Mobilization of CD34⁺-Progenitor Cells in Patients with Severe Trauma

Ulrike Ritz, Volker Spies, Isabella Mehling, Dominik Gruszka, Pol Maria Rommens, Alexander Hofmann*

BiomATICS-Group, University Medical Centre of the Johannes Gutenberg University, Center of Orthopaedic and Trauma Surgery, Mainz, Germany

Abstract

Circulating CD34⁺ progenitor cells (PSC) gained importance in the field of regenerative medicine due to their potential to home in on injury sites and differentiate into cells of both endothelial and osteogenic lineages. In this study, we analyzed the mobilization kinetics and the numbers of CD34⁺, CD31⁺, CD45⁺, and CD133⁺ cells in twenty polytrauma patients (n = 13 male, n = 7 female, mean age 46.5 ± 17.2 years, mean injury severity score (ISS) 35.8 ± 12.5 points). In addition, the endothelial differentiation capacity of enriched CD34⁺ cells was assessed by analyzing Dil-ac-LDL/lectin uptake, the expression of endothelial marker molecules, and the morphological characteristics of these cells in Matrigel and spheroid cultures. We found that on days 1, 3, and 7 after a major trauma, the number of CD34⁺ cells increased from 6- up to 12-fold (p < 0.0001) over the number of CD34⁺ cells from a control population of healthy, age-matched volunteers. The numbers of CD31⁺ cells were consistently higher on days 1 (1.4-fold, p < 0.01) and 7 (1.3-fold, p < 0.01), whereas the numbers of CD133⁺ cell did not change during the time course of investigation. Expression of endothelial marker molecules in CD34⁺ cells was significantly induced in the polytrauma patients. In addition, we show that the CD34⁺ cell levels in severely injured patients were not correlated with clinical parameters, such as the ISS score, the acute physiology and chronic health evaluation II score (APACHE II), as well as the sequential organ failure assessment score (SOFA-2). Our results clearly indicate that proangiogenic cells are systemically mobilized after polytrauma and that their numbers are sufficient for the development of novel therapeutic models in regenerative medicine.

Introduction

Musculoskeletal injuries are often accompanied by extensive vascular damage and local ischemia. In general, the processes of angiogenesis and de novo vasculogenesis represent key mechanisms of wound healing by restoring local blood supply and promoting tissue regeneration. Currently, it is generally understood that circulating stem and progenitor cells contribute to the repair of damaged tissues [1]. In particular, there is a wealth of evidence that circulating bone marrow-derived endothelial progenitor cells (EPCs) represent an important fraction of endothelial cells, as they have been shown to be involved in the repair and regeneration of blood vessels in animal models of ischemic tissue damage and myocardial infarction [2–4].

Circulating stem and progenitor cells have an innate ability to engage in vascular repair, but the mechanisms behind this are poorly defined. Endothelial progenitor cells were first discovered by Asahara et al. as CD34⁺ progenitor cells that derive from bone marrow [5]. CD34⁺ cells represent a heterogeneous population of cells with subpopulations that have different genetic and biological characteristics. These cells express a broad range of diverse surface markers and contain progenitor cells that are capable of differentiating into both endothelial and osteogenic lineages under the appropriate stimulating conditions [6]. However, it should be emphasized that the whole spectrum of bone-marrow derived CD34⁺ cells contains cell fractions that do not present functional characteristics of progenitor or of stem cells. Preclinical studies have shown that despite of their heterogeneity, human CD34⁺ cells can stimulate neovascularization in ischemic myocardium by increasing capillary density and improving function in models of acute and chronic myocardial ischemia [7]. Clinical trials in the field of cardiovascular medicine also provided evidence that enriched pools of autologous CD34⁺ cells can improve clinical outcome results when administered by intramyocardial, intravascular, or intramuscular injection and supported further clinical development of this treatment strategy [8,9].

Matsumoto et al. showed that CD34⁺ cells are recruited to fracture sites and contribute to fracture healing when they are intravenously injected into nude rats with non-healing femoral fractures [6]. Although the mechanism of EPC mobilization from bone marrow is not fully understood, there is accumulating evidence that musculoskeletal trauma may cause a systemic, provascular response in rodent bone marrow [10–12] and in human [13–15] bone marrow.

Although the number of publications on circulating progenitor cells has increased exponentially over the last few years, no data presently exist about the prevalence of these cells and their function in patients with multiple traumas. Patients who have been...
subjected to multiple traumas (or polytrauma) are described by a condition of multiple simultaneously occurring high-energy injuries of different body regions (for example serious head injury, blunt chest or abdomen trauma etc.) with one injury or the combination of injuries being dangerous to life. Despite of successful resuscitation, these patients are often at high risk to deteriorate unexpectedly due to “second hit” processes caused by severe tissue and organ damages. Therefore, this group of trauma patients in particular represents a potential candidate pool for therapeutic approaches in regenerative medicine.

The aim of this study was to analyze and specify mobilization kinetics, cell numbers, and the endothelial differentiation capacity of human CD34+ progenitor cells in severely injured patients. This study sheds new light on the question of whether CD34+ cells are systemically activated in patients with severe trauma and are available in sufficient numbers for the development of new therapeutic models.

Materials and Methods

Patient collective

Patients enrolled in the study fulfilled the criteria of having polytrauma at admission according to definitions of Tscherne et al.[16], Trentz [17], and the German S3-guideline on treatment of patients with severe and multiple injuries [18] (severe trauma-ST-Group; Table 1).

Ethics statement

The ethical committee of the “Landesärztekammer Rheinland-Pfalz” approved the investigations (No.: 837.046.03(3708)), which conformed to the principles of the Helsinki Declaration. Informed consent was taken from each patient according to the approved protocol, or in case of unconsciousness or death, from their legal representatives. Initial resuscitation, diagnostic procedures, and primary surgical interventions were performed according to the guidelines of the German Society of Trauma Surgery. Patients were recruited at the intensive care unit. Blood samples were obtained from a consecutive series of 20 patients in a non-randomized prospective manner. Transfusions with leukocyte-depleted, packed red blood cells, fresh-frozen plasma, and thrombocyte concentrates were provided to patients in accordance with their standard of care.

Quantification of circulating CD34+ progenitor cells in patients with severe trauma

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on days 1 (d1), 3 (d3), and 7 (d7). At each time point, samples of 20 ml of venous blood were collected in 7.5 ml S-Monovettes containing 1 ml CPDA (Sarstedt, Numbrecht, Germany). Blood samples were diluted 1:3 with phosphate buffered saline (PBS) containing 2 mM EDTA and separated on Ficoll-Paque (PAA Laboratories, Pasching, Austria) at 400 × g and 20°C for 40 min. To remove thrombocyte residues, the pellet was subsequently washed twice in PBS/EDTA and once in PBS/EDTA/BSA. Cell counts were performed using the standard Trypan blue staining in a Neubauer hemocytometer.

Cell populations were quantified by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, Heidelberg, Germany) using specific antibodies against the following cell surface markers: CD34 (FITC-mouse anti-human CD34 IgG2a, clone AC136, Miltenyi Biotec GmbH), CD133 (PE-mouse anti-human CD133 IgG1, clone AC133, Miltenyi Biotec GmbH), CD31 (APC-mouse

### Table 1. Polytrauma patients recruited to the study.

| Internal patient ID | Age | Gender | ISS | Injury pattern |
|---------------------|-----|--------|-----|----------------|
| 28                  | 55  | m      | 43  | TBI, multiple maxillofacial fractures, BCT |
| 29                  | 51  | m      | 34  | open extremity injuries, severe skin degloving, BCT, TBI |
| 30                  | 62  | m      | 18  | multiple extremity injuries, BCT |
| 33                  | 22  | m      | 33  | TBI, BCT, multiple extremity fractures |
| 34                  | 20  | m      | 42  | BCT, BAI, SR, multiple extremity fractures |
| 37                  | 51  | m      | 25  | pelvic ring injury, multiple extremity fractures, BAI |
| 46                  | 27  | m      | 34  | BCT, pelvic ring fracture, extremity fractures |
| 47                  | 74  | m      | 48  | BAI, stomach and colon rupture, BCT, extremity fractures |
| 52                  | 50  | m      | 29  | BCT, multiple extremity open fractures, pelvic ring injury |
| 53                  | 22  | m      | 45  | BCT, BAI, pelvic ring injury, urinary bladder rupture, lumbosacral plexus injury |
| 54                  | 26  | m      | 34  | BCT, BAI, multiple extremity fractures |
| 56                  | 62  | m      | 20  | pelvic ring injury, BAI, spine fractures, multiple extremity fractures |
| 57                  | 73  | m      | 41  | TBI, BCT, multiple extremity fractures |
| 38                  | 53  | f      | 25  | multiple open extremity injuries, spine injury, BAI |
| 39                  | 43  | f      | 48  | BAI, BCT, spleen and liver rupture, TBI, extremity fractures |
| 40                  | 67  | f      | 41  | TBI, BCT, multiple extremity fractures |
| 45                  | 31  | f      | 75  | TBI, BCT, pelvic ring fracture, multiple extremity fractures |
| 55                  | 54  | f      | 36  | BCT, pelvic ring fracture, extremity fractures |
| 60                  | 51  | f      | 34  | BAI, BAI, SR, spine fracture, extremity fractures |
| 61                  | 37  | f      | 37  | BAI, BCT, SR, LR, stomach rupture, pancreas rupture, multiple extremity fractures |

Median 35 (IQR 13) | TBI: n = 7, BCT: n = 15, BAI: n = 10, Pelvic ring: n = 7

TBI: traumatic brain injury; BCT: blunt chest trauma; BAI: blunt abdominal injuries; SR: spleen rupture; LR: liver rupture.

doi:10.1371/journal.pone.0097369.t001
Isolation and culture of CD34+ progenitor cells

CD34+ cells were isolated from PBMC suspensions using magnetically labeled CD34-antibodies (QBEND/10, CD34 MicroBead Kit, MiniMACS-Separator, Miltenyi Biotec, Germany) according to the manufacturer’s protocol. To ensure the purity of separated cells prior to the main experiments several pre-tests including FACS and immunofluorescence to specify the purity of the antibody were performed. The pre-tests revealed median purity of 96% of CD34+ cells. CD34+ cells were counted, resuspended in endothelial basal medium-2 (EBM-2 with supplement kit, 10% FCS; LONZA, Walkersville, USA; 100 U/ml penicillin, 100 μg/ml streptomycin sulpha) and cultured on fibronectin-coated 24-well plates at a density of 100,000 cells/well in a humidified atmosphere (5% CO₂, 37°C), with the media changed twice a week.

Analysis of the endothelial markers in CD34+PC cultures

Formation of colony forming units (CFU) of elongated, spindle-like cells was routinely monitored in CD34+ cultures using a phase contrast microscope three times a week. The endothelial differentiation capacity of CD34+ cells was verified in fifth-passage cultures using immunofluorescence staining for the following markers of mature endothelial cells: CD31 (PECAM-1, mouse anti-human CD31, clone Pecam1, DaKo; second antibody: anti-mouse IgG-FITC, Miltenyi), von Willebrand factor (vWF, mouse anti-human vWF, clone F8186, Dako, second antibody: anti-mouse IgG-FITC, Miltenyi), and CD45 (PE-mouse anti-human CD45, Miltenyi Biotec, Bergisch Gladbach, Germany) [19,20].

Assessment of clinical scores

The association of injury severity and clinical parameters with the number of circulating CD34+ cells, both the acute physiology and chronic health evaluation II score (APACHE II [24]) and the Sequential Organ Failure Assessment Score (SOFA [25]) were recorded and correlated with the number of circulating CD34+ cells. To make the severity classification more independent of treatment, the APACHE II score was determined at admission. The SOFA score, which is usually used to track a patient’s status during the course of treatment, was assessed at days 1, 3, and 7 and correlated with the respective cell numbers.

Statistical Analysis

The primary goals of this study were to determine the number and the differentiation capacity of CD34+ cells in patients with severe trauma. Other cell surface markers were investigated as secondary variables. The sample size calculation was performed based on a study published by Laing et al. [14], which described the number of CD34+ cells in patients with isolated, low-energy, closed tibial fractures. A total of 16 patients (8 patients per group) were needed to obtain a desired statistical power of 0.9 (anticipated effect size (Cohen’s d): 2.9; probability level for a two-tailed hypothesis (p): 0.001). All in vitro experiments were performed in a triplicate for each individual donor sample. Measurement values were expressed as the mean ± standard deviation (SD) of the mean or medians and quartiles/interquartile ranges (IQR), if reasonable. Data distributions were depicted in box plots. Differences between the means were compared using the non-parametric Mann-Whitney-U-Test. Correlation analyses were performed using the bivariate Pearson’s correlation test. Differences between the two groups were considered to be significant at α≤5%(number of comparisons), according to Bonferroni’s correction for multiple comparisons. Statistical analysis was performed using the SPSS 19.0 software.
Results

Patient’s characteristics

A total of twenty patients with severe trauma (n = 13 male, n = 7 female, mean age 46.5 ± 17.2 years) were included in the study. An age- and gender-matched population of fourteen healthy volunteers (10 male, 4 female, mean age 43.4 ± 19 years) was used as a control group (Table 2). There was no statistical difference in the mean age between the groups. Clinical characteristics such as age, gender, ISS score, and the number of RBC and FFP units within the first 7 days are depicted in Table 2. The median ISS Score in the ST group was 35 points (IQR 13 points). All of the patients survived the first seven days after trauma. One out of twenty patients died at day 10 after injury due to acute heart failure (female, 31 years old, ISS score 75 points).

Table 2. Clinical and laboratory data of the study cohort.

|                          | ST-Group | Control Group |
|--------------------------|----------|---------------|
|                          | male     | female        |
| Age (mean; ±SD)          | 45.8±20  | 48.0±12       |
| Gender (n)               | 13       | 7             |
| ISS (points, median (IQR))| 34 (16) | 37 (13)       |
| Diabetes (n)             | 0        | 1             |
| Hypertension (n)         | 3        | 1             |
| RBC (n)                  | 20.9±27.8| n.a.          |
| FFP (n)                  | 6.6±817  | n.a.          |
| APACHE II (points) predicted death rate | Day 1 | 16.9±4.5 [27.4±12.6%] |
| SAPS-2 (points)          | Day 1    | 52.9±15.8     |
| SOFA (points)            | Day 1    | 7.1±4.3       |
|                          | Day 3    | 6.0±4.5       |
|                          | Day 7    | 4.7±4.2       |
| CRP (mg/l)               | Day 1    | 1.4±1.2       |
|                          | Day 3    | 194.7±93.2    |
|                          | Day 7    | 130.9±74.9    |
| WBC (cells/nl)           | Day 1    | 14.4±6.9      |
|                          | Day 3    | 10.9±4.3      |
|                          | Day 7    | 9.1±2.0       |
| Thrombocytes (cells/nl)  | Day 1    | 262.4±107.1   |
|                          | Day 3    | 136.5±64.7    |
|                          | Day 7    | 193.2±91.7    |
| Hb (g/dl)                | Day 1    | 12.3±3.1      |
|                          | Day 3    | 10.1±2.2      |
|                          | Day 7    | 9.6±1.5       |
| Hematocrit (%)           | Day 1    | 36.1±9.0      |
|                          | Day 3    | 29.2±6.5      |
|                          | Day 7    | 28.4±4.0      |
| Lactate (mmol/l)         | Day 1    | 3.4±3.2       |
|                          | Day 3    | 2.4±2.3       |
|                          | Day 7    | 1.0±0.5       |
| PCT (ng/ml)              | Day 1    | n.a.          |
|                          | Day 3    | 1.4±1.1       |
|                          | Day 7    | 0.9±1.3       |

RBC: red blood cell concentrate; FFP: fresh-frozen plasma; CRP: C-reactive protein; WBC: white blood cell count; Hb: hemoglobin, PCT: procalcitonin.

doi:10.1371/journal.pone.0097369.t002

The numbers of CD34+ and CD31+ cells significantly increase after severe trauma

The first step in our study was to analyze the prevalence of CD34+ cells in the peripheral blood of severely traumatized patients (Fig. 1–2). The number of CD34+ cells was quantified and compared to the control group (Table 3, Fig. 2a). Significant physiological fluctuations of cell numbers of PBMCs, CD34+, CD31+, CD45+, CD133+, CD34+/CD45-, and CD31+/CD45- were excluded in the control group at p-values of >0.5 in all comparisons between different time points of measurements (Mann-Whitney-U-test for data distribution at days 1 vs. 3, days 1 vs. 7, and days 3 vs. 7).

We found that the number of CD34+ cells in the severely traumatized patients was significantly higher at all investigated...
time points (6, 10 and 12-fold increases on days 1, 3, and 7; \( p < 0.0001 \)) when compared with the control group, indicating that CD34+ cells are systemically recruited from the bone marrow or other niches of the body as a result of multiple injuries.

These results led us to further examine the prevalence of CD34\(^+\) cells that did not express the cell surface antigen CD45; therefore, they are unlikely of leukocyte origin. Interestingly, during the course of this investigation, the entire CD45\(^+\) population in the ST group significantly decreased in number (day 1: 89.5%; day 3: 80.6%; and day 7: 77.5% of PBMCs); however, the number of CD34\(^+\)/CD45\(^-\) cells increased 200-fold on days 1 and 3 and 300-fold on day 7 after injury \( (p < 0.001) \) in comparison to the control group (Fig. 2a, Table 3). The CD45\(^+\) population was significantly lower in relative number (%) in the ST group than the control group (day 3: 0.84-fold, day 7: 0.81-fold, \( p < 0.001 \), Fig. 2b).

To address the question of whether the numbers of circulating mature endothelial cells and endothelial progenitor cells are elevated in severely injured patients, we also assessed the prevalences of CD31\(^+\), CD31\(^+\)/CD45\(^-\), and CD133\(^+\) cells. The entire population of CD31\(^+\) cells was significantly elevated 1.42-fold.
and 1.26-fold on days 1 and 7 (p<0.01), respectively, in comparison with the control group (Table 3, Fig. 2c). The null hypothesis for temporal changes of cell numbers of CD31+/CD45− and CD133+ cells in the ST group was not rejected at p-values given in Table 3 after Bonferroni’s correction for multiple testing (Fig. 2d and e).

Table 3. Percentages of circulating cells detected by flow cytometry in PBMCs.

|                | ST-Group (%)                          | Control Group (%)          |
|----------------|---------------------------------------|----------------------------|
| **Day 1**      |                                       |                            |
| CD34           | 0.18±0.13 (p<0.0001)                  | 0.03±0.03                  |
| CD31           | 69.9±18.3 (p=0.001)                   | 492±11                     |
| CD45           | 89.5±9.5                              | 95.8±2.1                   |
| CD34+/CD45-    | 0.2±0.09 (p=0.001)                    | 0.01±0.004                 |
| CD31+/CD45-    | 0.13±0.6                              | 1.9±1.3                    |
| CD133          | 0.14±0.03                             | 0.08±0.04                  |
| **Day 3**      |                                       |                            |
| CD34           | 0.33±0.29 (p<0.00001)                 |                            |
| CD31           | 60.5±15                               |                            |
| CD45           | 80.6±13.4 (p<0.001)                   |                            |
| CD34+/CD45-    | 0.22±0.15 (p=0.001)                   |                            |
| CD31+/CD45-    | 1.5±0.7                               |                            |
| CD133          | 0.15±0.08                             |                            |
| **Day 7**      |                                       |                            |
| CD34           | 0.37±0.2 (p<0.0001)                   |                            |
| CD31           | 61.8±16 (p=0.007)                     |                            |
| CD45           | 77.5±13.5 (p=0.006)                   |                            |
| CD34+/CD45-    | 0.3±0.1 (p=0.001)                     |                            |
| CD31+/CD45-    | 3.6±2.8                               |                            |
| CD133          | 0.23±0.13                             |                            |

Significant differences between the experimental and the control groups are depicted in bold letters (Mann-Whitney-U-test).
Expression of endothelial marker molecules is significantly increased in CD34+ progenitor cells after severe trauma

Twenty-four hours after isolation and plating, ten percent of the cultured CD34+ cells from healthy donors revealed positive double staining with Dil-ac-LDL/lectin, which is generally considered to be specific for cells of endothelial lineage (Fig. 3a and 4b). In contrast, the CD34+ cell cultures from the severely traumatized patients contained 64 ± 14% (d1, p < 0.0001; Fig. 3b and 4b), 66 ± 12.7% (d3, p < 0.0001), and 63 ± 10.3% (d7, p < 0.0001) double positive cells, indicating that the CD34+ cells differentiated into the endothelial lineage in the patients with severe trauma.

CD34+ cells were subsequently expanded in vitro using standard protocols for endothelial cell culturing on fibronectin-coated surfaces. After 3–4 weeks in culture, the first colony forming units (CFUs) of elongated, spindle-like cells could be detected. In control cultures, only 1.5 ± 0.5 CFUc were detected after a culture period of 5 weeks (Fig. 5). In clear contrast, cell cultures isolated on days 1, 3, and 7 after severe trauma generated 12 ± 4, 19 ± 4, and 27 ± 4 CFUs, respectively (Fig. 5).

An analysis of endothelial cell marker expression in ST cultures by immunofluorescence revealed the positive expression of PECAM-1/CD34 and CD146 in these cells (Fig. 3c–f). Although the expression of endothelial cell marker vWF was found by PCR (Fig. 4a), we could not detect vWF expression by immunofluorescence in either group, probably due to a very low level of gene expression (not shown).

Analysis of cell morphology in Matrigel and spheroid cultures showed that CD34+ cultures in the ST-group contained an enriched population of elongated, spindle-shaped cells. In clear contrast to the control group, which revealed neither cord-like structures nor primitive network formations, cell cultures in the ST-group showed similar formation of cord-like structures as previously described for putative progenitors of endothelial cells (Fig. 6a–d) [26]. However, formation of both primitive capillary structures on Matrigel and endothelial sprouting from spheroids were to a much lower extent as found in HUVEC-cultures (Fig. 6d-g). These results indicate that CD34+ cells have the potential...
to differentiate into the endothelial lineage in vitro but that they likely possess a phenotype that may be different from mature endothelial cells.

**Correlation with injury severity and patient’s condition**

Pearson’s bivariate correlation analyses were performed to address the question of whether the number of CD34+ cells was
Table 4. Pearson’s correlation analysis.

| Pearson’s correlation | APACHE II (d1) | ISS (d1) | SOFA (d1) | SOFA (d3) | SOFA (d7) |
|-----------------------|----------------|----------|-----------|-----------|-----------|
| CD34+ (d1)            | 0.03           | 0.2      |           |           |           |
| r                     | 0.09           | 0.2      | 0.2       |           |           |
| r (2-tailed)          | 0.9            | 0.9      | 0.4       |           |           |
| CD34+ (d3)            | 0.22           | 0.3      | 0.1       |           |           |
| r                     | 0.6            | 0.3      | 0.3       |           |           |
| r (2-tailed)          | 0.6            | 0.3      | 0.005     |           |           |
| CD34+ (d7)            | 0.4            | −0.2     | 0.4       |           |           |
| r                     | 0.06           | −0.2     | 0.8       |           |           |
| r (2-tailed)          | 0.06           | −0.2     | 0.8       |           |           |

At the defined level of α, no statistically significant differences were detected.

doi:10.1371/journal.pone.0097369.t004

Discussion

CD34+ cells represent a heterogeneous fraction of enriched endothelial/hematopoietic progenitors in peripheral blood that give rise to endothelial cells [5,26] and mesenchymal cells, including osteoblasts [27]. These cells have been a focus of musculoskeletal research due to their supposed therapeutic potential in wound and fracture healing [6,11,13]. In this study, we showed for the first time that the numbers of pro-angiogenic CD34+ cells are significantly increased in severely traumatized patients during the first week after trauma. We also found that the number of circulating cells expressing CD31 (PECAM-1), a marker of mature endothelial cells, was consistently elevated in these patients. Furthermore, our in vitro analyses revealed significantly increased expression levels of endothelial marker molecules and endothelial cell growth in cultured CD34+ cells, as shown by Dil-ac-LDL/lectin double staining, by the expression of endothelial markers (CD31, CD34, and CD146, Fig. 3 a-b and 4a), and by increased numbers of CFUs (Fig. 5). With respect to the nature of CD34+ cells that do not exclusively represent the endothelial lineage, but which contain monocyte precursors as well; our results indicate that the differentiation of circulating CD34+ cells was clearly directed towards the endothelial lineage after severe trauma which might be due to increased numbers of endothelial progenitor cells within the fraction of CD34+ cells (e.g. CD34+/CD133+ cells).

Elements of EPC recruitment and homing are presumably promoted by the release of pro-angiogenic cytokines [28,29]. Henrich et al. showed that the sera from patients with multiple traumas contain soluble factors that promote the in vitro differentiation of EPCs and that TGF-beta1 (see also [30]) and VEGF may be involved in their recruitment [12,28]. Significant increases in the number of circulating EPCs have been detected in humans and animals after traumatic brain injuries [31], skin burns [32,33], sepsis [34,35], heart failure [36] and acute lung injury [37,38]. Furthermore, some authors reported the mobilization of CD34+ cells from bone marrow after an isolated bone fracture in rodents [10,11] and humans [14]. As a result, the stromal cell-derived factor-1/CXC chemokine receptor 4 (SDF-1/CXCR-4) axis has been recognized as a pivotal mechanism for the recruitment of EPCs to ischemic and damaged tissues [39,40].

A series of clinical and experimental studies, prompted by the discovery of circulating EPCs, has provided insights into these processes and rendered a new perspective for the application of new therapeutic approaches [15,41]. Although there is increasing evidence that upon stress, sepsis, and vascular injury, EPCs are systemically mobilized in humans [14,29], the biological function of these cells remains largely unknown. Interestingly, Matsumoto et al. showed that in mice, enriched fractions of EPCs were recruited to fracture sites following intravenous injection. Furthermore, the local transplantation of human CD34+ cells into the sites of fracture non-unions resulted in an improvement in fracture healing in nude rats. This effect may be explained by both the angiogenic and osteogenic potentials of CD34+ progenitor cells [6,27]. Kuroda et al. reported the successful transplantation of G-CSF-mobilized, autologous CD34+ cells in a patient with a tibial non-union [42], which resulted in a fracture union three months after the transplantation. Although these reports strongly suggest the therapeutic potential of CD34+ cells for alternative wound and fracture healing approaches, we cannot conclude from our results whether increased numbers of circulating CD34+ cells are beneficial or detrimental for patients with major trauma.

With regard to their prognostic value, the association of endothelial cells with clinical outcomes has been, in part, controversially discussed for different pathologic conditions. Numerous studies reported a strong association between the levels of circulating CD34+ cells with outcome parameters after cardiovascular events [2–4]. Liu et al. found a clear correlation between the levels of circulating CD34+/CD133+ cells and an improvement in the GCS scale in patients with traumatic brain injuries [31]. However, in patients with sepsis, the prognostic value of CD34+ cells remains under debate [29]. In a recent study, Xin-Long et al. investigated the correlation between the numbers of endothelial progenitor cells expressing cell surface antigens CD34 and CD133 and the injury severity score in patients with fractures [13]. According to the results of our investigation, they found no correlation between these two parameters. In our study, the number of circulating CD34+ cells did not correlate with any of the clinical outcome parameters, such as the injury severity score, survival, development of multiple organ failure, and the APACHE II scores. We suggest that the mobilization of CD34+ cells and/or endothelial cells may not be a dose-dependent effect, or it may reach maximum levels in patients with major trauma.
Study design

Focusing on the early post-traumatic phase, the patients enrolled in this study were not selected by the nature of their traumatic injury. To investigate the mobilization kinetics of CD34+ cells as a general response to multiple blunt injuries, rather than on specific injury patterns, the selection was met only by the definition of severe trauma, according to well-accepted ISS score criteria [43]. The time points for the collection of blood samples were chosen according to our pilot study, which revealed that levels of CD34+ recruitment peaked at day 3 after trauma. To obtain a purified fraction of CD34+cells for cell culture and for an analysis of their endothelial differentiation capacity, we used a magnet-activated cell sorting technique (MACS; Miltenyi Biotec). Because the volume of blood in our population of severely injured patients was restricted, further expression analyses of subsets of cell surface antigens could not be performed in this study to better characterize the cell phenotypes. However, the results of this study strongly suggest that this number of isolated CD34+ cells may be sufficient for the development of cell-based therapeutic approaches, even in patients with severe trauma.

Limitations of the study

Although we could clearly show that the number of CD34+ cells and their differentiation capacity significantly differs from those in the control group of healthy individuals, conclusions cannot be drawn from this study regarding the biological function of CD34+ cells. Furthermore, we cannot conclude that multiple blunt injuries are specific causes for the recruitment of CD34+ cells, taking into account that these cells may be associatively mobilized with other hematopoietic cells due to a release of different cytokines. In particular, we are also aware that factors such as drugs, surgery, and nutrition may have an impact on the recruitment kinetics of these cells. The sample size of twenty consecutive patients precludes further subgroup and multivariate analyses. However, we continue to recruit patients to define the prognostic value of CD34+ cells for patients with severe trauma.

Assessment of clinical scores in patients with severe trauma (APACHE II, SOFA, ISS) may be associated with serious limitations with respect to their predictive value for outcomes. The suitability of these scores and their advantages and disadvantages in comparison to other scores like TRISS, NISS etc. is still has been controversially discussed in the past. The results and conclusions of this study are therefore limited only to the assessed scores and do not describe a whole spectrum of possible relationships.

Important goals for future research are to elucidate the long-term temporal changes of CD34+PC numbers and their possible association with the outcome results and the roles of mobilization and the endothelial differentiation of CD34+ cells in soft tissue and organ recovery, as well as in post-traumatic systemic insults (such as sepsis), to establish critical determinants of morbidity and mortality and to evaluate the possibilities for therapeutic gain.

Conclusions

Our results clearly indicate that pro-angiogenic cells are systemically mobilized after severe trauma and that their numbers are sufficient for isolation and development of novel therapeutic models in regenerative medicine. However, the mobilization of these cells may not be a dose-dependent effect in patients with major trauma.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of Angelika Ackermann and thank Dr. Volker Mailänder for providing purified CD34+ cells for FACS analyses and for his support in analyzing and discussing our FACS-data.

Author Contributions

Conceived and designed the experiments: UR VS IM DG AH. Performed the experiments: UR VS. Analyzed the data: UR VS AH. Contributed reagents/materials/analysis tools: UR VS IM DG PAM AH. Wrote the paper: UR AH PAM. Acquired participants: AH IM.

References

1. Wu Y, Zhao RC, Tredget EE (2010) Concise review: bone marrow-derived stem/progenitor cells in cutaneous repair and regeneration. Stem Cells 28: 905–915.
2. Chang HW, Lee S, Sun CK, Hang CL, Youssef AA, et al. (2010) Level and value of circulating endothelial progenitor cells in patients with acute myocardial infarction undergoing primary coronary angioplasty: in vivo and in vitro studies. Trans Res 156: 251–263.
3. Maruyama S, Taguchi A, Iwashima S, Ozaki T, Yasuda K, et al. (2006) Low circulating CD34+ cell count is associated with poor prognosis in chronic hemodialysis patients. Kidney Int 74: 1603–1609.
4. Sobrino T, Hurtado O, Moro MA, Rodriguez-Yanez M, Castellanos M, et al. (2007) The increase of circulating endothelial progenitor cells after acute ischemic stroke is associated with good outcome. Stroke 38: 2759–2764.
5. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, et al. (1999) Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 85: 221–228.
6. Matsumoto T, Kawamoto A, Kuroda R, Ishikawa M, Mifune Y, et al. (2008) Therapeutic potential of vasculogenesis and osteogenesis promoted by peripheral blood CD34-positive cells for functional bone healing. Am J Pathol 169: 1440–1457.
7. Kawamoto A, Iwasa H, Kusano K, Maruyama T, Oyamada A, et al. (2006) CD34-positive cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction compared with total mononuclear cells. Circulation 114: 2163–2169.
8. Musialik P, Tekiel M, Kostkiewicz M, Majka M, Sotz W, et al. (2011) Randomized transcoronary delivery of CD34+ cells with perfusion versus stop-flow method in patients with recent myocardial infarction: Early cardiac retention of 59Fe(Tc)-labeled cells activity. J Nucl Cardiol 18: 104–116.
9. Pasquet S, Sovalat H, Henon P, Bischoff N, Arkam Y, et al. (2009) Long-term benefit of intracardiac delivery of autologous granulocyte-colony-stimulating factor-mobilized blood CD34+ cells containing cardiac progenitors on regional heart structure and function after myocardial infarct. Cytotherapy 11: 1002–1015.
10. Laing AJ, Dillon JP, Condron ET, Coffey JC, Street JT, et al. (2007) A systemic pro-angiogenic response in bone marrow to musculoskeletal trauma in mice. J Bone Joint Surg Br 89: 116–120.
11. Kawamoto A, Kuroda R, Kawamoto T, Mifune Y, Matsui T, et al. (2008) Fracture induced mobilization and incorporation of bone marrow-derived endothelial progenitor cells for bone healing. J Cell Physiol 215: 234–242.
12. Li R, Nauth A, Li C, Qamirani E, Aresko K, et al. (2012) Expression of VEGF gene isoforms in a rat segmental bone defect model treated with EPCs. J Orthop Trauma 26: 689–692.
13. Xin-Long M, Xiao-Lei S, Chau-You W, Jian-Xiong M, Peng T (2012) Significance of Circulating Endothelial Progenitor Cells in Patients with Fracture Healing Process. J Orthop Res.
14. Laing AJ, Dillon JP, Condron ET, Street JT, Wang JH, et al. (2007) Mobilization of endothelial precursor cells: systemic vascular response to musculoskeletal trauma. J Orthop Res 25: 44–50.
15. Kuroda R, Matsumoto T, Kawakami Y, Fukui T, Mifune Y, et al. (2014) Clinical Impact of Circulating CD34-Positive Cells on Bone Regeneration and Healing. Tissue Eng Part B Rev.
16. Tsoberne H, Regel G, Sturm JA, Friedl HP (1987) [Degree of severity and priorities in multiple injuries]. Schweiz Arch Chirurg. 124: 631–640.
17. Keel M, Trenz O (2005) Pathophysiology of polytrauma. Injury 36: 691–709.
18. German Trauma Society (2002) S3 – Guideline on Treatment of Patients with Severe and Multiple Injuries. AWMF-Registry No 012/019.
19. Fuchs S, Hofmann A, Kirkpatrick CJ (2007) MicrovesSEL-like structures from outgrowth endothelial cells from human peripheral blood in 2-dimensional and 3-dimensional co-cultures with osteoblastic lineage cells. Tissue Eng 13: 2577–2580.
20. Hofmann A, Ritz U, Verrier S, Eglin D, Alini M, et al. (2008) The effect of human osteoblasts on proliferation and neo-vessel formation of human umbilical vein endothelial cells in a long-term 3D co-culture on polyurethane scaffolds. Biomaterials 29: 4217–4226.

21. Casamassimi A, Balestrieri ML, Fiorito C, Schiano C, Maione C, et al. (2007) Comparison between total endothelial progenitor cell isolation versus enriched CD133+ culture. J Biochem 141: 503–511.

22. Korf T, Augustin HG (1998) Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. J Cell Biol 145: 1341–1352.

23. Laib AM, Bartol A, Alajati A, Korf T, Weber H, et al. (2009) Spheroid-based human endothelial cell microvessel formation in vivo. Nat Prostet 4: 1202–1215.

24. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13: 818–829.

25. Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, et al. (1998) Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Working group on “sepsis-related problems” of the European Society of Intensive Care Medicine. Crit Care Med 26: 1793–1800.

26. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, et al. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275: 964–967.

27. Chen JL, Hunt P, McElvain M, Black T, Kaufman S, et al. (1997) Osteoblast precursor cells are found in CD34+ cells from human bone marrow. Stem Cells 15: 308–317.

28. Henrich D, Hahn P, Wahl M, Wilhelm K, Dernbach E, et al. (2004) Serum derived from multiple trauma patients promotes the differentiation of endothelial progenitor cells in vitro: possible role of transforming growth factor-beta1 and vascular endothelial growth factor165. Shock 21: 13–16.

29. Rafat N, Hanusch C, Brinkkoetter PT, Schulte J, Brade J, et al. (2007) Increased circulating CD34+ cells in septic patients: correlation with survival. Crit Care Med 35: 1760–1765.

30. Liu L, Wei H, Chen F, Wang J, Dong J, et al. (2011) Endothelial progenitor cells correlate with clinical outcome of traumatic brain injury. Crit Care Med 39: 1760–1765.

31. Foresta G, Schipilliti M, De Toni L, Magagna S, Lancerotto L, et al. (2011) Blood levels, apoptosis, and homing of the endothelial progenitor cells after skin burns and escharectomy. J Trauma 70: 459–465.

32. Busnuiu CJ, Mogesam GD, Popescu FC, Lascar I, Parvanescu H, et al. (2013) Phases of the cutaneous angiogenesis process in experimental third-degree skin burns: histological and immunohistochemical study. Rom J Morphol Embryol 54: 163–171.

33. Patschan SA, Patschan D, Temme J, Koerst J, Wessels JT, et al. (2011) Endothelial progenitor cells (EPC) in sepsis with acute renal dysfunction (ARD). Crit Care 15: R94.

34. van Ierssel SH, Van Graevenbroeck EM, Hoymans YV, Vrints CJ, Conraads VM, et al. (2013) Endothelium dependent vasomotion and in vitro markers of endothelial repair in patients with severe sepsis: an observational study. PLoS One 8: e69499.

35. Qi Y, Qian L, Sun B, Chen G, Cao Y (2010) Circulating CD34+ cells are elevated in neonates with respiratory distress syndrome. Inflamm Res 59: 899–905.

36. Smadja DM, Maugue L, Nunes H, d'Audigier C, Juvin K, et al. (2013) Imbalance of circulating endothelial cells and progenitors in idiopathic pulmonary fibrosis. Angiogenesis 16: 147–157.

37. Copes WS, Champion HR, Sacco WJ, Lawnick MM, Keast SL, et al. (1988) The Injury Severity Score revisited. J Trauma 28: 69–77.