UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

AVALIAÇÃO DA *MILK FAT GLOBULE EPIDERMAL GROWTH FACTOR 8* (MFG-E8), DA INTEGRINA αvβ3 E DA *LEUKEMIA INHIBITORY FACTOR* (LIF) NA IMPLANTAÇÃO EMBRIONÁRIA HUMANA: ESTUDO EM MODELO *IN VITRO* E NO ENDOMÉTRIO DE MULHERES COM E SEM ENDOMETRIOSE

ALUNA CARLA REGINA SCHMITZ

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Orientador: Prof. Dr. João Sabino da Cunha-Filho. Tese apresentada como requisito parcial para obtenção do título de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

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DEDICATÓRIA

Dedico este trabalho aos meus pais, Paulo e Aneli Schmitz, aos meus irmãos, Gustavo e Leonardo Schmitz, e, especialmente, ao meu esposo, Michel Vigo, sem o qual eu certamente não teria alcançado esta conquista.

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EPÍGRAFE

"Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes."

Leonardo da Vinci (1476-1513)

BANCA EXAMINADORA

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RESUMO

Base teórica: O processo de implantação do embrião no ser humano é extremamente complexo e, ao mesmo tempo, essencial para que a mulher possa engravidar. Neste processo, em que o endométrio precisa sofrer uma série de mudanças para tornar-se receptivo, a adequada expressão de MFG-E8 (*milk fat globule epidermal growth factor* 8), seu receptor a integrina αvβ3 e LIF (*leukemia inhibitory factor*) parecem ter um papel importante. Além do mais, mulheres com infertilidade e endometriose podem apresentar a falha de implantação como uma grande barreira para obter seu sucesso terapêutico.

Objetivos: Avaliar o papel de MFG-E8 e do seu receptor integrina αvβ3 em um modelo de implantação in vitro com uma linhagem celular trofoblástica e outra de epitélio endometrial. Comparar a expressão de MFG-E8, de integrina αvβ3 e de LIF no endométrio de pacientes férteis e inférteis com endometriose durante a janela de implantação.

Métodos: No primeiro ensaio, utilizando-se uma linhagem celular bem diferenciada de adenocarcinoma de endométrio (células Ishikawa) e uma linhagem de coriocarcinoma de trofoblasto, o modelo *in vitro* de implantação humana foi estabelecido. Para investigação do impacto do bloqueio de MFG-E8 e integrina αvβ3, ambas linhagens celulares foram pré-tratadas com anticorpos contra estas proteínas em diferente concentrações antes do ensaio de adesão. No ensaio subsequente, para comparar a expressão de MFG-E8, de integrina αvβ3 e de LIF no endométrio humano, foram realizadas biópsias no período da janela de implantação (LH+7 a LH+10) com cateter de Pipelle. As amostras foram submetidas a imunohistoquímica, e analisadas através do HSCORE.

Resultados: Na avaliação *in vitro* observamos que as células Ishikawa pré-tratadas com anticorpo anti-MFG-E8 causaram diminuição da adesão das esferas Jar dose-dependente. Por outro lado, o pré-tratamento das esferas Jar não resultou em diminuição significativa da adesão. Pré-tratamento com anticorpos anti-integrina αvβ3, tanto de células Ishikawa como de esferas Jar, causaram inibição significativa, dose-dependente, da adesão das esferas. A análise imunohistoquímica das biópsias realizadas durante a janela de implantação mostrou uma expressão aumentada de MFG-E8 em pacientes com endometriose e infertilidade. Além do mais,

houve expressão diminuída de LIF no grupo em estudo. Contudo, não houve diferença estatisticamente significativa na expressão de integrina αvβ3 entre os grupos em estudo.

Conclusão: Este estudo demonstrou que, quando se bloqueia MFG-E8 ou seu receptor integrina αvβ3 em células Ishikawa em um modelo *in vitro*, ocorre uma diminuição de adesão das células Jar. Além do mais, bloqueando-se a integrina αvβ3 nas esferas Jar, também ocorre uma diminuição da adesão destas nas células Ishikawa. No entanto, quando estudamos o endométrio *in vivo* de pacientes com endometriose e infertilidade, encontramos a expressão aumentada de MFG-E8 e diminuída de LIF durante a janela de implantação no endométrio.

Palavras chave: MFG-E8, integrina αvβ3, LIF, implantação, endométrio, modelo *in vitro*, endometriose

ABSTRACT

Background: The human implantation process is very complex and, at the same time, it is essential for women to achieve pregnancy. In this process, where the human endometrium must go through a lot of changes in order to become receptive, an adequate expression of MFG-E8 (milk fat globule epidermal growth factor 8), integrin αvβ3 and LIF (leukemia inhibitory factor) appear to play an important role. Furthermore, women with endometriosis and infertility may have in their implantation process the key to achieve pregnancy.

Objectives: To investigate the role of MFG-E8 and its receptor integrin αvβ3 in the attachment of trophoblast cells to the endometrial epithelium, in an *in vitro* model. To compare endometrial expression of MFG-E8, integrin αvβ3 and LIF between fertile patients and patients with endometriosis and infertility during the window of implantation.

Methods: In our first assay, by using a well-differentiated endometrial adenocarcinoma cell line (Ishikawa cells) and choriocarcinoma human trophoblast cells (Jar cells), an in vitro model mimicking human implantation was established. To investigate the impact of blocking MFG-E8 and integrin αvβ3, the cell lines were pretreated with antibodies against those proteins at different concentrations before the attachment assay. Moreover, to compare endometrial expression of MFG-E8, integrin αvβ3 and LIF, endometrial biopsies were performed during the window of implantation (LH+7 to LH+10) with the Pipelle catheter. The samples were submitted immunochemistry, and analyzed with HSCORE.

Results: Pretreatment of Ishikawa cells with anti-MFG-E8 antibody caused a dose-dependent and significant inhibition of attachment is our *in vitro* assay. On the other hand, pretreatment of Jar spheroids did not result in a significant effect on the attachment rate. Pretreatment of Ishikawa cells as well as Jar spheroids with anti-integrin avb3 antibodies resulted in a dose-dependent, significant inhibition of attachment. The immunochemistry analysis of the endometrial biopsies performed during the window of implantation showed increased MFG-E8 expression in patients with endometriosis and infertility. Moreover, there was lower LIF expression in the study group.

Conclusion: This study showed that blocking MFG-E8 and its receptor integrin αvβ3 in

Ishikawa cells diminishes Jar spheroid attachment in an *in vitro* model. Moreover, blocking integrin αvβ3 in the trophoblastic cells also diminished their attachment to the Ishikawa monolayer. Nevertheless, when we studied the endometrium of patients with endometriosis and infertility, we saw an increased expression of MFG-E8 and decreased expression of LIF during the window of implantation.

Key words: MFG-E8, integrin αvβ3, LIF, implantation, endometrium, *in vitro* model, endometriosis

LISTA DE ABREVIATURAS

- ASRM – *american society for reproductive medicine*
- CD14 – *cluster of differentiation*
- EGF – *epidermal growth fator*
- FIV – fertilização *in vitro*
- HB-EGF – *heparin-binding epidermal growth fator*
- HOXA10 – *homeobox* A10
- ICSI – injeção intracitoplasmática do espermatozoide
- IL – interleucina
- JI – janela de implantação
- LH – hormônio luteinizante
- LIF – *leukemia inhibitory fator*
- LPS – lipopolissacarideo
- MFG-E8 – *milk fat globule-epidermal growth factor* 8
- MUC – mucina
- NK – *natural killer*
- PCAF – p300/CREB-binding protein-associated fator
- PGD – diagnóstico genético pré-implantacional
- PGS – *screening* genético pré-implantacional

- RIF – falha de implantação recorrente
- SOP – síndrome dos ovários policísticos
- TLR4 – *toll like receptor* 4
- TNF – fator de necrose tumoral
- VLP – videolaparoscopia

ÍNDICE

INTRODUÇÃO

A implantação do embrião humano na cavidade uterina tem sido uma das etapas mas difíceis de se atingir com sucesso dentro da reprodução assistida. Sabemos que para que isso ocorra é necessário um endométrio receptivo, um embrião funcional e de boa qualidade e um diálogo sincronizado entre o tecido materno e trofoblástico [1]. No entanto, vários fatores desconhecidos devem exercer um papel importante nesse processo [2, 3]. Além do mais, dentro da área da reprodução assistida, a taxa de gestação clínica por transferência de embriões em ciclos a fresco gira ao redor de apenas 31% [4, 5], sendo um dos fatores que contribui fortemente para esta baixa taxa, a falha no processo implantacional.

A proteína MFG-E8 (milk fat globule-epidermal growth factor 8) é uma molécula de adesão celular de 46 Kd que possui uma sequência ácida arginina-glicina-aspartato altamente conservada, através da qual ela é reconhecida pelas integrinas αvβ3/αvβ5 [6-8]. Recentemente, foi demonstrado aumento da expressão de MFG-E8 durante a janela de implantação no endométrio humano, quando se compara o perfil de expressão gênica da fase lútea média com a inicial [9]. Após este estudo, Franchi et al demonstrou que MFG-E8 é principalmente expressa na camada de células epiteliais do endométrio e durante a janela de implantação em mulheres hígidas ovulatórias [7]. O mesmo estudo demonstrou que seu ligante, a integrina αvβ3 possui sua maior expressão no endométrio na mesma fase, como já havia sido descrito anteriormente [10]. A participação da integrina αvβ3 no processo implantacional também vem sendo investigado e já foi demonstrado que sua a expressão inadequada está associada à infertilidade inexplicada [11, 12]. Alguns estudos *in vitro* reforçam o papel importante desta integrina na receptividade endometrial [13, 14].

Outro marcador de receptividade endometrial que vem sendo estudado é o LIF (leukemia inhibitory factor). O LIF é expresso predominantemente no epitélio glandular e luminal do endométrio humano durante a janela de implantação [15, 16]. A expressão alterada dessa glicoproteína já foi relacionada com infertilidade de causa idiopática [17].

Já foi demonstrado anteriormente que uma das causas de infertilidade em pacientes com endometriose é a diminuição da receptividade do endométrio e, portanto, dificuldade no processo

implantacional [18], o que acreditamos fazer sentido, uma vez que bons embriões parecem ter mais dificuldade de se implantar no endométrio dessas pacientes. No entanto, as causas da diminuição em sua receptividade permanecem pouco conhecidas. Portanto, a caracterização do endométrio dessas pacientes tem sido buscada em diferentes estudos [16, 19-24]. Talvez sejam falhas metodológicas e a própria dificuldade de estudar endometriose (devido a sua heterogeneidade) que mantém controverso este tema. Além disso, endometriose e infertilidade fazem parte da linha de pesquisa do nosso grupo, que já possui diversas publicações sobre o tema [25-30].

Sabe-se que o estudo do processo implantacional *in vivo* é muito limitado, especialmente por motivos éticos, pouca reprodutibilidade e dificuldade de manipulação. Sendo assim, os estudos *in vitro* são mais comumente realizados [13, 31, 32]. Para tal, modelos que mimetizam a implantação, com linhagens celulares endometriais e trofoblásticas, são largamente utilizadas. A linhagem celular Ishikawa, derivada de adenocarcinoma endometrial humano bem diferenciado [33], é considerado um bom modelo para estudo do endométrio fisiológico [34, 35]. Como modelo de tecido trofoblástico existe a linhagem de células Jar, as quais são derivadas de coriocarcinoma humano. Estas células tendem a crescer em esferas multicelulares quando em condições apropriadas, amplamente utilizadas como modelo de blastocisto [1, 36-38]. Apesar de estudos *in vitro* possuírem evidentes limitações, tem sido demonstrado que as células Jar e Ishikawa funcionam como bom modelo para estudo do processo implantacional [34, 39].

Uma vez que o melhor entendimento do processo implantacional é fundamental para o avanço das técnicas de reprodução assistida, o objetivo do nosso estudo é aumentar o conhecimento em relação à receptividade endometrial. Foi realizado um estudo da expressão proteica da MFG-E8, do seu receptor a integrina αvβ3 e de LIF no endométrio de pacientes férteis, e inférteis com endometriose durante a janela de implantação. Além disso, estudamos o papel da proteína MFG-E8 e do seu receptor a integrina αvβ3 no processo implantacional através de um modelo *in vitro*, utilizando as linhagens celulares Ishikawa e Jar.

ESTRATÉGIAS DE BUSCA DE INFORMAÇÃO

Esta revisão da literatura está focada no entendimento do processo implantacional, especialmente em relação aos marcadores endometriais MFG-E8, LIF e integrina αvβ3, e em uma possível associação de endometriose e infertilidade com a inadequada expressão destes. A estratégia de busca envolveu as seguintes bases de dados: SciELO e PubMed. Foram realizadas buscas através dos termos "Endometriosis", "Infertility", Endometrial Receptivity", "Implantation process", "MFG-E8", "LIF", "Integrin αvβ3", "*in vitro* model" e suas combinações.

REVISÃO DA LITERATURA

Processo implantacional e reprodução assistida

A implantação do embrião representa uma das etapas mais críticas do processo reprodutivo humano [5, 40]. Esse processo, tanto fisiológico como dentro da reprodução assistida, requer um embrião e um endométrio de boa qualidade, bem como um diálogo sincronizado entre os mesmos [2, 41]. O papel do endométrio já vem sendo estudado há décadas; um estudo realizado em 1992 demonstrou que transferindo embriões de boa qualidade para uma "barriga de aluguel" aumenta a chance de gravidez quando comparado à transferência de volta para as doadoras, o que ilustra a importância da qualidade do endométrio no sucesso do tratamento [42]. Além disso, uma metanálise recente de três ensaios clínicos (somando 633 ciclos) mostrou que ciclos com embriões congelados podem aumentar as taxas de gestação quando comparados a ciclos a fresco [43]. Outrossim, já está bem estabelecido que embriões de baixa qualidade comprometem significativamente as chances de gravidez [44, 45].

A despeito de o blastocisto possuir a capacidade de se implantar em diferentes tecidos, no endométrio humano, esse fenômeno precisa acontecer em um curto período de tempo, denominado "janela de implantação" (JI), que ocorre entre os dias 20 e 24 de um ciclo menstrual regular [40]. A implantação é dividida em três estágios: aposição, adesão e invasão [46, 47]. Durante a aposição ocorre a perda da zona pelúcida e a orientação correta do blastocisto [2, 46]. Após, o blastocisto entra em contato com a camada epitelial do endométrio, aderindo-se (fase de adesão), e após inicia a invasão da camada estromal (fase de invasão) [2].

Apesar dos avanços que ocorreram na área da reprodução assistida, a taxa de gestação clínica por embrião transferido gira ao redor de apenas 31%, chegando a 41% nos programas de doação de oócitos [4, 5]. A etiologia da falha de implantação neste processo parece complexo e ainda pouco compreendido [44]. Existe alguns fatores que reconhecidamente contribuem para que isso ocorra, como, por exemplo, anomalias genéticas do embrião, anormalidades anatômicas da cavidade uterina, doenças endocrinológicas, trombofilias e fatores imunológicos [48]. Estudos realizados com diagnóstico/screening pré-implantacional (PGD/PGS) mostram que a taxa de embriões com anormalidades genéticas é maior em pacientes com falha de implantação

recorrente submetidas a fertilização in vitro (FIV)/ injeção intracitoplasmática do espermatozoide (ICSI) do que naquelas submetidas ao tratamento por outras razões [49, 50].

Pacientes inférteis com endometriose possuem taxas ainda menores de sucesso na FIV/ICSI, sendo uma das razões para tal, o aumento da frequência de falha na implantação [44, 51]. Além disso, anormalidades da cavidade uterina como miomas, pólipos e malformações mullerianas, além de estarem associadas a aborto recorrente [52], também dificultam a implantação do embrião [44]. Distúrbios endocrinológicos como síndrome dos ovários policísticos (SOP) e defeito de fase lútea são mais frequentes em pacientes com falha de implantação recorrente [48]. Por fim, fatores imunológicos como produção adequada de citocinas, função das células T e NK também parece possuírem um papel importante dentro deste processo [48]. Apesar de ainda controverso, o uso de corticóide no período peri-implantacional parece aumentar as taxas de gravidez [53], o que corrobora a importância dos fatores imunes. Enfim, o processo implantacional precisa ser melhor compreendido e pesquisado para que se possam oferecer opções terapêuticas mais adequadas.

Receptividade do endométrio

Um endométrio adequadamente receptivo durante a JI é essencial para que ocorra a gestação [54]. Hoje em dia, uma definição exata para que o endométrio seja considerado receptivo ainda está longe de ser completa [3]. No entanto, ela certamente envolve a aquisição de receptores ou ligantes de adesão, bem como a perda dos componente inibitórios que podem atuar como barreira a implantação do embrião [55].

Durante muito tempo, o diagnóstico histológico de endométrio “fora de fase” [56] foi considerado uma causa de infertilidade, uma vez que se acreditava que um endométrio “adequado para fase” correspondia a um endométrio receptivo. No entanto, um trabalho de 2004 demonstrou que um endométrio “fora de fase” é mais frequente na população fértil (49%) do que na população infértil (43%) [57], fazendo com que esse critério diagnóstico caísse em desuso.

Sabe-se que a progesterona é essencial para receptividade do endométrio, uma vez que ela torna o endométrio previamente estimulado pelo estrogênio na estrutura secretória necessária

para sobrevivência do embrião [54]. A JI ocupa um período de 4 a 5 dias no ciclo menstrual (dia LH+7 a LH+11) [58], que é o mesmo período em que a progesterona atinge seu pico de concentração sérica [54].

Uma característica interessante da camada epitelial do endométrio durante a JI é o surgimento dos pinópodes, estruturas que se projetam no lúmen uterino acima do nível dos microvilos [59]. Sua expressão se inicia no dia 8 da fase lútea a termina ao redor do dia 10 [60], sendo aparentemente dependente de progesterona [40]. São suas mudanças morfológicas, mais do que sua presença ou ausência, que parecem ser importantes no processo de implantação [60].

Outra estrutura presente na camada epitelial que pode ter sua expressão influenciada pela progesterona é a MUC-1 (mucina-1) [61]. A MUC-1 é a principal mucina encontrada no endométrio, e sua função é a inibição de adesão celular [62]. Diferentemente de outras espécies, no endométrio humano, a expressão de MUC-1 parece aumentar na JI [63], o que é um paradoxo uma vez que sua função é inibitória. Sendo assim, estudos mais recentes com embriões humanos mostraram que o blastocisto deve liberar alguns fatores que fazem com que haja perda local de MUC-1 apenas na área de implantação [61, 64]. Embriões de baixa qualidade podem não produzir adequadamente esses fatores, o que contribui para falha na implantação [55].

Para que haja um adequado processo de implantação e receptividade endometrial, a família das integrinas possui um papel essencial [40]. A integrinas são uma família de receptores glicoproteicos expressos na membrana celular como heterodímeros que contém uma subunidade α e uma β, envolvidas em processo de adesão célula-célula e célula-substrato [10, 65]. A ligação das integrinas com seus ligantes ativa uma transdução de sinal intracelular clássica e dispara diversos eventos celulares [40]. Além do mais, sua ligação divalente cátion-dependente regula diversos processos celulares, como remodelamento ósseo, hemostasia, fertilização e implantação [6, 66, 67]. As integrinas sinalizam o recrutamento de uma rede de proteínas do citoesqueleto e complexos intracelulares, principalmente as quinases [40].

Uma grande variedade de integrinas tem sido descrita no endométrio durante todo o período menstrual, no entanto, algumas mudam o padrão regulatório dependendo da fase [65]. As integrinas que possuem sua expressão aumentada na fase lútea média, tem sido apontadas como marcadoras da JI, as quais são α1β1, α4β1 e αvβ3 [68]. Além disso, as integrinas também

são expressas no tecido trofoblástico durante o período peri-implantacional [69].

O padrão cíclico de expressão das integrinas no endométrio sugere regulação hormonal [40]. Sabe-se que durante a fase proliferativa, os altos níveis de estrogênio inibem a expressão das integrinas, e, por sua vez, a progesterona estimula, pois diminui a quantidade de receptores para o estrogênio e aumenta a produção de fatores parácrinos como EGF (epidermal growth factor) e HB-EGF (heparin-binding EGF) [70].

Integrina αvβ3

A integrante da família das integrinas mais estudada no endométrio humano, devido a sua importância no processo implantacional, é a integrina αvβ3 [71]. Ela foi detectada na superfície da camada epitelial do endométrio durante a JI, local onde se faz o primeiro contato com o blastocisto, tanto em humanos como em camundongos [10, 72]. Como mencionado anteriormente, a progesterona, hormônio fundamental para implantação, aumenta a produção de EGF e HB-EGF, os quais atuam aumentando a expressão da subunidade β3 desta integrina [70]. O próprio embrião também induz o aumento da produção desta mesma subunidade no endométrio [73]. Sabe-se que o fator de transcrição HOXA10 regula diretamente a produção da subunidade β3 [74], e tanto a HOXA10 como a integrina αvβ3 estão diminuídos nas pacientes com hidrossalpinge, as quais apresentam diminuição nas taxas de implantação [75, 76]. Inclusive, após remoção da hidrossalpinge, ocorre aumento dramático da expressão desta integrina no endométrio durante a JI [77].

Diversos estudos *in vitro* tem ajudado a elucidar o papel desta integrina no processo implantacional. Zhang et al demonstraram que o bloqueio da integrina αvβ3 e de Lewis Y (LeY), um oligossacarídeo difucosilado carregado por esta integrina, faz com que ocorra diminuição da adesão de tecido trofoblástico a uma linhagem de células epiteliais, as RL95-2 [13]. Outrossim, quando se silencia integrina αvβ3 em células Ishikawa (linhagem de adenocarcinoma endometrial humano), ocorre diminuição da adesão de blastocistos tanto de ratos, como de camundongos ou humanos, àquelas células [14, 78]. Kaneko et al também demonstraram que a integrina β3 está presente no embrião de ratos durante todo seu desenvolvimento, tendo sua expressão aumentada

na fase de blastocisto [14]. Ademais, foi demonstrado que o blastocisto de ratos submetidos a indução da ovulação tem sua expressão desta integrina diminuída [79].

A expressão endometrial inadequada de integrina αvβ3 já foi associada à infertilidade inexplicada, à RIF (falha de implantação recorrente) e a pacientes submetidas a indução da ovulação [11, 12, 71, 80]. Um recente estudo com modelo animal, mostrou que há alteração desta integrina tanto no endométrio como no blastocisto de camundongos que apresentam SOP no período da JI [81, 82]. A relação desta integrina com endometriose permanece controversa na literatura e será discutida a seguir [19-21].

Milk Fat Globule-Epidermal Growth Factor 8 (MFG-E8)

MFG-E8 é uma glicoproteína que foi primeiramente identificada na membrana dos glóbulos de gordura no leite de camundongo [8]. Em humanos, ela já foi identificada em diversos órgãos, fazendo então parte de diversos processos fisiológicos [83]. MFG-E8 contém dois domínios de fator de crescimento epidermal (EGF) N-terminal e dois domínios discóides C-terminal com homologia aos domínios C1 e C2 encontrados nos fatores de coagulação V e VIII [83]. A segunda repetição EGF possui uma sequência ácida arginina-glicina-aspartato altamente conservada, através da qual ela é reconhecida pelas integrinas αvβ3/αvβ5, enquanto os domínios C-terminal fator V/VIII like permitem sua ligação com as células apoptóticas via fosfatidilserina [6].

A maioria dos estudos realizados com MFG-E8 envolvem o sistema imune, devido ao importante papel que esta proteína exerce dentro desse sistema, promovendo a fagocitose de células apoptóticas pelos macrófagos e células dendríticas [84-86]. Assim, sua disfunção parece estar implicada em processos patológicos como progressão tumoral e lúpus eritematoso sistêmico [85, 87]. Nas área da cancerologia, estudos demonstraram que a proteína MFG-E8 possui importante papel no crescimento e proliferação celular [6]. Já foi demonstrado que patologias que promovem inflamação como a sepse, colite, lesão intestinal e insuficiência renal diminuem a expressão de MFG-E8 em tecidos como baço, pulmões, intestino, fígado e rins [6]. O próprio LPS, que é um potente agente pró-inflamatório, faz com que ocorra diminuição da expressão de

MFG-E8 em macrófagos e no baço de ratos e camundongos [88, 89]. A diminuição dessa proteína nos macrófagos de camundongo causada pelo LPS se dá via TLR4 e CD14 [88]. Além do mais, MFG-E8 promove angiogênese e é um mediador crucial para o efeito pró-angiogênico do fator de crescimento endotelial vascular [90]. Portanto, um melhor entendimento das funções da proteína MFG-E8 dentro do sistema imune tem sido apontada como uma potencial arma para futuros tratamentos de doenças como sepse e alguns tipos de câncer [91].

Apesar de sua função como mediadora do processo fagocítico ser o principal foco de investigação, o papel da proteína MFG-E8 no processo implantacional começou a ser estudado recentemente. Talvez o primeiro sinal que tenha sido encontrado sobre uma possível função dessa proteína na implantação foi sua expressão gênica endometrial aumentada durante a JI [9]. Após esse primeiro achado, Franchi et al demonstraram que a proteína também encontra sua maior expressão durante a JI, quase que exclusivamente no compartimento epitelial do endométrio [7]. Nesse mesmo estudo, foi realizada uma sequencia histológica da fase proliferativa para a fase lútea média, em que se observou uma progressão da imunolocalização da proteína da base da célula para o seu ápice, sugerindo sua secreção luminal [7]. Esses achados sugerem fortemente que a proteína MFG-E8 possui uma função na adesão do embrião no endométrio. Além do mais, um estudo recente mostrou que a produção de MFG-E8 pela linhagem celular Ishikawa é dependente de um citocina marcadamente associada ao processo inflamatório, o TNF-α [92].

Leukemia inhibitory factor (LIF)

LIF (leukemia inhibitory factor) é uma glicoproteína secretada por diversas células e tecidos humanos, possuindo atividade em diferentes processos, como por exemplo na hematopoise, na resposta inflamatória aguda e na supressão de células tronco no embrião [93]. Em 1992, um trabalho demonstrou pela primeira vez em camundongos, que a ausência de LIF no endométrio causa falha na implantação do blastocisto [94]. Logo após, outros estudos sugeriram a existência de um papel desse fator na implantação do blastocisto em humanos, através de sua expressão gênica e proteica aumentada na JI [23, 24]. Lalitkumar et al demonstraram falha de implantação de embrião humano *in vitro*, quando há bloqueio de LIF no tecido endometrial [95].

LIF é expresso predominantemente no epitélio glandular e luminal do endométrio humano durante sua fase secretória média, ou seja, durante a janela de implantação [15, 16]. Em 2007, outro trabalho demonstrou que a concentração de LIF está diminuída em pacientes inférteis, especialmente naquelas com causa idiopática, e que o nível de sua expressão está positivamente correlacionado com as chances de gravidez natural [17]. Além do mais, LIF também está diminuída no endométrio de úteros que apresentam anormalidade anatômica, podendo ser mais um fator que contribui com pior desfecho reprodutivo nessas pacientes [96]. A expressão diminuída desta proteína também está presente no endométrio de pacientes submetidas a indução da ovulação [80]. Um estudo recente demostrou uma série de alterações gênicas causada pela LIF no endométrio de camundongos (como de fatores de transcrição *Sox, Kfl, Hes e Hey*), responsáveis por uma adequada transformação endometrial, essencial para que ocorra implantação adequada [97].

Endometriose/Infertilidade e sua relação com Integrina αvβ3, MFG-E8 e LIF

A associação de endometriose e infertilidade tem sido amplamente estudada há muito tempo [98, 99]. Esse interesse se dá principalmente devido à alta prevalência de infertilidade (48.5 milhões de casais ao redor do mundo em 2010) [100], à alta taxa de endometriose que as pacientes inférteis apresentam, ao redor de 25-50% [101] e ao alto custo psicológico [102] e financeiro do seu tratamento [103].

O mecanismo através do qual endometriose causa infertilidade ainda não é completamente conhecido, mas vários aspectos dessa associação já foram estudados. Nosso grupo de pesquisa já demonstrou que essa doença está associada com diminuição da reserva ovariana, hiperprolactinemia e insuficiência lútea, com subseqüente alteração no padrão de secreção de progesterona na segunda fase do ciclo menstrual [28-30]. Além disso, pacientes com endometriose possuem distorção da anatomia pélvica, especialmente em estágios mais avançados da doença [104]. Alterações no fluido peritoneal como aumento de volume, maior número de macrófagos ativados, maiores concentrações de Interleucina-1 (IL-1), prostaglandinas, Fator de Necrose Tumoral (TNF) e proteases causam alteração em oócito, espermatozoide, embrião e função tubária [98, 105]. Alterações imunológicas no fluido folicular como aumento na

concentração de linfócitos B, macrófagos, células NK e interleucinas inflamatórias, também foram estudadas [106-109]. Além do mais, essas pacientes apresentam diminuição da receptividade do endométrio e, portanto, dificuldade no processo implantacional [18, 110].

Entre os marcadores de receptividade do endométrio está a integrina αvβ3, e, se sua expressão está ou não adequada em pacientes com endometriose, é um assunto que permanece controverso na literatura. Enquanto Lessey et al demonstraram diminuição significativa da sua expressão em pacientes com estágio I/II de endometriose [19], outros estudos falharam em encontrar diferença estatisticamente significativa [20, 21]. Uma das possíveis etiologias para a expressão alterada de integrina αvβ3, seria a produção de HOXA10 diminuída que essas pacientes apresentam [111, 112]. Lu et al demonstraram que essas pacientes, além de uma produção diminuída de HOXA10, também apresentam hipermetilação da mesma, o que atrapalha sua função. Já Zhu et al mostraram diminuição da integrina αvβ3 e da HOXA10, além de sua acetilação inadequada devido a super-expressão de PCAF (p300/CREB-binding protein-associated factor) [113].

A relação entre endometriose e ligantes desta integrina, como por exemplo a osteopoietina, já foi estudada [21, 22]. No entanto, nenhum estudo sobre a expressão endometrial do seu ligante MFG-E8 nessas pacientes foi publicado até o momento.

Outro importante marcador de receptividade endometrial estudado em pacientes com endometriose é a proteína LIF. Em 2006, Dimitriadis et al mostraram, através de imunohistoquímica, que a proteína esta diminuída em pacientes com endometriose grau I/II [114]. Já em 2007, outro estudo não mostrou diferença estatisticamente significativa na concentração de LIF no "lavado da cavidade uterina" em mulheres com endometriose, apenas em pacientes com infertilidade idiopática [17]. Por fim, recentemente, Alizadeh et al demonstraram, tanto em nível gênico, como em nível proteico, uma diminuição de LIF em paciente com endometriose grau I/II [115].

Modelo de implantação *in vitro*

Uma das causas que podem ser atribuídas ao nosso limitado conhecimento sobre os

processos que regulam a receptividade endometrial é a falta de um bom modelo de estudo [35]. Apesar de modelos *in vitro* não possuírem a capacidade de mimetizar perfeitamente o ambiente intrauterino, eles podem ser utilizados para estudos de algumas de suas interações específicas [61]. Portanto, linhagens celulares como as Ishikawa, utilizadas como modelo de epitélio endometrial, e Jar, utilizadas como modelo de tecido trofoblástico, tem sido amplamente utilizados [34, 39].

A linhagem celular Ishikawa é derivada de adenocarcinoma endometrial humano bem diferenciado [33, 116]. Essas células apresentam as mesmas enzimas e proteínas estruturais [34], bem como receptores hormonais encontrados no endométrio normal [117, 118]. Além disso, essas células apresentam a polarização apical-basal que influencia na resposta do tecido trofoblástico e, portanto, interfere no processo de implantação [39]. Além do mais, essas células apresentam adesividade apical com as células Jar [34, 39, 119]. Existem diversas linhagens celulares que pode ser usadas como modelo trofoblástico, entre elas estão as células Jar, as quais são derivadas de coriocarcinoma humano [34]. Essas células tendem a crescer em esferas multicelulares quando em condições apropriadas e são amplamente utilizadas como modelo de blastocisto [1, 36-38]. Enfim, diversos estudos mostram que as células Jar e Ishikawa funcionam como bom modelo para estudo do processo implantacional [34, 39, 119].

JUSTIFICATIVA

A prevalência de infertilidade ao redor do mundo no ano de 2010 foi de aproximadamente 48.5 milhões, e vem aumentando [100]. Muitos destes casais irão recorrer a técnicas de reprodução assistida, no entanto, a taxa de implantação por embrião transferido é de, no máximo, 20-60% [120]. Uma das razões para as baixas taxas de implantação é a falta de receptividade endometrial [120]. Mulheres com endometriose que apresentam infertilidade também parecem possuir um endométrio menos receptivo.

Portanto, uma melhor caracterização do endométrio no processo implantacional, especialmente em mulheres com endometriose, é fundamental para que se possa desenvolver um tratamento de melhor qualidade. Procuramos buscar alguns marcadores de receptividade já estudados, mas ainda controversos na literatura, como o LIF e a integrina, e um novo marcador, ainda não estudado anteriormente nessas pacientes, mas cuja patogênese teria plausabilidade biológica, que é o MFG-E8. Buscamos uma linha de estudo envolvendo pesquisa básica e clínica, para podermos demonstrar com mais consistência o papel dos marcadores no processo implantacional.

OBJETIVOS

Avaliar o papel de MFG-E8 e do seu receptor integrina αvβ3 em um modelo de implantação in vitro com uma linhagem celular trofoblástica e outra de epitélio endometrial.

Comparar a expressão de MFG-E8, de integrina αvβ3 e de LIF no endométrio de pacientes férteis e inférteis com endometriose durante a janela de implantação.

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ARTIGO 1

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Running title: MFG-E8 and integrin αvβ3 implantation roles

Role for the endometrial epithelial endometrial protein MFG-E8 and its receptor integrin αvβ3 in human implantation: results of an in vitro trophoblast attachment study using established human cell lines

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Capsule: MFG-E8 and its receptor integrin αvβ3 are involved in the attachment of trophoblasts to the endometrial epithelium

Abstract

Objective: To investigate the role of MFG-E8 and its receptor integrin αvβ3 in the attachment of trophoblast cells to the endometrial epithelium.

Design: Experimental *in vitro* study.

Setting: Academic center.

Patient(s): None

Interventions: By using a well-differentiated endometrial adenocarcinoma cell line (Ishikawa cells) and choriocarcinoma human trophoblast cells (Jar cells), an *in vitro* assay mimicking human implantation was established. To investigate the impact of blocking MFG-E8 and integrin αvβ3, we pretreated the cell lines with antibodies against those proteins at different concentrations prior to the attachment assay.

Main Outcome Measure(s): Attachment rate of Jar spheroids to the epithelial cell monolayer.

Results: Pretreatment of Ishikawa cells with anti-MFG-E8 antibody caused a dose-dependent and significant inhibition of attachment. On the other hand, pretreatment of Jar spheroids did not result in a significant effect on the attachment rate. Pretreatment of Ishikawa cells as well as Jar spheroids with anti-integrin αvβ3 antibodies resulted in a dose-dependent, significant inhibition of attachment.

Conclusions: This study showed that blocking MFG-E8 and its receptor integrin αvβ3 in Ishikawa cells diminishes Jar spheroid attachment. Moreover, blocking integrin αvβ3 in the trophoblastic cells also diminished their attachment to the Ishikawa monolayer.

Key words: Attachment, integrin αvβ3, implantation, human endometrium, Jar spheroids, MFG-E8

Introduction

It is well known that implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage of development, and a synchronized dialogue between them [1]. Nevertheless there are many unknown factors that play an important role in this process [2, 3]. Because human implantation studies *in vivo* are very limited (mainly owing to ethical reasons), have poor reproducibility, and lack capacity for manipulation/intervention, *in vitro* studies are the most common ones performed [4-6].

Integrins are a diverse family of glycoprotein receptors expressed on the cell membrane as heterodimeric α and β subunits involved in both cell-cell and cell-substratum adhesion [7, 8]. One particular integrin, the αvβ3, has been studied in many physiological/pathological processes, and it has been suggested to be involved implantation across many mammalian species, including mouse, rat, pig, rabbit and human [8-12]. In women, an adequate integrin αvβ3 receptor expression seems to be necessary for a receptive endometrium [7, 13-15]. Integrin's hallmark is the ability of individual family members to recognize multiple extracellular matrix (ECM) ligands, such as bone matrix proteins, collagens, fibronectins, fibrinogen, laminins, thrombospondins, vitronectin, and von Willebrand factor [16, 17]. Despite the structural diversity of their ligands, integrins recognize a core ligand motif centered on an acidic residue [18].

Recently, our group reported for the first time on a novel endometrial protein, milk fat globule-epidermal growth factor (MFG-E8). Initially it was found that MFG-E8 was up-regulated in the human endometrium during the window of implantation in a microarray study comparing the gene expression profile during the mid vs. early-luteal phase [19]. We showed that MFG-E8 protein is mostly expressed in the endometrial epithelial cells and during the window of implantation in healthy ovulatory women, both in luminal and glandular epithelial cells, and with intense staining at both apical and basal cellular compartments [20]. The MFG-E8 receptor, integrin αvβ3, was present in the epithelial and stromal compartments, with cycle-dependent and coincident peak expressions at mid-secretory phase, as previously described [20, 21].

The secreted glycoprotein, MFG-E8 (also known as lactadherin [SED1], and breast antigen 46 [BA46]) was initially described as a component of the milk fat globule membrane, which is mainly secreted in micro-vesicles [22]. It has also been suggested as a tumor marker in

breast carcinomas [23, 24]. Human MFG-E8 is a 46-kDa glycoprotein peripherally associated with the cell membrane having two N-terminal epidermal growth factors-like repeats, one of which includes an arginine-glycine-aspartic acid motif that serves as the ligand to its αvβ3 integrin receptor, facilitating cell adhesion and signal transduction. The two C-terminal discoidin C1-C2 domains are homologous to coagulation factors V and VIII and are responsible for binding to cell membrane phospholipids or cell surface carbohydrate moieties as well as to the extracellular matrix [25, 26].

MFG-E8 has a variety of functions in many extrauterine tissues related to apoptosis, cell adhesion and remodeling, neovascularization, and immunomodulation. In particular, it promotes phagocytosis of apoptotic cells by αvβ3/β5 integrin-expressing phagocytes [27], as well as endothelial and epithelial cells [28, 29]. In systemic endothelial cells, MFG-E8 binding to αvβ3/β5 integrins promotes vascular endothelial growth factor-induced survival and proliferation, leading to angiogenesis [30]. It stimulates cell/cell and cell/extracellular matrix adhesion during sperm-oocyte interaction [25, 31]**.** In intestinal, mammary gland, and epididyimal epithelium, MFG-E8 regulates migration of epithelial cells [25, 32, 33]. It also protects against symptomatic rotavirus infection [34].

On the basis of recent work from our laboratories, we postulated MFG-E8 as a novel glycoprotein with key roles in the regulation of endometrial function [20]. We showed that MFG-E8 protein was up regulated by prolactin in primary endometrial epithelial cell cultures. This finding supports a modulatory role for prolactin as a stromal-epithelial paracrine factor controlling MFG-E8 production. We also demonstrated that in epithelial Ishikawa cells, MFG-E8 protein secretion is up-regulated by human chorionic hCG and secreted via microparticles [35]. We also confirmed that MFG-E8 has proapoptotic activity in human endometrial stromal cells, suggesting that this molecule acts as a paracrine factor [36]. Furthermore, we reported that MFG-E8 protein is highly expressed in human chorionic villi at all trimesters of gestation (in both cytotrophoblasts and syncytiotrophoblasts) and in murine implantation sites [37]. Finally, we presented new evidence that recombinant MFG-E8 modulates endometrial endothelial cell proliferation and adhesion under *in vitro* conditions [38].

In the present experiments we investigated the role of MFG-E8 and integrin αvβ3 in the attachment of trophoblast cells to the endometrial epithelium, to further define its functional roles

during the early implantation events. We used an *in vitro* model with Jar spheroids and Ishikawa cells. The Ishikawa cell line is a human, well-differentiated endometrial adenocarcinoma cell line recognized as a well-characterized model for studying endometrial function as a surrogate for epithelial endometrial cells [39-41]. Jar cells are derived from a human choriocarcinoma, tend to grow as multicellular spheroids when cultured under appropriate conditions, and largely used in blastocyst/trophoblast attachment studies [1, 42-45]. Ishikawa cells/Jar spheroids interactions have been demonstrated to represent an adequate model for studying early implantation 3events [1, 40, 41].

Methods

Cell lines and culture conditions

Current work in our laboratories using endometrial tissue and cells has been approved by the Eastern Virginia Medical School institutional review board (#04-10-FB-0279 and #09-07-EX-0153, active projects). In this particular study consent was not required because the use of established cells lines is considered exempt. The human endometrial adenocarcinoma cell line Ishikawa was generously provided by C. Lockwood, M.D. from Yale University. Cells used in these experiments were from passage 10 and were characterized by immunostaining as cytokeratin-18^+ and vimentin^- cells, and were also estrogen receptor α^+ and β^+ as previously published by us (data not shown) [4, 35]. Ishikawa cells were cultured with Dulbecco´s modified essential medium/F12 containing 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and supplemented with 5% fetal bovine serum (Atlanta Biological) in 75-mL flasks at 37°C in a humid atmosphere with 5% CO_2. The cells were then plated in four- well plates (Thermo Fisher Scientific) until reaching 100% confluence and used in subsequent experiments.

The human choriocarcinoma Jar cell line was purchased from ATCC. Cells used in these experiments were from passage 6 and were characterized by immunostaining as cytokeratin-7^+ and hCG^+ as previously published by us (data not shown) [4, 46]. The Jar cells were cultured in RPMI 1640 (Gibco) with 5% fetal bovine serum (Atlanta Biological), and 100 U /ml penicillin and 100 µg/ml of streptomycin (Gibco). After reaching 80% confluence, Jar cells were detached

with 0.05% trypsin (Gibco). A single-cell suspension was transferred into an agarose-coated petri dish, and cultured for 2 days to form spheroids. All cultures were conducted in an incubator with 5% CO_2 at 37°C. The diameter of Jar spheroids as measured by inverted microscopy was between 150 and 200 μm [4, 46].

Immunocytochemistry

The Dako EnVision+ System-HRP kit (DAB, Dako) was used to perform immunocytochemistry. Ishikawa cells were grown on glass coverslips and fixed with methanol at -20°C for 10 minutes. After washing with phosphate buffered saline (PBS), endogenous peroxidase activity was removed with 3% hydrogen peroxide for 10 minutes. Nonspecific binding sites were blocked with 2% normal goat serum for 30 minutes at room temperature. Cells were incubated overnight at 4°C with the following mouse monoclonal primary antibodies prepared in 1% normal goat serum: anti-MFG-E8 (Abcam, catalog no. ab17787) at a 1:1000 dilution, and anti- αvβ3 integrin (Abcam, catalog no. ab78289) at a 1:10 dilution. The cells were then incubated with a goat anti-mouse secondary antibody labeled with horseradish peroxidase (Dako) for 30 minutes, then with 3,3-diaminobenzidine chromogen solution and substrate buffer for 5-10 minutes at room temperature. Slides were counter stained with Mayer's hematoxylin (Sigma) then dehydrated with ethanol, cleared in xylene, and mounted with mounting media. Negative controls included sections that were treated with a non-immune IgG control antibody (normal mouse ascites, clone NS-1, Sigma) at similar concentration as primary antibodies. Representative images were photographed with an Olympus microscope using an Olympus Q-color 3 camera [20].

Immunofluorescence Detection of MFG-E8 and Integrin αvβ3

Cells were cultured on glass coverslips and fixed with cold methanol, rinsed in phosphate-buffered saline, and blocked with normal goat serum at room temperature. Antibodies used for immunofluorescence were primary anti-MFG-E8 (1:1,000) and anti-integrin αvβ3 (1:50) antibodies as shown above, with a fluorescein isothiocyanate (FITC)-conjugated secondary goat anti-mouse antibody (Santa Cruz Biotechnology). Coverslips were mounted onto glass slides using mounting medium and were visualized using a fluorescence microscope, Olympus BX50, equipped with a DP-70 Olympus camera. Staining with 4′,6-diamidino-2-phenylindole (DAPI)

was performed to visualize nucleus of all cells (VECTASHIELD mounting medium with DAPI, Vector Laboratories).

In addition to standard cell fixation, we also performed staining of live, nonfixed cells following techniques described previously [47]. Jar cells were placed and cultured in eightwell chamber slides until 70%-80% confluence, then were incubated with primary antibodies diluted in complete growth medium in the incubator for 2 hours, followed by washing three times. An FITC-conjugated secondary goat anti-mouse antibody was incubated with cells for 2 hours at room temperature. A nonimmune IgG control antibody (normal mouse ascites; clone NS-1, Sigma) was used as negative control. Mounting and analysis were the same as above.

Protein Extraction and Immunoblotting

For total lysate protein extraction, Ishikawa and Jar cells grown in T-75 flasks were harvested in radio immunoprecipitation assay lysis buffer supplemented with Halt protease inhibitor cocktail (Pierce Thermo Scientific). Samples were sonicated, followed by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C. After centrifugation, supernatants were collected for total protein quantification by bicinchoninic acid protein assay using bovine serum albumin as the standard (Sigma-Aldrich). The protein extracts were loaded and were separated by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gel, followed by electrotransfer onto a polyvinylidene fluoride membrane. Immunoblotting was performed using standard techniques as previously reported by us (20, 35–38). The antibody used was anti-MFG-E8 (Abcam). Protein bands were detected by exposure to a chemiluminescent substrate.

Jar spheroids- Ishikawa cells attachment assay

The coculture system was set up into wells in four-well plates (Nunc). Ishikawa cells were plated as a monolayer; in parallel 20 Jar spheroids were placed in a Petri dish and carefully transferred into each well with a pipette tip and visualized under the microscope. Depending on the experiment, either the Ishikawa cells or the Jar spheroids were subjected to antibody pretreatment as presented below, and results compared with untreated controls. After 30 minutes of co-culture of Jar spheroids and Ishikawa cell monolayer, the 4-well plate was transferred into a bucket of a centrifuge (the top facing the bottom of the swinging bucket), and then centrifuged at

1,000 rpm for 5 min to remove unbound and any loosely attached spheroids. The attached Jar spheroids were counted under regular optic microscopy (x 40) [4, 46]. The number of seeded spheroids was constant (n = 20) per tested antibody dose (and control). There were 10 replicates per investigated condition (antibody dose and control) that were performed on three different experimental days. Additional experiments were performed in a similar fashion using a negative control with pretreatment with a nonimmune IgG control antibody (normal mouse ascites; clone NS-1 Sigma) at equal concentration as primary antibodies and compared with nontreated (non-IgG) conditions.

Treatment conditions

First, Ishikawa cells in the 4-well plates were pretreated with the anti-MFG-E8 antibodies at concentrations of 1 μg/mL and 2 μg/mL, or with the anti-integrin αvβ3 antibodies at concentrations of 1 μg/mL, 10 μg/mL and 50 μg/mL, for 2 hours, after which Jar spheroids were seeded on top of the monolayer for the attachment assay. When Ishikawa cells were treated, no treatment was added to Jar spheroids for 30 minutes.

Second, Jar spheroids were pretreated with either anti-MFG-E8 antibodies at concentrations of 1 μg/mL and 2 μg/mL or with anti-integrin αvβ3 antibodies at a concentration of 50 μg/mL, for 15 min, after which Jar spheroids were seeded on top of the (untreated) Ishikawa cell monolayer, for the attachment assay.

The anti-MFG-E8 and anti- integrin αvβ3 antibody doses used for treatment were chosen based on an in vitro human endometrial endothelial cell adhesion and proliferation study previously performed, where these concentrations showed stimulatory effects [37].

Statistical analysis

Ten replicates were run in three experiments, and 20 spheroids were seeded per replicate, for a total of 200 spheroids tested per investigated dose and for control. Overall effects of antibody preincubation on attachment were analyzed by analysis of variance, followed by a post hoc test (Tukey's test as per equal sample sizes per group) as appropriate. A P value of $<.05$ was considered statistically significant.

Results

Immunohistochemical studies confirmed the presence of MFGE8 and integrin αvβ3 in Ishikawa and also in Jar cells, as depicted in Figure 1. Figure 2 shows immunofluorescence images of Ishikawa and Jar cells for integrin αvβ3 and MFG-E8, depicting typically predominant membrane locations.

To address specificity of antibodies, two further experiments were performed. Figure 3A and B show immunofluorescence results of live, nonfixed Jar cells, confirming specific binding and showing similar localization of integrin αvβ3 and MFG-E8 on these cells as that observed in fixed slides. Figure 3C shows a representative photograph of a Jar spheroid attached to the Ishikawa cell monolayer during the in vitro attachment assay. In addition, Figure 3D presents results of immunoblotting for MFG-E8 in Ishikawa and Jar cells, confirming the expression of the expected 46-kDa protein in both cell types.

Figure 4 presents results of the attachment assays. For Ishikawa cells (upper panel), treatment with an anti-MFG-E8 antibody at doses of 1 μg/mL and 2 μg/mL was performed before the attachment assay. A dose-dependent inhibition of attachment was evidenced (overall effect, P<.0003), with an approximate decrease of Jar spheroids attachment rate by 49% for every one-unit (1 μg) increase in antibody dosage. Treatment with the highest antibody dose (2 μg) was significantly lower than controls (P<.0001), with a reduction of attachment by 76% (Fig. 4A). Treatment with anti-integrin αvβ3 antibodies at doses of 1 μg /mL, 10 μg /mL, and 50 μg /mL vs. controls was carried out before the attachment assay. A dose-dependent inhibition of attachment rate was observed (overall effect, P<.0001), and the attachment rate decreased by approximately 2% per every one-unit (1 mg) increase in antibody dosage. The treatment with 4 μg was significantly lower than controls (P<.0001), with a reduction of attachment by 81% (Fig. 4B).

For Jar spheroids (Fig. 4, middle panel), pretreatment of Jar spheroids with anti-MFG-E8 antibody did not result in a significant effect on attachment rate (Fig. 4C). Conversely, pretreatment of Jar spheroids with anti-integrin αvβ3 antibodies showed a dose-dependent inhibition of attachment (overall effect, P<.001), and treatment with the highest antibody dose (2 mg) was significantly lower than controls (P<.01), with a reduction of attachment by 44% (Fig. 4D).

Pretreatment with IgG antibody (negative control) showed no difference of attachment compared with control, untreated conditions (P>.7) in either Ishikawa (Fig. 4E) or Jar (Fig. 4F) cells.

Discussion

Previous studies have shown that integrin αvβ3 and MFG-E8 are expressed in primary human epithelial cells, more intensively during the window of implantation [7, 19, 20]. Our study confirmed that both of them are also expressed in Ishikawa cells [35, 48]. Furthermore, we demonstrated that both proteins are present in trophoblast Jar cells by immunochemistry and immunofluorescence. Integrin αvβ3 has been previously found to be expressed in mouse and human embryos [49, 50], and MFG-E8 has been shown to be expressed in human placental trophoblasts in all three trimesters of pregnancy [36]. Here we show for the first time that Jar cells express MFG-E8. After these characterizations we were able to proceed with our experiments to test our hypothesis that blocking MFG-E8 and integrin αvβ3 by specific antibodies might impair the attachment of Jar spheroids (mimicking the trophoblast/embryo) to the Ishikawa cell monolayer (simulating the endometrial epithelium).

Integrins divalent-cation-dependent binding to their ligands regulates many cellular processes, such as remodeling of bone, homeostasis, fertilization and implantation [15, 18]. Integrin αvβ3 and its ligands' roles in implantation have been subject to extensive investigation in the last few years. It was shown that blocking integrin αvβ3 and Lewis Y, a difucosylated oligosaccharide carried by this integrin, in epithelial RL95-2 cells diminishes Jar spheroids attachment [5]. Another study performed in Ishikawa cells knocked-down integrin αvβ3 and demonstrated diminished attachment of rat blastocysts to those cells [51] In agreement with these results, we found diminished attachment of Jar's spheroids to Ishikawa cells when this integrin was blocked. Additionally, we also showed that blocking integrin αvβ3 present in the trophoblast cells decreases attachment, an effect not reported before.

MFG-E8 is a glycoprotein that contains two epidermal growth factor domains, a proline/threonine-rich domain, and two factor-VIII-homologous domains [25, 26]. It has an

essential role in an adequate functioning immune system, promoting the engulfment of apoptotic cells by macrophages and dendritic cells [52]. MFG-E8 dysfunction seems to be implicated in some pathological processes, such as tumor progression and systemic lupus erythematosus [53, 54]. Furthermore MFG-E8 promotes angiogenesis and is a crucial mediator of vascular endothelial growth factor proangiogenic effect, and its therapeutic potential has been pointed out for future disease treatment, such as sepsis and certain types of cancers [55-57].

Although its function in mediating phagocytic engulfment seems to be the most investigated one, MFG-E8's role in the implantation process has also received some attention recently. It has been shown that there is an increase in its endometrial expression during the window of implantation in human [19, 20]. Here, we demonstrated that blocking MFG-E8 in Ishikawa cells significantly diminishes Jar spheroid attachment, although blocking the protein in the Jar spheroids did not have the same effect. To the best of our knowledge this is the first evidence of MFG-E8 adhesion properties in a trophoblast/endometrial epithelial cell model that simulates human implantation.

In conclusion, results demonstrated that independent blockage of MFG-E8 and its receptor integrin αvβ3 in Ishikawa cells significantly diminished Jar spheroid attachment. Moreover, we showed that blockage of integrin αvβ3 in the trophblastic tissue (Jar cells) also diminished their attachment to the Ishikawa monolayer. Further characterization of MFG-E8 and integrin αvβ3 intracellular pathways involved in this process is required for a better understanding of the roles of these proteins in normal and pathological human implantation.

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Legends to figures

Figure 1. Immunohistochemistry of Ishikawa and Jar cells (original magnification, x200). (A) Ishikawa cells stained with anti-MFG-E8 antibodies. (B) Ishikawa cells stained with anti-integrin αvβ3 antibodies. (C) Jar cells stained with anti-MFG-E8 antibodies (D) Jar cells stained with anti-integrin αvβ3 antibodies. Inserts: NC = respective negative controls.

Figure 2. Immunofluorescence of Ishikawa and Jar cells (FITC, green, DAPI-counterstained nuclei, blue, merged, original magnification, x 630). (A, B) Ishikawa cells stained with anti- integrin αvβ3 (A) and with anti-MFG-E8 (B). (C, D) Jar cells stained with anti-integrin αvβ3 antibody (C) and with anti-MFG-E8 antibody (D). Note predominantly membrane immunolocation of staining.

Figure 3. (A, B) Immunofluorescent images of live, nonfixed Jar cells (FITC, green; DAPI-counterstained nuclei, blue; merged, original magnification, x400). (A) Jar cells stained with anti-integrin αvβ3. (B) Jar cells stained with anti-MFG-E8. Inset: NC = negative control. (C) Multicellular Jar spheroid attached to the epithelial monolayer of Ishikawa cells in the in vitro attachment assay (inverted microscopy, original magnification, x200). (D) Immunoblotting of MFG-E8 in Ishikawa cells and Jar cells. MW = molecular weight. Note presence of single band at the expected 46-kDa location for both cell types.

Figure 4. Results of the attachment assay after antibody treatment, expressed as number of attached spheroids (mean ± SD). (A) For pretreatment of Ishikawa cells with anti-MFG-E8 antibodies, a dose-dependent inhibition of attachment was evident (overall effect, $P<.0003$). *$P<.0001$ vs. control. (B) For pretreatment of Ishikawa cells with anti-integrin αvβ3 antibodies, a dose-dependent inhibition of attachment rate was evident (overall effect, $P<.0001$). *$P<.0001$ vs. control. (C) Pretreatment of Jar spheroids with anti-MFG-E8 antibodies did not result in a significant effect. (D) On the other hand, pretreatment of Jar spheroids with anti-integrin αvβ3 antibodies resulted in a dose-dependent inhibition of attachment (overall effect, $P<.001$). *$P<.01$ vs. control. (E, F) No impact of pretreatment of Ishikawa or Jar cells on attachment, respectively, with an irrelevant IgG antibody vs. control.

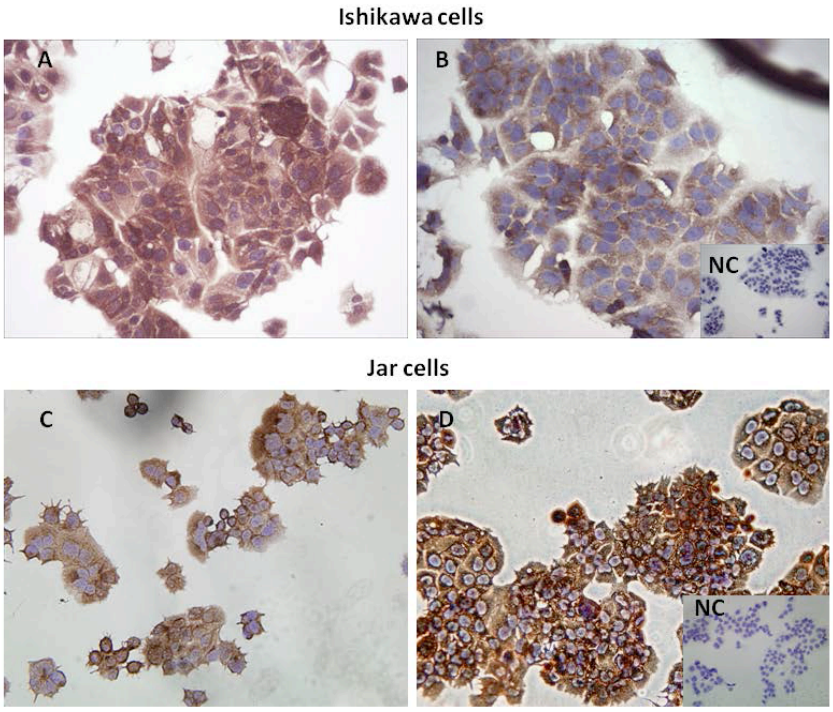

Figure 1

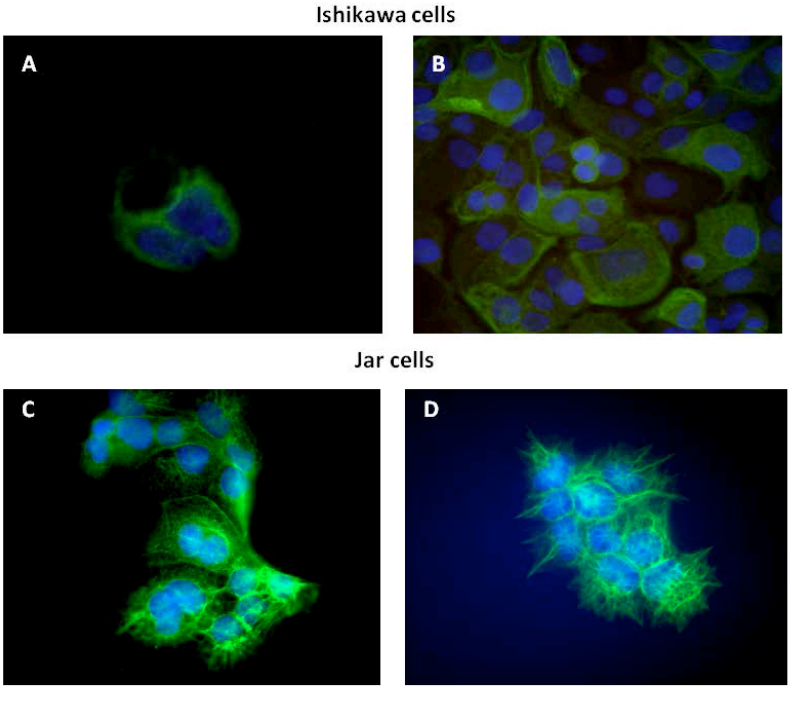

Figure 2

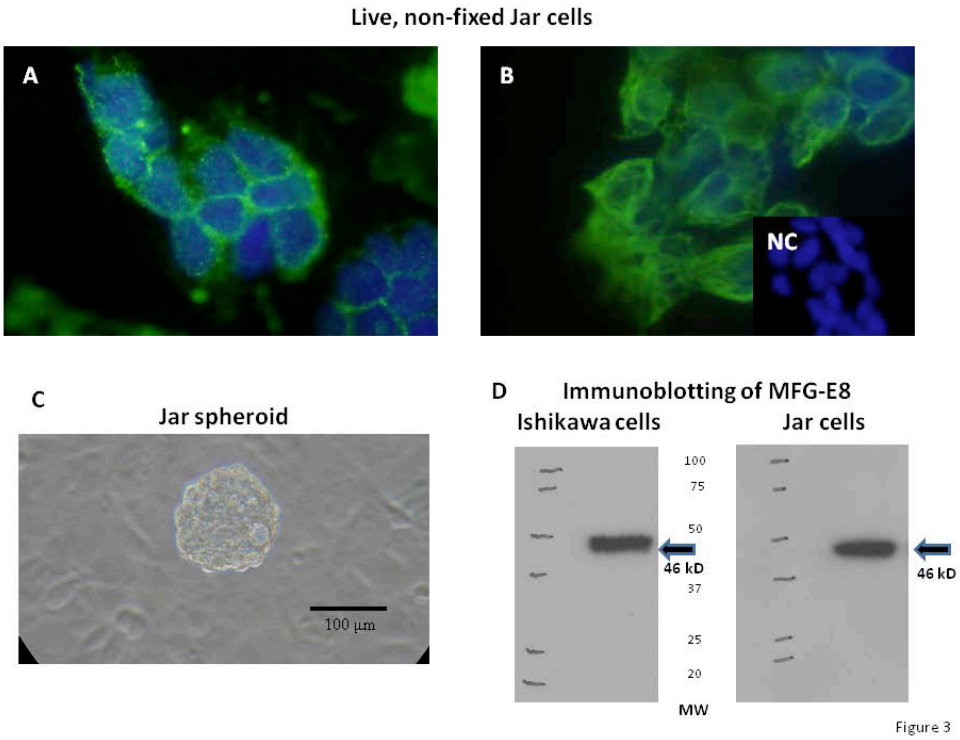

Figure 3

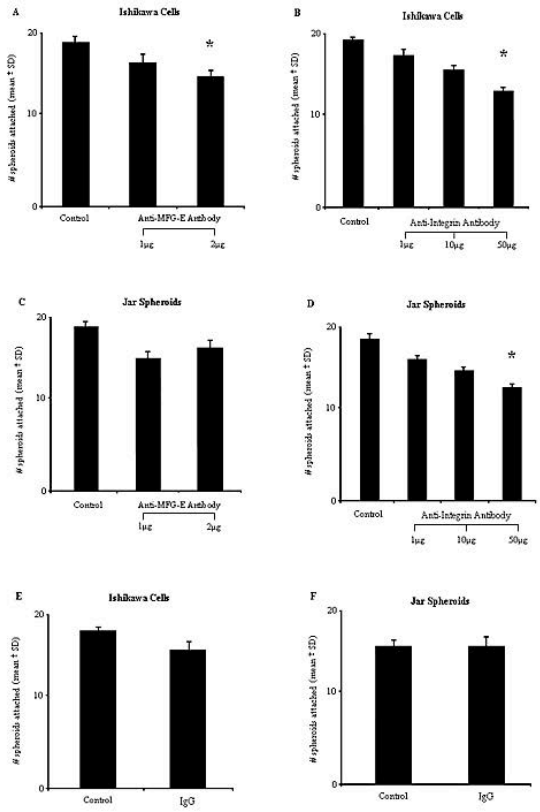

Figure 4

ARTIGO 2

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Running title: Receptivity markers in endometriosis

Alterations in expression of endometrial milk fat globule-EGF factor 8 (MFG-E8) and leukemia inhibitory factor (LIF) in patients with infertility and endometriosis

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Capsule: MFG-E8 and LIF expression are impaired in endometriosis/infertility patient´s endometrium.

Abstract

Objective: To compare the expression of milk fat globule-EGF factor 8 (MFG-E8), its receptor integrin αvβ3, leukemia inhibitory factor (LIF) in patients with endometriosis and infertility and healthy fertile patients.

Design: Prospective case control study.

Setting: Academic center.

Patients(s): Five patients with endometriosis and infertility (study group) and four healthy fertile patients (control group) were recruited

Intervention(s): Endometrial biopsies during the window of implantation (LH+8 to LH+10).

Main Outcome Measure(s): Immunochemistry for MFG-E8, integrin αvβ3 and LIF was performed and analyzed with HSCORE.

Result(s): In patients with endometriosis and infertility, expression of MFG-E8 was significantly increased in the glandular epithelium when compared to healthy fertile patients ($P<0.001$). Moreover, LIF expression was lower in patients with endometriosis and infertility ($P<0.05$). Nevertheless, we found no difference in integrin αvβ3 expression between the groups ($P=0.084$).

Conclusion(s): This study showed for the first time that MFG-E8 expression is impaired in the endometrium of patients with endometriosis and infertility during the window of implantation. Moreover, LIF is also diminished in these patients endometrium as shown before.

Key words: MFG-E8, integrin αvβ3, LIF, human endometrium, endometriosis

Introduction

Endometriosis is one of the most common gynecologic diseases, and its clinical manifestations include dyspareunia, dysmenorrhea, chronic pelvic pain and infertility [1, 2]. It is present in approximately 10% of women in fertile age [3], but among infertile patients, endometriosis prevalence can be as high as 25-50% [4]. Although it is known for decades that endometriosis is associated with infertility, the pathogenesis of this association is still not completely understood [3]. Nevertheless, several studies have shown that endometriosis is associated with abnormal folliculogenesis, with lutheal insufficiency [5, 6], and abnormal embryo fertilization and implantation [7], which contribute to infertility. Regarding lower implantation rates, it has been shown that some endometrial receptivity markers may be impaired in these patients endometrium [4, 8, 9].

Leukemia inhibitory factor (LIF) and integrin αvβ3 are well known endometrial receptivity markers. LIF is a polyfunctional pleiotropic cytokine, which belong to the IL-6 family [10]. In 1992, Stewart et al have shown for the first time that mice lacking a functional LIF gene fail to implant blastocysts in their endometrium [11]. Since then, many authors have demonstrated that a proper LIF expression by the endometrium is also important for women´s fertility [12, 13]. LIF is expressed mainly in the glandular and luminal epithelium of women´s endometrium during the window of implantation [14]. LIF´s impaired expression has been shown in patients with unexplained infertility, with uterine anatomical abnormalities and with endometriosis [9, 13, 15].

Integrin αvβ3 is a tansmembrane glycoprotein that has been extensively studied in the human endometrium. It can be detected in the epithelial layer of mice and human´s endometrium during the window of implantation [16-18]. Several studies have demonstrated that decreased expression of this protein can impair embryo´s implantation in vitro [19-22]. Moreover, patients with hydrosalpinx, unexplained infertility and recurrent pregnancy loss have presented impaired integrin αvβ3 expression [23-25]. Nevertheless, the relationship between this integrin and endometriosis has been controversial in the literature [26-28].

A novel protein that has been recently associated to implantation process is the milk fat globule epidermal growth factor 8 (MFG-E8) [29]. Franchi et al demonstrated for the first time

that MFG-E8 is expressed by human endometrial epithelium and that it is up-regulated during the window of implantation [18]. Besides, MFG-E8 histological sequence in epithelial cell location suggests luminal secretion of the protein [18]. Moreover, we have demonstrated that blocking this protein in an in vitro trophoblast/endometrial epithelium model can impair the implantation process [22]. Nevertheless this protein has never been studied in infertile patients´ endometrium.

Ultimately, an adequate expression of the endometrial receptivity markers integrin αvβ3 and LIF is controversial in endometriosis patients, while MFG-E8 has not yet been studied in these patients. Taking into account that endometriosis patients may have an impaired implantation process, the aim of our study was to compare endometrial expression of MFG-E8, integrin αvβ3, and LIF between patients with infertility and endometriosis and healthy fertile patients (controls) during the window of implantation.

Methods

Design

This was a prospective case-control study performed in the Gynecologic Department of Hospital de Clínicas de Porto Alegre and in the Jones Institute for Reproductive Medicine. STROBE guideline was used [30].

Subjects

Five patients with peritoneal endometriosis and infertility (study group) and four healthy fertile patients (control group) were recruited between January 2014 and November 2014 to take part in the study. Diagnosis of infertility was considered when the couple had not conceived after 12 months of contraceptive-free intercourse [31]. We selected consecutive patients who had peritoneal endometriosis diagnosed during laparoscopic surgery for infertility investigation and fulfilled the including criteria for study group (described below). Endometriosis degree was classified according to the revised American Society for Reproductive Medicine [32]. Patients being subjected to elective laparoscopic tubal ligation were asked to participate and constituted the control group. Therefore they had endometriosis diagnosis excluded (by laparoscopy), they

had also previous history of normal fertility, and were non-smokers.

The inclusion criteria in the study group as well as in the control group were: (i) age between 25 and 38 years (ii) regular menstrual cycles, (iii) presence of both ovaries, (iv) no endocrine disorder and (v) no family history of genetic disease. The study group had also a normal sperm analysis. We excluded patients with abnormal ovarian reserve (antral follicle count under 10), obesity (BMI ≥30), a history of miscarriage, or smoking habit.

The local ethics committee approved this study and a written informed consent was provided to all subjects prior to the sample collection (IRB equivalent).

Endometrial samples

Endometrial biopsies were performed during the natural cycle during the putative window of implantation (LH+8 to LH+10). LH + 1 was considered the day of ovulation [33]. Ovulation was detected by serial ultrasound exams, and it was defined as the 24-h period that separated the sigh of a mature follicle on one scan and either of the following on the second scan: (i) follicle rupture; (ii) the presence of an early corpus luteum; (iii) the presence of free fluid in the cul-de-sac [34].

The biopsies were performed with Pipelle® catheter (CCD, Paris, France). Each endometrial biopsy was fixed in formalin and embedded in paraffin in preparation for histological examination and immunostaining of MFG-E8, integrin αvβ3 and LIF.

Immunohistochemistry

Paraffin-embedded tissue blocks of the endometrial biopsies were cut into 5-μm sections. Immunohistochemistry was performed as previously described [35]. Briefly, the slides were deparaffinized, dehydrated, and rehydrated followed by retrieval solution 1:10 (Dako). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min and non-specific binding sites were blocked with 1.5% normal goat or horse serum (Vector Laboratories) for 30 min at room temperature. The sections were then covered by appropriate dilutions of primary antibody, MFG-E8 (Abcam) 1:100, integrin αvβ3 (Santa Cruz Biotechnologies) 1:10 or LIF (Sigma) 1:750, and placed in refrigerator overnight. After primary antibody incubation,

sections were washed with PBS and incubated with anti-mouse or anti-rabbit secondary antibody (Vector Lab) at a dilution of 1:200 for 30 min at room temperature. After secondary antibody, the tissue was incubated with ABC reagent (Vector Laboratories) for 30 min, followed by PBS wash. The antigens were localized by incubation with AEC chromogen–substrate (skyTek Labs) and finally mounted with Accergyl mounting media (Accurate Chemicals) with a cover slip. Negative controls included sections that were treated with a similar dilution of a non-immune IgG1 (isotype control, eBioscience, San Diego, CA, USA). Representative images were photographed with an Olympus BX50 microscope using an Olympus DP70 Q-color 3 camera [18].

Assessment of staining intensity and distribution for integrin and LIF was made using the semi-quantitative histologic score (HSCORE) system. HSCORE was calculated using the following equation: HSCORE: Σ Pi (i + 1), where i represents the intensity of staining ranging from 1, 2, or 3, (representing weak, moderate or strong, respectively) and Pi representing the percentage of stained endometrial stromal and epithelial cells for each intensity, varying from 0–100%, as previously done [36].

Assessment for staining intensity and distribution for MFG-E8 was made using computerized image analysis using a modification of Fuhrich et al, 2013 [37] that replaced the Image J program with Metamorph™ (Molecular Devices, ###). The original method was found to be highly correlated with HSCORE values obtained by expert evaluators. Color images collected using an Olympus 20x objective were automatically thresholded rather than manually circumscribed and thresholded areas from three different 20x fields were averaged and subtracted from total white (255) values on an eight byte scale, as published earlier [37]. A size filter setting was used to exclude stray pixels so that only cellular structures were analyzed.

Statistical analysis

The statistical analysis was carried out using the SPSS 18.0 software. The measure of central tendency used was the mean and the measure of variability was the standard deviation [38]. Categorical variables in the 2 groups were compared using the 2-sided Pearson Chi^2 test. Continuous variables were compared using the student t-test. We considered $P<5\%$ significant.

Results

Five patients with endometriosis and infertility composed the study group and four healthy fertile patients composed the control group. Demographic characteristics are shown in Table 1. In the study group, four patients presented endometriosis stage I and one patient endometriosis stage II.

MFG-E8 immunohistochemical analysis confirmed previous findings by showing predominantly localization to the glandular epithelium. MFG-E8´s receptor, integrin αvβ3, was localized in the epithelial, as well as in the stromal layer. Furthermore, LIF´s predominant immunolocalization was observed in the luminal epithelium (Figure 1).

Figure 2 shows the mean HSCORE for MFG-E8, integrin αvβ3 and LIF. There was statistical significant difference for MFG-E8 between control and study group ($P< 0.001$) and LIF ($P=0.033$). Nevertheless, there was no difference for integrin αvβ3 ($P=0.084$).

Discussion

This study showed for the first time that patients with endometriosis and infertility have an increased MFG-E8 expression in the endometrium during the putative window of implantation. Furthermore, we showed that patients also had a decreased LIF expression during the same period. Nevertheless, we found no difference in integrin αvβ3 expression between the groups.

MFG-E8 is a glycoprotein identified for the first time in 1990 [39]. Since then, it has been extensively studied in many physiological and pathological processes, especially in the immune system [40, 41]. A possible role for MFG-E8 in the implantation process was pointed out for the first time in 2005, when it was found up-regulated during the window of implantation [29]. After that, other studies suggested its participation in the implantation process [18, 22]; however, to the best of our knowledge, it was never studied in the endometrium of infertile patients.

MFG-E8 is known to be involved in the inflammatory processes [42], and it is regulated by TNF-α in the human endometrium [43]. Moreover, endometriosis patients are known to

present a chronic inflammatory state [44, 45]. Therefore, our hypothesis that endometriotic/infertile patients have increased MFG-E8 expression during the window of implantation was confirmed in the current study. We have previously showed that blocking MFG-E8 in an in vitro model impairs implantation process [22]. Nevertheless, it seems that either MFG-E8´s down regulation [46] as well as its up-regulation may impair physiological processes [47, 48].

MFG-E8´s receptor integrin αvβ3 is a well-established endometrial receptivity marker. Nevertheless, its expression in endometriosis patients' endometrium has been controversial in the literature. Our study found no difference in its expression between the groups, although there was a trend to be lower in the study group (P=0.084). In 1994, a large study with 241 endometriosis patients showed that patients with endometriosis stages I/II have decreased integrin´s expression [26]. However, this study included biopsies after day 19 of the cycle, not only during the window of implantation. Moreover, the study does not mention if all patients were infertile. On the other hand, two smaller studies failed to find such difference [27, 28], and they analyzed the protein´s expression during the window of implantation.

A possible cause of integrin αvβ3 decreased expression is the fact that these patients present an impaired HOXA10 production, which is responsible for β3 subunit expression [8, 49, 50]. Furthermore, may be the over expression of MFG-E8 down regulates its receptor production.

Another important endometrial receptivity marker that seems to be impaired in endometriosis patients' endometrium is LIF. In accordance to our results, Dimitriadis et al previously showed a diminished expression of LIF in stages I/II of endometriosis during the window of implantation [9]. In addition, Alizadeh et al also showed impaired LIF expression in the same group of patients [51]. Nevertheless, a study with 14 endometriosis patients showed no difference of LIF in uterine flushings when compared to fertile control (21 patients) [13].

Regarding our methods, our modification to a previously published technique [37] is more rapid and also more accurate because the Metamorph™ program thresholding removes holes in background objects, permitting quantitation of only the areas subjected to threshold establishment, while manually circumscribing objects, as in [37] is much slower and can also include holes while can reduce the averaged staining intensity in a variable fashion, depending on

the individual object's hole area. Metamorph™ size filter settings were also used to exclude stray pixels and small debris to allow a more accurate measurement of the cellular structures. Unfortunately, not all types of staining can be adequately analyzed by this method, as integrin and LIF. That is the reason we have used the traditional H-score.

Although it may be pointed out that the studied sample size is relatively slow, we found statistical significant differences. Moreover, the results are in agreement with what we were expecting, based on biological plausibility. Although we did not measure the serum or urinary LH to further define the ovulation day, previous studies have defined ovulation only with ultrasound [34].

In conclusion, our study showed for the first time that patients with endometriosis and infertility have an altered MFG-E8 expression in the endometrium during the putative window of implantation. Moreover, we also demonstrated that these patients have diminished LIF, as showed before. Nevertheless, there was no difference for integrin αvβ3 expression, although there was a trend to be lower in the study group. A better characterization of endometriosis patients' endometrium is essential for a better understanding of this disease associated infertility.

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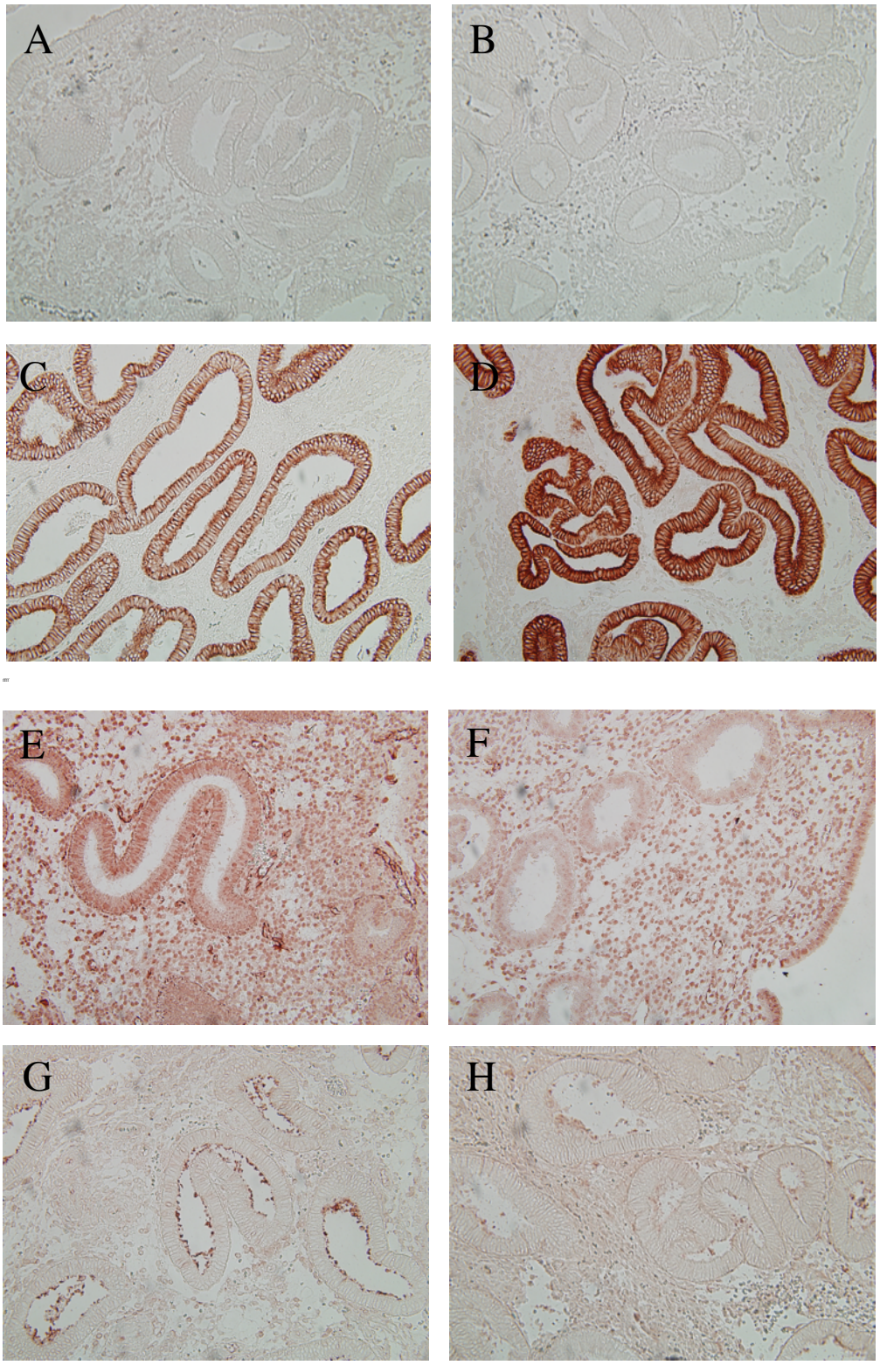
Legends to table and figures

Table 1. Demographic characteristics for women with infertility and endometriosis (study group) and healthy fertile women (control group)

	Control group (n = 4)	Study group (n = 5)	*P* value
Age (years)	35.2 ± 2.2	30.4 ± 4.3	.084[a]
Race			1.0[b]
Caucasian	4 (80%)	4 (80%)	
African-Brazilian	1 (20%)	1 (20%)	
BMI (Kg/m^2)	26.6 ± 2.8	22.8 ± 4.1	.156[a]
Menarche (years)	11.5 ± 1.3	12.6 ± 1.1	.216[a]
AFC	12.5 ± 1.0	13.6 ± 5.9	.727[a]

All values are means ± SD; BMI = body mass index; AFC = antral follicle count; a = student t test; b = Chi2 test.

Figure 1. Immunohistochemical localization of MFG-E8, integrin αvβ3 and LIF in the human endometrium. A negative control from the control group (endometriosis and infertility) (A) and from the study group (healthy fertile patients) (B). MFG-E8 staining from the control group (C) and a from the study group (D). Integrin αvβ3 staining from the control group (E) and from the study group (F). LIF staining from the control group (G) and from the study group (H).


A
B
C
D
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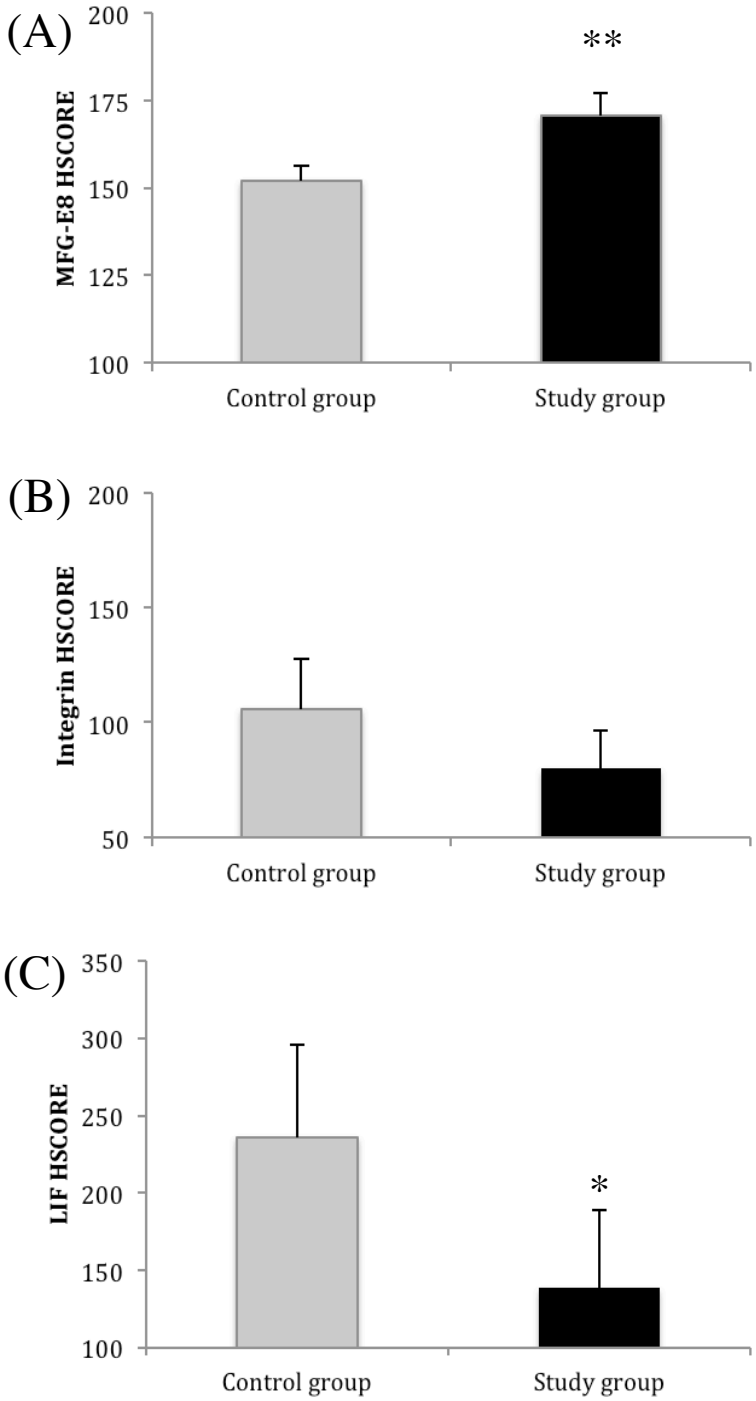
H

Figure 2. MFG-E8 (A), integrin αvβ3 (B) and LIF (C) HSCORE in healthy fertile patients (control group) and patients with endometriosis and infertility. **P<0.001 compared with controls. *P<0.05 compared with controls



CONSIDERAÇÕES FINAIS

Este estudo demonstrou pela primeira vez que, quando se bloqueia MFG-E8 em células Ishikawa, ocorre uma diminuição de adesão das células Jar. Também demonstrou que o mesmo efeito ocorre quando se bloqueia o seu receptor, a integrina αvβ3. Além do mais, bloqueando-se a integrina αvβ3 nas esferas Jar, também ocorre uma diminuição da adesão destas nas células Ishikawa.

Além disso, demonstramos pela primeira vez que a expressão de MFG-E8 está aumentada no endométrio de pacientes com endometriose e infertilidade durante a janela de implantação. Também demonstramos que estas pacientes apresentam expressão de LIF diminuída no grupo em estudo no mesmo período. No entanto, não encontramos diferença na expressão de integrina αvβ3 entre os grupos.

PERSPECTIVAS

Gostaríamos de estudar os mecanismos intracelulares que levam fazem com que bloqueio de MFG-E8 e da integrina αvβ3 nas células Ishikawa diminua a adesão das células Jar.

Além disso, planejamos aprofundar o estudo da proteína MFG-E8 no endométrio das pacientes com endometriose e avaliar fatores que regulam sua expressão. Também iremos estudar a expressão desta proteína no líquido peritoneal destas pacientes.

ANEXOS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (Controle)

Gostaríamos de convidá-la para participar de um estudo sobre proteínas e hormônios (substâncias normalmente presentes no organismo) expressos no endométrio (camada interna do útero) e sua relação com a infertilidade. Para a realização desta pesquisa é necessário comparar mulheres com infertilidade a mulheres que não apresentam este problema, como é o seu caso.

Sua participação neste estudo envolverá a realização de ecografias pélvicas transvaginais para visualização dos ovários e acompanhamento da ovulação, e a realização de uma biópsia de endométrio. A ecografia pélvica transvaginal não oferece nenhum risco a sua saúde, apenas um desconforto leve durante a sua realização. A biópsia de endométrio oferece uma baixa taxa de complicações, incluindo pequeno sangramento, infecção e raramente perfuração uterina, e ocorre principalmente em casos em que o médico não tem experiência para realizar o exame. Para minimizar este risco, os médicos que realizarão a biópsia para este estudo têm muita experiência neste procedimento.

Você não terá benefícios ao participar desta pesquisa, entretanto estará ajudando a entender melhor as causas de infertilidade e auxiliando no possível desenvolvimento de um tratamento mais eficaz no futuro para as mulheres que tem dificuldade de engravidar. Os resultados deste estudo serão publicados, entretanto os pesquisadores comprometem-se a manter a confidencialidade dos dados, ou seja, seu nome não será revelado. Além do mais, você não terá despesas decorrentes da participação nesta pesquisa.

Sua participação neste estudo deve ser voluntária. Você é livre para interromper sua participação a qualquer momento sem qualquer prejuízo ao atendimento recebido neste hospital.

Eu, , fui informada dos objetivos especificados acima e da justificativa desta pesquisa, de forma clara e detalhada. Recebi informações específicas sobre cada procedimento no qual estarei envolvida, dos desconfortos ou riscos previstos, tanto quanto dos benefícios esperados. Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer

momento. O pesquisador responsável por este estudo é o Prof. João Sabino da Cunha Filho do Serviço de Ginecologia Obstetrícia (CESGO), localizada no Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, 11º andar, sala 1117, e pode ser contatado pelo telefone 3359-8117. Se você tiver dúvidas sobre seus direitos como participante de pesquisa poderá entrar em contato com o Comitê de Ética em Pesquisa que aprovou este estudo pelo telefone 3359-7640. Este termo de consentimento foi elaborado em duas vias, uma delas será fornecida para a paciente e a outra ficará sob a guarda do pesquisador responsável.

O profissional , certificou-me de que as informações por mim fornecidas terão caráter confidencial.

Assinatura da participante:_____

Assinatura do pesquisador: _____

Porto Alegre, _____de _____ de _____.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (Grupo 1)

Gostaríamos de convidá-la para participar de um estudo sobre proteínas e hormônios (substâncias normalmente presentes no organismo) expressos no endométrio (camada interna do útero) e sua relação com a infertilidade.

Sua participação neste estudo envolverá a realização de ecografias pélvicas transvaginais para visualização dos ovários e acompanhamento da ovulação, e a realização de uma biópsia de endométrio. A senhora terá que utilizar preservativo ou não ter relações sexuais durante o ciclo em que será realizado o estudo, uma vez que a biópsia pode resultar em perda da gravidez. A ecografia pélvica transvaginal não oferece nenhum risco a sua saúde, apenas um desconforto leve durante a sua realização. A biópsia de endométrio oferece uma baixa taxa de complicações, incluindo pequeno sangramento, infecção e raramente perfuração uterina, e ocorre principalmente em casos em que o médico não tem experiência para realizar o exame. Para minimizar este risco, os médicos que realizarão a biópsia para este estudo têm muita experiência neste procedimento.

Se a senhora estiver em tratamento para infertilidade, o ciclo em que será realizada a biópsia de endométrio será anterior ao da fertilização in vitro, não implicando em atraso significativo ao seu tratamento. Além do mais, existem estudos que demonstram que lesão endometrial prévia ao tratamento (como a biópsia de endométrio) pode melhorar as taxas de gestação na fertilização in vitro.

Você terá benefícios indiretos ao participar desta pesquisa, pois estará ajudando a entender melhor as causas de infertilidade e auxiliando no possível desenvolvimento de um tratamento mais eficaz no futuro. Os resultados deste estudo serão publicados, entretanto os pesquisadores comprometem-se a manter a confidencialidade, ou seja, seu nome não será revelado. Além do mais, você não terá despesas decorrentes da participação nesta pesquisa.

Sua participação neste estudo deve ser voluntária. Você é livre para interromper sua participação a qualquer momento sem qualquer prejuízo ao atendimento recebido neste hospital.

Eu, , fui informada dos objetivos especificados acima e da justificativa desta pesquisa, de forma clara e detalhada.

Recebi informações específicas sobre cada procedimento no qual estarei envolvida, dos desconfortos ou riscos previstos, tanto quanto dos benefícios esperados. Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer momento. O pesquisador responsável por este estudo é o Prof. João Sabino da Cunha Filho do Serviço de Ginecologia Obstetrícia (CESGO), localizada no Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, 11º andar, sala 1117, e pode ser contatado pelo telefone 3359-8117. Se você tiver dúvidas sobre seus direitos como participante de pesquisa poderá entrar em contato com o Comitê de Ética em Pesquisa que aprovou este estudo pelo telefone 3359-7640. Este termo de consentimento foi elaborado em duas vias, uma delas será fornecida para a paciente e a outra ficará sob a guarda do pesquisador responsável.

O profissional , certificou-me de que as informações por mim fornecidas terão caráter confidencial.

Assinatura da paciente:_____

Assinatura do pesquisador: _____

Porto Alegre, _____de _____ de _____.

Ficha de coleta de dados projeto MFG-E8: GRUPO ENDOMETRIOSE

NOME_____PRONTUÁRIO_____

DN_____ IDADE_____ DATA_____

COR (1) branca (2) preta (3) parda (4) amarela ANOS DE ESTUDO_____

TABAGISMO (1) sim (2) não ETILISMO (1) sim (2) não

PESO_____ ALTURA_____ IMC_____

CLASSIFICAÇÃO EDT: (I) (II) (III) (IV) PONTUAÇÃO:___

DATA VLP_____

TIPO INFERTILIDADE (1) primaria (2) secundaria

TEMPO INFERTILIDADE_____

AVALIAÇÃO TUBÁRIA (1) HSG (2) VLP DATA_____

ESPERMOGRA VALORES: MORFOLOGIA (1) KRUGER (2) OMS DATA_____

MENARCA_____

GESTA ____ PARA_____ CESA_____ ABORTO_____

CICLOS (1) irreg (2) reg _____dias

FSH:_____ DATA_____

LH:_____ DATA_____

E2:_____ DATA_____

CA-125:_____ DATA_____

PRL:_____ DATA_____

TSH:_____ DATA_____

AMH:_____ DATA_____

AFC:_____ DATA_____

Ficha de coleta de dados projeto MFG-E8: GRUPO CONTROLE (LT)

NOME_____PRONTUÁRIO_____

DN_____ IDADE_____ DATA_____

COR (1) branca (2) preta (3) parda (4) amarela ANOS DE ESTUDO_____

TABAGISMO (1) sim (2) não ETILISMO (1) sim (2) não

PESO_____ ALTURA_____ IMC_____

DATA VLP_____

MENARCA_____

GESTA_____PARA____ CES____ ABORT_____

CICLOS (1) irreg (2) reg _____dias

FSH:_____ DATA_____

LH:_____ DATA_____

E2:_____ DATA_____

CA-125:_____ DATA_____

PRL:_____ DATA_____

TSH:_____ DATA_____

AMH:_____ DATA_____

AFC:_____ DATA_____