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CAMPUS DE BOTUCATU

POTENCIAL DE INIBIÇÃO DA PROLIFERAÇÃO DE LINFÓCITOS IN VITRO PELAS CELULAS TRONCO MESENQUIMAIS CANINAS DERIVADAS DO TECIDO ADIPOSO

DIEGO PETROCINO CAETANO

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Dissertação apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Mestre

Orientador: Prof. Dr. Rogério Martins Amorim

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LISTA DE FIGURAS

LISTA DE ABREVIAÇÕES

APC = Células Apresentadoras De Antígenos

AT-MSC = Células Tronco Mesenquimais de Tecido Adiposo

BDNF = Fator Neurotrófico Derivado do Cérebro

Breg = Célula B Reguladora

BTLA = Atenuador de Linfócitos B e T

CCL2 = Quimiocina De Motivo C-C Ligante 2

ConA = Concanavalina A

CTLA-4 = Receptores Antígeno 4 Associado ao Linfócito T Citotóxico

DC = Célula Dendrítica

EAE = Encefalomielite Autoimune Experimental

EM = Esclerose Múltipla

EPHB2 = Produtor De Eritropoietina B 2

EPHB4 = Produtor De Eritropoietina B 4

Fas = Proteína Transmembrânica Indutora de Apoptose

FasL = Ligante de Proteína Transmembrânica Indutora de Apoptose

HGF = Fator de Crescimento de Hepatócitos

HLA-G = Antígeno De Locus De Histocompatibilidade-g

HO-1 = Heme Oxigenase 1 (HO-1)

IDO = Indoleamina 2,3-dioxigenase

IFN-γ = Interferon-Gama

IL-1 = Interleucina 1

IL-10 = Interleucina 10

IL-17 = Interleucina 17

IL-1β = Interleucina-1 Beta

IL-2 = Interleucina 2

IL-33 = Interleucina 33

IL-6 = Interleucina 6

IL-7 = Interleucina 7

IL1RA = Antagonista Do Receptor De Interleucina-1

ILC = Célula Linfoide Inata

LAG3 = Gene de Ativação de Linfócitos 3

MDSC = Célula Mieloide Supressora Derivadas

MEN = Meningoencefalite Granulomatosa

MHC = Complexo Principal de Histocompatibilidade

MLR = Reação Mista de Leucócitos

MSC = Mesenchymal Stem Cells - Células Tronco Mesenquimais

MUO = Meningoencefalites de Origem Desconhecida

NGF = Fator De Crescimento Nervoso

NK = Natural Killer

NKT = Célula T Natural Killer

NO = Óxido Nítrico

NT-3 = Neurotrofina 3

PBMC = Células Mononucleares de Sangue Periférico

PD-1 = Proteína de Morte Celular Programada 1

PD-L1 = Ligante 1 de Proteína de Morte Celular Programada 1

PD-L2 = Ligante 2 de Proteína de Morte Celular Programada 1

PGE2 = Prostaglandina E2

TCR = Receptores de Células T

TGF-β = Fator de Crescimento Transformador β

Th = Célula T Helper

Th1 = Célula T Auxiliar Tipo 1

Th17 = Célula T Auxiliar Tipo 17

Th2 = Célula T Auxiliar Tipo 2

TIM-1 = Domínios de Mucina e Imunoglobulina de Célula T 1

TIM-2 = Domínios de Mucina e Imunoglobulina de Célula T 2

TIM-3 = Domínios de Mucina e Imunoglobulina de Célula T 3

TIM-4 = Domínios de Mucina e Imunoglobulina de Célula T 4

TNF-α = Fator De Necrose Tumoral Alfa

Treg = Células T Reguladoras

TSG6 = Gene 6 Estimulado Pelo Fator De Necrose Tumoral

VEGF = Fator De Crescimento Endotelial Vascular

µg = Microgramas

µL = Microlitros

RESUMO

A terapia com células-tronco mesenquimais (MSCs) tem se demonstrado uma biotecnologia promissora em medicina regenerativa, principalmente pelo seu potencial imunomodulador. Sua ação ocorre por diversos mecanismos, como secreção de fatores solúveis e interação direta entre células. Dentre as diversas interações, a redução da proliferação de linfócitos T é fundamental para controle de doenças autoimunes em geral. Dentre as formas de avaliar o potencial imunomodulador das MSCs *in vitro*, destaca-se o teste de reação mista de leucócitos (MLR), que avalia a inibição da proliferação de leucócitos, particularmente linfócitos. O objetivo deste estudo foi avaliar *in vitro* o ciclo celular e o potencial de inibição da proliferação de linfócitos estimulados com concanavalina A, quando em co-cultura (em contato direto e indireto) com MSCs derivadas do tecido adiposo (AT-MSCs) canino e células monoclueares de sangue periférico (PBMC). Para tal, foram realizados testes MLR utilizando-se PBMCs provenientes de cães doadores clinicamente sadios obtidas por meio de gradiente de separação com Histopaque 1077, em co-cultura com AT-MSCs caninas (n=4). Foram realizadas culturas em duplicatas de PBMCs (10^6 células por poço), PBMCs estimuladas com concanavalina A (10^6 células + 5ug/mL de ConA), PBMCs estimuladas com concanavalina A em co-cultura direta com AT-MSCs (10^6 PBMCs + 5ug/mL de ConA + $2x10^5$ AT-MSCs), e PBMCs estimuladas com concanavalina A em co-cultura indireta (membranas transwell) com AT-MSCs (10^6 PBMCs + 5ug/mL de ConA + $2x10^5$ AT-MSCs). As placas foram mantidas em cultivo por 96h, sendo adicionado BrdU nas últimas 24h. Em seguida, as amostras dos cultivos foram incubadas com anticorpos anti-CD3 canino, anti-BrdU e 7-AAD para avaliação por citometria de fluxo. As AT-MSCs caninas em co-cultura com as PBMCs induziram a redução da fase proliferativa dos linfócitos CD3+, possivelmente deslocando-os para a fase G0/G1 do ciclo celular. A redução da fase proliferativa dos linfócitos CD3+ foi evidenciada tanto nas co-culturas de AT-MSCs e PBMCs com contato direto quanto indireto, indicando que os mecanismos de ação também dependem de

mediadores químicos secretados no meio. Apesar de não observamos diferença estatística, houve tendência de inibição da proliferação de linfócitos CD3+ estimulados com concanavalina A quando em contato direto e indireto com as AT-MSCs. Nossos resultados indicam que as AT-MSCs caninas apresentam potencial imunomodulador. Novos estudos devem ser realizados para elucidar os mecanismos de ação imunomoduladores das AT-MSCs caninas sobre linfócitos.

Palavras-chave: PBMC, reação mista de leucócitos, autoimune, linfócito CD3+

ABSTRACT

Mesenchymal stem cells (MSCs) therapy has been shown to be a promising biotechnology in regenerative medicine, mainly due to its immunomodulatory potential. Its action occurs through several mechanisms, such as secretion of soluble factors and direct interaction between cells. Among the various interactions, the reduction of T lymphocyte proliferation is fundamental for the control of autoimmune diseases in general. Among the ways to evaluate the immunomodulatory potential of MSCs in vitro, the mixed leukocyte reaction test (MLR) is one of the most important, which evaluates the inhibition of leukocyte proliferation, particularly lymphocytes. The aim of this study was to evaluate in vitro the cell cycle and proliferation inhibition potential of lymphocytes stimulated with concanavalin A, when in co-culture (in direct and indirect contact) with canine adipose tissue-derived MSCs (AT-MSCs) and peripheral blood mononuclear cells (PBMC). For this purpose, MLR tests were performed using PBMCs from clinically healthy donor dogs obtained through separation gradient with Histopaque 1077, in co-culture with canine AT-MSCs (n=4). Cultures were performed in duplicates of PBMCs (10^6 cells per well), PBMCs stimulated with concanavalin A (10^6 cells + 5ug/ml ConA), PBMCs stimulated with concanavalin A in direct co-culture with AT-MSCs (10^6 PBMCs + 5ug/ mL of ConA + $2x10^5$ AT-MSCs), and PBMCs stimulated with concanavalin A in indirect co-culture (transwell membranes) with AT-MSCs (10^6 PBMCs + 5ug/mL of ConA + $2x10^5$ AT-MSCs). The plates were kept in culture for 96h, with BrdU being added in the last 24h. Then, culture samples were incubated with anti-CD3 canine, anti-BrdU and 7-AAD antibodies for evaluation in flow cytometry. Canine AT-MSCs in co-culture with PBMCs induced a reduction in the proliferative phase of CD3+ lymphocytes, possibly shifting them to the G0/G1 phase of the cell cycle. The reduction in the proliferative phase of CD3+ lymphocytes was evidenced both in co-cultures of AT-MSCs and PBMCs with direct and indirect contact, indicating that the mechanisms of action also depend on chemical mediators secreted in the medium. Although we did not observe any statistical difference, there was a tendency to inhibit the proliferation of CD3+ lymphocytes stimulated with

concanavalin A when in direct and indirect contact with AT-MSCs. Our results indicate that canine AT-MSCs have immunomodulatory potential. New studies must be carried out to elucidate the immunomodulatory mechanisms of action of canine AT-MSCs on lymphocytes.

Keywords: PBMC, mixed lymphocyte reaction, autoimmune, CD3+ lymphocyte

SUMÁRIO

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ABSTRACT

Mesenchymal stem cells (MSCs) therapy has been shown to be a promising biotechnology in regenerative medicine, mainly due to its immunomodulatory potential. Its action occurs through several mechanisms, such as secretion of soluble factors and direct interaction between cells. Among the various interactions, the reduction of T lymphocyte proliferation is fundamental for the control of autoimmune diseases in general. Among the ways to evaluate the immunomodulatory potential of MSCs in vitro, the mixed leukocyte reaction test (MLR) is one of the most important, which evaluates the inhibition of leukocyte proliferation, particularly lymphocytes. The aim of this study was to evaluate in vitro the cell cycle and proliferation inhibition potential of lymphocytes stimulated with concanavalin A, when in co-culture (in direct and indirect contact) with canine adipose tissue-derived MSCs (AT-MSCs) and peripheral blood mononuclear cells (PBMC). For this purpose, MLR tests were performed using PBMCs from clinically healthy donor dogs obtained through separation gradient with Histopaque 1077, in co-culture with canine AT-MSCs (n=4). Cultures were performed in duplicates of PBMCs (10^6 cells per well), PBMCs stimulated with concanavalin A (10^6 cells + 5ug/ml ConA), PBMCs stimulated with concanavalin A in direct co-culture with AT-MSCs (10^6 PBMCs + 5ug/ mL of ConA + $2x10^5$ AT-MSCs), and PBMCs stimulated with concanavalin A in indirect co-culture (transwell membranes) with AT-MSCs (10^6 PBMCs + 5ug/mL of ConA + $2x10^5$ AT-MSCs). The plates were kept in culture for 96h, with BrdU being added in the last 24h. Then, culture samples were incubated with anti-CD3 canine, anti-BrdU and 7-AAD antibodies for evaluation in flow cytometry. Canine AT-MSCs in co-culture with PBMCs induced a reduction in the proliferative phase of CD3+ lymphocytes, possibly shifting them to the G0/G1 phase of the cell cycle. The reduction in the proliferative phase of CD3+ lymphocytes was evidenced both in co-cultures of AT-MSCs and PBMCs with direct and indirect contact, indicating that the mechanisms of action also depend on chemical mediators secreted in the medium. Although we did not observe any statistical difference, there was a tendency to inhibit the proliferation of CD3+ lymphocytes stimulated with concanavalin A when in direct and indirect contact with AT-MSCs. Our results indicate that canine AT-MSCs have immunomodulatory

potential. New studies must be carried out to elucidate the immunomodulatory mechanisms of action of canine AT-MSCs on lymphocytes.

Keywords: PBMC, mixed lymphocyte reaction, autoimmune, regeneration

CONCLUSION

The Mixed Leukocyte Reaction test proved to be an important tool to investigate in vitro the immunomodulatory potential of MSCs, through the evaluation of the ability to inhibit lymphocyte proliferation.

Canine AT-MSCs induced a reduction in the proliferative phase (S phase of the cell cycle) of CD3+ lymphocytes, in co-cultures with and without direct contact.

Although we did not observe any statistical difference, there was a tendency towards a reduction in the proliferation of CD3+ lymphocytes stimulated with concanavalin A when in direct and indirect co-culture with AT-MSCs.

Our results indicate that canine AT-MSCs have immunomodulatory potential. New studies must be carried out to elucidate the immunomodulatory mechanisms of action of canine AT-MSCs on lymphocytes.

Data Availability Statement

The datasets used and/or analyzed during the currentstudy are available from the corresponding author on reasonable request.

Author Disclosure Statement

The authors declare no potential conflicts of interest.

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