Chemokine Receptors and Chemokine Production by CD34+ Stem Cell-derived Monocytes in Response to Cancer Cells

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Abstract. *Background: The chemokine-chemokine receptor (CR) network is involved in the regulation of cellular infiltration of tumours. Cancer cells and infiltrating macrophages produce a whole range of chemokines. This study explored the expression of some CR and chemokine production by cord blood stem cell-derived $CD34^+$ monocytes and their novel $CD14^{++}CD16^+$ and $CD14^+CD16^-$ subsets in response to tumour cells. Material and Methods: CR expression was determined by flow cytometry and their functional activity by migration to chemoattractants. Monocytes were cultured with tumour cells and the chemokine content was assessed in culture supernatants. Results: $CD14^{++}CD16^+$ monocytes exhibited increased expression of chemokine (C-C) receptor (CCR) 1, while $CD14^+CD16^-$ of CCR2, chemokine (C-X-C) receptor (CXCR) 1, 2 and 4. The increased expression of CCR2 on $CD14^+CD16^-$ monocytes was associated with their enhanced migration to monocyte chemoattractant protein–1 (CCL2), MCP-3 (CCL7), MCP-2 (CCL8) and MCP-4 (CCL13), while that of CXCR1 and 2 to interleukin 8 (CXCL8), and CXCR4 to stromal cell-derived factor-1 (CXCL12). Tumour cells induced production of macrophage inflammatory protein-1α (CCL3) MIP-1β and regulated on activation normal T-cells expressed and secreted (CCL5) but not CCL2 or CXCL8, monokine induced by gamma interferon (CXCL9), interferon gamma-induced protein 10 (CXCL10). Conclusion: The studied monocyte subsets, in comparison to those from blood, exhibit different expression of CRs and response to the stimuli that occur from tumour cells.*

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Key Words: Cord blood, $CD34^+$ haematopoietic stem cells, monocyte subpopulations, chemokines.

The chemokine–chemokine receptor (CR) network is involved in the regulation of leukocyte infiltration of tumours. Leukocytes, including monocytes, migrate to the tumour *via* the gradient of chemokines that are produced by tumour and stromal cells, including monocyte-derived macrophages (1-4). Several chemokines are found in the tumour microenvironment and are involved in the regulation of tumour-infiltrating macrophages (TIMs), by controlling their directed migration to the tumour and inhibiting their egress by regulation of angiogenesis and immune response to tumour cells and by controlling tumour cell movements, which is the first step in local tumour spread and metastasis (1). Chemokines that are found within the tumour microenvironment include: monocyte chemoattractant protein-1 (CCL2), macrophage inflammatory protein 1α (CCL3) and 1β (CCL4), regulated on activation normal T-cells expressed and secreted (CCL5), MCP-2 (CCL8), interleukin 8 (CXCL8), monokine induced by gamma interferon (CXCL9), interferon gamma induced protein 10 (CXCL10), stromal cell-derived factor-1 (CXCL12) and many others (1, 3, 4). In various types of tumors, production of a different range of chemokines occurs and there is no selectivity in their release, either by tumour cells or by TIMs. Many CRs are abundantly expressed on cancer cells and TIMs (1). CCR1 is mainly expressed on TIMs, while CCR7 is expressed on tumour cells (5). From this brief description, it becomes clear that very sophisticated chemokine–CR interactions occur in the tumours, which both foster and inhibit tumour growth and which are central to the regulation of immune response to cancer cells (1, 6). In tumours, the situation is further complicated by polarization of TIMs from M1 to M2 mononuclear phagocytes which exhibit different expressions of CR and production of chemokines (7).

During interactions with tumour cells and their tumour-derived microvesicles (TMVs), peripheral blood (PB) monocytes, which are precursors of TIMs, produce several chemokines (8). Two major subpopulations of PB monocytes: $CD14^{++}CD16^-$ (classical monocytes) and $CD14^+CD16^{++}$ (non-classical monocytes) (9) exhibit different expression of CRs (10).

We have previously described the protocol for the generation of monocytes from cord blood (CB) haematopoietic $CD34^+$ progenitors and provided evidence that these monocytes and their novel $CD14^{++}CD16^+$ and $CD14^+CD16^-$ subsets differ phenotypically and functionally from blood monocytes (11). On this background we wished to investigate the expression and function of CRs and the production of chemokines by CB $CD34^+$ stem cell-derived monocytes and their subsets. Stimulation with cancer cells was used to mimic the tumour microenvironment.

Materials and Methods

Isolation and culture of $CD34^+$ cells. $CD34^+$ cells were isolated using the EasySep Human CD34 Positive Selection Kit (StemCell Technologies, Vancouver, Canada) based on magnetic cell sorting from CB mononuclear cells obtained by standard density-gradient centrifugation. $CD34^+$ cells were expanded and differentiated to monocytes in two-step cultures in 24-well plates (Sarstedt, Numbrecht, Germany), as previously described (11). Firstly, the cells were expanded for 7-10 days by culturing 1×10^5 $CD34^+$ cells/well/ml in the expansion medium: X-VIVO 10 medium (BioWhittaker, Verviers, Belgium) supplemented with 4% of foetal bovine serum (FBS; Gibco, Paisley, UK) and recombinant human stem cell factor (SCF, 50 ng/ml), thrombopoietin (TPO, 15 ng/ml), interleukin (IL)-3 (30 ng/ml) and Fms-related tyrosine kinase 3 ligand (FLT-3L, 30 ng/ml), all from PeproTech (London, UK). Then cells were harvested and seeded at 1×10^5/ml in the differentiation medium: Iscove's Modified Dulbecco's Medium (IMDM; Gibco) with 20% of FBS and SCF (25 ng/ml), macrophage colony-stimulating factor (M-CSF; 30 ng/ml, PeproTech), IL-3 (30 ng/ml) and FLT-3L (30 ng/ml), and cultured for 7-10 days.

Isolation of monocyte subpopulations. Cells cultured in the differentiation medium were harvested, washed, and suspended at a density of 10×10^6/ml in phosphate buffered saline (PBS; Gibco). After staining with anti-CD14 allophycocyanin (APC) and anti-CD16 phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs; both from BD Pharmingen San Diego, CA, USA), cells were sorted into $CD14^+$ (total monocytes), $CD14^{++}CD16^+$ and $CD14^+CD16^-$ populations in a fluorescence-activated cell sorter FACS Aria II (BD Biosciences, Immunocytometry Systems, San Jose, CA, USA), using a 100 μm nozzle tip. Sorted cells were collected into polystyrene Falcon 2057 tubes (BD Biosciences) pre-coated with FBS to avoid plastic charging and cell attachment to the wall.

Analysis of CR expression. The expression of CRs on monocytes was performed by three-colour flow cytometric analysis after staining with the following PE-conjugated (BD Pharmingen) mAbs against: chemokine (C-C) receptor (CCR) 1, 3, 5 and 7; chemokine (C-X-C) receptor (CXCR) 1, 2 and 4; and PE-conjugated mAb to CCR2 (R&D, Minneapolis, MN, USA), allophycocyanin (APC)-conjugated anti-CD14 and PE-Cyanine 5 (PE-Cy5)-conjugated anti-CD16. In parallel, staining with appropriate isotype-matched mouse immunoglobulins were used as negative controls. After incubation for 30 min at 4˚C with mAb or isotype controls,monocytes were washed, re-suspended in 0.3 ml of phosphate buffred saline (PBS) containing 0.1% sodium azide and analysed by flow cytometry (FACS Canto; BD Biosciences) using the FACS Diva software. List mode data for 20,000 events were acquired and statistical analysis was performed according to green, orange or red fluorescence of cells stained with isotype controls.

Migration to tumour-derived chemoattractants. Costar Transwell 24-well plates with 8-μm pore-size filter (Costar Corning, Cambridge, MA, USA) were used for chemotactic assays. RPMI-1640 medium (PAA Laboratories, Pasching, Germany) supplemented with 0.5% bovine serum albumin (Sigma, St. Louis, MO, USA) (control) or with the addition of CCL2, 3, 7, 8, 13, 19 or CXCL8 or 12, (all at 100 ng/ml; PeproTech) in a final volume of 650 μl was added to the lower chamber. Monocytes ($CD14^+$) and subpopulations ($CD14^{++}CD16^+$ and $CD14^+CD16^-$) were placed in the upper chamber (1×10^5/100 μl). As controls, 2×10^4 cells (*i.e.* 20% of these in the upper chamber) were put into the lower chamber. After 4 h, the cells in the lower chamber of the Transwell were harvested and their number was determined by flow cytometry. The cells were gated according to their forward scatter (FSC)/side scatter (SSC) parameters and counted during 20 s acquisition time at a high-flow rate. Data are expressed as the percentage of migrating cells according to the formula:

$$\text{migrating cells (\%)} = \frac{\text{Number of cells in the test well} \times 20\%}{\text{Number of cells in the control well}}$$

Determination of chemokine production. The total population of $CD14^+$ monocytes and their subpopulations were cultured in flat-bottom 96-microtitre plates (Nunc, Roskilde, Denmark) at 1×10^5/100 μl/well in RPMI-1640 medium with 5% FBS. Cells were cultured alone or were stimulated with human pancreatic carcinoma cells (HPC-4) cultured, as previously described (12), at a ratio of 1:0.3 for 18 h at 37˚C in a humidified atmosphere with 5% CO_2. The concentrations of chemokines (CCL2-5 and CXCL8-10) in the supernatants were measured using the FlexSet system (BD Biosciences) followed by flow cytometric analysis (FACS Canto). The FlexSet beads were discriminated in FL-4 and FL-5 channels, while the concentration of specified chemokines was determined by the intensity of FL-2 fluorescence, using the respective standard reference curve and FCAP Array software (BD Biosciences). For all chemokines, the detection level was 10 pg/ml.

Statistical analysis. Non-parametric one-way ANOVA test with the use of Microcal Origin version 5.0 software (Northampton, MA, USA) was used for the analysis. Differences were considered significant at $p<0.05$.

Results

Expression of CRs on monocyte subsets. It is well-established that monocytes arriving at the tumour site interact with tumour-associated chemokines (1) and that blood monocytes differ in the expression of some CRs (10). We studied the expression of some CRs, mostly those which interact with chemokines produced by tumour cells, on monocyte subsets $CD14^{++}CD16^+$ and $CD14^+CD16^-$ generated from CB $CD34^+$ stem cells. Table I shows that CCR3 and 5 and CXCR1 were mostly expressed on the $CD14^{++}CD16^+$ subset, as judged by mean fluorescence intensity (MFI), and CCR1 was mostly expressed on the $CD14^{++}CD16^+$ both as percentage of positive

Table I. *Expression of chemokine receptors on monocyte subsets. Data represent the mean±SD from six independent experiments.*

Receptor	$CD14^{++}CD16^{+}$		$CD14^{+}CD16^{-}$	
	%	MFI	%	MFI
CCR1	14±6[a]	485±226[a]	4.3±2.5	63.5±43.6
CCR2	4.8±2.7	499±193	38.7±28[b]	364±301
CCR3	15±14	567±323[a]	9.7±6.4	86±17
CCR5	3.1±1.7	594±355[a]	1.97±1.2	280±195
CCR7	1.32±1.28	360±284	0.48±0.4	51±3.5
CXCR1	8.3±2.2	256±168[a]	29±8.6[a]	26±9
CXCR2	3.5±1.8	318±173	31±6.4[a]	399±155
CXCR4	6.1±3.7	498±17	68±9.6[a]	1241±262[a]

[a]Significantly different between the subsets at $p<0.05$, [b]at $p=0.055$.

Table II. *Migration of monocytes and their subsets to some chemokines. Data are expressed as the percentage of migrating cells. The mean±SD from four different experiments is shown.*

	$CD14^{+}$	$CD14^{++}CD16^{+}$	$CD14^{+}CD16^{-}$
CCL2	20±8	2.5±2.7	20±17[a]
CCL7	53±30	6±3	51±28[a]
CCL8	12±7	1.9±1.9	31±18[a]
CCL13	54±31	1.5±0.6	46±21[a]
CCL19	5.6±4.7	5.6±4.7	2.3±2.1
CXCL8	29±16	13±5	40±16[a]
CXCL12	7.5±5	19.2±1.4	33.8±8[a]

[a]Significantly different from $CD14^{++}CD16^{+}$ cells.

cells and as MFI. The $CD14^{+}CD16^{-}$ monocytes exhibited a higher number of CCR2-, CXCR1-, 2-, and 4-expressing cells. CCR7 was not detected on either subpopulation.

Migration to tumour cell-derived chemokines. It is well-known that PB monocytes are attracted to the tumour site by chemokines, and are kept there by chemotactic inhibitory proteins that are produced by tumour or inflammatory cells in the local microenvironment. Therefore, the migration of monocytes to some products of cancer cells was studied (Table II). $CD14^{+}$ cells exhibited a range of degrees of chemotaxis, but migrated to all chemokines. Comparison of both subsets revealed significantly higher migration of $CD14^{+}CD16^{-}$ cells to CCL2, 7, 8, 13 and CXCL8 and 12, which are produced by cancer cells (1). Chemotaxis of $CD14^{++}CD16^{+}$ cells was lower than that of the total population of $CD14^{+}$ cells.

Production of chemokines. The $CD14^{+}$ monocytes (total population) and their subpopulations were cultured for 18 h in medium, or stimulated with HPC-4 tumour cells and the chemokine content in the culture supernatants was determined. No induction of expression of CCL2 or CXCL8-10 by tumour cells was observed (data not shown). No difference in the spontaneous production of CCL3 and -5 between the subsets was observed, but CCL4 release by $CD14^{+}CD16^{-}$ cells was lower. Tumour cells stimulated CCL3-5 production by $CD14^{+}$ monocytes and their subsets, but there was no difference between them (Figure 1A-C).

Discussion

It is well-established that the chemokine-CR network plays an important role in cancer, infection and inflammation (1, 4). In the present study, the CR pattern and chemokine production exhibited by novel monocyte subsets $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{-}$ generated from CB $CD34^{+}$ haematopoietic stem cells, were determined. As it is unestablished whether the number of positive cells or the MFI is important, and to our knowledge, there is a difference between these parameters within a given cell population, no clear conclusions about the receptor expression can be made. Therefore, we presented both the percentages of CR-expressing cells and the MFI. This study showed that $CD14^{++}CD16^{+}$ cells were characterized by an enhanced expression of CCR1 (percentage of positive cells and MFI) and CCR3 and 5 and CXCR1 (MFI only). The $CD14^{+}CD16^{-}$ subset contained an increased percentage of CCR2-, CXCR1-, 2- and 4-positive cells. CCR7 was not expressed by either subset, although it is known that it is abundantly expressed on polarized M1 macrophages (7). Our findings provide further evidence that these monocyte subsets are clearly different from that of PB monocytes, as CCR2 is mostly expressed on blood $CD14^{++}CD16^{-}$ cells and CCR5 on $CD14^{+}CD16^{++}$ monocytes, with equivalency of CCR1 expression on both subsets (10). The only consistently expressed CCR on TIMs is CCR1 in ovarian cancer (1, 5), but it is unknown whether it is limited to a particular macrophage subset. It is also unknown whether monocytes/macrophages are generated *in situ* from $CD34^{+}$ progenitors. Furthermore, there is a dynamic variation in the cycling of haematopoietic stem cells in steady-state and inflammation (17).

CR expression is a pre-requisite for recruitment of blood monocytes to the tumour and inflammatory sites and is governed by a whole range of chemokines, mostly by CCL2 (1, 10), but also by CCL12, which is produced by cancer cells (1). Hence, CXCR4 expression is important for migration of monocytes to the tumour site. Furthermore, the CXCR4-CXCL12 axis is also involved in tumour progression, angiogenesis and metastasis (14). CCR1 and CXCR2 may also be involved as their ligands CCL1, 3 and 5, and CXCL8, respectively, are abundantly produced by cancer and inflammatory cells (4, 7, 15). Therefore, the expression of

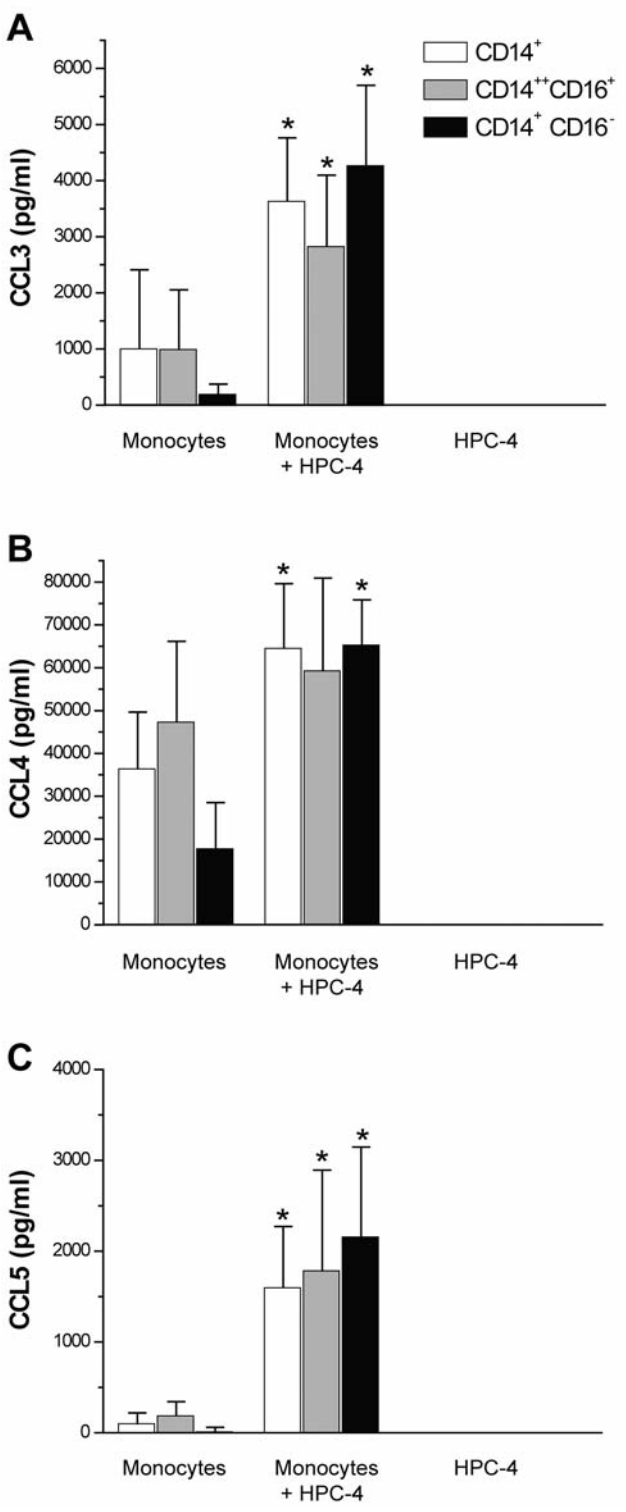

Figure 1. *Production of chemokines by monocytes and their subpopulations following stimulation with tumour cells: Chemokine CCL3 (A), CCL4 (B), CCL5 (C). Chemokine concentrations in the culture supernatants were analysed using BD™ FlexSet System. Data from five independent experiments are shown and expressed as the mean±SD. *Significantly different from unstimulated monocytes.*

relevant CRs is crucial for interactions with appropriate ligands. For migration studies the chemokines that are produced by tumour cells (CCL2, 7, 8, 13 and 19, and CXCL8 and 12) were chosen. The increased percentage of CCR2-, and CXCR1-, 2- and 4-positive cells within the $CD14^{+}CD16^{-}$ population was in keeping with the enhanced migration of this subset to the appropriate ligands. Thus, expression of CCL2, 7, 8 and 13 which are ligands of CCR2, CXCL8, ligand of CXCR1 and 2 and CXCR4, ligand of CXCL12, were observed. It indicates that these CRs are of functional relevance. There was no difference between the subsets in the migration to CCL19, for which CCR7 receptor was virtually absent and chemotaxis was very low. However, it should be noted that CRs are not always selective for a given chemokine, and more than one CR may exist for several chemokines. Thus, CCR1 interacts with CCL2, 3, 5 and 7, and CCR5 with CCL3, 4 and 5, while CXCL8 with both CXCR1 and 2 (16). Surprisingly, $CD14^{++}CD16^{+}$ cells which exhibited an increased expression (MFI) of CCR1, 3 and 5, and CXCR1, did not migrate to CCL2, 7, 8, 13 and 19. There are two possible explanations for this. Either MFI determination is not a good marker for the chemotactic ability of cells, or simply, as these $CD14^{++}CD16^{+}$ monocytes are larger (11) and more adherent to human umbilical vein endothelial cells (unpublished observation), their movement is limited. Hence, according to our data, the number of CR-positive cells, rather than the magnitude of their expression, as defined by MFI, seems to be the decisive factor for the chemotactic ability of a given cell population. Based on the functional data, we wish to suggest that $CD14^{++}CD16^{+}$ monocytes are characterized by enhanced CCR2 expression, while $CD14^{+}CD16^{-}$ cells by increased CCR2, and CXCR1, 2 and 4. CRs and their chemotactic ligands play a number of roles in several steps of tumour metastasis, including adherence of cancer cells to vascular endothelium, extravasation from blood vessels, metastatic colonization, angiogenesis and proliferation (17). Therefore, these observations, indicating differences in expression of CRs on the studied monocytes subsets, may further support the existence of complicated chemokine-CR networks operating in cancer.

As stated above, chemokines are produced at inflammatory and tumour sites, not only by cancer cells but also by TIMs. Hence, to mimic this situation we also studied the production of chemokines by monocytes stimulated with tumour cells by which they are affected *in situ*. Tumour cells induced the production of CCL3, 4 and 5. A whole spectrum of chemokines is found in the tumour microenvironment and play different roles in cancer (1). At the tumour site, TIMs are the major producers of CCL3, 4 and 5 (1). At the invasive site, macrophages enhance tumour cell migration and invasion through their secretion of chemotactic factors (18). It becomes clear that several chemokines produced within tumour may attract blood monocytes that give rise to TIMs.

Recently, it has been demonstrated that solid head and neck cancer is infiltrated by CD34+ stem cells and that when these are isolated from CB, following stimulation with these cancer cells, the CD34+ cells produced IL-6, known to exert pro- and anti-inflammatory activities (19). Since stem cells can multiply into different types of cell (20), the generation of monocytes or TIMs *in situ* cannot be excluded. The present study may provide some insight into the behavior of stem cell-derived monocytes/TIMs within the tumour.

Acknowledgements

This study was supported by the Ministry of Science and Higher Education (grants no. N402 110 32/3560 and N N401 052538). We wish to thank Ms I. Ruggiero for excellent technical support.

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Received July 20, 2012
Revised September 26, 2012
Accepted October 1, 2012