490b Nitric Oxide Inhibits Endothelial Receptor Expression and Sickle Red Blood Cell Adhesion Induced by Cytokine Stimulation

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Sickle cell anemia results from the homozygous inheritance of the sickle β hemoglobin gene and affects over 70,000 people in the United States. Sickle hemoglobin is distinct from normal in that upon deoxygenation in the microcirculation, sickle hemoglobin molecules interact hydrophobically to form stiff, polymer-like chains inside red blood cells. These chains distort red cells into the "sickle" shape for which the disease is named and damage the red cells such that the lifespan of sickle erythrocytes is reduced from the normal 120 days to only 12 to 15 days. As a result, sickle patients have higher percentages of young red blood cells, or reticulocytes, which can interact with the vascular wall.

Complications associated with sickle cell anemia are systemic, as bones, lungs, brain, spleen, kidneys, heart, eyes, and other organs may be damaged in some patients. While the disease affects a broad scope of organs, the common etiology of many of these symptoms is vascular occlusion, caused in part by adhesion of sickle red blood cells, especially reticulocytes, to the endothelium on the vascular wall. Current therapies for clinical symptoms are merely palliative and do not address causes of occlusion; however, the inhibition of red cell adhesion to the vascular wall may be a useful therapeutic for sickle patients.

At physiologic shear stresses, sickle erythrocyte adherence to endothelium occurs via specific, high-affinity receptor/ligand interactions. Sickle patients demonstrate chronically activated endothelium and higher levels of expression of receptors VCAM-1 (vascular cell adhesion molecule-1) and E-selectin, both of which bind sickle red cells. Stimulation of endothelial cells with inflammatory cytokines such as tumor necrosis factor-α (TNF-α) *in vitro* also induces expression of these receptors and increases sickle red cell adhesion. Interestingly, occlusive events in sickle cell anemia patients are frequently preceded or accompanied by infection and the associated inflammatory response, suggesting a link between inflammation, receptor expression, and occlusion *in vivo*. Also suggested is that strategies to control inflammation may reduce sickle red cell adherence and occlusive events in sickle cell patients. Towards this end, we hypothesize that receptor expression and sickle red cell adhesion due to cytokine stimulation can be inhibited by interrupting endothelial cell signaling cascades initiated by cytokines. More specifically, we hypothesize that increasing endothelial nitric oxide (NO) concentrations provides such an interruption and results in reduced capability of cytokines to promote VCAM-1 and E-selectin expression as well as sickle erythrocyte adhesion.

Nitric oxide has recently received much attention by sickle cell researchers and is one of the most promising therapeutic target molecules in the field to date. It is a soluble gas synthesized in endothelial cells upon activation of the family of nitric oxide synthase enzymes, and one of its primary physiological roles is the regulation of vascular tone. Increasing nitric oxide results in vasodilation and increase in survival rates of hypoxic sickle mice, and has been shown to have several other potentially beneficial effects including inhibition of platelet aggregation and thrombotic complications and inhibition of receptor expression. However, little is known about the effects of nitric oxide on sickle red cell adhesion.

To test the effects of this molecule on cytokine-induced receptor expression and sickle red cell adhesion *in vitro*, confluent monolayers of microvascular endothelial cells were stimulated with TNF-α for 6 hrs, and some monolayers were treated with either sodium nitroprusside (SNP) or diethylenetriamine/nitric oxide adduct (DETA-NO) for 30 minutes prior to and during TNF-α stimulation to increase NO concentrations. Untreated endothelial cells served as negative controls for all experiments. Monolayers were then rinsed to remove reagents and perfused with washed sickle red cells at 1 dyne/cm^2 shear stress

for 40 minutes at 37°C. Firmly adherent sickle red cells were counted in 20 microscopic fields each at 1, 3, 5, 10, 20, 30, and 40 minutes after initiation of perfusion, and results were averaged and normalized per area at each time point. Additionally, ELISA was used to test the effects of these reagents on endothelial VCAM-1 and E-selectin expression.

ELISA results show minimal expression of VCAM-1 and E-selectin on unstimulated endothelial cells, but significant expression of both after 6 hrs of TNF-α stimulation. Similarly, studies in the flow chamber reveal that adhesion of sickle red cells to unstimulated endothelial cells is minimal (12 ± 1.6 cells/mm^2) after 40 minutes of red cell perfusion. However, adhesion of sickle cells to TNF-α stimulated endothelial cells increases with perfusion time and plateaus at 52 ± 12 cells/mm^2 after 30 to 40 minutes of perfusion. Treatment of endothelial cells with SNP or DETA-NO to increase nitric oxide content before and during TNF-α stimulation inhibits adhesion of sickle erythrocytes by 61% and 65%, respectively. Comparable studies using ELISA show that treatment with either reagent also inhibits VCAM-1 and, to a lesser extent, E-selectin expression. No effect on either receptor expression or sickle cell adhesion is observed when endothelial cells are treated with either SNP or DETA-NO alone (without TNF-α). Together these data suggest that one important role of nitric oxide may be suppression of inflammation and the associated cell signaling. Given the potential role of inflammation in the progression of sickle cell adhesion, nitric oxide may represent a useful therapeutic target for the prevention or treatment of vaso-occlusive events in sickle cell anemia.