no máximo 24 horas após o parto e armazenados a 4°C em frasco com PBS e glicose 0,111 M pH 6,5 estéril. Sob câmara de fluxo laminar vertical, as veias dos cordões foram canuladas nas duas extremidades com cânulas de metal, lavadas com PBS rico em glicose a 37°C. As células foram descoladas da parede da veia com a adição de colagenase 0,1% em PBS rico em glicose. Os cordões foram mantidos a 37°C por 10 minutos e após este tempo as células foram coletadas em tubo cônico contendo meio 199 (M199) complementado com 20% de soro fetal bovino (SFB), L-glutamina 2mM, bicarbonato de sódio 26mM, fungizona 2,5µg/ml, penicilina 500U/ml e gentamicina 40µg/ml. A suspensão celular foi centrifugada a 180 x g durante 10 minutos e ressuspensas em M199 com SFB a 20%. A suspensão celular foi distribuída em garrafas de cultura de 25cm2 de área previamente recoberta com gelatina 1% estéril. As garrafas foram incubadas a 37°C em atmosfera 5% de CO2 até atingirem o estado de confluência.

Isolamento de células progenitoras endoteliais humanas do sangue periférico de adultos. As células mononucleares (MN) de baixa densidade foram obtidas por fracionamento de 10 mL de buffy coat (diluído 1:4 em PBS) em Histopaque 1077 (Sigma), na proporção de 4 mL de buffy coat para 3ml de Ficoll, como descrito a seguir. Após centrifugação (400 x g) por 30 minutos, o anel de células MN foi recuperado em PBS contendo 0,1% de BSA. Durante as lavagens a velocidade e o tempo de centrifugação foram reduzidos para 200 x g por 10 minutos. Após duas lavagens, as células foram ressuspensas em 12 mL de meio basal endotelial-2 (EBM-2; Clonetics, Walkersville, MD, USA, cc-3156), suplementado com meio de crescimento endotelial (EGM-2 –Single Quots, Clonetics, Walkersville, MD, USA, cc-4176) contendo fator de crescimento de endotélio vascular (VEGF-A), fator de crescimento de fibroblasto humano (hFGF-B), fator de crescimento de epiderme (EGF), fator-1 de crescimento tipo-insulina (IGF-1), soro fetal bovino (FBS), e ácido ascórbico. Visando otimizar a obtenção em massa de células precursoras endoteliais de sangue periférico de adultos utilizamos um meio de cultura híbrido composto por EGM-2 enriquecido (EGM-2/ENR), desenvolvido em nosso laboratório. A suspensão (106 células/cm2) foi semeada sobre 1µg/cm2 de fibronectina (Gibco/Invitrogen,USA) e mantida durante 4 dias em cultura a 37°C/5% de CO2. Após esse período de incubação, as células não aderentes foram removidas e o meio passou a ser renovado a cada dois dias, até atingirem o estado de confluência.

Peptídeos derivados da TSP-1. A síntese dos peptídeos derivados do domínio N-terminal da TSP-1 – TSP HepI/A1 ou TSP HepII/A2 - foi realizada pelo Dr. Luiz Juliano no

Departamento de Biofísica da UNIFESP (Escola Paulista de Medicina, São Paulo), utilizando um sintetizador automático de fase sólida (PSSM 8; Shimadzu, Tokyo, Japão). Os peptídeos foram purificados por HPLC e o material eluído foi monitorado por absorbância a 220 nm. O peso molecular e a pureza foram checados por espectrometria de massa (MALDI-TOF MS) e/ou por sequenciamento, utilizando-se um sequenciador protéico (PPSSQ-23 - Shimadzu Corp.). Foram sintetizados também peptídeos que sofreram modificações nos aminoácidos essenciais para o reconhecimento do sítio de ligação a glicosaminoglicanos (GAGs) - S/TSP HepI ou S/TSP HepII - para análise de interações das célula progenitoras endoteliais com a matriz extracelular. Para obter a concentração proteica, utilizamos o *kit* para dosagem de proteína BCAssay (Upitma). Dez miligramas do peptídeo foram pesados e solubilizados em 1 ml de água ultra pura. Para 25µl de amostra (diluída 1:20 em tampão Tris/NaCl) foi depositado 200µl de reativo BCAssay/poço na placa de 96 poços e incubados por 30 minutos a 60°C. A absorbância foi lida a 550nm. A concentração foi calculada baseada na curva padrão de BSA (estabelecida em mg/ml). Os peptídeos devidamente dosados foram aliquotados e mantidos em freezer a -80°C.

Adesão de células progenitoras endoteliais humanas. Analisamos a adesão de pEPCs, utilizando o ensaio colorimétrico MTT, que também permite avaliar a viabilidade celular. Essa técnica consiste na capacidade da enzima mitocondrial succinato-desidrogenase converter o sal de tetrazolium (brometo de 3-4,5-dimetiltiazol-2yl-2,5-diphenyltetrazólio) de coloração amarela em um precipitado de coloração púrpura. Essa reação ocorre em células viáveis, refletindo o estado funcional da cadeia respiratória, não identifica, portanto células mortas. Os peptídeos TSP HepI/A1 ou TSP HepII/A2 foram diluídos em 100µl PBS sem Ca++ e Mg++ ,em uma concentração de 20µg/ml (6µg/cm2) e depositados na placa de 96 poços que foram mantidas à 4°C - overnight. No dia seguinte, o excesso de proteína foi retirado com uma pipeta e os poços foram saturados com 0,1% de albumina fetal bovina (BSA) durante 1h à temperatura ambiente. Após a lavagem dos poços, as células foram semeadas em 200µl de M199/BSA 0,1%, em uma densidade de 1.5x105 cells/cm2, na ausência ou na presença de proteína suporte. Após 2h de adesão, os poços foram lavados com M199 para remoção das células não aderentes e 100µl de M199/BSA 0,1% foi adicionado. Em seguida 100µl de MTT (2 mg/ml) foi diluído em M199 sem soro e adicionado aos poços. Após 2h de reação no escuro, a 37°C/5%CO2, a solução foi substituída por 200 µl de álcool isopropílico para a solubilização dos cristais formados pela reação de redução. A absorbância foi lida a 595 nm. O número de células foi calculado baseado na curva padrão de pEPCs. O

controle basal do experimento foi realizado com BSA 0,1% utilizado como suporte para a adesão.

Proliferação de células endoteliais progenitoras. O ensaio foi realizado utilizando-se o método colorimétrico MTT. As pEPCs ($1x10^4$) foram semeadas em 100µl de M199 contendo 20% de SFV na placa de 96 poços revestida com 2% de gelatina. Após 2h de incubação a 37°C/5%CO2. O peptídeo TSP HepI/A1 foi diluído a 10µg/ml em 100µl de M199 sem SFV e acrescentado aos poços. Portanto, a concentração final de soro nos poços foi de 10%. As pEPCs foram incubadas por 72h a 37°C/5%CO2. Após este tempo, 1mg/ml de MTT foi acrescentado aos poços, sendo realizada a incubação por 90 minutos. Neste momento, as células viáveis catalisavam o MTT, gerando os cristais de coloração púrpura. Em seguida os cristais foram dissolvidos vigorosamente em isopropanol e a absorbância foi lida a 595nm. O experimento foi realizado em quadriplicata e o número de células foi calculado baseado na curva padrão de pEPCs. O controle basal do experimento foi realizado com M199 10% SFV.

Migração de células progenitoras endoteliais humanas. O ensaio de migração a favor do gradiente de concentração (quimiotaxia) foi realizado utilizando insertos de transmigração com porosidade de 8 µm. As membranas dos insertos foram tratadas previamente com gelatina 2% por 30 minutos e inseridas na placa de 24 poços. Os peptídeos TSP HepI/A1 ou TSP HepII/A2, ambos na concentração de 20µg/ml, foram diluídos em M199/BSA 0,1% e adicionados na parte inferior da câmara de Boyden. As pEPCs ($7x10^4$ células/poço) foram semeadas em M199/BSA 0,1% no compartimento superior (interior do inserto) e permitidas migrar por 6 horas à 37ºC. Após a incubação, as células não migrantes, aderidas na parte superior da membrana, foram removidas com auxílio de uma haste de algodão. As células migrantes, localizadas na parte inferior da membrana foram fixadas e tratadas com o kit de coloração Panótico rápido (Laborclin), levadas ao microscópio e quantificadas. Foram analisados nove campos aleatórios por filtro em objetiva de 20x em duplicata. O FGF-2 recombinante (bFGF 10ng/ml, R&D Systems, França) foi utilizado como controle positivo. O M199/BSA 0,1% foi utilizado como controle basal.

Ensaio de tubulogênese. O MatrigelTM (BD Bioscience) com fator de crescimento reduzido foi misturado aos peptídeos TSP HepI/A1 ou TSP HepII/A2 (10µg/ml) e depositado na placa de 24 poços. Durante o período de polimerização, que variou de 30 minutos a 1h em 37°C, as células foram descoladas com tripsina e ressuspendidas em M199 contendo 5% SFB.

As pEPCs foram semeadas em uma densidade de 1,5 x 10^5 células/cm2 na ausência ou na presença dos peptídeos. Após 6h de diferenciação a 37°C os poços contendo os géis foram fixados com glutaraldeído 1,1% por 15 minutos e corados com Giemsa. As imagens foram fotografadas em microscópio invertido Olympus e o número de estruturas formadas foi quantificado com Image J. Foram quantificados 6 campos, em duplicata, na objetiva de 100 ×.

Quantificação de fibronectina (FN) celular por ELISA. A avaliação de fibronectina solúvel, secretada no meio de cultura das células endoteliais foi realizada pelo ensaio imunoenzimático (ELISA). As células HUVECs ou pEPCs foram semeadas em placas de 24 poços na densidade de 2x10^5 células/poço em M199 suplementado com SFV 20% ou EGM-2, respectivamente, e foram incubadas durante 24h a 37°C/5%CO2. Os poços foram lavados com M199 sem soro e 1ml/poço de M199 contendo BSA 0,1% foi acrescentado. Após 24h de incubação a 37°C/5%CO2 o sobrenadante foi coletado para a dosagem da fibronectina (FN). Para tal, a placa de ELISA (contendo 96 poços) foi sensibilizada com 100µL de anticorpo policlonal anti-FN [Dako Patts, (10µg/mL), EUA] diluído em TBS, e mantida overnight a 37°C. No dia seguinte, os poços foram saturados com 0,1% de BSA em salina tamponada (TBS) por 2 horas a 37°C. Após lavagens com salina contendo Tween 20 0,05%, 100µl dos sobrenadantes das células foram adicionados em 6 poços e incubados por 1h a 37°C. Novamente os poços foram lavados com salina+ Tween 20 0,05%. O anticorpo anti-FN conjugado à peroxidase (Dako Patts, HPR - 1:4000) foi diluído em TBS e incubado por 1h a 37°C. Em seguida, os poços foram igualmente lavados e foram adicionados 100µL de OPD (*o*-fenilenodiamina - reagente cromogênico que produz uma coloração amarelo-alaranjada, ao reagir com produtos de degradação do peróxido de hidrogênio, catalisada pela peroxidase) na concentração de 1mg/mL diluído em tampão citrato 0,1 M, pH 4,5 com 0,03% H_2O_2. Após o aparecimento da cor, a reação foi paralisada com H_2SO_4 3M. A placa foi lida no leitor de ELISA a 490nm. O cálculo foi realizado baseado na curva padrão de FN purificada, contendo concentrações de 10ng/ml a 1000ng/ml.

Detecção e quantificação de FN na matriz extracelular por imunofluorescência. As células HUVECs ou pEPCs foram semeadas em lamínulas revestidas com 2% de gelatina, dentro de placas de 24 poços, na densidade de 1x105 células/poço em M199 suplementado com SFV 20% ou EGM-2, respectivamente, e foram incubadas durante 72h a 37°C/5%CO2. Os poços foram lavados com PBS com Ca^{++} e Mg^{++} e o tampão de extração de matrix extracelular (NH_4OH, 0,1M, Triton X-100, 0,1%, 1mM PMSF, leupeptina 40µM e PBS

0,1M com Ca^{++} e Mg^{++}) foi adicionado por cerca de 3 minutos até que as células fossem visualmente (ao microscópio) lisadas. Em seguida, os poços foram lavados com PBS com Ca^{++} e Mg^{++} e foram saturados com 5% de BSA. Após 1h de incubação à temperatura ambiente, os poços foram lavados com PBS/BSA 0,1%. O anticorpo primário anti-FN (1:50), diluído em PBS/BSA 3%, foi adicionado aos poços que foram novamente incubados por 1h à temperatura ambiente. Em seguida, após os poços terem sido lavados com PBS/BSA 0,1%, o anticorpo sencundário anti IgG de coelho, conjugado a isotiocianato de fluoresceína (FITC - 1:400), diluído em PBS/3%BSA foi adicionado. Finalmente, os poços foram lavados com PBS/BSA 0,1% e a lamínulas foram montadas com Vectashield. A quantificação da FN foi realizada através da análise morfométria, calculando a área marcada com FITC (fluorocromo verde). Dez campos de cada lamínula foram fotografados em objetiva de 100 × e analisados usando o programa Image-Pro Plus 5.0 (MediaCybernetics, USA).

Detecção de filamentos de actina (actina-F). Para este ensaio foi utilizada uma placa de 24 poços, contendo lamínulas estéreis revestidas com gelatina 2%. As células (HUVECs ou pEPCs/uEPCs) foram plaqueadas (2 x 10^5 células/poço) e mantidas em meio com SFB 20% a 37°C em atmosfera contendo 5% de CO2 até atingirem a semiconfluência. O primeiro passo foi a lavagem da placa uma vez com PBS, para retirar as células mortas ou que não aderiram a superfície do poço. A seguir as células foram fixadas em paraformaldeído 3,7% diluído em PBS por 10 minutos. A membrana plasmática das células foi permeabilizada com PBS contendo 0,1% de Triton X-100 por 6 minutos, para permitir a entrada de faloidina acoplada a isotiocianato de fluoresceína (ou faloidina/FITC) e sua ligação aos filamentos de actina (actin-F) no citoesqueleto. Em seguida, as células foram incubadas por 30 minutos com PBS contendo 1% de BSA, a fim de evitar assim interações inespecíficas. As células foram lavadas 3 vezes em PBS e incubadas por 1 hora com 1µg/mL faloidina/FITC, no escuro. Em seguida foi adicionado o corante nucelar DAPI por 5 minutos. Após a incubação, as lamínulas foram lavadas 3 vezes com PBS e por fim foram montadas com Vectashield® (Vector Laboratories, USA) para posterior visualização ao microscópio de fluorescência.

Análise fenotípica por citometria de fluxo. A expressão dos antígenos de superfície celular foi analisado em células progenitoras endoteliais humanas isoladas do sangue periférico de adultos (pEPCs) ou do sangue de cordão umbilical (uEPCs) pela medida de imunofluorescência com a técnica de citometria de fluxo. As EPCs foram descoladas com PBS EDTA 5nM, centrifugadas a 1.100 rpm (200 x g) por 10 minutos e ressuspendidas em

PBS 1x, gelado. Em seguida, as células foram fixadas com paraformaldeído 2% diluído em PBS durante 15 minutos. Após duas centrifugações em PBS contendo 1% de BSA, as células foram incubadas em tubos separados por 1h/temp. ambiente, com os anticorpos diretamente conjugados à ficoeritrina (PE): CD34, CD31 (PECAM-1) e CD133 ou com os anticorpos primários não conjugados: anticorpo monoclonal anti-sindecan (SDN-4 5G9, Santa Cruz Biotech, EUA). Após a centrifugação (200 x g – 10min) os tubos contendo anticorpos não conjugados foram incubados com o anticorpo secundário: antimouse TRITC (Dako), 1:100, por 1h/temp. ambiente. Finalmente as células foram centrifugadas (200 x g – 10min), ressuspendidas em PBS e analisadas no citômetro (FACSCalibur, Becton Dickinson). Em cada experimento utilizamos como controle o anticorpo IgG antimouse, correspondendo ao isotipo do anticorpo específico. A intensidade de fluorescência das células marcadas foi analisada com o software CellQuestTM. Na análise de expressão do SDN4, HUVECs foram utilizadas para comparação. As células foram utilizadas entre 30 a 40 dias de cultura.

RESULTADOS COMENTADOS

Isolamento e expansão de colônias de PB-ECFCs (células endoteliais formadoras de colônia isoladas do sangue periférico)

Um dos problemas relatados na literatura referente ao uso terapêutico de progenitores endoteliais é a reduzida quantidade destas células na circulação de adultos - tanto para estudar suas propriedades biológicas quanto para expansão visando sua reinjeção em tecidos afetados - aliada a um número limitado de duplicações *in vitro*, quando comparadas às CB-ECFCs (Ingram, 2004). No entanto, foi recentemente demonstrado que CB-ECFCs são capazes de provocar forte resposta imune alogênica em indivíduos receptores (Suárez *et al*., 2007), sugerindo, portanto, que a inoculação direta de CB-ECFCs em indivíduos adultos possa gerar rejeição. Assim, melhoramentos técnicos que visem otimizar a expansão de PB-ECFCs isoladas do sangue periférico são extremamente valiosos para a o desenvolvimento de transplantes autólogos, sem riscos de provocar rejeição imune dos progenitores inoculados em humanos.

Trabalhos prévios por outros autores sugerem que o contato com HUVECs em cultura podem servir como adjuvantes para o crescimento e expansão de PB-ECFCs em cultura (Yildirim *et al*., 2005; Li *et al*., 2006). Esses efeitos benéficos foram observados tanto com o cocultivo, quanto pela adição de meio condicionado por HUVECs. Dessa forma, decidimos

comparar o desenvolvimento de culturas de progenitores endoteliais na presença de meio condicionado de HUVECs confluentes, em cultura primária, produzidas rotineiramente em nosso laboratório, com o objetivo de otimizar a expansão da PB-ECFCs.

As HUVECs foram colocadas em cultura em M199, 20% de soro fetal bovino (SVF). Os meios condicionados foram incubados por 48 horas, centrifugados para remoção de debris e mantidos congelados a -20°C até sua utilização. O meio EGM-2, tradicional para cultivo de progenitores endoteliais, preparado com um coquetel de suplementos fornecido pelo fabricante. Preparamos uma mistura 1:1 (v/v) com o meio condicionado de HUVEC (M199/20%SFV), meio denominado EGM-2/ENR. Esse meio EGM-2/ENR foi utilizado como meio padrão para a cultura das células plaqueadas sobre a fibronecina, nas etapas de isolamento (cultura da porção mononucleares, não fracionada pela expressão de CD34) e de expansão (cultura de células ECFCs).

A Figura 1 mostra o desenvolvimento de uma cultura de PB-ECFCs. Colônias precoces (*early outgrowth cells*) surgem entre o 8º e 14º dias de cultura, acompanhadas de células fibroblastóides que tendem a desaparecer da cultura, coincidentemente com a detecção das primeiras colônias de células com morfologias em seixos (em torno do 20º dia de cultivo), compatíveis com o fenótipo endotelial (*late outgrowth progenitor cells*).

A Tabela 1 mostra a análise semiquantitativa dos diversos fenótipos celulares observáveis ao longo do desenvolvimento das culturas até o aparecimento de colônias de PB-ECFCs compatíveis com a morfologia endotelial, conforme analisado em quatro procedimentos de isolamento independentes. Os dados confirmam que a inclusão de meio condicionado de HUVECs propicia o aparecimento de colônias endoteliais em torno do 20º dia de cultura, enquanto que nenhuma colônia foi detectável utilizando-se somente o meio tradicional consagrado pela literatura da área.

Caracterização fenotípica das PB-ECFCs expandidas *in vitro*

Inicialmente, analisamos as PB-ECFCs por citometria de fluxo, quanto à expressão de um antígeno de superfície presentes em células progenitoras hematopoiéticas, o CD34. Investigamos ainda a expressão de antígenos de células endoteliais maduras, como o CD31, e um marcador de células consideradas imaturas, CD133. Para fins comparativos, alguns destes marcadores também foram avaliados em HUVECs, consideradas como um padrão de células endoteliais de vasos maduros.

A Tabela 2 apresenta o percentual de células positivas para uma série de marcadores de superfície que precisam ser monitorados nas culturas de progenitores, a fim de se

confirmar sua natureza endotelial. Foram analisadas populações de PB-ECFCs em passagem cinco (PB-ECFCs/p5), em comparação com HUVECs: as HUVECs, conforme esperado, possuem uma elevada proporção de células CD34+ e reconhecidamente não expressam CD133+, aqui confirmado. Por outro lado, as PB-ECFCs expandidas in vitro (p5, Tabela II; p4 e p3 - dados não mostrados) não apresentam baixa positividade para o marcador hematopoiético CD34 (5,72%).

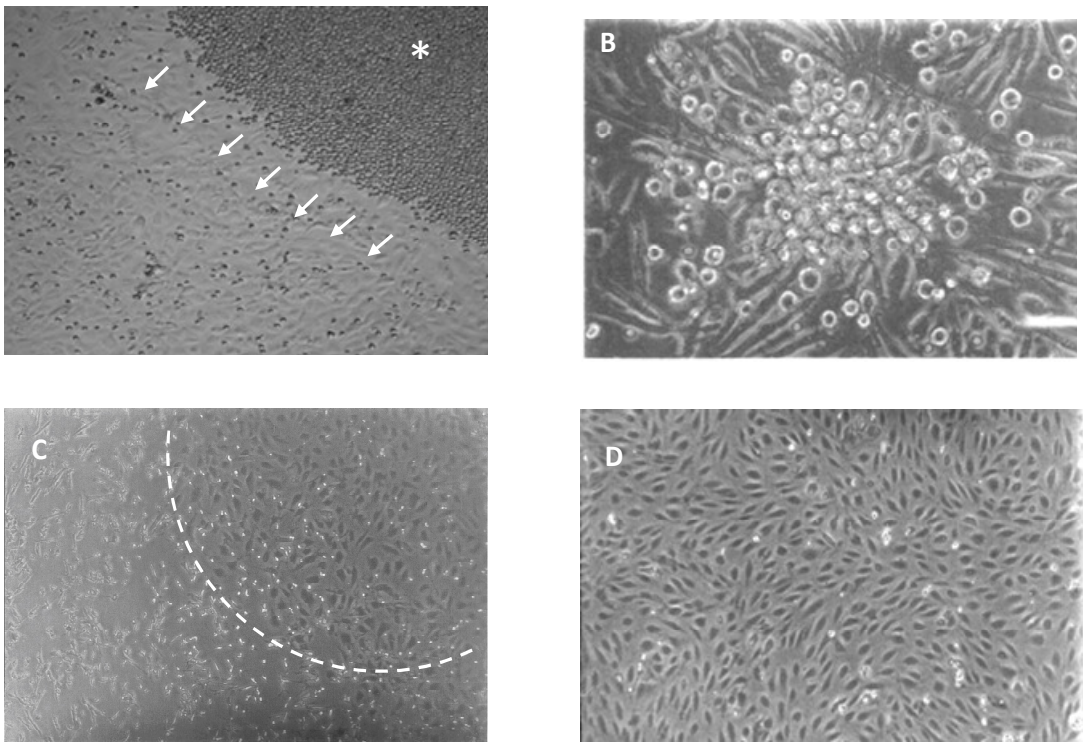

Figure 1 – Ex-vivo expansion of ECFC isolated from human peripheral blood, in a mixture of EBM-2 medium and conditioned medium from confluent HUVEC (1:1). Mononuclear cells isolated by fractioning 10 mL of buffy coat on a Histopaque 1077 density gradient were plated on fibronectin-coated culture flasks for up to 20 days (A-C). (A) and (B) By day 14, colonies (P_0) are detectable as clusters of round cells (star), bearing elongated, spindle-like cells at their borders (arrows); (C) By day 20, most round and spindle cells have disappeared from the culture, leaving in place colonies of cobblestone-like cells with typical endothelial morphology (border of the colony limited by the dashed line); (D) By day 30, after one replating round on fibronectin (P_1), colonies generate cobblestone endothelial-like monolayers. Magnification 100 × (A, C and D), and 200 × (D).

Table 1: Comparison of different culture media combinations for the obtention of late outgrowth colonies of peripheral blood endothelial progenitors

Days in culture	*Adhering cells*		*Spindle-shaped cells*		*Early outgrowth colonies*		*Isolated cobblestone cells*		*Cobblestone cell colonies (Late outgrowth colonies)*	
	EGM-2	*EGM-2/ENR*	*EGM-2*	*EGM-2/ENR*	*EGM-2*	*EGM-2/ENR*	*EGM-2*	*EGM-2/ENR*	*EGM-2*	*EGM-2/ENR*
01	++	++	0	0	0	0	0	0	0	0
04	++	+++	+	++	0	0	0	0	0	0
06	++	++++	+	++++	0	0	0	0	0	0
07	+++	+++++	++	++++	0	0	0	0	0	0
08	+++	+++++	++	+++++	+	+++	0	0	0	0
09	+++	+++++	++	+++++	++	+++++	0	0	0	0
10	+++	+++++	++	+++++	++	+++++	0	0	0	0
13	+++	++++++	+++	++++++	0	0	0	0	0	+
14	+++	++++++	+++	++++++	0	0	0	0	0	+
15	+++	++++++	+++	++++++	0	0	0	+++	0	++
16	++	++++++	+++	+++++++	0	0	0	+++++	0	+++++
17	++	++++++	+++	+++++++	0	0	0	+++++	0	+++++
18	++	++++++	+++	++++++++	0	0	0	+++++++	0	++++++
19	++	++++++	+++	++++++++	0	0	0	+++++++	0	++++++
20	++	++++++	+++	+++++++++	0	0	0	++++++++	0	+++++++

(+) represents a proportional incidence score, obtained by counting the indicated cells figures and/or colonies in five high-power fields when observing the adhesion, proliferation and differentiation of mononuclear cells isolated from buffy coats of human peripheral blood from healthy donors (a representative profile; n = 4)

A falta de uniformidade na expressão de marcadores hematopoiéticos, como CD34, mesmo dentro de um lote de PB-ECFCs já foi descrita por outros (Ingram *et al*., 2004). Para PB-ECFCs/p5, a expressão de CD45 foi praticamente indetectável. O antígeno CD146 (MUC 18), uma molécula de adesão interjuncional envolvida no tráfego de leucócitos e células tumorais através do endotélio, tem sido considerado um marcador da natureza endotelial também em ECFCs (Timmermans *et al.*, 2009). Neste trabalho, detectamos a expressão deste marcador em PB-ECFCs (92,3%).

Dados obtidos previamente em nosso laboratório mostram que as PB-ECFCs/p5, quando comparadas a HUVECs (células endoteliais consideradas maduras), apresentam ainda uma forte marcação nuclear para o fator transcripcional GATA-2, normalmente detectado em células de origem hematopoiéticas que guardam elevada capacidade proliferativa (Kocher *et al*., 2001). As PB-ECFCs/p5 expressam ainda o receptor endotelial VE-caderina, além do marcador endotelial fator de Von Willebrand (dados não mostrados).

É preciso considerar que, na literatura, não existe um consenso quanto à caracterização definitiva das PB-ECFCs, visto que os muitos marcadores descritos estão presentes também em células endoteliais de vasos maduros (Richardson & Yoder, 2011), conforme confirmamos aqui. O fato de não ser detectada a expressão de CD133 nas PB-ECFCs cultivadas não está em desacordo com a literatura atual da área: existem estudos que demonstraram que as EPCs $CD133^{+}$ não dão origem a células endoteliais maduras (EC) *in vitro* (Timmermans *et al.*, 2009), além de outros sugerirem que a perda do marcador CD133 define o momento em que PB-ECFCs se tornam EC (Janic *et al.*,2010). Porém, parece existir um consenso que as PB-ECFCs que originam ECs não expressam os marcadores de leucócitos, CD14 e CD45 (Smadja *et al*., 2011).

Table 2. Flow cytometric analysis of some phenotypic markers relevant of significance in HUVEC and endothelial colony forming cells from human peripheral blood (ECFC)

	HUVEC/p1	PB-ECFC/p5
Phenotypical cell markers	*Positive cells (%)*	
CD31	99,05	99,55
CD45	18,9	23,3
CD133	0,01	0,02
CD105	99,3	97,0
KDR	53,25	70,5
CD146	72,35	92,3
CD34	49,6	5,72
CXCR4	35,0	36,8
CD117(c-Kit)	0,58	3,65

Dessa forma, as PB-ECFCs aqui caracterizadas, quando expandidas *in vitro*, são populações $CD45/CD133^{-}/CD34^{+/-}$ que expressam antígenos extremamente relacionados às funções endoteliais especializadas, como CD31, VE-caderina e KDR/VEGFR2. Além destas características, deve-se observar que estes progenitores são células formadoras de colônias de crescimento tardio, de elevado potencial proliferativo, com morfologia e funções endoteliais

reprodutíveis *in vitro* e *in vivo*, portanto compatíveis com as PB-ECFCs descritas na literatura.

As PB-ECFCs secretam três vezes menos fibronectina (FN) no meio e na matriz extracelular (MEC) do que HUVECs.

Observações anteriores feitas pelo nosso grupo estabeleceram que um dos mecanismos centrais do efeito estimulatório da tubulogênese endotelial, pelos peptídeos TSP Hep I e TSP Hep II é a competição pelo domínio Hep II da fibronectina (FN) pela ligação ao sindecan-4 (Nunes *et al*, 2008). Estimamos que tal interferência leve ao relaxamento da adesão mediada por adesões focais e, consequentemente, à adoção de uma maior plasticidade celular, a qual seria favorecedora da migração celular e da tubulogênese.

Após a confirmação de que as PB-ECFCs disponíveis para este estudo possuem marcadores importantes de células endoteliais funcionais - pela expressão de CD31 e VE-caderina - bem como se caracterizam pela ausência de marcadores CD14 e CD45, voltamos nossa atenção para as características funcionais destas células progenitoras, particularmente o perfil adesivo relevante para a interpretação dos efeitos biológicos dos peptídeos pró-angiogênicos derivados da TSP-1, que podem interferir funcionalmente com a adesão celular mediada por FN.

Considerando ainda que o relaxamento coordenado da adesão celular em áreas específicas da superfície celular é um dos principais fatores que predispõem as células ao estado proliferativo e migratório, resolvemos verificar se haveria diferença na expressão de FN pelas PB-ECFCs. Para tanto, avaliamos quantitativamente a presença de FN no sobrenadante e na matriz secretada por essas células. Como descrito em “Materiais e Métodos”, o sobrenadante de HUVECs e PB-ECFCs foi dosado pelo método de ELISA. De forma não esperada, observamos que as HUVECs secretam cerca de três vezes mais FN do que as PB-ECFCs (Figura 2A). Quantificamos ainda a FN da MEC após a lise da camada celular e revelação por imunofluorescência. Mais uma vez observamos que a quantidade de proteína depositada na matriz de HUVECs é muito maior quando comparamos com as PB-ECFCs (Figura 2B-C).

A informação de que a quantidade de FN secretada por células endoteliais progenitoras é menor do que a produzida por endotélio oriundo de vasos maduros ainda não foi descrita na literatura e está em concordância com o fenótipo de elevado potencial proliferativo das PB-ECFCs: há bastante tempo já é conhecido que a expressão desta

glicoproteína adesiva matricial também se encontra diminuída em diversas células proliferativas/migratórias de caráter tumoral (Hynes e Yamada, 1982).

As PB-ECFCs exibem um rearranjo de filamentos de actina compatível com um fenótipo migratório.

Schmidt e colaboradores (2007) propuseram que angioblastos e ECFCs possuem uma capacidade de migrar muito maior que células mais maduras. Essa hipótese é consistente com o fato de que as PB-ECFCs originadas da medula óssea sejam recrutadas para a circulação durante uma situação de lesão vascular ou isquemia. Pelo fato de as PB-ECFCs serem células que circulam no fluxo sanguíneo e de as HUVECs apresentarem o fenótipo de células aderentes, fomos buscar se, em paralelo com o padrão modificado de expressão de da FN, a principal proteína envolvida na adesão focal, as PB-ECFCs também apresentavam modificações importantes no arranjo do citoesqueleto de actina, principal componente intracelular deste complexo juncional.

Realizamos a marcação com faloidina marcada com FITC para verificar a organização dos filamentos de actina no citoesqueleto de PB-ECFCs, em comparação com HUVECs. Observamos nas HUVECs a presença de filamentos reativos para a faloidina espalhados por toda a extensão celular, organizados paralelamente à membrana plasmática e dispostos em várias direções (Figura 2D). Nas PB-ECFCs, ocorre uma significativa diminuição de fibras de estresse, com os microfilamentos se localizando preferencialmente na proximidade da membrana plasmática, em disposição cortical e pouca marcação na região central das células. Além disso, foi observada uma grande proporção de células exibindo evidentes lamelipódios e filopódios, características marcantes de células de fenótipo migratório. Tais estruturas não são observadas em HUVECs semeadas nas mesmas condições. Apesar dessas importantes diferenças fenotípicas, as PB-ECFCs expandidas em vitro conservam a capacidade de se diferenciar em estruturas tubulares, considerada uma das principais propriedades de células endoteliais com fenótipo angiogênico (Figura 3), comparáveis às formadas por células endoteliais de fenótipo mais maduro, como as HUVECs.

Papel de peptídeos derivados do domínio N-terminal (NH_2) da TSP-1 (TSP Hep I e TSP Hep II) na modulação angiogênica de PB-ECFCs.

Dessa maneira, tendo sido comprovado que nosso protocolo de isolamento e expansão modificados resulta em PB-ECFCs que conservam as características endoteliais primordiais,

passamos a avaliar se estes progenitores podem ter suas propriedades angiogênicas otimizadas pelos peptídeos pró-angiogênicos de TSP-1, já estudados por nosso grupo em células de endotélio maduro.

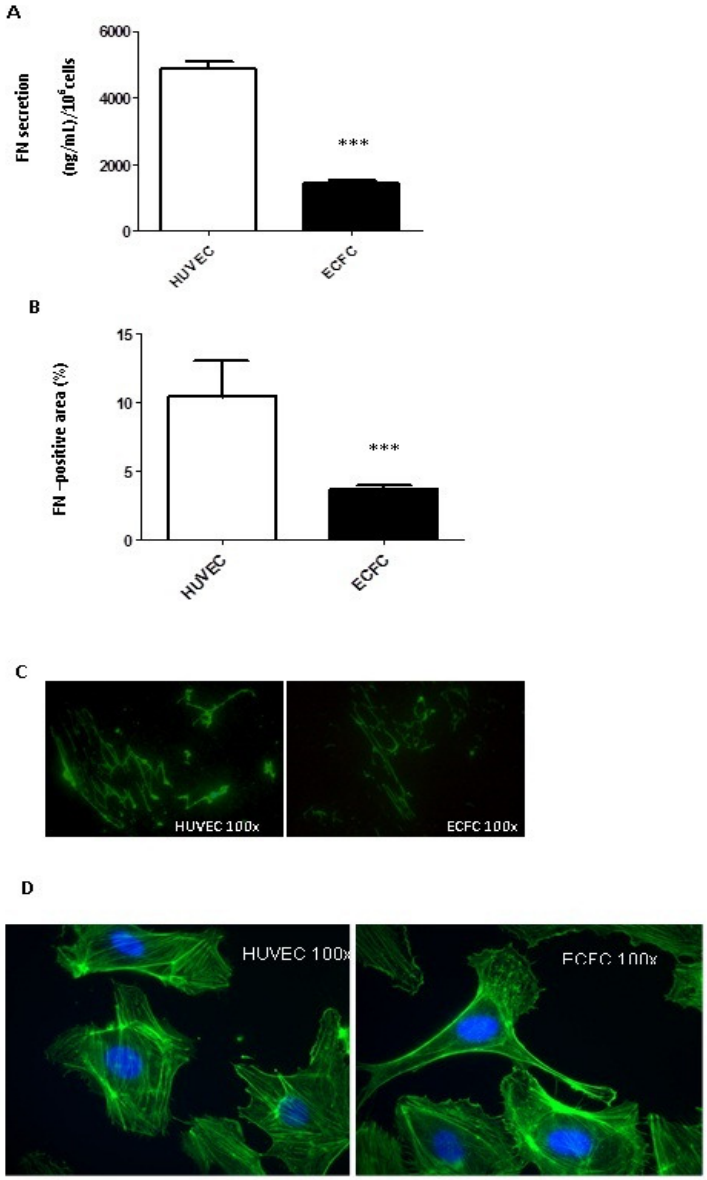

Figure 2: Quantification of fibronectin (FN) secretion and incorporation to the PB-ECFC extracellular matrix. (A) After 24 hours in culture, in serum-free M199 supplemented with 0.1% BSA, conditioned media from PB-ECFC (passage 11) or HUVEC were collected and analysed by a quantitative direct ELISA, as described in Methods. (B) Morphometric analysis of matrix-bound FN, immunostained with an anti-FN polyclonal antibody followed by incubation with anti-rabbit/FITC conjugate (green). (C) Images of at least 10 fields were captured and fluorescence intensity was quantified using the Image-Pro 5.0 software; (D) Microfilament organization of PB-ECFC, as compared to HUVEC, was visualized following incubation of adhering cells with 1 mg/mL phalloidin-FITC reagent, using a Nikon Eclipse E400 epifluorescence microscope (100 ×). Each experiment was done at least three times, with comparable results. ***$\rho < 0.001$.

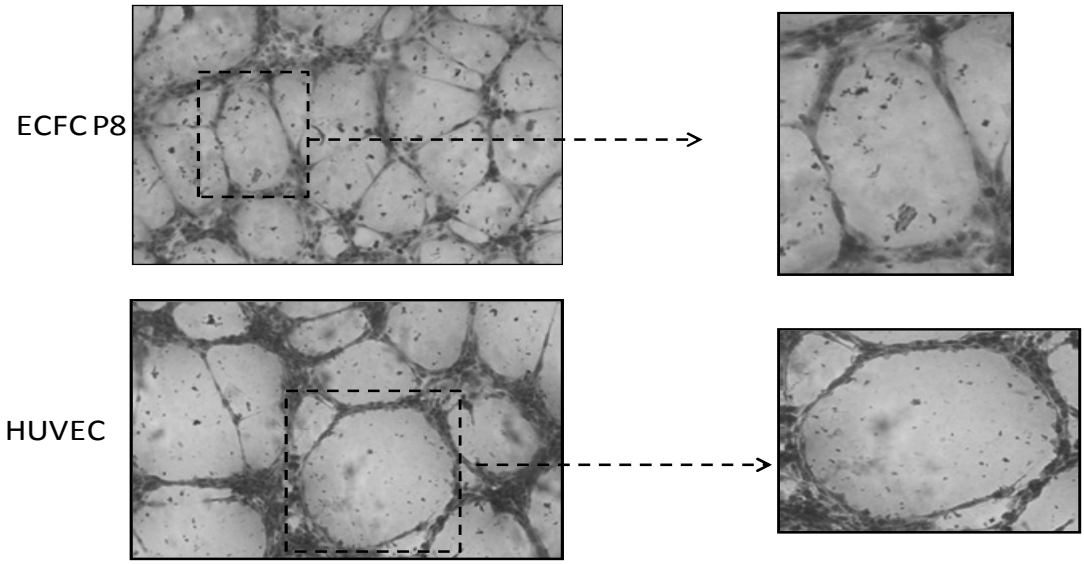

Figure 3 - Tubulogenesis of in vitro expanded peripheral blood ECFC/p8 and HUVEC in Matrigel. Cells ($1x10^5$ células/cm^2) were seeded onto growth factor-reduced Matrigel ™ (BD Bioscience) and allowed to differentiate for 6 hours. Gels were fixed with 1.1% glutaraldehyde and stained with the Panotic histochemical dye system. Magnification: 200 ×.

Conforme já mencionado, nosso grupo identificou propriedades angiogênicas tanto em um fragmento de 18 KDa do domínio N-terminal da TSP-1 (TSP18, sequência de aminoácidos 1-174), quanto em peptídeos derivados desta região da proteína (TSP HepI/A1 ou TSP HepII/A2), que possuem motivos estruturais de caráter básico de ligação de alta afinidade a glicosaminoglicanos. Demonstramos ainda que os efeitos são mediados pelo proteoglicano heparan-sulfato, sindecan-4 (SDN4) (Ferrari do Outeiro-Bernstein *et al*., 2002; Nunes *et al*., 2008).

Primeiramente, realizamos uma análise por citometria de fluxo para avaliar o perfil das PB-ECFCs quanto à expressão do SDN4 na superfície celular e observamos que estas células expressam o receptor em níveis comparáveis às HUVECs (Figura 4), bem como da isoforma sindecan-2 (SDN2), um outro proteoglicano que não está envolvido na modulação de contatos focais dependentes de fibronectina (Tckachenko *et al*., 2005). Passamos então a avaliar os possíveis efeitos dos peptídeos sobre o comportamento adesivo, migratório e proliferativo de PB-ECFCs.

Os peptídeos TSP HepI/A1 e TSP HepII/A2 foram imobilizados em suportes de poliestireno e as PB-ECFCs foram semeadas e permitidas aderir por 2h. Observamos que ambos os peptídeos foram capazes de mediar a adesão das PB-ECFCs quando comparados ao suporte controle não aderente (Figura 5A).

Dados prévios de nosso grupo mostraram que os peptídeos TSP Hep I e TSP Hep II foram capazes de estimular a migração de HUVECs e que o efeito obtido foi comparável à migração induzida pelo fator angiogênico $VEGF_{165}$ (dados não publicados).

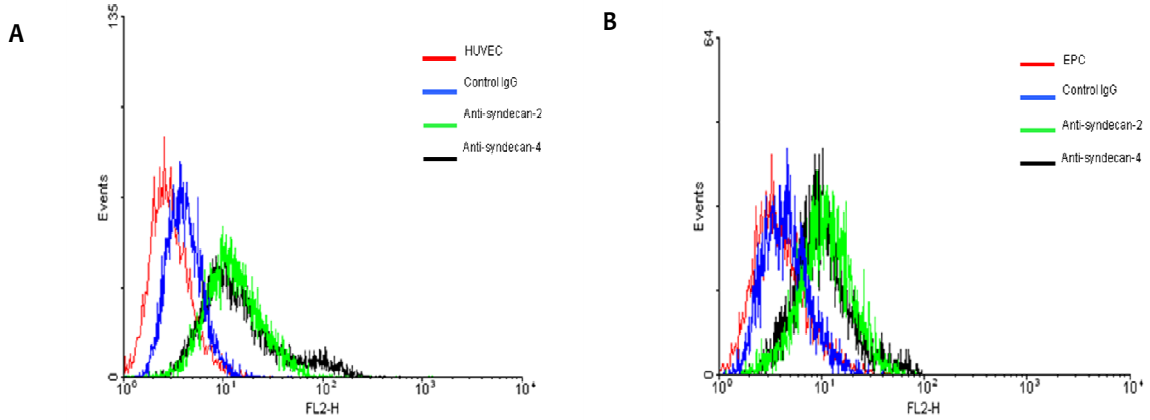

Figure 4 – Expression of syndecans 2 and 4 by ECFC, as compared to HUVECs. ECFC cells expanded for 5 passages (ECFC/p5), were analyzed by FACS for the expression of SND2 (A) and SND4 (B), as described in the Methods section.

A fim de verificar o efeito desses peptídeos como fatores quimiotáticos para EPCs, foram utilizados ensaios de migração celular, usando-se insertos de transmigração com porosidade de 8 µm. As PB-ECFCs foram semeadas na parte superior e os peptídeos a serem testados foram depositados no compartimento inferior delimitado pelo inserto. Pudemos observar que ambos os peptídeos TSP HepI/A1 e TSP HepII/A2 foram capazes de estimular a migração celular, quando comparado ao controle. Esse resultado foi comparável à quimiotaxia celular induzida pelo FGF-2 (Figura 5B).

Ao lado da regulação do comportamento adesivo e migratório, é importante estudar outras etapas que são fundamentais para a diferenciação angiogênica de PB-ECFCs, que pudessem ser reguladas pelos peptídeos TSP HepI e TSP Hep II, como a proliferação e tubulogênese endoteliais. Testamos o efeito dos peptídeos TSP Hep I/A1 solúvel sobre a proliferação de PB-ECFCs, avaliada após 72 horas de crescimento. As PB-ECFCs foram semeadas em meio contendo soro e o peptídeo TSP HepI/A1 (10µg/ml). Não observamos alteração da proliferação de PB-ECFCs na presença do peptídeo TSP HepI/A1 (Figura 5C). O ensaio de diferenciação angiogênica em Matrigel permite avaliar o potencial angiogênico de células endoteliais, com base na capacidade destas de formarem estruturas semelhantes a

tubos (*capillary-like*), portadoras de lúmen. Nesse experimento, estudamos os peptídeos TSP HepI/A1 e TSP HepII/A2 imobilizados no Matrigel na concentração de 10µg/ml. Observamos que a presença de ambos os peptídeos foram capazes de induzir uma intensa diferenciação PB-ECFCs em estruturas tubulares, quando comparados ao controle (Figura 5D). Esse resultado está de acordo com prévios trabalhos do grupo que demonstrou os efeitos pró-angiogênicos destes peptídeos sobre a diferenciação de HUVECs, já comentados.

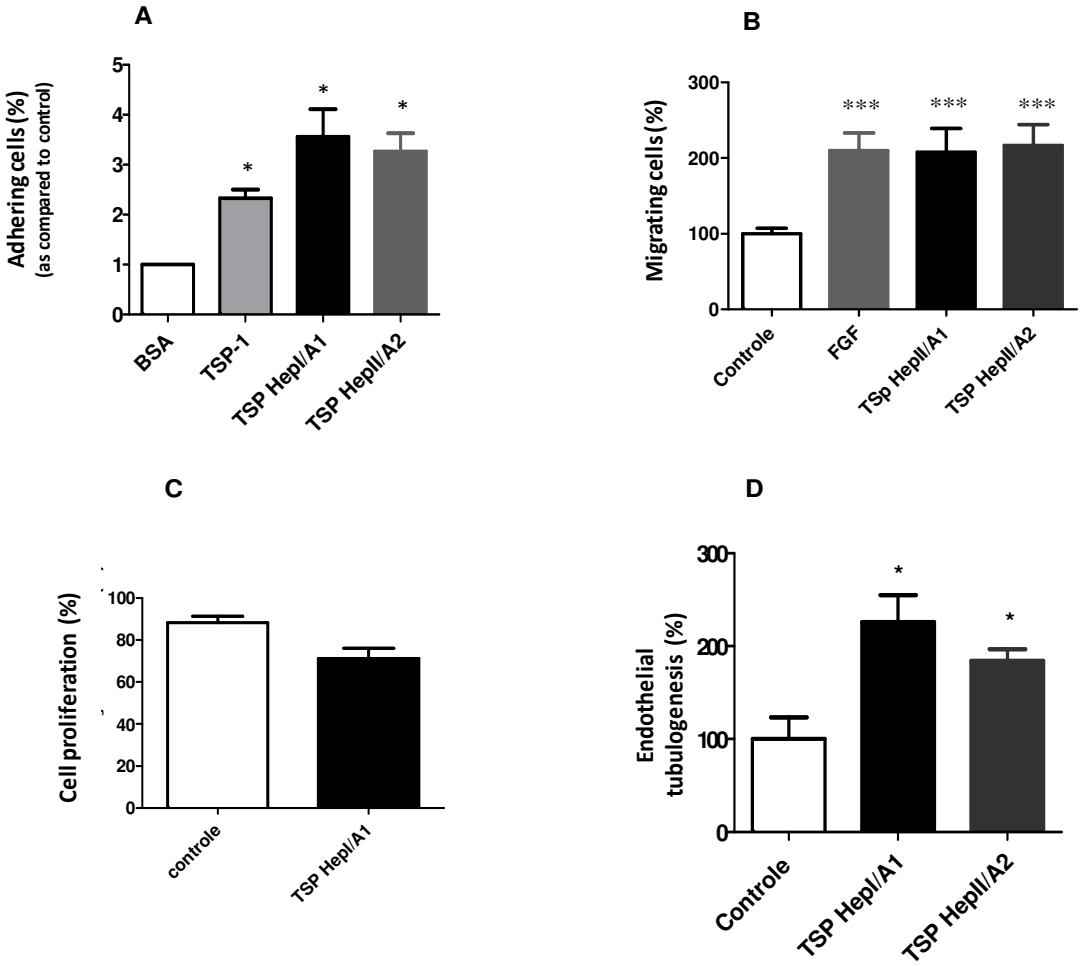

Figure 5: TSP Hep I and TSP Hep II peptides modulate pro-angiogenic properties of ECFCs from human peripheral blood. (A) ECFC were seeded onto purified TSP-1 or peptides immobilized to plastic dishes for 2 hours and adhering cells were then quantified by the MTT method. Cells adhering on BSA-coated wells were set as 01 arbitrary adhesion unit; (B) Chemotaxis of ECFC (7 x 10^4) towards soluble TSP HepI/A1, TSP HepII/A2 (both at 20µg/ml) or FGF-2 (10 ng/ml) was measured using 8µm-porous inserts, after 6 hours of incubation, as described in the Methods section; (C) ECFC (1 x10^4 cells/well) proliferation in the presence of TSP HepI peptide (10µg/ml) was measured after 72 hours by the MTT method; (D) ECFC (1x10^5 cells/cm2) were seeded onto growth factor-reduced Matrigel™ (BD Bioscience) and allowed to differentiate for 6 hours. Gels were fixed with 1.1% glutaraldehyde and colored with the Panotic histochemical dye system. Tube-like structures were counted in 3-6 fields in each well, as described in the Methods section.). ***p<0.001 and *p<0.05, as compared to the control condition.

3.2 **A trombospondina-1 é um marcador plasmático para a doença arterial periférica (DAP) que modula as propriedades angiogênicas das células progenitoras endoteliais**

Título: Thrombospondin-1 is a plasmatic marker of peripheral arterial disease that modulates endothelial progenitor cell angiogenic properties. Arterioscler Thromb Vasc Biol. Mar 2011. Mar;31(3):551-9.

Autores: Smadja DM, d'Audigier C, Bièche I, Evrard S, Mauge L, Dias JV, Labreuche J, Laurendeau I, Marsac B, Dizier B, Wagner-Ballon O, Boisson-Vidal C, Morandi V, Duong-Van-Huyen JP, Bruneval P, Dignat-George F, Emmerich J, Gaussem P.

Considerações Gerais:

Este trabalho foi desenvolvido em colaboração com a Université René Descartes (Paris V - França), como parte de um acordo de cotutela apoiado pelo Programa CAPES-COFECUB, sob a coorientação da Dr. Catherine Boisson-Vidal.

No artigo 2, descrevemos o trabalho realizado em colaboração com o grupo dos Drs. Joseph Emmerich e Dr. Pascale Gaussem. Este grupo faz parte do projeto multicêntrico OPTIPEC (Optimization of Progenitor Endothelial Cells in the Treatment of Critical leg ischemia) focado na terapia celular para pacientes com doença arterial periférica (do inglês peripheral arterial disease ou PAD). O objetivo deste trabalho foi encontrar fatores angiogênicos que poderiam estar alterados no plasma de pacientes PAD que poderiam ser utilizados como um biomarcador plasmático dessa doença isquêmica. Os resultados descritos aqui fazem parte do estudo clínico de fase 1 (clinical trial, phase 1) realizado com tais pacientes portadores de PAD, submetidos ou não à terapia com células mononucleares da medula óssea (do inglês Bone Marrow Endothelial Cells ou BM-MNCs).

Neste trabalho, colaboramos para o entendimento do papel da trombospondina-1 (TSP-1) e seus domínios na regulação do processo angiogênico envolvido na PAD. Como relatado na revisão da literatura, a TSP-1 foi primeiramente descrita como uma proteína antiangiogênica. Em contrapartida, o efeito pró-angiogênico da TSP-1 é localizado em sequências peptídicas dentro do domínio N-terminal (NH_2) da TSP-1, que foram descritos no capítulo anterior (Artigo 1), serem capazes de estimular a diferenciação angiogênica de HUVEC e de ECFCs isoladas de sangue periférico. Aqui nós demonstramos que pacientes PAD apresentaram altos níveis de TSP-1 no plasma. Além disso, após o tratamento de tais pacientes com BM-MNCs, a TSP-1 estava presente nos tecidos que apresentavam intensa

neovascularização. Como relatado em um prévio trabalho do grupo (Smadja et al, 2008), as células endoteliais presentes nos vasos recém-formados (neovascularização) de pacientes que receberam injeção terapêutica de BM-MNCs parecem apresentar um fenótipo do tipo ECFC. Portanto, questionou-se o papel da TSP-1 na modulação da angiogênese induzida pelas ECFCs.

Neste estudo utilizamos ECFCs isoladas do sangue de cordão umbilical com base na expressão da molécula de superfície CD34. As ECFCs, previamente caracterizadas pelo grupo da Dra. Catherine Boisson-Vidal, expressam os marcadores endoteliais CD31/Tie-2/ KDR (Flk-1 ou VEGFR2) / Flt-1(VEGFR1) /CD144. Além disso, as ECFC não expressam CD14 e CD45 moléculas de superfície presente em leucócitos (Dados suplementares deste artigo 2 – Figura 1). Para demonstrar o papel da TSP-1 na modulação destas células utilizamos além da proteína monomérica, o peptídeo TSP-HepI (que ao longo de nossos estudos demonstrou possuir um potencial mais angiogênico que o TSP-HepII, resultados apresentados no Capítulo III, como dado suplementar). Avaliamos, portanto, o efeito do peptídeo TSP-HepI e da TSP-1 sobre as ECFCs isoladas do sangue de cordão umbilical nos seguintes ensaios in vitro: (a) determinação da adesão estática e proliferação das ECFCs, ensaio no qual o peptídeo ou a TSP-1 foram imobilizados; (b) estudo da tubulogênese induzida pelas ECFCs, no qual o peptídeo ou a TSP-1, foi misturada ao Matrigel antes da polimerização e as células foram permitidas diferenciar na presença ou na ausência destas proteínas.

O artigo foi publicado na Artheriosclerosis Thrombosis and Vascular Biology em 2011 e se encontra nas páginas a seguir, tendo como principal conclusão a confirmação de que a TSP-1 possui um duplo papel na angiogênese e que provavelmente esta propriedade é relevante no contexto fisiopatológico, como no da lesão isquêmica. O efeito antiangiogênico se dá pela inibição da proliferação e da tubulogênese induzida por ECFCs isoladas de sangue de cordão umbilical, efeitos que são provavelmente mediados pela porção C-terminal, enquanto a adesão estática destas células foi estimulada. Este último efeito também foi observado utilizando o peptídeo TSP-HepI, sugerindo que os efeitos positivos são modulados pelo domínio NH_2. Além disso, o peptídeo foi capaz de estimular a tubulogênese de ECFCs e não interferiu na proliferação endotelial. Esses resultados foram considerados motivadores, visando aplicações futuras da TSP-1 e seus fragmentos do domínio NH_2 como agentes terapêuticos capazes de modular a atividade angiogênica das ECFCs recrutadas para sítios de isquemia, que precisam aderir e se diferenciar em novos vasos.

Thrombospondin-1 Is a Plasmatic Marker of Peripheral Arterial Disease That Modulates Endothelial Progenitor Cell Angiogenic Properties

David M. Smadja, Clément d'Audigier, Ivan Bièche, Solène Evrard, Laetitia Mauge, Juliana-Vieira Dias, Julien Labreuche, Ingrid Laurendeau, Bérengère Marsac, Blandine Dizier, Orianne Wagner-Ballon, Catherine Boisson-Vidal, Verônica Morandi, Jean-Paul Duong-Van-Huyen, Patrick Bruneval, Françoise Dignat-George, Joseph Emmerich, Pascale Gaussem

Objective—We examined whether plasma levels of angiogenic factors are altered in plasma of patients with peripheral arterial disease (PAD) and whether these factors affect endothelial progenitor cell–induced angiogenesis.

Methods and Results—Plasma was collected from 184 patients with PAD and 330 age-matched healthy controls. Vascular endothelial growth factor and placental growth factor concentrations did not differ between the groups, whereas we found a linear correlation between PAD disease and thrombospondin (TSP)-1 plasma level. TSP-1 was expressed in newly formed vessels in PAD patients having received local injections of bone marrow mononuclear cells. To analyze the functional role of TSP-1 during neoangiogenesis, we used a Matrigel plug assay and showed that vascularization of implanted Matrigel plugs was increased in *TSP-1*$^{-/-}$ mice. Moreover, injections of TSP-1 in C57Bl6/J mice after hindlimb ischemia induced a significant decrease of blood flow recovery. To investigate the effects of TSP-1 on human endothelial colony-forming cell (ECFC) angiogenic potential, recombinant human TSP-1 and a small interfering RNA were used. In vitro, TSP-1 N-terminal part significantly enhanced ECFC adhesion, whereas recombinant human TSP-1 had a negative effect on ECFC angiogenic potential. This effect, mediated by CD47 binding, modulated stromal cell–derived factor 1/CXC chemokine receptor 4 pathway.

Conclusion—TSP-1 is a potential biomarker of PAD- and ECFC-induced angiogenesis, suggesting that TSP-1 modulation might improve local tissue ischemia in this setting. (Clinical trial registration: NCT00377897.) (***Arterioscler Thromb Vasc Biol*. 2011;31:00-00.)**

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Key Words: angiogenesis ■ arterial thrombosis ■ endothelial progenitor cells

Peripheral arterial disease (PAD), characterized by atherosclerosis of the lower extremities, affects up to 15% of people older than 55 years.[1] The main clinical manifestations of PAD are intermittent claudication and critical limb ischemia (CLI). Intermittent claudication is characterized by reproducible pain on exertion that is relieved by rest. CLI is the most severe form of PAD and is characterized by the inability of arterial blood flow to meet the metabolic demands of resting muscle or tissue, resulting in rest pain and/or tissue necrosis and frequently necessitating amputation. Currently, PAD diagnosis is based on the ankle–brachial systolic pressure (ABI), but the ABI is a poor marker of PAD severity. There are no other reliable diagnostic tests for PAD, and new biomarkers would therefore be useful.

Atherosclerosis induces occlusion of the arterial tree and tissue hypoxia, which is a strong stimulus for angiogenesis. Collateral vessels develop physiologically in patients with CLI, mainly driven by an enhanced angiogenic response.[2] However, the capacity of this compensatory mechanism is rapidly exceeded, and normal flow is not restored. Autologous endothelial progenitor cells (EPCs) are candidates for angiogenic therapy. Because of their scarcity in human samples, EPCs have been characterized by culture methods. At least 2 populations of EPCs have been described.[3] "Early" EPCs appear within 4 to 7 days of culture, whereas "late" EPCs, also called endothelial colony-forming cells (ECFCs),[4,5] develop after 2 to 3 weeks and have the characteristics of precursor cells committed to the endothelial lineage. We have previously shown, in amputation specimens from patients with CLI who had received local therapeutic injections of bone marrow mononuclear cells (BM-MNCs), that endothelial cells from newly formed vessels have an ECFC pheno-

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From the Université Paris Descartes (D.M.S., C.d'A., I.B., S.E., L.M., I.L., B.M., J.-P.D.-V.-H., P.B., J.E., P.G.), Paris, France; INSERM UMRS 765 (D.M.S., C.d'A., S.E., L.M., J.-V.D., B.M., B.D., C.B.-V., J.E., P.G.), Paris, France; AP-HP (D.M.S., C.d'A., S.E., L.M., J.-P.D.-V.-H., P.B., J.E., P.G.), Hôpital Européen Georges Pompidou, Paris, France; INSERM UMRS 745 (I.B., I.L.), Paris, France; Departamento de Biologia Celular (J.-V.D., V.M.), Universidade do Estado do Rio de Janeiro, Brazil; INSERM UMRS 698 (J.L.), Paris, France; INSERM UMRS 1009 (O.W.-B.), Paris, France; INSERM UMRS 872 (J.-P.D.-V.-H., P.B.), Paris France; and INSERM UMRS 608 (F.D.-G.), Paris, France.

Correspondence to Dr David Smadja, Faculté of Pharmacie, 4 Avenue de l'Observatoire, 75006 Paris, France. E-mail david.smadja@egp.aphp.fr

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type.[6] This is in keeping with results obtained with preclinical models showing that ECFCs are the cell type responsible for blood vessel formation.[5]

A number of angiogenic growth factors may modulate vascular growth and EPC angiogenic potential during PAD. Vascular endothelial growth factor (VEGF) is probably 1 of the most important proangiogenic factors.[7,8] Elevated VEGF plasma levels were found in a study of 46 PAD patients.[9] Placental growth factor (PlGF), which is not required for normal embryonic vascular development, could also play a critical role in pathological angiogenesis.[10] Antiangiogenic factors could likewise be involved in PAD. Thrombospondin (TSP)-1 has been found to be overexpressed in histological sections of human and mouse ischemic leg tissues.[11,12] However, plasma TSP-1 levels have not yet been measured in PAD patients.

Here, we compared concentrations of the circulating angiogenesis-related factors VEGF, PlGF, and TSP-1 in 184 PAD patients enrolled in the PALLAS clinical study[13–15] and in 330 paired healthy controls. Because TSP-1 levels were higher in the patients than in the controls, we further investigated the effect of TSP-1 on the ECFC angiogenic properties.

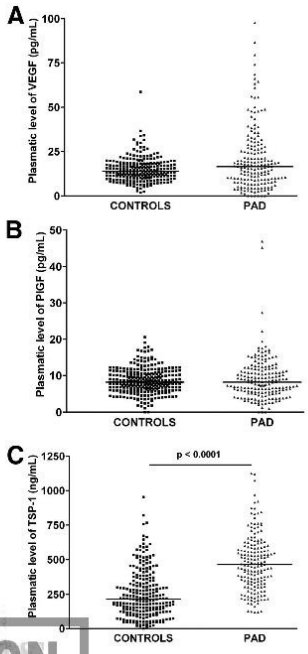

Figure 1. Plasmatic levels of TSP-1 are increased in patients with peripheral arterial disease. No significant difference in plasmatic levels of VEGF (A) or PlGF (B) was found between PAD patients and age-matched controls (n=330). PAD patients had higher TSP-1 levels (C) than controls.

Methods

Study Population for Plasma Concentrations of VEGF, PlGF, and TSP-1

PAD patients (n=184) were enrolled in our vascular medicine department over a 2-year period. Patients were eligible if they were white men younger than 70 years with symptomatic atherosclerotic disease of the lower limbs and an ankle–brachial systolic pressure index (ABI) of <0.90, or a history of surgical or endovascular revascularization, as previously reported.[13–15] Age-matched control subjects (n=330) with no history of arterial disease were randomly selected as described previously.[16]

Local Injection of BM-MNCs in PAD Patients With CLI

The OPTIPEC clinical trial was a phase I nonrandomized study. Briefly, patients were eligible for the protocol if they had CLI associated with limited gangrene or a nonhealing ischemic ulcer and if they were not eligible for surgical revascularization or percutaneous angioplasty, or if such a procedure had little chance of success. The cell therapy protocol was similar to that initially published by Tateishi-Yuyama et al.[17]

ELISA Assays, ECFC Culture, In Vitro Angiogenesis Assays, In Vivo Matrigel Plug Assay, and Hindlimb Ischemia Model

Experiments are described in detail in the online Data Supplement at http://atvb.ahajournals.org.

Results

Angiogenic-Related Factors in PAD

The baseline demographic and clinical characteristics of the study population are described elsewhere.[13–15] Briefly, smoking, hypertension, diabetes, serum lipids, lipid-lower therapy, and glucose level were significantly different between patients with PAD (n=184) and controls (n=330) (Table I in the online Data Supplement).

Plasma concentrations of VEGF, PlGF, and TSP-1 were determined in patients and controls. VEGF and PlGF levels did not significantly differ between cases and controls (Figure 1A and 1B), whereas TSP-1 levels were significantly higher in PAD patients (Figure 1C; $P<0.0001$). After categorization in tertiles of plasma levels of VEGF, PlGF, and TSP-1, we found a U-shaped relationship between PAD and VEGF or PlGF plasmatic levels (supplemental Table II). Indeed, we found that upper and lowest tertiles were associated with disease. Concerning TSP-1, we found a linear correlation between occurrence of PAD disease and TSP-1 plasma level. Similar results were found after adjustment for common PAD risk factors (hypertension, hypercholesterolemia, diabetes, and smoking status), lipid-lowering treatment, and C-reactive protein. We next assessed whether common PAD risk factors affected VEGF, PlGF, and TSP-1 concentration. VEGF and PlGF significantly correlated with C-reactive protein. In addition, PlGF correlated with age, hypertension, and antiplatelet therapy. TSP-1 plasmatic levels were not associated with clinical parameters (supplemental Table III). Whereas TSP-1 levels were associated with the occurrence of PAD, we found no significant difference in plasma levels of VEGF, PlGF, and TSP-1 according to disease severity (supplemental Table IV).

Taken together, these results suggest that TSP-1 plasma level is the only angiogenic factor among those tested that is linearly associated to PAD.

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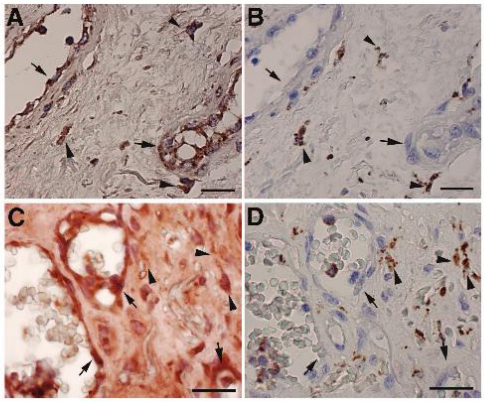

Figure 2. TSP-1 are expressed in newly formed vessels after cell therapy product in peripheral arterial disease. TSP-1 was expressed by endothelial cells (black arrows) and macrophages (black arrowheads) in amputation specimens from CLI patients treated (C) and untreated (A) with local injections of BM-MNCs. Scale bar=50 μm. Macrophage CD68-positive cells are shown in serial sections of amputation specimens from CLI patients treated (D) and untreated (B) with local injections of BM-MNCs. Scale bar=50 μm.

TSP-1 Is Expressed by Endothelial Cells in Critical Leg Ischemia Patients and in Newly Formed Vessels After BM-MNC Therapy

TSP-1 expression was explored in distal tissues from amputated patients with CLI and also in patients treated with BM-MNCs for CLI in the OPTIPEC trial, in whom extensive neoangiogenesis was observed in distal tissues.[2,6] We performed serial sections of paraffin blocks prepared from patients who had amputation. Then, immunostaining for CD68 and TSP-1 was visualized. TSP-1 was predominantly expressed in endothelial cells and macrophages regardless of the patient group, treated or not by the cell therapy product (Figure 2).

TSP-1 Negatively Regulates Angiogenesis in Matrigel Plug and Hindlimb Ischemia Models

To analyze the functional role of TSP-1 during neoangiogenesis, we used a Matrigel plug assay in *TSP-1*$^{-/-}$ mice. Basic fibroblast growth factor–containing plugs were implanted subcutaneously in *TSP-1*$^{-/-}$ and C57Black6/J wild-type mice for 14 days. Plugs from *TSP-1*$^{-/-}$ animals (Figure 3A) had a higher blood vessel content than those from wild-type animals (Figure 3A). This was further confirmed by measuring the hemoglobin content of the plugs (Figure 3B; $P=0.01$). We have also determined the effect of 10 μg/mL TSP-1 administered to wild-type and to *TSP-1*$^{-/-}$ mice on the Matrigel plug vascularization. Figure 3C and 3D shows that TSP-1 induced a strong decrease in plug vascularization in both wild-type and *TSP-1*$^{-/-}$ mice ($*P=0.017$ and $*P=0.016$ respectively).

The hindlimb ischemia model in *TSP-1*$^{-/-}$ mice has been previously reported by Brechot et al,[12] who found that *TSP-1*$^{-/-}$ mice were clinically and histologically protected from necrosis compared to wild-type controls. Tissue protection was associated with increased postischemic angiogenesis and muscle regeneration. In the present work, we have explored the effects of recombinant human TSP-1 in the model of hindlimb ischemia induced in C57Bl6/J mice by femoral artery and vein excision. As shown in Figure 3E and 3F, intramuscular injections of recombinant human TSP-1 induced a significant decrease in blood flow recovery 14 days after surgery ($*P=0.04$).

TSP-1 Inhibition in ECFCs Induces a Proangiogenic Phenotype In Vitro and In Vivo

Because postnatal angiogenesis is thought to involve EPCs,[8] and given the expression of TSP-1 found in newly formed vessels in human, we further explored whether TSP-1 inhibition was able to modulate ECFC angiogenic potential. We focused on ECFCs because this cell type is currently proposed as the cell type at the origin of newly formed vessels.[5,6] ECFCs express endothelial markers such as CD146, but they do not express leuko-monocytic markers such as CD14 and CD45. We found that ECFCs expressed a high surface density of the TSP receptor CD47, whereas no expression of CD36 was observed (supplemental Figure I).

We verified that TSP-1 mRNA and protein expression remained strongly inhibited by the specific small interfering (si)RNA during the days following transfection in cord and adult ECFCs (supplemental Figures II and IV). We then explored the effect of TSP-1 inhibition on the angiogenic properties of ECFCs in vitro. TSP-1 inhibition induced a strong increase in ECFC proliferation (Figure 4A; $P<0.0001$), associated with a 4-fold increase in the gene expression of the nuclear proliferation marker Ki67 (Figure 4B; $P=0.03$). TSP-1 inhibition also induced ECFCs to adopt a proangiogenic phenotype, as reflected by enhanced pseudotube formation in Matrigel (Figure 4C).

TSP-1 Inhibition in ECFCs Upregulates Stromal Cell–Derived Factor 1/CXC Chemokine Receptor 4 Pathway

To examine the possible transcriptional effect of TSP-1 inhibition, we used reverse transcription–quantitative polymerase chain reaction to measure mRNA levels of several angiogenic factors and their receptors, including VEGFA, angiopoietin-2, stromal cell–derived factor (SDF)-1, and endothelial nitric oxide synthase. We also verified that TSP-1 inhibition did not modify TSP-2 expression. After 48 hours of transfection, TSP-1 inhibition resulted in a significant 2-fold increase in SDF-1 mRNA and a 4-fold increase in its receptor CXC chemokine receptor (CXCR)-4 mRNA (Figure 4D). Increased ECFC surface expression of CXCR4 was shown by flow cytometry (supplemental Figure III). This upregulation, first observed in cord blood–derived ECFCs, was also found in adult ECFCs, but not in human umbilical vein endothelial cells (supplemental Figure IVC). To explain the effect of TSP-1 inhibition on ECFC differentiation in pseudotubes and to explore the potential involvement of the SDF-1/CXCR4 pathway, we used 12G5, a monoclonal antibody recognizing an epitope located in the second extracellular loop of CXCR4 that we had previously used to reduce the angiogenic potential of ECFCs related to the SDF-1/CXCR4 pathway.[18] The increase in Matrigel tube formation induced by TSP-1 inhi-

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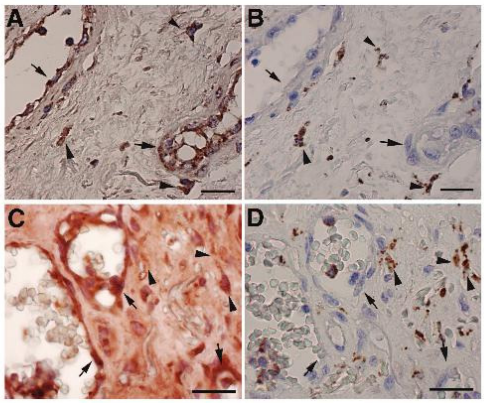

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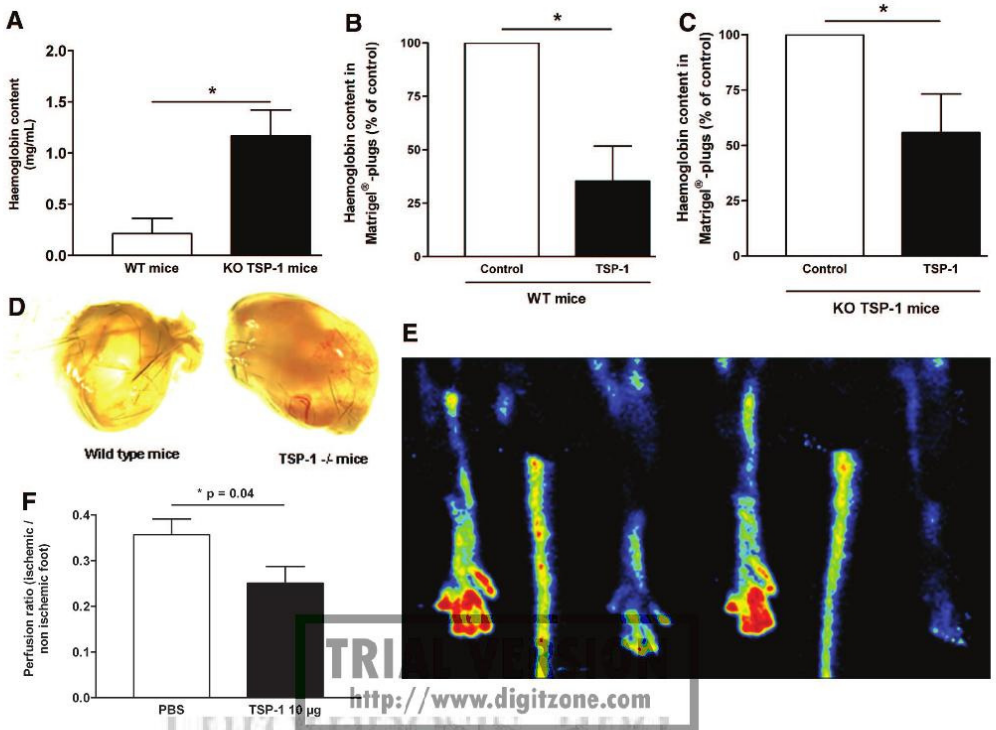

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Figure 3. TSP-1 modifies Matrigel plug vascularization in TSP-1 knockout mice and in wild-type mice. A, Matrigel plugs containing basic fibroblast growth factor 750 ng/mL implanted in C57/Bl6/J wild-type mice and *TSP-1* $^{-/-}$ mice. B, Hemoglobin quantification with the Drabkin method in C57/Bl6/J wild-type and *TSP-1*$^{-/-}$ mice. Values are expressed as means±SEM; n=10 per group. C, Hemoglobin quantification in C57/Bl6/J wild-type mice with and without additional recombinant TSP-1 at 10 μg/mL. Values are expressed as means±SEM; n=10 per group. D, Hemoglobin quantification in C57/Bl6/J *TSP-1*$^{-/-}$ mice with and without additional recombinant TSP-1 at 10 μg/mL. Values are expressed as means±SEM; n=10 per group. E, Laser Doppler perfusion imaging at day 14 in the C57Bl6/J mouse hindlimb ischemia model. Left, Mouse receiving 20 μL of PBS per day by intramuscular injections during 5 days. Right, Mouse receiving 10 μg of recombinant human TSP-1 per day, in 20-μL intramuscular injections, during 5 days. F, Perfusion ratio means for the C57Bl6/J mouse hindlimb ischemia model. P=0.04; n=10 for each group.

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bition was abrogated by monoclonal antibody 12G5 (P=0.0007; Figure 4E). Finally, we tested TSP-1–inhibited ECFCs in a nude mouse model of hindlimb ischemia. ECFCs transfected with TSP-1 siRNA or control siRNA were intravenously injected into nude mice, 6 hours after femoral artery ligature. On day 7, foot perfusion was improved significantly more by TSP-1–inhibited ECFCs than by control ECFCs (P=0.03; Figure 4F).

TSP-1 N Terminus Mediates ECFC Adhesion

We chose to test the effect of recombinant human TSP-1 on ECFC angiogenic properties. In keeping with reports that TSP-1 acts as an adhesion molecule, ECFC adhesion was significantly enhanced by recombinant human TSP-1 (Figure 5A). TSP-1 N terminus has been described to be responsible for proangiogenic function of TSP-1. We thus compared the effect of TSP-1 to that of A1 peptide (TSP Hep I), a short peptide derived from the N-terminal part of TSP-1.[19,20] A1 peptide induced a strong increase in ECFC adhesion, similar to that observed with TSP-1 (Figure 5A).

TSP-1 Decreases ECFC Proliferation and Pseudotube Formation by an N-Terminal–Independent Mechanism

To examine TSP-1–proliferative effect, ECFCs were plated in the presence or in the absence of precoated TSP-1 or A1 peptide. As shown in Figure 5B, TSP-1 significantly reduced ECFC proliferation, whereas no effect was observed with A1 peptide. TSP-1 inhibitory effect on proliferation could be explained in part by an increase of apoptosis, as reflected by a significant increase in proapoptotic protein p53 and FasL transcription on TSP-1 incubation (supplemental Figure V). The upregulation of these 2 pathways was previously proposed to explain antiangiogenic and/or antimetastatic properties of TSP-1.[21–23] In an in vitro Matrigel model, an increase in pseudotube formation was observed with A1 peptide (Figure 5C and supplemental Figure VII), whereas TSP-1 significantly inhibited ECFC organization into branched structures and pseudotubes. This effect was further attributed to a downregulation of SDF-1 and CXCR4 (supplemental Figure VI).

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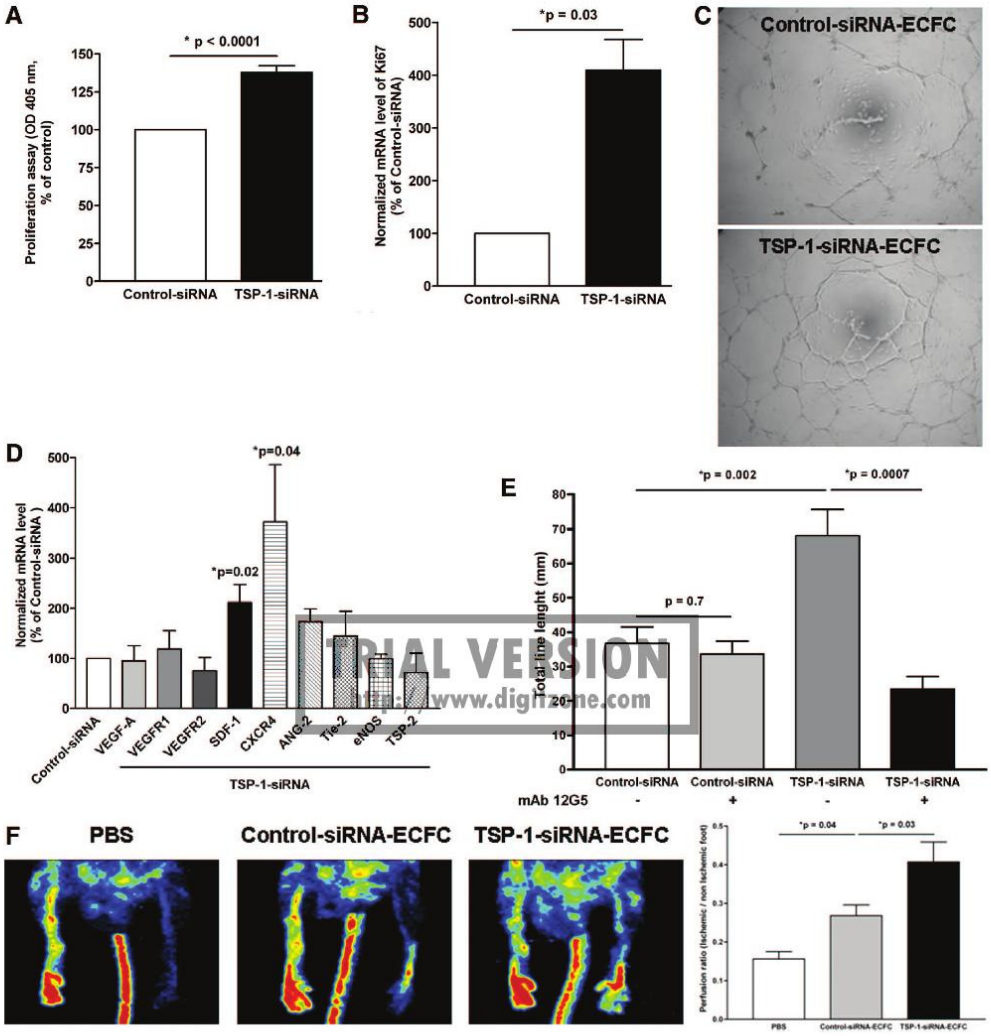

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Figure 4. Inhibition of TSP-1 in ECFCs increases their angiogenic potential in vitro and in vivo. A, Inhibition of TSP-1 with siRNA promotes ECFC proliferation. The effect of TSP-1 siRNA on ECFC proliferation was evaluated by measuring the release of the paranitrophenol (pNP) (optical density [OD] at 405 nm) in EBM2 medium containing 5% FBS (mean±SEM) ($P<0.001$). B, Quantitative analysis of the proliferative nuclear antigen Ki67 mRNA by reverse transcription–quantitative polymerase chain reaction, after 72 hours of transfection with control siRNA (AllStars Negative Control; Qiagen) or TSP-1 siRNA (Santa Cruz Biotechnology). The mean and SEM of 3 experiments are shown ($P=0.03$). C, siRNA-transfected ECFCs (3×10^4) were seeded on Matrigel, and tube formation was measured by phase-contrast microscopy. The mean and SEM of 3 experiments are shown ($P=0.0002$). D, Effect of TSP-1 inhibition with siRNA on the mRNA levels of VEGF, angiopoietin-2, and SDF-1 and their receptors. Values are expressed as means±SEM and are the result of 3 independent experiments. E, Inhibition of pseudotube formation in response to TSP-1 inhibition with siRNA in ECFCs by monoclonal antibody 12G5. The mean and SEM of 3 experiments are shown. Quantitative analysis of network length was determined with Videomet software (Microvision). Values are expressed as means±SEM and are the result of 3 independent experiments. F, Inhibition of TSP-1 in ECFCs increases their proangiogenic potential in hindlimb ischemia. Representative photomicrographs and quantitative evaluation of foot perfusion in mice injected with PBS or control siRNA– or TSP-1 siRNA–transfected ECFCs. Values are means±SEM; n=10 per group.

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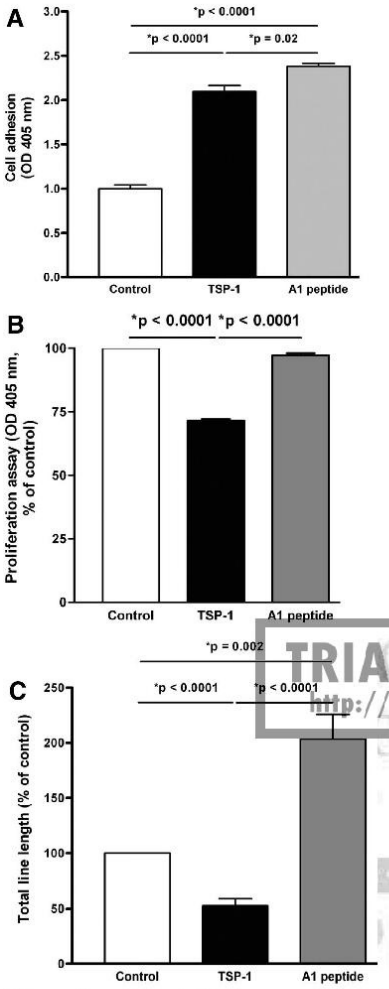

Figure 5. Recombinant human TSP-1 increases ECFC adhesion by its N-terminal extremity. A, Adhesion assays were performed in 24-well polystyrene plates (Becton-Dickinson) with or without recombinant TSP-1 or A1 peptide at 10 μg/mL. After incubation for 20 minutes, nonadherent cells were discarded, and the number of adherent cells was determined measuring the release of pNP (OD at 405 nm). Each data point is the average of 3 wells, and each experiment was performed at least 3 times. B, Proliferation assays were performed in 24-well plates with or without TSP-1 or A1 peptide at 10 μg/mL. After 72 hours, nonadherent cells were washed off, and the number of adherent cells was determined by measuring the release of pNP (OD at 405 nm). Each data point is the average of 3 wells, and each experiment was performed at least 3 times. C, ECFCs are plated in an 18-hour Matrigel tubule-formation assay, with or without TSP-1 or A1 peptide at 10 μg/mL. Quantitative analysis of network length was determined with Videomet software (Microvision). The mean and SEM of 3 experiments are shown.

CD47 Inhibition in ECFCs Induces the Same Proangiogenic Phenotype As TSP-1 Inhibition

The differences observed between TSP-1 pro- and antiangiogenic properties are probably the result of an imbalance between the proadhesive effect of the TSP-1 N-terminal part and another mechanism involving TSP-1 receptors. Because CD36 was not found on ECFCs, such as described previously in human umbilical vein endothelial cells (supplemental Figure I),[24] we inhibited CD47 gene expression in ECFCs. CD47 protein expression is strongly inhibited by the CD47 siRNA (Figure 6A). We explored the effect of CD47 inhibition on the angiogenic properties of ECFCs in vitro. ECFC adhesion was not modified by recombinant human TSP-1 between control siRNA– and CD47 siRNA–transfected ECFCs (Figure 6B). However, as for TSP-1 inhibition, CD47 siRNA induced a significant increase in ECFC proliferation (Figure 6C; $P<0.0001$) and pseudotube formation (Figure 6D and 6E; $P<0.0001$).

Discussion

Our findings suggest that the balance between angiogenic and antiangiogenic factors are altered in peripheral artery disease and that TSP-1 is associated with a loss of EPC angiogenic potential.

Plasma concentrations of TSP-1 in 184 patients with PAD were significantly higher than in controls, and we found a linear correlation along the different tertiles between PAD disease and TSP-1 plasma level. The increased TSP-1 plasma concentrations observed here may reflect an overexpression by ischemic tissues, as previously reported for cultured endothelial cells subjected to hypoxia.[25] In line with this hypothesis, Favier et al previously showed by in situ hybridization an absence of TSP-1 labeling in control muscles by comparison to ischemic tissues of PAD patients and an intense TSP-1 labeling in the ischemic samples.[11] Moreover, we found that newly formed vessels in patients treated with BM-MNCs were positive for TSP-1, pointing to an autocrine in situ effect during neoangiogenesis. However, it would be overstated to give any further conclusion about the role of TSP-1 in vessel function. Our aim was to discover an angiogenic biomarker potentially detectable in PAD patient plasma and to correlate it with the neoangiogenesis process in patients treated with a cell therapy product. One explanation is that treated patients who had to receive amputations had a neoangiogenic process inefficient to allow the vessel salvage. In this case, TSP-1 expression could represent a signal to prevent neoangiogenesis.

TSP-1 and TSP-2 are very similar structurally and functionally; both have been implicated as inhibitors of angiogenesis, endothelial cell survival, and endothelial cell migration.[26] Loss of TSP-1 or TSP-2 during embryonic development is not lethal but results in increased vascular density.[27,28] Double knockout for TSP-1 and -2 were shown to modulate proangiogenic activity of thrombopoietic cells.[29] However, because of contradictive results about TSP-2 implication in ischemic injury response, we focused our work on TSP-1.[30] TSP-1 is a heterogeneous molecule in terms of its cellular origin and functions, different parts of the molecule showing either pro- or antiangiogenic properties. Platelets may be an important source of circulating TSP-1 in PAD

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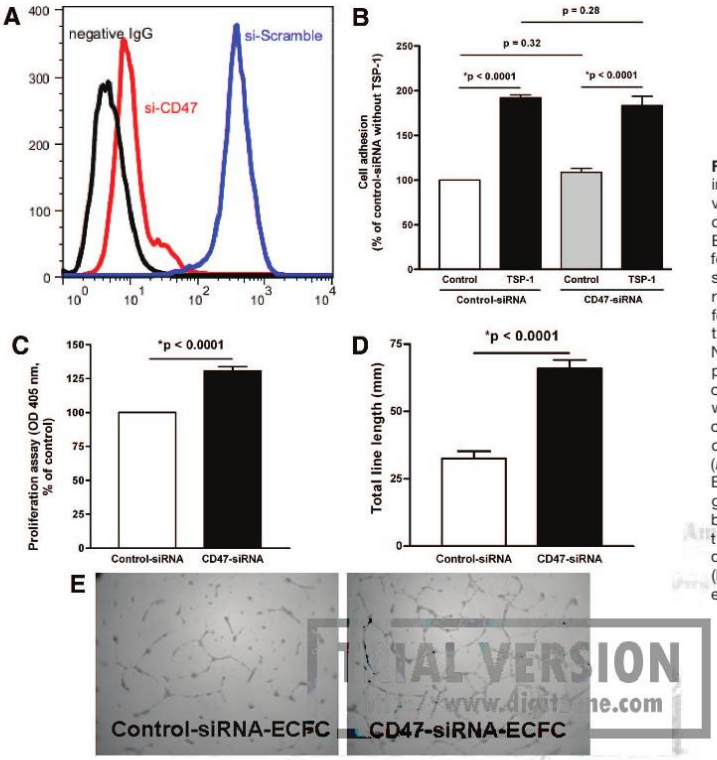

Figure 6. Inhibition of CD47 in ECFCs increases their angiogenic potential in vitro. A, Flow cytometry. CD47 siRNA decreased expression of CD47 on ECFCs. B, Adhesion assays were performed as described above, on control siRNA and CD47 siRNA, with or without recombinant TSP-1 at 10 μg/mL. No difference was observed on TSP-1 condition, for control siRNA– and CD47 siRNA–transfected ECFCs. C, CD47 siRNA promoted ECFC proliferation. The effect of CD47 siRNA on ECFC proliferation was evaluated by measuring the release of pNP (OD at 405 nm) in EBM2 medium containing 5% FBS (mean±SEM) ($P<0.0001$). D and E, Transfected ECFCs (3×10^4) were seeded on Matrigel, and tube formation was measured by phase-contrast microscopy. Quantitative analysis of network length was determined with Videomet software (Microvision). The mean and SEM of 3 experiments are shown ($P<0.0001$).

patients. Indeed, TSP-1 is stored in α-granules and is released during platelet activation, an important phenomenon in PAD underlined by the efficacy of antiplatelet agents in this setting. During various pathological situations, and according to thrombin concentration level, platelets have been shown to release either their α-granule–containing angiogenic factors or those containing antiangiogenic molecules[31] or soluble CD40 ligand.[32] Moreover, activated platelets[33,34] or their derived microparticles[35] have been shown to promote recruitment, differentiation, and/or angiogenic potential of progenitor cells, including also transfer of membrane receptors such as CXCR4.[35] The role of TSP-1 in CLI has been explored in TSP-1 knockout mice, in which macrophages infiltrating ischemic tissues were found to have a less proinflammatory phenotype than those in wild-type mice. TSP-1 knockout led to enhanced tissue regeneration and prevented necrosis.[12]

We investigated in vitro and in vivo the effect of TSP-1 on the angiogenic properties of cultured ECFCs, cells known to be involved in neoangiogenesis process. Interestingly, we found that TSP-1 had a double-edged effect on ECFCs, enhancing ECFC adhesion probably mediated by its N-terminal part, while reducing their proliferation and differentiation in pseudotubes, probably a consequence of TSP-1 binding to CD47. These 2 effects could contribute to the vessel phenotype in patients treated with BM-MNCs. TSP-1 is expressed in PAD ischemic tissues, to which it is thought to attract progenitor cells. Indeed, TSP-1 enhances the expression of adhesion molecules,[36] and can also act via proteoglycans. TSP-1 also binds to CD36 and/or CD47, that have important roles in cell migration, proliferation or apoptosis.[37] It has also been suggested that TSP-1 blocks the activation of matrix metalloproteinases such as matrix metalloproteinase 9, and thereby prevents the release of VEGF protein from extracellular stores.[38] ECFCs were negative for CD36, whereas a strong expression of CD47 was observed. CD47 inhibition in ECFCs gave the same proangiogenic phenotype than TSP-1 inhibition. These results are in line with those from several studies that propose CD47 as a target to improve ischemia caused by aging or PAD.[37]

However, adhesive properties of TSP-1 did not allow EPCs to form viable and normal vessels in patients with CLI who had received BM-MNCs. Indeed, newly formed vessels showed disorganized structures. In diabetic mice, TSP-1 mRNA expression by EPCs is significantly upregulated, whereas EPC angiogenic properties are downregulated in vitro and in vivo.[39] We found that TSP-1 inhibition enhanced the ECFC angiogenic potential both in vitro and in vivo and that increased differentiation in pseudotubes could be attributed to SDF-1/CXCR4 pathway upregulation. In line with these observations, we show that vascularization of implanted Matrigel plugs was significantly more efficient in *TSP-1*$^{-/-}$ mice than in their wild-type counterparts.

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TSP-1 could potentially serve as a therapeutic target in PAD patients. The antiangiogenic effects of TSP-1 are classically attributed to the C-terminal domain, whereas its proangiogenic effects are attributed to its N-terminal domain.[20] Peptides mimicking different parts of the TSP-1 molecule[20] might modulate angiogenesis in PAD or be used to prime EPC or BM-MNC products, as reported with SDF-1.[40] Moreover, the SDF-1/CXCR4 pathway upregulation observed here after TSP-1 inhibition suggests that *TSP-1* gene suppression could be used to produce an ECFC-based cell product effective in PAD. In addition, such an approach might be used to prevent restenosis after arterial interventions in diabetic patients. Alternately, pharmacological TSP-1 modulation in vivo might be beneficial in PAD.[12] TSP-1 has also been reported to modulate blood flow in elderly subjects and in patients with atherosclerosis, by modifying NO levels.[41] Drugs targeting TSP-1 might restore blood flow, enhance tissue healing, improve surgical outcomes, and reverse age-related vascular changes.[41]

In conclusion, the increased plasma levels of TSP-1 found in PAD patients might contribute to the inadequate neovascularization observed in this setting. TSP-1 targeting ex vivo or in vivo might have the potential to modulate angiogenesis.

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Disclosures

None.

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Thrombospondin-1 is a plasmatic marker of peripheral arterial disease that modulates endothelial progenitor cell angiogenic properties

David M. Smadja et al. Supplementary data

Study population

Consecutive PAD patients (n = 184) were enrolled in our vascular medicine department over a 2-years period. Patients were eligible if they were Caucasian men under 70 years of age with symptomatic atherosclerotic disease of the lower limbs and an ankle-brachial systolic pressure index (ABI) < 0.90, or a history of surgical or endovascular revascularization, as previously reported [1-3]. They were excluded if they had non-atherosclerotic causes of PAD (cardioembolic disease, thromboangiitis obliterans, vasculitis, or congenital or metabolic vascular disease). Critical leg ischemia (CLI) was defined by pain at rest or non healing ulceration (> 2 weeks) plus an ankle systolic blood pressure below 50 mm Hg. Walking distance was defined as the maximum walking distance reported by the patient before stopping because of claudication. Smoking, hypertension and diabetes were significantly more frequent in the cases than in the controls. Cases were five times more likely to be on lipid-lowering drugs; as a result, they had lower total and LDL-cholesterol levels than the controls. Triglyceride levels were higher and HDL-cholesterol levels were lower in the cases. Interestingly, the cases had a lower mean body mass index (BMI), together with a lower hematocrit, a lower plasma protein concentration, and higher leukocyte and platelet counts. Renal function, based on serum creatinine levels, was similar in the two groups. Age-matched control subjects (n = 330) with no history of arterial disease (stroke, MI, angina, or PAD) were randomly selected among 703 Caucasian men composing a previously described control group used to study genetic risk factors for vascular thrombosis [4]. They were recruited in a

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healthcare center specializing in cardiovascular prevention, to which they had been referred for a routine check-up. All participants gave their written informed consent and the study protocol was approved by the Paris-HEGP -Broussais ethics committee.

Blood collection

Blood samples were collected in Vacutainer tubes (Becton-Dickinson Diagnostics, Le Pont-de-Claix, France) containing 0.105 M sodium citrate (1 vol/9 vol). Plasma was obtained by centrifugation at 2300 *g* for 10 minutes and was immediately stored at - 80°C until analysis.

Local injection of bone marrow mononuclear cells (BM-MNC) in PAD patients with CLI

The OPTIPEC clinical trial (Optimization of Progenitor Endothelial Cells in the Treatment of Critical Leg Ischemia; *clinicaltrials.gov*: NCT00377897) was a multicenter, phase I non randomized study. The clinical findings have been published elsewhere [5]. Briefly, patients were eligible for the protocol if they had CLI associated with limited gangrene or a non healing ischemic ulcer and if they were not eligible for surgical revascularization or percutaneous angioplasty, or if such a procedure had little chance of success. The cell therapy protocol was similar to that initially published by Tateishi-Yuyama et al [6]. Control histological sections were obtained from age- and sex-matched patients with critical limb ischemia who were not included in the cell therapy protocol and who were amputated during the same period.

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ELISA Assays

Plasma levels of VEGF, PlGF and TSP-1 were measured with enzyme-linked immunosorbent kits from R&D Systems® (Minneapolis, MN, USA).

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ECFC culture, *in vitro* angiogenesis assays, and *in vivo* Matrigel-plug assay

ECFC culture, *in vitro* angiogenesis assay and *in vivo* Matrigel-plug assay conditions have been described in detail elsewhere.[7-12] *TSP1*$^{-/-}$ mice, in which the *TSP-1* gene was disrupted by homologous recombination [13], were kindly provided by Dr. A Bonnefoy [14]. For this study the *TSP-1* gene deficiency was bred into a C57Bl6/J background.

A1 peptide (TSP Hep I [15]) derived from N-terminus of TSP-1 was synthesized in the Department of biophysics at UNIFESP ((Federal University of São Paulo, Escola Paulista de Medicina), using an automated bench-top simultaneous multiple solidphase peptide synthesizer (PSSM 8 System; Shimadzu, Tokyo, Japan).

ECFC transfection with siRNA against TSP-1

Short interfering RNA silencing *TSP-1* gene expression (sc-36665, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was mixed with the Primefect® reagent (LONZA) at 10 µM to obtain transfection complexes, which were added to $2x10^5$ ECFC in complete EGM2 medium in 6-well plates. Control siRNA (Allstars Neg. control siRNA, Qiagen, Cambridge, MA, USA) was used in parallel.

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Real-Time - quantitative Polymerase Chain Reaction (RT-qPCR)

The theoretical and practical aspects of RT-qPCR on the ABI Prism 7900 Sequence Detection System (Applied Biosystems) are described in detail elsewhere [10]. Rapidly, we quantified transcripts of the TBP gene, which encodes the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and target gene expression was normalized on the basis of its TBP content. Primers for TBP and the target genes (sequences available on request) were chosen with the assistance of Oligo 5.0 software (National Biosciences, Plymouth, MN, USA).

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Hindlimb ischemia model.

Nude mice underwent surgery to induce unilateral hindlimb ischemia as previously described [16] and were then randomly allocated to intravenous injection of ECFC that had been transfected with control or TSP-1 siRNA. After 1 week, the ischemic/normal limb blood flow ratio was determined by using a laser Doppler perfusion imaging system (Moor instruments). C57Bl6/J mice underwent surgery to induce unilateral hindlimb ischemia left femoral artery and vein ligation and excision. Laser Doppler Perfusion Imaging was performed at day 14 in the C57Bl6/J mice Hindlimb ischemia model. Figure 3E: left panel: mice receiving 20 µL PBS per day by intra-muscular injections during 5 days; right panel: mice receiving 10 µg rhTSP-1 (R&D systems) per day, in 20 µL intra-muscular injections, during 5 days. Figure 3F: Perfusion ratio means for the C57Bl6/J mice Hindlimb ischemia model. n = 10 for each group, p = 0.04.

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Statistical analysis

The nonparametric Mann-Whitney *U* test was used to analyze differences between PAD patients and controls. Data from *in vitro* ECFC assays are reported as means ± SEM. Significant differences were identified by ANOVA followed by Fisher's protected least-significant-difference test. StatView statistical software (Cary, NC 27513, USA) was used for all analyses, and p values below 0.05 were considered to denote significant differences.

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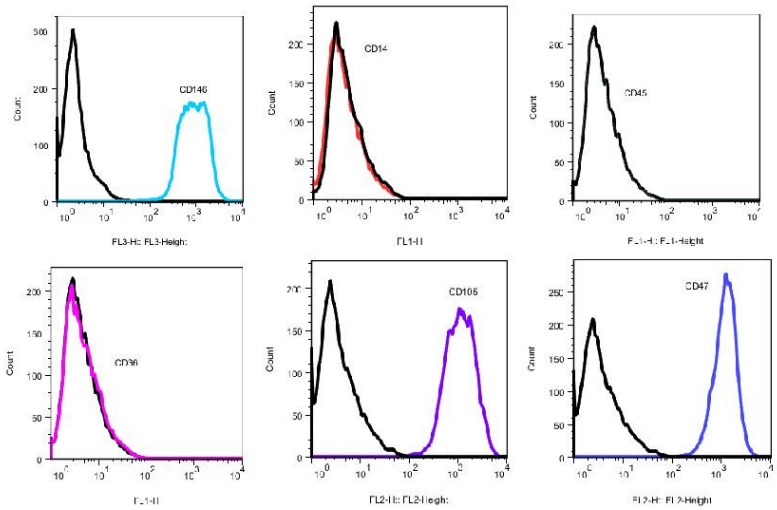
Supplementary Figure I: ECFC express endothelial markers and TSP-1 receptor CD47 but not CD36



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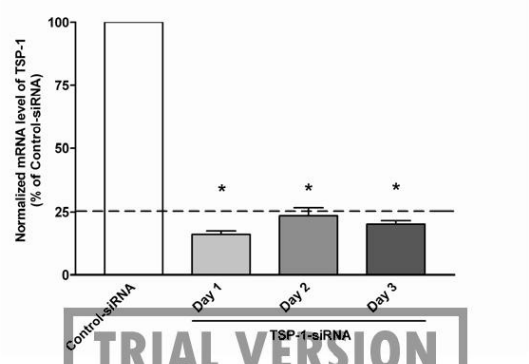
Supplementary Figure II: TSP-1 inhibition in cord blood derived ECFC

A. Quantitative analysis of TSP-1 mRNA by RT-qPCR, after 24, 48 and 72 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA). The mean and SEM of three experiments are shown.*: $p < 0.05$



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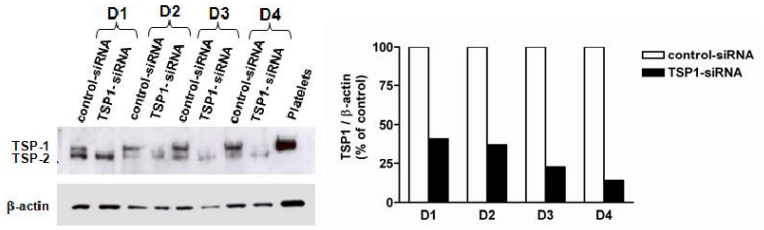
B. Quantitative analysis of TSP-1 protein by western blot, after 48 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA).



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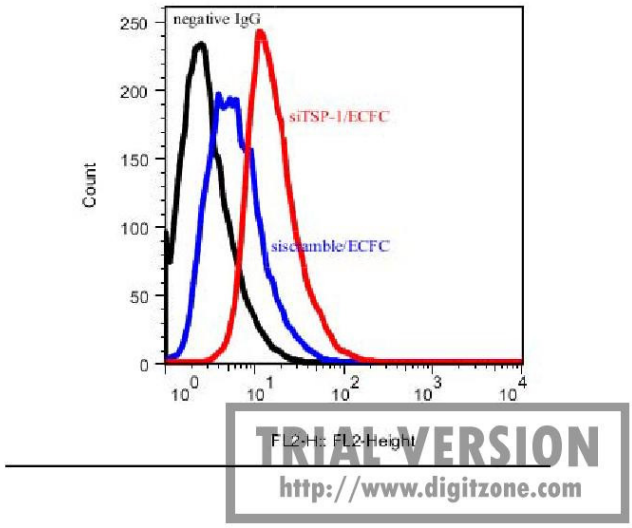
Supplementary Figure III :Expression of CXCR4 on transfected cord blood-ECFC



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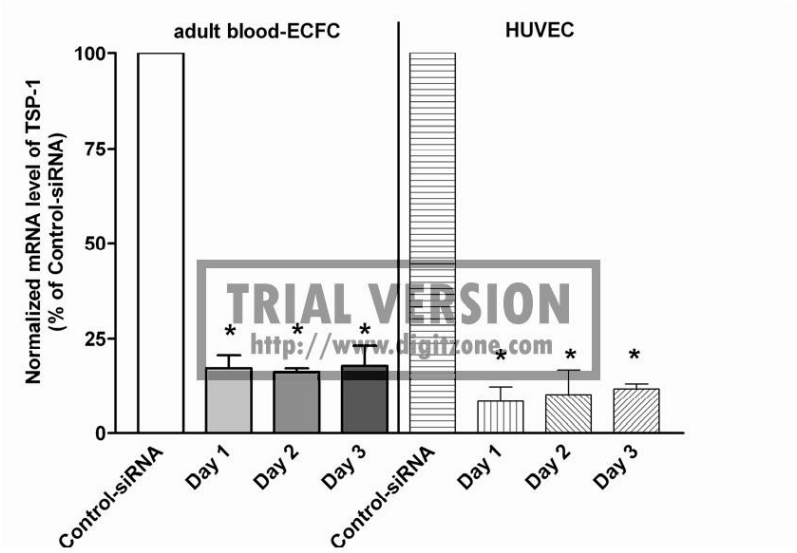
Supplementary Figure IV: TSP-1 inhibition in adult blood derived ECFC and in HUVEC

A. Quantitative analysis of TSP-1 mRNA by RT-qPCR, after 24, 48 and 72 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA). The mean and SEM of three experiments are shown. *:$p < 0.05$



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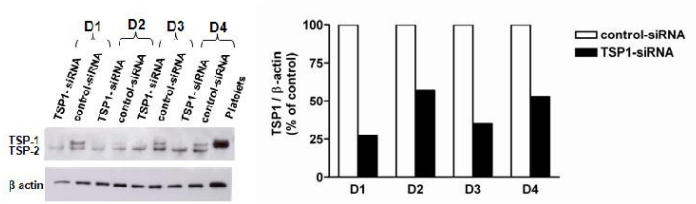
B. Quantitative analysis of TSP-1 protein by western blot, after 48 hours of transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA).



C. Quantitative analysis of SDF-1 and CXCR4 after transfection with control-siRNA (All Star negative control, Qiagen®) and TSP-1-siRNA (Santa Cruz Biotechnologies®) using the Primefect® reagent kit (LONZA) in adult blood derived ECFC and HUVEC.

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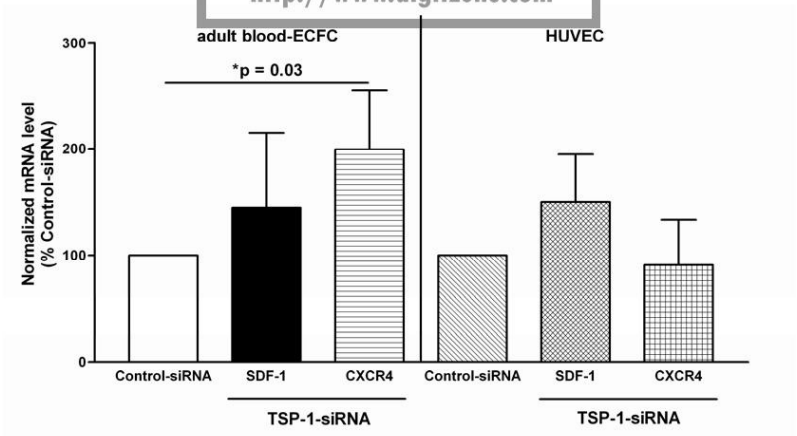

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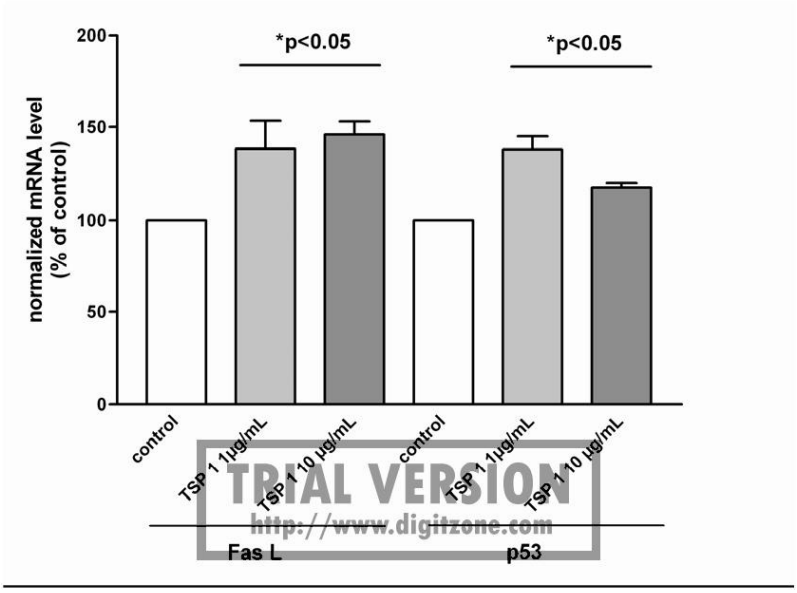
Supplementary Figure V: effect of 1 and 10 µg/mL human recombinant TSP-1 on apoptosis related genes in cord blood derived ECFC.



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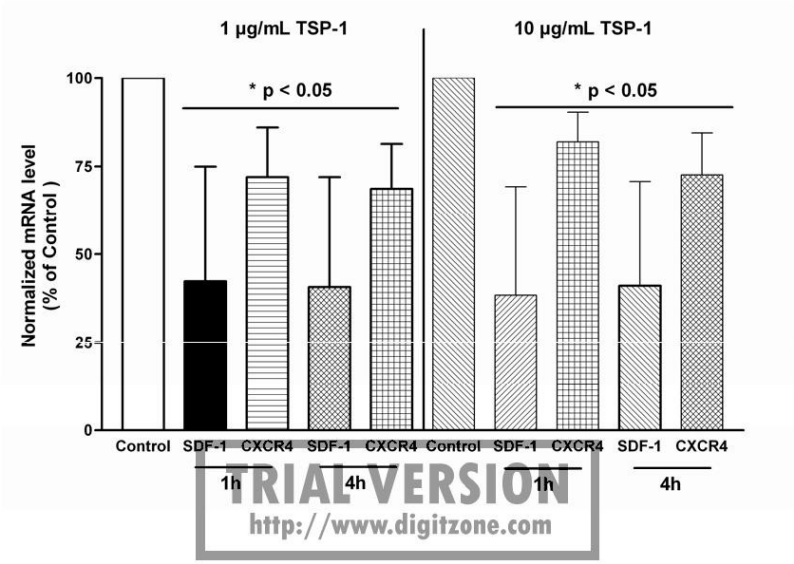
Supplementary Figure VI: effect of 1 and 10 µg/mL human recombinant TSP-1 on cord blood derived ECFC.



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Supplementary Figure VII: Effect of TSP-1 N-terminal peptide (10 µM) on cord blood derived ECFC differentiation in Matrigel *in vitro*.

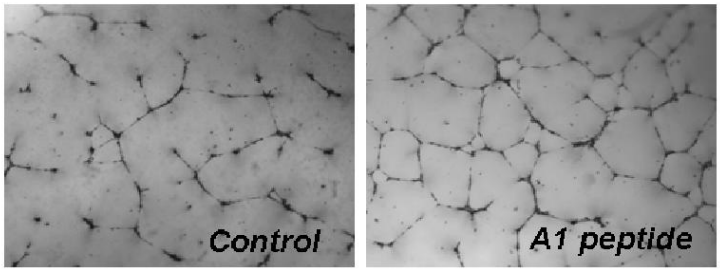

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Table 1. Characteristics of the Study Population

	Cases (n=184)	Control Subjects (n=330)	*P*
Age, y	57.1±7.2	56.7±7.6	0.6
Current+past smokers, %	97.8	47.0	<0.0001
Hypertension, %	43.5	16.1	<0.0001
Hypercholesterolemia, %	59.2	61.2	0.9
Lipid-lowering treatment, %	43.0	8.4	<0.0001
Total cholesterol, mmol/L	4.97±1.23	6.10±0.93	<0.0001
LDL cholesterol, mmol/L	3.04±1.03	3.94±0.87	<0.0001
HDL cholesterol, mmol/L	1.09±0.33	1.54±0.41	<0.0001
Triglycerides, mmol/L	1.96±1.35	1.41±0.87	<0.0001
Diabetes, %	24.5	8.5	<0.0001
Fasting blood glucose, mmol/L	6.5±2.4	5.9±0.7	<0.0001
Asymptomatic, %	10.3	...	...
Intermittent claudication, %	75.6	...	...
Critical ischemia, %	14.1	...	...
Prior revascularization, %	55.4	...	...
Ankle-brachial systolic pressure index	0.64±0.14	...	...
Coronary heart disease, %	23.9	...	...
Cerebrovascular disease, %	10.4	...	...

Values are given as mean±SD or percentages. BMI indicates body mass index.

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Table 2. Association of peripheral arterial disease with VEGF, PlGF, TSP-1 tertiles

	CASES	CONTROLS	P	OR (95%CI)	OR (95%CI)*
VEGF					
<11.2	36.4 (67)	31.0 (77)	<0.0001	2.62 (1.59-4.31)	4.11 (1.80-9.43)
11.2-18.0	19.6 (36)	44.0 (109)		1.00 (reference)	1.00 (reference)
>18.0	44.0 (81)	25.0 (62)		3.95 (2.39-6.53)	2.28 (0.99-5.21)
PlGF					
<6.7	39.1 (72)	29.9 (89)	0.005	2.09 (1.32-3.33)	5.03 (2.09-12.09)
6.7-9.8	24.5 (45)	38.9 (116)		1.00 (reference)	1.00 (reference)
>9.8	36.4 (67)	31.2 (93)		1.84 (1.16-2.94)	0.84 (0.35-2.02)
TSP-1					
<215	9.1 (16)	50.4 (122)	<0.0001	0.17 (0.09-0.32)	0.23 (0.09-0.60)
215-432	34.9 (61)	32.6 (79)		1.00 (reference)	1.00 (reference)
>432	56.0 (98)	16.9 (41)		3.09 (1.88-5.07)	3.25 (1.40-7.08)

Odd ratios for the lowest and upper tertiles relative to the middle tertile were computed using unconditional logistic regression analysis adjusted on age

* Additional adjustment for PAD risk factors (hypertension, hypercholesterolemia, diabetes and smoking status), lipid-lowering treatment and CRP.

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Table 3. Association of PAD patient clinical characteristics with VEGF, PlGF, TSP-1 levels

	VEGF	PIGF	TSP-1
Continuous factors			
Age	0.02	0.12 *	-0.07
BMI	0.01	0.08	-0.05
Glycaemia	-0.02	0.05	-0.003
Total cholesterol	-0.004	0.04	0.01
HDL cholesterol	-0.07	-0.03	0.07
LDL cholesterol	-0.004	0.04	-0.004
Triglycerides	0.10	0.05	-0.004
CRP	0.16 *	0.24 *	0.001
Categorical factors			
Diabetes			
No	219 ± 7	238 ± 7	219 ± 6
Yes	214 ± 16	258 ± 17	219 ± 13
Current smoking			
No	209 ± 8	237 ± 9	214 ± 7
Yes	232 ± 10	247 ± 11	227 ± 8
Hypertension			
No	221 ± 8	225 ± 9 *	217 ± 7
Yes	214 ± 9	262 ± 10	223 ± 8
Hyperlipidemia			
No	219 ± 9	246 ± 10	223 ± 8
Yes	217 ± 8	238 ± 8	217 ± 7
Lipid-lowering treatment			
No	212 ± 7	242 ± 8	224 ± 6
Yes	234 ± 13	242 ± 14	208 ± 11
Antiagregants			
No	206 ± 9	256 ± 10 *	226 ± 8
Yes	233 ± 11	220 ± 12	212 ± 9

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* $p < 0.05$ (Partial spearman correlation analysis or nonparametric analysis of variance adjusted on case-control status). Partial Spearman correlation or adjusted mean (±SEM) ranks are shown after pooled PAD cases and controls together.

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Table 4. Association of severity of peripheral arterial disease with VEGF, PlGF, TSP-1 levels

	Grade of Severity			P
	1, n=102	2, n=44	3, n=38	
VEGF	17.1 (9.7-25.9)	12.9 (7.0-25.6)	15.0 (6.4-30.8)	0.43
PlGF	7.1 (5.1-10.6)	9.3 (5.3-12.1)	8.7 (6.9-13.4)	0.07
TSP-1	430 (308-588)	468 (345-584)	513 (379-628)	0.59

Median (interquartile range) are shown and compared using nonparametric analysis of variance

We classified cases according to clinical criteria of PAD severity (Fontaine-Leriche stages, WHO classification): grade 1 = intermittent claudication with maximal walking distance > 100 meters; grade 2 = intermittent claudication with maximal walking distance < 100 meters; grade 3 = critical leg ischemia.

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3.3 **Um peptídeo derivado do domínio N-terminal da trombospondina-1 potencializa as atividades pró-angiogênicas das células progenitoras endoteliais formadoras de colônia.**

Titulo: A motif within the N-terminal domain of TSP-1 specifically promotes the proangiogenic activity of endothelial colony-forming cells. Submetido à *Angiogenesis* (Novembro/2011)

Autores : Dias JV, Benslimane-Ahmim Z, Egot M, Lokajczyk A, Grelac F, Galy-Fauroux I, Juliano L, Le Bonniec B, Takiya CM, Fischer AM, Blanc-Brude O, Morandi V, Boisson-Vidal C

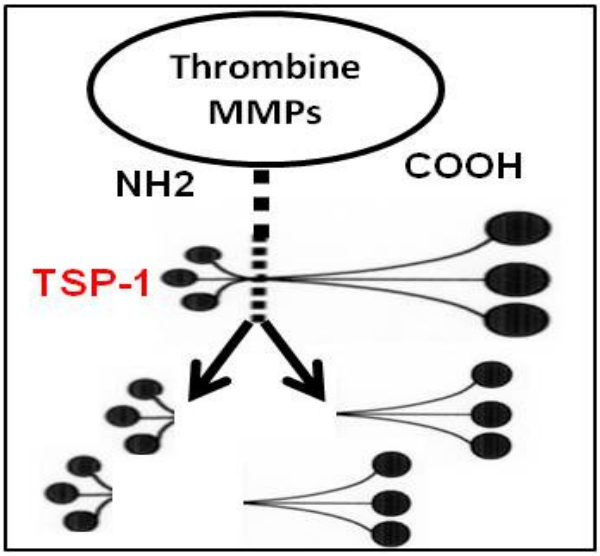
Considerações Gerais:

Neste artigo, aprofundamos o estudo dos efeitos do peptídeo TSP-HepI na angiogênese induzida pelas ECFCs isoladas do sangue de cordão umbilical.

Os resultados precedentes obtidos (Artigo 2) que demonstraram que a TSP-1 está presente em uma concentração significativamente mais elevada no plasma e no tecido de pacientes portadores de doença arterial periférica (DAP), sugere que o envolvimento desta molécula no tecidos isquêmicos pode estar ligada tanto à sua forma associada a matriz extracelular quanto à sua forma solúvel. De fato, a TSP-1 associada à matriz induz a adesão das células e favorece a angiogênese. De outra maneira, devido à produção em grande quantidade de citocinas inflamatórias nos tecidos isquêmicos a TSP-1 pode ser secretada em forte proporção nestes sítios por células endoteliais e plaquetas. Nestes contextos fisiopatológicos, a TSP-1 pode ser rapidamente clivada após ter ido secretada, e libera os peptídeos dos domínios N-terminal (NH_2) e C-terminal (ou fragmentos/peptídeos que não pertencem ao domínio NH_2) (Esquema 1). Uma parte da proteína ou dos fragmentos pode permanecer sob a forma solúvel e desta maneira modular, de forma diferencial – inibindo ou ativando - a proliferação, a quimiotaxia e a tubulogênese de células endoteliais.

Durante este trabalho, nós propomos que os peptídeos do domínio NH_2 da TSP-1 pudessem estar presentes em sítios de isquemia que necessitam de neovascularização, agindo como moduladores positivos dos efeitos angiogênicos. Avaliamos em um primeiro momento, a capacidade quimiotática do peptídeo TSP-HepI em atrair as ECFCs do sangue de cordão umbilical in vitro no ensaio utilizando insertos de transmigração com porosidade de 8 µm

(migração com gradiente de concentração) e in vivo utilizando o modelo murino de revascularização da matriz extracelular (Matrigel plug). Em seguida nós estudamos o efeito do pré-condicionamento das ECFCs com o peptídeo sobre a capacidade da interação destas células com o endotélio e de migração celular. Enfim nós caracterizamos o mecanismo de ação do peptídeo avaliando o efeito dos GAGs, em particular do sindecan-4 sobre estas propriedades angiogênicas. O conjunto de resultados derivados desta estratégia constitui o Artigo 3, apresentado nas páginas a seguir.



Esquema 1: A TSP-1 secretada por plaquetas e por células endoteliais é clivada por proteases como a trombina e MMPs, liberando dois fragmentos que podem estar solúveis no plasma (adaptado de Taraboletti et al., 2000).

Os resultados que serão apresentados nos ajudaram a perceber o duplo efeito da TSP-1 e a complexidade de seus mecanismos de ação que dependem de sua biodisponibilidade, solúvel ou associado à matriz extracelular. Apesar de possuir propriedades anti-angiogênicas, nós observamos que esta proteína é capaz de estimular a angiogênese agindo através do domínio N-terminal. Além disso, como demonstrado anteriormente (Artigo 2), a TSP-1 é fortemente expressa nos vasos recém-formados de amostras de tecidos isquêmicos de pacientes PAD, tratados pela terapia celular. Nossos resultados nos permitiram entender melhor esta observação e confirmaram a importância do domínio NH2 e em particular dos fragmentos ligantes de heparina nas ações pró-angiogênicos da TSP-1.

Ainda que a importância fisiológica desses fragmentos N-terminais permanece a ser demonstrada, nossos resultados sugerem que peptídeos biologicamente ativos, derivados deste

domínio da TSP-1, possam ser utilizados para pré-tratar as células progenitoras expandidas ex vivo, antes de sua administração em sítios isquêmicos, podendo representar uma via interessante para melhorar as propriedades funcionais e a capacidade de adesão dessas células.

Artigo 3 – A motif within the N-terminal domain of TSP-1 specifically promotes the proangiogenic activity of endotelial colony-forming cells

*Manuscript Dias JV et al. NH2-TSP1_ECFC
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A motif within the N-terminal domain of TSP-1 specifically promotes the proangiogenic activity of endothelial colony-forming cells

Juliana Vieira Dias [a,b,c,1], Zahia Benslimane-Ahmim [b,c], Marion Egot [b,c], Anna Lokajczyk [c], Françoise Grelac [b], Isabelle Galy-Fauroux [c], Luiz Juliano [d], Bernard Le-Bonniec [b], Cristina Maeda Takiya [e], Anne-Marie Fischer [c,f], Olivier Blanc-Brude [c,g], Verônica Morandi [a] * and Catherine Boisson-Vidal [b] *.

[a] Departamento de Biologia Celular, Laboratório de Biologia da Célula Endotelial e da Angiogênese (LabAngio), Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, UERJ, Rio de Janeiro, RJ, Brazil

[b] INSERM U765, Paris, France

[c] Université Paris Descartes, Sorbonne Paris Cité, France

[d] Departamento de Biofísica, Escola Paulista de Medicina, UNIFESP, São Paulo, SP, Brazil

[e] Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

[f] AP-HP, Haematology Department, Hôpital Européen Georges Pompidou, Paris, France

[g] INSERM, U970, Paris Cardiovascular Research Center – PARCC, Paris, France

* Verônica Morandi and Catherine Boisson-Vidal contributed equally to this study.

Corresponding author: Dr Juliana Vieira Dias, INSERM U1034, Université Bordeaux Ségalen , 125, Av. du Haut Lévêque, 33600 Pessac, France. Tel +33 (0)5 57 89 19 79, Fax +33 (0)5 56 36 89 79

e-mail: juvbiodias@gmail.com

Short title: Proangiogenic effect of the TSP-1 N-terminal domain

[1] Permanent address to Juliana Vieira Dias at : INSERM U1034, Université Bordeaux Ségalen - 125, Av. du Haut Lévêque, 33600 Pessac, France. Tel +33 (0)5 57 89 19 79, Fax +33 (0)5 56 36 89 79

Abstract

Thrombospondin-1 (TSP-1) gives rise to fragments that have both pro- and anti-angiogenic effects *in vitro* and *in vivo*. The TSP-HepI peptide (2.3 kDa) located in the N-terminal domain of TSP-1 has proangiogenic effects on endothelial cells. We have previously shown that TSP-1 itself exhibits a double-edged effect on endothelial colony-forming cells (ECFC), enhancing their adhesion through its TSP-HepI fragment while reducing their proliferation and *in vitro* differentiation into vascular tubes (tubulogenesis), likely through CD47 binding to the C-terminal domain of TSP-1. Here we investigated the effect of TSP-HepI peptide on the angiogenic properties of ECFC *in vitro* and *in vivo*. TSP-HepI peptide potentiated FGF-2-induced neovascularization in a Matrigel plug assay enhancing ECFC chemotaxis and tubulogenesis. ECFC exposure to 20 µg/ml TSP-HepI peptide for 18 h enhanced their migration ($p<0.001$ versus VEGF exposure), upregulated alpha 6-integrin expression, and enhanced their adhesion to activated endothelium in physiological shear stress conditions, at levels compared with SDF-1α. This latter effect appeared to be mediated by the heparan sulphate proteoglycan (HSPG) syndecan-4, as ECFC adhesion was significantly reduced by a syndecan-4-neutralizing antibody. ECFC migration and tubulogenesis were not stimulated by a TSP-HepI peptide with a modified heparin-binding site (S/TSP-HepI), or when the GAGs moieties were removed from ECFC surface by the treatment of cells with specific enzymes. *Ex vivo* TSP-HepI priming could potentially serve to enhance the effectiveness of therapeutic neovascularisation with ECFC.

Key words: thrombospondin-1, endothelial colony-forming cells, glycosaminoglycans, angiogenesis

Abbreviations: EPC, endothelial progenitor cells; ECFC, endothelial colony-forming cells; FGF-2, basic fibroblast growth factor; FACS, fluorescence-activated cell sorting; FCS, foetal calf serum; HUVEC, human umbilical vein endothelial cell; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor.

1. Introduction

Angiogenesis triggered by vascular injury or tissue ischemia is a multistep process involving mobilization, migration, proliferation, adhesion and differentiation of endothelial cells, and the release of cytokines, soluble growth factors, proteases, and extracellular matrix proteins [1,2]. Endothelial progenitor cells (EPC) from bone marrow migrate to ischemic tissues and participate in neovascularisation [3], representing a potential tool for autologous cell therapy of vascular diseases such as heart and leg ischemia [4]. EPC targeting to sites of neovascularisation involves their migration, adhesion, and differentiation into mature endothelial cells [5,6]. Various cell populations play a role in angiogenesis [7,8], but only one subset, endothelial colony-forming cells (ECFC), has been shown to possess all the characteristics of a true endothelial progenitor capable of forming neovessels *in vivo* [9].

Thrombospondin-1 (TSP-1) is a homotrimeric 180-kDa cell matrix protein, first identified as a natural inhibitor of angiogenesis. The anti-angiogenic activity of TSP-1 is mainly exerted through the binding of type I repeats (TSR) to CD36 receptor on microvascular cells, and also by its pro-collagen homology domain [10,11]. The C-terminal domain of TSP-1, containing the integrin-associated protein/CD47-binding sites may also play a role [12]. There is growing evidence, however, that TSP-1 also has pro-angiogenic properties, both *in vitro* and *in vivo*. In particular, TSP-1 expression is enhanced at sites of vascular injury [13]. In addition, we have previously reported that TSP-1 is expressed in newly formed vessels in patients with peripheral arterial disease having received local injections of bone marrow mononuclear cells, suggesting a possible modulation of tissue ischemia by TSP-1 [14]. We also found that TSP-1 had a double-edged effect on ECFC: its N-terminal domain enhanced ECFC adhesion, while the whole protein reduced ECFC proliferation and *in vitro* differentiation into vascular tubes, these last effects probably being mediated by CD47 binding to the C-terminal domain of TSP-1 [14].

We and others have independently attributed the pro-angiogenic activities of TSP-1 to its N-terminal heparin-binding domain or HBD [15–20], through its binding to several receptors, including calreticulin/LDL-related receptor [15], integrins $\alpha_3\beta_1$ [16], $\alpha_4\beta_1$ [17], and $\alpha_6\beta_1$ [18], and, as reported by our group, the cell-surface heparan sulphate proteoglycan (HSPG) syndecan-4 [19]. We characterized two specific motifs within the HBD of TSP-1, namely TSP-HepI/A1 (spanning residues 17-35) and TSP-HepII/A2 (spanning residues 78-94), that can interact with mature human umbilical cord endothelial cells (HUVEC) and induce their

differentiation into vascular tube-like structures when immobilized in fibrin matrices [20] or Matrigel [19]. These pro-angiogenic peptides both exhibit high affinity for glycosaminoglycans (GAG) and interact with syndecan-4 [19].

In vitro studies have shown that the HBD is readily released from TSP-1 and can be detected in platelet aggregation supernatants [21,22] as well as in endothelial cell-conditioned medium [22,23]. Thrombin, plasmin, cathepsins and matrix metalloproteinases, specifically ADAMTS1, cleave TSP-1 *in vitro*, releasing its HBD [21,23,24]. A 25-kDa fragment of the HBD has also been show to induce angiogenesis *in vivo* (rabbit cornea model) by enhancing the effect of FGF-2 [25].

In keeping with our finding that recombinant human TSP-1 acts as an adhesion molecule for ECFC and reduces ECFC proliferation and differentiation into vascular tubes, we subsequently showed that, in the same experimental conditions, TSP-HepI peptide, mimicking part of the TSP-1 N-terminal domain, strongly increased ECFC adhesion, similarly to TSP-1, but without affecting cell proliferation [14]. The aim of the present study was to investigate the effects of TSP-HepI peptide on the angiogenic properties of ECFC *in vitro* and *in vivo*.

2. Methods

2.1. Animals

Animal care conformed to French guidelines (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture, Paris, France), and all experiments were performed in keeping with the guidelines of Université Paris Descartes and the Institutional Committee on Animal Care and Use (C75.06.02).

2.2. Reagents

Recombinant human thrombospondin-1 (TSP-1) was from R&D Systems Europe (3074-TH-050). The peptides TSP-HepI and S/TSP Hep I (modified at amino acid positions essential for GAG binding), derived from the N-terminus of TSP-1, were synthesized at the Department of Biophysics at UNIFESP (Federal University of São Paulo, Escola Paulista de Medicina, Brazil), using an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 System; Shimadzu, Tokyo, Japan), followed by HPLC purification. Molecular weight was determined by MALDI-TOF mass spectroscopy and the sequences were verified with a PPSQ-23 (Shimadzu) sequencer. Matrigel was from Becton-Dickinson (BD; Pont de Claix,

France). Basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1 alpha (SDF-1α) were from R&D Systems Europe (Lille, France). Heparinase II (Heparin lyase; EC4.2.2.7), heparinase III (HS lyase; EC4.2.2.8), and chondroitinases ABC (chondroitinases ABC lyase; EC4.2.2.4) were from Sigma. R-PE-conjugated monoclonal antibody directed against α6-integrin (CD49f, clone G0H3), mouse R-PE-conjugated anti-human IgG_2a, secondary R-PE-conjugated anti-mouse and mouse R-PE conjugated anti-human IgM were from Becton-Dickinson. Monoclonal antibody directed against human heparan sulphate (clone F-58-10E4) was from AMS Biotechnology (Abingdon, UK). R-PE-conjugated monoclonal antibody directed against the ectodomain of human syndecan-4 (SDN4, clone 5G9), and mouse anti-human IgG_2a were from Santa Cruz Biotechnology (Le Perray en Yvelines, France).

2.3. Cell isolation, culture and pretreatment

Umbilical cord blood was collected with the mothers' consent (n=20). The study was approved by the ethics committee of Hôpital des Instructions et des Armées de Begin (France) (201008043234797) and the protocol conformed to the ethical guidelines of the Declaration of Helsinki. ECFC were isolated from human umbilical cord blood, expanded and characterized as previously described [26]. HUVEC were isolated by enzymatic digestion as previously described [27]. The endothelial cell phenotype was shown by double positivity for DiI-AcLDL uptake and BS-1 lectin binding. Combined expression of cell-surface antigens of the endothelial lineage, namely CD31, KDR, Tie-2, CD144, CD34 and Flt-1, was shown by FACS analysis (FACSCalibur, Becton Dickinson). In some cases, one day before experiments, cells were growth-arrested for 18 hours in EBM2, 2% FCS and released from growth arrest by adding EBM2, 5% FCS, with or without 20 µg/mL TSP-HepI for various times at 37°C. They were then washed, detached with accutase and washed twice with buffered Hank's, 0.5% BSA before use in angiogenesis assays *in vitro*. All assays were performed in triplicate with cells cultured for less than 30 days.

2.4. Migration assay

A directional migration (chemotaxis) assay was carried out in modified Boyden chambers as previously described [26]. All conditions used in a given experiment were tested in triplicate. A migration index was calculated as the ratio of the number of cells that migrated in control conditions (M199, 2%BSA; without protein).

2.5. Shear-flow adhesion assays

Flow adhesion experiments were conducted with a parallel-plate flow chamber in physiological shear stress conditions as previously described [28]. HUVEC monolayers ($3x10^5$) were seeded on coverslips, maintained at 37°C for 4 days, placed in the flow chamber, and then stimulated by exposure to a shear rate of 50 s^{-1} for 30 min. To distinguish adherent pretreated ECFC from detached endothelial cells (HUVEC), ECFC were stained with calcein (Fluoroprobes). Then, calcein-labeled ECFC ($3x10^6$) in adhesion buffer (cation-free HBSS, 10 mmol/L HEPES, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 2 mg/mL BSA, pH 7.4) were perfused for 15 min at 37°C at a shear rate of 50 s^{-1}, and the coverslips were washed with adhesion buffer for 10 min. Adherent cells were examined by phase-contrast microscopy. All experiments were observed in real-time and videotaped for offline analysis. Fluorescence micrographs of 40 random microscopic fields (obj. x10) were collected with Replay software (Microvision Instruments, France). Data were expressed as the number of adherent cells per field. Results of 3 different experiments were pooled for each study. Adherent ECFC were tested for resistance to detachment from the model endothelium by increasing the flow rate from 50 to 5000 s˙1 over one minute, and by counting the number of remaining adherent cells at one-minute intervals.

2.6. FACS analysis

Expression of cell-surface antigens on TSP-HepI-treated and untreated ECFC was analysed after immunofluorescent labelling in a FACS-SORT flow cytometer (BD Biosciences). Labelling with mouse anti-human heparan sulphate was visualized with a PE-conjugated anti-mouse antibody. A PE-conjugated anti-α6 antibody was used to visualize human #α6-integrin. In each immunofluorescence experiment an isotype-matched IgG antibody was used as a control, and the fluorescence intensity of stained cells was gated according to established methods. Data were analysed with CellQuestTM software (Becton Dickinson).

2.7. *In vitro* tube formation assay

Basement-membrane gels used for three-dimensional assays were prepared by Matrigel polymerization (8 mg/ml) for 30 min at 37°C. When required, 10 µg/ml TSP-HepI was included in the gels before polymerization. ECFC (10^5 cells/cm^2) diluted in EBM2 containing 2% FCS were seeded and allowed to form pseudotubes for 18 h at 37°C with 5% CO2. The cells were then fixed with 1.1% glutaraldehyde for 15 min and stained with Giemsa. The total length of the tube structures was quantified with Videomet software (Microvision Instruments, France). The results are reported as the average of three different experiments in each condition. When appropriate, ECFC were pretreated for 2 h at 37°C with a mixture of 0.5 U/ml

heparinase I, 0.1 U/ml heparinase III and 0.2 U/ml chondroitinases ABC before inclusion of TSP-HepI in the Matrigel.

2.8. Murine angiogenesis assay

Ice-cold Matrigel (8 mg/ml) mixed with PBS and FGF-2 (350 ng/ml), alone or supplemented with TSP-HepI peptide (200 µg/ml), was prepared and maintained in liquid form at 4°C. The solution was injected subcutaneously to C57Bl/6 mice (8 weeks old, from Janvier, France) and the Matrigel plug was recovered 14 days later. Haemoglobin content was measured as previously described [29]. Functional vessels were identified by light microscopy as vessels containing red blood cells.

2.9 Statistical analysis

Significant differences between means were identified by ANOVA with the Tukey post-test for multiple comparisons. Results are expressed as mean ± SEM of at least 3 experiments. P values below 0.05 were considered to denote significant differences.

3. Results

ECFC were isolated from human umbilical cord blood on the basis of CD34 expression. The presence of Weibel-Palade bodies and combined expression of endothelial markers (CD31, Tie-2, KDR, Flt-1, CD144) unequivocally confirmed the endothelial phenotype of the ECFC thus obtained (data not shown). Furthermore, ECFC did not express leuko-monocytic markers such as CD14 and CD45 [14].

3.1. TSP-HepI peptide enhances FGF-2-induced neoangiogenesis *in vivo*

In order to evaluate the proangiogenic potential of TSP-HepI *in vivo*, we used a mouse model of implanted Matrigel plugs. In this model, host endothelial cells and ECFC migrate into the implanted plugs, attracted by growth factors, mainly FGF-2 and/or peptides included in the gel, and form a capillary network within two weeks [30]. Matrigel (0.5 ml) containing either PBS (control), TSP-HepI alone (200 µg/ml), FGF-2 (350 ng/ml) alone, or a mixture of TSP-HepI and FGF-2, was injected into the flank of C57BL/6 mice. As shown in **Fig. 1A**, after two weeks, plugs from the PBS control group were mostly translucent and pale in colour, indicating little or no vessels formation and plugs containing TSP-HepI alone had a similar aspect (**Fig. 1B**). In contrast, plugs containing FGF-2 alone were redder (**Fig. 1C**), indicating new vessels formation.

presence of abundant new capillary vasculature formation (**Fig. 1D**). Analysis of haemoglobin content confirmed that TSP-HepI and FGF-2 together enhanced neoangiogenesis ($p<0.001$ versus TSP-HepI alone, **Fig. 1E**). Thus, TSP-HepI peptide appeared to enhance FGF-2-induced recruitment of circulating cells, suggesting a synergistic effect on angiogenesis *in vivo*.

3.2. TSP-HepI promotes ECFC chemotaxis and tubulogenesis. Role of HSPG

Having previously shown that TSP-1 is present at sites of neovascularization [14] and that TSP-HepI potentiate FGF-2-induced neoangiogenesis, we suspected that local release of fragments of N-terminal domain of TSP-1 by protease cleavage might participate in the recruitment of circulating progenitor cells and in their differentiation into mature endothelial cells, in sites of neovascularization. We therefore first examined whether TSP-HepI peptide promoted ECFC chemotaxis in a standard migration assay. We found that TSP-HepI peptide significantly enhanced ECFC migration (**Fig. 2**), to an extent similar to that observed with the proangiogenic chemotactic factor FGF-2. In the same experimental conditions, we showed that S/TSP-HepI peptide which was modified in the GAG-binding consensus motif, does not have any effect (Fig. 2). We then evaluated *in vitro* the effect of TSP-HepI on ECFC tubulogenesis. Capillary-like tube formation was enhanced when 10 µg/ml TSP-HepI was added to Matrigel gel prior to polymerization, whereas TSP-1 significantly inhibited capillary-like tube formation in the same conditions (**Fig. 3A**). As we had previously shown that the effect of TSP-HepI on HUVEC tubulogenesis involved the HSPG syndecan-4 binding [19], we then examined whether the proangiogenic effects of TSP-HepI on ECFC involved GAG binding. ECFC were incubated for 2 h at 37°C with enzymes that selectively removed heparan and chondroitin sulphates, and then seeded on Matrigel containing TSP-HepI peptide (**Fig. 3B**). As shown in **Fig. 3A and 3C**, ECFC treatment with enzymes that specifically remove heparan sulphate reduced tube formation in Matrigel by 45% ($p<0.001$). Similarly, ECFC treatment with the same enzymes reduced tubulogenesis in TSP-HepI-containing Matrigel by 72% ($p<0.001$). Our S/TSP-HepI peptide induces a reduction of pseudotube formation by 30% ($p<0.01$) in the same experimental condition (**Fig. 3A**). Thus, the interaction of TSP-HepI peptide with HSPG seems essential to promote ECFC migration and tubulogenesis, likely through activation of specific HSPG-dependent signalling pathways.

3.3. TSP-HepI peptide preconditioning promotes ECFC adhesion to activated HUVEC monolayers in physiological shear stress conditions

We then investigated whether TSP-HepI had a direct effect on ECFC adhesion to endothelium, one of the key steps of endothelial progenitor recruitment to ischemic sites. ECFC were incubated overnight in EBM2/2% FCS, then stimulated for 18 h with various concentrations of TSP-HepI in medium supplemented with 5% FCS, prior to adhesion. We used a flow-based adhesion assay using HUVEC monolayers to investigate the binding of TSP-HepI-stimulated ECFC to an activated endothelium. The experimental conditions mimicked the shear forces encountered by ECFC adhering to vascular endothelial cells *in vivo*. ECFC pretreated with TSP-HepI adhered much more rapidly to HUVEC than control cells (**Fig. 4A**), while TSP-1 pretreatment did not affect ECFC adhesion. As shown in **Fig. 4B**, TSP-HepI–treatment increased the percentage of adhered ECFC (265% versus 100% control after 10 min; $p<0.001$), an effect similar to that observed with SDF-1 pretreatment. Only 110% of TSP-1-treated ECFC adhered in the same conditions ($p<0.01$) (**Fig 4B**). In addition, TSP-HepI-stimulated ECFC adhered more strongly and were more resistant to washing than control untreated cells, at shear rates up to 2000 s^{-1} (**Fig. 4C**).

3.4. Syndecan-4 is involved in TPS-HepI-stimulated ECFC adhesion to HUVEC monolayers

To assess the possible role of syndecan-4 (SDN4) in the effects of TSP-HepI, we first used FACS analysis to examine SDN4 expression on the ECFC surface. As shown in **Fig. 5A** (right panel), ECFC expressed SDN4 at levels similar to HUVEC (**Fig. 5A**, left panel). We then pre-incubated ECFC with an anti-SDN4 or control antibody prior to TSP-HepI stimulation and adhesion assay. ECFC stimulated with TSP-HepI adhered as in the previous experiments (**Fig. 5B-C**), and anti-SDN4 preincubation reduced their adhesion by 84% ($p<0.001$, **Fig. 5C**). The control antibody had no noteworthy effect on ECFC adhesion after TSP-HepI stimulation (**Fig. 5B-C**).

3.5. TSP-HepI preconditioning enhances ECFC expression of the #α6-integrin subunit.

We have previously reported that increased vascular tube formation by HUVEC is associated with α6-integrin subunit overexpression [26]. Furthermore, we have demonstrated that this α6-integrin plays a major role in the proangiogenic properties of ECFC [31]. We therefore suspected that α6 subunit modulation might also occurs after ECFC stimulation with TSP-HepI. We used flow cytometry to measure surface expression of the α6-subunit on ECFC, before and after TSP-HepI stimulation for 18h (**Fig. 6**). Expression of the α6-subunit

was 1.5-fold higher on TSP-HepI-stimulated ECFC than on control ECFC ($p<0.001$), suggesting that TSP-HepI might enhance the proangiogenic properties of ECFC also by modulating α6-subunit expression.

3.6. TSP-HepI preconditioning enhances ECFC motility

Finally, we examined the effect of pretreatment with TSP-HepI for 18h on ECFC motility. As shown in **Fig. 7**, TSP-HepI-treated ECFC migrated much more rapidly that untreated ECFC, and about twice as fast as ECFC pretreated with VEGF. To determine the role of GAG in this effect, we used the S/TSP-HepI peptide devoid of the GAG-binding consensus motif. S/TSP-HepI-stimulated ECFC indeed had a reduced migratory capacity compared to TSP-HepI-stimulated ECFC ($p<0.001$). Thus, TSP-HepI binding to GAGs may contribute to the observed enhancement of ECFC angiogenic potential.

4. Discussion

The N-terminal domain of TSP-1 has a proangiogenic effect on mature endothelial cells, whereas the C-terminal region, as well as the intact protein, has antiangiogenic effects [10,12,13]. We have previously reported that TSP-HepI, a 2.3-kDa fragment of the TSP-1 N-terminal domain or HBD, markedly enhances ECFC adhesion, to an extent similar to intact TSP-1, whereas it has no effect on ECFC (14). Here, we demonstrate that TSP-HepI also modulates the angiogenic properties of ECFC. Additionally, the data presented here demonstrate that TSP-HepI potentiates FGF-2-induced neovascularization in an *in vivo* Matrigel plug model, suggesting that a synergy between the pro-angiogenic factor FGF-2 and TSP-HepI would lead to a better vascularization. It has been already reported that a combined administration of two pro-angiogenic molecules seems to be the most recommended strategy for vascular therapy, since stimulation with a single angiogenic factor may be insufficient for induction and maintenance of functional blood vessels [32]. In agreement with those reports, a recent study showed that the combined administration of FGF-2 and syndecan-4 enhanced neovascularization of ischemic muscle [33], by optimizing both FGF-2 uptake and signalling in the target tissue. These observations suggest that the activation of syndecan-4 as a co-receptor for FGF-2 may greatly impact the efficacy of FGF-2-based cell therapies. The combined administration of TSP-HepI – which also binds to and activates syndecan-4 signalling [19] - and FGF-2 could thus represent another important approach for therapies of neovascularisation based on this angiogenic factor.

TSP-HepI modulated the main angiogenic properties of ECFC *in vitro*. Our peptide stimulated ECFC chemotaxis with similar potency to FGF2, an effective stimulator of cell motility. In addition, it enhanced

ECFC differentiation into vascular tubes when incorporated in Matrigel, whereas TSP-1 had no such effect. Furthermore, TSP-HepI-pretreated ECFC adhered tightly to activated endothelium (HUVEC monolayers) in dynamic conditions, and were resistant to high shear rates, while their adhesion was reduced by pretreatment with the whole TSP-1 molecule. Importantly, the effect of TSP-HepI pretreatment in this assay was comparable to that obtained with SDF-1, a chemokine which potently recruits progenitor cells to ischaemic lesions [34]. Finally, ECFC migrated faster when treated with TSP-HepI than with VEGF, a well-known growth factor essential for endothelial cell recruitment.

We have reported that the HSPG syndecan-4 contributes to the proangiogenic activity of HUVEC by interacting with motifs within the HBD of TSP-1 [19]. Additionally, Roberts and co-workers identified several endothelial integrins, such as α3β1, α4β1 and α6β1, involved in the pro-angiogenic effects of the N-terminal domain of TSP-1 on microvascular endothelial cells [16–18]. Cooperation between syndecan-4 and such integrins that are able to modulate the response of HBD to angiogenesis, is therefore a possibility. Our present findings highlight the importance of TSP-HepI-HSPG interaction, as (i) S/TSP-HepI peptide (possessing a modified GAG-binding site) reduced chemotaxis and vascular tube formation, (ii) pre-incubation with a monoclonal antibody directed against syndecan-4 reduced TSP-HepI-pretreated ECFC adhesion to HUVEC monolayers, and (iii) pretreatment with S/TSP-HepI had no effect on ECFC motility. A study of the crystal structure of N-terminal domain of TSP-1 showed that the R29 residue at the TSP-HepI region is exposed and available for interactions. Thus, TSP-HepI peptide could effectively mimic HBD in its interaction with syndecan-4 as stated by Tan et al. [35].

The α6-integrin subunit has been implicated in ECFC recruitment to sites of ischemia [31], and α6-integrin overexpression by ECFC enhances *in vitro* vascular tube formation [26]. We observed a moderate increase in ECFC α6-integrin expression after TSP-HepI stimulation, although the functional relevance of this effect remains to be established. The interaction of α6-integrin with HSPG is known to induce proangiogenic activity in HUVEC [36]. Interestingly, it has been recently shown that TSP-1 induces the expression of α6-integrin chain on breast carcinoma cells, which in turn become more adherent towards laminin-rich matrices [37]. Thus, one could expect that α6-integrin also play a role in ECFC adhesion to target subendothelial matrices exposed by activated endothelium in sites of neovascularisation. These authors were not able to block

this induction effect with an antibody against anti-TSP-1 type I repeat, suggesting that other TSP-1 domains are responsible for the increase in α6-integrin chain expression [37].

Together, our results suggest that syndecan-4, α6-integrin and TSP-HepI interaction enhances the proangiogenic activity of ECFC. Syndecan-4 is strongly expressed in ischemic tissues and at sites of vascular injury. It acts as a co-receptor in focal adhesion, via heparin-binding growth factors (e.g. VEGF and FGFs) and extracellular matrix proteins, and binds to integrins such as α4β1 and α5β1 [38]. Two studies have suggested that syndecan-4 enhances HUVEC motility by activating Rac1 in a protein kinase-C-α (PKC-α) activation-dependent manner [39,40]. In the other hand, we have previously observed that the adhesion of HUVECs activate the PKC-dependent activation of Akt phosphorylation [19]. Nevertheless, we found no evidence that TSP-HepI affected the pERK-1/2 or pAkt signalling pathway in ECFC (data not shown), even if both are known to be activated by syndecan-4 and are involved in endothelial cell adhesion, migration and survival [19,41,42]. We showed that PKC-α inhibition led to a 4-fold decrease in the spreading ratio of HUVEC adhering to TSP-HepI, findings that support the participation of syndecan-4 in intracellular signalling in response to the N-terminal domain of TSP-1 [19]. Further work is needed to determine whether TSP-HepI also activates other signalling pathways in ECFC.

The two-edged effect of TSP-1 on angiogenesis seems to depend on the availability of its soluble proteolytic products, whether free in plasma or bound to extracellular matrix proteins and/or HSPG. We have previously observed elevated plasma TSP-1 levels in patients with peripheral artery disease [14]. Furthermore, despite its anti-angiogenic properties, TSP-1 is expressed on newly formed vessels following local injections of bone marrow mononuclear cells. We demonstrated here that the soluble proteolytic fragments of HBD of TSP-1 may modulate local angiogenesis and thus improve tissue ischemia. This HBD is rapidly cleaved by proteases relevant to the vascular process into 20-40kDa fragments recognized by specific antibodies [22]. However, the presence of smaller peptides could not be addressed since monoclonal antibodies directed against NH2-terminal domain of TSP-1 were unable to recognize the smaller fragments and peptides derived from HBD. No TSP-1 isoforms resulting from alternative splicing have so far been identified [43]. The known active fragments of TSP-1 therefore appear to arise from *in vivo* proteolysis of the mature protein. Indeed, TSP-1 cleavage with production of the N-terminal fragment (40 kDa) was shown to occur *in vivo* in a wound healing situation [24]. The physiological relevance of these fragments remains to be formally demonstrated.

In summary, local release of TSP-HepI during neovascularization could be an important factor in ECFC recruitment to sites of ischemia, by enhancing their capacity to adhere to the endothelium, migrate and form an extensive tubular network. TSP-HepI priming might be an interesting strategy to improve the efficiency of therapeutic neovascularisation using bone-marrow-derived endothelial precursors. Also, the combined use of TSP-HepI and FGF-2 could be designed as a new approach for increasing the efficacy of angiogenic growth factors therapies. Our results also open new perspectives for the understanding of the clinical significance of TSP-1 at sites of angiogenesis.

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Author contributions

JVD, ZBA, ME, CMT, VM and CBV designed and performed the research, analysed the data and wrote the manuscript

ZBA, JVD and CBV performed Matrigel plug assays, data collection and image analysis

FG performed zymography, data collection and image analysis

AL: technical assistance with cell culture

IGF: cell culture supervisor