Formation of new blood vessels and capillaries is a key feature of wound healing and repair of damaged tissues. In the last decade, understanding the process of new blood vessel and capillary formation has changed dramatically. Postnatal vasculogenesis requires a coordinated pattern of endothelial-progenitor-cell (EPC) recruitment, migration, and differentiation. These cells contribute to blood vessel regeneration by incorporating themselves into newly-generated vessels and participating in the sub-

**Surgical Wound Fluid From Elderly Patients Shows a Dramatically Reduced Potential to Stimulate In-vitro Recruitment and Differentiation of Endothelial Progenitor Cells: Role of VEGF-165 and TGF-β1**

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**Abstract:** Purpose. Elderly patients are susceptible to wound healing problems due to impaired neovascularisation in tissue repair. We investigated influence of surgical wound fluid (WF) obtained from both young and aged patients after musculoskeletal surgery on in-vitro recruitment and differentiation of endothelial progenitor cells (EPCs). Further, VEGF and TGF-β1 in WF were measured and blockade experiments were performed to analyze the role of both cytokines in EPC recruitment. Methods. WF was obtained from young (28 ± 5 years, n = 14) and elderly (74 ± 8 years, n = 15) patients at 3, 8, and 24 hours after surgery. EPCs were isolated from healthy donors and incubated (72 hours) in medium substituted by WF. EPC number/differentiation was determined by fluorescence-microscopy and flow-cytometry after staining for DiLDL and lectin. CBF or ELISA was used to measure VEGF and TGF-β1 in WF. For blockade experiments, WF was mixed with antibodies against VEGF/TGF-β1 before incubation. Results. A significantly higher number and increased differentiation of EPC can be observed after incubation with WF from young compared to elderly individuals. VEGF and TGF-β1 were higher in young patients’ WF, and blockade of both cytokines reduced EPC numbers significantly. Conclusion. Impaired wound healing in the elderly could be a result of dampened recruitment of EPC to site of affliction, possibly due to low VEGF/TGF-β1 levels.
sequent chemoattraction of mature endothelial cells due to paracrine effects. Thus, reduced or insufficient recruitment of EPCs to ischemic and damaged tissue could result in impaired postnatal vasculogenesis with consequent delays in wound healing, a higher susceptibility to delayed wound closure, and higher risk for wound infections.

Geriatric patients are more susceptible to postsurgical wound healing complications than adolescent or middle-aged persons. The aging process produces multiple changes in tissues and organs rendering elderly people more susceptible to injury and less able to heal. Clinical studies have documented that compromised macrophage function leads to a decreased inflammatory response, limited mitogenic activity of keratinocytes and fibroblasts, reduced rates of collagen synthesis, slower epithelialization, increased tissue fragility, and decreased number of capillaries. However, since two key events that underlie successful wound regeneration are ingrowth and formation of healthy new blood vessels, the authors investigated the possibility that alterations in wound microenvironment following surgery in elderly patients may reduce EPC differentiation and recruitment of EPCs from cultured peripheral blood mononuclear cells (PBMCs), thereby mitigating wound healing ability in these patients. Using an in-vitro model, incubated PBMCs were isolated from healthy blood donor buffy coat with surgical wound fluid from young and elderly patients and the magnitude of EPC recruitment (number) and endothelial differentiation (uptake of DiL labelled low density lipoprotein) were compared. Concentrations of VEGF-165 and TGF-β1 in wound fluid samples were compared, and blockade experiments were performed using neutralizing antibodies, since both mediators are known to play a pivotal role in angiogenesis and are involved in EPC recruitment and differentiation.

Methods

Patients and wound fluid samples. Surgical wound fluid was collected from young (28 ± 5 years, n = 14) and elderly (74 ± 8 years, n = 15) patients undergoing surgical limb treatment after single long bone fracture. All patients were treated by open reduction and stabilized with internal fixation. Samples were taken from closed suction drains 3, 8, and 24 hours after surgery. The samples were centrifuged immediately after collection at 1300 g for 10 minutes and then frozen at -80°C. Neither hematoma nor infection complicated any of the wounds.

Exclusion criteria for this study included patients receiving corticosteroids, preoperative chemotherapy, or radiation therapy before or during the time of sample collection. All participants gave written consent to participate in the study. The local ethics committee at Johann Wolfgang Goethe University (Frankfurt, Germany) approved the study.

Isolation, identification, and quantification of EPCs. EPCs were isolated as previously described. PBMCs were briefly isolated from buffy coat by density gradient centrifugation (20 minutes, 600 g) with Ficoll (1.077 g/mL, [Biochrom, Berlin, Germany]). PBMCs were washed twice with cold phosphate buffered saline (PBS) without calcium and magnesium (PBSw/o, [10 minutes, 350 g]), and 8 x 10⁶ cells were cultivated on fibronectin-coated (10 µg/mL, Sigma, Deisenhofen, Germany) 12-well culture dishes in 800 µL of endothelial basal medium ([EBM], Cambrex, Verviers, Belgium) supplemented with 20% wound fluid at 37°C, 5% CO₂. After 72 hours, nonadherent cells were removed by washing twice with PBS/+/. EPCs were identified using previously described methods. Cells were incubated for 1 hour with 2.4 µg/mL 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine labeled acetylated low density lipoprotein (DiLDL, Cell-Systems, St. Katharinen, Germany) and 8 x 10⁶ cells were cultivated on fibronectin-coated (10 µg/mL, Sigma, Deisenhofen, Germany) 12-well culture dishes in 800 µL of endothelial basal medium ([EBM], Cambrex, Verviers, Belgium) supplemented with 20% wound fluid at 37°C, 5% CO₂. After 72 hours, nonadherent cells were removed by washing twice with PBS/+/. EPCs were identified using previously described methods. Cells were incubated for 1 hour with 2.4 µg/mL 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine labeled acetylated low density lipoprotein (DiLDL, Cell-Systems, St. Katharinen, Germany) in EBM supplemented with 20% FCS. Cells were fixed with 2% paraformaldehyde for 10 minutes, and after washing with PBS/+/, FITC-labelled Ulex europaeus agglutinin-1 ([10 µg/mL], Lectin, Sigma, Deisenhofen, Germany), were incubated for 1 hour. Cells exhibiting double-positive fluorescence were considered to be EPC. EPC numbers were determined by counting 5 randomized fields of view (x200 magnification) of lectin-positive cells using
Figure 1. Evaluation of EPC number and DiLDL uptake. A) DiLDL positive cells after incubation with wfy. B) WFE (200 x). C) Dot plots show ratio of DiLDL/Lectin double positive cells (EPCs) to total cells after incubation with wfy. D) WFE. E) Histogram illustrates differences in DiLDL uptake in EPCs after incubation with wfy (right peak) and WFE (left peak).
an inverse fluorescence microscope (Olympus, Hamburg, Germany). Thereafter counted number was multiplied with ratio of double positive cells (EPC) to total cells determined with FACScan (see below). The ability to take up DiLDL is considered a prototypic endothelial function. For determination of DiLDL uptake/cell, and of the ratio of DiLDL/lectin double-positive cells, cells were removed by gently scraping. After two washes (10 minutes, 300 g) cells were subjected to flow cytometry using a FACScan (Becton Dickinson, Heidelberg, Germany). DiLDL-uptake could be measured as changes in the mean fluorescence intensity of the second channel.

Detection of VEGF-165 using CBA. Levels of VEGF-165 in wound fluid samples were determined using Cytometric Bead Array (CBA) (Becton Dickinson, Heidelberg, Germany) following the manufacturer’s instructions. Samples were diluted 1:4 with dilution buffer. CBA analyses were performed using a dual laser, 4-color FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Detection of TGF-β1 using ELISA. Levels of TGF-β1 in wound fluid samples were determined using ELISA-Kits (R&D Systems, Wiesbaden, Germany) following the manufacturer’s instructions. Samples were diluted 1:20 with dilution buffer, after TGF-β1 activation with 1N HCl as recommended by the manufacturer.

Neutralization of TGF-β1 and VEGF165 in WF. Specific monoclonal anti-TGF-β1 IgG1 ([10 µg/mL WF, clone 9016.2], R&D Systems, Wiesbaden, Germany) was used to block TGF-β1 bioactivity of each individual WF sample. VEGF165 bioactivity was blocked using polyclonal IgG ([0.03 µg/mL WF], R&D Systems). Neutralization dosage for each antibody was calculated on the basis of a neutralization/concentration curve, provided by the manufacturer. As isotype control for TGF-β1, a KHL-specific monoclonal IgG1 ([clone 11711.11], R&D-Systems, Wiesbaden, Germany) was used. Normal goat IgG with appropriate concentration was used for VEGF isotype control ([AB-108-C], R&D Systems). WFs were incubated with each antibody at least for 30 minutes, before adding to the cell culture.

Statistical Analysis
Statistical evaluation was conducted using Wilcoxon-Mann-Whitney-U test with Bonferroni correction if multiple testing was applied. Results are presented as boxplots (Figure 2A and 2B) or as mean ± SD; P < 0.05 was considered statistically significant.

Results
EPC in-vitro recruitment and differentiation after incubation with surgical wound fluid from young versus elderly patients. After culturing periph-

Figure 2. Comparison of EPC number and DiLDL uptake after incubation with surgical wound fluid from young and old individuals. A) EPC number was significantly higher after incubation with wfy isolated 3, 8, and 24 hours after surgery in comparison to WFE isolated at the same points of time. B) DiLDL uptake per cell of DiLDL/Lectin double-positive cells was higher for wfy when isolated 3 and 8 hours after surgery in comparison to WFE isolated at the same points of time. A statistical significance was not observed in samples taken 24 hours after surgery. *P < 0.05
eral blood mononuclear cells (PBMCs) from healthy donors with wound fluid from young (wfy) or wound fluid from elderly patients (WFE) EPCs were identified by double staining for DiLDL and lectin. Figure 1A and 1C show representative data from PBMCs incubated wfy, while Figure 1B and 1D depict data obtained following incubation of identical cells with WFE. The upper right region of depicted dot plots demonstrates that ratio between DiLDL/Lectin double positive cells (EPCs) and total cells (PBMCs) did not differ significantly between groups. The overlay (Figure 1E) shows a representative rating of different DiLDL uptake in EPCs after incubation with wfy (right peak) versus WFE (left peak). Figure 2A depicts the average EPC-number per field of view (corrected with ratio of DiLDL/Lectin double positive cells [EPC] to total cells) after incubation of PBMCs with wfy versus WFE. A significant lower number of EPCs were observed following incubation with wound fluid from elderly patients when compared with wound fluid from young patients for WF isolated at any point of time after surgery.

To investigate the ability of WF from young versus elderly patients to stimulate EPC differentiation, PBMCs were cultured with wfy and WFE and DiLDL uptake was measured in EPCs by cytometric analysis (Figure 1E). Figure 2B demonstrates that DiLDL uptake in EPCs was significantly lower for WFE at 3 and 8 hours post-surgery samples.

**Comparison of VEGF-165 and TGF-β1 concentrations in wound fluid samples of young and old individuals.** TGF-β1 concentrations in WFE were consistently and significantly lower than those observed in wfy (Figure 3A). For both groups, TGF-β1 concentration decreased from 3–24 hours post-surgery (Figure 3A). In contrast to TGF-β1, VEGF-165 concentrations in wound fluid were found to increase during the post-surgical study period. Significantly reduced concentrations of VEGF-165 were found in elderly patients only in wound fluid obtained at 24 hours post-surgery (Figure 3B).

**Incubation of EPCs with wound fluid from young patients after inactivation of VEGF-165 and TGF-β1 with neutralizing antibodies.** To investigate whether the differences observed in the potential to recruit and stimulate differentiation of EPCs in wfy versus WFE may be associated with the observed differences in concentrations of VEGF-165 and/or TGF-β1, blockade experiments were performed for both cytokines. Inactivation of VEGF-165 in wfy with neutralizing antibodies resulted in a decrease of total EPCs versus control (unblocked wfy) after incubation (Figure 4B). Neutralization of TGF-β1 in wfy also resulted in a lower total EPC number versus control (Figure 4B). The effect of EPC number reduction could not be amplified when using antibodies against both TGF-β1 and VEGF-165. Cytometric analysis of DiLDL uptake in EPCs did not differ significantly versus control, neither for VEGF-165 nor for TGF-β1 neutralized wfy (data not shown).
Discussion

One of the key features underlying ability of post-surgical patients to recover and heal wounds without complications is an appropriate revascularization response in early phase of wound repair. Two principal components underlying neovascularization have been identified and studied: angiogenesis and post-natal vasculogenesis. While angiogenesis is defined as the sprouting of new capillaries from existing vessels and endothelial cells, post-natal vasculogenesis encompasses the extravasation of circulating EPCs, their ability to direct and participate in tissue regeneration due to paracrine proangiogenic properties and their characteristic features allowing them to incorporate themselves into newly-forming vessels. Impaired formation of new capillaries, described for rodent models and in elderly people, with resultant decreased supply of resident cells with nutrition and oxygen and depletion of metabolites, is one of the causes underlying a post-operative decrease in collagen synthesis, lower mitotic activity of parenchymal and mesenchymal cells, attenuated cell differentiation, reduced macrophage function and recruitment, delayed immune response with higher tissue fragility.

The results of the present study suggest that following a 72-hour incubation period of PBMCs with wound fluid obtained from young versus elderly surgical patients, the ability to recruit EPCs from PBMCs significantly declines with patient age. Both experimental studies and early clinical trials have underscored the importance of EPCs in regeneration of damaged tissue. Takahashi et al demonstrated that systemic transfer of human EPCs or cytokine-induced mobilization of bone marrow-derived EPCs leads to a significant improvement of blood circulation in a rodent model of hind limb ischemia. Moreover, injection of ex vivo cultured and multiplied EPCs into the left ventricular coronary artery has been shown to increase left ventricular cardiac output after cardiac infarction in humans, while increasing local EPC concentrations in human ischemic limbs improved transcutaneous oxygen pressure and pain-free walking time.

In addition to the observed alterations in EPC recruitment ability of WFE, the present study also showed significantly reduced DiLDL uptake per DiLDL/Lectin double positive cell (EPC) after incubation with wound fluid from the same elderly population. Previously, EPC DiLDL uptake has been shown to correlate with expression of CD31, von Willebrand Factor (vWF), VE-Cadherin, endothelial nitric oxide synthase (eNOS), VEGF Receptor 2 (KDR), and the ability of EPCs to form tube-like structures in matrigel. Hence, DiLDL uptake by EPCs is considered to be a powerful marker of EPC differentiation and maturation, indicative of a strong neovasculargenic response. Furthermore, differentiation of EPCs renders these cells to potent paracrine effectors with important functions in microenvironmental cytokine regulation. Elevated mRNA levels of insulin like growth factor-1 (IGF-1), stromal derived factor-1 (SDF-1/CXCL12) and different vascular endothelial growth factor isoforms (VEGF-A, VEGF-B) have been reported for differentiated EPCs. All three cytokines are known to be very potent chemotactic mediators for cells involved in wound healing processes. IGF-1 is known to be a chemoattractant for both fibroblasts and mesenchymal stem cells and may exert anabolic effects important for tissue regeneration. VEGF exhibits migration inducing properties for mature endothelial cells, pericytes and, as part of an autocrine amplifying loop together with SDF-1/CXCL12, EPCs. To date, the VEGF family is also one of the most important angio- and vasculogenic-regulating peptides.

The authors have previously shown that VEGF is capable of inducing recruitment of EPCs from PBMCs incubated with serum from multiple trauma patients. Moreover, we have previously demonstrated a
dose-dependent recruitment of EPCs by TGF-β1. These findings encouraged us to measure both cytokines in wfy versus WFE in order to ascertain molecular changes underlying known age-dependent alterations in wound healing. Indeed, both TGF-β1 and VEGF-165 were down regulated in WFE when compared with young patients wound fluid.

Although it has been previously reported that the physiological response to cytokines and cytokine secretion by activated cells declines commensurately with increasing age,15,20,27 most published studies have been performed in vitro with aging cell cultures or cell culture supernatants or in vivo in aging animal models.15,20,27 To the best of our knowledge, a prospective evaluation of age-dependent changes in total VEGF-165 and TGF-β1 concentrations in human WFE has not been reported elsewhere.

**Conclusion**

The present study has shown that TGF-β1 is reduced in WFE over 3 successive post-surgical points in time ranging from 3 to 24 hours following surgery. VEGF-165 was also significantly reduced in WFE when compared with wfy at the 24-hour post-surgery time point. These data confirm and extend previous observations that both growth factors are involved in recruiting EPCs when PBMCs are incubated with serum from multiple trauma patients.7 Knockout studies using neutralizing antibodies for VEGF-165 and TGF-β1 confirmed their role in EPC recruitment. The authors’ research, however, has yet to reveal the underlying factors involved in mediating differential effects of wfy versus WFE on EPC differentiation. The factors mediating these changes remain to be determined and further studies in this important area are warranted.

**References**


