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PAPEL DOS LEUCÓCITOS NA FISIOPATOLOGIA DA ANEMIA FALCIFORME

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Tese de Doutorado apresentada à Pós Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Fisiopatologia Médica, área de concentração em Biologia Estrutural, Celular, Molecular e do Desenvolvimento

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RESUMO

Anemia falciforme (AF) é um distúrbio na síntese de hemoglobina causada por uma mutação pontual que leva a produção de uma hemoglobina anormal, HbS. Em locais com baixa concentração de O_2, a HbS se polimeriza resultando em uma série de alterações que podem culminar em processos vaso-oclusivos, a principal causa de dor e mortalidade entre os pacientes falciformes. A AF é uma doença inflamatória crônica caracterizada por níveis de citocinas alterados, além de um elevado número de leucócitos e lesões nas células endoteliais. Os leucócitos apresentam um papel importante na AF uma vez que, seu recrutamento pode levar a um bloqueio do fluxo sanguíneo da microvasculatura, já que são células grandes, pouco deformáveis e que aderem facilmente ao endotélio ativado, além de interagirem com eritrócitos circulantes. As estratégias terapêuticas utilizadas e desenvolvidas para o tratamento da doença baseiam-se em 3 pontos principais: 1) diminuir a concentração intracelular de HbS como, por exemplo, aumento da HbF; 2) reduzir processos inflamatórios e o estresse oxidativo; 3) inibir a adesão celular e consequentemente, reduzir a vaso-oclusão (VO). A Hidroxiuréia (HU) é um agente quimioterápico que vem sendo utilizada com sucesso no tratamento da AF. Essa droga é capaz de elevar a HbF e também de reduzir o número de células brancas. Entretanto, além dos efeitos adversos provocados por ela, nem todos os pacientes respondem à terapia. Assim, faz-se necessária uma melhor compreensão da fisiopatologia da AF, incluindo o papel dos leucócitos e os mecanismos envolvidos no processo de VO, para o desenvolvimento de terapias alternativas. Neste estudo, tivemos como objetivos principais 1) verificar a ação de fatores presentes no soro de pacientes com AF quanto à modulação do número de neutrófilos e à geração de estresse oxidativo; 2) melhor compreensão do papel dos neutrófilos na iniciação e propagação de processos inflamatórios e consequentemente a participação dessas células no processo de VO. Dados indicaram que fatores presentes no soro de pacientes com AF são capazes de alterar a apoptose de neutrófilos e também iniciar a geração de espécies reativas de oxigênio (ROS), possivelmente pela ativação da enzima NADPH presente

nestas células. Adicionalmente, resultados obtidos com um modelo de camundongo transgênico falciforme indicaram que a ativação da via dependente de GMPc pode ser uma abordagem interessante para a inibição da adesão de neutrófilos à parede vascular sob condições inflamatórias. Além disso, os dados mostram que o uso de drogas que amplificam os efeitos da HU poderiam ser úteis na prevenção da VO.

ABSTRACT

Sickle Cell Disease (SCD) is a disorder of hemoglobin synthesis, caused by a point mutation, and resulting in the production of abnormal sickle hemoglobin, HbS. The consequence of low oxygen levels is HbS polymerization, which is responsible for the vaso-occlusive phenomenon that is the hallmark of the disease. SCD is a chronic inflammatory disease characterized by alterations in cytokine levels and an increase in leukocytes number and endothelial cell injury. Leukocytes play an important role in SCD since their recruitment to the microvasculature, consequent adhesion to the vessel wall, and interactions with other cells may interrupt the blood flow and culminate in vaso-occlusion. Therapeutic strategies for SCD treatment are based on three points: 1) reduction of intracellular HbS concentration, for example, by increasing the erythrocytic HbF concentration; 2) reduction of inflammatory processes and oxidative stress; 3) inhibition of cellular adhesion and, as a consequence, a reduction in vaso-occlusion. HU is a chemotherapeutic drug used for SCD treatment. This drug is able to increase HbF concentrations and reduce white blood cells counts, although it has some adverse effects and some patients do not respond to this therapy. Thus, it is necessary to fully understand the mechanisms that contribute to the vaso-occlusive process, including the role of the leukocytes in this phenomenon. The goals of this study are: 1) To check the influence of factors present in SCD serum on mechanisms that may affect the neutrophil count and oxidative stress generation; 2) understand the role of neutrophils in the initiation and propagation of vascular inflammation and vaso-occlusion. Date indicate that factors present in the SCD serum are able to alter neutrophil apoptosis and induce reactive oxygen species (ROS) generation, possibly by the activation of the NADPH enzyme expressed in these cells. Additionally, results obtained from a SCD inflammatory mouse model indicate that an activation of the cGMP - dependent pathway can inhibit neutrophil adhesion to the vascular endothelium cells after inflammatory induction and that drugs that amplify the effects of HU may represent a potential new therapy for SCD treatment.

SUMÁRIO

Introdução

INTRODUÇÃO

ANEMIA FALCIFORME

A anemia falciforme (AF) foi descrita pela primeira vez em 1910 em um estudante de odontologia, que apresentava um quadro de anemia, icterícia, úlceras nos membros inferiores e complicações pulmonares. Este paciente foi tratado pelo Dr. Herrick, um médico cardiologista de Chicago, o qual utilizou o termo "formato de foice" para descrever a aparência peculiar das células vermelhas encontradas nesse paciente (Herrick, 1910). Em 1945, hipotetizou-se que a doença poderia ser originada de anormalidades na molécula de hemoglobina (Hb) e, quatro anos mais tarde, a hipótese foi demonstrada por uma diferença na velocidade de migração eletroforética entre a HbS e a Hb normal (HbA) (Pauling *et al*., 1949). No mesmo ano, verificou-se que se tratava de uma doença genética com herança autossômica recessiva (Neel, 1949). Mais tarde, comprovou-se que a mutação responsável por originar a HbS altera apenas um aminoácido da HbA, ou seja, uma mutação no sexto códon que codifica a cadeia polipeptídica da globina β, presente no cromossomo 11, que leva à substituição de um resíduo do aminoácido ácido glutâmico por uma valina (Ingram, 1958). Sob condições de hipóxia, a HbS se polimeriza, levando à formação de estruturas tubulares que resultam em deformação e diminuição da flexibilidade das hemácias, as quais são impedidas de transitarem livremente na microvasculatura, e por isso apresentam maior tendência a formar agregados celulares na microcirculação, ocasionando diversas manifestações clínicas presentes nos pacientes com doenças falciformes, tais como anemia, episódios recorrentes de vaso-oclusão, infecções, síndrome torácica aguda, hipertensão pulmonar, priapismo, osteonecrose, insuficiência renal, úlceras de perna, retinopatia e doenças cardíacas (Nahavandi *et al.*, 2000; Frenette & Atweh, 2007). A figura 1 representa esses aspectos fisiopatológicos da AF.

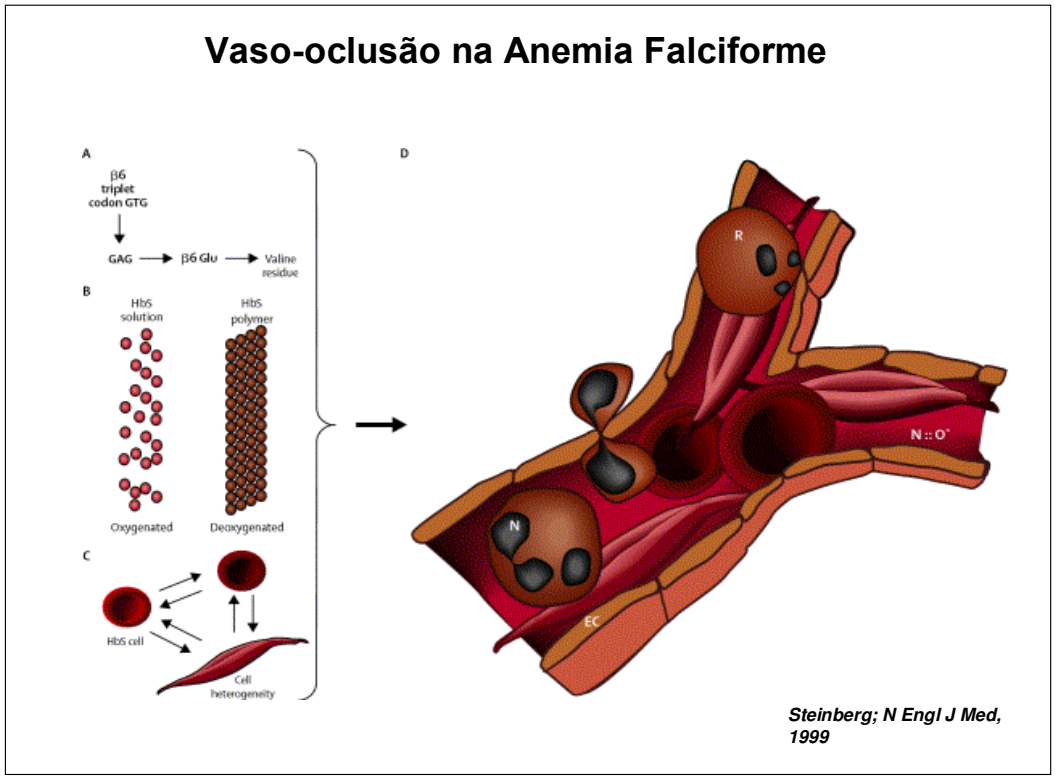

Figura 1: Fisiopatologia da anemia falciforme (Steinberg, 1999*).*

O conceito de vaso-oclusão evoluiu após a descoberta de que os eritrócitos falcizados têm uma forte propensão a aderir a células do endotélio (Hebbel *et al*., 1980). Mais tarde, descobriu-se que reticulócitos imaturos também podem participar desse processo de adesão, e que essas células parecem ser mais aderentes às células do endotélio cultivadas *in vitro* do que os eritrócitos maduros (Barabino *et al.*, 1987; Mohandas & Evans, 1985). É certo que, com base em todas as evidências recentes, o processo de vaso-oclusão é o resultado de uma complexa interação que envolve reticulócitos falcizados e normais, células endoteliais, leucócitos e plaquetas, além de fatores de coagulação e outras proteínas do plasma (Chiang & Frenette, 2005).

HEMOGLOBINA FETAL (HbF)

A HbF ($\alpha_2\gamma_2$) está presente durante a vida intrauterina e é caracterizada, principalmente, pela alta afinidade ao oxigênio. Tanto em humanos quanto em alguns outros mamíferos, a HbF é substituída pela hemoglobina adulta (HbA – $\alpha_2\beta_2$) fazendo com que a distribuição de oxigênio nos tecidos e órgãos seja mais eficiente na vida adulta. No momento do nascimento, aproximadamente metade da HbF já foi substituída pela HbA e provavelmente algum passo no período pós-natal (por exemplo a respiração ou algum outro estímulo que ocorre durante o nascimento) desencadeia o silenciamento do gene que codifica a globina γ (Papayannopoulou & Abkowitz, 1991; Oneal *et al.*, 2006). A HbF tem um papel importante na AF, uma vez que, em alta concentração, inibe a polimerização da HbS (Boyd & Neldner, 1991) e, consequentemente, reduz a frequência de crises vaso-oclusivas, síndrome torácica aguda, as taxas de hospitalizações e de mortalidade dos pacientes (Francheschi & Corrocher, 2004). Em estudos realizados com pacientes que apresentavam AF e persistência hereditária de HbF, observou-se que esses indivíduos possuíam sintomas clínicos menos dramáticos (Jacob & Raper, 1958).

ESTADO INFLAMATÓRIO CRÔNICO NA ANEMIA FALCIFORME

Ciclos repetidos de falcização e desfalcização das células vermelhas provocam danos na membrana dos eritrócitos levando à exposição de algumas proteínas epítopes e à produção de espécies reativas de oxigênio (ROS) (Frenette & Atweh, 2007; Switzer *et al*., 2006). Células falciformes podem causar danos ao endotélio, e algumas dessas lesões, juntamente com a hemólise intravascular, parecem ativar as células endoteliais com consequente expressão de alguns mediadores inflamatórios como endotelina-1 (Graido-Gonzalez *et al*., 1998), um vasoconstritor e agonista pró-inflamatório, e ativação do fator de transcrição NFκB (*nuclear factor* κB) (Belcher *et al*., 2005).

A família NFκB/Rel, quando ativada, também apresenta um papel central no desenvolvimento da inflamação, devido à sua habilidade de induzir a

transcrição de genes que codificam mediadores pró-inflamatórios. Quando NFκB está ativado no endotélio de vasos sanguíneos inflamados, há um aumento na produção de TNF-α (*tumoral necrosis factor-α*), IL-1 (interleucina-1) e IL-8 (interleucina-8), além da expressão das moléculas de adesão como selectinas, VCAM-1 (*vascular cell adhesion molecule-1*) e ICAM-1 (*intercellular adhesion molecule-1*) (Tak & Firestein, 2001; Sun & Andersson, 2002; Conran *et al*., 2004; Kato *et al*., 2005). Uma superativação de NFκB pode resultar em uma resposta inflamatória exagerada e como consequência pode ocasionar lesões inflamatórias agudas em determinados órgãos (Sun & Andersson, 2002).

Além disso, outras interações adesivas do endotélio com as células vermelhas, leucócitos e plaquetas levam a uma ativação pancelular que resulta na liberação de mais citocinas e quimiocinas pró-inflamatórias, perpetuando um ciclo vicioso de repetida ativação celular, adesão celular e produção de moléculas inflamatórias que proporcionam um estado inflamatório crônico fundamental no processo de vaso-oclusão. A figura 2 é um esquema representativo do estado inflamatório crônico e esse processo (Conran *et al*., 2009).

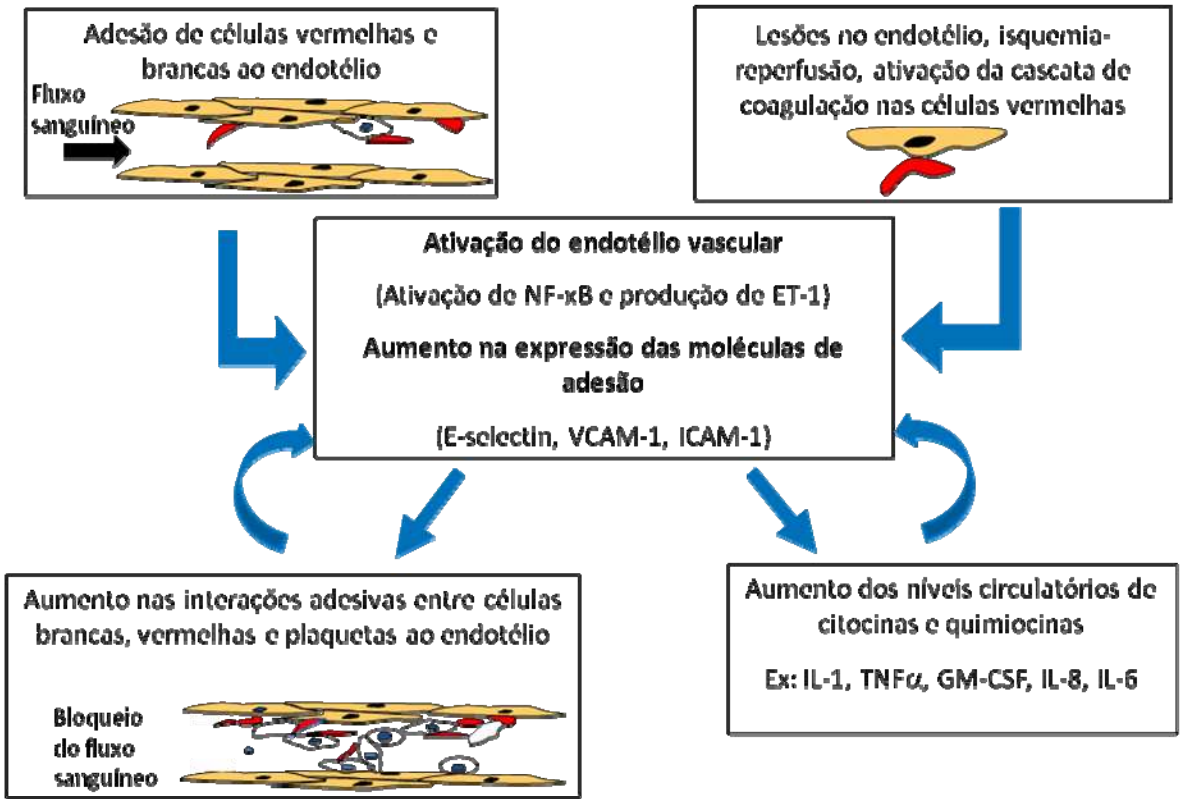

Figura 2: Estado inflamatório crônico e vaso-oclusão na anemia falciforme. A formação de polímeros de HbS leva a exposição de glicolipídeos e proteínas epítopes na superfície da membrana das células vermelhas. Como consequência, células vermelhas e, possivelmente, células brancas aderem-se ao endotélio, ocasionando danos endoteliais e ativando a cascata de coagulação, a qual, por sua vez, ativa o endotélio. A ativação endotelial aumenta a atividade de NFκB e a produção de endotelina-1 (ET-1) em associação com o aumento da expressão das moléculas de adesão da superfície. Além disso, interações adesivas do endotélio com células vermelhas, leucócitos e plaquetas, são induzidas em conjunto pela ativação pancelular que resulta na super-regulação de vários mediadores inflamatórios. Portanto, um ciclo repetitivo de ativação e adesão celular e produção de moléculas inflamatórias se perpetua no estado inflamatório crônico e tem um papel fundamental no processo de vaso-oclusão. (adaptado Conran *et al;* 2009).

O papel das citocinas e proteínas inflamatórias na anemia falciforme é um tema de contínua pesquisa, uma vez que as diversas formas de atuação dessas substâncias são bastante questionadas. Há relatos de que essas citocinas pró-inflamatórias estão elevadas no plasma de pacientes AF, assim como a expressão do RNAm dessas citocinas também está aumentada nos monócitos

(Francis & Haywood, 1992; Belcher *et al*., 2000; Selvaraj *et al*., 2003; Conran *et al*., 2007; Lanaro *et al*., 2009). Os níveis plasmáticos de GM-CSF estão elevados nos pacientes portadores de AF que apresentam baixas taxas de HbF (Croizat, 1994; Croizat & Nagel, 1999).

PAPEL DOS LEUCÓCITOS NA ANEMIA FALCIFORME

Há várias evidências sugerindo que os leucócitos possuem um papel importante na anemia falciforme e no processo de vaso-oclusão, uma vez que a leucocitose é frequentemente observada nessa doença e está associada com uma taxa maior de morte precoce, síndrome torácica aguda e AVC (Charache *et al*., 1995A; Miller *et al*., 2000; Assis *et al*., 2005). Os leucócitos parecem apresentar várias características que contribuem para a vaso-oclusão, como, por exemplo, o fato de serem células relativamente grandes (12-15 μm) e rígidas, fazendo com que seu recrutamento para a microcirculação reduza o fluxo sanguíneo dos vasos. Além disso, os leucócitos teriam um papel iniciador no processo de vaso-oclusão. Técnicas de microscopia intravital aplicadas em camundongos que expressam HbS humana demonstraram que após o estímulo inflamatório, os leucócitos são recrutados para o endotélio ativado e subsequentemente ocorrem interações adesivas com o endotélio e com as células vermelhas circulantes. Células falcizadas são então retidas, diminuindo ainda mais o fluxo sanguíneo e finalmente causando vaso-oclusão (Frenette, 2002; Turhan *et al*., 2002).

Aparentemente, os neutrófilos são os leucócitos que mais participam do processo de vaso-oclusão, pelo menos em camundongos transgênicos para AF (Chiang *et al*., 2007); em humanos é possível que os eosinófilos também estejam envolvidos no fenômeno. Além dessas células serem encontradas em elevado número nos pacientes falciforme, ensaios de adesão estática mostraram que as propriedades adesivas dos eosinófilos estão aumentadas nesses pacientes (Canalli *et al*., 2006).

Além disso há dados na literatura que sugerem que monócitos de indivíduos com AF encontram-se ativados devido, possivelmente, a fagocitose de células vermelhas falcizadas senescentes ou de restos e micropartículas celulares (Belcher *et al*., 2000). Esses monócitos ativados estimulam, por sua vez, as células endoteliais através da liberação de citocinas pró inflamatórias como TNF-α, IL-1β ou ambos (Belcher *et al*., 2000). As células endoteliais ativadas resultam no aumento da expressão das moléculas de adesão endotelial para neutrófilos, como por exemplo, E-selectina, P-selectina e ICAM-1 (Segel *et al*., 2011). Outros dados indicam que essas células, em situações de estresse oxidativo, também induzem a migração transendotelial (Rathan *et al.,* 1997), podendo até mesmo interagir com plaquetas. A interação desses múltiplos mecanismos pode levar à perda da integridade vascular podendo culminar na vaso-oclusão (Pathare *et al*., 2003).

No entanto, são os neutrófilos que apresentam um papel mais importante na anemia falciforme, já que são o tipo de leucócito mais abundante na vaso-oclusão. Os neutrófilos possuem uma meia-vida que pode variar entre 6 a 12 horas na circulação. Entretanto, esse tempo para a apoptose pode ser prolongado por alguns agentes ou processos, chegando a estender a vida dessas células a alguns dias. Os fatores capazes de provocar a interrupção da apoptose são: citocinas pró-inflamatórias, hipóxia, adesão celular, transmigração e lipossacarídeos de bactérias. (Moulding *et al*., 1998; Cross *et al*., 2005). A interrupção da apoptose pode levar a um aumento do número de neutrófilos contribuindo, por consequência, com a propagação do estado inflamatório e o processo de vaso-oclusão (Conran *et al*., 2007).

Em ensaios *in vitro* de adesão estática e dinâmica, observou-se que os neutrófilos de pacientes com AF apresentam uma adesão aumentada à fibronectina e às células endoteliais ativadas com TNF-α (Assis *et al*., 2005; Canalli *et al*., 2007; Finnegan *et al*., 2007). Além disso, quando esses pacientes estão em crise, os neutrófilos são ainda mais aderentes às células endoteliais do que os neutrófilos de indivíduos controle (Fadlon *et al*., 1998).

MORTE CELULAR E LEUCÓCITOS

A apoptose é um processo de morte celular não inflamatória cuja característica é a manutenção da integridade da membrana celular, prevenindo a liberação do conteúdo tóxico presente nos grânulos intracelulares dos neutrófilos. Os aspectos morfológicos das células que entram no processo apoptótico são: condensação da cromatina, fragmentação do DNA, diminuição do volume citoplasmático e deformidades da membrana celular (Walker *et al*., 2005).

A morte celular ocorre principalmente por um processo dependente de caspases (*cysteine-dependent aspartate-specific protease*). A ativação da cascata de caspases pode ocorrer por 2 mecanismos independentes; o primeiro seria pela via de morte do receptor extrínseco, mediado principalmente pelo receptor do fator de necrose tumoral (TNFR) e Fas, o qual pode guiar a ativação da cascata através da caspase 8. O segundo caminho é conhecido como apoptose intrínseca, e é um processo celular mediado pela mitocôndria e caracterizado pela perda do potencial de membrana mitocondrial e liberação de fatores pró-apoptóticos, como citocromo c, no citosol. A caspase 9 é ativada pela liberação do citocromo c e atua como a caspase iniciadora nessa via (Luo & Loison, 2008). Assim sendo, a ativação das caspases iniciadoras (caspases 8, 9 e 10) nos neutrófilos leva a ativação das caspases efetoras (caspases 3, 6 e 7), as quais possuem como função primária quebrar as proteínas celulares para desfazer as células (Walker *et al.,* 2005).

A via apoptótica dos neutrófilos é regulada por uma complexa rede de moléculas. Além disso, várias citocinas encontradas no sítio inflamatório são capazes de inibir ou prolongar a sobrevivência dessas células. GM-CSF e IFN-γ, por exemplo, aumentam a sobrevida dos neutrófilos via indução da PI3K (*phosphoinositide 3-kinase*) e ativação de Akt, NFκB e ou CREB (*cAMP response element binding protein*) (Cowburn *et al*., 2002; Kilpatrick *et al*., 2002; Scheel-Toellner *et al*., 2002). Já o TNF-α é a única citocina que apresenta tanto uma atividade pró quanto anti-apoptótica, dependendo dos meios extra e intracelular. A sobrevivência celular é geralmente induzida por baixas doses de TNF-α, enquanto o efeito pró-apoptótico é observado em altas doses da citocina (Luo & Loison,

2008). Também foi demonstrado que agentes capazes de elevar os níveis intracelulares de AMPc, como certas prostaglandinas e forskolin, apresentam um efeito inibitório da apoptose dos neutrófilos devido a uma via dependente de PKA (Ottonello *et al*., 1998; Rossi *et al*., 1995).

Recentemente, um novo tipo de morte celular independente de caspase, conhecido como necroptose, foi identificado por Degterev e cols (2005). Esses pesquisadores demonstraram que esse tipo de morte celular compartilha características semelhantes à necrose e à apoptose. Por um lado, há indícios de que as células em necroptose são morfologicamente similares às células em necrose, as quais seguem para a condensação do núcleo e inchaço das organelas. Por outro lado, esses pesquisadores verificaram que a necroptose é um tipo de morte celular programada como a apoptose (Xu *et al*., 2007; Teng *et al*., 2005). Importante também foi que Degterev e cols (2005) descobriram que a morte celular por necroptose pode ser inibida por uma pequena molécula chamada Necrostatin-1 (Nec-1), a qual é específica para necroptose.

Conran e cols (2007) demonstraram uma inibição da apoptose espontânea nos neutrófilos isolados de pacientes com AF, quando essas células foram cultivadas *in vitro* por 20 horas. Essa inibição da apoptose, por sua vez, parece ser mediada por uma via dependente de AMPc pois, quando essas células foram co-incubadas por 20 horas com um inibidor de proteína kinase dependente de AMPc (PKA), houve uma reversão da inibição da apoptose. Foi hipotetizado que fatores presentes em níveis aumentados no soro de indivíduos AF, tais como as citocinas GM-CSF, IL-8, TNF-α, e as prostaglandinas E1 e E2 poderiam elevar níveis intracelulares de AMPc e, portanto, aumentar a sobrevida dos neutrófilos na anemia falciforme. Além disso, há indícios de que a adesão celular possa levar a uma inibição da apoptose. Como os neutrófilos de pacientes com AF exibem propriedades adesivas elevadas, tal fato poderia também estar influenciando na inibição da apoptose.

Pouco se sabe sobre a apoptose dos neutrófilos na anemia falciforme. Se essas células possuem a maquinaria celular alterada e, consequentemente,

isso acarreta em uma alteração na taxa de apoptose, ou se o soro desses pacientes apresenta citocinas ou substâncias capazes de alterar a apoptose das células, são questões que ainda precisam ser respondidas.

ESTRESSE OXIDATIVO E ESPÉCIES REATIVAS DE OXIGÊNIO (ROS)

O estresse oxidativo é o resultado do desequilíbrio entre o aumento da produção de ROS e a baixa composição celular de antioxidantes enzimáticos, tais como glutationa peroxidase (GSH) e superóxido dismutase (SOD), e antioxidantes não enzimáticos, incluindo as vitaminas A, C e E (Natta *et al.,* 1990; Wood & Granger, 2007). O aumento de ROS na anemia falciforme pode ser consequência de vários mecanismos, tais como o aumento da atividade das enzimas NADPH oxidase e xantina oxidase endotelial devido à leucocitose e ativação endotelial. Repetidos ciclos de falcização das células vermelhas, processos de isquemia na microcirculação, auto-oxidação da hemoglobina polimerizada e aumento da função da enzima NOS (*nitric oxide synthase*), são processos que contribuem para a produção de ROS na AF (Amer *et al.,* 2006; Aslan & Freeman, 2007; Nur *et al*., 2011). O estresse oxidativo apresenta um papel significativo em vários mecanismos fisiopatológicos da AF, contribuindo para a hemólise, danos endoteliais, redução da biodisponibilidade do NO (óxido nítrico), formação de peroxinitrito, aumento das propriedades adesivas das células vermelhas e brancas e ativação da arginase, entre outros efeitos (Kuypers *et al.,* 1990; Sultana *et al.,* 1998; Wood & Granger, 2007; Iyamu *et al.,* 2008).

ROS também são conhecidas pelo seu papel de mediador intracelular de apoptose em neutrófilos. Uma grande quantidade de ROS, principalmente ânions superóxido, é produzida pela enzima NADPH oxidase em neutrófilos ativos, o que facilita a neutralização de bactérias. Além disso, a produção de ROS pode ser gerada por um mecanismo independente de NADPH oxidase, no qual a produção de superóxido e peróxido de hidrogênio pode ser induzida pela ativação de canais de cálcio e potássio, diminuindo os níveis de citocromo c, levando à redução da respiração mitocondrial, resultando em um acúmulo de elétrons na

cadeia respiratória e, subsequentemente, na formação de ROS. Estas, por sua vez, são reconhecidas como mediadores da morte de neutrófilos (Luo & Loison, 2008). Há evidências de que, nos neutrófilos, a produção de radicais livres citotóxicos pode ser elevada pela produção de NO através da enzima NOS, o que acarretaria em um aumento da apoptose dessas células (Ozdogu *et al*., 2007; Luo & Loison, 2008).

TERAPIA PARA PREVENÇÃO DA VASO-OCLUSÃO - HIDROXIUREIA (HU)

As estratégias terapêuticas utilizadas e desenvolvidas para o tratamento da anemia falciforme são baseadas em três pontos: o primeiro seria diminuir a concentração intracelular de HbS com agentes que ativem a síntese de HbF ou agentes que impeçam a falcização da hemácia; o segundo seria diminuir os eventos de adesão celular e, consequentemente, a vaso-oclusão; e o terceiro ponto seria reduzir o processo inflamatório e o estresse oxidativo.

Nas últimas décadas, vários compostos têm sido estudados com o intuito de aumentar a concentração de HbF em pacientes AF. No entanto, apenas a HU é aprovada pelo FDA (*Food and Drug Administration*) para o tratamento da anemia falciforme, uma vez que ela é capaz de modificar o curso da doença, melhorando os parâmetros hematológicos e o número de hospitalizações (Charache *et al*., 1995 A; Charache *et al*., 1995 B; Rosse *et al*., 2000). Acredita-se que essa droga atua, principalmente, aumentando os níveis de HbF nos eritrócitos e impedindo, assim, a polimerização da HbS nessas células (Rosse *et al*., 2000).

A HU é um agente quimioterápico que diminui a frequência de crises vaso-oclusivas, síndrome torácica aguda e necessidade de transfusão (Charache *et al*., 1995A; Maier-Redelsperger *et al*., 1999). Há estudos demonstrando que a HU diminui significativamente o número de neutrófilos nos pacientes falciformes (Castro *et al*., 1994; Rogers, 1997) e há alguns autores que postularam que os neutrófilos são o alvo primário dessa droga. Existem indícios de que a terapia com HU diminui as propriedades adesivas e a expressão de receptores das móleculas

de adesão VLA-4 e CD36, presentes nos reticulócitos (Joneckis *et al.,* 1993; Gambero *et al*., 2007). Além disso, neutrófilos ativados de pacientes com AF apresentam um aumento na exposição de fosfatidilserina, presente nas células vermelhas, o que facilita a adesão destas ao endotélio. Recentemente, Haynes e cols (2008) observaram que a HU é capaz de atenuar esse processo. Assim, a HU vem sendo utilizada com sucesso no tratamento da anemia falciforme.

Enquanto a HU é conhecida por inibir a sintese de DNA via inativação da enzima ribonucleotideo redutase (Yarbro, 1992), existem dados que indicam que a HU, quando oxidada, produz moléculas de NO tanto *in vitro* quanto *in vivo* (Gladwin & Schechter, 2001; Gladwin *et al.*, 2002; King, 2004), sendo capaz de aumentar a biodisponibilidade de NO na circulação de pacientes com AF (Lepoivre *et al.*, 1994; Cokic *et al*., 2007) e de induzir a expressão de globina γ em progenitores de células eritróides *in vitro*, através da ativação da via dependente de GMPc (Cokic *et al*., 2003). Outros dados mostram que a HU pode ser importante para reestabelecer a biodisponibilidade vascular de NO, facilitando a vasodilatação dependente de NO e reprimindo a atividade das moléculas de adesão (Conran *et al.,* 2009)

Em um estudo recente, camundongos transgênicos falciformes, que não produzem HbF na vida adulta, foram tratados com HU por 10 a 18 semanas a fim de avaliar se haveria benefícios clínicos pelo uso dessa droga, independentemente dos níveis de HbF. Os resultados obtidos mostraram que não houve melhora nos parâmetros avaliados. Porém, quando esses camundongos sem HbF e tratados com HU foram comparados à camundongos falciformes que apresentavam elevação da concentração de HbF, houve melhora com relação aos mesmos parâmetros avaliados. Os autores concluiram então que, pelo menos nos camundongos falciformes, a indução da HbF é aparentemente um fator primordial para que ocorra os diversos efeitos benéficos observados com o uso crônico da HU (Lebensburger *et al*., 2010).

É certo que nem todos os pacientes com AF respondem positivamente ao tratamento com HU. Além disso, a mielosupressão é um efeito adverso bastante comum nos pacientes sob tratamento com HU. Estudos com animais mostraram que a HU é embriotóxica e teratogênica, podendo causar inúmeras malformações, além de reduzir a contagem de espermatozóides (Wiles & Howard, 2009). Assim sendo, faz-se necessário o estudo de alternativas para o tratamento da anemia falciforme.

REDUÇÃO DA BIODISPONIBILIDADE DE ÓXIDO NÍTRICO (NO) NA ANEMIA FALCIFORME

O NO é um gás com meia-vida de alguns segundos, produzido pelas células endoteliais vasculares e que atua como um potente vasodilatador. Este gás é sintetizado através da oxidação do aminoácido L-arginina em uma reação catalisada pela enzima eNOS. O NO se difunde através da membrana celular para se ligar à enzima GCs (guanilato ciclase solúvel), a qual é responsável por converter o GTP (trifosfato de guanosina) em GMPc (monofosfato de guanilato cíclico). A produção deste segundo mensageiro cíclico leva a diminuição da contração das células do músculo liso devido à redução da concentração intracelular de cálcio livre (Rongen *et al*., 1994; Lundberg *et al*., 2008; Akinsheye & Klings, 2010).

Recentemente, o NO tornou-se uma substância de grande interesse devido, principalmente, aos seus efeitos benéficos na manutenção do tônus muscular, diminuição da pressão sanguínea (Stamler *et al*., 1994), prevenção da agregação plaquetária (através do aumento de GMPc e diminuição do Ca^{2+} intraplaquetário) (Nathan, 1992), inibição da adesão de monócitos e neutrófilos ao endotélio (inibindo VCAM-1 e ICAM-1) e, consequentemente, aumento do fluxo sanguíneo na microcirculação (Dusse *et al.*, 2003; Mack *et al*., 2008) e redução dos eventos dolorosos (Head *et al*., 2010).

Pacientes com AF apresentam uma elevada concentração de hemoglobina descompartimentalizada, produto da hemólise da hemácia. Ao observar que a biodisponibilidade de NO nesses pacientes era menor, Reiter e cols (2002) hipotetizaram que a Hb livre da célula consumiria o NO disponível produzindo metahemoglobina e nitrato. Como consequência da diminuição de NO disponível, há indícios de que ocorreria facilitação na vasoconstricção, aumento da ativação das plaquetas e da expressão e função das moléculas de adesão nos leucócitos e células endoteliais (Kato *et al*., 2007). Todos esses efeitos contribuiriam para o processo de vaso-oclusão e, por consequência, para hipertensão pulmonar e úlcera cutânea na perna (Reiter *et al*., 2002; Kato *et al*., 2007).

VIA DE SINALIZAÇÃO NO/GMPc

Há dados indicando que a modulação dos níveis intracelulares do mensageiro secundário do NO, GMPc, pode representar um possível alvo terapêutico para o tratamento da anemia falciforme, uma vez que a indução da via de sinalização dependente de GCs/ PKG (fosfoquinase G) pode constituir um mecanismo para aumentar a expressão de globina γ e, consequentemente, aumentar a concentração de HbF em células eritróides (Ikuta *et al*., 2001). Há relatos ainda de que a HU induz a expressão de globina γ por um mecanismo dependente de GMPc (Cokic *et al*., 2003). A figura 3 é uma representação gráfica dessa via de sinalização.

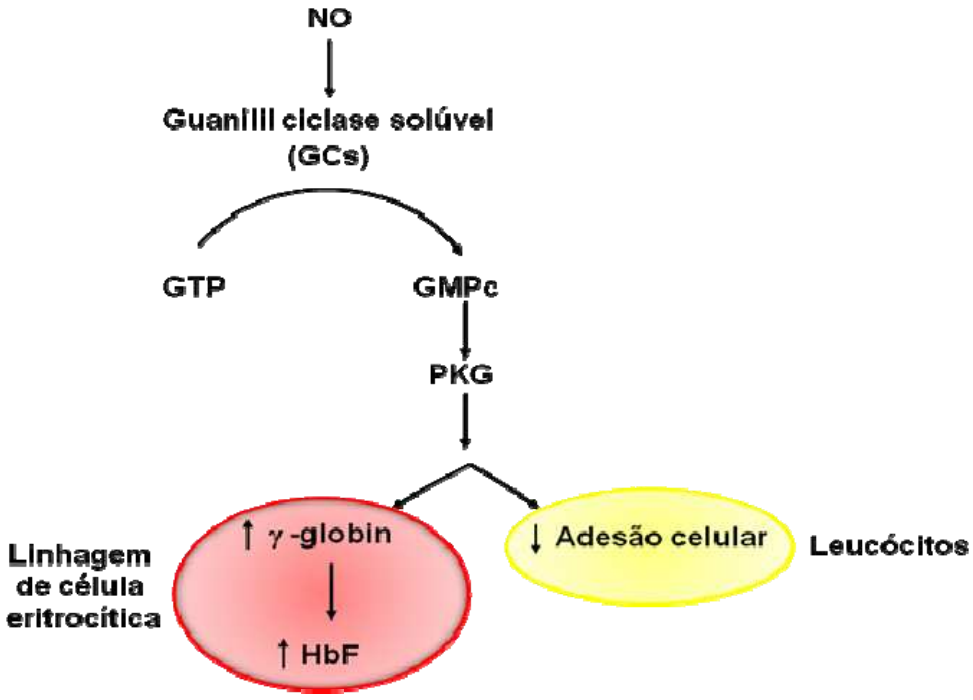

Figura 3: Via de sinalização NO/GMPc. Figura baseada em Ikuta *et al*., 2001; Cokic *et al*., 2003; Almeida *et al.*, 2008.

Um estudo recente do nosso grupo demonstrou que uma estimulação farmacológica dessa via dependente de GMPc pode ser um caminho em potencial para reduzir a adesão dos neutrófilos ao endotélio. Nossos estudos demonstraram que a incubação dos leucócitos *in vitro* com doadores de NO (nitroprussiato de sódio e DETA-NONOate) e com o ativador de GCs, BAY41-2272, diminuiu a adesão à fibronectina e ICAM-1 encontrada previamente aumentada nos neutrófilos de pacientes com AF (Canalli *et al.,* 2008). Além disso, ativadores de GCs têm sido utilizados com sucesso no tratamento de doenças cardiovasculares, hipertensão pulmonar e inflamação em modelos animais (Evgenov *et al*., 2006; Boerrigter & Burnett, 2007; Freitas *et al*., 2007). Como alternativa, drogas que apresentam um efeito célula-específica, como os inibidores de fosfodiesterases (PDE), poderiam proporcionar um efeito pontual, revertendo as funções alteradas dos neutrófilos.

FOSFODIESTERASES (PDE) E A VIA DE SINALIZAÇÃO NO/GMPc

As PDEs desempenham um papel fundamental na regulação dos níveis de GMPc e da monofosfato de adenosina cíclica (AMPc), atuando também na transdução de sinal, catalisando a hidrólise intracelular e a degradação de GMPc e AMPc. As enzimas PDE podem ser agrupadas em 11 famílias baseado-se na especificidade do substrato; além disso, constituem um importante alvo para a inibição farmacológica, já que a expressão dessas enzimas geralmente está restrita a alguns tipos celulares (Essayan, 2001). Os inibidores de PDE bloqueiam a capacidade de hidrólise exercida pelas PDEs, causando um acúmulo de nucleotídeo cíclico correspondente ao tipo de inibidor utilizado. Esses inibidores de PDE têm sido avaliados como potenciais antidepressivos, antiinflamatórios, antiapoptóticos, antiproliferativos, antihipertensivos e cardiovasculares. Uma das razões mais importantes pela qual essas PDEs foram reconhecidas como um bom alvo terapêutico é o fato de existirem diferentes isoformas. Assim, um inibidor de uma isoforma seletiva permite alcançar o alvo de funções específicas e condições fisiopatológicas com baixo risco de efeitos em sítios não específicos (Bender & Beavo, 2006).

Um bom exemplo de inibidor de PDE utilizado como medicamento é o citrato de sildenafil (Viagra®), que inibe a PDE5, responsável pela hidrólise do GMPc. Assim, esse medicamento mantém elevados níveis de GMPc, que permitem o relaxamento da musculatura lisa do corpo cavernoso e o aumento do influxo de sangue facilitando a ereção peniana (Dusse *et al.*, 2003). Existem casos na literatura em que esse mesmo medicamento foi utilizado com sucesso no tratamento de outras doenças como, por exemplo, hipertensão pulmonar, sendo um eficaz vasodilatador pulmonar (Cea-Calvo *et al*., 2003), ou até mesmo após cirurgias de próstata, tratamento de ejaculação precoce, combinado com outras terapias (Mitka, 2003).

Em nosso estudo prévio, demonstramos que a enzima PDE9A é altamente expressa em reticulócitos e neutrófilos humanos, com um aumento ainda mais proeminente nos reticulócitos e neutrófilos de pacientes com AF

(Almeida *et al*., 2008). Além disso, a inibição *in vitro* dessa enzima (com BAY73-6691, um potente inibidor de PDE9 (Wunder *et al*., 2005)) nas células de linhagem eritróide e leucócitos induziu um aumento na expressão de globina γ e diminuiu as propriedades adesivas, respectivamente. A distribuição dessa proteína está relativamente limitada aos tecidos hematopoiéticos, sugerindo a possibilidade dessa enzima representar um novo alvo terapêutico (Almeida *et al*., 2008). Tal fato justifica novos estudos para avaliar se fármacos doadores de NO e outras drogas que atuam na via de sinalização NO/GMPc podem ser benéficos para o tratamento de pacientes com anemia falciforme.

Objetivos

OBJETIVOS

1) Investigar os processos que podem contribuir com a alteração no número e na atividade de leucócitos na anemia falciforme, tais como fatores presentes no soro que influenciam a apoptose e a produção de estresse oxidativo, e a maturidade das células na circulação de indivíduos AF;

2) Avaliar os efeitos, com relação ao processo vaso-oclusivo, da administração aguda de HU, do inibidor de PDE9 (BAY73-6691), ou da combinação das duas drogas em um modelo inflamatório de vaso-oclusão em camundongos falciformes. Além disso, verificar se os efeitos observados são mediados pela via NO-GMPc-PKG.

Resultados

RESULTADOS

CAPÍTULO 1:

"Sickle cell disease serum induces NAPDH enzyme subunit expression and oxidant production in leukocytes."

CAPÍTULO 2:

"Alterations in cell maturity and serum survival factors may modulate neutrophil numbers in sickle cell disease."

CAPÍTULO 3:

"Hydroxyurea and a cGMP-amplifying agent have immediate benefits on acute vaso-occlusive events in sickle cell disease mice."

CAPÍTULO 1

Sickle cell disease serum induces NAPDH enzyme subunit expression and oxidant production in leukocytes

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Sickle cell disease serum induces NADPH enzyme subunit expression and oxidant production in leukocytes

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Oxidative stress plays a significant role in sickle cell disease (SCD), contributing to haemolysis, vaso-occlusive processes and endothelial dysfunction. To study the effects that the serum of SCD individuals has on the oxidative state of blood cells, sera were pooled from control individuals, steady-state SCD patients and SCD patients on hydroxyurea therapy (SCDHU), and their effects on markers of oxidative stress and damage in neutrophils isolated from healthy individuals observed. Incubation of control neutrophils, but not platelets nor red blood cells, with SCD serum (10% v/v; 2 hours) significantly augmented their production of reactive oxygen species (ROS). Increased ROS production in SCD serum-incubated neutrophils was associated with increased superoxide anion generation, apoptosis and increased nicotinamide adenine dinucleotide phosphate oxidase subunit expression. Although serum from SCDHU individuals also induced ROS generation in neutrophils, its oxidative capacity appeared to be lower. Results suggest that factors in the serum of SCD individuals contribute to ROS generation and oxidative damage in leukocytes.

Keywords: NADPH oxidase, leukocytes, oxidative stress, sickle cell disease

Introduction

Sickle cell disease (SCD) is a disorder characterized by sickling and haemolysis of red blood cells (RBCs);[1] however, the disease is now recognized to induce a chronic inflammatory and oxidative state that plays a fundamental role in much of the pathology of the disease. The mechanisms leading to chronic inflammation in SCD are not well understood, but are certainly multifactorial and appear to involve interactions between activated endothelium, cell adhesion mechanisms, inflammatory mediator expression and the production of reactive oxygen species (ROS).[2–4] Chronic inflammation, in turn, participates in the initiation of vaso-occlusive processes that involve the adhesion of red cells, leukocytes and, possibly, platelets to the vascular endothelium.[3] The production of ROS, with ensuing oxidative stress, further augments endothelial activation, cellular and tissue damage as well as contributing to reduce vascular nitric oxide bioavailability.[5]

Oxidative stress results from the imbalance between enhanced generation of ROS produced and a low cellular content of enzymatic antioxidants such as glutathione peroxidase and superoxide dismutase (SOD) and non-enzymatic antioxidants including vitamins A, C and E.[5,6] Increased ROS generation, in SCD, may be the consequence of numerous mechanisms. For example, activities of enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and endothelial xanthine oxidase may be increased due to leukocytosis and endothelial activation. Repeated sickling and unsickling of SCD RBCs, ischemia–reperfusion processes in the microcirculation, auto-oxidation of

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sickle haemoglobin and aberrant nitric oxide synthase function are all thought to contribute to ROS production in SCD.[7,8] Oxidative stress may play a significant role in a number of the pathophysiological mechanisms of SCD, contributing to haemolysis, endothelial damage and uncoupling of nitric oxide synthase, inactivation of nitric oxide and formation of damaging peroxynitrite, augmentation of red and white cell adhesive properties and activation of arginase, amongst other effects.[9–11]

To date, the complex interplay between mechanisms that contribute to oxidative stress in SCD is not fully understood. To study the effects that the serum of SCD individuals has on the oxidative state of leukocytes, we observed the effects of serum pooled from healthy control individuals (CON), steady-state SCD patients (SCD) and SCD patients on hydroxyurea (20–30 mg/kg/day) therapy (SCDHU) on markers of oxidative stress and damage in neutrophils isolated from healthy individuals. Results suggest that factors present in the serum of SCD individuals may contribute to the generation of ROS and oxidative damage in leukocytes.

Patients and methods

Subjects

A total of 36 SCD patients diagnosed as homozygous for sickle haemoglobin (using haemoglobin electrophoresis methods and high pressure liquid chromatography) in steady state and attended at the Haematology and Haemotherapy Center, University of Campinas (Campinas, Brazil), participated in the study. Patients were not in crisis and had not received blood transfusions in the preceding 3 months. Patients on hydroxyurea (HU) therapy had been taking 20–30 mg/kg for at least 3 months. Healthy individuals (18) were used as controls (aged 20–50 years). Informed consent was obtained from all patients and controls and the study was approved by the Ethics Committee of the University of Campinas, in accordance with the Helsinki Declaration of 1975. For clinical characteristics of all subjects participating in the study, see Table 1.

Collection of serum

Whole blood samples from controls and patients were collected in glass tubes. Serum was separated from samples by centrifugation (1000*g*; 15 minutes) and filtered through 0·22 μm filters (Millipore, Bedford, MA, USA) to remove any cell debris. Serum samples were manipulated under sterile conditions and pools of serum were prepared by mixing serum from 18 individuals from each subject group and storing frozen (−20°C) for posterior use in cell culture or incubation.

Isolation of neutrophils from peripheral blood

Neutrophils were separated from fresh peripheral blood collected in ethylenediaminetetraacetic acid (EDTA), following a previously described method with adaptations.[12] Briefly, whole blood was laid over two layers of Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) of densities of 1·119 and 1·077 g/ml, respectively. After separation of granulocytes by centrifugation at 700*g* for 30 minutes, the cells were washed once in phosphate-buffered saline (PBS; pH 7·4) before lysing contaminating red cells (10 minutes, 4°C, and lysis buffer; 155 mM/l NH_4Cl and 10 mM/l $KHCO_3$). Cells were washed once again in PBS before resuspending in Roswell Park Memorial Institute medium or other appropriate media. Neutrophil suspensions were utilized immediately in assays and only when their purity was greater than 92%.

Neutrophil culture

Neutrophils (99% viable, according to trypan blue exclusion) were purified under sterile conditions, as

Table 1 Clinical details of SCD patients participating in the study

	CON (n=18)*	SCD (n=18)	SCDHU (n=18)
Male/female	11/7	4/14	11/7
Age (years)	39·0 (20, 40·5, 63)	39·6 (27·0, 37·5, 65·0)	34·2 (21, 35, 48)
RBC ($\times 10^6$/μl)	N/D	2·9 (1·6, 3·0, 4,6)	2·5 (1·6, 2·6, 4,0)
Haematocrit (%)	42·1 (38, 42, 48)	25·4 (16·0, 25·2, 34·3)	25·7 (18·4, 26·6, 36·7)
Hb (g/dl)	N/D	8·0 (3·3, 8·1, 11·2)	8·7 (6·0, 8·8, 13·1)
Mean corpuscular volume (fl)	N/D	89·7 (67·9, 91·8, 105·2)	103 (68·1, 101·7, 127·6)
Mean corpuscular Hb (pg)	N/D	29·7 (20·5, 30·4, 36·8)	34·9 (22·5, 34·4, 44·1)
WBC ($\times 10^3$/μl)	N/D	8·8 (4·5, 8·3, 15·0)	9·6 (4·1, 9·6, 16·7)
HbF (%)	N/D	4·5 (1·9, 3·1, 11·5)	15·9 (5·3, 16·4, 27·7)

CON, healthy control individuals; SCD, steady-state SCD patients not taking HU; SCDHU, steady-state SCD patients on hydroxyurea therapy (20–30 mg/kg/day for at least 3 months); RBC, red blood cell; Hb, haemoglobin; WBC, white blood cell; HbF, fetal haemoglobin; N/D, not determined.
Data presented (except male/female value) are mean (median, minimum, maximum).

described above, and resuspended (4×10^6 cells/ml) in Roswell Park Memorial Institute medium (GIBCO-Invitrogen Corp., Carlsbad, CA, USA), containing 100 U/ml penicillin and 10 μg/ml streptomycin (GIBCO-Invitrogen Corp.). Pooled serum was added (to a final concentration of 10% v/v) and the cells were incubated in flat-bottomed 24-well plates for 16 hours or in tubes for 2 hours at 37°C in a 5%CO_2 atmosphere. Neutrophils were also co-cultured in the presence of superoxide dismutase (300 U/ml), vitamin C (10–1000 μg/ml) and N-acetyl L-cysteine (NAC; 10–1000 μg/ml). All substances were purchased from Sigma-Aldrich.

Measurement of intracellular ROS formation

ROS was measured in RBCs, platelets and neutrophils from peripheral blood samples of healthy control individuals, collected in EDTA. After the incubation of whole blood with pooled serum (10% v/v) for 2 hours at 37°C in 5%CO_2, RBC, platelets and neutrophils were marked with anti-glycophorin A-phycoerythrin (PE; clone CLB-ery-1; Caltag Laboratories, Burlingame, CA, USA; RBC marker), anti-CD42b-PE (clone HIP-1; BD Pharmingen, San Diego, CA, USA; platelet marker) or anti-CD16-PE-Cy5 (clone MOPC-21; BD Pharmingen; neutrophil marker), respectively. Samples were then incubated with 2′-7′-dichlorofluorescin diacetate (DCF; 100 μM final concentration; Invitrogen Corp., Carlsbad, CA, USA) for 15 minutes at 37°C in a humidified atmosphere of 5%CO_2 in air. The cells were then pelleted by centrifugation before resuspending in PBS. Cell populations were identified by gating PE-binding cells and cell fluorescence (10 000 events) was determined by flow cytometry at a wavelength of 530 nm. Isolated neutrophils were also incubated with DCF using the same protocol, but in the absence of anti-CD16-PE-Cy5, and intracellular ROS production determined.

Measurement of superoxide anion ($O_2^{\bullet -}$) production

After co-incubation of control neutrophils with pooled serum (10% v/v) for 2 hours at 37°C in 5%CO_2, cells were resuspended in Hank's balanced salt solution supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7·4), and diluted to a concentration of 2×10^6 cells/ml. The production of $O_2^{\bullet -}$ was assayed spectrophotometrically by measuring the superoxide dismutase inhibitable reduction of ferricytochrome c.[13] As a positive control, $O_2^{\bullet -}$ generation was also analyzed after 1 hour of incubation with phorbol myristate acetate (10 nM) in the presence of serum. Spectrophotometric measurements at 550 nm were performed to determine the degree of reduction of ferricytochrome by superoxide anion.

Superoxide dismutase assay

SOD was determined in individual plasma samples using a commercial SOD assay (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

Assessment of annexin-V binding by flow cytometry

Annexin-V binding was assessed by flow cytometry. Cultured neutrophils were washed in PBS and then resuspended in annexin-V binding buffer (BD Pharmingen; 1×10^5 cells/ml). The cell suspension (100 μl) was incubated with fluorescein isocyanate-labelled recombinant annexin V (5 μl; BD Pharmingen) for 15 minutes at room temperature in the dark. Samples were then diluted in binding buffer and analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA, USA) within 1 hour. The neutrophil population was gated and 10 000 events were analyzed. Cells marked with fluorescein isothiocyanate were considered to be annexin-V binding cells, indicating that cells were undergoing apoptotic processes.

Reverse transcription of messenger ribonucleic acid (mRNA)

RNA was isolated from neutrophils pre-treated using TRIzol reagent. RNA samples (5 μg) were incubated with 1 U deoxyribonuclease I (DNase I; Invitrogen, Rockville, MD, USA) for 20 minutes at room temperature and EDTA was added to a final concentration of 2 mM to stop the reaction. DNase I enzyme was subsequently inactivated by incubation at 65°C for 5 minutes. DNase I-treated RNA samples were then reverse transcribed with SuperScript III and RNaseOUT (Invitrogen, Carlsbad, CA, USA) for 50 minutes at 50°C and 15 minutes at 70°C. Complementary deoxyribonucleic acid (cDNA) samples were quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA).

Amplification and quantification of p22phox, p40phox, p47phox, p67phox *and* gp91phox *gene expression by real-time polymerase chain reaction (PCR)*

Synthetic oligonucleotide primers were designed to amplify cDNA for conserved regions of the *p22phox*, *p40phox*, *p47phox*, *p67phox* and *gp91phox* genes (Primer Express™; Applied Biosystems, Foster City, CA, USA). Specific primers were synthesized by Invitrogen (*see* Table 2 for primer sequences). Primers designed to amplify genes encoding beta-actin

(*BAC*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were synthesized as internal controls. All samples were assayed in a 12 µl volume containing 10 ng cDNA (3 µl), 6 µl SYBR Green Master Mix PCR (Applied Biosystems) and respective primers (3 µl) (for primer concentrations, *see* Table 2) in a MicroAmp Optical 96-well reaction plate (Applied Biosystems) using the StepOnePlus™ Real-Time PCR System (Applied Biosystems), as described previously. To quantify gene expression, the geNorm mathematical model[14] was used, employing calibration data and *BAC* and *GAPDH* expressions as internal controls. Two replicas were run for each sample and results are expressed as relative gene expression when compared to *BAC* and *GAPDH* expressions.

Statistical analysis

Data are reported as mean ± SEM and were analyzed by repeated-measures analysis of variance in association with the Bonferroni post-test. A P value of $<0{\cdot}05$ was considered statistically significant.

Results

Incubation of healthy control neutrophils, but not RBCs and platelets, with SCD serum, induces intracellular ROS production

Whole blood from healthy control individuals was incubated in the presence of 10% (v/v) pooled serum from CON, SCD or SCDHU, for 2 hours at 37°C in 5%CO_2. The presence of intracellular ROS was then determined in neutrophils (identified by CD16-PE binding) by the addition of DCF in association with flow cytometry. After 2 hours, SCD and SCDHU serum had induced significantly higher levels of ROS production in control neutrophils, compared to CON serum (data not shown). Following 16 hours of incubation of control blood with all serum types, ROS levels were high in neutrophils of all groups and levels were not found to differ significantly according to serum type (2227·0 ± 345·2, 1986 ± 214·1 and 2035 ± 243·3 mean fluorescence intensity (MFI) for CON, SCD and SCDHU, respectively; $n=6$, $P>0{\cdot}05$). ROS production in isolated control neutrophils was also determined following incubation for 2 hours (37°C; 5%CO_2) with CON, SCD or SCDHU serum (10% v/v); results were analogous to those seen in whole blood-incubated neutrophils (Fig. 1A), and results for isolated neutrophils are presented in the rest of the manuscript.

In contrast, ROS production was not significantly induced in healthy control RBC or platelets following incubation of whole blood for 2 hours with pooled serum from CON, SCD and SCDHU individuals (37°C; 5%CO_2). ROS production by RBC following incubation with CON, SCD and SCDHU serum was 29·8 ± 2·3, 29·7 ± 1·9 and 43·6 ± 11·9 MFI, respectively; $n=6$, $P>0{\cdot}05$. ROS production by platelets following incubation with CON, SCD and SCDHU serum was 21·1 ± 2·0, 20·5 ± 1·2 and 23·7 ± 1·8 MFI, respectively; $n=6$, $P>0{\cdot}05$.

ROS production is reduced when control neutrophils are co-incubated with pooled serum and anti-oxidants

Isolated control neutrophils were co-incubated with CON, SCD and SCDHU serum (10% v/v) in the presence of the anti-oxidants, vitamin C (100 µg/ml) or NAC (100 µg/ml), for 2 hours at 37°C in 5%CO_2. Vitamin C was able to reduce ROS production independently of the type of serum (Fig. 1B).

Production of superoxide anion ($O_2^{\bullet-}$) from control neutrophils incubated with SCD serum

Isolated control neutrophils were incubated with pooled CON, SCD and SCDHU serum (10% v/v) for 2 hours at 37°C in 5%CO_2, and $O_2^{\bullet-}$ production evaluated. SCD serum induced a significantly higher level of $O_2^{\bullet-}$ production in control neutrophils, when

Table 2 Primer sequence for performance of real time quantitative PCR

Gene	Primer sequence (5′-3′)	Primer concentration (nM)
p22phox	F - GGGGAAGAGGAAGAAGGGC R - GGCACCGAGAGCAGGAGAT	70
p40phox	F - TGCCCTCAACGCCTACATG R - TGGTAAAAGAAGATCCGGACG	70
p47phox	F - TGGCAAGAGTACCGCGACA R - CCCCCGTGGACAGAGCC	70
p67phox	F - AGAAGGGCAATGATAACTGGG R - GGTGGATCCGCAGCTCAA	35
gp91phox	F - TTCCTCAGCTACAACATCTACCTCA R - CTCCTCATCATGGTGCACAGC	150
BAC	F - AGGCCAACCGCGAGAAG R - ACAGCCTGGATAGCAACGTACA	150
GAPDH	F - GCACCGTCAAGGCTGAGAAC R - CCACTTGATTTTGGAGGGATCT	150

F, forward; R, reverse; *BAC*, encoding beta-actin; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

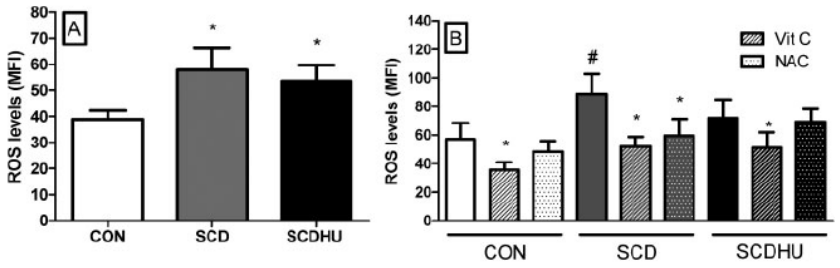

Figure 1 (A) ROS levels in control neutrophils co-incubated with pooled serum from CON, SCD and SCDHU (20–30 mg/kg/day) (10% v/v) for 2 hours; the cells were incubated with 100 μM DCF and fluorescence measured by flow cytometry. *$P<0{\cdot}05$, compared to CON; $n=18$. (B) ROS levels in control neutrophils co-incubated with pooled CON, SCD or SCDHU serum (10% v/v) together with 100 μg/ml vitamin C or 100 μg/ml NAC, for 2 hours. *$P<0{\cdot}05$, compared to culture without vitamin C or NAC; #$P<0{\cdot}05$, compared to CON without vitamin C or NAC; $n=7$

compared to CON serum (Fig. 2A). SCDHU serum also augmented neutrophil $O_2^{\bullet-}$ production, but this increase was not significant. Phorbol myristate acetate (10 nM) stimulation of neutrophils was able to markedly increase $O_2^{\bullet-}$ production in the presence of all three serum types ($41{\cdot}2\pm2{\cdot}7$, $37{\cdot}5\pm2{\cdot}8$ and $36{\cdot}1\pm2{\cdot}6$ pM $O_2^{\bullet-}/10^6$ cells/minute, for CON, SCD and SCDHU serum, respectively; $n=8$, $P>0{\cdot}05$).

SOD levels are significantly lower in SCD plasma

Levels of SOD, an endogenous anti-oxidant, were determined in the plasma of CON, SCD and SCDHU individuals. A significantly lower level of this enzyme was observed in the plasma of SCD individuals not on HU therapy (Fig. 2B), compared to the plasma of CON and SCDHU individuals. Data indicate that the anti-oxidizing capacity of SCD plasma or serum may be impaired.

Induction of neutrophil ROS production by SCD serum may contribute to membrane phosphatidyl serine exposure

To look at the damaging effects that ROS production may have on leukocytes, we determined the ability of serum from SCD individuals to induce apoptotic effects in control neutrophils. Cells were cultured for 16 hours in the presence of 10% (v/v) pooled CON, SCD or SCDHU serum. SCD pooled serum induced a significantly higher rate of annexin-V binding to control neutrophils than CON or SCDHU serum, indicative of a higher apoptotic rate (Fig. 3A).

The anti-oxidants, vitamin C (100 µg/ml) and NAC (100 µg/ml), significantly reduced annexin-V binding to neutrophils following their culture in the presence of SCD serum for 16 hours ($52{\cdot}7\pm2{\cdot}7\%$ reduced to $50{\cdot}1\pm2{\cdot}5\%$ with vitamin C, $P<0{\cdot}05$, $n=11$; $50{\cdot}2\pm2{\cdot}9\%$ reduced to $45{\cdot}9\pm3{\cdot}5\%$ with NAC, $P<0{\cdot}05$, $n=9$). A combination of the use of both vitamin C and NAC in co-culture with neutrophils and CON, SCD and SCDHU serum significantly reduced the apoptotic rate (as indicated by annexin-V binding) of all neutrophil groups (Fig. 3B), indicating that ROS production may contribute to apoptosis of neutrophils cultured in the presence of serum.

Neutrophils were also cultured for 16 hours in the presence of 300 U/ml SOD together with CON, SCD or SCDHU serum. The presence of SOD significantly and drastically decreased the rate of annexin-V binding to neutrophils incubated with all three types of serum (Fig. 3C), indicating that intracellular superoxide production probably contributes significantly to neutrophil cell death during culture. It may be speculated that the SOD contained in serum is not sufficient to inhibit the effects of increased superoxide production.

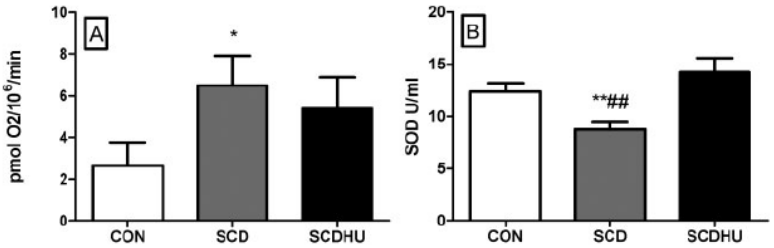

Figure 2 (A) Superoxide ($O_2^{\bullet-}$) production in supernatant of control neutrophils co-incubated for 2 hours with pooled CON, SCD or SCDHU serum. Production expressed as mean$\pm$SEM of pM $O_2^{\bullet-}/10^6$ cells/minute; *$P<0{\cdot}05$, compared to CON; $n\geq6$. (B) Plasma SOD was determined in plasma from CON, SCD and SCDHU individuals, utilizing a colorimetric assay. **$P<0{\cdot}01$, compared to CON; ##$P<0{\cdot}01$, compared to SCDHU; $n\geq11$

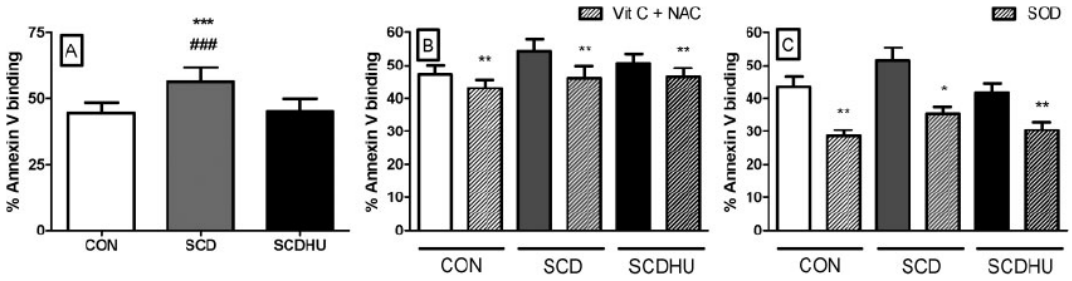

Figure 3 (A) Annexin-V binding to control neutrophils following culture of these cells with pooled CON, SCD or SCDHU serum (10% v/v) for 16 hours; ***$P<0.001$ compared to CON; ###$P<0.001$ compared to SCDHU; $n=8$. (B) Cells were cultured in the presence of pooled serum and with or without 100 μg/ml vitamin C and 100 μg/ml NAC. **$P<0.01$, compared to culture without vitamin C and NAC, $n\geq 8$. (C) Cells were also cultured in the presence of pooled serum, with or without 300 U/ml SOD. *$P<0.05$, **$P<0.01$ compared to culture without SOD; $n\geq 8$

p22phox, p40phox, p47phox, p67phox *and* gp91phox *gene expressions in control neutrophils incubated with pooled serum from control and SCD individuals*

The *p22phox*, *p40phox*, *p47phox*, *p67phox* and *gp91phox* genes encode the subunits of NADPH oxidase, the principal enzyme responsible for superoxide anion production in neutrophils. After incubation of control neutrophils with pooled CON, SCD and SCDHU serum for 2 hours (37°C; 5%CO_2), gene expressions were evaluated by real time PCR; results demonstrated a significantly higher expression of *p22phox*, *p47phox* and *gp91phox* in neutrophils incubated with SCD serum. *p22phox* expression was also significantly elevated in neutrophils incubated with SCDHU serum. In contrast, no significant differences were observed in the expressions of *p40phox* and *p67phox* in neutrophils co-incubated with different serums (Fig. 4).

Discussion

The production of ROS has major pathophysiological consequences. Oxidative stress modulates numerous cell functions, including signal transduction, cell death pathways and enzyme activity. NADPH oxidases are a major source of ROS in blood vessels[15] and, in neutrophils, the superoxide anion produced by the NADPH oxidase complex is subsequently converted to other oxidants, including H_2O_2. ROS may also be converted by neutrophil myeloperoxidase, leading to the production of the potent oxidant, hypochlorous acid.[16]

It has been recognized for some time that patients with SCD are subject to increased oxidative stress, especially during vaso-oclusive crises and acute chest pain.[17] Previous studies have demonstrated that neutrophils, RBC and platelets of SCD individuals generate significantly higher quantities of ROS than

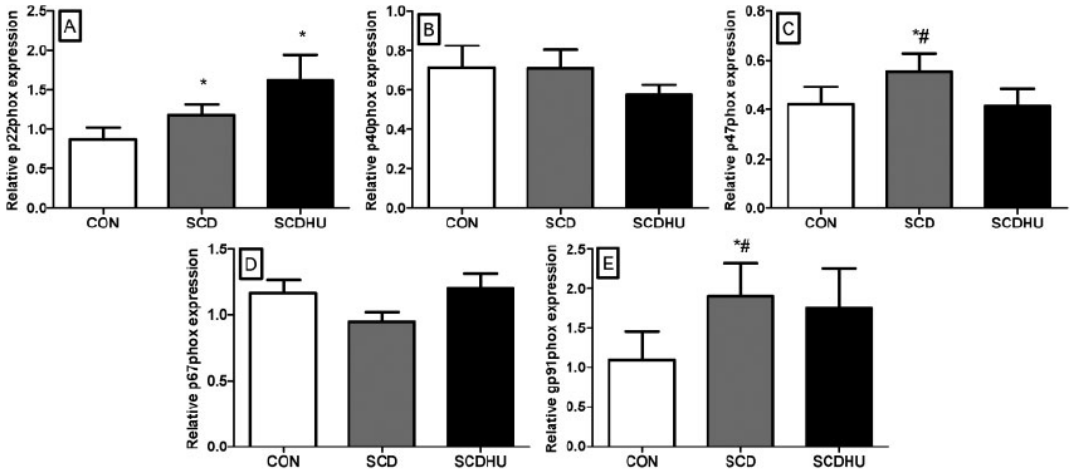

Figure 4 Expressions of (A) ***p22phox***, (B) ***p40phox***, (C) ***p47phox***, (D) ***p67phox*** and (E) ***gp91phox*** mRNA in control neutrophils co-incubated for 2 hours with pooled CON, SCD or SCDHU serum. Values recorded were normalized according to the quantity of ***BAC*** and ***GAPDH*** mRNA in each sample measured in the same reverse transcriptase PCR run. *$P<0.05$, compared with CON; #$P<0.05$, compared with SCDHU; $n\geq 6$

control cells,[18] an effect that could be reversed by exposure of cells to anti-oxidants. The data presented herein demonstrate that factors present in the serum of SCD individuals appear to be capable of inducing production of ROS in healthy control neutrophils, but not healthy control RBC nor platelets.

Production of ROS by control neutrophils, in the presence of SCD serum, was associated with a significant increase in superoxide anion production in these cells. Interestingly, levels of the SOD enzyme in the circulation of our population of steady-state SCD patients were found to be significantly decreased (as previously observed in other SCD populations),[6] and it may be hypothesized that decreased $O_2^{\bullet -}$ breakdown, due to reduced SOD, may contribute to increased $O_2^{\bullet -}$ levels in SCD serum incubated leukocytes.

The production of ROS by neutrophils is essential to the function of these cells, which employ these molecules to destroy pathogens.[19] The neutrophil NADPH oxidase enzyme consists of a membrane-bound flavocytochrome b_{558} (formed by *p22phox* and *gp91phox*/NOX2) and several proteins present in the cytosol (*p47phox*, *p67phox*, *p40phox* and RAC2), which interact with each other to form a complex.[20–22] In response to stimulation, the cytosolic components migrate to the membrane where they assemble with the flavocytochrome b_{558} to form the activated enzyme, a process regulated by phosphorylation of *p47phox*[23] and also by phosphorylation of *gp91phox*/NOX2.[21] *p47phox* phosphorylation appears to be absolutely required for NADPH oxidase activation, since this subunit is responsible for transporting the cytosolic complex to the membrane[24] and phosphorylation of *gp91phox* also participates in the regulation of phagocyte NADPH oxidase activity.[21] Importantly, the expression of *gp91phox*/NOX2, and some of its regulatory proteins, may be upregulated in certain circumstances, as observed in the presence of cardiovascular risk factors.[15] Interestingly, our findings demonstrate an up-regulation of the expressions of the genes encoding the *p47phox*, *gp91phox* and *p22phox* subunits in control neutrophils after incubation with pooled SCD serum, compared to control serum. Relative expressions of genes encoding *p40phox* and *p67phox* were not significantly increased by incubation of CON neutrophils with SCD serum, compared to CON serum; both these subunits appear to play a less essential role in the activation of NADPH oxidase.[20,25]

The evidence for a role of oxidative stress in the induction of apoptosis comes mainly from observations that anti-oxidants inhibit or delay the onset of apoptotic cell death.[26] Reports indicate that ROS production may block the sensitivities of the caspase enzymes that mediate apoptotic processes and, therefore, trigger novel oxidant-dependent neutrophil death pathways.[27–29] Serum from SCD individuals was shown to increase the apoptotic rate of neutrophils from healthy control individuals, and this increase was reversed by the presence of anti-oxidants such as vitamin C and NAC, as well as by the presence of SOD. Results indicate a role for ROS production in the SCD serum-induced cell death observed and, indeed, under the conditions utilized, data suggest a major role for the superoxide anion in neutrophil apoptotic processes. The apoptotic death of activated neutrophils is proposed to be a critical component in the resolution of the inflammatory process.[30] Small alterations in apoptotic rates, may be capable of inducing differences in cell counts; previous reports demonstrate that apoptotic pathways may be inhibited in neutrophils of SCD individuals,[31] possibly contributing to leukocytosis in these patients. As such, whilst data indicate that one of the consequences of the oxidizing potential of SCD serum may be the induction of cell death, it may be that this effect may contribute beneficially to counter the attenuation of apoptotic processes that appears to be innate to neutrophils of these individuals.

Some effects of HU therapy upon the oxidizing potential of the serum of SCD individuals were observed. Total ROS production and superoxide anion generation was reduced, but not significantly, in leukocytes incubated with SCDHU serum, compared to SCD serum; however, HU therapy was found to be associated with significantly higher levels of plasma SOD and a lower serum apoptotic-inducing capacity, compared to SCD serum. Thus, HU therapy may be associated with some anti-oxidant effects in SCD.

The factors that may contribute to the leukocyte-oxidizing capacity of SCD serum are numerous. Certainly data indicate that the lack of SOD in the serum of SCD individuals may well contribute to increase levels of superoxide anion in leukocytes incubated in serum. Furthermore, previous reports indicate that anti-oxidants such as vitamins A, C and E, and zinc[29,32–34] are decreased in the circulation of SCD patients. High levels of haemolysis result in the liberation of free haemoglobin to the plasma that, together with high levels of free iron, may make an important contribution to the oxidizing potential of SCD serum.[35] Since leukocyte numbers are elevated in SCD, the induction of ROS production in leukocytes by factors present in the serum of these individuals may

make an important contribution to the oxidative stress that is characteristic in SCD and, in turn, may aggravate the pathophysiology of the disease. Supplementation with anti-oxidants, such as vitamin E, zinc and NAC, has shown some effect in reducing oxidative stress in small groups of SCD patients[32,34,36] and is reported to reduce ROS production in RBC from SCD and beta-thalassemia individuals.[7,37] Further studies to observe the benefits of such agents in association with therapies for SCD should be considered.

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CAPÍTULO 2

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Alterations in cell maturity and serum survival factors may modulate neutrophil numbers in sickle cell disease

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Running title: Modulation of neutrophil numbers in SCD

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Abstract

Leukocytes are known to exacerbate inflammatory and vaso-occlusive processes in sickle cell disease (SCD). The aim of this study was to determine whether alterations in neutrophil maturity and/or cell-death modulating factors in the circulation contribute to the increased leukocyte counts and leukocyte survival observed in SCD. The maturity of circulating neutrophils from healthy control individuals (CON), SCD and SCD patients on hydroxyurea (SCDHU) was determined immunophenotypically. Serum factors affecting neutrophil apoptosis (determined by annexin-V binding) were analyzed by culturing control neutrophils (CON neutrophil) with pooled serum from CON, SCD and SCDHU individuals. Immunophenotypic characterization of neutrophils suggested a slight, but significant, increase in the circulation of immature neutrophils in SCD. Whilst SCD neutrophils cultured in the presence of CON serum presented delayed apoptosis, unexpectedly, the culture of CON neutrophil with SCD serum demonstrated significantly augmented apoptosis and caspase-9 activity. Inhibition of the activity of serum interleukin-8, a neutrophil-apoptosis-inhibiting cytokine, significantly increased SCD serum-induced CON neutrophil apoptosis, indicating that SCD serum may have both apoptotic and anti-apoptotic properties. The decreased maturity of SCD neutrophils observed is suggestive of an accelerated immigration of leukocytes from the bone marrow to the circulating pool that, together with inherent alterations in SCD leukocytes, may contribute to an increase in cell survival, subject to modulation by a complex balance of both anti- and pro-apoptotic factors contained in the circulation of SCD individuals.

Keywords: Apoptosis; Cytokine; Inflammation; Neutrophil; Sickle cell disease

Introduction

Sickle cell disease (SCD) is a genetic disorder caused by a single mutation in the β-globin gene.[1] As the consequence of hemoglobin S polymerization and red cell alterations, individuals with SCD present a characteristic chronic inflammatory state that, in conjunction with endothelial dysfunction, oxidative stress and cell adhesiveness, initiates vaso-occlusion, a multi-step process that appears to involve the participation of several cell types. Whilst the adhesion of erythocytes to the vascular endothelium of post-capillary venules is known to be a crucial event in the pathophysiology of vaso-occlusion,[2] animal models suggest that augmented leukocyte adhesion to the endothelium and the consequent formation of heterocellular aggregates may initiate this process.[3]

Increased white cell counts are associated with augmented mortality and morbidity in SCD;[4] however the mechanisms responsible for leukocytosis remain unclear. Increased levels of circulating granulocyte macrophage-colony stimulating factor (GM-CSF) have been found to correlate significantly with leukocyte and neutrophil counts in SCD patients,[5] suggesting that increased granulopoiesis may contribute to leukocytosis. However, alterations in the rate of the immigration of leukocytes from the bone marrow to the circulating pool and their subsequent emigration into tissues are both mechanisms that are also known to contribute to leukocytosis.[6] In addition, small alterations in apoptotic rates may also be capable of inducing differences in cell counts.[7]

Neutrophil apoptosis is initiated by the activation of caspases (caspase-3 and the effector caspases-8 and-9), whose cleavage of cellular proteins disassembles the cell.[8] In a previous study, we observed an inhibition of caspase-dependent apoptotic processes in neutrophils from SCD individuals when cultured for 20 h,[9] suggesting that an inhibition of

programmed cell death pathways could lead to an increase in leukocyte survival in SCD and, therefore, increase leukocyte numbers in this disease.[4] The relevance of cell death to the pathogenesis of inflammatory disease is now recognized, since alterations in the apoptotic and death processes of leukocytes may affect their cellular function and inflammatory processes. Necroptosis, for example, is a recently-identified caspase-independent alternative cell death pathway that resembles a programmed cellular necrosis mechanism.[10] Necroptosis may occur following TNF-α-receptor stimulation, under certain conditions, and may play a role in inflammatory processes due to the liberation of cell contents in a non-controlled manner.[11] Importantly, the presence of leukocytes at the vaso-occlusive site, in addition to participating in the physical obstruction of the vessel, probably also makes a considerable contribution to local inflammation due to the production of inflammatory mediators, including interleukin (IL)-8, Interferon γ, tumor necrosis factor α (TNF-α), IL-1β and Macrophage Inflammatory Protein (MIP)-1α/β.[12,13]

The first aim of this study was to determine whether alterations in neutrophil maturity and/or cell-death modulating factors in the circulation contribute to the increased leukocyte counts and survival observed in SCD. Subsequently, since the serum of SCD individuals was found to contain factors that both increased and inhibited neutrophil death, we investigated whether SCD serum was able to induce programmed necroptic death in neutophils, a mechanism that could contribute to inflammation in SCD, and also determined whether neutrophils undergoing cell death could carry out potentially inflammatory activities, such as cell adhesion.

Patients and Methods

Subjects

A total of 63 SCD patients diagnosed as homozygous for HbS (using hemoglobin electrophoresis methods and high pressure liquid chromatography) in steady state and attended at the Hematology and Hemotherapy Center, University of Campinas, participated in the study. Patients were not in crisis and had not received blood transfusions in the preceding 3 months. Patients on hydroxyurea therapy (SCDHU) had been taking 20-30 mg/Kg for at least 3 months. Of the 63 SCD individuals participating in the studies, 36 (18 SCD and 18 SCDHU) provided serum for the serum pools, 7 individuals provided both serum and neutrophils for *in vitro* studies, and 23 patients provided both serum and neutrophil samples. A total of 63 healthy individuals were used as controls (CON) (aged, 20-50) in the study; of these 18 provided serum that was used in a "control serum pool"; while the remaining individuals provided neutrophil for use in all the assays control individual neutrophil assays. Informed consent was obtained from all patients and controls and the study was approved by the Ethics Committee of the University of Campinas, in accordance with the Helsinki Declaration of 1975. For clinical characteristics of all subjects participating in the study, see Table 1.

Immunophenotypic characterization of neutrophils

For determination of neutrophil maturation marker expression, peripheral blood samples (100µl) collected in EDTA were incubated with anti-CD45-fluorescein isocyanate (FITC)

(Invitrogen, CA, USA) and anti-CD16-PE (Invitrogen, CA, USA) for 20 min, RT in the dark. After lysis of red cells (155 mM NH4Cl, 10 mM KHCO3, 10 minutes, RT in the dark), cells were pelleted and washed once in PBS before resuspending in 1% paraformaldehyde. Fluorescence data for erythrocyte-depleted leukocytes (10 000 events) were acquired in a FACSCalibur flow cytometer (Becton-Dickinson Biosciences, San Jose, CA) using the CellQuest software program. For data analysis, DIVA software (Becton-Dickenson) was used. The granulocytic population was gated and subpopulations of interest were quantified as percentages of total gated granulocytes; eosinophils were excluded from the population based on CD45/SSC and CD16/CD45 dot plots. Fluorescence was compared to that of cells stained with negative isotype antibodies. CD16/CD45 immunophenotypic characterization of granulocytic maturation permitted the discrimination of at least two granulocytic differentiation stages; immature neutrophils ($CD16^{neg}/CD45^{low}$) and mature neutrophils ($CD16^{pos}/CD45^{pos}$).[14]

Collection and pooling of serum

Whole blood samples from CON, SCD and SCDHU individuals were collected in glass tubes. Serum was separated from samples by centrifugation (1000 g, 15 min) and filtered through 0.22 µm filters (Millipore, Bedford, MA, USA) to remove any cell debris. Serum samples were manipulated under sterile conditions and pools were prepared by mixing serum from 18 individuals from each subject group and storing frozen (-20°C) for posterior use in cell culture.

Neutrophil culture

Neutrophils were purified from peripheral blood (collected in EDTA), under sterile conditions,[9,14] and resuspended ($4x10^6$ cells/mL; 99% viable, according to Typan blue exclusion; > 92% neutrophil purity) in RPMI (Gibco™, Grand Island, NY, USA), containing 100 U/mL penicillin and 10 µg/mL streptomycin (Gibco™ Grand Island, NY). Pooled serum (Gibco™, Grand Island, NY, USA) was added (to a final concentration of 10% v/v) and the cells were incubated in flat-bottomed 24-well plates at 37°C in a 5% CO_2 atmosphere for 16 h. Neutrophils were also co-cultured in the presence of cytokine activity-neutralizing antibodies (R&D Research, Minneapolis, MN, USA); anti-GM-CSF (1; 10; 500ng/mL), anti-TNF-α (0.1; 1; 10µg/mL); anti-IL-8 (0.01; 0.1; 10 µg/mL); anti-IL-6 (0.5; 1; 10 µg/mL) or with IL-8 (R&D Research, Minneapolis, MN, USA; 500ng/mL) or Necrostatin (Merck, Darmstadt, Germany) (10µM).

Assessment of viability by MTS assay

Viability of cells was assessed using the MTS colorimetric assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit; Promega Corporation, Madison, WI, USA). Following 16-h culture of neutrophils, MTS solution (20 µl) was added directly to the culture wells ($4x10^5$ cells) and incubated for 1 h at 37°C, 5% CO_2. Absorbance at 490nm was then recorded in an ELISA microplate reader. Control experiments were carried out using empty wells and fully vital cells.

Assessment of annexin-V and propidium iodide binding by flow cytometry

Annexin-V binding was assessed by flow cytometry. Cultured neutrophils were washed in PBS and then resuspended in annexin-V binding buffer (BD Pharmingen, San Diego, CA, USA; $1x10^5$ cells/mL). The cell suspension (100 μL) was incubated with FITC-labelled recombinant annexin V (5 μL) and propidium iodide (PI; 5 μL) (BD Pharmingen, San Diego, CA, USA) for 15 min, RT, in the dark. Samples were then diluted in binding buffer and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA) within one hour. The neutrophil population was gated and 10 000 events were analyzed. Cells marked with FITC were considered to be annexin-V binding cells, indicating that they were undergoing the apoptotic process. Staining of cells with PI alone indicated that cells were undergoing necrosis.

Caspase-3, -8 and -9 activity assays

Neutrophils were cultured under the conditions described above for 16 h. Cell samples (3-$5x10^6$ cells) were taken at 0 and 16 h, pelleted and stored at -80°C until assay. At the time of assay, samples were resuspended in chilled lysis buffer (Biosource, Carlsbad, CA, USA) and incubated on ice for 10 minutes. Following centrifugation (1 min, 10 000 g), supernatant was removed and the cytosol extract was then diluted to a protein concentration of 1 mg/mL (determined by Bradford assay[15]). Caspase-3, -8 and -9 activities were then determined in each sample using Caspase Apo-Target kits (Biosource International, Camarillo, CA, USA), according to the instructions of the manufacturer. The sample's ability to cleave synthetic peptide substrates was quantified by measuring absorbance at 405 nm.

Caspase-3, -8 and -9 gene expression

RNA was isolated from neutrophil pre-cultured under the conditions described above, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the samples were reverse transcribed in cDNA using SuperScript III (Invitrogen, Carlsbad, CA, USA). Caspase gene expressions were quantified by real time PCR, using synthetic olygonucleotide primers, the sequences of which may be found in Table 2. Beta-actin (BAC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized as internal controls. All samples were assayed in a 12 µL volume containing 10 ng cDNA (3 µL), 6 µL SYBR Green Master Mix PCR (Applied Biosystems) and respective primers (3 µL) using the StepOnePlus Real-Time PCR System (Applied Biosystems). GeNorm was used to quantify gene expression.

Neutrophil adhesion assay

Neutrophil static adhesion assays were performed as previously described,[16,17] where neutrophils ($2x10^6$ cells/ml in RPMI medium) were allowed to adhere to immobilized fibronectin (20 µg/ml) for 30 min (37^oC, 5%CO_2).

Statistical analysis

Data are reported as median (Min/Max), or mean ± SEM, and were compared the Wilcoxon test for non-parametric paired data or the Mann-Whitney test for non-parametric unpaired data, as indicated. A *p*-value of less than <0.05 was considered as statistically significant.

Results

Immunophenotypic characterization of peripheral blood neutrophils indicates a higher incidence of immature cells in the SCD circulation

The expression of markers of neutrophil maturity was determined on the surface of neutrophils from the peripheral blood of CON, SCD and SCDHU patients (Table 3 and Supplementary Figure 1). A significant increase in immature neutrophils, as well as a decrease in mature circulating neutrophils, was observed in SCD, compared to CON individuals (Table 3 and Supplementary Figure 1). No significant correlations of neutrophil immaturity with neutrophil WBC numbers were observed for any of the groups (results not shown).

Neutrophils from SCD patients present a lower apoptotic rate than CON neutrophils, when cultured for 16h in the presence of serum from CON individuals

To define whether factors present in the serum of SCD patients or properties inherent to the cells themselves influence neutrophil cell death processes, neutrophils were first isolated from CON individuals, SCD or SCDHU patients and all were cultured in the presence of serum pooled from CON individuals (CON serum, 16 h, 10% v/v). Similarly to our previous findings, [9] SCD neutrophils presented a significantly lower rate of annexin V

binding than CON neutrophils, indicative of decreased apoptosis and, therefore, increased cell survival (Figure 1A).

Serum from SCD patients augments the death rate of CON neutrophils

To determine whether factors present in the serum of SCD patients are responsible for the inhibition of SCD neutrophil apoptosis, neutrophils were isolated from CON individuals and cultured for 16 h in the presence of pooled serum from CON, SCD or SCDHU individuals. Unexpectedly, SCD serum induced a significantly higher rate of annexin V binding on CON neutrophils than serum from CON individuals and from SCDHU patients, indicative of a higher apoptotic rate (Figure 1B). Viability of CON neutrophils, as determined by MTS assay, was not significantly different when cultured in the presence of CON, SCD or SCDHU serum (0.83±0.08; 0.86±0.05; 0.86±0.05 OD^{490nm}, MTS assay, respectively; n=8). Increased annexin V binding on CON neutrophils co-cultured with SCD serum (16 h) was associated with a significantly higher caspase-9 activity, compared to neutrophils cultured in the presence of pooled CON or SCDHU serum (0.016±0.001 OD^{405nm}; 0.025±0.003**# OD^{405nm}; 0.017±0.001 OD^{405nm}, respectively for caspase 9; n=12; **, $p<0.01$ compared to CON; #, $P<0.05$ compared to SCDHU). In contrast, the activities of the caspases-3 and -8 were not observed to be significantly altered in groups of neutrophils following culture with different serums (data not shown). Gene expressions of caspase 3, 8 and 9 were determined in CON neutrophil co-cultured with CON, SCD or SCDHU serum, however no significant differences between the groups was observed (data not shown).

IL-8 in SCD serum may exert an anti-apoptotic effect on CON neutrophils

CON neutrophils were cultured in the presence of CON, SCD and SCDHU serum and also in the presence, or absence, of antibodies that neutralized the activities of the chemo/cytokines, GM-CSF, IL-6, IL-8 and TNF-α. Representative data for one concentration of each antibody are depicted in Figure 2. The GM-CSF, IL-6 and TNF-α cytokine-inhibiting antibodies did not significantly alter annexin-V binding to CON neutrophils following 16 h–culture in the presence of the different serums tested. In contrast, the anti-IL-8 antibody slightly, but significantly, augmented annexin V binding of CON neutrophils cultured for 16h in the presence of SCD serum (Figure 2C); accordingly co-incubation of CON neutrophils with 500ng/ml IL-8 significantly inhibited neutrophil apoptosis (data not shown).

Necroptosis makes some contribution to neutrophil cell death during culture in the presence of serum

Since the induction of cell death by SCD serum appears not to be mediated by increases in the caspase-3 dependent pathway, we hypothesized that CON neutrophils cultured in the presence of SCD serum may be undergoing an alternative cell death process, such as necroptosis. Propidium iodide staining was determined after incubation of CON neutrophils with CON, SCD or SCDHU serum in the presence of the programmed necrosis (necroptosis) inhibitor, Necrostatin (10µM). A reduction in death cell was observed in

neutrophils, independently of serum type (Figure 3), although non-necroptotic death of CON neutrophils incubated with SCD serum was significantly higher than for CON neutrophils when incubated with CON serum (Figure 3).

Adhesive properties of CON neutrophils are higher following culture for 16h in the presence of SCD serum

CON neutrophils were cultured for 16 h in the presence of CON, SCD or SCDHU serum, before comparing their adhesion to fibronectin (FN) using static adhesion assays. Cells that had been cultured in the presence of SCD or SCDHU serum for 16 h demonstrated a higher capacity to adhere to FN (Figure 4).

Discussion

Leukocytosis is frequently observed in SCD and elevated leukocyte counts are associated with increased mortality, constituting a risk factor for crisis, acute chest syndrome and stroke.[4,18-20] In addition, white cell adhesion to the vessel wall may play an initiating role in the vaso-occlusive process.[3] As such, an understanding of the mechanisms that contribute to increase leukocyte counts in the circulation is imperative, as is the comprehension of cell survival and death pathways in SCD. There is evidence that the chronic inflammatory state, observed in SCD, probably contributes to increase granulopoiesis in the bone marrow; [5] however, alterations in the migration of leukocytes

and the rate of their apoptosis in the circulation may also contribute to increase leukocyte numbering SCD.

Neutrophils from SCD individuals, when cultured in the presence of control individual (CON) serum, rather than autologous serum, presented a lower apoptotic rate (i.e. increased cell survival) compared to control individual neutrophils, suggesting that inherent alterations in the cell, rather than circulating factors, could be responsible for delayed apoptosis. An accelerated release of leukocytes from the bone marrow, leading to the circulation of younger cells, could explain changes in apoptotic neutrophil rate. Accordingly, flow cytometric analysis of markers of maturation on the surface of peripheral blood neutrophils demonstrated that there is a small but significant increase in the presence of immature neutrophils in the circulation of SCD individuals, when compared to healthy control individuals and to SCDHU patients, possibly indicating an increase in the mobilization of leukocytes from the bone marrow. Although further data are required to substantiate this hypothesis, IL-8, a chemokine found in elevated concentrations in the circulation of SCD patients[12,21] is known to promote the mobilization and egress of hematopoietic stem cells into the peripheral circulation.[22]

In additional experiments, we investigated whether substances in the serum of SCD individuals can alter neutrophil apoptotic pathways. Unexpectedly, when we cultured CON neutrophils with serum from SCD individuals for 16 h, a higher apoptotic rate was observed than when the same CON neutrophils were cultured in the presence of CON serum and serum from SCDHU individuals. This increase in SCD-serum induced apoptotic rate (as indicated by annexin V binding) was not associated with any significant changes in caspase-3, -8 and -9 gene expression nor in the activities of the effector caspases, caspase-

3 or caspase-8, which mediates the extrinsic death receptor pathway.[23] In contrast to the lack of alteration in caspase-3 activity, neutrophil caspase-9 activity, generally believed to be induced by mitochondrial-mediated cellular processes,[24] was augmented in neutrophils following their culture in SCD serum. It has been shown that SCD serum can induce the production of reactive oxygen species (ROS) in neutrophils[25] and that the production of ROS may participate in the activation cell death pathways in these cells. Importantly, serum from SCDHU had similar effects on neutrophil cell death parameters to CON serum, suggesting that HU therapy appears to reverse alterations in the factors present in the serum that may play a role in the modulation of SCD leukocyte death.

Since elevated TNF-α has been observed in our SCD patient population,[12] and SCD-serum induced annexin V binding occurred in the absence of caspase-3 and -8 activity, we investigated a possible contribution of necroptosis to SCD-serum-induced neutrophil death. Co-culture of CON neutrophils with a necroptosis inhibitor significantly decreased nuclear membrane permeability in the presence of all three serums (CON, SCD and SCDHU), indicating that necroptosis may constitute one of the minor cell death pathways utilized by the neutrophil during *in vitro* culture conditions, although there was no evidence that SCD serum increases neutrophil necroptosis any more than the CON and SCDHU serums.

Cytokines are known to alter neutrophil apoptotic processes;[24,26] a number of inflammatory cytokines, including GM-CSF, IL-8, IL-6 and TNF-α, have been reported as elevated in the circulation of our and other populations of SCD individuals.[5,12,27] GM-CSF and IL-8[28] have important inhibitory effects on neutrophil apoptosis, whilst TNF-α may either induce or suppress neutrophil apoptosis, depending on its concentration.[24] Whilst

GM-CSF, IL-6 and TNF-α-inhibiting antibodies had no effect on SCD-serum induced neutrophil apoptosis, the IL-8-inhibiting antibody significantly augmented apoptosis, indicating that the increased levels of circulating IL-8, previously reported in our population of patients,[12] are sufficient to exert an inhibition of neutrophil apoptosis and that both anti- and pro- apoptotic factors present in the circulation of SCD individuals may regulate neutrophil death. A number of inflammatory diseases are now being treated with cytokine-inhibiting therapies and it may be postulated that therapies[29-31] that successfully reduce inflammation and the production of granulopoietic and apoptosis-inhibiting cytokines could also be an important approach for reducing leukocytosis in SCD.

Alterations in cell death processes, coupled with continual functional activity, and the production of inflammatory molecules may exacerbate the inflammatory state in these individuals. Whilst the serum of SCD individuals appears to contain both factors that induce and inhibit cell death, we sought to clarify whether SCD serum factors can contribute to increase aberrant leukocyte activity. Although SCD serum increased neutrophil apoptosis processes, compared to CON serum, the ability of cells to adhere to the integrin ligand, fibronectin, was not diminished following the incubation of cells for 16 h, indicating that these cells are still able to carry out important adhesive functions that make a significant contribution to vaso-occlusive and inflammatory processes.

In conclusion, the decreased maturity of SCD neutrophils observed is suggestive of an accelerated immigration of leukocytes from the bone marrow to the circulating pool that may contribute to an increase in cell survival. Furthermore, the serum of SCD individuals appears to contain both anti-apoptotic proteins, such as IL-8, and pro-apoptotic agents, such as ROS generating factors, indicating that leukocytes may be

subject to both anti- and pro-apoptotic stimuli in the circulation of SCD individuals that modulate cell death pathways. Further investigations are required in the search to identify molecular drug targets that may be useful for the control of leukocyte numbers in SCD.

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The authors report no potential conflicts of interest relevant to this study.

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Figure Legends

Figure 1: Annexin V binding to **(A)** neutrophils from healthy control individuals (CON, N= 16), SCD patients (SCD, N=13) and SCD patients on HU therapy (SCDHU, N=14), following culture of cells (16 h, 37^{o}C, 5% CO_2) in the presence of serum pooled from control individuals (CON serum, 10% v/v). Data depict percentage of neutrophils binding annexin V (median, max, min), as determined by flow cytometry. *, p <0.05, compared to CON; #, p <0.05, compared to SCDHU; Mann-Whitney test. **(B)** CON neutrophils cultured (16 h, 37^{o}C, 5% CO_2) in the presence of serum (10% v/v) pooled from CON (N=18); SCD (N=18) or SCDHU (N=18) individuals. Data depict percentage of neutrophils binding annexin V (median, max, min); *** p <0.001 compared to CON; ### p <0.001 compared to SCDHU; Wilcoxon test; N=17 different control individual neutrophil populations for each serum pool tested.

Figure 2: Percentage of CON neutrophils binding annexin V following culture of cells with pooled CON, SCD or SCDHU serum (10% v/v) in the absence or presence of **(A)** 10ng/mL anti-GM-CSF; p >0.05; N= 4. **(B)** 1μg/mL anti-IL-6; P>0.05; N=4. **(C)** 10μg/mL anti-IL-8; * p <0.05 compared to w/out anti-IL-8, N=7. **(D)** 1μg/mL anti-TNF-α; p >0.05; N=11. Wilcoxon test. N, refers to the number of independent control individual neutrophil populations utilized in assays.

Figure 3: Nuclear permeability, as assessed by propidium iodide (PI,) staining, of CON neutrophils (N=9), following culture (16 h, 37^{o}C, 5% CO_2) with pooled CON, SCD and

SCDHU serum (10% v/v) and in the presence or absence of necrostatin-1 (Nec-1, 10µM). Graph depicts percentage (Median, Max, Min) of cells staining for PI. **, P<0.01; *** P<0.001, compared to cells incubated with Nec-1; ## *p* <0.01, compared to CON with Nec-1. Wilcoxon test. N, refers to the number of independent control individual neutrophil populations utilized in the assay.

Figure 4: Effect of culture (16 h, 37^{o}C, 5% CO_2) with CON, SCD or SCDHU serum (10% v/v) on the adhesive properties of CON neutrophils (N=7). Following culture, neutrophils were allowed to adhere to immobilized fibronectin (FN; 30 min, 37^{o}C, 5% CO_2), and adhesion expressed as the percentage of cells adhered (Median, Max, Min). * $p < 0.05$, compared to adhesion of neutrophils cultured with CON serum. Wilcoxon test, comparing each group with the CON serum group. N, refers to the number of independent control individual neutrophil populations utilized in the assay.

Supplementary Figure 1: Immunophenotypic characterization of neutrophil maturity: Representative dot plots of FACS analysis for control and SCD patient neutrophils. Neutrophils were identified in the peripheral blood of CON, SCD or SCDHU individuals. The granulocytic population was gated (P1) and eosinophils were excluded from the population based on CD45/SSC and CD45/CD16 dot plots. Immature neutrophils (P3; $CD16^{neg}/CD45^{low}$) and mature neutrophils (P2; $CD16^{pos}/CD45^{pos}$) were classified according to CD16 and CD45 antibody binding.

Table 1: Details and clinical characteristics of all controls and patients participating in the study.

	Control	SCD	SCDHU
Male/female	38/25	13/21	13/16
Age (years)	37.5 (19; 37, 63)	40.7 (19, 42, 65)	34.7 (21, 33, 64)
Red blood cell count (10^{12}/L)	N/D	2.87 (1.77, 2.67, 4.64)	2.53 (1.61, 2.56, 3.56)
Hematocrit (%)	44.22 (38.00, 45.20, 51.00)	25.08 (16.40, 24.40, 38.40)	26.08 (18.40, 26.60, 36.70)
Hemoglobin (g/dl)	N/D	8.29 (5.10, 8.25, 13.20)	8.86 (6.20, 9.10, 13.10)
Mean corpuscular volume (fl)	N/D	88.90 (69.10, 88.90, 111.50)	104.10 (74.90, 100.40, 125.10)
Mean corpuscular hemoglobin (pg)	N/D	29.40 (22.10, 28.80, 39.10)	35.40 (25.40, 35.40, 44.10)
WBC (x10^9/L)	N/D	9.31 (4.50, 9.64, 15.33)	8.36 (4.30, 8.47, 15.56)
HbF (%)	N/D	5.47 (0.50, 4.40, 11.50)	15.11 (5.30, 14.90, 24.70)

SCD, steady-state SCD patients not taking HU; SCDHU, steady-state SCD patients on hydroxyurea therapy (20-30 mg/kg/day for at least 3 months); HbF, fetal hemoglobin; N/D, not determined. Data present (except M/F value) are mean (min, median, max).

Table 2: Primer sequences for the performance of real time quantitative PCR

Gene	Primer sequence (5´-3´)	Primer concentration (nM)
Caspase 3	F- GTGGAATTGATGCGTGATGTTT	300
	R- TTCACCATGGCTCAGAAGCAC	
Caspase 8	F- CCAGTCACTTTGCCAGAGCC	600
	R- CACTTCAGTCAGGATGGTGAGAAT	
Caspase 9	F- AGGCCCCATATGATCGAGG	150
	R- GGAGATGAACAAAGGAAGAGCC	
BAC	F - AGGCCAACCGCGAGAAG	300
	R - ACAGCCTGGATAGCAACGTACA	
GAPDH	F - GCACCGTCAAGGCTGAGAAC	300
	R - CCACTTGATTTTGGAGGGATCT	

F, forward; R, reverse; BAC, encoding beta-actin; GAPDH, encoding glyceraldehyde-3-phosphate dehydrogenase.

Table 3: Immunophenotypic determination of neutrophil maturity in the peripheral blood of study subjects.

	Control (n=7)	SCD (n=10)	SCDHU (n=13)
Immature Neutrophils (%)	0.40 (0.10; 0.50; 1.10)	1.35 (0.60; 1.43 2.70) **	1.00 (0.20; 1.05; 2.00)
Neutrophils (%)	99.60 (98.90;99.50; 99.90)	98.65 (97.30; 98.57; 99.40) **	99.00 (98.00; 98.95; 99.80)

Phenotypic characterization was carried out by flow cytometry and data are expressed as percentages of the gated granulocytic population Median (Max, Mean, Min). $CD16^{neg}/CD45^{low}$ cells were classified as immature neutrophils; $CD16^{pos}/CD45^{pos}$ were classified as mature neutrophils. ** $p<0.01$, compared to control individuals; Mann-Whitney test.

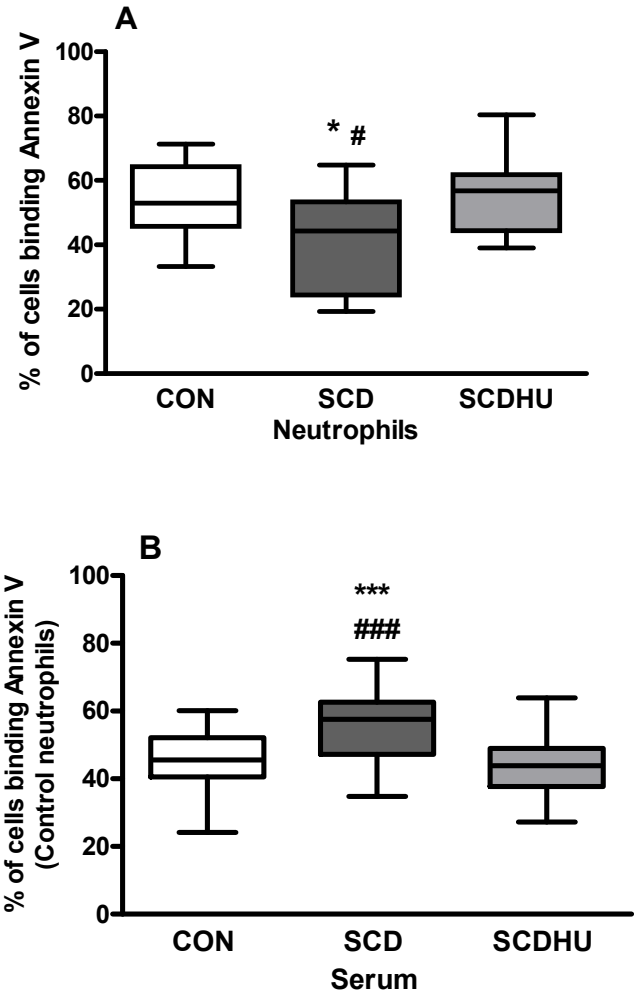

A
% of cells binding Annexin V
100
80
60
40
20
0
* #
CON
SCD
SCDHU
Neutrophils
B
% of cells binding Annexin V
(Control neutrophils)
100
80
60
40
20
0
***
###
CON
SCD
SCDHU
Serum

Figure 1

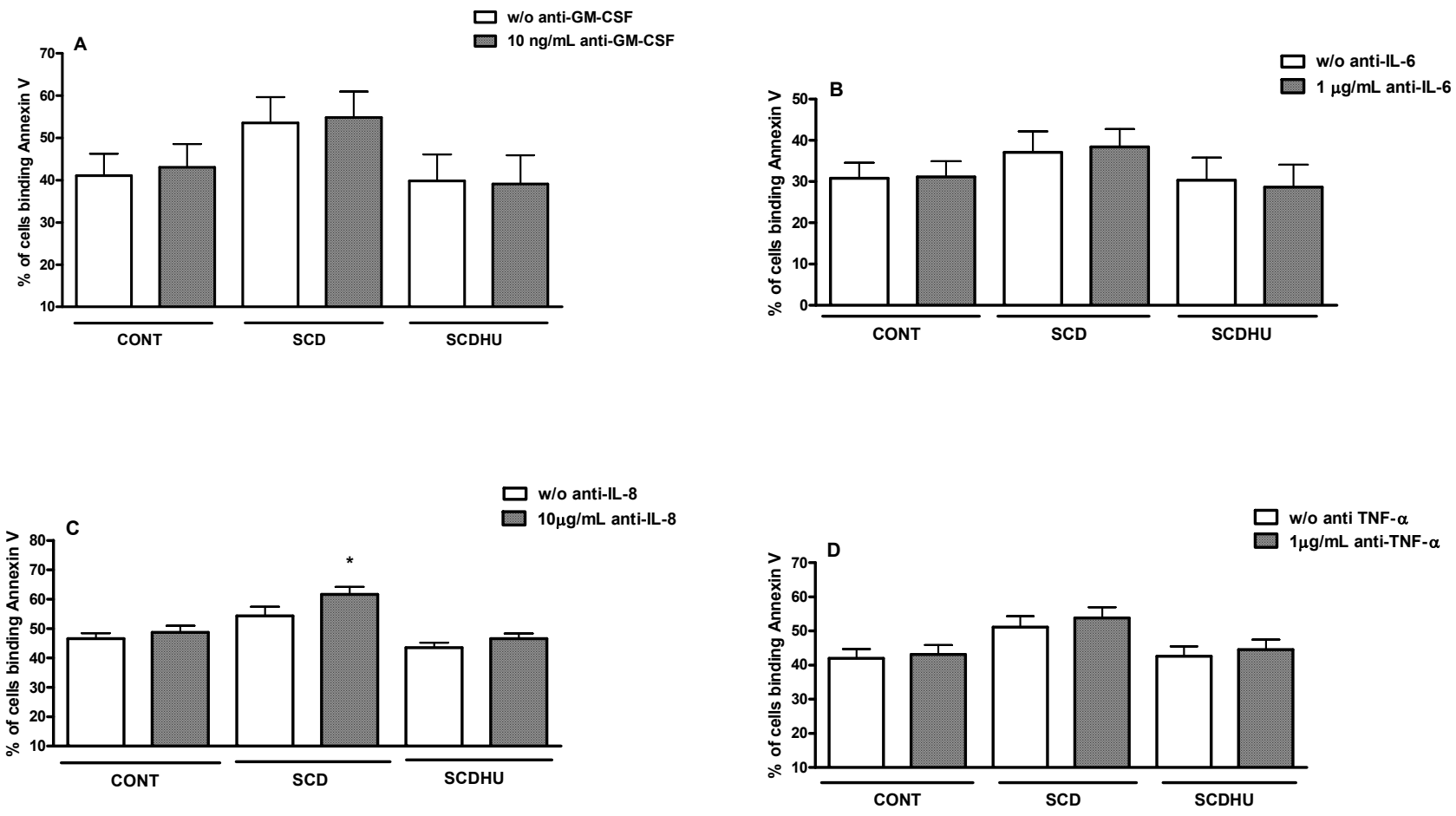

A
w/o anti-GM-CSF
10 ng/mL anti-GM-CSF
% of cells binding Annexin V
CONT
SCD
SCDHU
B
w/o anti-IL-6
1 μg/mL anti-IL-6
C
w/o anti-IL-8
10μg/mL anti-IL-8
*
D
w/o anti TNF-α
1μg/mL anti-TNF-α

Figure 2

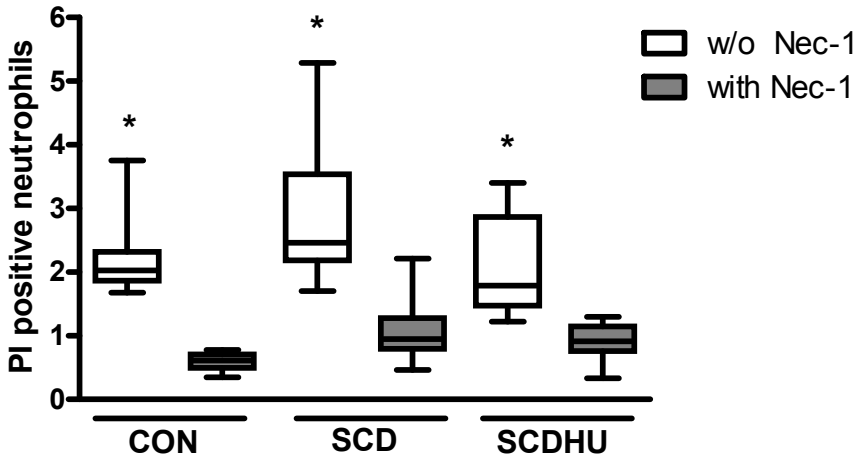

PI positive neutrophils
6
5
4
3
2
1
0
*
*
*
w/o Nec-1
with Nec-1
CON
SCD
SCDHU

Figure 3

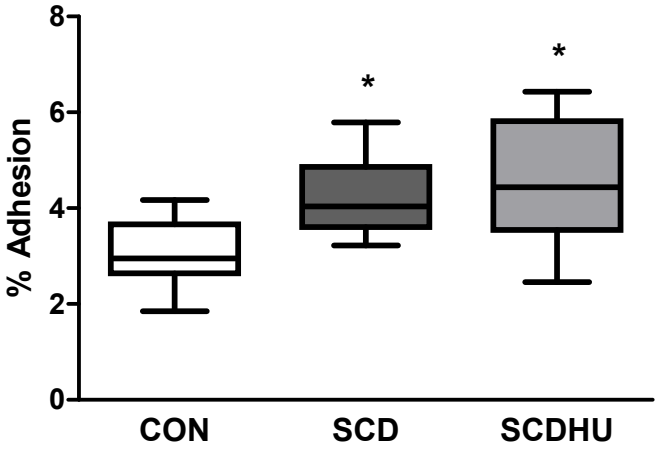
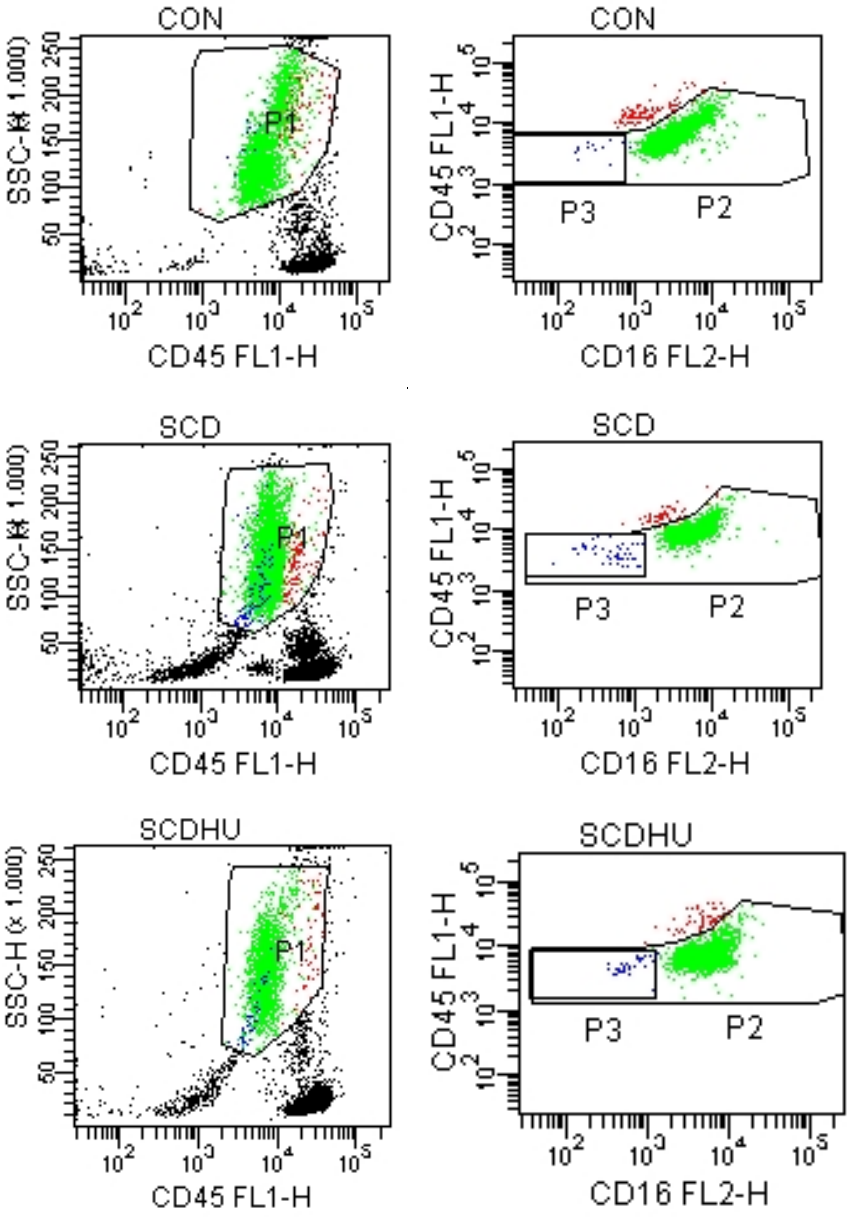


Figure 4



Supplementary Figure 1

CAPÍTULO 3

Hydroxyurea and a cGMP-amplifying agent have immediate benefits on acute vaso-occlusive events in sickle cell disease mice

Artigo em elaboração

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Hydroxyurea and a cGMP-amplifying agent have immediate benefits on acute vaso-occlusive events in sickle cell disease mice

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Running Head: Hydroxyurea and sickle cell vaso-occlusion.

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Abstract

Inhibition of leukocyte adhesion to the vascular endothelium represents an important approach for decreasing sickle cell disease (SCD) vaso-occlusion. Using a humanized SCD mouse-model of TNF-α-induced acute vaso-occlusion, we herein present data demonstrating that short-term administration of either hydroxyurea (HU) or the specific phosphodiesterase 9 (PDE9) inhibitor, BAY73-6691, results in reduced leukocyte adhesion to the microvasculature. Notably, the administration of both agents led to synergistic improvements in leukocyte rolling and adhesion and decreased heterotypic red blood cell (RBC)-leukocyte interactions, coupled with a prolonged survival. Mechanistically, these rheological recuperations were associated with a decrease in leukocyte Mac-1 integrin activity and cGMP-signaling-mediated reductions in leukocyte adhesion to the endothelium. Our findings indicate that HU has immediate beneficial effects on the microvasculature in acute sickle cell crises that are independent of the drug's fetal hemoglobin-elevating properties. In addition, inhibition of PDE9, an enzyme that is highly expressed in hematopoietic cells, amplified the effects of HU and may represent a promising and more tissue-specific therapy for this disease.

Introduction

Sickle cell disease (SCD) is a genetic disorder caused by a point mutation in the β-globin gene, resulting in the production of abnormal sickle hemoglobin (HbS).[1,2] HbS polymerizes at low oxygen levels, making the red cell more rigid and eventually irreversibly sickled. This causes the complex pathophysiology of SCD that includes hemolysis, chronic inflammation, elevated cell adhesion, leukocytosis, increased oxidative stress and endothelial activation/dysfunction, which can culminate in the acute vaso-occlusive processes that are responsible for much of the morbidity observed in these patients.[1,2]

Vaso-occlusion comprises a multi-step and multi-cellular process that appears to be initiated by the adhesion of red cells and leukocytes to activated endothelium via a mechanism in which inflammation, hypoxic events, oxidative stress and reduced nitric oxide availability probably also play roles.[3-9] Data from *in vivo* studies using sickle cell mice[6,10,11] and *in vitro* studies[12,13] indicate that the recruitment of large, less deformable leukocytes to the vessel wall, and their consequent interaction with circulating red cells, may initiate vaso-occlusion. As such, drugs that inhibit the adhesion of leukocytes to vascular endothelium may represent an important approach for decreasing, or even preventing, vaso-occlusive processes.[14]

Research over recent years indicates that decreased nitric oxide (NO) bioavailability, due to mechanisms that include cell-free hemoglobin NO scavenging, may contribute to manifestations of SCD such as pulmonary hypertension and cutaneous leg ulceration.[15,16] Whether reduced NO signaling has a direct role in the vaso-occlusive process is currently unknown; however a number

of studies indicate that nitric oxide-based therapies may be beneficial for increasing regional blood flow,[17] reducing pain[18] and treating stroke[19] in SCD. Furthermore, studies demonstrate that elevation of NO, or supplementation of its substrate, arginine, can reduce SCD neutrophil adhesive properties *in vitro*, and can improve microvascular function,[20] increase survival and prevent lung injury during hypoxia[21,22] in sickle mice.

Hydroxyurea (HU) is a drug approved by the U.S. Food and Drug Administration for use in adults with SCD and is currently the only drug proven to modify the disease process by improving hematologic parameters and hospitalization.[23-25] The drug is thought to act, principally, by increasing fetal hemoglobin (HbF) production in erythrocytes, thereby inhibiting HbS polymerization. While HU is known to inhibit DNA synthesis via inactivation of ribonucleotide reductase,[26] data exist to indicate that HU is a donor of NO *in vivo,*[27] and can induce γ-globin expression in erythroid progenitor cells, *in vitro*, via a cyclic guanosine monophosphate (cGMP)-dependent pathway (Figure 1).[28] Whilst numerous studies indicate that HU might have benefits in SCD that could be independent of its HbF-inducing properties, including reductions in leukocyte counts and increased erythrocyte cation transport,[2,25] to date no immediate short-term benefits have, to our knowledge, been reported following its administration in SCD patients or mouse models.

Importantly, modulation of intracellular levels of the NO second messenger cGMP[29] may represent an effective and cell-specific approach for amplifying intracellular NO-dependent signaling.[29] In addition to the induction of γ-globin

production in erythroid lineage cells,[30,31] activation of this pathway results in a reduction in the adhesive properties of leukocytes, *in vitro*.[32] Recent data demonstrate that the cGMP degrading enzyme, phosphodiesterase 9 (PDE9), may be highly expressed in hematopoietic cells, possibly providing a more tissue-specific drug target.[33] *In vitro*, the specific PDE9 inhibitor, BAY73-6691, both increases γ-globin expression in K562 erythroleukemic cells and reduces SCD neutrophil adhesive properties (Figure 1).[33,34]

The aim of this study was to investigate the effects of acute administration of HU alone, and in combination with the PDE9-inhibiting agent, BAY73-6691, in an *in vivo* model of inflammation-induced vaso-occlusion in SCD mice, and to further determine whether these effects are mediated by a cGMP-dependent pathway.

Methods

Animals

C57BL/6 mice were commercially obtained from the Charles River Breeding Laboratories. Chimeric male sickle cell mice (SCD mice) were generated by transplanting nucleated bone marrow cells harvested from Berkeley sickle cell mice into lethally-irradiated C57BL/6 mice, as previously described.[6,14] Only chimeric SCD mice expressing > 97% human globin were utilized in intravital microscopy techniques at 3-5 months after transplantation. All experimental procedures were approved by the Animal Care and Use Committees of the Mount Sinai School of Medicine, New York, and the Albert Einstein College of Medicine, the Bronx.

Intravital Microscopy

Mice were anesthetized with a mixture of 2% α-chloralose and 10% urethane in PBS (6 ml/kg) and a polyethylene tube was inserted into the trachea to facilitate spontaneous respiration. The cremaster muscle was surgically exteriorized and continuously superfused with bicarbonate-buffered saline (37°C, pH 7.4), equilibrated with a mixture of 95% N_2 and 5% CO_2. Prior to surgery, an inflammatory process was induced (150 min before surgery) in mice by the injection of TNF-α (0.5 µg *i.p*) and mice were concomitantly treated with BAY73-6691 (3mg/Kg *i.v.*), hydroxyurea (100mg/Kg *i.v.*), a combination of the two drugs (3 mg/Kg BAY73-6691 and 100 mg/Kg HU *i.v.*) or vehicle (2% DMSO in PBS), as illustrated in Figure 3A. Some experiments were also carried out employing non-transplanted C57BL/6 mice (Figure 2A). Mice were pre-treated (30 minutes before TNF-α) with the guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 15 mg/kg *i.v.*), or the proten kinase G inhibitor (PKG), KT5823 (1 mg/kg *i.v.*), 30 minutes before the administration of HU or BAY73-6691, respectively. For both protocols, immediately after surgery, microvessels (8-15 for each mouse) were visualized using a custom-designed intravital microscope (MM-40, Nikon; 60X magnification) and videotaped continuously for 2 minutes. Images were recorded using a charge-coupled video camera device (Hamamatsu, Bridgewater, NJ) and a Sony SVHS SVO-9500 video recorder. Leukocyte (WBC) rolling, adhesion and extravasation were monitored and analyzed for 30 minutes after surgery. The definitions of rolling, adhesion, extravasation and interactions between red blood cells (RBC) and WBC, which were analyzed by videotape

playback, have been described previously. [6,14] All SCD mice were monitored until death or up to 660 min, at which time they were sacrificed. Blood cells counts were determined using an automatic cell counter Beckman Coulter (A^cT) Fullerton, CA.

Fluorescence intravital microscopy and Mac-1 activity assay

Mice were prepared for intravital microscopy as described above. Inflammation was induced with TNF-α (0.5 µg *i.p*) and mice were treated *i.v*. with a combination of BAY73-6691 and HU or vehicle. Five min before analysis, mice were injected *i.v*. with 10^9 BSA-coated fluorospheres (Invitrogen) as described before[11] and binding of spheres to adherent leukocytes was quantified using Slidebook software (Intelligent Imaging Innovations, Denver, CO) on a Zeiss Examiner microscope equipped for epifluorescence imaging.

Quantification of plasma cGMP and inflammatory cytokines

Blood was collected from mice at the time of death (time of death varied between 330 to 660 min after TNF-α injection). Plasma was separated from the blood by centrifuging at 2500g for 10 minutes. Samples were then stored at -80°C. Plasma cGMP, as well as CXCL-1 and CXCL-2 levels, were quantified using commercially-available ELISA kits (GE Healthcare, Piscataway, NJ, USA and R&D Systems, Minneapolis, MN, USA, respectively).

Statistical analyses

Values are expressed as mean ± standard error of mean (SEM). Parametric comparisons were performed using ANOVA with Newman-Keuls post-test.

Statistical significance for the Kaplan-Meier survival curve was assessed using a log-rank (Mantel-Cox) test. Differences among groups were considered significant at $p<0.05$.

Results

Hydroxyurea and BAY73-6691 alter TNF-α-induced leukocyte adhesion and extravasation

Intraperitoneal administration of TNF-α in mice induces a well-described inflammation in the cremasteric microcirculation,[6,11,35] associated with increased leukocyte adhesion to the vessel wall. To investigate the effects of HU and BAY73-6691 on leukocyte recruitment, we injected each drug together with TNF-α and quantified the leukocyte responses by intravital microscopy at 3h post treatment (Figure 2A). While neither drug showed an effect on leukocyte rolling (Figure 2B and E), leukocyte adhesion (Figure 2C and F) and extravasation (Figure 2D and G) were significantly reduced. To test whether the observed effects on leukocyte-endothelial cell interactions were mediated by cGMP-dependent pathways, we administered the soluble guanylate cyclase (sGC) inhibitor ODQ; (Figure 1) or KT5823, (a PKG inhibitor). Both ODQ and KT5823 were each able to prevent the effects of HU and BAY73-6691, respectively (Figure 2), indicating that, under these conditions, HU and BAY73-6691 are exerting their effects via a cGC/PKG signaling pathway.

Hydroxyurea and BAY73-6691 inhibit leukocyte recruitment in TNF-α inflamed SCD mice

We next investigated whether the drugs had an effect on leukocyte recruitment in a mouse model of sickle cell disease. While treatment of SCD mice with either HU or BAY73-6691 (Figure 3A) did not alter TNF-α-induced rolling (Figure 3B), a significant reduction was observed for both leukocyte adhesion (~40% inhibition) and extravasation (~40% for HU and 70% for BAY73-6691) (Figure 3C and D). Interestingly, co-administration of both drugs was found to synergistically increase leukocyte rolling along the vessel wall, and decrease leukocyte adhesion even further. Elevated blood flow was only observed after HU treatment and no significant alteration in venule diameter was observed following any of the treatments, suggesting that HU and BAY73-6691 do not induce vasodilation in the microcirculation, under the conditions used (venule diameters; 21.6 ± 0.38 μm, 22.2 ± 0.47μm, 22.4 ± 0.46μm, 21.6 ± 0.45μm, respectively for control, HU, BAY73-6691, HU and BAY73-6691, respectively; n= 52-93 venules).

Hydroxyurea and BAY73-6691 elevate cGMP in the plasma of SCD mice

cGMP levels were determined at the time of death in the plasma of the SCD mice that had received an acute administration of HU, BAY73-6691 or a combination of the two drugs (Figure 4A). No significant alteration in plasma cGMP was observed following the administration of each of these drugs alone, conversely, the combination of the two drugs significantly augmented plasma levels of this cyclic nucleotide.

Hydroxyurea and BAY73-6691 reduce TNF-α-induced WBC-RBC interactions and Mac-1 integrin activation

Since drug treatment inhibited leukocyte endothelial cell interactions we hypothesized that the activation status of leukocytes was reduced. Indeed, BAY73-6691 by itself and in combination with HU significantly decreased heterotypic WBC-RBC interactions (Figure 4B). More specifically co-administration of both drugs resulted in a significantly diminished activity of the leukocyte integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) (Figure 4C, D and E) as demonstrated by the reduced ability to bind albumin-coated fluorospheres, an assay developed in the lab to assess leukocyte activation, *in vivo*. [11]

Hydroxyurea and BAY73-6691 prolong survival after TNF-α treatment in SCD mice

Following TNF-α administration, SCD mice showed mortality with an average survival period of 423 minutes (Figure 4F). Mice were monitored until 660 min post TNF-α, in the absence or presence of both drugs. Importantly, co-administration of HU and BAY73-6691 significantly improved the survival of SCD mice (550 minutes) (Figure 4F). No significant effect of either drug on survival was observed when given alone (data not shown).

Hydroxyurea and BAY73-6691 do not act by systemic alterations of major inflammatory cytokines.

To test whether HU and BAY73-6691 exhibit direct effects on cell signaling, consequently reducing leukocyte adhesion, rather than affecting the systemic inflammatory response elicited by the administration of TNF-α, we determined the

levels of major neutrophil-activating cytokines in the circulation of SCD mice following TNF-α injection. After stimulation, plasma levels of CXCL-1 (Suppl. Figure 1A) and CXCL-2 (Suppl. Figure 1B) were not altered after co-administration of HU and/or BAY73-6691. These results infer that the effects of HU and BAY73-6691 are not due to systemic alterations in inflammation.

Discussion

Vaso-occlusive processes account for much of the morbidity of SCD,[1,2] and therapeutic approaches to prevent or reduce such mechanisms in SCD patients are continually sought. Experiments in mouse models of SCD indicate that vascular obstruction can be triggered by hypoxia, trauma and/or inflammatory events.[6,11,36,37] In a humanized mouse model of SCD, the administration of TNF-α initiates the recruitment of leukocytes to the microvascular endothelium, followed by the capture of sickle RBC to adherent leukocytes, leading to a reduction in blood flow and, frequently, to the death of the animal.[11] Using the TNF-α-induced SCD mouse-model of acute vaso-occlusion, our data indicate that the administration of either HU or BAY73-6691, are able to reduce leukocyte adhesion to the microvascular endothelium. Notably, co-administration of both drugs, synergized and resulted in significant improvements in leukocyte/endothelial cell and RBC/WBC interactions. These changes were associated with a decrease in leukocyte Mac-1 integrin activity and, importantly, a significant increase in survival.

In non-transplanted C57BL/6 mice, our data indicate that HU-mediated alterations in leukocyte behavior, following the induction of inflammation, are

mediated via activation of the soluble guanylate cyclase (as shown by the reversal of the effects of HU by ODQ). Similarly, in the same model, the effects of BAY73-6691 were prevented by the inhibition of cGMP-dependent protein kinase. Thus, the data indicate that both drugs exert their effects, under these experimental conditions, via a central cGMP-dependent pathway. HU is reported to stimulate sGC, *in vitro*, by direct nitrosylation of the enzyme, by NO donation as well as by stimulation of nitric oxide synthase; [38-40] however, whether the rapid *in vivo* stimulation of sGC by HU reported herein occurs via the production of NO or by direct stimulation of the enzyme remains to be elucidated. BAY73-6691 inhibits PDE9, an enzyme that degrades cGMP. Thus, the concomitant use of BAY73-6691 probably amplifies the cGMP-elevating effect of HU and, accordingly, circulating cGMP levels were found to be elevated in the circulation of SCD mice following the administration of the two drugs.

HU, usually used in a chronic regimen, can improve SCD disease progression by increasing HbF production, in addition to other effects that include the reduction of leukocyte and platelet counts and, to some extent, inflammation.[2,23] Effects of this drug are generally believed to result mainly from its HbF-elevating capacity and its marrow cytotoxicity that decreases neutrophil and reticulocyte counts.[41] In a recent study, transgenic Berkeley SCD mice (which do not express HbF during adulthood) were treated for 20 weeks with a maximally-tolerated dose of HU. While significant reductions in the neutrophil and platelet counts were observed, no improvements in hemolytic anemia and end-organ damage were found in treated mice. In contrast, mice expressing high levels of HbF, due to gene transfer, showed improvements in anemia and organ damage;

authors concluded that HbF induction is essential for the clinical benefit of HU.[42] On the other hand, HU is known to have NO donor properties *in vivo*,[27] inducing nitric oxide synthase activity and NO production in endothelial cells.[40] Thus, given the contribution of reduced NO bioavailability to SCD pathophysiology,[43] we hypothesized that HU could exert immediate positive effects that are not mediated by HbF elevation and the other long term effects of the administration of this drug. We, herein, present data that indicate, for the first time in an *in vivo* setting, that HU may have acute and immediate benefits.

The PDE9 enzyme constitutes an attractive therapeutic target, due to its apparently limited tissue expression, being expressed in the brain[44] and hematopoietic cells,[33] and with very low-level expression in other tissues.[33] Furthermore, endothelial gene expression of PDE9 appears to be low, but augments following cell activation (Almeida, unpublished observations). Inhibition of PDE9 may have cognition-enhancing effects and the use of PDE9 inhibitors has been postulated for the treatment of Alzheimer's disease. BAY73-6691, a potent inhibitor of PDE9,[45] has demonstrated significant effects *in vitro*, inducing erythroid cell γ-globin gene production and decreasing leukocyte adhesive properties and adhesion molecule expression.[33,34] In our SCD mouse model, BAY73-6691 amplified the cGMP-dependent effects of HU in the absence of any apparent vasodilatory effect. While the mechanism for the HU/ BAY73-6691-mediated decrease in leukocyte adhesion and RBC-WBC interactions observed is not clear, data indicate that a decrease in the activity of the major leukocyte integrin, Mac-1, may play a role in this mechanism. Leukocyte integrin activity is mediated by

changes both in the surface expression of the integrin, as well as by alterations in its affinity and avidity.[46,47] Interestingly, the *in vitro* inhibition of PDE9 in human neutrophils from SCD individuals was found to decrease surface expression of Mac-1, in association with a decrease in their capacity to adhere to recombinant ICAM-1.[34] Whilst evidence of the down-regulation of Mac-1 activity by mechanisms of integrin shedding is scarce,[48] the possibly that cGMP-dependent signaling reduces leukocyte adhesive functions via shedding events should not be ruled out.

Our data suggest that drugs that stimulate the NO/cGMP pathway may be capable of diminishing vaso-occlusive processes, at least in SCD mice, when administered acutely. Notably, our findings indicate that HU could have important, immediate and direct beneficial effects on the microvasculature that are mediated by cGMP-dependent mechanisms. HU is administered in a chronic therapeutic regimen for SCD, with proven results. While the administration of hydroxyurea by the *i.v.* route is possible, it does not appear to present any clear advantages over oral administration, with regard to bioavailability.[49] Our data, however, indicate that the use of hydroxyurea (either via oral or *i.v*. routes) as an option for the treatment of acute vaso-occlusive crisis should be considered and carefully investigated. Since the PDE9 enzyme is highly expressed in hematopoietic cells, the employment of cGMP-potentializing PDE9 inhibiting drugs may constitute a promising means of intensifying the NO-mediated effects of HU, in a tissue-specific manner.

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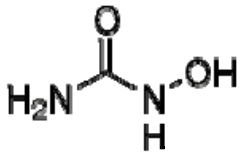
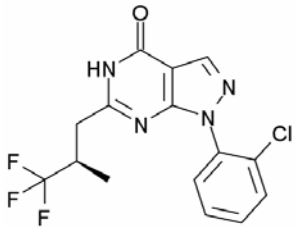
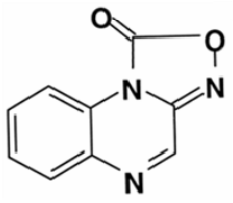
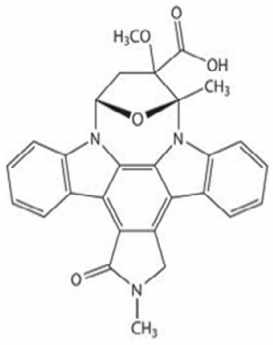
Table 1: Chemical structures of drugs

HU	BAY73-6691	ODQ	KT5823
			

Legends to Figures

Figure 1. The NO-cGMP pathway. Hydroxyurea (HU) is thought to act as a nitric oxide (NO) donor *in vivo* and/or may directly activate intracellular soluble guanylate cyclase (sGC): NO stimulates intracellular sGC to produce cGMP (cyclic guanosine monophosphate) from GTP (guanosine-5'-triphosphate). Stimulation of cGMP-dependent kinase (PKG) by cGMP in erythroid lineage cells is reported to elevate γ-globin and fetal hemoglobin production and decreases leukocyte (WBC) adhesive mechanisms. PDE9 (phosphodiesterase 9) degrades intracellular cGMP (icGMP) and is highly expressed in hematopoietic cells; BAY73-6691 is a selective inhibitor of PDE9, and therefore elevates icGMP in cells that express PDE9. ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) is a selective inhibitor of sGC, while KT5823 selectively inhibits PKG.

Figure 2. Hydroxyurea and BAY73-691 reduce leukocyte adhesion and extravasation via cGMP-dependent mechanisms. (A) Experimental protocol used for C57BL/6 mice. ODQ , KT5823 or vehicle were administered i.p. 30min before TNF-α was injected, simultaneously with HU, BAY-73-6691 or vehicle. (B) Leukocyte rolling, (C) adhesion and (D) extravasation in TNF-α-treated mice after vehicle or HU administration, in the presence or absence of ODQ. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to vehicle alone; ##, $p<0.01$; ###, $p<0.001$, compared to HU alone; n= 4-10 per group. (E) Leukocyte rolling, (F) adhesion and (G) extravasation in TNF-α-treated mice after vehicle or BAY73-6691 administration in the presence or absence of KT5823. *, $p<0.05$; ***, $p<0.001$, compared to vehicle alone; #, $p<0.05$; ###, $p<0.001$, compared to BAY73-6691 alone; n= 4-10 per group.

Figure 3. Effects of HU and BAY73-6691 on leukocyte recruitment in TNF-α-treated SCD mice. (A) Experimental protocol used for SCD mice. (B) Leukocyte rolling; (C) adhesion; and (D) extravasation in TNF-treated SCD mice. *,$p<0.05$; **,$p<0.01$; ***,$p<0.001$, compared to vehicle; n=5-7 per group. (E) Representative images of SCD mice venules at 180 min after TNF-α stimulation. Black dots indicate adherent leukocytes; white arrowheads designate RBC adhesion to the endothelium; white arrow, RBC-WBC interaction; black arrow, direction of blood flow; Scale bar, 10 μM.

Figure 4. Co-administration of HU and BAY73-6691 improves blood rheology in TNF-α-treated SCD mice. (A) plasma cGMP levels at the time of death or sacrifice, as determined by ELISA. *, $p<0.05$, compared to vehicle; n= 5-6 per group. (B) Quantification of RBC-WBC interactions in SCD mice. *, $p<0.05$ compared to vehicle; n= 5-7 per group. (C) Mac-1 activity on adherent leukocytes, as assessed by fluorescent sphere assay, in TNF-α-stimulated SCD mice. *, $p<0.05$ compared to vehicle-treated SCD mice; n= 2-4, per group. Representative images of fluorescent spheres bound to leukocytes in TNF-treated SCD mice that received drug vehicle alone (D) or HU and BAY-73-6691 (E); scale bar, 10 μm; arrow indicates direction of blood flow. (F) Kaplan-Meier survival curve after treatment with HU and BAY73-6691 or vehicle. Log-rank (Mantel-Cox) test, p=0.04. n= 5- 7 per group.

Supplementary Figure 1. Levels of inflammatory cytokines in the circulation of TNF-α –induced SCD mice. Plasma levels of (B) CXCL-1 and (C) CXCL-2 in SCD mice at the time of death or sacrifice, as determined by ELISA.

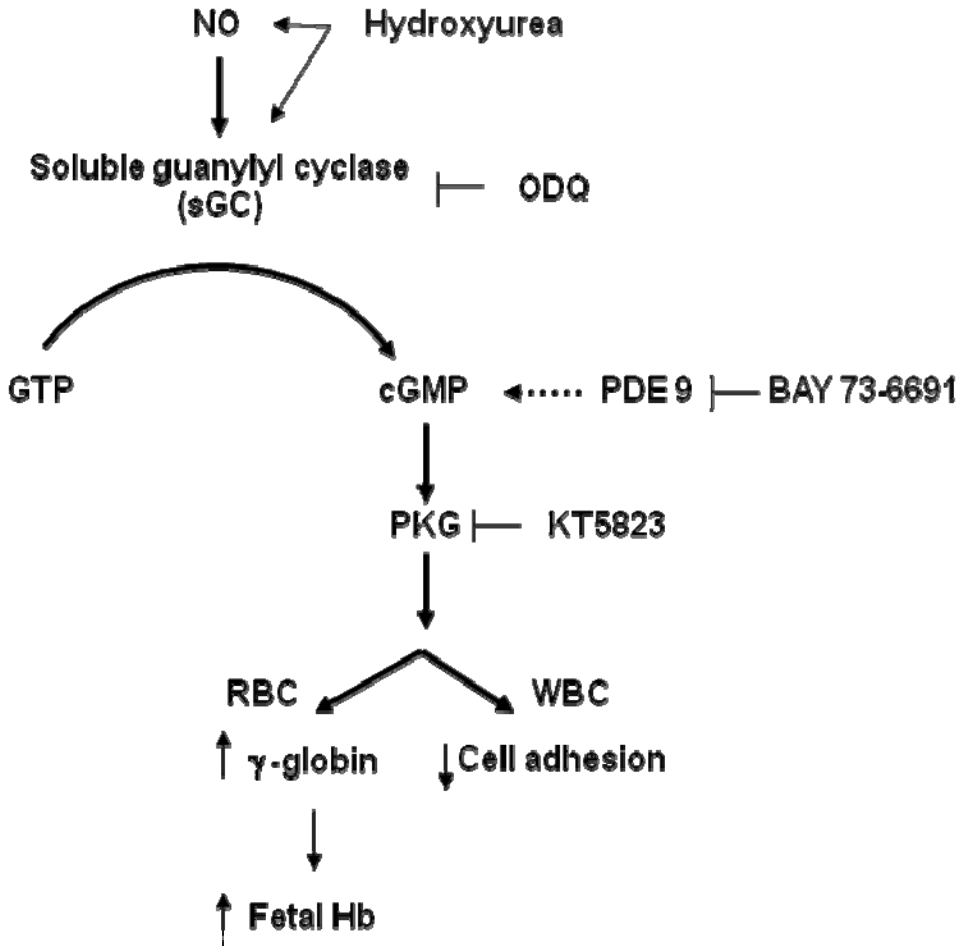

NO
Hydroxyurea
Soluble guanylyl cyclase
(sGC)
ODQ
GTP
cGMP
PDE 9
BAY 73-6691
PKG
KT5823
RBC
WBC
γ-globin
Cell adhesion
Fetal Hb

Figure 1

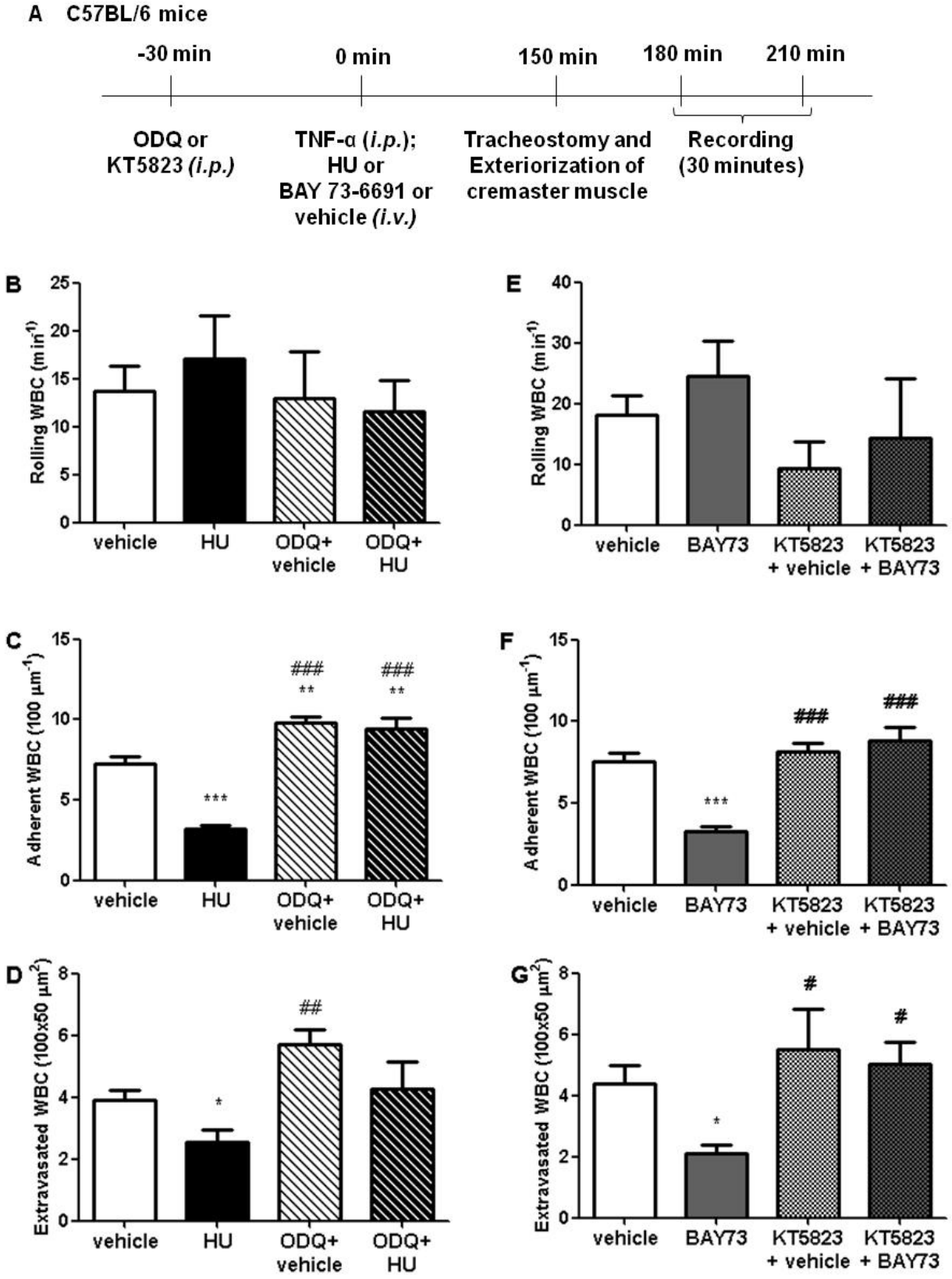

A C57BL/6 mice
-30 min
0 min
150 min
180 min
210 min
ODQ or KT5823 (i.p.)
TNF-α (i.p.); HU or BAY 73-6691 or vehicle (i.v.)
Tracheostomy and Exteriorization of cremaster muscle
Recording (30 minutes)
B
Rolling WBC (min-1)
vehicle
HU
ODQ+ vehicle
ODQ+ HU
E
Rolling WBC (min-1)
vehicle
BAY73
KT5823 + vehicle
KT5823 + BAY73
C
Adherent WBC (100 µm-1)
F
Adherent WBC (100 µm-1)
D
Extravasated WBC (100x50 µm2)
G
Extravasated WBC (100x50 µm2)

Figure 2

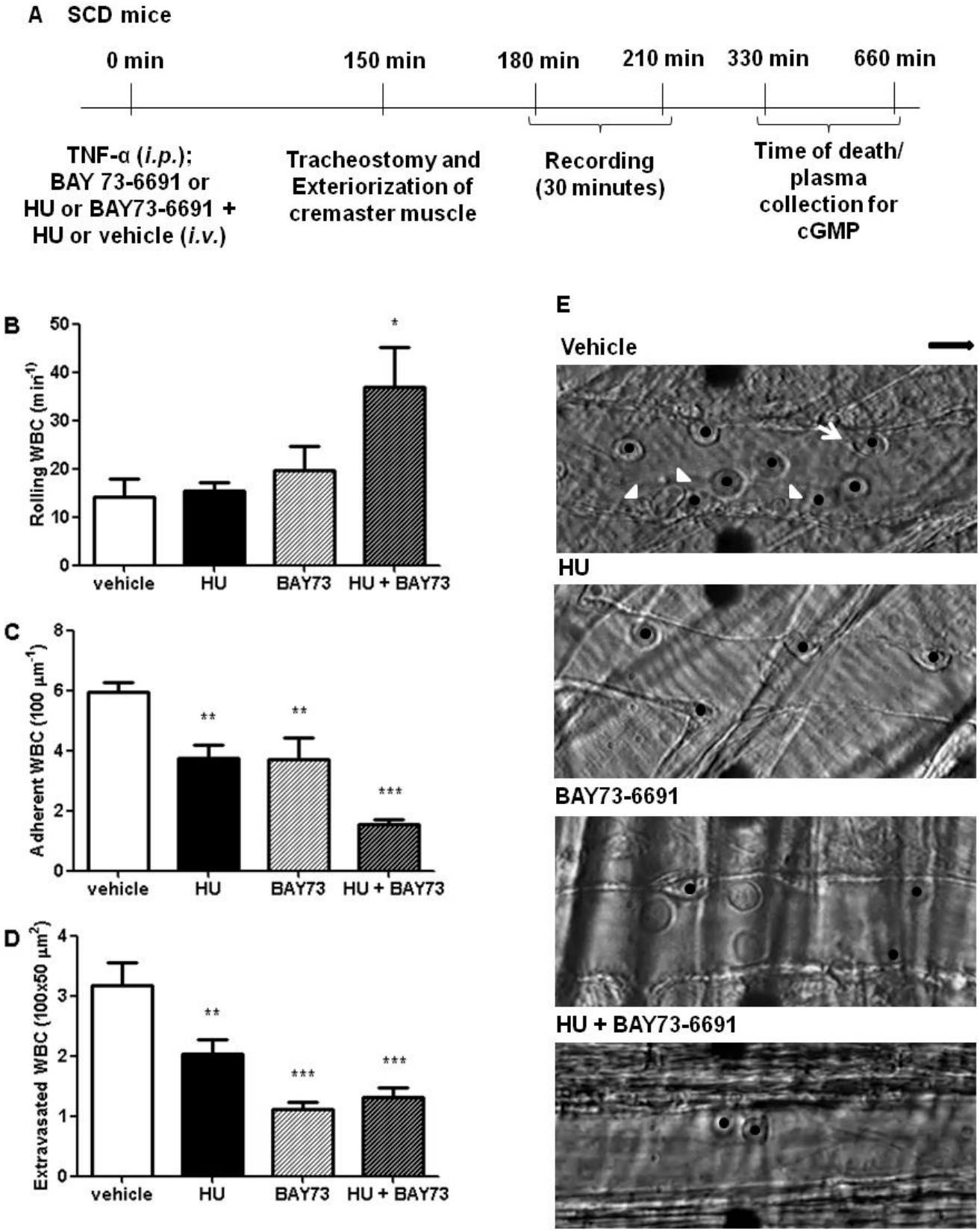

A SCD mice
0 min
150 min
180 min
210 min
330 min
660 min
TNF-α (i.p.); BAY 73-6691 or HU or BAY73-6691 + HU or vehicle (i.v.)
Tracheostomy and Exteriorization of cremaster muscle
Recording (30 minutes)
Time of death/ plasma collection for cGMP
B
Rolling WBC (min-1)
vehicle
HU
BAY73
HU + BAY73
C
Adherent WBC (100 µm-1)
D
Extravasated WBC (100x50 µm2)
E
Vehicle
HU
BAY73-6691
HU + BAY73-6691

Figure 3

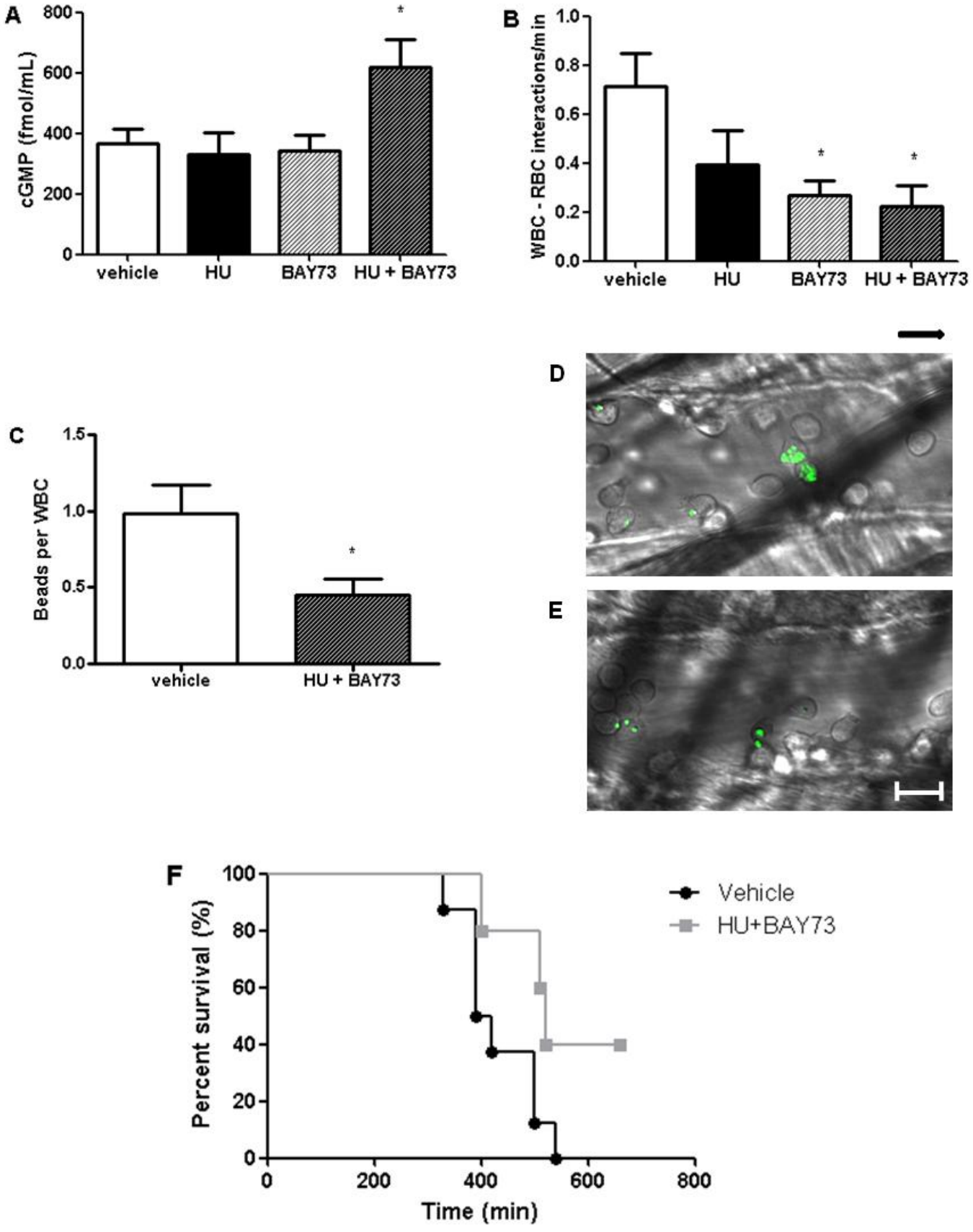
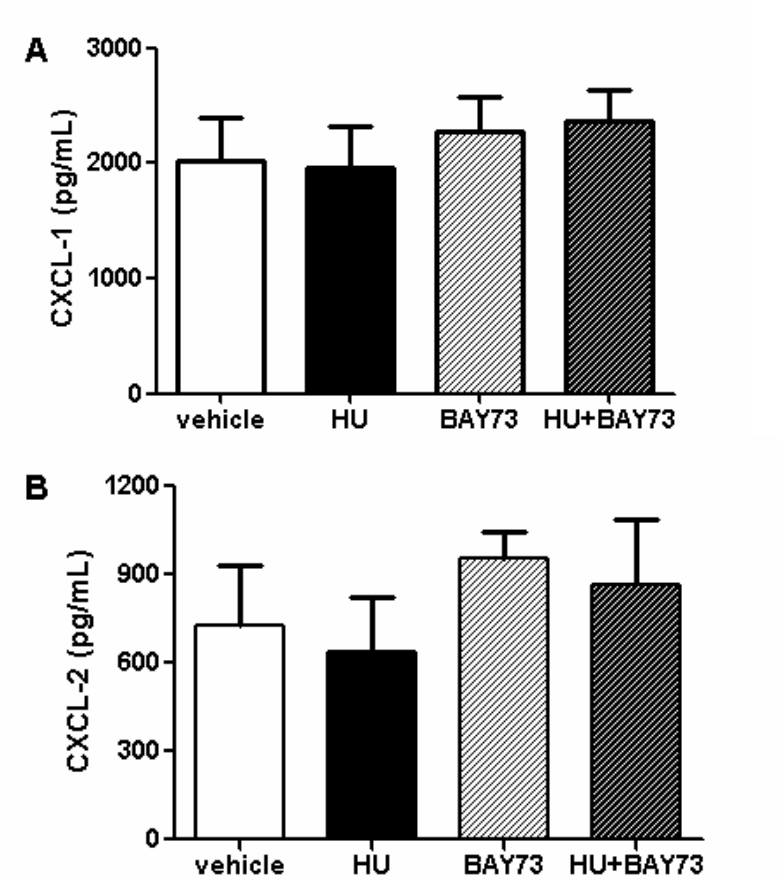

A
cGMP (fmol/mL)
800
600
400
200
0
*
vehicle
HU
BAY73
HU + BAY73
B
WBC - RBC interactions/min
1.0
0.8
0.6
0.4
0.2
0.0
*
*
vehicle
HU
BAY73
HU + BAY73
C
Beads per WBC
1.5
1.0
0.5
0.0
*
vehicle
HU + BAY73
D
E
F
Percent survival (%)
100
80
60
40
20
0
0
200
400
600
800
Time (min)
Vehicle
HU+BAY73

Figure 4



Supplementary Figure 1

Discussão Geral

DISCUSSÃO GERAL

O controle do número de leucócitos é essencial para a resolução do processo inflamatório. Alterações na quantidade de leucócitos circulantes podem estar relacionadas a uma rotatividade acelerada das células ou ao atraso da apoptose, contribuindo, assim, com a leucocitose que é frequentemente observada na anemia falciforme, constituindo um fator de risco capaz de propiciar crises vaso-oclusivas e síndrome torácica aguda, podendo culminar na morte desses indivíduos (Platt *et al*., 1994; Abboud, *et al*., 1998; Ohene-Frempong, *et al*.,1998; Segel *et al*., 2011).

Há evidências demonstrando que o estado inflamatório crônico, observado nos pacientes com AF provavelmente contribui com o aumento da granulopoiese na medula óssea (Conran *et al*., 2007). Avaliamos, então, se haveria uma liberação acelerada das células imaturas da medula óssea para a circulação periférica. Nossas análises mostraram um aumento significativo de neutrófilos imaturos na circulação de pacientes com AF, quando comparados aos indivíduos controles. As alterações encontradas podem contribuir para o atraso inerente da apoptose dos neutrófilos na anemia falciforme. Uma possível hipótese que poderia explicar a liberação precoce dessas células na circulação seria a quimiocina IL-8 (Conran *et al*., 2007), uma vez que esta se encontra em níveis elevados na circulação desses pacientes (Gonçalves *et al*., 2001; Lanaro *et al*., 2009), e sabe-se que essa substância é capaz de promover a mobilização e a saída das células-tronco hematopoiéticas para a circulação periférica (Laterveer *et al*., 1996).

Pequenas alterações nas taxas de apoptose também podem ser capazes de induzir diferenças na contagem de células. Em um estudo prévio do nosso grupo, foi demonstrado um atraso na via apoptótica dos neutrófilos de pacientes com AF (Conran *et al*., 2007), possivelmente contribuindo com a leucocitose presente nesses pacientes. Assim sendo, resolvemos verificar se essas alterações estavam relacionadas ao mecanismo celular ou a fatores presentes no soro de pacientes falciformes que induziriam o atraso na apoptose.

Surpreendentemente, o soro de pacientes com AF foi capaz de aumentar os níveis apoptóticos de neutrófilos controles, enquanto que na presença de soro controle, a apoptose de neutrófilos provenientes de indivíduos falciformes foi menor do que a apoptose de neutrófilos de indivíduos controle, sugerindo que alterações inerentes às células, ao invés de fatores circulantes, seriam os responsáveis pelo atraso da apoptose. Isso nos fez questionar quais seriam os fatores envolvidos na aceleração e no retardo da apoptose.

Há dados na literatura indicando que a produção de ROS e o estresse oxidativo estão diretamente ligados à indução da apoptose, principalmente devido ao fato de que a presença de antioxidantes atrasa ou bloqueia a morte por apoptose das células (Jacobsen *et al*., 1996). Além disso, há anos é sabido que pacientes com anemia falciforme apresentam aumento no estresse oxidativo, especialmente durante crises vaso-oclusivas e síndrome torácica aguda (Hebbel *et al*., 1982). Nesse trabalho observamos que o soro de pacientes falciforme foi capaz de induzir a produção de ROS e ânions superóxido em neutrófilos provenientes de indivíduos controle. Interessantemente, os níveis de SOD encontram-se reduzidos no plasma da população falciforme estudada, o que possivelmente contribui com os níveis de ânions superóxido alterados. Além disso, estudos prévios mostraram que a quantidade de antioxidantes como vitaminas A, C e E e zinco está reduzida na circulação de pacientes falciformes (Gbenebitse *et al*., 2005; Hasanato, 2006; Bao *et al*., 2008 Ren *et al*., 2008). Corroborando esses dados prévios, quando acrescentamos agentes antioxidantes ou SOD na cultura de neutrófilos controle com soro de pacientes com AF, observamos uma reversão no aumento da apoptose previamente constatado, sugerindo, assim, um papel fundamental dos ânions superóxido no processo apoptótico dos neutrófilos. A hemólise, característica desses pacientes, resulta na liberação de hemoglobina livre no plasma, a qual, juntamente com altos níveis de ferro livre, representa uma importante contribuição para o potencial oxidante do soro desses pacientes (Kato & Taylor, 2010).

A produção de ROS é necessária à função dos neutrófilos, uma vez que essas moléculas são requeridas no momento de destruição de patógenos (Yasui & Baba, 2006). A enzima NADPH oxidase é essencial para catalizar a produção de ânions superóxido através da redução de um elétron do oxigênio e a doação de um elétron da NADPH (Babior, 1999). Interessantemente, nossos dados mostram que as subunidades fundamentais para a ativação da enzima encontram-se superexpressas nos neutrófilos controles cultivados com soro de pacientes com AF.

As citocinas são conhecidas por alterarem os processos apoptóticos dos neutrófilos (Luo & Loison, 2008; Hallett *et al*., 2008) e várias citocinas inflamatórias, incluindo GM-CSF, IL-8, IL-6, TNF-α, encontram-se em níveis elevados na circulação de nossa e de outras populações de pacientes falciformes (Taylor *et al*., 1995; Conran *et al*., 2007; Lanaro *et al*., 2009). GM-CSF e IL-8 apresentam um importante efeito inibitório da apoptose de neutrófilos (Hofman, 2004), enquanto o TNF-α pode tanto induzir quanto reprimir a apoptose de neutrófilos, dependendo da concentração (Luo & Loison, 2008). Nosso trabalho revelou que a cultura de neutrófilos controle incubados com soro de pacientes com AF e neutralizadores de diferentes citocinas não alterou a taxa de apoptose dos neutrófilos, com exceção do neutralizador de IL-8, que induziu um aumento na apoptose, indicando que níveis elevados dessa citocina circulante, presente nos pacientes falciformes (Lanaro *et al*., 2009), são responsáveis por exercer uma inibição na apoptose dos neutrófilos. O certo é que existem fatores pró- e anti-apoptóticos, presentes na circulação dos pacientes, que podem regular a morte dos neutrófilos.

Outra forma de morte celular avaliada foi a necroptose, mecanismo de necrose celular regulada, que pode ocorrer, sob certas condições, após a estimulação do receptor de TNF-α (Hitomi *et al*., 2008), citocina esta que se apresenta em níveis elevados nos pacientes (Lanaro *et al*., 2009). A co-cultura de neutrófilos controle com o inibidor de necroptose diminuiu significativamente a morte celular na presença tanto de soro de pacientes quanto de indivíduos

controle, indicando que a necroptose pode constituir um modo menos significativo de morte celular utilizado pelos neutrófilos *in vitro*. Entretanto, o número de neutrófilos em necrose, mesmo na presença do inibidor, apresenta um aumento significativo, embora discreto, nos neutrófilos controle incubados com soro de pacientes com AF demonstrando que esse tipo de soro pode induzir baixos níveis de necrose nos neutrófilos.

Tanto a produção de ROS e o estresse oxidativo, quanto a inibição da apoptose são fatores que estão diretamente relacionados ao processo de vaso-oclusão na anemia falciforme, já que a inibição da produção de ROS em modelos de camundongos transgênicos falciformes resulta numa diminuição da adesão de leucócitos e o reestabelecimento do fluxo sanguíneo (Wood *et al*., 2006). O prolongamento da sobrevida dos neutrófilos nos pacientes está correlacionado à amplificação da resposta inflamatória nesses indivíduos e, consequentemente, aos processos vaso-oclusivos, os quais contribuem com a grande mortalidade entre os pacientes (Platt *et al*., 1994; Stuart & Nagel, 2004).

Em um modelo de camundongo humanizado falciforme, a administração da citocina TNF-α inicia o recrutamento de leucócitos e consequente adesão dos mesmos ao endotélio da microcirculação. Em seguida, ocorre a captura de células vermelhas falcizadas, o que leva a uma redução do fluxo sanguíneo e, frequentemente, à morte do animal (Hidalgo *et al*., 2009). Em nosso trabalho, utilizando esse modelo animal, observamos que tanto a HU quanto o inibidor de PDE9, BAY73-6691, são capazes de reduzir a adesão de leucócitos ao endotélio da microvasculatura. Notavelmente, a co-administração das duas drogas resulta em uma melhora significativa nas interações leucócito/endotélio e células vermelhas/células brancas. Essas mudanças estão associadas com a diminuição da atividade da integrina Mac-1 e, importantemente, com o aumento significativo da sobrevida desses animais.

Utilizando o mesmo modelo inflamatório em camundongos selvagens, observamos que as alterações provocadas pela HU são mediadas pela via de ativação da enzima GCs, uma vez que o ODQ (inibidor da enzima GCs) foi capaz

de reverter os efeitos benéficos observados previamente com o uso apenas da HU. Esses dados estão de acordo com resultados previamente observados *in vitro* (Cokic *et al*., 2006; Cokic *et al*., 2008; Xu *et al*., 2011). Entretanto, ainda não se sabe se a rápida estimulação *in vivo* da enzima GCs pela HU ocorre via produção de NO ou pela direta estimulação da enzima GCs. De maneira similar à HU, observamos que os efeitos obtidos com o uso de BAY73-6691 foram revertidos pela inibição da enzima PKG. Assim, os dados indicam que ambas as drogas atuam em uma via dependente de GMPc.

Acredita-se que os efeitos da HU, usualmente utilizada em tratamentos crônicos, estão relacionados à sua capacidade de elevar os níveis de HbF e seus efeitos citotóxicos na medula que reduzem o número de neutrófilos e reticulócitos (McGann & Ware, 2011). Entretanto, é sabido que a HU tem propriedades doadoras de NO *in vivo* (King, 2004) induzindo a atividade da enzima NOS e a produção de NO pelas células endoteliais (Cokic *et al*., 2006). Assim, levando-se em consideração a redução da biodisponibilidade de NO na anemia falciforme (Kato *et al*., 2009), nós hipotetizamos que a HU pode exercer um efeito positivo imediato independente do aumento de HbF ou de um tratamento crônico. Nesse trabalho, apresentamos, pela primeira vez, dados que indicam que a HU pode apresentar efeitos agudos e imediatos em um modelo *in vivo*.

Experimentos *in vitro* demonstaram que BAY73-6691, um potente inibidor de PDE9 (Wunder *et al*., 2005), é capaz de induzir a expressão de globina γ em células eritróides e reduzir as propriedades adesivas e a expressão das moléculas de adesão dos leucócitos (Almeida *et al*., 2008; Miguel *et al*., 2011). No presente trabalho, os resultados indicam uma diminuição na atividade da principal integrina dos leucócitos, Mac-1, após a adminstração de HU e BAY73-6691 no modelo estudado *in vivo*; dados estes condizentes com resultados prévios obtidos *in vitro* (Miguel *et al*., 2011). A atividade da integrina Mac-1 pode ser mediada por mudanças na expressão superficial da integrina, assim como por alterações em sua afinidade (Fagerholm *et al*., 2006; Luo *et al*., 2007). Interessantemente, a inibição *in vitro* da PDE9 em neutrófilos de pacientes falciformes diminuiu a

expressão superficial de Mac-1 (Miguel *et al*., 2011) indicando que, possivelmente, a diminuição da atividade dessa integrina, observada nos experimentos *in vivo* com camundongos, está relacionada a um mecanismo de liberação dessa proteína da superfície da célula. Entretanto, novos estudos devem ser realizados.

Conclusões Gerais

CONCLUSÕES GERAIS

Vários fatores contribuem com a modulação do número de leucócitos na anemia falciforme. Entre eles, estão:

- Aumento significativo de neutrófilos imaturos sugere uma emigração acelerada de leucócitos da medula óssea para a circulação periférica, podendo contribuir com o aumento no número de células vivas;
- Alterações inerentes às células são responsáveis pelo atraso na apoptose;
- O soro de pacientes com AF induz a produção de ROS em neutrófilos podendo contribuir com o estresse oxidativo característico da doença;
- Aumento nos níveis de ânions superóxido, que talvez possa ser explicado pelos índices baixos de SOD no plasma de pacientes falciformes, leva as células a apoptose;
- O soro de pacientes com AF apresenta fatores anti apoptóticos como, por exemplo, altos níveis da citocina IL-8 e fatores pró apoptóticos como altos índices de ROS.

Observamos que em um modelo inflamatório de camundongo falciforme humanizado:

- HU e BAY73-6691 (inibidor de PDE9), quando administrados agudamente, alteram significativamente as interações leucócito/endotélio/células vermelhas;
- Combinação de HU e BAY73-6691 reduz episódios de vaso-oclusão e aumenta a sobrevida dos camundongos falciformes;

- HU e BAY73-6691, neste modelo, parecem atuar pela via NO-GMPc- PKG, indicando que possivelmente a estimulação dessa via de sinalização pode constituir um novo caminho terapêutico para o tratamento da AF;

- HU, quando administrada de maneira aguda, apresenta efeitos benéficos imediatos (redução dos processos de vaso-oclusão) independentes das propriedades de aumentar os níveis de HbF, revelando uma possível alternativa para o uso dessa droga.

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Anexos

ANEXOS

Resumos apresentados em congressos, simpósios e prêmios recebidos relacionados à tese

Almeida, CB; Scheiermann, C; Jang, J; Prophete, C; Costa FF; Frenette, PS; Conran, N. "The cGMP pathway as a drug target for the reduction of vaso-occlusion in sickle cell anemia mice: acute effects of hydroxyurea and phosphodiesterase 9 inhibitor". Apresentação oral - 16th Congress of the European Hematology Association – Londres (UK), Jun/2011. Contemplado com "Travel Award".

Almeida, CB; Favero, ME; Saad, STO; Costa, FF; Conran, N. "Alterations in cell turnover and pro-apoptotic serum factors may modulate neutrophil numbers in sickle cell disease". Apresentação em pôster - 16th Congress of the European Hematology Association – Londres (UK), Jun/2011. Contemplado com "Travel Award".

Almeida, CB; Scheiermann, C; Jang, J; Prophete, C; Costa FF; Frenette, PS; Conran, N. "Via NO/GMPc – possível alvo terapêutico na redução da vaso-oclusão em camundongos falciformes: efeitos da hidroxiuréia e do inibidor de PDE9 BAY73-6691". Apresentação oral - Hemo 2010 - Brasília, Nov/2010. Contemplado com o prêmio de "Melhor Trabalho do Congresso Hemo 2010".

Almeida, CB; Pereira, FG; Lorand-Metze, I; Saad, ST; Costa, FF; Conran, N. "Sickle cell disease serum has both apoptotic and anti-apoptotic effects on leukocytes; role for reactive oxygen species generation and cytokines". Apresentação em pôster - V Simpósio Brasileiro de Doença Falciforme e outras Hemoglobinopatias – Belo Horizonte, Out/2009.

Almeida, CB; Pereira, FG; Lorand-Metze, I; Franco-Penteado, C; Saad, ST; Costa, FF; Conran, N. "Neutrophils are subject to both anti and pro apoptotic stimuli in the serum of sickle cell disease individuals". Apresentação em pôster -

14th Congress of the European Hematology Association – Berlim (Alemanha), Jun/2009.

Almeida, CB; Pereira, FG; Lorand-Metze, I; Saad, ST; Costa, FF; Conran, N. “Induction of caspase-independent apoptosis by sickle cell disease (SCD) serum in non-SCD leukocytes”. Apresentação em pôster - 50th ASH – São Francisco (EUA), Dez/2008.

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Miguel, LI; **Almeida, CB**; Traina, F; Canalli, AA; Dominical, VM; Saad, STO; Costa, FF; Conran, N. Inhibition of Phosphodiesterase 9A (PDE9A) Reduces Cytokine-Stimulated *in vitro* Adhesion of Neutrophils from Sickle Cell Anemia Individuals; *Inflammation Research*; 60(7):633-642; 2011.

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