

Editorial

Neutrophils and Neovascularization

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Editorial

Neovascularization (NV) via growth of collateral arteries (arteriogenesis) and capillaries, arterioles and venules (angiogenesis) becomes impaired as ischemia from vascular disease progresses [1-3]. Our hypothesis is that overcoming hemodynamic and cellular obstacles to NV would yield an effective and durable option to surgical or catheter revascularization. A novel approach has been tested clinically that seeks to restore innate NV by overcoming obstacles that arise due to the multilevel arterial occlusive disease and progenitor cell deficits characteristic of Chronic Limb-Threatening Ischemia (CLTI) [4-7].

Patients with CLTI were treated with Filgrastim, a granulocyte colony stimulating factor, 8-10 mcg/kg every 72 h for up to a month (N = 8 and N = 6, at two institutions). All wore an infragenuate Programmed Compression Pump (PCP) on each leg for at least 3 hours daily to offset the hemodynamic impairment of multi-level arterial occlusive disease. Additional patients (N=19) were treated with PCP alone as a control group. The PCP was used to increase endothelial shear stress (needed to initiate arteriogenesis) and to increase delivery of oxygenated nutritive blood flow and clearance of toxic metabolic by-products. Besides enhancing the biochemical milieu, the PCP was used to help disseminate ischemic molecular signals from the ischemic tissue to reach bone marrow niches where subsequently mobilized salutary progenitor cells could then home back to the activated endothelium in the ischemic tissue.

Enzyme-Linked Immunosorbent Assay was used to measure the plasma concentrations of plasmin and of Fibrin Degradation Products (FDP) and the serum concentrations of proteins associated with NV. Cytometry and cell blood counts were used to measure the impact of

Filgrastim. In the PCP-alone group, blood was sampled on Day 1 (baseline) and after 30 days of PCP. In the Filgrastim and PCP group, blood was drawn on Day 1 and 1 day after the 5th and the 10th Filgrastim doses. Each blood draw occurred before and after 2 hours of supervised PCP.

Significant ($p < 0.01$) PCP independent increases in the plasma concentration of plasmin (>10-fold) and FDP (>5-fold) were observed 1 day after both the 5th and the 10th Filgrastim doses, as compared to Day one. Significant ($p < 0.05$) increases in the concentration of pro-angiogenic proteins (Hepatocyte Growth Factor, Membrane Metalloproteinase-9, Vascular Endothelial Growth Factor A and others) were also observed [7].

Each patient's blood specimen was obtained the day after the 5th and 10th Filgrastim doses. Filgrastim has a half-life of 3.5 hours. Significant increases in the circulating number of progenitor cells (CD34+ and VEGFR2+) and of leukocytes were confirmed, as expected per the product label. What was remarkable was that the proteomic data obtained at the same time supported the evolution of a pro-angiogenic environment, as well as evidence for a physiologic level of fibrinolysis. This new information helped explain improving arterial hemodynamic measurements over 6 months. Angiography showed growth of corkscrew collaterals (arteriogenesis) and segmental arterial recanalization (fibrinolysis). The contrast transit time through the previously profoundly ischemic tissue significantly improved, supporting NV evolution. Healing of ischemic wounds occurred.

Filgrastim, a Granulocyte-Colony Stimulating Factor, produced significant acute neutrophilia in our CLTI patients. Less than a decade ago, neutrophilia was not considered pro-angiogenic. The anti-angiogenic activity of neutrophil elastase biased against use of agents like Filgrastim as a neovascularization strategy. This was despite Filgrastim's beneficial correction of the typical circulatory progenitor cell deficit observed in vascular patients, particularly in the elderly and the diabetic ones [8-20]. Filgrastim also increased the monocyte count in our CLTI patients. Monocytes and progenitor cells participate directly in vascular remodeling. New data now show that neutrophil elastase does not directly inhibit angiogenesis, but rather disrupts vascular endothelial integrity and stabilization by interfering with angiopoietin expression [21]. A specific neutrophil elastase inhibitor (sivelestat sodium) is being developed to enhance angiogenesis [21].

Cancer patients receive Filgrastim to correct leukopenia following cytotoxic chemotherapy. While their leukopenia improves, dramatic leukocytosis and neutrophilia does not typically occur. On the other hand, our CLTI patients start off with a normal leukocyte population. Brisk leukocytosis and neutrophilia occur following Filgrastim administration. Both resolve within 72 hours (hence the unusual dosimetry of one dose every 3 days). Permission was granted by the US-FDA to test Filgrastim in CLTI based on its other indication, progenitor cell mobilization. At that time (2008-2013) the pro-angiogenic potential of neutrophils had not been established. A plurality of studies in the oncology literature now relates neutrophils to pro-angiogenic mediators [10-24]. Our 2015 report highlighted increases in serum VEGF-A, ANGPT1, HGF and MMP-9 in CLTI patients with neutrophilia one day following Filgrastim administration [6]. These same proteins are what are reportedly produced by activated human neutrophils in the oncology literature [9]. A reasonable new hypothesis is that neutrophilia contributes to the pro-angiogenic spectrum that was documented by ELISA in each of our CLTI patients.

Furthermore, an association between Filgrastim and fibrinolysis was previously reported [22-24], but was not evaluated clinically until our CLTI study. In 1989 Filgrastim was reported to stimulate activity of plasminogen activator in both extracellular and intracellular milieus of endothelial cells obtained from bovine arteries [22]. This effect was dependent on the concentration of Filgrastim added to the culture medium and on the treatment time. Analyses by fibrin and reverse fibrin autography revealed that activity of plasminogen activator increased more than its inhibitor in endothelial cells treated with Filgrastim. In 2006, *in-vitro* experiments suggested Filgrastim associated fibrinolysis was directly attributable to the increase in circulating neutrophils [23]. Work that still needs to be done is to more precisely identify the plasma plasmin and FDP levels following Filgrastim administration. Plasmin has a short half-life *in-vivo*. The plasma concentration of plasmin-alpha2 antiplasmin and tissue plasminogen activator-plasmin activator inhibitor complexes will more precisely define plasmin generation in vivo in response to Filgrastim. Furthermore, FDP measured in EDTA-anticoagulated plasma would correct for FDP release during clot retraction in serum separator tubes. The neutrophil contribution to plasmin release following Filgrastim administration then can be systematically derived.

Phase II Clinical trials attempting to orchestrate discrete cellular or molecular aspects of NV have not yet yielded a widely accepted solution to promoting this complex process [24]. An alternative approach is to strategically overcome obstacles to NV so this vital compensatory process can function as nature intended. We chose this latter approach, guided by proteomic and cytometry data and were rewarded by positive clinical results. We identified neutrophilia as a likely facilitator in promoting NV in chronic ischemia. Safely promoting NV while simultaneously generating a physiological level of fibrinolysis offers the promise of durable revascularization, relevant to CLTI and to other tissue beds (heart, lung, brain).

Keywords: Angiogenesis; Arteriogenesis; Neovascularization; Neutrophil; Neutrophilia; Granulocyte Colony Stimulating Factor; Neupogen; Filgrastim; Cell therapy; Ischemia; Chronic Ischemia; Limb Threatening Ischemia; Amputation Prevention

Conflict of Interest

The authors have no conflict of interest to declare.

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