Insulin-like growth factor binding proteins-2 and -4 enhance the migration of human CD34$^-$/CD133$^+$ hematopoietic stem and progenitor cells

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Abstract. The insulin-like growth factor (IGF) system is involved in cell migration, which plays an important role in cancer progression. It has been shown that cancer progression correlates with the level of circulating human hematopoietic stem and progenitor cells (HSPCs) expressing CD34 and/or CD133. However, it is unknown whether factors released from cancer cells, including soluble compounds of the IGF system, recruit these HSPCs via enhancing their migration. Our study showed the expression of type I IGF receptor (IGF-IR) in human HSPCs expressing CD34 and/or CD133. In an indirect co-culture model, soluble factors released from human lung epithelial cancer cells (H358, H322) increased the migration of CD34$^-$/CD133$^+$ cells towards cancer cells, whereas migration of CD34$^+$/CD133$^+$ or CD34$^+$/CD133$^-$ cells remained unchanged. The lung epithelial cancer cell lines H358 and H322, exhibited a high expression of IGFBP-2, -4 and -6 but not IGF-I and IGFBP-3. Subsequent analyses with those soluble compounds of the IGF system revealed a dose-dependent stimulating effect of the IGFBP-2 and -4 on the migration of CD34$^-$/CD133$^+$ cells. In contrast, IGF-I and IGFBP-3 and -6 did not influence the migration of CD34$^-$/CD133$^+$ cells. Because IGFBPs are involved in cell migration via IGF-dependent and -independent mechanisms, our study indicates that IGFBP-2 and -4, which are expressed in lung epithelial cancer cells, enhance the migration of CD34$^-$/CD133$^+$ HSPCs independent of IGF-I.

Introduction

Cell migration contributes to physiological processes such as development and regeneration of tissues as well as to pathological processes like cancer progression (1). The migration of various types of cells is modulated by the insulin-like growth factor (IGF) system (2). Key compounds of the IGF system are the multifunctional peptides IGF-I and IGF-II (3). Both IGF peptides mediate their cellular action via binding to the insulin-like growth factor I receptor (IGF-IR), which binds IGF-I with higher affinity than IGF-II. The cellular action of IGFs is modified by the type II IGF receptor (IGF-IIR) and a family of IGF binding proteins (IGFBP-1 to -6) (3). The IGFBPs both reduce and enhance IGF action, either by sequestering or releasing IGFs (4). Moreover, the IGFBPs mediate an IGF-independent function following interaction with other extracellular/cell surface partners, such as fibrinogen (5) and $\alpha_5\beta_1$ integrin (6). In adults, both IGF peptides and IGFBPs are expressed and released into the circulation and extracellular fluids (7,8). Altered local or systemic levels of those soluble compounds of the IGF system have been linked to the progression of cancer (9,10) including lung cancer (11-14).

Increasing evidence suggests an important role of endothelial progenitor cells (EPCs), which are derived from hematopoietic stem and progenitor cells (HSPCs), in the development of tumor vasculature and metastatic progression (15-18). In the case of lung cancer, animal studies demonstrated the importance of EPCs for the increased formation of new blood vessels (neovascularization) in tumors, increased tumor metastasis and poorer survival of the tumor-bearing animals (19). This observation corresponds to human studies showing a relation between the increased level of circulating EPCs in peripheral blood and the poorer survival of patients with non-small cell lung carcinoma (NSCLC) (20). Immunohistochemical studies mainly detected EPCs in the small vessels of NSCLC tissues (20,21). Nonetheless, it is unknown whether EPCs reach the cancer tissue indirectly following preceding vascularization events and/or directly following active recruitment by cancer cells and/or stromal cells of cancer tissue. Among the variety of cells forming the tumor stroma, mesenchymal fibroblasts have been identified as important immune modulators (22).

EPCs are an immature subset of CD34-expressing cells that co-express the five-transmembrane glycoprotein CD133 (also termed AC133) (23,24). It is suggested that these CD34$^+$/CD133$^+$ cells are derived from CD34$^-$/CD133$^+$ HSPCs (24-26), whereas CD34$^+$/CD133$^-$ cells are mature EPCs (27-29). This suggestion is still debated because HSPCs expressing CD133 but not CD34

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also mediate an efficient endothelialization *in vivo* thereby indicating $CD34^-/CD133^+$ cells as another type of EPC (30).

Gammaitoni *et al* (32) described an *ex vivo* system for the expansion of human HSPCs from peripheral blood that results in the development of cells expressing CD34 and/or CD133 (31). By use of this *ex vivo* expansion system and subsequent migration assays we aimed to ivestigate whether soluble factors released from human lung epithelial cancer cells or fibroblasts recruit human HSPCs depending on the expression of CD34 and CD133 in HSPCs. If so, our study aimed at the importance of soluble compounds of the IGF system on the migration of these HSPCs.

Materials and methods

Isolation, ex vivo expansion and sorting of HSPCs. Human HSPCs were isolated from buffy coat peripheral blood (BCPB) cells, which were obtained from volunteer-healthy blood donors. All samples were processed within 24 h of collection. The investigation conforms to the principles outlined in the Declaration of Helsinki and the Guiding Principles in the care and use of human tissue or subjects. The studies were approved by the local ethics committee of the Martin Luther University Halle-Wittenberg, Germany. Firstly, mononuclear cells were isolated from BCPB using standard density centrifugation through Ficoll (Lymphosep, D=1.077, C.C.pro GmbH, Oberdorla, Germany). The mononuclear cell fraction was enriched for $CD34^+$ cells using the magnetic micro-bead-conjugated anti-CD34 (Miltenyi Biotech, Bergisch Gladbach, Germany) with subsequent separation by the mini-magnetic activated cell sorter system (MACS, Miltenyi Biotech). The MACS provided an 85-95% pure $CD34^+$ cell fraction as evaluated by antibody staining with the fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (BD Biosciences, Heidelberg, Germany) and subsequent flow cytometry using the FACSCalibur and CellQuest Pro software (BD Biosciences). In addition, cells were stained for CD133 using the phycoerythrin (PE)-conjugated anti-CD133 (anti-AC133/2; Miltenyi Biotech). Antibody staining was carried out for 30 min at 4˚C in the dark, and isotype-matched antibodies served as controls.

Ex vivo expansion of $CD34^+$ cell fraction was performed according to a previously described protocol for long-term culture of HSPCs (31,32). In short, MACS-isolated $CD34^+$ cells were seeded at 1-2x10^5 cells per well in 12-well cell culture dishes containing 2 ml of Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, CA, USA). IMDM was supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT, USA), 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 100 ng/ml recombinant (rh) stem cell factor (SCF), 100 ng/ml rh FMS-like tyrosine kinase 3 ligand (Flt-3L), 10 ng/ml rh interleukin (IL)-6, and 10 ng/ml rh thrombopoietin (TPO) (all cytokines were from CellGenix, Freiburg, Germany). When not specified the cells were always cultured under these conditions. Cell cultures were maintained in a humidified atmosphere at 37˚C and 5% CO_2. The cell number was estimated after counting the total number of living cells using the Casy® cell counter (Schärfe System GmbH, Reutlingen, Germany).

After 7 days in culture, cells were collected, washed with phosphate-buffered saline (PBS) and stained with FITC-conjugated anti-CD34 and PE-conjugated anti-CD133. The different subsets of cells were sorted into IMDM by fluorescence activated cell sorting (FACS) using the FACSVantage flow cytometer (BD Biosciences). The purity of the sorted cell fractions was ~95%. After FACS-mediated cell separation, HSPCs expressing CD34 and/or CD133 were washed in PBS, resuspended in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and subjected to further analysis.

Cells and tissues from human lung. As human lung cells we studied National Cancer Institute (NCI) lung epithelial cancer cell lines (H358, H322) and fetal lung fibroblasts from the Wistar Institute (WI-38) (ATCC cell bank, Manassas, VA, USA). All cell types were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 10% CO_2 and 37˚C. In addition, we studied human lung specimens from tumor and paired non-tumor tissue of 16 patients with non-small cell lung carcinoma (NSCLC), who underwent pulmonary resection surgery. The local ethics committee of the Martin Luther University Halle-Wittenberg, Germany approved this study and the subjects gave informed consent.

In vitro cell migration. The migration of HSPCs expressing CD34 and/or CD133 was studied by use of the transwell system according to a previously described protocol (33). The migration system consisted of either 3- or 8-μm pore membrane cell culture inserts placed in a 12-well cell culture plate (ThinCerts, Greiner Bio-One, Frickenhausen, Germany). HSPCs (10,000) in DMEM were seeded into the pore membrane insert and allowed for transwell migration into the bottom chamber of the 12-well cell culture plate, which either contained compounds of the IGF system or co-cultured human lung cells.

For transwell migration in response to compounds of the IGF system, we used rh IGF-I and several rh IGFBPs (R&D Systems, Wiesbaden, Germany). According to the range of concentration in human plasma or serum (8,34,35), 100-500 ng/ml rh IGF-I, 100-900 ng/ml rh IGFBP-2, 2,000-3,000 ng/ml rh IGFBP-3, 250-750 ng/ml rh IGFBP-4 or 100-500 ng/ml rh IGFBP-6 were applied. Because these rh IGF compounds are stored in 0.1% bovine serum albumin (BSA) solution, each control experiment was performed with the same amount of BSA. For migration in response to co-cultured human lung cells, H358, H322 or WI-38 cells were seeded before into the bottom chamber of the 12-well cell culture plate and allowed to proliferate in DMEM containing 10% FCS until they reached cell confluence. To create cell culture medium saturated only with soluble factors from human lung cells, DMEM with 10% FCS was replaced for DMEM without FCS 24 h (in the case of 2 h experiments) or 2 h prior to starting the migration (in the case of 24 h experiments).

At the end of each experiment, non-migrated cells were removed from the insert by a pad of cotton wool. The insert was washed several times in PBS, the migrated cells were fixed on the pore membrane with methanol and fluorescence labeled by DNA staining with 1 μg/ml 4',6-diamidino-2-phenyl-indol-dihydrochloride (DAPI) in PBS (Sigma, Taufkirchen, Germany). After washing the insert with PBS, the pore membrane was removed from the insert, transferred to a glass slide and embedded with Glycergel® mounting medium (Dako, Cambridgeshire, UK).

Table I. Human primers for mRNA expression analysis by PCR.

Name	Gene bank entry	5'-3' Primer sequence		Primer annealing	PCR cycles	PCR fragment
		Sense	Antisense	(˚C)	(n)	(bp)
IGF-I	M29644	CAG CAG TCT TCC AAC CCA AT	TCC TGC ACT CCC TCT ACT TG	58	36	429
IGF-II	NM_000612	CGG CTT CTA CTT CAG CAG	TTG GAA GAA CTT GCC CAC	55	36	202
IGFBP-1	NM_000596	GAA AGC CCA GAG AGC ACG GAG ATA	CCT CTT CCC ATT CCA AGG GTA GAC	58	36	351
IGFBP-2	NM_000597	CAC GTG GAC AGC ACC ATG AAC ATG	GTA GAA GAG ATG ACA CTC GGG GTC	58	38	447
IGFBP-3	M31159	TCC AGG AAA TGC TAG TGA GTC GGA G	CTT GCT CTG CAT GCT GTA GCA GTG C	58	33	475
IGFBP-4	NM_001552	AGC ACT TCG CCA AAA TTC GAG	CTC ACT CTC GAA AGC TGT CAG	58	38	357
IGFBP-5	NM000599	TCT CTG CAC CTG AGA TGA GAC	CAA CGT TGC TGC TGT CGA AGG	55	36	282
IGFBP-6	NM_002178	GTG TCC AAG ACA CTG AGA TGG	CCT CTA TCC CCC AGC TTT AGC	55	36	279
IGF-IR	NM_000875	CAC GAG GCT GAG AAG CT	AGG CAT ACA GCA CTC CA	50	35	501
IGF-IIR	NM_000876	GAG TGG CTG ATG GAA GAG AT	GGA GTC AGA TGT GTA AGA GG	55	36	379
18S rRNA	M10098	GTT GGT GGA GCG ATT TGT CTG G	AGG GCA GGG ACT TAA TCA ACG C	60	17	345

The number of HSPCs, which migrated into the pores of the transwell membrane, was estimated according to the DAPI fluorescence by using the Zeiss Axiovert microscope (Zeiss, Jena, Germany) equipped with a Spot Camera and Metamorph 4.6.5. software (Visitron Systems, Puchheim, Germany). The number of HSPCs, which migrated across the transwell pore membrane into the bottom chamber, was estimated by flow cytometry using the FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences).

RNA isolation and reverse-transcription (RT). Total RNA of HSPCs was isolated according to the protocol of the RNeasy Micro Kit (Qiagen, Hilden, Germany) and total RNA of lung cells was isolated according to the protocol of the RNeasy Mini Kit (Qiagen). Isolated RNA was controlled for integrity by agarose gel-electrophoresis and the concentration was calculated from the absorption at 260 nm using the NanoDrop® ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). Thereafter, 200 ng RNA was reverse transcribed into cDNA with 50 ng random primers (Promega, Mannheim, Germany) and 100 units Superscript II™ reverse transcriptase (Invitrogen).

Polymerase chain reaction (PCR) and oligonucleotide microarray analysis. PCR was established for cDNA amplification of compounds of the IGF system. One tenth volume of the cDNA reaction was used for PCR containing 2xPCR mix (Promega) and 5 pmol of each gene-specific primer pair (Table I). After PCR amplification in a thermocycler (Biometra, Göttingen, Germany) and subsequent agarose gel-electrophoresis, PCR products were estimated by use of the LAS 3000 computer-based imaging system (FujiFilm, Tokyo, Japan) and AIDA 3.5 software (Raytest, Straubenhardt, Germany). Finally, cDNA amplification of compounds of the IGF system was normalized per amplification of 18S rRNA.

In addition, mRNA expression analysis of HSPCs was performed with high-density oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA). Biotinylated target cRNA was prepared from 5 μg total RNA as described (36) and then hybridized to the microarrays according to Affymetrix GeneChip technology (37). Microarrays were washed and stained with the GeneChip Fluidics Station 400. The intensity of hybridization signals was scanned with the GeneArray Scanner 7G and analyzed by using the GeneChip® Operating System (Affymetrix). In the case of several hybridization signals per gene the signal intensities were averaged.

Statistical analysis. Student's t-test was used for statistical comparison of two groups and the one-way ANOVA procedure was applied for multiple comparisons (Microcal™ Origin® 6.0, Microcal Software Inc., Northampton, MA, USA). All data are reported as mean ±SD (n as given), and P-values <0.05 were accepted as a significant difference in mean values.

Results

Characterization of HSPCs expressing CD34 and/or CD133. Human HSPCs from peripheral blood were isolated according to their CD34 expression. Subsequent flow cytometry analyses showed that these $CD34^+$ cells also express CD133 (Fig. 1, top left). In the presence of FCS, SCF and other cytokines (IL-6, TPO, Flt-3L) the $CD34^+/CD133^+$ cells partially develop into cells either expressing CD34 or CD133 after cell cultivation

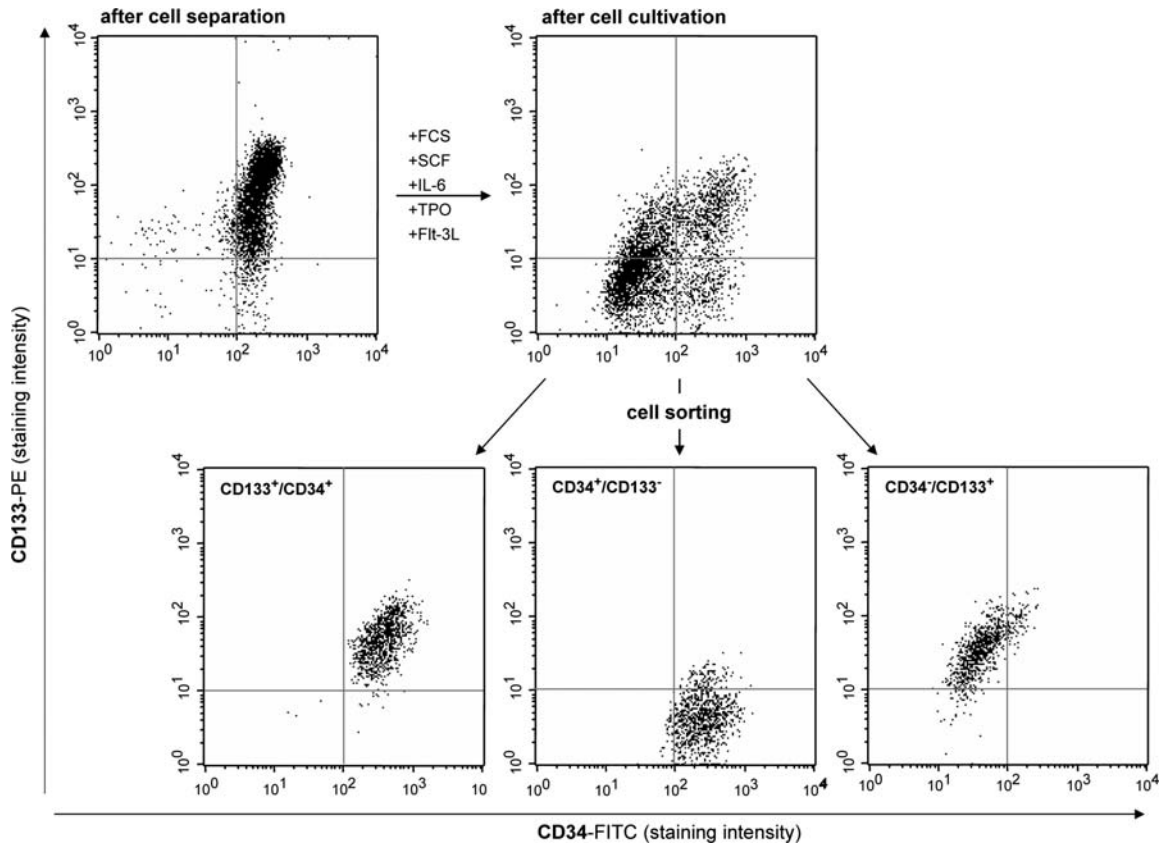


Figure 1. Flow cytometry detection of human HSPCs following antibody staining for CD34 and CD133 after the MACS-mediated cell isolation, after cell cultivation for 7 days, and after FACS-mediated cell separation.

for 7 days (Fig. 1, top right). According to their expression of CD34 and/or CD133 the HSPCs were separated by FACS method into three cell fractions ($CD34^+/CD133^+$, $CD34^+/CD133^-$, $CD34^-/CD133^+$) for further analysis (Fig. 1, below). The cell cultivation longer than 10 days resulted in the loss of $CD34^+/CD133^+$ cells (data not shown).

Oligonucleotide microarray analyses revealed a well detectable expression of the type I IGF receptor (IGF-IR) in each HSPC fraction, whereas the expression level of IGF-IIR and both IGF peptides (-I and -II) was less detectable (Table II). The microarray data were confirmed by RT-PCR analysis and by use of individual cell samples from other donors (data not shown).

Thereafter, we established the transwell migration assay for two different pore sizes of the transwell membrane (8-μm pores, i.e. less selective; 3-μm pores, i.e. more selective). To avoid the influence of growth factors and additional cytokines present in the cell culture medium, the migration of HSPCs was analyzed under FCS- and cytokine-free conditions at the earliest possible time. Thus, we exclusively analyzed the number of cells being migrated into the pores of the transwell membrane but not yet across the membrane (33). After preliminary tests we found an optimal migration time of 2 h for the 8-μm pores and 24 h for the 3-μm pores. Subsequent analysis revealed no differences in the basal migration into the pores of the transwell membrane between the three fractions of HSPCs (Table II).

Epithelial cells co-exist with other types of cells such as fibroblasts in the normal and cancer tissues. When indirectly co-culturing each fraction of HSPCs with human lung epithelial cancer cell lines (H358, H322) or human primary lung fibroblasts (WI-38) for 2 h the $CD34^-/CD133^+$ cells but not the $CD34^+/CD133^+$ or $CD34^+/CD133^-$ cells increased their transwell migration towards the co-cultured lung cells (8-μm pores of the transwell membrane; Fig. 2). The migration of $CD34^-/CD133^+$ cells towards the co-cultured lung cells was even more

Table II. Characteristics of human HSPCs expressing CD34 and/or CD133.

	$CD34^+/CD133^+$	$CD34^+/CD133^-$	$CD34^-/CD133^+$
IGF-R level (average signal intensity)[a]			
IGF-IR	495.4	310.0	370.7
IGF-IIR	142.2	94.8	163.2
IGF level (average signal intensity)[a]			
IGF-I	10.3	39.5	65.0
IGF-II	13.9	12.7	13.2
Basal migration (% cells)[b]			
into 8-μm pores, 2 h	4.51±1.07	5.22±2.71	4.99±0.47
into 3-μm pores, 24 h	not determined	2.70±046	2.77±0.73

[a]Analyzed by Affymetrix oligonucleotide microarray (sample mix of n=5 donors); [b]Mean data of n=7 cell cultivations.

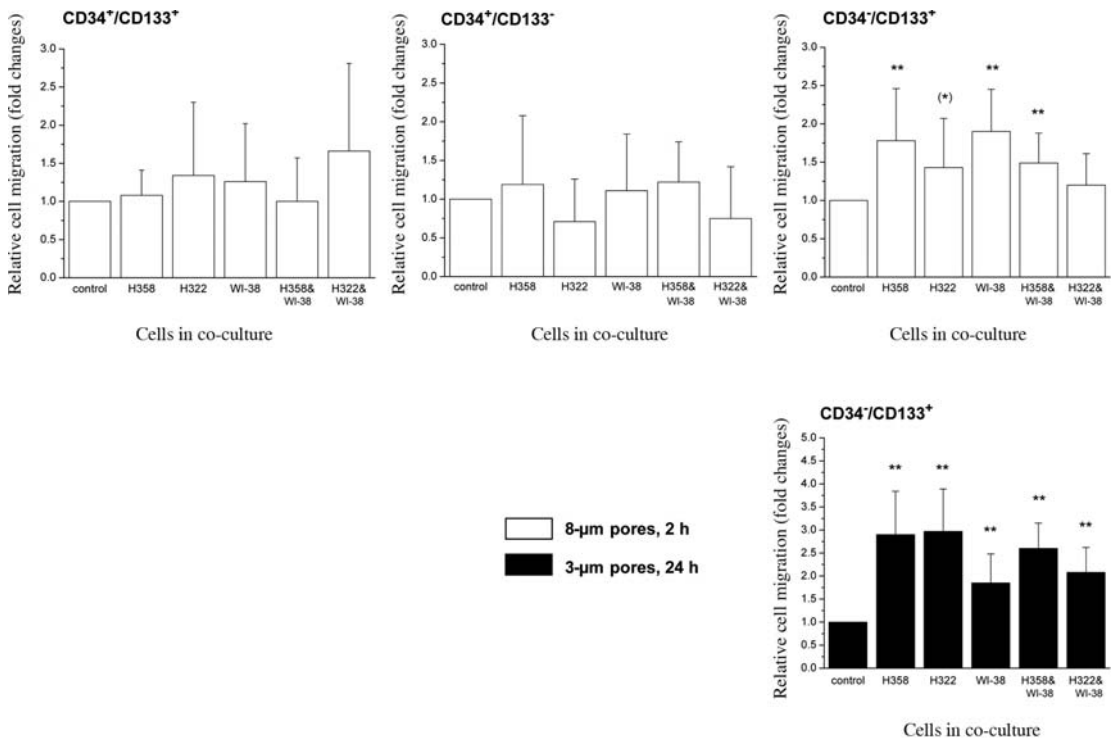


Figure 2. Migration of human HSPCs expressing CD34 and/or CD133 in the presence of different human lung cells, which were indirectly co-cultured in the bottom chamber of the transwell migration system. The cell migration into 8-μm pores of the transwell membrane was analyzed after 2 h and into 3-μm pores after 24 h. The relative migration was evaluated per control without co-cultured cells. Data are given as mean ±SD (n≥4) with $^{(*)}$P≤0.1, *P≤0.05 and **P≤0.01.

Table III. mRNA expression of compounds of the IGF system in human lung cells.

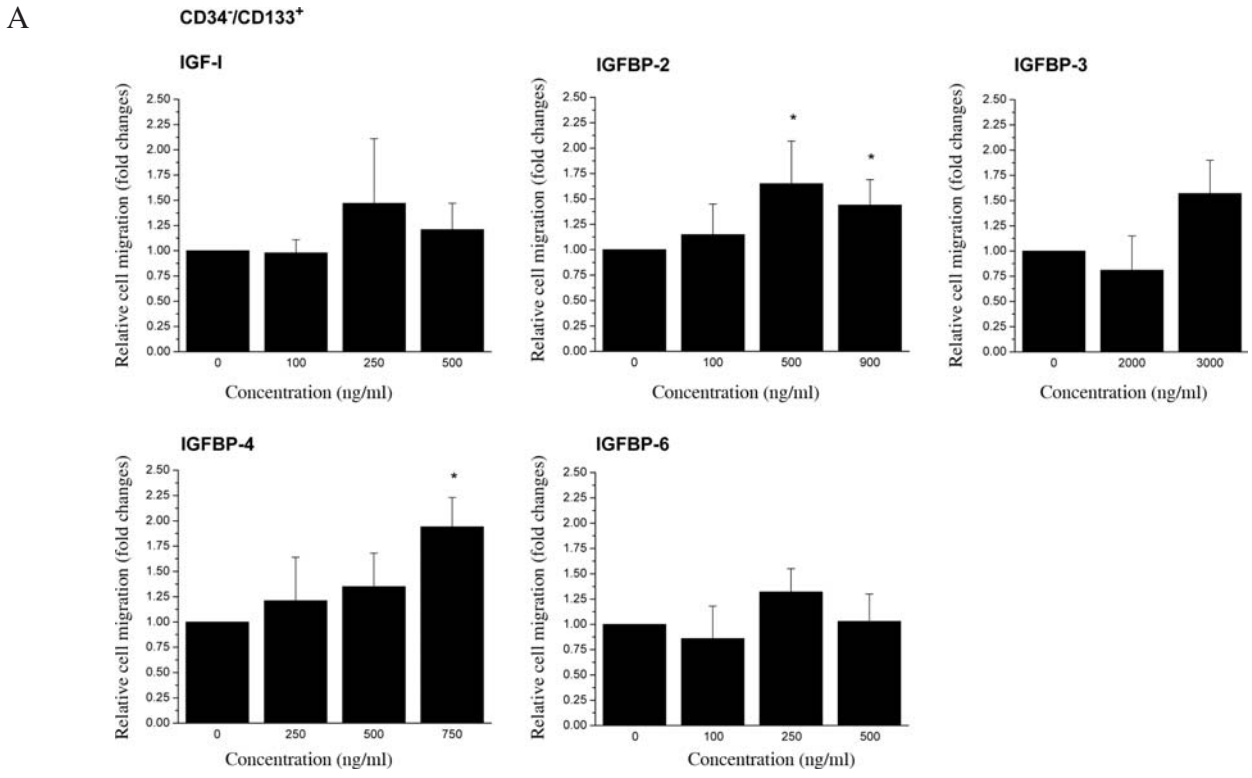
Name	Lung tissues[a]		Lung epithelial cell lines[b]		Primary lung fibroblasts[b]
	Normal	Carcinoma	H358	H322	WI-38
IGF-I	1.90	2.37	0.02±0.02	0.06±0.05	0.27±0.14
IGF-II	0.17	0.29	0.10±0.05	0.17±0.12	0.03±0.02
IGFBP-1	0.01	0.04	0.02±0.01	0.02±0.02	0.05±0.05
IGFBP-2	1.02	2.09	1.43±0.65	1.47±0.66	0.92±0.41
IGFBP-3	0.15	0.42	0.02±0.02	0.03±0.03	1.07±0.75
IGFBP-4	1.25	1.50	0.36±0.35	0.54±0.49	1.32±1.14
IGFBP-5	1.11	1.14	0.96±0.34	0.85±0.46	1.29±0.31
IGFBP-6	0.22	0.22	0.28±0.25	0.89±0.88	1.54±0.71

Level of the mRNA expression was analyzed by RT-PCR and normalized per level of 18S rRNA for inter-sample correction. [a]Mean data of a mixture of n=16 tissues; [b]mean data ±SD of n=3 cell cultivations.

increased in the case of 3-μm pores of the transwell membrane and 24 h of co-culture (Fig. 2). Co-culture experiments using a mixture of the lung epithelial cancer cell lines and lung fibroblasts (H358 and WI-38, H322 and WI-38) did not indicate changes in the migration of the CD34$^-$/CD133$^+$ cells compared with co-culture experiments using each lung cell type alone (Fig. 2).

Influence of compounds of the IGF system on the migration of CD34$^-$/CD133$^+$ HSPCs. The co-culture experiments suggested the stimulating effect of soluble factors released from human lung epithelial cancer cells and fibroblasts on the migration of human CD34$^-$/CD133$^+$ HSPCs. Therefore, we analyzed the expression of soluble compounds of the IGF system in human lung cells and tissues. RT-PCR analyses showed a well detectable expression of both types of IGFs (-I and -II) and several IGFBPs (-2 to -6) in human normal lung and lung carcinoma tissues (Table III). In contrast, the expression of IGF-I was less detectable in human lung epithelial cancer cell lines (H358, H322) and WI-38 primary lung fibroblasts (Table III). The IGFBP-2, -4, -5 and -6 were expressed in both lung epithelial cell lines and WI-38 lung fibroblasts except for IGFBP-1 (Table III). The expression of IGFBP-3 was mainly detected in WI-38 fibroblasts (Table III).

On the basis of the expression data, we selected recombinant factors for IGF-I and the IGFBP-2, -3, -4 and -6 to study the individual importance of these soluble compounds of the IGF system on the migration of CD34$^-$/CD133$^+$ cells. The applied



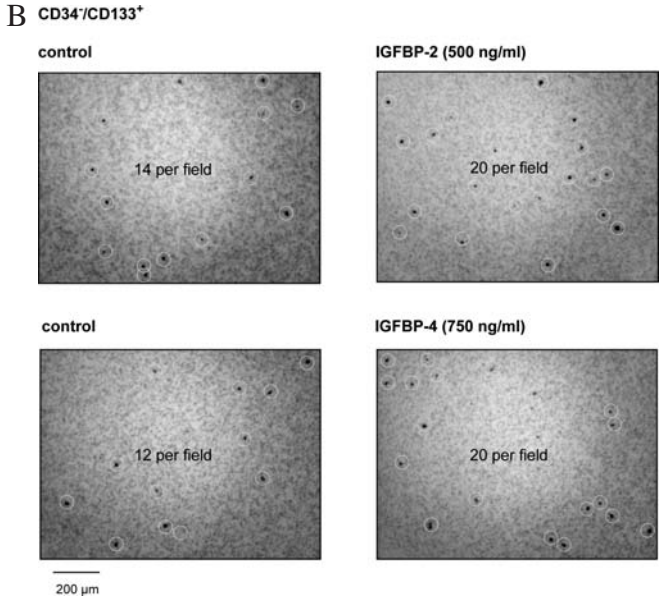


Figure 3. Transwell migration of human CD34-/CD133+ HSPCs in the presence of IGF-I or several IGFBPs. The cell migration into 3-μm pores of the transwell membrane was analyzed after 24 h. (A) The relative migration was evaluated per internal control for each experiment. Each control contained the same amount of BSA as included in the IGF-I/IGFBP solutions. Data are given as mean ±SD (n=3) with $^{*}P \leq 0.05$. (B) Inverted photos of the fluorescence microscopy show examples of the migration of CD34-/CD133+ cells (labeled by DAPI staining of nuclear DNA) into the 3-μm pores of the transwell membrane.

concentrations of each factor were chosen according to the concentration range detected in human plasma or serum (8,34,35). For these experiments we used the transwell membrane with 3-μm pores for 24 h because of the more selective effect on cell migration in co-culture as compared to experiments with 8-μm pores membrane for 2 h (Fig. 2). As demonstrated in Fig. 3A and B, we found an increased migration of CD34-/CD133+ cells in the presence of IGFBP-2 or IGFBP-4. The pro-migratory effect of both IGFBPs was determined at a higher concentration of IGFBP-2 (500-900 ng/ml) and IGFBP-4 (750 ng/ml) respectively. In contrast, IGF-I and the IGFBP-3 and -6 had no influence on the migration of CD34-/CD133+ cells at the given concentrations (Fig. 3A).

We also studied the individual effect of IGF-I, IGFBP-2 or -4 on the migration of CD34+/CD133- cells, which did not increase their cell migration in response to co-cultured lung cells (Fig. 2). In accordance with the co-culture experiments, the migration of CD34+/CD133- cells into the 3-μm pores of the transwell membrane was not influenced by any of these soluble compounds of the IGF system (data not shown).

Discussion

Hematopoietic stem and progenitor cells (HSPCs) are a source for endothelial progenitor cells (EPCs) expressing the surface molecules CD34 and/or CD133 (38,39), which contribute to the progression of non-small cell lung carcinoma (NSCLC) (19-21). Our study showed that soluble factors released from human lung epithelial cancer cells and fibroblasts enhance the migration of human HSPCs depending on the expression of

CD34 and CD133 in HSPCs. The pro-migratory effect of lung epithelial cancer cells has been demonstrated for two types of NSCLC cell lines, H358 and H322 (40), which only enhanced the migration of $CD34^-/CD133^+$ HSPCs but not $CD34^+/CD133^+$ and $CD34^+/CD133^-$ cells.

Although the identification of stem/progenitor cells by detection of surface molecules is debated, $CD34^+/CD133^+$ and $CD34^+/CD133^-$ cells from peripheral blood are primarily suggested to be EPCs (38,39). Because EPCs contribute to tumor neoangiogenesis (17,18), we primarily expected an enhanced migration of $CD34^+/CD133^+$ and/or $CD34^+/CD133^-$ cells in the presence of lung epithelial cancer cells. However, our study found a primary effect of lung epithelial cancer cells on the migration of $CD34^-/CD133^+$ HSPCs that indicates a preferred involvement of this type of HSPCs in cancer. The additional effect of primary fibroblasts from human lung (WI-38) on the migration of $CD34^-/CD133^+$ cells corresponds to the stimulating effect of tumor-associated fibroblasts on the migration of immune cells into the tumor tissue (41). Lung epithelial cancer cells and fibroblasts might influence each other in the tumor tissue thereby enhancing or abolishing the migration of $CD34^-/CD133^+$ cells, but our studies did not find a co-stimulating effect of either type of lung cells.

Collectively, our findings give a partial explanation for the immunohistochemical detection of $CD34^-/CD133^+$ cells in tumor tissues of NSCLC patients (42). Although the immunohistochemistry of NSCLC tissues cannot clarify the origin of $CD34^-/CD133^+$ cells, further immunohistochemical studies detected CD133-expressing cells in the NSCLC (20,21,43). These studies often detected CD133-expressing cells at the site of NSCLC neovascularization (20,21,43). In this context, $CD34^-/CD133^+$ HSPCs have been observed to mediate a better endothelialization following vascular injury than $CD34^+/CD133^+$ cells do (30). This observation at least indirectly indicates the particular capability of $CD34^-/CD133^+$ cells for migration to sites of action.

The three populations of HSPCs ($CD34^+/CD133^+$, $CD34^+/CD133^-$, $CD34^-/CD133^+$) generated by the *ex vivo* expansion system of $CD34^+/CD133^+$ cells from peripheral blood (31,32) did not exactly correspond to those existing *in vivo*. However, some cytokines used in this *ex vivo* expansion system are also elevated in the blood of NSCLC patients, such as SCF (44) and IL-6 (45), that indicates their potential importance in tumor progression. In addition to circulating cytokines in the peripheral blood, both lung epithelial cancer cells and fibroblasts express a variety of soluble compounds that might act locally. Among these we identified the expression of soluble compounds of the IGF system.

The multifunctional peptide IGF-I was strongly expressed in NSCLC tissues but not in the lung carcinoma cell lines (H322, H358) and WI-38 lung fibroblasts. Although this might also be based on the individual expression of the human lung cells studied, our expression data suggest other cells than lung epithelial cancer cells and fibroblasts as a source of the IGF-I expression in NSCLC. Moreover, we did not find an IGF-I-mediated migration of $CD34^-/CD133^+$ HSPCs despite the type I IGF receptor (IGF-IR) expression in $CD34^-/CD133^+$ cells. The IGF-I-mediated migration of $CD34^-/CD133^+$ cells due to a strong basal expression of IGF-I by the cells themselves can also be excluded, because $CD34^-/CD133^+$ cells express IGF-I at a marginal level only. Despite the fact that we cannot absolutely exclude the sequestration of few IGF-I molecules by any IGFBP, our findings are in contrast to other types of cells including mature endothelial cells (46,47). However, we observed an enhanced migration of $CD34^-/CD133^+$ HSPCs in the presence of the IGFBP-2 and -4. Because the expression levels of IGF-I and IGF-II are marginal in $CD34^-/CD133^+$ cells and the migration of $CD34^-/CD133^+$ cells does not respond to IGF-I, our observations strongly support the IGF-independent action of certain IGFBPs.

The IGF-independent stimulation of cell migration by IGFBP-2 has been described previously for cancer cells (48). It is suggested that the IGFBP-2-mediated cell migration is induced following binding of IGFBP-2 to $\alpha_5ß_1$ integrin via its carboxy-terminal RGD (Gly-Arg-Asp) peptide motif (48). The greatest effect of IGFBP-2 on the migration of $CD34^-/CD133^+$ cells we observed at a concentration of 500 μg/ml that corresponds to the blood level in a healthy human (8,34). Although IGFBP-2 was also well expressed in the lung cancer cells and tissues of our and other studies (49,50), nothing is known about the local concentration of IGFBP-2 in tumor tissues. In contrast to IGFBP-2, our experimental study is the first to determine an IGF-independent stimulating effect of IGFBP-4 on the cell migration. An IGFBP-induced cell migration independent of IGFs has also been described for the IGFBP-6 in cancer cells (51). Although lung epithelial cancer cells and fibroblasts express IGFBP-6, the IGFBP-6 did not influence the migration of $CD34^-/CD133^+$ cells. The IGFBP-3, which is the major IGFBP in peripheral blood (8,35), had no influence on the migration of $CD34^-/CD133^+$ HSPCs. In contrast to $CD34^-/CD133^+$ cells, the migration of $CD34^+/CD133^-$ HSPCs was not influenced by the IGFBP-2 and -4. These findings again support the fact that $CD34^+/CD133^-$ cells are not influenced by soluble factors released from lung epithelial cancer cells or fibroblasts.

Targeted cell migration is a critical mechanism for the specific recruitment of cells at the site of action. Our study suggests that cancer and stromal cells contribute to the recruitment of $CD34^+/CD133^+$ cells into the tumor tissue. Moreover, our study suggests that the recruitment of $CD34^-/CD133^+$ cells can be mediated, at least in part, by the IGF-independent action of the IGFBP-2 and -4.

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