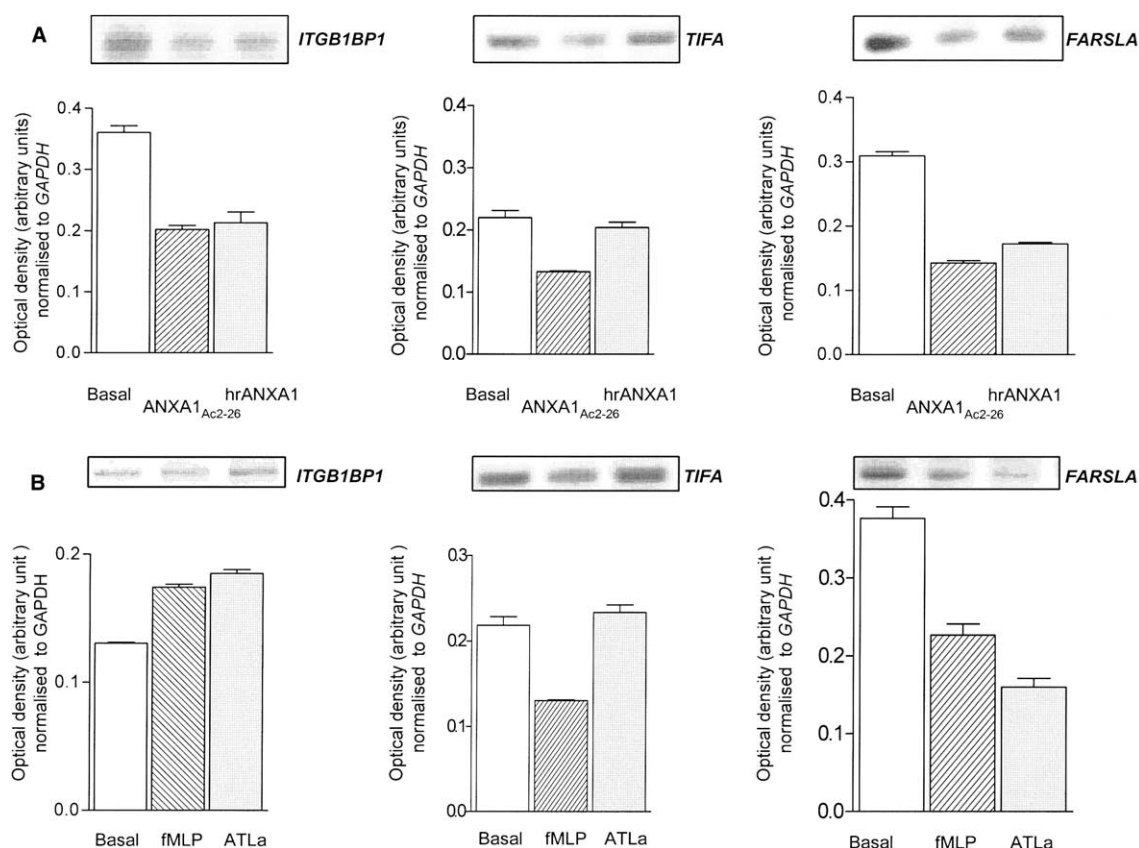


Fig. 1. Comparison of the effect of ANXA1 and FPRs ligands: fMLP and ATLa on *ITGB1BP1*, *TIFA* and *FARSLA* in Hep-2 genes isolated by RaSH. Cells were plated and treated as reported for RaSH analysis. After 72 h of stimulation with (A) ANXA1 (100 nM) or ANXA1_{Ac2-26} (2 μM) or (B) fMLP (100 nM) and ATLa (100 nM) total RNA was extracted and RT-PCR performed using specific oligos for the above genes. Semi-quantitative normalization was performed using GAPDH expression. The data are representative of six independent experiments.

mediating the genomic effects of ANXA1 reported above. First, we used RT-PCR and FACS analysis to determine whether Hep-2 cells express FPR and FPRL1. Our data demonstrated mRNA and protein for both receptors in Hep-2 cells (Fig. 2A and B). Subsequently, we used ligands of FPR and FPRL1, fMLP and the lipoxin A4 analogue (ATLa), to explore whether activation of these receptors modulated the expression of *ITGB1BP1* (Fig. 1B and Table 3B supplement

data). Neither fLMP nor ATLa, ligands for FPR and FPRL1, modified the expression of *ITGB1BP1* (Fig. 1B). Although we cannot exclude the possibility that higher concentrations of these compounds may have been effective they would be expected to act at the concentrations tested [13] and indeed have been shown to modify the expression of other genes in this study. We therefore suggest that the regulatory actions of ANXA1 and ANXA1_{Ac2-26} on the expression of *ITGB1BP1*

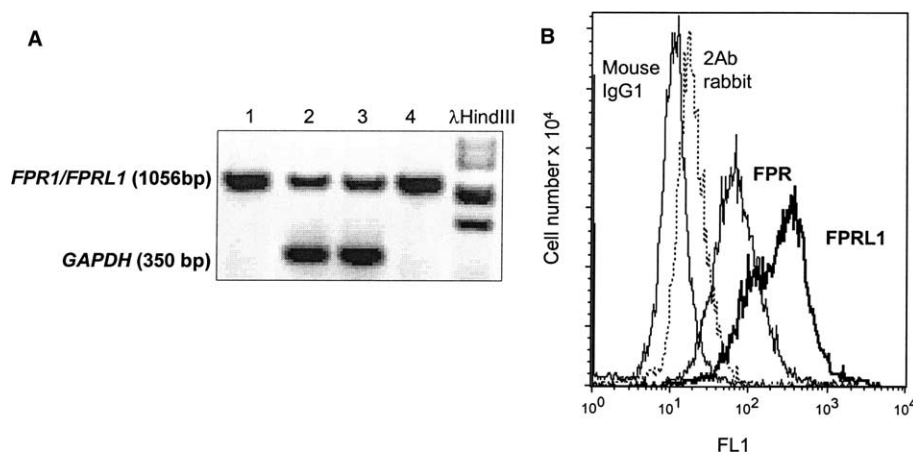


Fig. 2. Hep-2 cells express both FPR and FPRL1 receptors. (A) RT-PCR analysis lanes 1 and 4 represent the control amplified plasmids containing the entire coding sequences for FPR and FPRL1, respectively. Lanes 2 and 3 amplified product in Hep-2 cells for FPR and FPRL1, respectively. (B) FACS analysis of FPR and FPRL1 expression on the surface of the cells. The data are representative of three independent analyses.

were mediated via a mechanism independent of FPR or FPRL1.

A second gene downregulated by both hrANXA1 and ANXA1_{Ac2-26} was a phenylalanine-tRNA synthetase (*FARSLA*). Unlike ITGB1BP1 this gene was also downregulated by fMLP and ATLa, suggesting a role for the FPR family in mediating the response (Fig. 1A and B). In vivo FARSLA is expressed in a tumour-selective, cell cycle stage and differentiation-dependent manner and its expression is augmented in tumourigenic versus non-tumourigenic variant of the same cell line [29]. Interactions between ANXA1 and aminoacyl-tRNA synthase have been reported in patients with dermatomyositis [30] and between ANXA1 and tryptophanyl-tRNA synthetase [31]. Together, these results raise the possibility that ANXA1 plays a role in cell transformation as well as in protein translation and may explain why it is highly expressed in some cancers and not in others [32]. Interestingly, it has been proposed that ANXA1 and tRNA synthase share some physiological role in process related to myositis and immune response [31].

A third gene downregulated by ANXA1_{Ac2-26} and fMLP, but not by ANXA1 or ATLa (Fig. 1A and B) was a TRAF-interacting protein with a forkhead-associated domain termed *TIFA* or *T2BP*. Although merely speculative, as we do not know how the acetylation may affect the structure and function of the peptide, the downregulation of *TIFA* suggests that ANXA1 may modulate inflammatory processes via TNF α and *TIFA*, supporting our previous findings on the role of ANXA1 in systemic endotoxemia [33]. The data on the full protein (hrANXA1) or ATLa (ligand of FPRL1 receptor), which did not affect the expression of this gene, suggest that the full-length protein (hrANXA1) and the N-terminal peptide (ANXA1_{Ac2-26}) act via different mechanisms and thus support a preferential binding of ANXA1 to FPRL1 receptor [13]. We have recently published data on the differential effects of the full-length protein and its peptide in the firm adhesion of human PMNs on the endothelium under flow [20] and the results demonstrated here reinforce the dichotomy in the biological activity of the acetylated peptide versus the entire molecule.

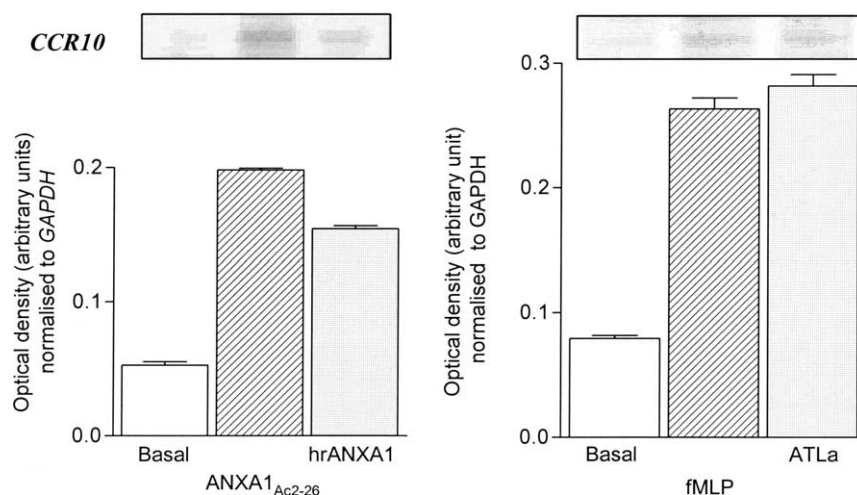


Fig. 3. CCR10 expression in Hep 2 cells. RT-PCR analysis of *CCR10* expression after stimulation with ANXA1, ANXA1_{Ac2-26}, fMLP and ATLa for 72 h. The data are representative of six independent experiments.

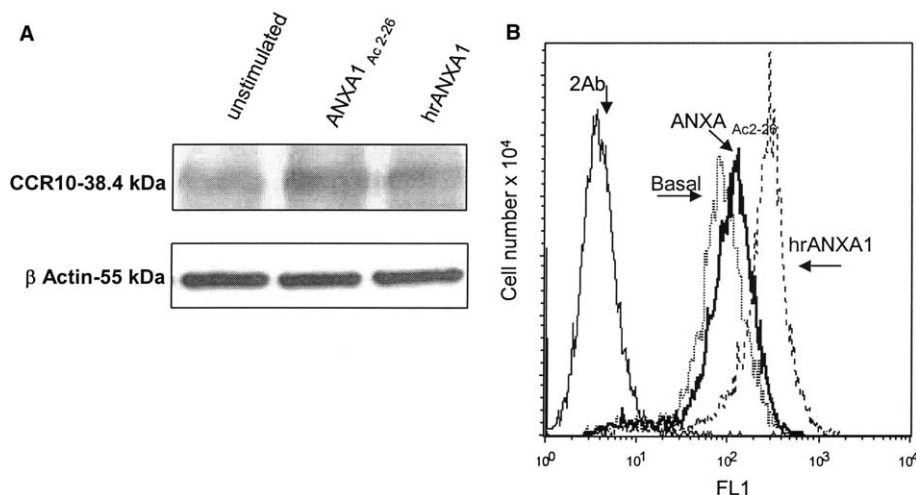


Fig. 4. Western blot and FACS analysis of CCR10 expression on Hep-2 cells treated with recombinant hANXA1 and ANXA1_{Ac2-26}. (A) Total protein cell extract from treated or untreated cells were analysed by Western blot. (B) FACS analysis: histogram (intensity of fluorescence versus number of cells) showing the data from one of three independent experiments which all showed similar results.

In contrast to its effects on *ITGB1BP1*, *FARSLA* and *TIFA*, ANXA1 (which is strongly expressed in epithelial cells and skin [34]), had a positive effect on the expression of the

CCR10 gene (Table 1), a skin homing receptor for chemokine CCL27 [35]. This effect was mimicked by ANXA1_{Ac2–26}, fMLP and ATLα (Fig. 3 A and B), supporting a role for the FPR

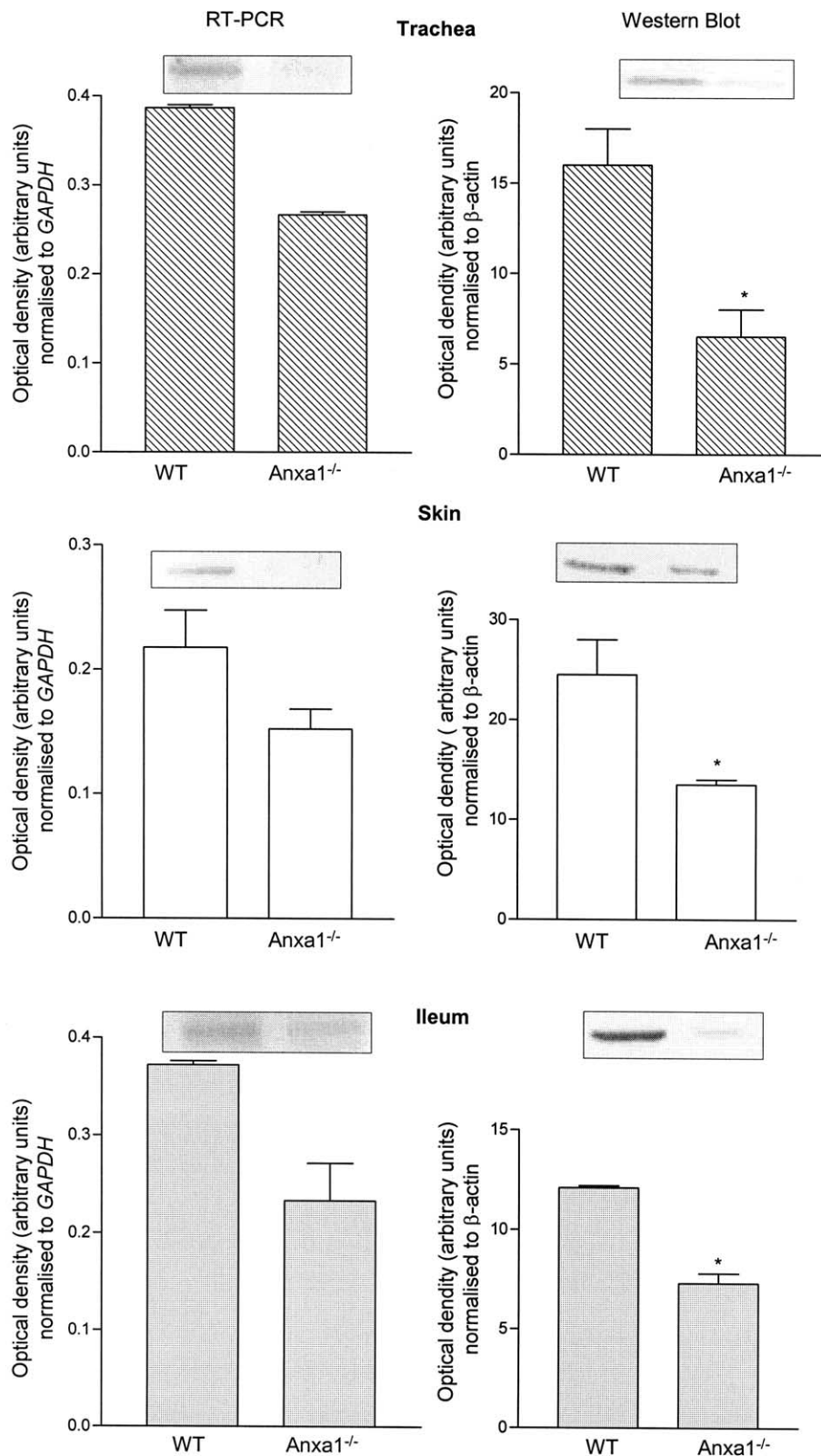


Fig. 5. CCR10 expression in Anxa1 null mice. Left: RT-PCR analysis and right: Western blot analysis of CCR10 in trachea, skin and ileum from Anxa1 null and wild type control mice. Histograms represent a semi-quantitative analysis performed on 6 mice per genotype, * $P < 0.005$ versus relative WT.

family in mediating the positive effect of ANXA1. Support for a role of ANXA1 in CCR10 gene regulation was provided by the Western blot and FACS analysis of the protein expression and membrane localisation (Fig. 4A and B) indicating the potential for a ligand binding activity.

3.2. Analysis of CCR10 expression in vivo

Our studies on *Anxa1*-null mice and wild type controls reveal a marked reduction in CCR10 expression in the *Anxa1*-null mice versus control (Fig. 5) and show that this is reversed by treatment with hrANXA1 or ANXA1_{Ac2-26} (Fig. 6). How-

ever *Anxa1* null mice did not show any phenotype that could readily be linked to downregulation of CCR10 and further studies are necessary to explore the significance of these findings. However, our own data (John et al. manuscript in preparation) and a recent report showing the LPS susceptibility of the *Anxa1* null mice [36] open new avenues on the possible significance of changes in CCR10 expression.

As ANXA1 is commonly viewed as an anti-inflammatory protein and CCL27 is pro-inflammatory, it might have been predicted that ANXA1 would downregulate CCR10 expression. However recent studies have reported that CCR10

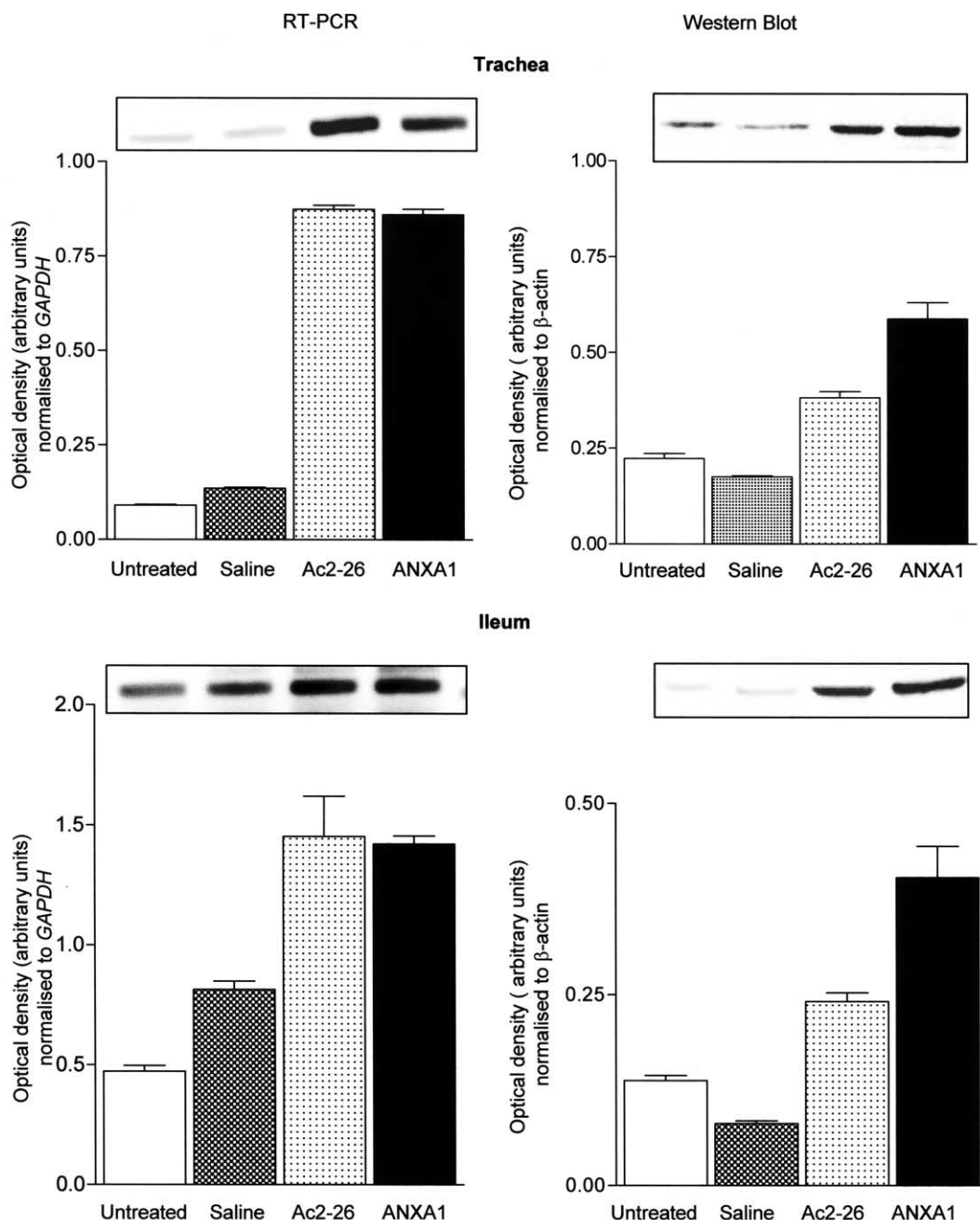


Fig. 6. Exogenous recombinant ANXA1 augments the expression of CCR10 in trachea and ileum from *Anxa1* null mice. Left panel: RT-PCR analysis Right panel: Western blot of CCR10 in trachea and ileum of wild type (WT) and *Anxa1* null mice treated with human recombinant ANXA1 or ANXA1_{Ac2-26} (10 ng/mouse, and 14 ng/ mouse, respectively, iv, 24 h before animals were killed). The data are representative of two independent experiments performed on three animals per group.

induction on pathogen-specific IgA-secreting B cells may be an important factor for successful protection following local mucosal infection [37].

4. Conclusions

Taken together, the data provide new evidence that ANXA1 modulates gene expression. The mechanisms responsible require further investigation. While our data support a role for the FPR family in mediating some of the effects, the failure of fLMP and ATLa to suppress *ITGB1BP1* suggest other mechanisms are involved. Furthermore, the dissociation between the activities of hrANXA1 and ANXA1_{Ac2–26} support the premise that the actions of ANXA1, but not ANXA1_{Ac2–26}, are akin to those of the FPRL1 ligand LXA4.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2006.01.072](https://doi.org/10.1016/j.febslet.2006.01.072).

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3.2 Annexin 1: differential expression in tumor and mast cells in human larynx cancer*

ABSTRACT

Annexin 1 protein (ANXA1) expression was evaluated in tumor and mast cells in human larynx cancer and control epithelium. The effect of the exogenous ANXA1 (peptide Ac 2-26) was also examined during the cellular growth of the Hep-2 human larynx epidermoid carcinoma cell line. This peptide inhibited the proliferation of the Hep-2 cells within 144 hr. In surgical tissue specimens from 20 patients with larynx cancer, ultrastructural immunocytochemistry analysis showed in vivo down-regulation of ANXA1 expression in the tumor and increased in mast cells and Hep-2 cells treated with peptide Ac2-26. Combined in vivo and in vitro analysis demonstrated that ANXA1 plays a regulatory role in laryngeal cancer cell growth. We believe that a better understanding of the regulatory mechanisms of ANXA1 in tumor and mast cells may lead to future biological targets for the therapeutic intervention of human larynx cancer.

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Annexin 1: differential expression in tumor and mast cells in human larynx cancer

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Annexin 1 protein (ANXA1) expression was evaluated in tumor and mast cells in human larynx cancer and control epithelium. The effect of the exogenous ANXA1 (peptide Ac 2-26) was also examined during the cellular growth of the Hep-2 human larynx epidermoid carcinoma cell line. This peptide inhibited the proliferation of the Hep-2 cells within 144 hr. In surgical tissue specimens from 20 patients with larynx cancer, ultrastructural immunocytochemistry analysis showed *in vivo* down-regulation of ANXA1 expression in the tumor and increased in mast cells and Hep-2 cells treated with peptide Ac2-26. Combined *in vivo* and *in vitro* analysis demonstrated that ANXA1 plays a regulatory role in laryngeal cancer cell growth. We believe that a better understanding of the regulatory mechanisms of ANXA1 in tumor and mast cells may lead to future biological targets for the therapeutic intervention of human larynx cancer.

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Key words: human mast cells; Hep-2 cells; annexin 1; larynx tumor; ultrastructural immunocytochemistry

Larynx cancer is one of the most common types of tumors with high mortality rates and poor prognosis.¹ This cancer (159,000 new cases and 90,000 deaths globally per year) is predominantly a cancer affecting men, in whom it comprises 2.4% of cases and 2.1% of deaths.² This evidence demonstrates that the development of better treatment, diagnostic and preventive approaches requires an improved understanding of the complex processes of larynx tumorigenesis.

The anti-inflammatory protein annexin 1 (ANXA1) is a 37 kDa member of the annexin superfamily that share the function to bind phospholipids in the presence of calcium ions. Previous studies have emphasized that the N-terminal peptide of ANXA1 (peptide Ac2-26) can reproduce the anti-inflammatory actions of the full-length protein.³ ANXA1 is described as a steroid-regulated protein and is implicated in some of the beneficial actions of glucocorticoids, including inhibition of cell proliferation, anti-inflammatory effects, the regulation of cell differentiation⁴ and membrane trafficking.⁵ The molecular mechanisms by which ANXA1 modulates these cellular responses have not been fully determined. The studies indicate that ANXA1 inhibits cytosolic phospholipase 2 activity and arachidonic acid generation,⁴ which may explain some of its anti-inflammatory and antiproliferative functions.⁶ Due to these antiproliferative effects, ANXA1 protein has been studied in some types of cancer. ANXA1 expression in tumor cells has been investigated *in vitro*⁷ and *in vivo* in cancers of the head and neck,⁸ liver,⁹ esophagus,¹⁰ lung¹¹ and prostate.^{10,12} Antiproliferative actions of ANXA1 have been reported in experiments *in vitro*. The synthetic glucocorticoid dexamethasone inhibits A549 human lung adenocarcinoma cell line proliferation, a process that is mediated by induction of ANXA1.¹³

Studies *in vivo* have demonstrated that the pattern of ANXA1 expression in human cancer is not well defined. While up-regula-

tion of ANXA1 was previously observed in glioblastoma¹⁴ and mammary adenocarcinoma,¹⁵ many investigators have reported that ANXA1 expression was reduced or lost in other cancer tissues. Down-regulation of ANXA1 was reported in many types of cancers, including in head and neck,^{8,10} esophageal,¹⁰ gastric,¹⁶ prostate^{10,12} and breast.¹⁷ The altered expression of annexins has also been associated with cell line transformation, tumor progression and metastasis, and interpreted to suggest either a homeostatic or possible tumor suppressive role for annexins.⁸

ANXA1 also has been reported in inflammatory cells, particularly in mast cells involved in the inflammatory responses.^{18,19} Mast cells have been reported in various types of the cancer such as breast,²⁰ skin,²¹ liver²² and gastric.²³ These cells accumulate at sites of tumor growth in response to numerous chemoattractants. Mast cell infiltration has been implicated in tumor growth and is associated with poor prognosis.²⁴ Evidences indicate that mast cells could have a dual role, to promote or inhibit tumor growth depending on the local stromal conditions. This potential dual role of mast cells is probable because of their ability to secrete either the content of individual granules or distinct mediators selectively.²⁵ Trypsinase, a serine proteinase, is one important mast cell mediator stored in cytoplasmic granules and is a specific marker of human mast cells investigated in cancer diseases.²¹

In the present study we evaluated ANXA1 protein expression *in vivo* in tumor and mast cells in invasive human larynx cancer together with patient-matched normal epithelium using ultrastructural immunocytochemistry analysis. The effect of the exogenous ANXA1 (peptide Ac2-26) was also investigated during the cellular growth of the Hep-2 human larynx epidermoid carcinoma cells.

Material and methods

Tissue specimens

Human invasive laryngeal cancer tissues were collected from 20 patients in the Hospital de Base, São José do Rio Preto, São

Abbreviations: ACTB, actin, beta; ANXA1, annexin 1; cPLA2, cytosolic phospholipase A2; EGF, epidermal growth factor; FPR, formyl peptide receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBEA, phosphate buffer egg albumin; TBAE, chicken egg albumin in tris buffer; TUBA6, tubulin, alpha 6.

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Paulo, Brazil, according to protocols approved by the Committee of Ethics in Research of the Federal University of São Paulo, School of Medicine, Brazil (CONEP—process 128/01). Surgical samples were collected from the center of the laryngeal tumor, peritumoral (clinically normal adjacent tissue) and control regions (normal laryngeal tissue) of the same patient. The patients were all male and the age at surgery was in the range of 50–80 years and none of them had received radiotherapy or chemotherapy before intervention.

Cell culture and conditions

The Hep-2 cell line used was derived from a human larynx epidermoid carcinoma cells (ATCC Rockville, Maryland USA). Hep-2 cells were cultured at 37°C in 5% CO₂ in complete medium: MEM-Glutamax (Invitrogen, Paisley, UK), pH 7.5, containing 10% fetal calf serum (PAA Laboratories, Teddington, Middlesex, UK), 1% nonessential amino acids (Invitrogen) and 0.1% gentamycin (Sigma-Aldrich, Poole, Dorset, UK). The cells were plated in 10-cm plates (Corning, High Wycombe, UK), and were incubated in the conditions described above until the cells attained 70% confluence.

For experiments, Hep-2 cells were seeded at a concentration of 10⁴ cells/35 mm plates in two sets of triplicates. By 24 hr, the cells were adhered and treated with N-terminal peptide of ANXA1 (peptide Ac2-26, 2 µM) in complete medium. Control cells were treated with boiled peptide Ac2-26 or complete medium. During protocol optimization, 3 different concentrations (5, 7.5 and 10 µg/mL) of Ac 2-26 peptide were used. Since the cells died with the last 2 concentrations, the former concentration was used in all experiments. Fresh drugs were added every 2 or 3 days, when the medium was changed. Cell morphology was observed every day. After 1, 24, 48, 96 and 144 hr, control and treated cells were harvested, counted using a Neubauer hemocytometer, fixed and embedded for ultrastructural immunocytochemical microscopy analysis to investigate cell-associated ANXA1. In the second set of triplicates total RNA was extracted. The experiment was repeated 4 times.

RNA extract and real time PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies, Inc.) and treated with DNase. Aliquots (10 g) of total RNA from control and treated cells were used for double-stranded cDNA synthesis by using High Capacity cDNA Archive kit (Applied Biosystems) as described by the manufacturer.²⁶

PCR amplifications were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixture consisted of a 20 µL volume solution containing 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems), and 500 nM of each primer. For every experiment, 10 ng cDNA were used and each sample was tested in duplicate. The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve analysis was also performed for each gene to check the specificity and identity of the RT-PCR products. The gene expression stability over different samples was analyzed using the geNorm software.²⁷ Each transcript level was normalized by division with the expression values of the *GAPDH*, *ACTB* and *TUBA6* used as internal standards.

Fixation, processing and embedding for immunohistochemistry and ultrastructural immunocytochemistry

Fragments (0.5 mm) of laryngeal tumor, peritumoral and control tissues (*n* = 20 patients) of the same patient and Hep-2 cells were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 24 hr at 4°C. They were then washed in sodium cacodylate buffer, dehydrated through a graded series of methanol, and embedded in LR Gold resin (London Resin Company, Reading, Berkshire, UK). Sections

(1 µm thick) for immunohistochemistry and (70 nm thick) for ultrastructural immunocytochemistry were cut on an ultramicrotome (Reichert Ultracut; Leica, Austria) and placed on nickel grids for immunogold labeling.

Immunohistochemical analysis

To quantify human mast cells²¹ anti-tryptase antibody (specific marker of human mast cells) was used. The sections (1 µm thick) were prepared onto histological slides,²⁸ washed in 0.1 M Tris buffer (Sigma Chemical), pH 8.2, incubated for 30 min with 3% hydrogen peroxide and then washed 3 times in Tris buffer for further 30 min. Nonspecific binding sites were then blocked with 10% Chicken Egg Albumin in Tris buffer (TBAE). Sections were incubated overnight at 4°C in primary antibody, a mouse monoclonal anti-tryptase (Novocastra, Benton Lane, UK), diluted 1:500 in 1% TBAE. As control, sections were incubated with nonimmune mouse serum. Sections were washed in 1% TBAE for 40 min before incubation for 1 hr at room temperature with the secondary antibody rabbit anti-mouse IgG (Fc fragment specific) antibody (1:50 in TBEA) conjugated to 5 nm colloidal gold (British BioCell International, Cardiff, UK). Sections were washed in Tris buffer (30 min) and in distilled water (30 min). Silver enhancing solution (SEKL, British BioCell International, Cardiff, UK) was used to augment gold particle staining. At the end of the reaction, sections were washed thoroughly in distilled water, counterstained with hematoxylin and eosin and mounted in BIO-MOUNT (British BioCell International, Cardiff, UK). Finally, sections were examined using an AXIOSKOP2 microscope (ZEISS, GR) and photographed.

Ultrastructural immunocytochemistry

For ANXA1 detection, we used an established immunogold procedure.¹⁸ Ultrathin sections (70 nm) of laryngeal tissues (tumor, peritumoral and control) and Hep-2 cells were prepared and incubated at room temperature with 0.1 M phosphate buffer containing 5% egg albumin (PBEA) for 30 min. The sections were then incubated for 2 hr with a polyclonal antibody anti-ANXA1 named LCPS1, a sheep polyclonal antibody raised against the N-terminal peptide of human ANXA1 (peptide Ac2-26), diluted 1:200 in 1% PBEA. Sections incubated with normal sheep serum were used as control (1:200). After 20 min washing in PBEA, a 1:50 dilution in PBEA donkey anti-sheep IgG (Fc-fragment specific) antibody conjugated to 15 nm colloidal gold (British BioCell International, Cardiff, UK) was added and sections were incubated for 1 hr at room temperature. Sections were extensively washed in PBEA and then in distilled water. Ultrathin sections were stained with uranyl acetate and lead citrate before examination on a ZEISS-LEO 906 electron microscope (IBILCE-UNESP, São José do Rio Preto, São Paulo, Brazil).

Data handling and statistical analysis

Analyses of ANXA1 protein expression were realized in electron micrographs (*n* = 10 cells) of the tumor, mast cells (laryngeal tumor, peritumoral and control tissues) and Hep-2 cells. The area of each cell compartment (nucleus and cytoplasm) was determined with the Axiovision software. The density of immunogold (number of gold particles per µm²) was calculated and expressed for each cell compartment. Values are reported as mean ± SEM of gold particles per µm². The mean gold particle density was determined by analysis of variance followed, if significant, by the Bonferroni test (tissue) and Student's *t*-test (Hep-2 cells); *p* values less than 0.05 were taken as significant.

Quantitative analysis of the intact and degranulated mast cells of human larynx (*n* = 20 patients) was performed in histological sections of the laryngeal tumor, peritumoral and control tissues stained for detection of tryptase in human mast cells. The quantification of the mast cells was analyzed in 4 regions of mast cells

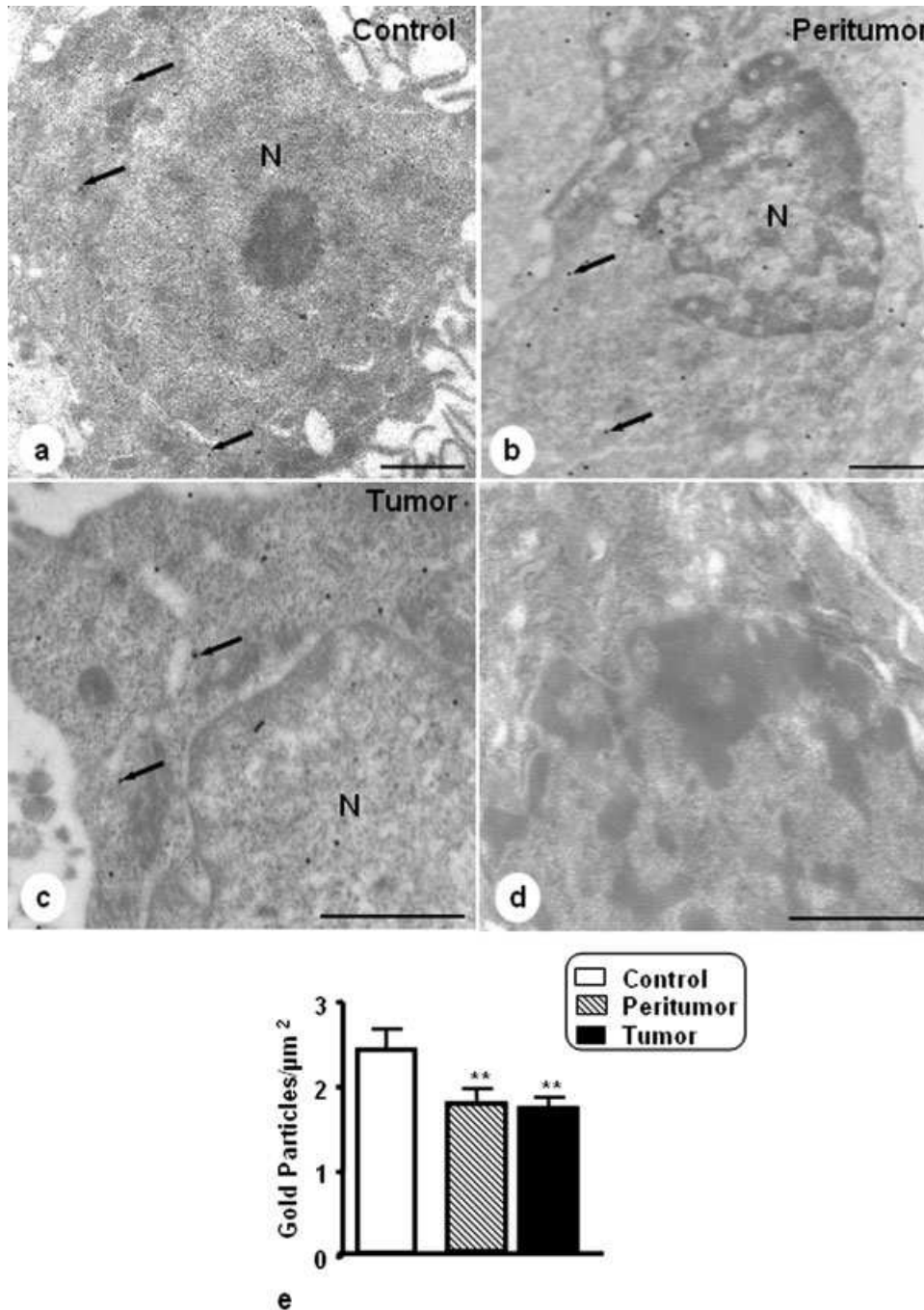


FIGURE 1 – Immunocytochemical analysis of ANXA1 immunoreactivity in human laryngeal tissues *in vivo*. (a) The epithelial laryngeal cells (control tissue) showed widespread immunoreactivity in nucleus (N) and cytoplasm (arrows). (b) A low degree of ANXA1 immunoreactivity was observed in nucleus (N) and cytoplasm (arrows) of the epithelial cells in peritumor tissue. (c) ANXA1 expression in tumor cells was to a lower degree of immunoreactivity in nucleus (N) and cytoplasm (arrows). (d) Absence of gold labeling in section incubated with nonimmune sheep serum. (e) Density of ANXA1 immunogold particles in control epithelium cells (control and peritumor laryngeal tissues) and tumor cells. Data represent mean $\pm$ SEM of gold particles per μm^2 localized in the control, peritumor and tumor laryngeal tissues cells ($n = 200$ cells for each group obtained from 20 patients). * $p < 0.05$ versus Control Tissue. Scale bars, 1 μm .

was performed with a high-power objective (40 $\times$) counting the cells number in mm^2 areas. The mean mast cell number was used for statistical analysis by the independent Student's *t*-test; *p* values less than 0.05 were taken as significant.

Results

Expression of ANXA1 in tumor and epithelial cells of the laryngeal tissues *in vivo*

ANXA1 was detected by post-embedding immunogold labeling in tumor and normal epithelium. The successful fixation protocol of these tissues was evaluated by the good preservation of ultrastructural morphology and antigenicity for ANXA1. The ANXA1 expression was observed in epithelial cells from control and peri-

tumoral tissues (Figs. 1a and 1b) and tumor (Fig. 1c). No gold labeling was detected in control sections incubated with nonimmune sheep serum (Fig. 1d). The significant decrease ($p < 0.05$) in the expression of ANXA1 was observed in tumor and epithelial cells from peritumoral tissue in relation to control laryngeal tissue (Fig. 1e).

Effect of exogenous ANXA1 (peptide Ac2-26) on Hep-2 cell proliferation

We monitored the growth of the Hep-2 cells *in vitro* during a course of treatment with peptide Ac2-26 for 144 hr. The growth curve showed that peptide Ac2-26 (2 μM) inhibited cell proliferation in Hep-2 cells treated for 144 hr compared to both vehicle and boiled Ac2-26 peptide controls (Fig. 2).

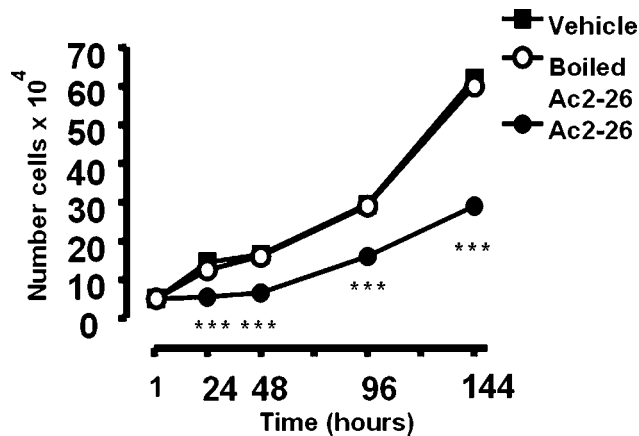


FIGURE 2 – Growth curve of Hep-2 cells treated with peptide Ac2-26. Proliferation of the cells was inhibited by peptide Ac2-26 (2 μ M) during 144 hr. Vehicle group (Hep-2 cells treated with complete medium), boiled Ac2-26 (second control) and Ac2-26 (Hep-2 cells treated with peptide Ac2-26). Data are expressed as means $\pm$ SEM of number cells $\times 10^4$. *** $p \leq 0.001$ versus vehicle.

Exogenous Ac2-26 peptide regulates expression of ANXA1

The real time PCR data showed greater expression of ANXA1 mRNA in Hep-2 cells after 6 hr of peptide Ac2-26 treatment. This was followed by a decrease in expression at 12 and 24 hr, and a second peak at 48 hr prior to a subsequent decline up to 144 hr, when compared to untreated Hep-2 cells (Fig. 3a). However, protein expression was observed to be significantly increased at 24 and 96 hr, maintaining a stable amount up to 144 hr (Fig. 3b). Ultrastructural immunocytochemistry analysis showed positive endogenous ANXA1-staining in the nucleus and cytoplasm of untreated Hep-2 cells (Fig. 3c) and treated with peptide Ac2-26 for 144 hr (Fig. 3d). No gold labeling was detected in control sections incubated with nonimmune sheep serum (Fig. 3e).

Expression of tryptase in the mast cells of the laryngeal tissues in vivo

The immunohistochemical reaction using the monoclonal antibody anti-tryptase, was used as a specific marker for human mast cells, in order to quantify their appearance in the laryngeal tissues studied (Figs. 4a–4c). In the control tissue, we observed intact mast cells in connective tissue, showing intense tryptase immunoreactivity within cytoplasmic granules (Fig. 4a). In contrast, no immunoreactivity was observed in sections incubated with nonimmune mouse serum (data not shown). The mast cells within the peritumoral and tumor tissue in some cases showed the characteristic signs of degranulation and were also immunoreactive for tryptase (Figs. 4b and 4c). The intraepithelial regions of invasive tumor tissue showed frequent infiltration by immunoreactive mast cells (Fig. 4c). The tumor and peritumoral tissues showed a significant ($p < 0.05$) decrease in the number of intact mast cells when compared with control tissue (Fig. 4d). However, the number of degranulated mast cells was significantly ($p < 0.05$) higher in the tumor and peritumoral tissues than in the control tissue. Mast cell number in the tumor was significantly ($p < 0.05$) lower than in the peritumoral tissue (Fig. 4d).

Subcellular localization of ANXA1 in mast cells in vivo

The mast cells in the laryngeal tissues showed immunoreactivity for ANXA1 within the nucleus, cytosol and in the matrix of the electron-dense cytoplasmic granules (Figs. 5a–5c). Some immunoreactivity was also found in association with the plasma mem-

brane. Clusters of ANXA1 immunoreactivity in the matrix of cytoplasmic granules were selectively detected in the mast cells of laryngeal tumor (Fig. 5c). No gold labeling was detected in control sections incubated with nonimmune sheep serum (Fig. 5d). The quantitative analysis of ANXA1 immunogold expression in mast cells showed subcellular localization of gold particles in each tissue (Table I). The data obtained in the cellular compartments examined (nucleus, cytosol and cytoplasmic granules) in mast cells indicated differences in the expression of the protein in each tissue. A significant increase ($p < 0.05$) in the immunoreactivity of ANXA1 was observed in the nucleus and cytoplasmic granules of mast cells in the laryngeal tumor and peritumor tissues compared to the control. The peritumoral tissue also showed an increase significantly ($p < 0.05$) of immunogold particles in the cytosol of the mast cells compared to the laryngeal tumor and control tissues (Table I).

Discussion

Cancer of the larynx shows a high rate of mortality, despite advances in diagnosis and treatment.¹ There is evidence that in certain clinical conditions, changes in annexin expression levels or localization may contribute to the pathological consequences and sequelae of disease. In this way, annexins are indirectly linked to some of the most serious human disease including cancer.²⁹ Here we have explored that endogenous ANXA1 expression in tumor and mast cells infiltrates would regulate the function of both cell types in the tumor development. Also investigated the effect exogenous ANXA1 *in vitro* in Hep-2 human larynx epidermoid carcinoma cells.

The density of ANXA1 immunoreactivity showed a significant down-regulation in the laryngeal tumor and peritumoral cells compared with control tissue. Several other studies have shown that ANXA1 expression also changes during the development and progression of cancer.¹⁷ It is down-regulated in head and neck,⁸ prostate¹² and breast cancers.¹⁷ The ANXA1 protein expression was decreased in these cancers compared with normal epithelium suggesting that this protein is an essential component for maintenance of the normal epithelial phenotype, a fact that may be important in early events in invasive cancer.¹⁰

Based on our findings demonstrating the down-regulation of ANXA1 expression in laryngeal tumor cells *in vivo*, we went on to investigate the effect of exogenous ANXA1 on Hep-2 larynx cancer cells. The results demonstrated that addition of peptide Ac2-26 inhibited cellular growth of the Hep-2 cells in 144 hr. These antiproliferative actions of ANXA1 in Hep-2 cells suggest that this protein has an important role in larynx tumor suppression.

We also investigated whether the mechanism of ANXA1 induction by peptide Ac2-26 in Hep-2 cells was at the transcriptional level. The real time PCR results combined with ultrastructural immunocytochemistry demonstrated that the peptide Ac2-26 treatment of Hep-2 cells induced a peak of ANXA1 mRNA expression at 6 and 48 hr, whereas the peak in protein expression was observed at 24 and 96 hr. Other studies have also demonstrated difference in ANXA1 expression patterns at mRNA and protein levels.^{28,30} This data correlated with the antiproliferative response, suggesting that peptide Ac2-26 can trigger Hep-2 cell growth regulation by direct interaction with the ANXA1 FPR receptor. The peptide may also have activated endogenous ANXA1 expression, resulting in growth regulation by an unclarified autocrine/paracrine mechanism. The ANXA1 autocrine/paracrine role at the membrane has been also shown in the invasion of a model epithelial cell line, SKCO-15, derived from colorectal adenocarcinoma through nFPR activation.³¹

The yet unknown mechanism by which ANXA1 regulates proliferation might involve gene expression modulation since recent data support such a role for this protein.³² It is also possible that

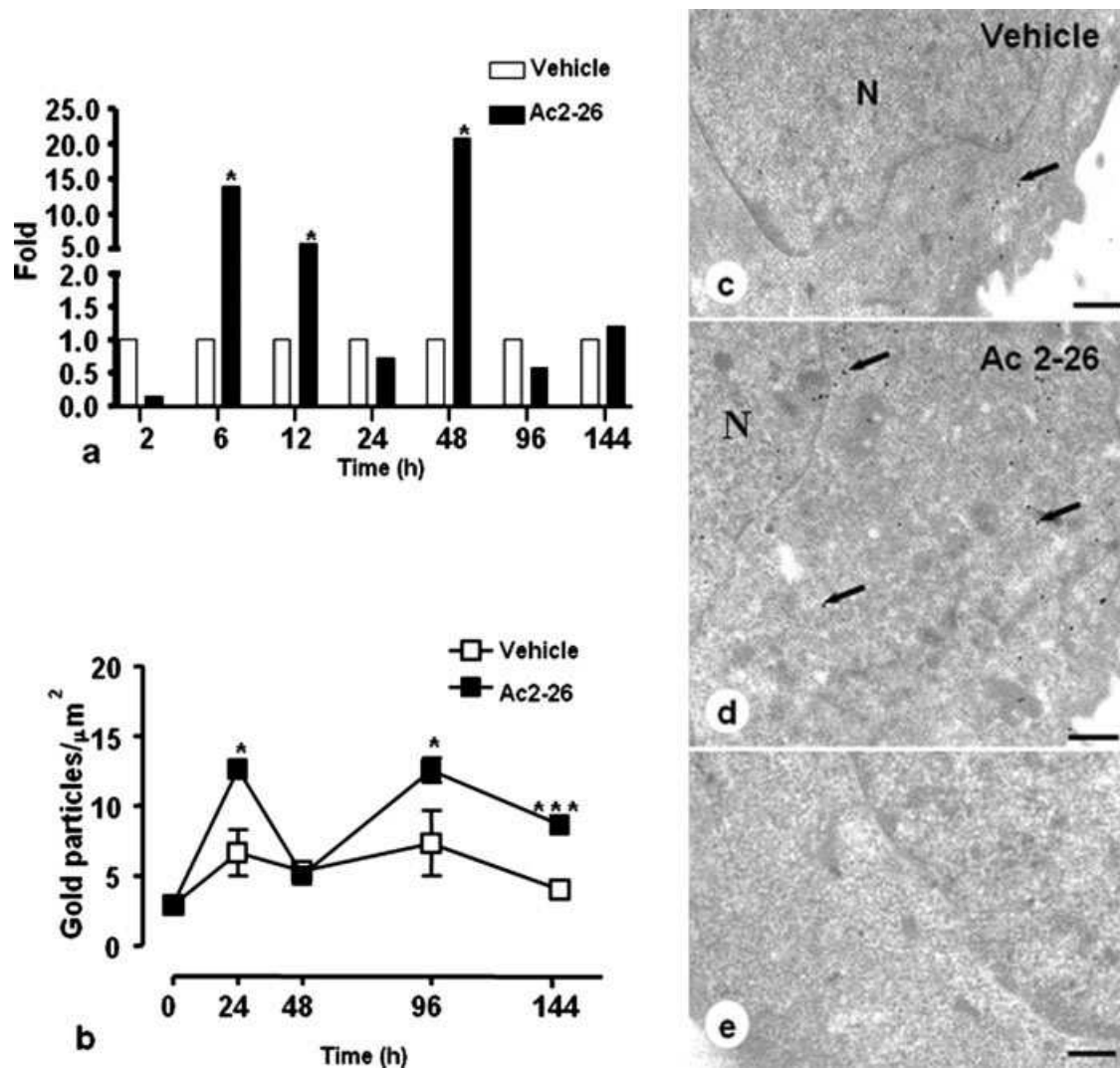


FIGURE 3 – Transcriptional and translational expression of ANXA1. (a) mRNA expression level of ANXA1 from Hep-2 cells treated with peptide Ac2-26 was determined by real time PCR and compared with vehicle group (Hep-2 cells treated with complete medium). Each transcript level was normalized by division with the expression values of the *GAPDH*, *ACTB* and *TUBA6* used as internal standards. (b) Protein expression of ANXA1 in Hep-2 cells over 144 h. Representative time course showing the 37 kDa of ANXA1 in Hep-2 cells. Values (mean $\pm$ SEM) were obtained by immunocytochemical analysis for Hep-2 cells at 24, 48, 96 and 144 hr. ($n = 10$ distinct cells for each group). * $p \leq 0.05$ versus vehicle; *** $p \leq 0.001$ versus vehicle. Electron micrographs showing ANXA1 immunogold labeling in Hep-2 cells. (c) Vehicle group (untreated Hep-2 cells) shows some immunoreactivity in the nucleus (N) and cytoplasm (arrows). (d) Hep-2 cells after treatment with peptide Ac2-26 (144 hr) showed intense ANXA1 immunoreactivity localized to the nucleus (N) and cytoplasm (arrow). (e) Absence of gold labeling in sections incubated with nonimmune sheep serum. Scale Bars: (a–c) 1 μ m.

the antiproliferative actions of ANXA1 are mediated *via* stimulation of apoptosis. ANXA1 has been shown to stimulate apoptosis, mainly in cells of the myelo-monocytic lineage,^{33,34} neutrophils³⁵ and in broncho-alveolar epithelial cells.³⁶ In addition, ANXA1 expression is strongly induced in thyroid carcinoma cells during “tumor necrosis factor-related apoptosis inducing ligand” (TRAIL)-induced apoptosis.³⁷ Preliminary experiments by us to investigate if treatment with peptide Ac2-26 (2 μ M) influenced apoptosis in Hep-2 cells revealed that there was no increase in apoptotic cell number as identified by ANXA5 binding (data not shown). Another possible mechanism for the antiproliferative action of ANXA1 could be *via* activation of the extracellular receptor kinase (ERK) signaling cascade, as previously demonstrated in a variety of cell types.⁶ The precise mechanism by which ANXA1 regulates cellular proliferation in laryngeal carcinoma cells remains to be determined.

Differentiation induction by ANXA1 was also reported *in vitro* for A549 cells¹³ and in the human squamous cell carcinoma cell line SqCC/Y1. Studies demonstrated that ANXA1 added exogenously to SqCC/Y1 cells are capable of inducing cellular differentiation presumably through its capacity to decrease phospholipase A2 (PLA2) activity and arachidonic acid formation.³⁸ Most studies of ANXA1 regulation of cellular proliferation have focused on glucocorticoid-mediated suppression of growth ANXA1 influences dexamethasone-induced changes in cell proliferation, probably through inhibition of cPLA2 activity and subsequent suppression of prostaglandin E2 and arachidonic acid release.¹³ This mechanism potentially involves rapid changes in the phosphorylation of ANXA1.⁶ One common element in the induction and phosphorylation of ANXA1 shared between the process of liver development and regeneration is epidermal growth factor (EGF), which exerts its mitogenic effects through phosphorylation and upregulation.

ANXA1 also has been shown to be a substrate for epidermal growth factor receptor kinase, a recognized indicator of poor prognosis and reduced survival in head and neck cancer.⁸ ANXA1 may be correlated with these findings since this protein has also been associated with the development of metastasis in

some invasive malignancies suggesting a role for this protein in regulating epithelial cell migration/invasion.³⁹ While we cannot exclude the possibility that some of the positive staining reflected uptake of peptide Ac2-26 into the cells, these data suggest that application of peptide Ac2-26 to Hep-2 cells up-regulates the endogenous expression of the protein in both the cytoplasm and the nucleus. We suggest that the nuclear localization of the protein supports the premise that ANXA1 may influence gene expression directly.

Analyzing the stroma surrounding the tumor we observed mast cells infiltrating intraepithelially within the larynx cancer and in the connective tissue near the invasive tumor. The number of these cells was quantified using the immunohistochemistry reaction for serine proteinase tryptase in the laryngeal tissues. This technique is extensively used for human mast cell identification.²¹

The presence and increase in degranulated mast cell number observed in the laryngeal tumor and peritumoral tissues suggests that tumor cells are recruiting and activating mast cells to release their secretion of biological mediators that could be detrimental to the tumor or contribute to the tumor development. In cancer, some authors have suggested that inflammatory cells including mast cells can stimulate the epithelial cells during the events of promoting tumor growth through release of mediators that alter the microenvironment.⁴⁰ Previous studies have described an increase in the mast cell number of the peritumoral tissue in patients with melanoma²¹ and basal cell carcinoma⁴¹ but mast cell degranulation was not evaluated. Evidence indicates that mast cells accumulate at sites of tumor growth in response to numerous chemoattractants released by tumor cells and could either promote or inhibit tumor growth depending on the local stromal conditions. Several authors have discussed the dual role of mast cell in cancer. Mast cells can be beneficial or detrimental to the tumor depending on the tumor stroma microenvironment altering its secretory process.²⁵

A number of studies have provided evidence that mast cells express the anti-inflammatory protein ANXA1^{18,19,42} and this protein has been implicated in playing an important role in tumor development, directly or indirectly, and could be a diagnostic marker for certain types of tumors.⁷ Our results have demonstrated the intracellular localization and disposition of human mast cell ANXA1 by ultrastructural immunocytochemical analysis in laryngeal tissues (tumor, peritumoral and control tissues) obtained from the same patient. In the tumor, mast cells showed granules with varying electron density, characteristic of degranulated mast cells. In these cells the immunoreactivity to ANXA1 showed characteristic clusters in the interior of the granules. The quantitative analysis of the ANXA1 expression showed a significant increase of the protein in the cytoplasmic granules and nucleus of the mast cells in laryngeal tumor and peritumoral tissues. Previous studies demonstrated that increased ANXA1 expression in rat mast cells after dexamethasone treatment might be one of the potential mechanisms by which glucocorticoids inhibit the mast cells degranulation.^{18,42} Also local administration of the ANXA1 N-terminal peptide inhibits mast cell degranulation in rats inflamed by infection of ovalbumin into the pleural cavity.¹⁹

The increase in ANXA1 expression observed in mast cells in the tumor and peritumoral region compared to control tissue, suggests that the endogenous protein may act as a feedback response in the modulation of mediators release. Since mast cells mediators

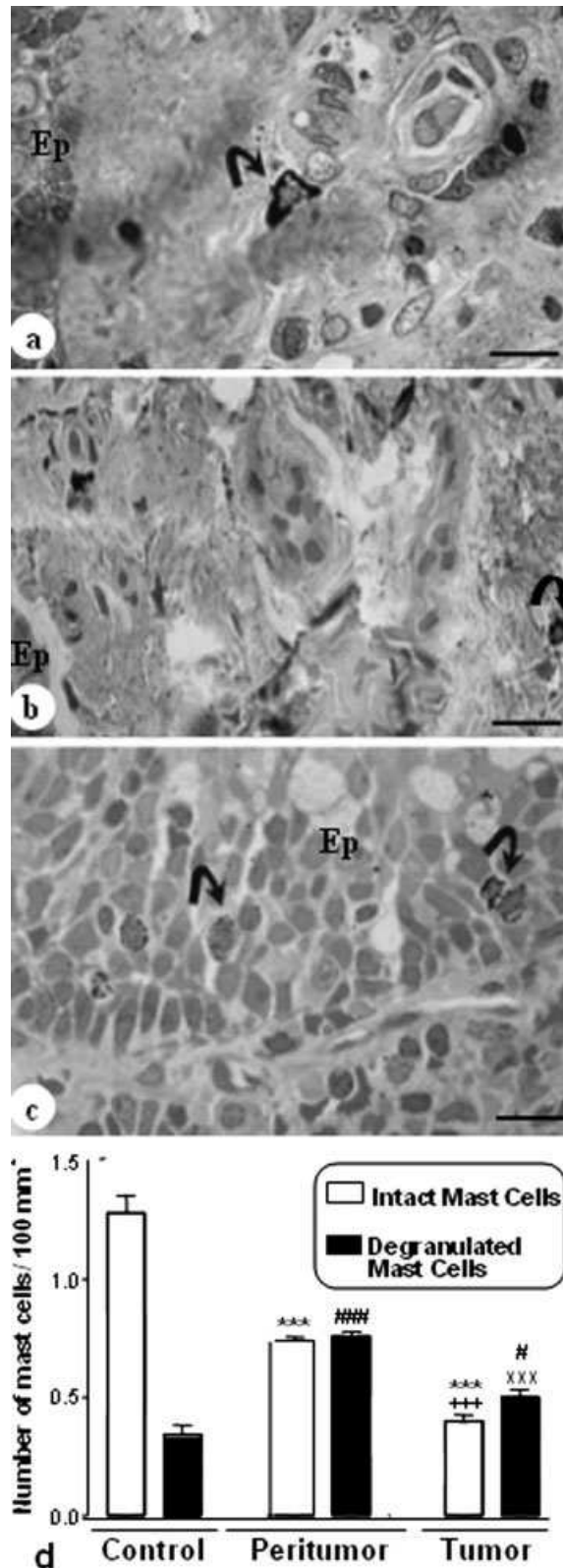


FIGURE 4 – Immunohistochemical analysis of tryptase in the mast cells of the laryngeal tissues *in vivo*. Sections (1 μ m) were stained with hematoxylin and eosin. (a) Control, (b) peritumor and (c) tumor tissue showed mast cells immunoreactive by tryptase (curve arrow) and epithelium (Ep). (d) Quantitative analysis of intact and degranulated mast cells ($n = 20$ patients). *** $p \leq 0.001$ versus intact mast cells, control; +++ $p \leq 0.001$ versus intact mast cells, peritumor; # $p \leq 0.05$, ### $p \leq 0.001$ versus degranulated mast cells, control; xxx $p \leq 0.001$ versus degranulated mast cells, peritumor. Scale bars 10 μ m.

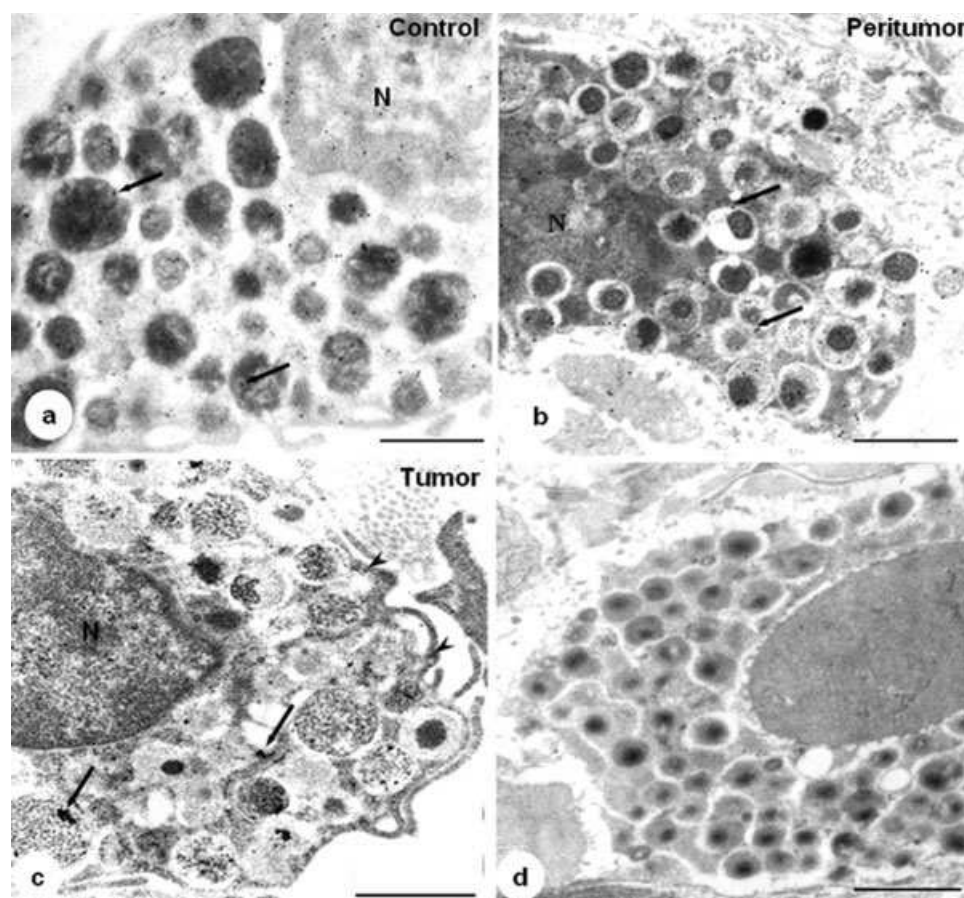


FIGURE 5 – Modulation of ANXA1 protein expression in laryngeal human mast cells. (a) Control, (b) peritumor and (c) tumor tissue ($n = 20$ patients). Mast cells with gold particles in the nucleus (N), cytosol, cytoplasmic granules (arrows) and plasma membrane (arrowheads). Clusters of ANXA1 immunoreactivity in the matrix of cytoplasmic granules were selectively detected in the mast cells of tumor tissues (arrows). (d) Absence of gold labeling in section incubated with normal sheep serum. Scale bars, 1 μm .

TABLE I – DENSITY OF ANNEXIN 1 IMMUNOGOLD PARTICLES IN MAST CELLS IN CONTROL, PERITUMOR AND TUMOR LARYNGEAL TISSUES

Tissue	ANXA1 immunoreactivity in mast cells (gold particles/ μm^2)		
	Nucleus	Cytosol	Granule
Control	0.19 ± 0.02	0.31 ± 0.02	0.26 ± 0.02
Peritumor	$0.81 \pm 0.09^{***}$	$0.68 \pm 0.05^{***\cdot\#\#\#}$	$0.75 \pm 0.05^{***\cdot\#\#\#}$
Tumor	$0.92 \pm 0.2^{***}$	0.35 ± 0.02	$0.46 \pm 0.04^*$

Data represent mean $\pm$ SEM of gold particles per μm^2 localized in different mast cells analyzed in the control, peritumor and tumor laryngeal tissues ($n = 200$ cells for each group obtained from 20 patients).

* $p < 0.05$ versus control tissue; # $p < 0.05$ versus tumor tissue.

have a role in cancer growth, ANXA1 may contribute to the modulation of mediators release.

In conclusion, by *in vivo* and *in vitro* approaches we have demonstrated that ANXA1 may have a regulatory role in laryngeal cancer development. Mast cell-derived ANXA1 might contribute to the regulation of *in vivo* tumoral growth by a paracrine mechanism. A better understanding of the regulatory mechanisms of

ANXA1 in tumor and mast cells may lead to future biological targets for the therapeutic intervention of human larynx cancer.

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3.3 ANXA1Ac2-26 peptide reduces ID1 expression in cervical carcinoma cultures*

ABSTRACT

Cervical cancer is the second most frequent cancer in women worldwide and is associated with genetic alterations, infection with human papilloma virus (HPV), angiogenesis and inflammatory processes. The idea that inflammation is involved in tumorigenesis is supported by the frequent appearance of cancer in areas of chronic inflammation. On the other hand, the inflammatory response is controlled by the action of anti-inflammatory mediators, among these mediators, annexin A1 (ANXA1), a 37 kDa protein was detected as a modulator of inflammatory processes and is expressed by tumor cells. The study was carried out on the epithelial cancer cell line (SiHa) treated with the peptide of annexin A1 (ANXA1Ac2-26). We combined subtraction hybridization approach, Ingenuity Systems software and quantitative PCR, in order to evaluate gene expression influenced by ANXA1. We observed that ANXA1Ac2-26 inhibited proliferation in SiHa cells after 72h. In these cells, 55 genes exhibited changes in expression levels in response to peptide treatment. Six genes were selected and the expression results of 5 up-regulated genes (TPT1, LDHA, NCOA3, HIF1A, RAB13) and one down-regulated gene (ID1) were research by real time quantitative PCR. Four more genes (BMP4, BMPR1B, SMAD1 and SMAD4) of the ID1 pathway were investigated and only one (BMPR1B) shows the same down regulation. The data indicate the involvement of ANXA1Ac2-26 in the altered expression of genes involved in tumorigenic processes, which could potentially be applied as a therapeutic indicator of cervical cancer.

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Research paper

ANXA1_{Ac2-26} peptide reduces *ID1* expression in cervical carcinoma cultures

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The study was carried out on the epithelial cancer cell line (SiHa) treated with the peptide of annexin A1 (ANXA1_{Ac2-26}). We combined subtraction hybridization approach, Ingenuity Systems software and quantitative PCR, in order to evaluate gene expression influenced by ANXA1.

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The data indicate the involvement of ANXA1_{Ac2-26} in the altered expression of genes involved in tumorigenic processes, which could potentially be applied as a therapeutic indicator of cervical cancer.

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Abbreviations: [LaCit2]3, lanthanum citrate complex; µg, microgram; ACTB, beta-actin; ANX, annexin; ANXA1, annexin A1; BMP4, bone morphogenetic protein 4; BMPRI1B, bone morphogenetic protein receptor, type IB; cDNA, complementary DNA; CO₂, carbon dioxide; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide-solution mix; DTT, DL-Dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCL, hydrochloric acid; HIF1A, hypoxia inducible factor 1, alpha subunit; HPV, human papilloma virus; ID1, DNA-binding protein inhibitor; kDa, kilodalton; LDHA, lactate dehydrogenase A; Mbol, enzyme gene from *Moraxella bovis*; MEM, minimum essential medium; MgCl₂, magnesium chloride; mL, milliliter; mRNA, messenger RNA; NCOA3, nuclear receptor coactivator 3; ng, nanogram; nM, nanomolar; p53, tumor suppressor p53; PCR, polymerase chain reaction; PLA2, phospholipase A2; pRb, retinoblastoma protein; RAB13, member RAS oncogene family; RaSH, Rapid Subtraction Hybridization; RNA, ribonucleic acid; SiHa, cells epidermoid carcinoma of the cervix; SMAD1, SMAD family member 1; SMAD4, SMAD family member 4; TPT1, tumor protein, translationally-controlled 1; XhoI, enzyme gene from *Xanthomonas holcicola*.

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1. Introduction

Carcinoma of the cervix, or cervical cancer, is the second most frequent neoplasm in women worldwide. Each year, approximately 530,000 new cases of cervical cancer arise. Cervical cancer is the fourth leading cause of death in developing countries, with approximately 275,000 deaths per year (Jemal et al., 2011).

A factor directly related to cervical cancer is infection with human papilloma virus (HPV), and approximately 90% of cervical cancer cases are associated with HPV as a causative agent (Jaafar et al., 2009; Walboomers et al., 1999). A common feature of this type of tumor is the high expression of two oncogenes, *E6* and *E7*, which encode multifunctional proteins known for their ability to inactivate the tumor suppressors p53 and pRb, respectively (Narisawa-Saito and Kiyono, 2007). This altered expression of oncogenes leads to instabilities in the genome by blocking the cell cycle and checkpoints deregulation in these cells, due to the loss of these tumor suppressors genes. These cells then can progress towards the tumorigenic process (Incassati et al., 2006).

Other factors that contribute to the risk of developing cervical cancer have been described. These factors include age; the number of sexual

partners, which has been especially associated with co-infection; prolonged use of hormonal contraceptives; a large number of full-term pregnancies; and cigarette smoking (Soto-De Leon et al., 2011).

The inflammatory response is controlled by the action of anti-inflammatory mediators, which act to maintain the homeostasis of the immune response to prevent tissue damage. These mediators include annexin-A1 (ANXA1), a 37 kDa protein expressed by tumor cells that act as a modulator of inflammation (Flower, 1988; Silistino-Souza et al., 2007; Alves et al., 2008).

The Annexins (ANX) are a well-conserved superfamily of structurally related proteins characterized by a C-terminal homologous domain containing four to eight repeats of 70–75 amino acids that are responsible for the calcium and phospholipid binding properties. The variable N-terminal region is unique in length and sequence and includes potential sites of phosphorylation, glycosylation and peptidase action (Gerke and Moss, 2002). Annexins are present in many organisms, including molds, plants, fungi and mammals. They are preferentially localized in the cytoplasm, where they associate with the plasma membrane or cytoskeleton, but some annexins, including ANXA1, have also been found to localize to the outside of the plasma membrane (Solito et al., 1994, 2003a). Though the function of the annexins is far from clear, they have been shown to be involved in signal transduction (Alldridge et al., 1999; De Coupade et al., 2000), vesicle transport (Diakonova et al., 1997), cell transformation (Violette et al., 1990; Solito et al., 1998), inflammation (Perretti and Gavins, 2003), cell matrix interactions and apoptosis (Raynal and Pollard, 1994; Solito et al., 2003b).

Annexin A1 (ANXA1), originally known as lipocortin 1, was the first member of this family to be cloned (Wallner et al., 1986). ANXA1 mimics the anti-inflammatory activity of glucocorticoids by inhibiting the synthesis of eicosanoids and the activity of phospholipase A2 (PLA2), affecting the components of the inflammatory reaction and the release of arachidonic acid (Flower, 1988; Perretti and Gavins, 2003). This protein is especially found in many cell types related to defensive processes, which include neutrophils (Oliani et al., 2001), mast cells (Oliani et al., 2008), eosinophils (Oliani et al., 2002) and monocytes (Goulding et al., 1990).

The expression and localization of ANXA1 have been investigated in experimental models of acute inflammation induced by carrageenan in the rat mesentery through the analysis of extravasated leukocytes (Oliani et al., 2001; Gil et al., 2006). The vascular endothelium and chronic granulomatous inflammation have also been examined in the skin of mice (Oliani et al., 2008; Gibbs et al., 2002).

After it was discovered that the biological activity of ANXA1 could be reproduced by the first twenty-six amino acids in the N-terminus (peptide Ac2-26) as well as some smaller peptides (Cirino et al., 1993; Perretti et al., 2001), the use of these molecules in experimental models of acute (Damazo et al., 2006; Gavins et al., 2007), chronic (Gibbs et al., 2002) and systematic inflammation has become commonplace (Damazo et al., 2005). The management of these mimetic ANXA1 peptides causes the activation of the abundant ANXA1 receptors found in leukocytes, promoting cell activation and intracellular signaling, provoking detachment of adherent leukocytes (Gavins et al., 2003; Gastardelo et al., 2009).

In comparison to HPV negative squamous cell penile carcinoma samples, ANXA1 protein expression was significantly increased in high-risk HPV squamous cell penile carcinoma samples independently of the subtype of penile squamous cell carcinoma. ANXA1 is likely to play an oncogenic role in penile cancer associated with high-risk HPVs (Calmon et al., 2013). By contrast, the ANXA1 expression in cervical carcinogenesis has been scarcely investigated. The levels of this protein and its mRNA decrease as the cancer develops, suggesting that ANXA1 may be regarded as a biological marker in tumor progression (Lee et al., 2008). The down-regulation of ANXA1 expression in [LaCit2]3 (Lanthanum citrate complex)-treated SiHa cells suggests that ANXA1 might regulate growth factors

related to apoptosis to mediate programmed cell death (Shen et al., 2010).

Though the works related to ANXA1 have contributed to a greater understanding of the role of this protein in inflammatory and tumorigenic process, the molecular mechanisms by which ANXA1 modulates cellular responses in the inflammatory process have not yet been fully determined. Available data suggest that this protein may be significantly involved in cancer in addition to its important role in the inflammatory process, most likely through signaling cascades that include genes related to the cell cycle, differentiation and apoptosis.

To investigate the effects of ANXA1 in cervical cancer, we evaluated the influence of this peptide on the morphology, cell proliferation and gene expression in SiHa cells. We also identified the signaling pathways that genes modulated by ANXA1_{Ac2-26} participate which are important for tumorigenic and the inflammatory process.

2. Materials and methods

2.1. SiHa culture conditions

The SiHa cell line originally established from an epidermoid carcinoma of the cervix (ATCC, Rockville, Maryland, USA) was obtained and seeded at a density of 1×10^6 cells/mL per 75 cm² culture flask (Corning, NY, USA). MEM-Earle medium (Cultilab, Campinas, SP, Brazil) was used at pH 7.5 and supplemented with 10% fetal calf serum (Cultilab), 1% non-essential amino acids and 1% antibiotic/antimycotic (Invitrogen Corporation, Carlsbad, CA, USA) and cultured at 37 °C under an atmosphere of 5% CO₂.

2.2. Pharmacological treatment

The SiHa cells were subdivided into a control group that was not manipulated and an experimental group to which 3 μM of ANXA1_{Ac2-26} was added. The experiments were conducted for time periods of 2, 4, 24, 48 and 72 h to determine the statistically significant time for further use in the methodology.

2.3. Growth curve

SiHa cells were seeded in triplicate at a density of 5×10^4 cells in plastic 6-well plates and divided in two groups. The control group was grown on complete medium whereas peptide ANXA1_{Ac2-26} was added to the experimental group. 24 h later, when the cells had already adhered, SiHa cultures were incubated with serum free medium (MEM 0%). After an additional 24 h, peptide was added to the experimental group whereas the control group was maintained in complete medium without any pharmacological manipulation.

The cell morphology was observed on each day. The cytotoxic effects of peptide on the proliferation of SiHa cells were investigated in two experimental groups at 2, 4, 24, 48 and 72 h. The cells were harvested, stained with trypan blue and counted using a Neubauer hemocytometer. Significant differences between the groups were determined by one-way analysis of variance (one-way ANOVA) and if found to be significant, further analyzed using the Bonferroni test. The same experiment was repeated twice.

2.4. RNA extraction for Rapid Subtraction Hybridization (RaSH) and real time PCR experiments

SiHa cells were seeded at a density of 1×10^6 cells/mL in 75 cm² culture flasks in complete medium (control group) and incubated with 10 μg/mL of peptide Ac2-26 for 72 h (experimental group). The RNA from control and ANXA1_{Ac2-26} treated SiHa cells was extracted using Trizol (Sambrook and Russel, 2001) following treatment with DNase (Invitrogen). cDNA synthesis was performed using a High Capacity

cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.5. Rapid Subtraction Hybridization (RaSH)

The RaSH technique (Jiang et al., 2000) was performed with two subtractions. The first was termed “Sub I”, in which the SiHa control cells were referred to as *tester* and the cells treated with peptide Ac2-26 were termed *driver*. In the second subtraction, “Sub II”, the opposite test was performed.

The first cDNA strand was obtained by reverse transcriptase with 2 µL of oligo (dT) (50 mM) and 2 µL of 10 mM dNTPs at 65 °C for 5 min. The samples were immediately placed on ice for 1 min and 2.0 µL of 0.1 mM DTT, 1 µL of 40 U/µL RNAout and 4.0 µL of 5X First-Strand Buffer [250 mM Tris–HCL (pH 8.3), 375 mM KCL and 15 mM MgCl₂] were added. The samples were incubated at 42 °C for 2 min before 1.0 µL of Superscript™ II reverse transcriptase (RT) (200 U/µL) was added. The mixture was incubated again at 42 °C for 50 min and then at 70 °C for 15 min. Segments of β-actin cDNA were also amplified to verify the quality of these reactions.

The second strand of cDNA was synthesized in reactions containing 30 µL of 5X Second Strand Buffer, 3 µL of 10 mM dNTPs, 1 µL of DNA ligase (10 U/µL), 4 µL of DNA Pol I (10 U/µL) and 1.0 µL of RNase H (2 U/µL). The samples were incubated for 2 h at 16 °C before the addition of 2 µL of T4 DNA Pol (5 U/µL) and further incubation for an additional 5 min. The action of T4 DNA Pol was inhibited by the addition of 10 µL of 0.5 M EDTA. Phenol:chloroform extraction was used to purify the sample and the pellet was resuspended in 45 µL of water. The cDNA was used in amplification reactions for segments of GAPDH and was subsequently digested with 2 µL of MboI enzyme (10 U/µL) and incubated for 1 h at 37 °C. Finally, over 1 µL of this enzyme was added to the reaction, and the samples were incubated overnight at 37 °C.

To this cDNA was added 4.1 and 4.5 µL, respectively, of the adaptors XDPN-14 5'-CTGATCACTCGAGA and XDPN-12 5'-GATCTCTCGAGT (Sigma Chemical, final concentration 10 mM), 8 µL of 5X T4 DNA ligase buffer Invitrogen®, heated at 55 °C for 1 min, and cooled down to 14 °C within 1 h. The cDNA received 3 µL (9 U) of T4 DNA ligase (3 U/µL), ligation was carried out overnight at 14 °C. After phenol:chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100 µL with TE buffer (10 mM Tris/1 mM EDTA); 40 µL of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 µM XPDN-18 5'-CTGATCAC TCGAGAGATC, 0.4 mM dNTPs, 10 × PCR buffer, 1.5 mM MgCl₂ and 1 U Taq DNA polymerase (Invitrogen). Thermocycler conditions were 1 cycle at 72 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, ending in a final extension at 72 °C for 3 min. Ten microgram of purified PCR product (*tester*) was digested with 10 U/µL XhoI (Invitrogen) for 6 h at 37 °C and followed phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the *tester* cDNA were mixed with 5 µg of the *driver* cDNA in hybridization solution (0.5 M NaCl, 50 mM Tris/HCl, SDS2% and 40% formamide) and, after heating at 99.9 °C for 5 min, incubated at 42 °C for 1 h, and 42 °C for 48 h. After extraction and precipitation, the hybridization mixture (1 µg) was ligated with XhoI-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as DNA template for PCR. Clones were sequenced using an automated DNA sequence and homologies sequences were searched using the BLAST program. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes using terms from Gene Ontology database.

We further analyzed the genes through IPA (Ingenuity Systems®) to relate the differentially expressed genes with biological functions and processes in which these genes are involved and evaluate what the main networks of interaction between them.

2.6. Quantitative PCR – RT-qPCR

The RNA from control and ANXA1_{Ac2-26} treated SiHa cells was extracted and cDNA synthesis was performed as described above.

Six differentially expressed genes were selected for validation by quantitative real time PCR experiments according to their direct or indirect involvement in tumorigenesis. Their expression was checked in treated samples relative to matched non-treated samples.

The primers were manually designed with: 19–23 bp length, 30–80% GC content and a short amplicon size (80–120 bp). Their sequences are available upon request (Table 1). Real time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixture consisted of a 20 µL volume solution containing 100–500 ng of Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer and 100 ng cDNA (control and treated with peptide ANXA1_{Ac2-26} [3 µM]). The PCR conditions were 95 °C for 10 min followed by 40 cycles of 95° for 15 s and 60° for 1 min. Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products.

For each primer set, the efficiency of the PCR reaction (linear equation: $y = \text{slope} + \text{intercept}$) was measured in triplicate on serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays.

Two control genes (*GAPDH*, *ACTB*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001). Statistical analysis was performed by a two-tailed unpaired *t* test using GraphPad prism software.

To investigate the pathway of *ID1* gene, were analyzed four genes (*BMP4*, *BMPR1B*, *SMAD1* and *SMAD4*) following the methodology described above. The *ID1* gene is connected with more four genes (*BMP4*, *BMPR1B*, *SMAD1* and *SMAD4*) in TGF-Beta signaling pathway, from KEGG (<http://www.kegg.jp/pathway/ko04350>), according to Pathways from Biosystems (<http://www.ncbi.nlm.nih.gov/gene/3397>).

3. Results

3.1. Cellular morphology

SiHa cells were initially cultured and observed by phase contrast microscopy. These cells have a fusiform appearance and central core, with multiple nucleoli and a eucromatic position, which are characteristics of a cell with high metabolic activity (Fig. 1A). In cells treated with ANXA1_{Ac2-26} [3 µM], there was no morphological change, and cytoplasmic processes were more clearly observed due to reduced cell proliferation (Fig. 1B).

3.2. Proliferation assay

In the proliferation assay, one-way ANOVA using the GraphPad Prism 5 program was used to observe the growth inhibitory effect

Table 1
Primers for the interesting genes.

Primer	Sequence
HIF1A <i>right</i>	5' TCCTCACACGCAATAGCTG 3'
HIF1A <i>left</i>	5' TGCTCATCAGTTGCCACTTC 3'
ID1 <i>right</i>	5' TAGTCGATGACGTGCTGGAG 3'
ID1 <i>left</i>	5' AAACGTGCTGCTCTACGACA 3'
LDHA <i>right</i>	5' GCCGTGATAATGACCAGCTT 3'
LDHA <i>left</i>	5' TGGCAGCCTTTTCCTTAGAA 3'
NCOA3 <i>right</i>	5' AAGTCCCCACACCTTCACTG 3'
NCOA3 <i>left</i>	5' TTCTGGCAATACTGGGAACC 3'
RAB13 <i>right</i>	5' CTGCAAGCGAATGATCAGA 3'
RAB13 <i>left</i>	5' AGCTACGACCACTCTTCA 3'
TPT1 <i>right</i>	5' TAGAGCAACCATGCCATCTG 3'
TPT1 <i>left</i>	5' GCTGCAGACAAATCAAGCA 3'

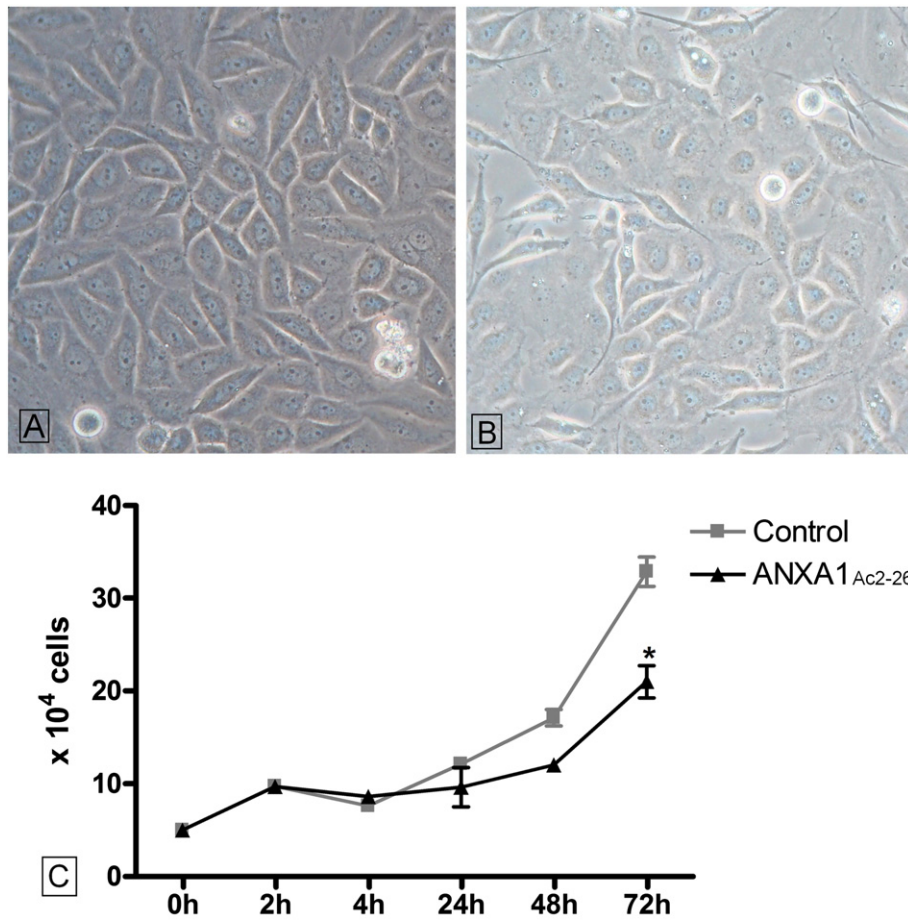


Fig. 1. Morphological analysis and growth curve of ANXA1_{Ac2-26} peptide treatment in SiHa cell line. SiHa cells were seeded in complete MEM 10% at a concentration of 5×10^4 per well (6 well plate). The cell morphology observed in (A) the control group, characterized by a monolayer of nucleated cells was not altered in (B) the ANXA1_{Ac2-26} peptide [10 $\mu\text{g/mL}$] treated group. 400 $\times$ magnification. (C) SiHa cells were cultured, counted after 2, 4, 24, 48 and 72 h and these results were plotted on a growth curve. Assays were performed in triplicate. Graph $\times$ = time (hours) and y = number of cells $\times 10^4$. P values < 0.05 were significant.

under ANXA1_{Ac2-26} treatment. The peptide was shown to decrease the proliferation in treated cells in comparison to the control group (Fig. 1C). Statistical analysis showed that these differences were most significant ($P \leq 0.05$) at a concentration of 10 $\mu\text{g/mL}$ of peptide and a culture time of 72 h.

3.3. Genes identified using the RaSH approach

Rapid subtractive hybridization (RaSH) compares two distinct experiments to find differential gene expression. RaSH was performed on SiHa cells from the control group and those treated with ANXA1_{Ac2-26} for 72 h, and 82 clones were isolated and sequenced. By screening these sequences through GenBank (*Blast*) and by rigorous classification and selection, 55 genes were found to be differentially expressed. Two libraries of genes (Sub I and Sub II) were acquired. Twenty-three genes of the “Sub I” library exhibited changes in expression levels in the control cells, whereas 32 genes of the “Sub II” library changed in expression under treatment with peptide. Therefore, it was expected that the gene expression of one library would be lower than that of the other one. The libraries of the genes obtained along with their different functions and processes described in the Gene Ontology (GO) databases.

IPA (Ingenuity Systems©) was used to further analyze the 55 differentially expressed genes, which were especially associated with tumorigenesis, apoptosis, necrosis and cell proliferation (Supplements 1 and 2). Moreover, some of these genes were chosen because they form a network of important interactions with other genes such as *NF- κ B*, *Ubiquitin* and *HSP* in the “Sub I” library or with genes associated with cancer,

inflammatory diseases, the cell cycle, senescence, replication and cell death in the “Sub II” library.

Six differentially expressed genes, *TPT1*, *LDHA*, *ID1*, *NCOA3*, *HIF1A* and *RAB13*, were selected for validation by quantitative real time PCR experiments (Table 1). These genes are associated with calcium ion transport, cellular response to extracellular stimulus, apoptotic process, regulation of transcription, response to hypoxia and regulating membrane trafficking, respectively and were chosen according to their functions related to their direct or indirect involvement in tumorigenic and inflammatory processes.

3.4. Real-time PCR validation of differentially expressed genes

The RaSH analysis indicated the down-regulation of four genes (*ID1*, *LDHA*, *RAB13* and *TPT1*) and up-regulation of two genes (*HIF1A* and *NCOA3*) in SiHa cells treated with ANXA1_{Ac2-26}. However, the quantitative PCR experiment showed that only the expression of *ID1* was significantly reduced in these cells after peptide treatment (Fig. 2). The expression of other genes oscillated without statistical significance, which was most likely because the peptide does not quantitatively modify the expression of the selected genes despite its interaction with the cell.

3.5. Real-time PCR gene-related pathway gene *ID1*

Four genes (*BMP4*, *BMPRI1B*, *SMAD1* and *SMAD4*) of *ID1* pathway were analyzed, however, the quantitative PCR experiment showed that only the expression of *BMPRI1B* was significantly reduced in SiHa

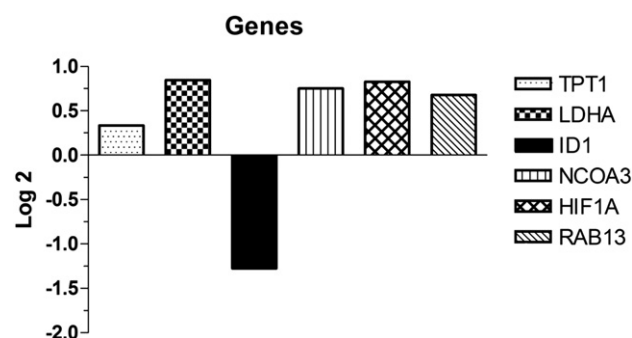


Fig. 2. Quantitative real time PCR gene expression in SiHa cells treated with ANXA1_{Ac2-26} peptide. Expression of *HIF1A*, *ID1*, *LDHA*, *NCOA3*, *RAB13* and *TPT1* genes in SiHa cells treated with ANXA1_{Ac2-26} peptide (10 µg/mL), compared with control cells. Assays were performed in triplicate to confirm the results and the data represented in base 2 logarithm.

cells after ANXA1_{Ac2-26} treatment (Fig. 3), like the *ID1* gene expression. The expression of other genes oscillated without statistical significance (*BMP4*, *SMAD1* and *SMAD4*).

4. Discussion

Although cervical cancer can be prevented, it still represents a major public health problem for women, especially in underdeveloped countries where there is little monitoring and prevention.

The anti-inflammatory protein Annexin A1 (ANXA1) could be used as an indicator of treatment. Though the literature on the in vitro (D'Acquisto et al., 2008) and in vivo (Gastardelo et al., 2009; Facio et al., 2011) pharmacological effects of this protein and its mimetic peptides in inflammatory processes is extensive, the expression of this protein in cervical carcinogenesis has been scarcely investigated. Some studies found the low expression of ANXA1 in cervical cancer, suggesting that this protein could be considered a biological marker in the progression of invasive cervical cancer or regulates apoptosis-related growth factors, point to a negative effect of ANXA1 in the cervical cancer, low expression worst prognostic (Lee et al., 2008; Shen et al., 2010). Thus, we explored the action of ANXA1_{Ac2-26} on SiHa cells derived from cervix squamous cell carcinoma, a cell line that provided the best conditions for the experiments. These cells contained HPV16, with an estimated 10 copies of HPV 16 DNA integrated into the cellular DNA (Yee et al., 1985).

In analyzing the SiHa cell line, we observed that the morphology did not change after treatment with peptide ANXA1_{Ac2-26}. However, cell proliferation decreased after 72 h of treatment. ANXA1 mediates the disruption of normal cell morphology and inhibits cyclin D1 expression, reducing cell proliferation through proximal modulation of the ERK signal transduction pathway (Alldridge and Bryant, 2003). ANXA1 has direct anti-proliferative effects on cells possibly through the constitutive

over-activation of ERK1/2. Thus, the anti-proliferative role of ANXA1 may be directly responsible, at least in part, for the growth arrest. These findings suggest that ANXA1 may act as a tumor suppressor (Ang et al., 2009).

But in the other hand, this anti-proliferative effect can be explained by apoptosis too, Parente and Solito (2004) comment that ANXA1 promotes inflammatory cell apoptosis associated with transient rise in intracellular calcium and caspase-3 activation. Then in these SiHa cells, how there are inflammatory cells in cancer process, we can suppose that this negative effect can be explained by apoptosis (Parente and Solito, 2004).

Apoptosis was also a criterion for selecting the differentially expressed genes for validation by real-time RT-PCR. Of the 55 genes identified by RaSH approach was very difficult choose only six genes to validation, because all of them have some interesting function, but IPA (Ingenuity Systems©) was helpful to choose which genes were especially associated with tumorigenesis, apoptosis, necrosis, regulation of transcriptional and in the same gene network.

After an analysis of the literature, six genes (*HIF1A*, *ID1*, *LDHA*, *NCOA3*, *RAB13* and *TPT1*) were selected for validation by real-time RT-PCR using treated and non-treated cell lines. These genes show potential involvement in signaling cascades related to tumorigenesis and/or the interaction between inflammation and tumor cells through angiogenesis, metastasis, lactate dehydrogenase activity, regulation of gene expression, cell adhesion and binding and calcium ion transport. The RT-PCR results for the *ID1* gene were consistent with the RaSH data. For the gene expression of *NCOA3* and *HIF1A*, the results were not significantly increased. Thus, these genes can be considered to be good molecular markers if their high expression levels are confirmed in other cells. *LDHA*, *RAB13* and *TPT1* genes still require further testing. These results are most likely due to the higher concentrations of the peptide that are required in these cells to observe the mimetic effects.

The different expression levels of the *ID1* gene are related to angiogenesis, differentiation and metastasis. The overexpression of *ID1* was detected in esophageal squamous cell carcinoma, which suggests that in our study, ANXA1 may regulate this gene by decreasing its expression and may be used as a possible therapeutic target. *ID1* belongs to a family of small molecules of short length (13 to 20 kDa) and acts as an inhibitor of differentiation or DNA binding (Benezra et al., 1990). The *ID1* gene is not a transcription factor, it is a transcriptional regulator (lacking a basic DNA binding domain) which negatively regulates the basic helix-loop-helix (bHLH) transcription factors by forming heterodimers and inhibiting their DNA binding and transcriptional activity (Zheng et al., 2004). Thus, *ID1* regulates the effects of transcription factors on target genes. Infection by high-risk human papilloma viruses (HPVs) is considered to be the central cause of invasive cervical cancer, suggesting that high-risk HPVs can enhance the progression of human cervical cancer through *ID1* regulation (Darnel et al., 2010).

The overexpression of *ID1* alone can induce angiogenic processes in endothelial progenitor cells (EPCs) in ovarian cancer. The expression of *ID1* and matrix metalloproteinase-2 (MMP-2) was increased in EPCs isolated from ovarian cancer patients compared to those obtained from healthy subjects. It can thus be concluded that *ID1* can enhance EPC angiogenesis in ovarian cancer, which is mainly mediated by the PI3K/Akt and NF-κB/MMP-2 signaling pathways (Su et al., 2013).

In most metastatic cancer patients, epithelial tumor cells from primary tumor samples express any grade *ID1* positivity. In addition, higher protein expression levels seem to correlate with poorer prognoses in prostate cancer (Ponz-Sarvisé et al., 2014). The overexpression of *ID1* protein is associated with more aggressive behavior of tumor cells in human cervical cancer and is an independent marker for the progression of this tumor type (Schindl et al., 2001).

The *ID1* is considered as a BMP (bone morphogenetic protein) specific target gene that binds either to a type 1 or a type 2 receptor to form the ternary signaling complex (Chang et al., 2002). BMPs belong to the TGF-β superfamily and act via their specific receptors, the

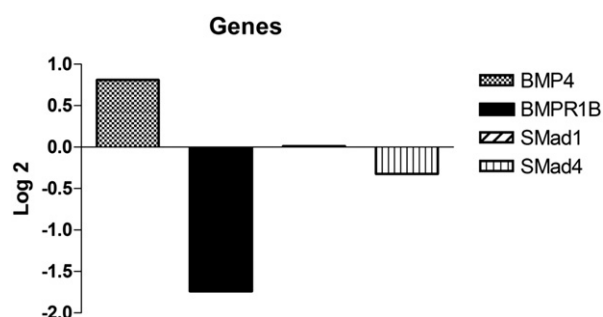


Fig. 3. Quantitative real time PCR gene expression in SiHa cells treated with ANXA1_{Ac2-26} peptide. Expression of *BMP4*, *BMPR1B*, *SMad1* and *SMad4* genes in SiHa cells treated with ANXA1_{Ac2-26} peptide (10 µg/mL), compared with control cells. Assays were performed in triplicate to confirm the results and the data represented in base 2 logarithm.

BMPs. Among the receptors, BMP1B has been previously analyzed and shown to be linked to the advancement of breast cancer and poor prognosis. Analysis revealed lower expression levels of *BMP1B* transcript in poor prognosis patient samples compared to good prognosis samples, as well as, down-regulation of *BMP1B* in MDA-MB-231 cells and leads to increased cell proliferation of breast cancer cells in vitro (Bokobza et al., 2009). Probably the down expression of the *BMP1B* receptor is according to the *ID1* gene expression.

In the literature, the overexpression of *ID1* gene is observed in cervical cancer, but our data showed decreased expression of *ID1* and *BMP1B* genes after the peptide treatment, suggesting that this peptide can modify the expression of some genes in cervical carcinoma cells. As consequence of the decreased expression of *ID1* gene by the peptide, *ID1*-targeted transcription factors will be relieved to regulate their downstream signals.

The genes evaluated in this study showed differential expression in SiHa cells in a manner dependent on ANXA1_{Ac2-26} treatment. In summary, this study highlights the importance of the RaSH technique as a fast and efficient method for identifying genes modulated by ANXA1 in cells of epithelial origin. Further experiments are needed to determine whether other genes obtained by subtraction are regulated by ANXA1 and whether these are causally related to treatment-induced changes in this protein. The functions of the genes identified in this study are important in tumor progression and are amenable to changes through altering the metabolism of the cervical epithelial cells.

The cervix is difficult to access, and treatments for cervical carcinoma commonly involve surgery, chemotherapy or radiotherapy. However, new therapeutics such as the use of gene therapy associated with nanoparticles tested in cervical cancer derived HeLa cells are being developed, allowing the delivery of drugs into target cells (Zheng et al., 2013).

Finally, the ANXA1_{Ac2-26} peptide alters the proliferation and gene expression of SiHa cells. These changes are most likely related to inflammatory and tumorigenic processes. Thus, we suggest that the peptide has modulatory effects on the metabolism of this cell type. Few investigations have evaluated the effects of this peptide on the expression of selected genes. Our data suggests the mechanisms of action of this anti-inflammatory protein, and future molecular studies must be conducted to verify these processes. Our findings suggest the importance of future studies using SiHa cells for the development of new therapies. These studies should focus on gene activities that involve the action of ANXA1 and applications of this peptide as a possible therapeutic in the treatment cervical carcinoma.

5. Conclusions

These findings show that the ANXA1_{Ac2-26} alters the proliferation of SiHa cells and the expression of genes with roles in signaling cascades involved in the inflammatory and tumorigenic processes. Thus, our results point to possible applications of the ANXA1_{Ac2-26} peptide as an indicator of innovative therapies for the treatment of cervical carcinoma.

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Disclosures

The authors have declared that no competing interests exist.

Author contributions statement

JP carried out the cell culture experiments, RaSH assay, real-time PCR assay, performed the statistical analysis, and drafted the manuscript. GBF-S assisted with the cell culture experiments and RaSH assay. ARDS and WASJr assisted with the RaSH assay. BRC assisted with the real-time PCR assay. EHT and SMO were involved in drafting the manuscript or revising it critically for important intellectual content. FCR-L

conceived the study, and participated in its design and coordination and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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3.4 ANXA1_{Ac2-26} peptide: as a possible therapeutic approach in inflammatory ocular diseases? *

ABSTRACT

The eye is immunologically privileged when inflammatory responses are suppressed. One component responsible for the suppression of inflammatory responses is the blood retinal barrier, which comprises the retinal pigment epithelium. The destruction of this barrier initiates inflammation, which can affect any part of the eye. Therefore, inflammatory response is controlled by the action of anti-inflammatory mediators, among these mediators, annexin A1 (ANXA1) protein acts as a modulator of inflammation. In this study we aimed to improve the knowledge of this area by investigating how a peptide of the ANXA1 protein (ANXA1_{Ac2-26}) modulates the morphology, proliferation, migration and expression of genes and proteins in human retinal pigment epithelium cells (ARPE-19). Determining how signaling pathways (NF- κ B and UBC) are modulated by the ANXA1_{Ac2-26} peptide could be important for understanding the inflammatory process. ARPE-19 cells were activated by bacterial lipopolysaccharide endotoxin (LPS) and treated with ANXA1_{Ac2-26} peptide, in a concentration of 1.7 μ M and 33.8 μ M. We observed that the LPS activation diminished the levels of endogenous ANXA1 after 2h and 24h and ANXA1_{Ac2-26} peptide decreased the proliferation and re-establishes the migration of ARPE-19 cells. After using a hybridization approach, 80 differentially expressed genes were found. Five of these genes were selected (LRAT, CTGF, MAP1B, ALDH1A3 and SETD7) and all were down-regulated after treatment with the peptide. The genes CTGF and LRAT would be considered as potential molecular markers of ophthalmologic inflammation. The expression of pro-inflammatory cytokines was also decreased after the treatment, indicating the efficiency of the anti-inflammatory peptide at high concentrations, since the reduction in the levels of these mediators were observed after the treatment with ANXA1_{Ac2-26} peptide at 33.8 μ M. Our results suggest that the retinal pigment epithelial cells are a potential target of the ANXA1 protein and point to possible applications of the ANXA1_{Ac2-26} peptide as an innovative therapy for the treatment of ocular inflammation.

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Research paper

ANXA1_{Ac2–26} peptide, a possible therapeutic approach in inflammatory ocular diseases

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ABSTRACT

The eye is immunologically privileged when inflammatory responses are suppressed. One component responsible for the suppression of inflammatory responses is the blood retinal barrier, which comprises the retinal pigment epithelium. The destruction of this barrier initiates inflammation, which can affect any part of the eye. Therefore, inflammatory response is controlled by the action of anti-inflammatory mediators, among these mediators, annexin A1 (ANXA1) protein acts as a modulator of inflammation. In this study we aimed to improve the knowledge of this area by investigating how a peptide of the ANXA1 protein (ANXA1_{Ac2–26}) modulates the morphology, proliferation, migration and expression of genes and proteins in human retinal pigment epithelium cells (ARPE-19). Determining how signaling pathways (NF-κB and UBC) are modulated by the ANXA1_{Ac2–26} peptide could be important for understanding the inflammatory process. ARPE-19 cells were activated by bacterial lipopolysaccharide endotoxin (LPS) and treated with ANXA1_{Ac2–26} peptide, in a concentration of 1.7 μM and 33.8 μM. We observed that the LPS activation diminished the levels of endogenous ANXA1 after 2 h and 24 h and ANXA1_{Ac2–26} peptide decreased the proliferation and re-establishes the migration of ARPE-19 cells. After using a hybridization approach, 80 differentially expressed genes were found. Five of these genes were selected (*LRAT*, *CTGF*, *MAP1B*, *ALDH1A3* and *SETD7*) and all were down-regulated after treatment with the peptide. The genes *CTGF* and *LRAT* would be considered as potential molecular markers of ophthalmologic inflammation. The expression of pro-inflammatory cytokines was also decreased after the treatment, indicating the efficiency of the anti-inflammatory peptide at high concentrations, since the reduction in the levels of these mediators were observed after the treatment with ANXA1_{Ac2–26} peptide at 33.8 μM. Our results suggest that the retinal pigment epithelial cells are a potential target of the ANXA1 protein and point to possible applications of the ANXA1_{Ac2–26} peptide as an innovative therapy for the treatment of ocular inflammation.

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Abbreviations: ACTB, Beta-actin; ALDH1A3, Aldehyde Dehydrogenase 1 Family, member A3; ANX, Annexin; ANXA1, Annexin A1; ANXA1_{Ac2–26}, peptide of the ANXA1; ARPE-19, human retinal pigment epithelium cells; BRB, blood retinal barrier; CTGF, Connective Tissue Growth Factor; DMEM, Dulbecco Minimum Essential Medium; DTT, DL-Dithiothreitol; ECL, Electrochemical luminescence; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HCL, hydrochloric acid; HIF1A, hypoxia inducible factor 1, alpha subunit; HRP, horseradish peroxidase; IL, interleukin; IgG, Immunoglobulin; LPS, bacterial lipopolysaccharide endotoxin; LRAT, Lecithin Retinol Acyltransferase (phosphatidylcholine-retinol O-acyltransferase); MAP1B, Microtubule-Associated Protein 1B; MAPK, Mitogen-Activated Protein kinase; *Mbol*, enzyme gene from *Moraxella bovis*; MgCl₂, magnesium chloride; MYC, v-myc avian myelocytomatosis viral oncogene; NLRs, NOD-like receptors (NLRs); NF-κB, factor nuclear kappa B; PLA2, phospholipase A2; RaSH, rapid subtraction hybridization; RLRs, RIG-I-like receptors; RPE, retinal pigment epithelium; SETD7, SET domain-containing (lysine methyltransferase) 7; SMAD2, SMAD family member 2; TLRs, Toll-like receptors; UBC, Ubiquitin C.

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1. Introduction

The eye is an immunologically privileged organ where inflammatory responses are suppressed and many factors are responsible for this suppression, including the presence of the blood retinal barrier (BRB) (Cai and Brandt, 2008). The retinal pigment epithelium (RPE) is a major component of the BRB that controls the nutrient flow to photoreceptors (Leung et al., 2009) and makes an essential contribution to ocular immune privilege (Streilein, 2003).

Inflammation is the main contributing factor to the functional impairment of the RPE (Leung et al., 2009). The epithelium is involved in various metabolic functions and supports the sensory retina; thus, inflammation of this area may lead to the appearance of ophthalmologic diseases (Hamilton and Leach, 2011; Steinberg, 1985; Strauss, 2005). The first line of defense against pathogens is mediated by receptors, which recognize the invaders. These receptors may initiate the inflammatory pathway of NF- κ B and mitogen-activated protein kinase (MAPK) to trigger the expression of pro-inflammatory cytokines and chemokines (Corn and Vucic, 2014).

Bacterial lipopolysaccharide (LPS) can stimulate the RPE cells at concentrations of 100 ng/mL and 1 mg/mL to produce an inflammatory response (Elner et al., 2003; Garcia-Cabanes et al., 2001; Girol et al., 2013; Koga et al., 2003; Zanon et al., 2015) and in the retina, LPS increases the concentration of pro-inflammatory cytokines (Wang et al., 2005; Yang et al., 2007). RPE cells express diverse LPS receptors, including CD14 and TLR4, which play an important role in defense against a variety of ocular pathologies (Elner et al., 2005; Kindzelskii et al., 2004; Paimela et al., 2007).

Inflammation is a major contributing factor to many blinding disorders (Leung et al., 2009). Corticosteroids are frequently administered to treat this intraocular process, nevertheless, there is the risk of side effects (Jonas et al., 2003; McGhee et al., 2002; Yoshikawa et al., 1995). Corticosteroids induce the expression of the protein annexin A1 (ANXA1), which is one of the endogenous anti-inflammatory mediators (Blackwell et al., 1980; Flower, 1988). Anti-inflammatory agents block specific pathways related to inflammation (Serhan et al., 2008), these molecules are different from pro-resolving agents, which present pro-resolving properties together with anti-inflammatory actions (Sugimoto et al., 2016). The protein Annexin A1 (ANXA1) is one of these inflammatory pro-resolving mediators (Blume et al., 2011), and has been investigated as an anti-inflammatory therapeutic agent.

The annexins (ANX) comprise a well conserved super-family of proteins that are structurally related and characterized by a homologous C-terminal domain that is responsible for the properties of calcium and phospholipids binds (Yazid et al., 2010). The N-terminal domain, which is unique in length and sequence for each family member, includes potential phosphorylation, glycosylation and peptidase activity sites (Gerke and Moss, 2002). The ANXA1 anti-inflammatory and anti-proliferative actions have been shown in *in vivo* and *in vitro* studies that used administration of exogenous ANXA1 or its mimetic peptide of the N-terminal domain (Ac2–26 and Ac-AMVSEFLKQAWFIENEQEYVQTVK) (Gastardelo et al., 2014, 2009; Gimenes et al., 2015; Girol et al., 2013; Prates et al., 2015).

There is a wide variety of literature on the pharmacological effects of ANXA1 and its mimetic peptides on inflammatory processes *in vitro* (D'Acquisto et al., 2008; Gastardelo et al., 2014; Prates et al., 2015) and *in vivo* (de Paula-Silva et al., 2016; Facio et al., 2011; Gastardelo et al., 2009; Gimenes et al., 2015; Machado et al., 2016). However, few studies have tracked their actions on ocular inflammation (Girol et al., 2013). The purpose of the ANXA1 treatments was to maintain the anti-inflammatory properties and to avoid the side effects of glucocorticoids (Perretti and Gavins, 2003). Identification of the ANXA1 mechanism of action, along with the development of new therapeutic agents with the potential to mimic specific endogenous pathways, has increased the development of drug discovery programs (Gavins and Hickey, 2012).

For decades, scientists developed knowledge to prevent vision loss or restore vision in patients affected by retinal degeneration through drug therapy, gene enhancement or cell transplantation (Wiley et al., 2015). In this study we aimed to improve the knowledge of this area by investigating how a peptide of the ANXA1 protein modulates the morphology, proliferation, migration and expression of genes and proteins in ARPE-19 cells. Determining how signaling pathways, NF- κ B and Ubiquitin C (UBC), are modulated by the ANXA1_{Ac2–26} peptide and how this could be important for understanding the inflammatory process.

2. Materials and methods

2.1. ARPE-19 culture conditions

ARPE-19 cells (derived from human RPE; American Type Culture Collection) were grown in culture dishes, in a mixture of DMEM and HamF-12 (1:1) (Cultilab, Campinas, Brazil) (Dunn et al., 1996) supplemented with 10% FBS, 200 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin (Invitrogen) and were incubated at 37 °C and 5% CO₂. The morphology was observed by inverted microscopy.

2.2. Pharmacological treatments

ELISA and Western blotting assays were carried out to optimize the duration of inflammation activation by LPS for use in the following two groups: a) control and b) bacterial lipopolysaccharide endotoxin (LPS, *Escherichia coli*, serotype 0127: B8, Sigma Chemical Co. Poole, Dorset, UK); a concentration of 10 µg/mL (Paimela et al., 2007) was used for periods of 1, 2, 4, 24 and 48 h.

ARPE-19 cells were subdivided into three groups: a) control; b) activation by 10 µg/mL LPS (Paimela et al., 2007) for 24 h (Girol et al., 2013); and c) activation by LPS and treatment with two concentrations of the ANXA1_{Ac2–26} peptide (Ac-MVSEFLKQAWFIENEQEYVQTVK) of the ANXA1 protein (Raynal and Pollard, 1994). Treatment concentrations were chosen based on the results previously obtained by our research group: 1.7 µM (Rodrigues-Lisoni et al., 2006), was used in the growth curve, migration, rapid subtraction hybridization, quantitative PCR and multiplex assays, and 33.8 µM (Girol et al., 2013) was used in the quantitative PCR and multiplex assays.

2.3. ELISA assay

The evaluation of cytokines levels in the supernatant of ARPE-19 cells was performed using the immunoassay kit for IL-6 and IL-8 (R&D, Minneapolis, MN, USA) using the conditions described above. The quantification of cytokine levels was determined by an optical density reader (Molecular Devices Sunnyvale, CA) using standard curves according to the manufacturer's instructions. The results were shown as the mean $\pm$ standard error of the cytokine levels in pg/mL.

2.4. Western blotting

ARPE-19 cells were cultivated in 75 cm² culture flasks, in accordance with the description above. The cells were collected in 1 mL of lyses buffer (1 complete mini tablet of EDTA-free protease inhibitor [Roche Applied Science, Mannheim, Germany], 50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X), subjected to centrifugation, and the supernatant was collected. Aliquots of these samples were subjected to the Bradford assay for quantification of proteins by an optical density reader (Molecular Devices Sunnyvale, CA). Equal amounts of protein (30 µg) were separated by gel electrophoresis according to the protocol in a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, USA) and transferred to nitrocellulose membranes (Hi-Bond C; Amersham Biosciences, Little Chalfont, UK). Membranes were blocked with 5% powdered milk diluted in TBS-T (Bio-Rad Reagent, Hercules, USA). Then, the membranes were incubated overnight with

anti-ANXA1 (rabbit polyclonal IgG, 1:1000, Zymed Laboratories, San Francisco, CA, USA) and anti- α -tubulin (mouse monoclonal IgG, 1:1000, Zymed Laboratories, San Francisco, CA, USA). The following day, the membranes were incubated with anti-rabbit IgG (1:2000) conjugated with horseradish peroxidase (HRP) (Serotec, Oxford, UK). The HRP reaction product was visualized on photographic film (Hyperfilm, Amersham, Little Chalfont, UK) after application of the chemiluminescent ECL kit (Amersham, Little Chalfont, UK). The densitometry levels of ANXA1 were quantified using the Axioskop 2 software. As a control of the reaction, tubulin was detected by mouse monoclonal IgG antibody, 1:1000 (Zymed Laboratories, San Francisco, CA, USA).

2.5. Growth curve

ARPE-19 cells were seeded in triplicate in plastic 6-well plates in accordance with the description above. At 24 h later when the cells adhered, ARPE-19 cultures were incubated with serum free medium. After an additional 24 h, the cells were activated using LPS. The control group was maintained without any pharmacological manipulation. After 24 h of induction, one group was treated with the ANXA1_{Ac2–26} peptide for 1, 2, 4, 24, 48 and 72 h (Gastardelo et al., 2014).

The cytotoxic effect of the peptide with regard to the proliferation and viability of ARPE-19 cells was investigated in all experimental conditions. The cells were harvested and quantified using the Countess® Automated Cell Counter (Invitrogen®) and Trypan Blue. Significant differences between the groups were determined by one-way analysis of variance (one-way ANOVA) and groups that were significantly different were further analyzed using the Bonferroni test. The same experiment was repeated three times.

2.6. Cell migration assay

ARPE-19 cells were seeded in triplicate in 24-well plates and processed using the growth curve assay. After treatment with the ANXA1_{Ac2–26} peptide, grooves were made in all wells using a 10 μ L pipette tip. Analyses were performed at 0, 8, 24, 48 and 72 h (these times were chosen according to standard laboratory procedures). The migration was monitored using images that were taken, and the number of cells was measured from three different microscope areas. Significant differences between the groups were determined by *t*-test. The same experiment was repeated twice.

Based on statistical analysis, treatment for 72 h was chosen as the time for the subsequent techniques: rapid subtraction hybridization, quantitative PCR and multiplex assay.

2.7. Rapid subtraction hybridization (RaSH)

The RaSH technique (Franco-Salla et al., 2016; Jiang et al., 2000; Prates et al., 2015; Rodrigues-Lisoni et al., 2006) was performed with two subtractions (Sub). The first was termed “Sub A”, in which the ARPE-19 cells activated by LPS were referred to as *tester*, and the cells stimulated and treated with ANXA1_{Ac2–26} were termed *driver*. In the second subtraction, “Sub B”, the opposite test was performed.

Aliquots of 20 μ g of total RNA were used for double-stranded complementary DNA (cDNA) synthesis. The first cDNA strand was obtained by reverse transcription, 50 mM oligo (dT) and 10 mM dNTPs at 65 °C for 5 min. The samples were placed on ice and 0.1 mM DTT, 40 U/ μ L RNAout, 5 $\times$ Superscript III Buffer [250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂] and 3 μ L of Superscript III reverse transcriptase (RT) (200 U/ μ L) were added. The mixture was incubated at 42 °C for 60 min and then at 70 °C for 15 min. RNase H (2 U/ μ L) was added and the reaction was incubated at 37 °C for 20 min. Segments of β -actin cDNA were amplified to verify the quality of these reactions.

The second strand of cDNA was synthesized in reactions containing 5 $\times$ Second Strand Buffer, 10 mM dNTPs, DNA ligase (10 U/ μ L) and DNA Pol I (10 U/ μ L). The samples were incubated for 2 h at 16 °C before T4

DNA Pol (5 U/ μ L) was added, and the sample was incubated for an additional 5 min. The action of T4 DNA Pol was inhibited by the addition of 0.5 M EDTA. Phenol:chloroform extraction was used to purify the samples, and the pellet was resuspended in 45 μ L of water. The cDNA was used in amplification reactions for the segments of β -actin and it was digested with *Mbo*I enzyme (10 U/ μ L) and incubated for 1 h at 37 °C. Finally, > 1 μ L of this enzyme was added to the reaction and incubated overnight at 37 °C.

The cDNA was then supplemented with molecular adaptors XDPN-14 5'-CTGATCACTCGAGA and XDPN-12 5'-GATCTCTCGAGT (Sigma Chemical, final concentration 10 mM), 10 $\times$ T4 DNA ligase buffer (Invitrogen®) and water, heated at 55 °C for 1 min, and cooled to 14 °C within 1 h. The cDNA received 9 U of T4 DNA ligase (3 U/ μ L) and ligation was carried out overnight at 14 °C. After phenol:chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100 μ L with TE buffer (10 mM Tris/1 mM EDTA). 40 μ L of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 μ M XDPN-18 5'-CTGATCACTCGAGAGATC, 0.4 mM dNTPs, 10 $\times$ PCR buffer, 1.5 mM MgCl₂ and 1 U Taq DNA polymerase (Invitrogen). Thermocycler conditions were 1 cycle at 72 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 3 min. A total of 10 μ g of purified PCR product (tester) was digested with 10 U/ μ L *Xho*I (Invitrogen) for 6 h at 37 °C, followed by phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the tester cDNA was mixed with 5 μ g of the driver cDNA in hybridization solution (0.5 M NaCl, 50 mM Tris/HCl, 2% SDS and 40% formamide) and, after heating at 95 °C for 5 min, was incubated at 42 °C for 48 h. After extraction and precipitation, the hybridization mixture (1 μ g) was linked with the *Xho*I-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as the DNA template for PCR. Clones were sequenced using an automated DNA sequencer, and homologous sequences were identified using the BLAST program. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes using terms from the Gene Ontology database.

IPA (Ingenuity Systems®) software was used to connect the differentially expressed genes with biological functions, to relate the process in which these genes are involved and to recognize the pathways of interaction between them.

2.8. Quantitative PCR – RT-qPCR

The gene expression levels were measured in ARPE-19 cells plated in 75 cm² culture flasks, and the experiment was conducted according to the description above. Following treatment with DNase, RNA extraction was performed with TRIzol, according to the manufacturer's instructions (Invitrogen). cDNA synthesis was performed using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Five differentially expressed genes were selected for validation by quantitative real time PCR experiments, according to their direct or indirect involvement in inflammation and eye metabolism. The genes are: Connective Tissue Growth Factor (CTGF), Lecithin Retinol Acyltransferase (phosphatidylcholine-retinol O-acyltransferase) (LRAT), Microtubule-Associated Protein 1B (MAP1B), Aldehyde Dehydrogenase 1 Family, member A3 (ALDH1A3) and SET domain-containing (lysine methyltransferase) 7 (SETD7).

The primers were manually designed using the following criteria: 19–23 bp length, 30–70% GC content and a short amplicon size (90–110 bp) (Table 1). Real time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture consisted of a 20 μ L volume solution containing 100–500 ng of Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer and 100 ng cDNA. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 1
Primers for the genes analyzed by real time PCR.

Oligonucleotide	Sequence
LRAT right	5' AGATGCCATAGTGGGTCAGG 3'
LRAT left	5' CGAAGACAAAGGGAGGAACA 3'
CTGF right	5' TGGAGATTTTGGGAGTACGG 3'
CTGF left	5' GCAGGCTAGAGAAGCAGAGC 3'
MAP1B right	5' CAGGATGGGTGGTGTCTAGT 3'
MAP1B left	5' AGTCCGGCTCTTCTCTCTC 3'
ALDH1A3 right	5' GTCCGATGTTTGAGGAAGGA 3'
ALDH1A3 left	5' GAATACGCTTTGGCCGAATA 3'
SETD7 right	5' ACATACGTGCCCTGGAGAAC 3'
SETD7 left	5' GCACCTGGAGGGGTATTAT 3'

Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products.

For each primer set, PCR efficiency (linear equation: $y = \text{slope} + \text{intercept}$) was measured in triplicate for serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays.

Two control genes (*GAPDH* and *ACTB*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001).

2.9. Multiplex assay

The cytokine levels were quantified from the supernatants of ARPE-19 cells, and the experiment was conducted according to the description in Section 2.2. The MILLIPLEX MAP HCYTOMAG 60K kit and Magpix Luminex xMAP equipment (Millipore Corporation, Billerica, MA, USA) were used according to the manufacturer's instructions. The metabolites analyzed included interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 1 beta (IL-1 β), regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP-1), tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). The assays were performed in quintuplicate to confirm the results. Metabolite concentrations were determined using Magpix Xponent (Millipore Corporation, Billerica, MA, USA) software.

3. Results

3.1. Inflammatory mediators and annexin A1 protein expression in ARPE-19 cells

The presence of IL-6 and IL-8 cytokines was measured in the supernatant of ARPE-19 cells by ELISA assay. Cells activated with LPS showed a significant increase in the release of IL-6 (Fig. 1A) and IL-8 (Fig. 1B)

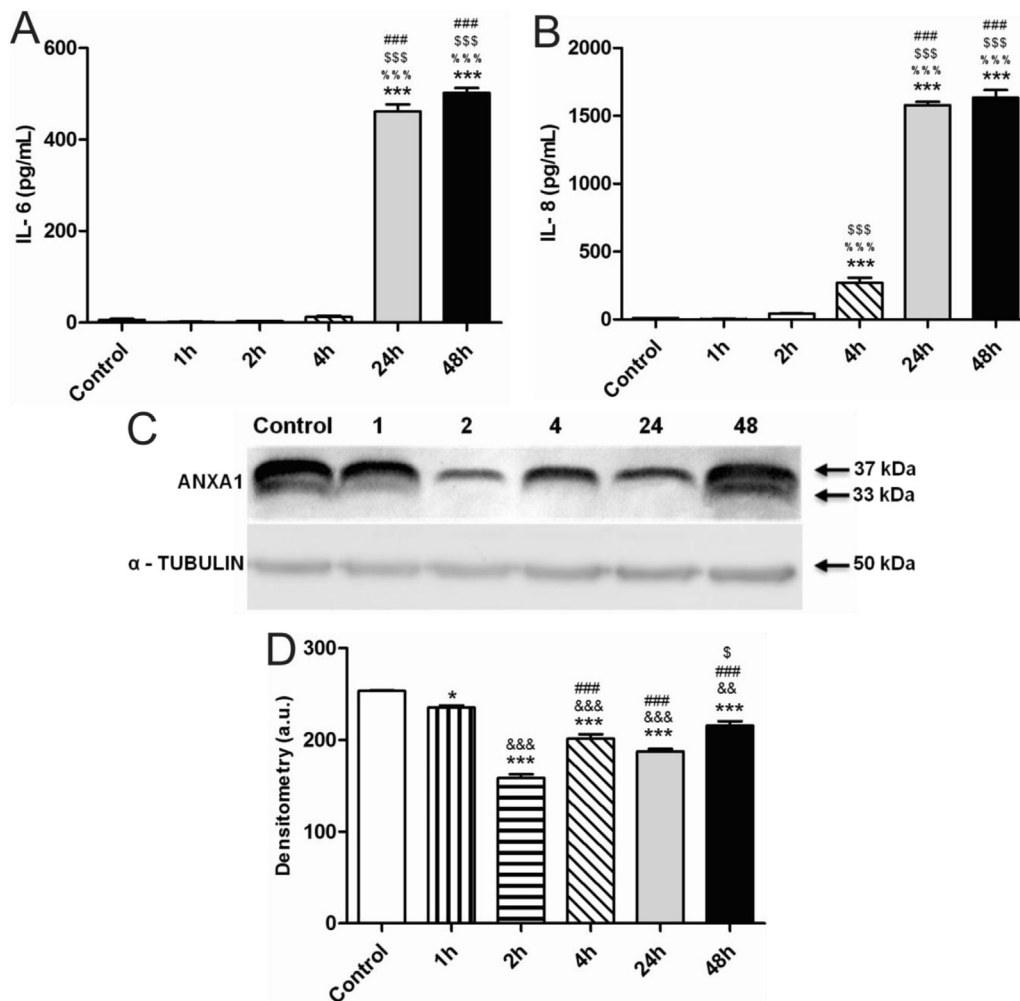


Fig. 1. Analysis of pro-inflammatory mediators and ANXA1 expression in ARPE-19 cells after activation by LPS. Dosages of [A] IL-6 and [B] IL-8 were monitored by ELISA; [C] Representative Western blotting illustrating the intact (37 kDa) and N-terminal cleaved (33 kDa) forms of ANXA1 and [D] Densitometry analyses of endogenous ANXA1 protein. The times used were 1, 2, 4, 24 and 48 h. The results were obtained as mean $\pm$ standard error of the cytokine levels in pg/mL (ELISA) with five independent experiments and, the index densitometry of the bands (Western blotting) with three independent experiments. 1 symbol = $p < 0.05$; 2 symbols = $p < 0.01$; 3 symbols = $p < 0.001$ [A] and [B] vs. control; % vs. 1 h; \$ vs. 2 h; # vs. 4 h; [D] * vs. control; & vs. control; # vs. 2 h; \$ vs. 24 h (ANOVA followed by Bonferroni's test).

cytokines after 24 h compared to the control group. High levels of cytokines were maintained at 48 h. The expression of ANXA1 in these cells under control conditions and after the activation with LPS by Western blotting was determined (Fig. 1C). The immunoreactivity of ANXA1 was detected in cells under all experimental conditions, and low levels of intact protein (37 kDa) were observed at 2 and 24 h compared to those of the controls at 1, 4 and 48 h after induction (Fig. 1D). Tubulin showed similar immunoreactivity in all experimental conditions (Fig. 1C). Therefore, the time of 24 h was chosen for the induction of inflammation by LPS.

3.2. Migration and proliferation in ARPE-19 cells

The cellular morphology is characterized by a monolayer of elongated cells ordered in hexagonal form in the control group. There was no change after activation by LPS or treatment with the anti-inflammatory peptide ANXA1_{Ac2-26} (Figs. 2A–C). ARPE-19 cells showed a progressive growth between 1 and 72 h. The three groups did not exhibit an intense difference in growth (Fig. 2D). Nonetheless, there was a significant difference after 72 h, and proliferation was diminished in the cells treated with the ANXA1_{Ac2-26} in comparison to the control group. The cell migration was influence by the treatment. ARPE-19 activated with LPS exhibited a decrease in migration and an increase in migration after ANXA1_{Ac2-26} treatment (Fig. 2E). After 24 to 72 h of LPS activation, migration was affected in these cells, but treatment with ANXA1_{Ac2-26} re-establishes the migration similar to that of the control group. Thus, the assays showed that the period of 72 h was the most statistically significant ($P \leq 0.05$).

3.3. Genes identified using the RaSH approach

RaSH was performed on ARPE-19 cells from the group activated by LPS and those stimulated and treated with ANXA1_{Ac2-26} peptide for 72 h. A total of 142 clones were isolated and sequenced. By screening

these sequences through GenBank (Blast), followed by rigorous classification and selection, 80 differentially expressed genes were identified. The research acquired two libraries. Twenty-one genes of the “Sub A” library exhibited changes in expression levels in response to LPS activation, whereas 59 genes of the “Sub B” library exhibited changes in expression with ANXA1_{Ac2-26} treatment. Therefore, it was expected that the gene expression of one library would be lower than that of the other one. The gene libraries and their different functions and processes are described in the Gene Ontology (GO) databases.

The 80 differentially expressed genes were further analyzed through IPA (Ingenuity Systems®). Genes in the “Sub A” library were found to be associated with cellular organization, cell cycle and DNA repair and replication. Genes in the “Sub B” library were found to be associated with immunological and inflammatory disease, cell cycle and cellular development. Moreover, some of these genes were chosen because they form a network of important interactions with genes such as MYC, SMAD2 and UBC in the “Sub A” library (Fig. 3A and Supplement 1A) and HIF1A and NF- κ B in the “Sub B” library (Fig. 3B and Supplement 1B).

Five differentially expressed genes, CTGF, LRAT, MAP1B, ALDH1A3 and SETD7, were selected for validation by quantitative real time PCR experiments (Table 1) because of their direct or indirect involvement in the visual cycle, nervous and visual system development and function, and ophthalmic and inflammatory disease.

3.4. Real-time PCR validation of differentially expressed genes

The RaSH analysis indicated the down-regulation of two genes (CTGF and LRAT) and the up-regulation of three genes (MAP1B, ALDH1A3 and SETD7) in ARPE-19 cells treated with the peptide. However, the quantitative PCR experiment with 1.7 μ M of ANXA1_{Ac2-26} showed oscillation in the gene expression without statistically significant alterations (Fig. 4A). Nevertheless, the experiment using 33.8 μ M showed significantly reduced expression of the five selected genes (Fig. 4B).

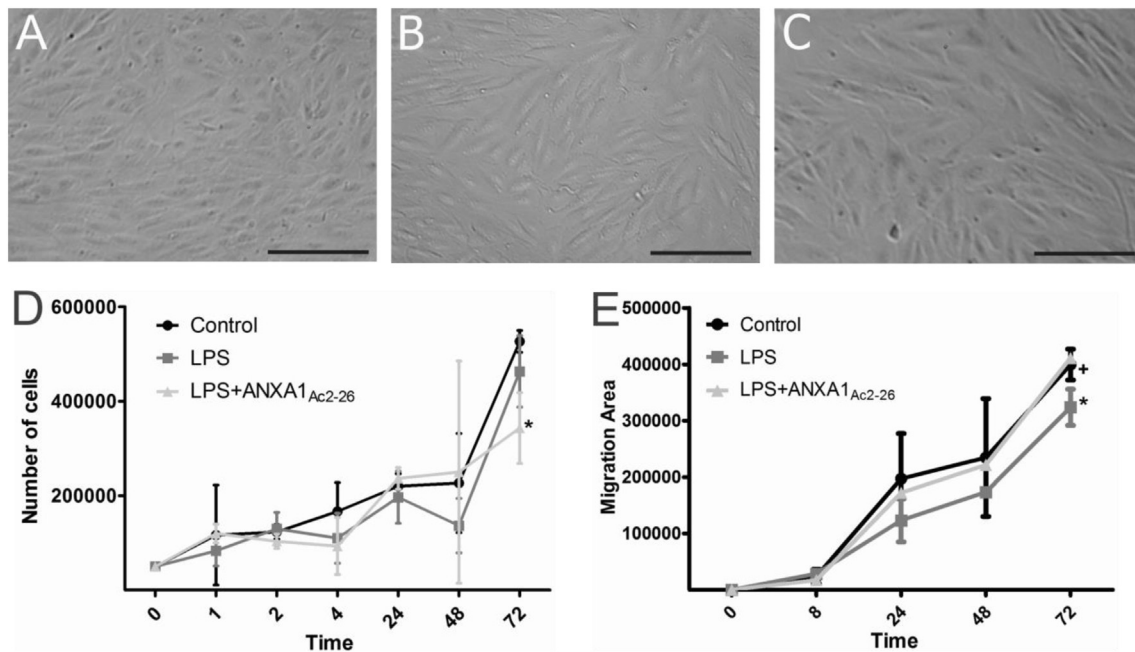


Fig. 2. Morphological analysis and effects of treatment with ANXA1_{Ac2-26} peptide on the proliferation and migration of ARPE-19 cells. The cell morphology was observed by inverted microscopy in the [A] control group, that is characterized by a monolayer of elongated cells ordered in hexagonal form, was not altered in the groups [B] activated by LPS [10 μ g/mL] and [C] activated by LPS and treated with ANXA1_{Ac2-26} [1.7 μ M]. [D] Cell growth showed a progressive growth between 0 and 72 h, and did not differ between the experimental groups. [E] Cell migration decreased after activation by LPS and increased after administration of the ANXA1_{Ac2-26} peptide. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 5×10^4 per well (6 well plate) and activated by LPS [10 μ g/mL] and treated with ANXA1_{Ac2-26} [1.7 μ M]. Assays were performed with three independent experiments. Bar: 100 μ m. [D] Graph with x = time (h) and y = number of cells. * $p < 0.05$ LPS + ANXA1_{Ac2-26} vs. Control. [E] Graph with x = time (h) and y = migration area (mm²). * $p < 0.05$ LPS vs. control; + $p < 0.05$ LPS vs. LPS + ANXA1_{Ac2-26}.

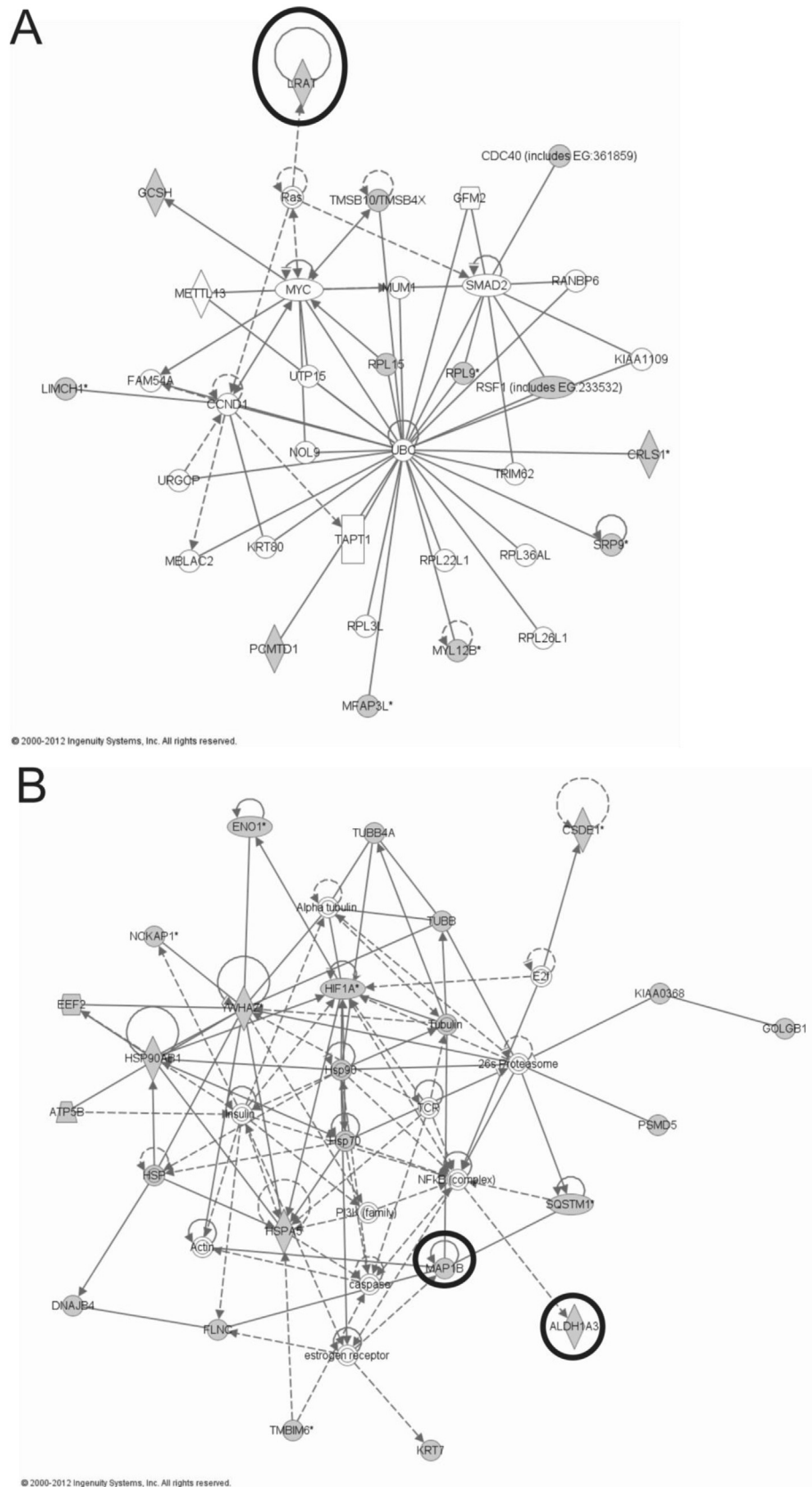


Fig. 3. Differentially expressed genes were analyzed using a manually curate structured network tool (Ingenuity Pathway Analysis). [A] Subtraction A (Sub A), after LPS activation, *LRAT* gene network showed associated functions related to tumorigenesis, apoptosis, cell proliferation and necrosis. [B] Subtraction B (Sub B), after treatment with ANXA1_{Ac2-26} peptide, Gene network showed associated functions related to cancer, inflammatory diseases, cell cycle, senescence, death and cell replication. White nodes: genes identified by IPA; grey nodes: genes identified by IPA and RaSH; black node: identified by IPA and validated by Real Time PCR.

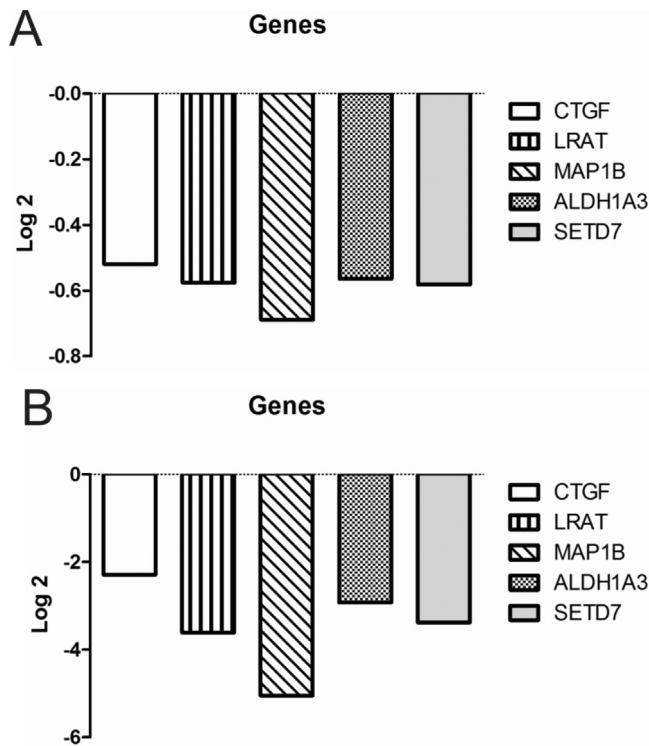


Fig. 4. Gene expression by real time PCR. [A] The genes showed oscillation in expression without statistically significant alterations in cells treated with ANXA1_{Ac2-26} peptide [1.7 μ M] compared with cells activated by LPS. [B] Significantly reduced expression of the *CTGF*, *LRAT*, *MAP1B*, *ALDH1A3* and *SETD7* gene in cells treated with ANXA1_{Ac2-26} peptide [33.8 μ M] compared with cells activated by LPS. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 1×10^6 per flask, activated by LPS [10 μ g/mL] and treated with ANXA1_{Ac2-26} peptide. Assays were performed with three independent experiments. Data represent logarithm base 2 and relative expression ≥ 1.0 and ≤ -1.0 was statistically significant.

3.5. Metabolite analysis in the supernatant of ARPE-19 cells by multiplex assay

The LPS activation increased the concentration of IL-6, IL-8, IL-1 β , Rantes, MCP-1, TNF- α , INF- γ and VEGF (Figs. 5 and 6) in the supernatant obtained from ARPE-19 cells. The treatment with 1.7 μ M ANXA1_{Ac2-26} for 72 h (Fig. 5) did not reduce the levels of the inflammatory mediators. Nonetheless, the treatment with 33.8 μ M (Fig. 6) significantly diminished the levels of IL-6, IL-8, IL-1 β , Rantes, MCP-1, TNF- α , INF- γ and VEGF, indicating the efficiency of the anti-inflammatory peptide at high concentrations.

4. Discussion

The inflammation is triggered through the pathogen recognition receptors (PRRs) and the activation of diverse pathways, which induce the secretion of pro-inflammatory molecules (Kauppinen et al., 2016; Kumar et al., 2011), it could be related to the development of ocular pathogenesis. Our in vitro analysis showed that the ANXA1_{Ac2-26} may play an important role in gene cascades and a regulatory role in ocular inflammation. The protective role of ANXA1_{Ac2-26} peptide was observed in retinal ganglion cell line (RGC-5), where the treatment decreased the expression of caspase 3 and bax, while increased the expression of bcl2 in cell lysates (Shao et al., 2012). Thus, our data demonstrate the importance of RPE cells as a potential target for gene activities involving the action of ANXA1, and its applications should be considered as a possible therapeutic approach in inflammatory ocular diseases.

In this study, we observed that after the treatment, the morphology was not modified, cells proliferation decreased, and cell migration

increased. The constitutive over-activation of ERK1/2 may directly interfere with the anti-proliferative effects of ANXA1 on cells; thus, this characteristic may be partially responsible for growth arrest (Prates et al., 2015). The presence of the protein ANXA1 at different concentrations promotes cell migration, which suggests that the presence of the protein modulates cell invasiveness (Kang et al., 2012). Together, these data show us that the protein may influence different processes in inflammatory environments to restore homeostasis.

The analysis of the inflammatory mediators after activation with LPS showed an increase in the release of TNF- α , IL-10, IL-8, IL-6 and MCP-1, which are involved with inflammation. Several investigations indicate that in ocular inflammation, pro-inflammatory cytokines are mainly produced by the inflammatory endothelial cells and the RPE (Ooi et al., 2006; van Laar and van Hagen, 2006; Zenkel et al., 2010). Other studies have also demonstrated the release of some inflammatory mediators, such as IL-6 and IL-8, in RPE cells activated by LPS (Girol et al., 2013; Leung et al., 2009; Paimela et al., 2007; Zanon et al., 2015). The treatment with 33.8 μ M of ANXA1_{Ac2-26} peptide decreased the release of these mediators, it was also seen in our research group, where the ANXA1 had modified the expression of pro- and anti-inflammatory cytokines (Damazo et al., 2011; Girol et al., 2013). Our data show that ANXA1_{Ac2-26} reduces the release of inflammatory mediators by epithelial cells, consequently preventing the development of inflammation.

The RaSH technique generated 80 differentially expressed genes, showing that ANXA1 may have a regulatory effect on gene expression after activation with LPS and treatment with ANXA1_{Ac2-26} peptide. Genes selected for validation were *LRAT*, *CTGF*, *MAP1B*, *SETD7* and *ALDH1A3*, which would indicate involvement with the important functions associated with ocular disease, retinal fibrosis syndrome, nervous system functions and development, eye system function and development, and inflammatory disease.

The treatment with the ANXA1_{Ac2-26} peptide revealed a reduction in the expression of all the analyzed genes, confirming the down-regulation of the *CTGF* and *LRAT* genes found in RaSH. Furthermore, a significant reduction in expression occurred at the concentration of 33.8 μ M, indicating that the mimetic peptide is probably more effective at higher concentrations in these cells. Thus, the *CTGF* and *LRAT* genes would be considered as potential molecular markers of ophthalmologic inflammation, while *MAP1B*, *ALDH1A3* and *SETD7* genes require further testing.

The *CTGF* gene is correlated with different degrees of retinal fibrosis syndrome that depend on expression levels (Chen et al., 2012). It was demonstrated that its expression at high levels was present in age-related macular degeneration, where it was related to it the production of matrix proteins through the ERK signaling pathway (p42/p44^{MAPK}) and p38^{MAPK} (Nagai et al., 2009). The treatment with the peptide decreased the expression of this gene, indicating that ANXA1 may alter its expression through the NF- κ B signaling pathway, supported by the fact that these genes are related by the IPA system. The down-regulation of *CTGF* may be an alternative to homeostasis restoration. On the other hand, it was shown that a time-dependent increase in the levels of *CTGF* was concomitant with the retinal vascularization development in neonatal mice (Chintala et al., 2012). The expression of this gene may be important for the development and remodeling of retinal vessels, explaining its increase after LPS induction, and decreased, after the treatment with 33.8 μ M, as an attempt to restore homeostasis.

LRAT is important in the retinoid regeneration process, known as the visual cycle, where it esterifies all-*trans*-Retinol to all-*trans*-Retinyl Ester (Mondal et al., 2000; Muñiz et al., 2014). This protein is expressed in diverse fetal and adult tissues, including the RPE (Mondal et al., 2000; Ruiz and Bok, 2010). Mutations in this gene are associated with the early onset of severe retinal dystrophy (Tang et al., 2013; Trevino et al., 2005) and are also associated with progressive retinal atrophy (Dev Borman et al., 2012). The down-regulation of this gene, which is related to the UBC pathway, as shown by the IPA system, may be related to the inflammatory environment, perhaps when there is impairment in the visual system or to help activate the inflammatory signaling cascades.

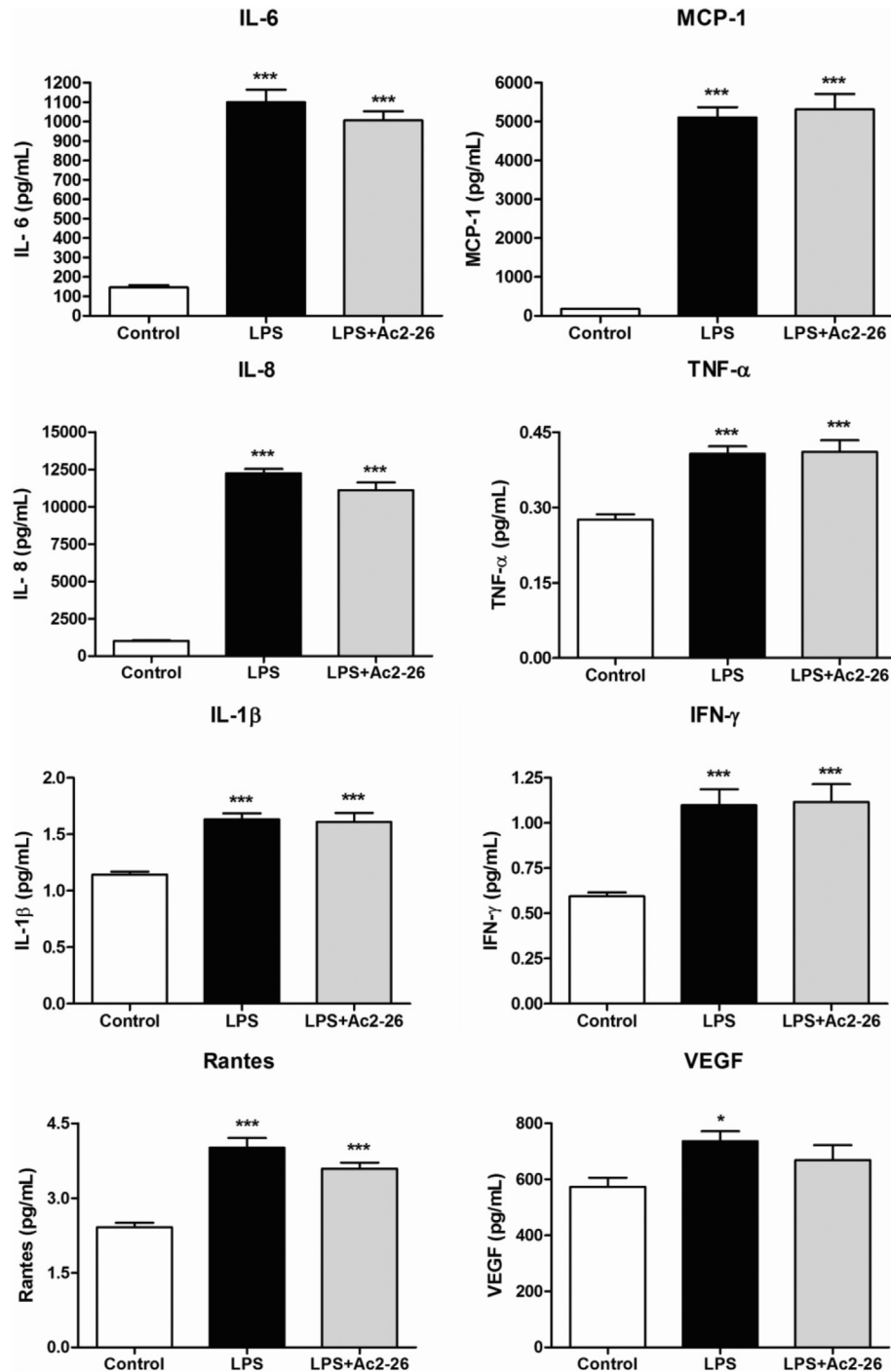


Fig. 5. Effect of ANXA1_{Ac2-26} [1.7 μ M] peptide on the expression of pro- and anti-inflammatory metabolites. Analysis of IL-6, IL-8, IL-1 β , Rantes, MCP-1, TNF- α , IFN- γ and VEGF, were monitored after the treatment for 72 h. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 5×10^4 per well (6-well plate), activated by LPS [10 μ g/mL] and treated with the ANXA1_{Ac2-26} peptide. Assays were performed with three independent experiments. Data represent mean $\pm$ standard error. Metabolite concentrations were determined using Magpix Xponent software. * $p < 0.05$ LPS and LPS + ANXA1_{Ac2-26} vs. control.

The studied genes in this work showed differential expression in ARPE-19 cells in an ANXA1_{Ac2-26} treatment-dependent manner. The peptide altered cell proliferation and migration, and gene and protein expression. Few studies have evaluated the effect of ANXA1_{Ac2-26} in the expression of the selected genes, and future molecular studies are needed to show the mechanisms of action of this anti-inflammatory protein. Other experiments will be needed to determine whether the other genes, obtained from the RaSH technique, are causally related or associated with changes induced by the treatment.

5. Conclusion

Our findings show that ANXA1 is involved in the signaling cascades of inflammatory processes, NF- κ B and UBC, which decrease cell proliferation and increase migration, modulating *CTGF* and *LRAT* gene expression in RPE cells. Therefore, our results indicate that RPE cells are potential targets for the gene activity of the ANXA1_{Ac2-26} peptide, and their possible use for innovative therapies in the treatment of ocular inflammations should be considered.

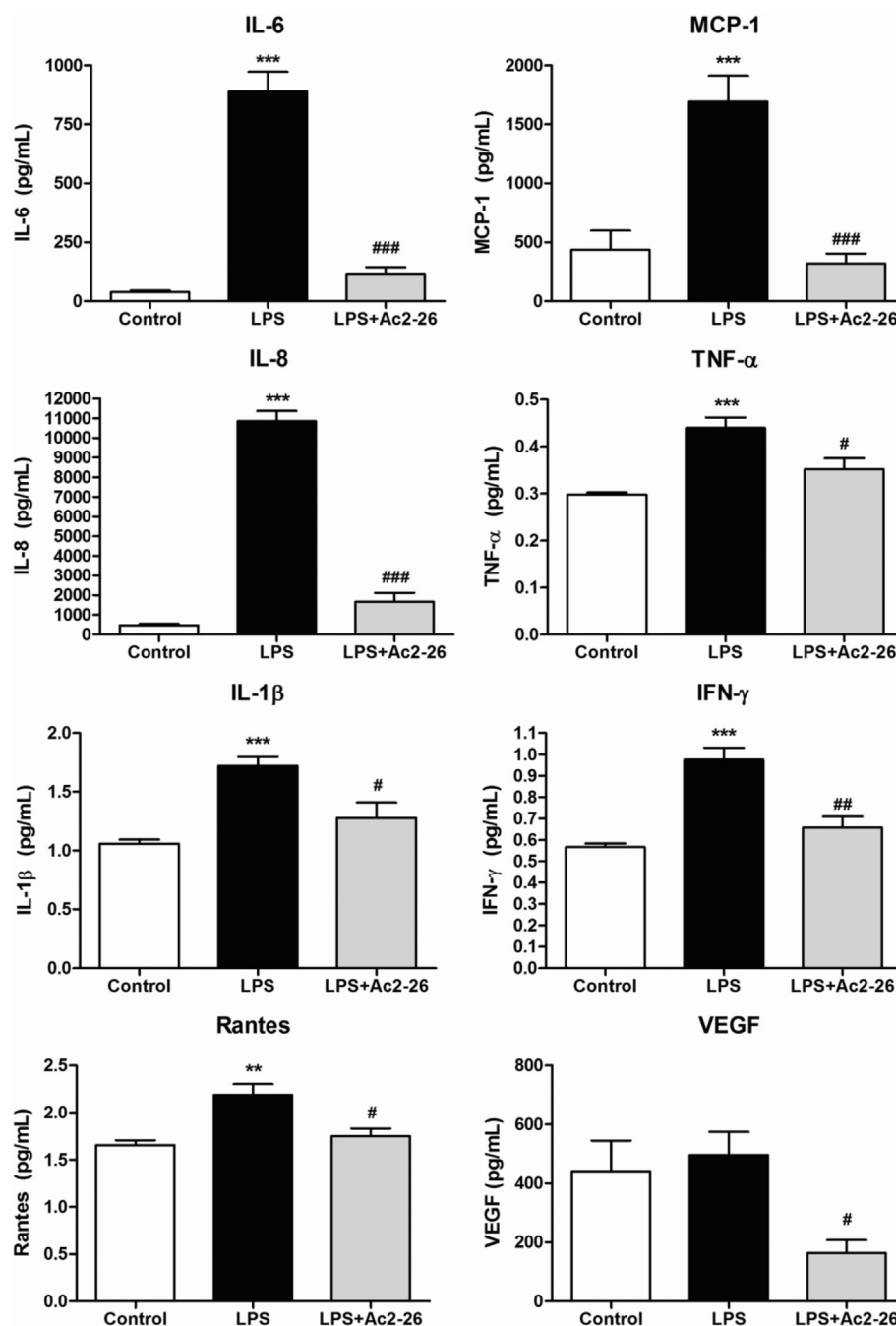


Fig. 6. Effect of ANXA1_{Ac2-26} [33.8 μ M] peptide on the expression of pro- and anti-inflammatory metabolites. Analysis of IL-6, IL-8, IL-1 β , Rantes, MCP-1, TNF- α , IFN- γ and VEGF, were monitored after treatment for 72 h. ARPE-19 cells were seeded in complete DMEM:F-12 medium at a concentration of 5×10^4 per well (6-well plate), activated by LPS [10 μ g/mL] and treated with the ANXA1_{Ac2-26} peptide. Assays were performed with three independent experiments. Data represent mean $\pm$ standard error. Metabolite concentrations were determined using Magpix Xponent software. * $p < 0.05$ LPS and LPS + ANXA1_{Ac2-26} vs. control; # $p < 0.05$ LPS + ANXA1_{Ac2-26} vs. LPS.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2017.02.032>.

Author contributions statement

LTC carried out the cell culture experiments, RaSH assay, real-time PCR assay, assisted with the ELISA and Western blotting assays, performed the statistical analysis, and drafted the manuscript. NMS assisted with the cell culture experiments. KKOM carried out the ELISA and Western blotting assay. LMS and AML carried out the Migration assay. ARDS and WASjr assisted with the RaSH assay.

BRC assisted with the real-time PCR assay and Ingenuity® Systems analysis. EHT and SMO were involved in drafting the manuscript and revising it for important intellectual content. FCR-L conceived the study, participated in its design and coordination, and helped draft the manuscript. All of the authors read and approved the final manuscript.

Disclosures

The authors have declared that no competing interests exist.

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4. CONSIDERAÇÕES FINAIS

Os efeitos pró e anti-inflamatórios da proteína ANXA1, glicocorticoide endógeno, dependente do tipo de ligante, já foram descritos (GUO; LIU; SUN, 2013), essa proteína é encontrada no citoplasma celular, está associada à membrana plasmática e dentro de vesículas secretórias e endossomais (GREWAL et al., 2016). Dependendo da célula que ativa a via da ANXA1, cascatas gênicas diferentes são desencadeadas. Dentre essas cascatas temos a MAPK/ERK; quando essa é ativada em monócitos e células T, há uma resposta de diferenciação e proliferação, e quando ativada em neutrófilos, a resposta pode levar a apoptose e/ou necrose (D'ACQUISTO; PIRAS; RATTAZZI, 2013; PERRETTI; D'ACQUISTO, 2009; POETER et al., 2013). A proteína ANXA1 no organismo foi demonstrada por ativar a cascata p38 ativada por mitógeno e o peptídeo Ac2-26 promove a ativação da via quinase N-terminal da c-Jun (JNK) (COORAY et al., 2013).

Nos nossos trabalhos houve uma diminuição da proliferação quando os diferentes tipos celulares foram tratados com o peptídeo da ANXA1, portanto o possível mecanismo para a ação antiproliferativa da ANXA1 poderia ser via ativação da cascata de sinalização MAPK/ERK, conforme demonstrado anteriormente em uma variedade de tipos celulares (ALLDRIGDE et al., 2003).

No capítulo 3.2 observamos a resposta antiproliferativa nas células Hep-2, sugerindo que o peptídeo da ANXA1 pode desencadear a regulação do crescimento por interação direta com o receptor FPR/ANXA1. O peptídeo também pode ter ativado a expressão ANXA1 endógena, resultando em regulação do crescimento por um mecanismo autocrino/parácrino. O papel autocrino/parácrino da ANXA1 na membrana também foi mostrado na invasão com modelo de linhagem celular epitelial, SKCO-15, derivada do adenocarcinoma colorretal através da ativação da interação dessa com os receptores FPR (BABBIN et al., 2006). O mecanismo pelo qual a ANXA1 regula a proliferação ainda é desconhecido, mas pode estar relacionado com a modulação da expressão gênica, uma vez que os dados suportam esse papel para essa proteína (RODRIGUES-LISONI et al., 2006). Também é possível que as ações antiproliferativas da ANXA1 sejam mediadas por meio da estimulação da apoptose. A ANXA1 demonstrou estimular a apoptose, principalmente nas células da linhagem mielo-monocítica (SOLITO et al., 2001), neutrófilos (SOLITO et al., 2003) e nas células epiteliais bronco-alveolares (DEBRET et al., 2003).

No capítulo 3.3 observamos que também houve diminuição na proliferação de células de câncer cervical após tratamento com o peptídeo da ANXA1, agindo como um supressor tumoral, possivelmente por meio da cascata MAPK/ERK. Essa ação pode acarretar na expressão aumentada dessa via, a qual altera expressão de moléculas envolvidas no ciclo celular, fazendo com que haja parada na fase G1/S (MCCUBREY et al., 2007). A proteína ANXA1 pode assim possuir ação antiproliferativa nas células de câncer cervical por meio das cascatas MAPKs. A ação dessa proteína na resposta imune, por meio de diversos mediadores moleculares e receptor FPR2, não é bem conhecida, mas há indicativas de que as cascatas MAPKs (ERK1/2, p38MAPK e quinase N-terminal da c-Jun) estão envolvidas (GREWAL et al., 2016). A diminuição da proliferação de células de câncer é ocasionada pela regulação aumentada dessa proteína (BIAOXUE; XIGUANG; SHUANYING, 2014). Alguns trabalhos também corroboram com essa ação antiproliferativa da ANXA1, por exemplo, estudos com linhagem celular de adenocarcinoma de pulmão, mostrou que bloqueio da ANXA1, utilizando siRNA, diminuiu a capacidade de invasão dessas células (LIU et al., 2011), assim como estudos com camundongos ‘nude’, os quais tiveram expressão da ANXA1 bloqueada, apresentaram redução significativa da tumorigênese em glioblastoma; em adição, bloqueio da proteína e do receptor FPR1 fizeram com que crescimento tumoral diminuísse ainda mais (YANG et al., 2011).

No capítulo 3.4, analisando as células cultivadas da linhagem celular ARPE-19, observamos que a proliferação celular diminuiu após indução pelo LPS e após o tratamento com o peptídeo da ANXA1, a proliferação e a migração celular aumentaram com relação ao LPS. O teste de migração celular também foi realizado por Kang e colaboradores (2012), em diferentes linhagens celulares de câncer de mama humano, e foi visto que cada tipo celular expressa a ANXA1 em diferentes concentrações, promovendo a migração celular. Esses pesquisadores, ainda relataram que após a administração do micro RNA de interferência da ANXA1 ocorre diminuição da migração e proliferação celular, sugerindo assim que a presença da proteína modula a invasividade e o crescimento celular.

Investigação realizada no nosso laboratório, também com a linhagem celular ARPE-19 (MIMURA, 2012), mostrou que a ANXA1 está envolvida na fisiologia do Epitélio Pigmentar da Retina nas condições normais e após o estímulo inflamatório com LPS e o tratamento possui ação anti-inflamatória nessas células. Outros trabalhos mostraram que a expressão da ANXA1, bem como as atividades anti-inflamatórias e

protetoras dessa proteína e dos seus peptídeos miméticos, têm sido exploradas (GAVINS et al., 2007; D'ACQUISTO, 2009) em vários modelos experimentais, como inflamações pulmonares (DA CUNHA; OLIANI; DAMAZO, 2012), regulação da atividade da COX-2 em células da macula densa renal (SEIDEL et al., 2012), tratamento de colite (OUYANG et al., 2012), efeito protetor após lesão do nervo cavernoso (FACIO JUNIOR; BURNETT, 2012), entre outros.

Além da ação antiproliferativa da ANXA1, também relatamos nos nossos trabalhos a ação dessa proteína na modulação da expressão gênica. No capítulo 3.1 observamos que a ANXA1 exógena modifica a expressão gênica, em cultura de células de carcinoma de laringe (Hep-2), porém os mecanismos responsáveis requerem mais investigação. Neste trabalho, relatamos, pela primeira vez, os efeitos da anexina A1 (ANXA1) e seu peptídeo N-terminal (ANXA1Ac2-26) na expressão gênica, utilizando análise genômica em linhagens de carcinoma e de células foliculoesteladas de adenoma pituitário (PDFS). Também foram utilizadas análises *in vivo* da pituitária de camundongos selvagens e *knockouts* para o gene da ANXA1. Os dados do trabalho, obtidos por técnicas de eletroforese 2D, PCR semi-quantitativa, imuno-histoquímica, western blot e citometria de fluxo, mostraram que a proteína regula sua própria expressão e inibe a expressão de um grande número de proteínas. Tais efeitos são comparáveis à ação poderosa do tratamento com esteróides e reforçam a função proposta da ANXA1 como um mensageiro secundário da ação de glicocorticóides (FLOWER, 1988).

Pela técnica de RaSH (*Rapid Subtractive Hybridization*) realizada em células tratadas e não tratadas com a proteína ANXA1 ou seu peptídeo foi observada a regulação positiva da expressão do gene *CCR10*, um receptor para citocina CCL27 (HOMEY et al., 2002). O mesmo resultado foi observado em camundongos *knockouts* para ANXA1. Tanto as análises por PCR semi quantitativa como por Western Blot mostraram que as células de pele e do trato respiratório desses animais exibem níveis reduzidos de *CCR10* em relação aos camundongos selvagens. A citocina CCL27 e seu receptor *CCR10* mediam respostas quimiotáticas no recrutamento de células T *in vitro* (LEUNG, SOTER, 2001; SOLER et al., 2003). A CCL27, cuja expressão é constitutiva e elevada em pele normal e inflamada, é regulada positivamente pelo fator de necrose tumoral TNF- α e pelo fator de transcrição nuclear kappa B (NF-kB) *in vitro* e negativamente pelos glicocorticóides *in vivo* (VESTERGAARD et al., 2005; HOMEY et al., 2002). Como a ANXA1 é vista frequentemente como uma proteína anti-

inflamatória, seria esperada uma baixa expressão do CCR10, que modula efeitos pró-inflamatórios. Entretanto, como a ANXA1 está presente na pele (SCHLAEPFER, HAIGLER, 1990), é possível que ela module positivamente a expressão desse receptor para facilitar o acúmulo local de linfócitos. Um efeito similar é observado em relação aos glicocorticóides, um outro grupo de fatores anti-inflamatórios que aumentam, em eosinófilos, a expressão de um dos membros desta família de receptores, o CXCR4, provavelmente resultando no sequestro de eosinófilos circulantes para tecidos não inflamados (NAGASE et al., 2000). Os eosinófilos, cuja migração é uma característica marcante da inflamação alérgica, possuem sítios ligadores de glicocorticóides, que parecem atuar diretamente sobre essas células e reduzir sua longevidade pela indução de apoptose (WALLEN et al., 1991).

A ANXA1 está fortemente expressa durante o desenvolvimento do trato respiratório superior e da pele (WELLS, et al. 2004) e exibe, em fibroblastos, significantes mudanças na expressão relacionadas com a idade (BORALDI et al., 2003), com níveis elevados em fibroblastos humanos de doadores adultos comparados a doadores idosos. Este fato pode se refletir na expressão e na funcionalidade do CCR10 como parte de uma falha geral, relacionada com o envelhecimento, em responder ao estresse ambiental e/ou fisiológico. A mesma falha pode ocorrer em câncer, considerando a semelhança de muitos mecanismos envolvidos na senescência e na tumorigênese. O fato do receptor CCR10 ser um mediador do recrutamento de linfócitos específicos da pele e o fato da ANXA1 ser expressa na pele e inibir a L-selectina em neutrófilos (STRAUSBAUGH, ROSEN, 2001) e em monócitos (de COUPADE et al., 2003) reforçam o papel dessa proteína na inflamação cutânea.

Em resumo, esse trabalho mostrou a técnica de RaSH como um método rápido e eficiente identificando genes modulados pela ANXA1. Para o gene CCR10, sua expressão foi claramente relacionada com a presença da ANXA1 *in vivo*. Apesar da sua influência sobre um grande número de genes, a perda da ANXA1 em animais *knockout* não comprometeu sua sobrevivência, suportando uma função adaptativa para a ANXA1 em uma variedade de padrões metabólicos. Outros experimentos são necessários para determinar se os demais genes regulados pela ANXA1 estão casualmente relacionados ou associados com mudanças induzidas pelo tratamento com esta proteína, embora isto pareça improvável, pois nossos achados foram observados em duas linhagens celulares independentes. Estes estudos, embora desafiantes, fornecerão informações importantes a

respeito do fenótipo de organismos *knockout* para anexina A1 e, portanto, da função fisiológica desta proteína.

No capítulo 3.2, ainda trabalhando com a linhagem celular de carcinoma epidermóide de laringe (Hep-2) tratadas com o peptídeo da ANXA1, também foi avaliada a expressão da ANXA1 endógena em células tumorais e em mastócitos, no qual foi observado a imunoreatividade da ANXA1 mostrando baixa regulação nos tumores de laringe e células peritumorais, comparadas com o tecido controle. Vários outros estudos demonstraram que a expressão de ANXA1 também se altera durante o desenvolvimento e a progressão do câncer (SHEN et al., 2005) e está regulada negativamente em tumores de cabeça e pescoço (PEDRERO et al., 2004), próstata (XIN et al., 2003) e câncer de mama (SHEN et al., 2005). A expressão da proteína ANXA1 foi diminuída nesses tumores em comparação com o epitélio normal sugerindo que esta proteína é um componente essencial para a manutenção do fenótipo epitelial normal, um fato que pode ser importante em eventos iniciais em câncer invasivo (PAWELETZ et al., 2000).

Com base em nossos achados demonstrando a baixa expressão de ANXA1 em células tumorais de laringe *in vivo*, investigamos o efeito da ANXA1 exógena em células de câncer de laringe Hep-2. Os resultados de PCR em tempo real combinados com imunocitoquímica ultraestrutural demonstraram que o tratamento com peptídeo em células Hep-2 induziu um pico de expressão de mRNA de ANXA1 a 6 e 48 h, enquanto que o pico na expressão de proteína foi observado a 24 e 96 h. Outros estudos também demonstraram diferença nos padrões de expressão ANXA1 nos níveis de mRNA e proteína (GIBBS et al., 2002).

No capítulo 3.3, no estudo de células de carcinoma de colo de útero tratadas com o peptídeo da ANXA1, os resultados mostraram que o tratamento resultou em 82 genes diferencialmente expressos (pela técnica de RaSH). A expressão da proteína ANXA1 foi diminuída nas células SiHa tratadas com o peptídeo pelas análises imunocitoquímicas ultraestruturais. O gene *ID1* foi validado por RT-qPCR, confirmando a sua baixa expressão após o tratamento com o peptídeo. Também foram interligadas, pelo *Ingenuity Systems*, as vias de sinalização celular, das quais a ANXA1 participa, importantes para o desenvolvimento tumoral.

A seleção dos genes validados contribui para um melhor entendimento dos processos tumorigênicos. Na literatura, os níveis de expressão do gene *ID1* estão relacionados com angiogênese, diferenciação e metástase, e a superexpressão foi

encontrada em carcinoma esofágico de células escamosas (LUO et al., 2012), sugerindo que, no nosso trabalho, a ANXA1 pode ter regulado este gene diminuindo sua expressão, podendo ser utilizada como um possível alvo terapêutico. O gene *ID1* é um inibidor de diferenciação ou de ligação de DNA que pertence a uma família de pequenas moléculas de curto comprimento (13 a 20 KDa). O inibidor de diferenciação contém um domínio hélice-alça-hélice pelo qual inibe os fatores de transcrição básica de ligação ao DNA. Desta maneira esse gene regula os fatores de transcrição de genes alvos (BENEZRA, et al., 1990). Em um estudo, Darnel e colaboradores (2010) correlacionaram tipos de HPVs, em câncer cervical, com a expressão gênica dos genes *ID*, onde verificaram que a expressão da oncoproteína E6 correlaciona com a expressão de *ID1* em 95% dos carcinomas cervicais invasivos, sugerindo que os HPVs de alto risco contribuem para a progressão tumoral pela regulação do gene *ID1*.

No capítulo 3.4 estudamos o efeito do tratamento com o Peptídeo da ANXA1 em um modelo experimental de uveíte *in vitro*, no qual observamos pela tecnologia de RaSH 80 genes diferencialmente expressos após o tratamento. Cinco desses genes (*LRAT*, *CTGF*, *MAP1B*, *ALDH1A3* e *SETD7*), devido às suas funções estarem relacionadas com doenças oculares e/ou inflamatórias, foram validados por RT-qPCR, mas apenas dois deles (*LRAT*, *CTGF*) confirmaram a baixa expressão. Também foram interligadas, pelo *Ingenuity Systems*, as vias de sinalização celular, das quais a ANXA1 participa, importantes para o processo inflamatório. A seleção dos genes validados contribui de forma significativa para o entendimento dos processos inflamatórios oculares, pois na literatura os níveis de expressão do gene *CTGF* estão relacionados com os diferentes graus da síndrome da fibrose retiniana (CHEN et al., 2012) e, ainda, com a expressão elevada na Doença Macular relacionada com a idade, pois induz a produção de proteínas da matriz pelas vias de sinalização ERK (p42/p44^{mapk}) e p38^{mapk} (NAGAI et al., 2009). Chintala e colaboradores (2012) estudaram a retinopatia induzida por oxigênio em ratos neonatais, e injetaram vetores virais recombinantes do gene *CTGF*. Esses pesquisadores observaram que o desenvolvimento da vascularização da retina foi concomitante com o aumento, progressivo e dependente do tempo, nos níveis de *CTGF*, e a supressão da expressão ou atividade do *CTGF* atrasou a expansão do plexo capilar da retina. O *CTGF* possui efeito direto no desenvolvimento e remodelação dos vasos da retina, sendo importante no reparo e adaptabilidade vascular após lesões. Além da relação com o metabolismo ocular, esse gene possui função na diferenciação e

proliferação dos condrócitos, adesão celular e está relacionado com o fator de crescimento derivado de plaquetas.

No gene *LRAT* estudado, ressaltamos a importância relacionada por codificar uma proteína que catalisa a estratificação do *All-trans*-Retinol para *All-trans*-Retinil Ester, uma reação essencial para o ciclo dos retinóides no sistema visual. Essa proteína é expressa em diversos tecidos em adultos e fetos, incluindo o Epitélio Pigmentar da Retina (RUIZ; BOK, 1999). Mutações nesse gene estão relacionadas com o início adiantado de severas distrofias na retina (TREVINO et al., 2005; TANG et al., 2012). Estudos de Borman e colaboradores (2012) mostraram que mutações no gene *LRAT* causam atrofia progressiva de retina. Eles avaliaram, pela tecnologia de *microarray*, 149 pacientes com Amaurose Congênita de Leber ou distrofia precoce de retina (caracterizada pela perda da visão desde o nascimento). Nesse estudo identificaram um paciente com a mutação homozigota nesse gene e outros três pacientes com novas mutações no exon 2, sugerindo que alterações no gene *LRAT* podem mostrar uma boa resposta à novas terapias.

Os genes estudados mostraram expressão diferencial nas células do Epitélio Pigmentar da Retina de uma maneira dependente do tratamento com o peptídeo. Em resumo, esse trabalho evidencia a importância da técnica utilizada e pelas funções dos genes encontrados podemos observar que são importantes nas cascatas dos processos inflamatórios, podendo ter sua expressão modificada pela possível interação no metabolismo das células epiteliais oculares. O olho possui características que o torna um órgão alvo para terapia gênica, por ser imuno privilegiado, ter tamanho pequeno, e ser facilmente visualizado e examinado. Os fotorreceptores e o Epitélio Pigmentar da Retina são os principais alvos para terapia gênica no tratamento de doenças da retina, devido seu importante papel no ciclo visual (LIPINSKI; THAKE; MACLAREN, 2013).

Em relação à análise da expressão das citocinas, no capítulo 3.4 foi observado que o tratamento com o peptídeo da ANXA1 diminuiu a expressão de moléculas pró-inflamatórias. Analisamos nos sobrenadantes dos cultivos a liberação dos mediadores pró-inflamatórios IL-6, IL-8, IL-1 β , Rantes, MCP-1 e TNF- α e anti-inflamatórios IL-10, EGF, VEGF e INF- γ , nas células ARPE-19 após ativação pelo LPS, com e sem tratamento. O estímulo pela endotoxina nessas células induziu a liberação dos mediadores envolvidos na resposta inflamatória, como TNF- α , IL-10, IL-8, IL-6 e MCP-1, mostrando aumento significativo em relação ao controle, e mantendo altos níveis após 72 horas.

Várias investigações indicam que, na inflamação ocular, as citocinas pró-inflamatórias são produzidas principalmente pelas células inflamatórias, endoteliais e, pelo epitélio pigmentado da retina (OOI *et al.*, 2006; ZENKEL *et al.*, 2010). Outros estudos também mostram a liberação de alguns mediadores inflamatórios, como a IL-6 e IL-8, em células do epitélio pigmentar da retina ativadas pelo LPS (LEUNG; BARNSTABLE; TOMBRAN-TINKA, 2009; MIMURA, 2012). Pacientes com doenças do segmento anterior do olho também apresentaram alta expressão de IL-6 e IL-8 em fluido lacrimal (FODOR *et al.*, 2006). Há evidências de que essas citocinas podem induzir a degeneração celular e tecidual, pois, camundongos nocautes para IL-6 apresentam maior sobrevida de células ganglionares da retina em relação aos selvagens após lesão do nervo óptico (FISHER *et al.*, 2001).

Anteriormente, já foi demonstrado que a ANXA1 está relacionada com a modificação da expressão de citocinas pró e anti-inflamatórias em estudos do nosso grupo (DAMAZO *et al.*, 2011; GIROL, 2012, MIMURA, 2012) e, também, de Yang e colaboradores (2006) usando linhagens celulares de fibroblasto de pulmão (com e sem o gene da ANXA1), observaram que essa proteína regula negativamente a expressão da citocina IL-6, via efeitos na cascata $p38^{mapk}$. A sinalização da IL-6 leva ao aumento da produção de citocinas inflamatórias, quimiocinas, proteases e fatores de crescimento (IL-6, IL-1 β , IL-23, CCL5, CXCL8, MCP-1, MMP9, MMP2, VEGF e HIF) (AGGARWAL; GEHLOT, 2009; SANSONE; BROMBERG, 2011). Os nossos dados mostram que o ANXA1_{Ac2-26} reduz a liberação de mediadores inflamatórios pelas células epiteliais, consequentemente impedindo o desenvolvimento inflamatório.

Concluimos que o peptídeo da ANXA1 altera a proliferação, migração e expressão gênica e proteica nas diferentes linhagens celulares estudadas (Hep-2, PDFS, SiHa e ARPE-19) e, essas alterações podem provavelmente estar relacionadas com os processos inflamatórios, devido a indução realizada por endotoxina. Assim, sugerimos que ANXA1 tem ação moduladora no metabolismo dessas células. Poucas investigações têm avaliado o efeito da ANXA1 na expressão desses genes que foram selecionados em nossos trabalhos e, futuros estudos moleculares serão necessários para mostrar os mecanismos de ação dessa proteína anti-inflamatória.

As análises *in vitro* mostraram que o ANXA1_{Ac2-26} pode ter um importante papel nas cascatas gênicas e um papel regulatório no desenvolvimento tumoral e na inflamação ocular. Dessa maneira, os nossos dados apontam a importância das células Hep-2, SiHa e ARPE-19 como um alvo potencial de atividades gênicas envolvendo a

ação da ANXA1, tendo em vista as aplicações dessa proteína como uma possível alternativa terapêutica no carcinoma de laringe, de colo uterino e nas doenças inflamatórias oculares.

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