

Appendix E-1 Details of the Study Methodology

Surgical and Pharmacological Procedures

The surgical procedure was similar for all patients. None of the patients required an intraoperative blood transfusion; 10% of the patients received one unit of packed red blood cells postoperatively. None of the patients required ICU (intensive care unit) admission. Patients were not discharged unless anemia, pain, and fever were absent. All patients followed the same pharmacological protocol. Lorazepam was administered preoperatively (1 mg every 12 hours), and anesthesia was achieved by intradural blockade (infiltration) plus sedation with benzodiazepine. While in the recovery room after surgery, patients underwent infiltration of the femoral and sciatic nerves with local anesthetic and also received an intravenous dose of dexketoprofen (50 mg). Low-molecular-weight heparin was administered to prevent deep venous thrombosis. Each patient also received the specific medications that had been taken regularly.

Determination of CD31+/Annexin V+ EMPs

Platelet-free plasma was obtained by centrifugation at 1500 ×g for ten minutes at room temperature; this was followed by centrifugation at 13,000 ×g for twenty minutes to separate the EMPs, which were stored at −20°C until use. The EMPs were resuspended, incubated with monoclonal antibody against phycoerythrin (PE)-labeled anti-CD31 (Caltag Laboratories, Burlingame, California), and incubated with a fluorescein isothiocyanate (FITC)-conjugated annexin V kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. The negative control was obtained with use of the anti-isotype antibodies. An equal volume of Flow-Count Fluorospheres (Beckman Coulter) was added. Fluorescence-activated cell sorting was performed in a Cytomics FC 500 flow cytometer with CXP software (Beckman Coulter). Each result (single reported value) was the average of three independent determinations of the same sample.

Characterization of EPCs

Immediately after blood extraction, PBMCs (peripheral blood mononucleated cells) were separated by density-gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway), washed in phosphate-buffered saline solution (PBS; Life Technologies, Grand Island, New York), and resuspended in standard endothelial cell basal medium (EBM; Lonza, Walkersville, Maryland) plus endothelial cell growth medium supplements (EGM; Lonza) and 10% fetal calf serum (FCS; Life Technologies). Thereafter, 2×10^6 cells were incubated for thirty minutes in the dark at 4°C with saturated concentrations of anti-VEGFR-2 (Human VEGF R2/KDR phycoerythrin MAb; R&D Systems), anti-CD31 FITC, and anti-CD14 (TRICOLOR; Life Technologies) monoclonal antibodies and the corresponding isotype controls. The cells were then washed in PBS and resuspended in 500 µL of CellFIX (Becton Dickinson). Quantitative analysis was performed on an FACSCalibur cytometer (Becton Dickinson) measuring 200,000 events per sample. Data were analyzed with use of CellQuest software (Becton Dickinson) by side-scatter fluorescence dot-plot analysis. EPCs were defined as events that were triply positive for CD31, CD14, and VEGFR-2. The negative control (zero value) was the value obtained with use of the isotype antibodies.

Cytometric Bead Array Immunoassay

Plasma cytokine concentrations were evaluated with use of a Cytometric Bead Array immunoassay (Becton Dickinson), which is based on the binding of beads labeled with various fluorescence intensities to antibodies. This method allowed the simultaneous determination of several different cytokines in the same sample: IL-8, IL-1β, IL-6, IL-10, TNF α, and IL-12p70. ■