Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: A mechanism for cellular human immunodeficiency virus 1 infection

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The release of microparticles from eukaryotic cells is a well-recognized phenomenon. We demonstrate here that the chemokine receptor CCR5, the principal co-receptor for macrophage-tropic human immunodeficiency virus (HIV)-1, can be released through microparticles from the surface of CCR5$^+$ Chinese hamster ovary cells and peripheral blood mononuclear cells. Microparticles containing CCR5 can transfer the receptor to CCR5$^-$ cells and render them CCR5$^+$. The CCR5 transfer to CCR5-deficient peripheral blood mononuclear cells homozygous for a 32-base-pair deletion in the CCR5 gene enabled infection of these cells with macrophage-tropic HIV-1. In monocytes, the transfer of CCR5 could be inhibited by cytochalasin D, and transferred CCR5 could be downmodulated by chemokines. A transfer of CCR5 from peripheral blood mononuclear cells to endothelial cells during transendothelial migration could be demonstrated. Thus, the transfer of CCR5 may lead to infection of tissues without endogenous CCR5 expression. Moreover, the intercellular transfer of membrane proteins by microparticles might have broader consequences for intercellular communication beyond the effects seen for HIV-1.

To efficiently infect target cells, human immunodeficiency virus (HIV)-1 requires CD4 together with a specific chemokine co-receptor[1]. *In vivo*, the chemokine receptor CCR5 is central to the transmission and propagation of HIV-1 (ref. 2) and is the principal co-receptor for macrophage-tropic (M-tropic) HIV-1 strains. The essential involvement of CCR5 in HIV infection is supported by the observation that individuals homozygous for a 32-base-pair deletion in the CCR5 gene (Δ32/Δ32) that disrupts expression of CCR5 are very resistant to HIV infection. Apart from T cells and macrophages, other cell types, such as endothelial cells[3], astrocytes[4,5,6] and possibly renal cells[7], seem to be infected with M-tropic HIV-1 and may be important for the chronicity of the infection[6,8,9]. Although T cells and macrophages express CCR5, there is still debate about expression of CCR5 by endothelial cells[10,11], astrocytes[12,13] and renal cells[11]. It remains uncertain if, and how, cells that do not express CCR5 could be infected by M-tropic HIV-1. Here we studied the possibility of a temporary transfer of HIV co-receptors from one cell to another. A growing body of evidence has described the release of microparticles from a variety of cell types including lymphocytes and monocytes. We sought to determine whether CCR5 could be released from the cell surface as microparticles and subsequently be transferred to other cells. CCR5 microparticles might thereby enable infection of cells with M-tropic HIV strains in the absence of endogenous CCR5 expression.

Microparticles are small membrane vesicles released from the plasma membrane of various cell types[14,15], and contain cell surface proteins and cytoplasmic components of the original cell[16]. Such microparticles, which typically range in size from 0.1 to 2 μm (ref. 17), are able to interact with other cell types and with extracellular matrix proteins. The release of microvesicles was first described from platelets after activation by diverse stimuli including thrombin, collagen and shear stress[18]. These microparticles can activate platelets and endothelial cells through the transcellular delivery of arachidonic acid and increase the binding of monocytes to endothelial cells by upregulation of adhesion molecules[19,20]. Microparticles derived from B lymphocytes and dendritic cells have the capacity to present antigens and to induce antigen-specific T-cell responses[21,22]. Release of microparticles has also been demonstrated in granulocytes[23], T cells, monocytes and endothelial cells[24,25]. The release of microparticles from eucaryotic cells seems to be part of a normal cellular function[15], but also is involved in apoptosis and altered cellular viability[19,24]. Moreover, gradient-enriched HIV-1 preparations from infected peripheral blood mononuclear cells (PBMCs) and H9 cells contain many (50–75%) membrane vesicles derived from the infected cells[26]. Budding of virus and the release of microparticles co-localized at distinct regions of the cell surface.

Here we show that various cell types, including PBMCs and stably transfected Chinese hamster ovary (CHO) cells, released microparticles that contained large amounts of CCR5. These microparticles were taken up by other CCR5$^-$ cells, leading to a cel-

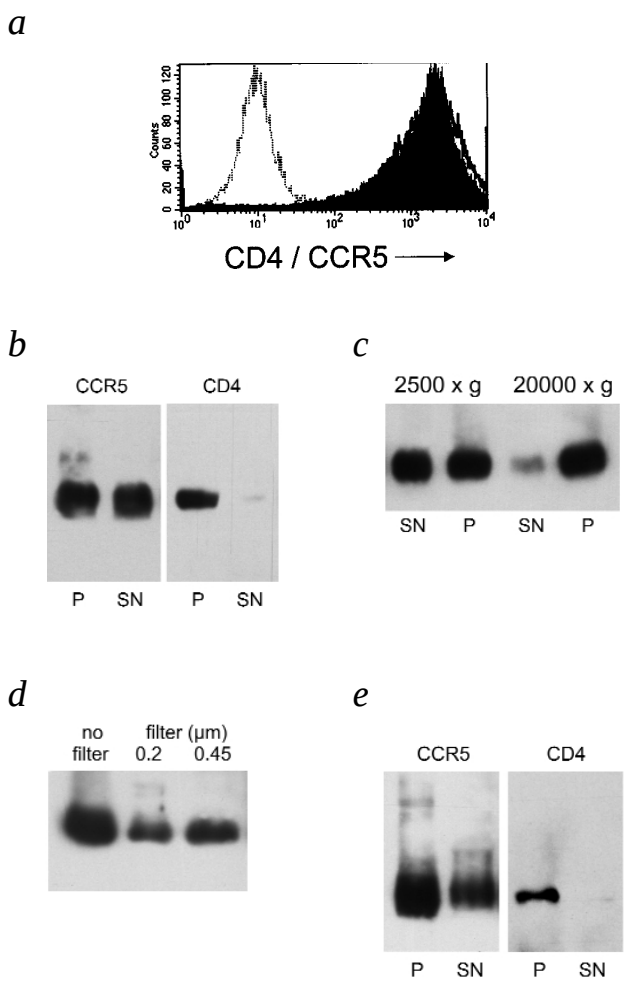

Fig. 1 Release of CCR5$^+$ microparticles from stably transfected CHO cells and PBMCs. ***a***, FACS analysis showing equal expression of CCR5 (thick line) and CD4 (shaded area) on stably transfected CHO cells. Dotted line, staining with an isotype control antibody. ***b***–***e***, Detection of CCR5 and CD4 by western blot analysis in the cell pellet (P) and cell-free supernate (SN) of CHO cells and PBMCs. CCR5$^+$ CD4$^+$ CHO cells (*b*) and PBMCs (*e*) release considerable amounts of CCR5 into the supernate, whereas CD4 is mainly found in the cell pellet and shows little signal in the supernate. *c*, Centrifugation of cell-free supernate from CCR5$^+$ CHO cells demonstrates that the CCR5$^+$ microparticles are partially pelleted by centrifugation at 2,500*g* and are almost completely removed from the supernate after centrifugation at 20,000*g*. *d*, CCR5$^+$ microparticles from the supernate of CHO cells are partially retained by 0.45-µm and 0.2-µm filters.

lular transfer of the chemokine receptor CCR5. Transcellularly delivered CCR5 was downmodulated by its ligands from the surface of monocytes. Moreover, CCR5 was transferred from PBMCs to endothelial cells during transendothelial migration. Finally, CCR5 transfer by microparticles from CCR5$^+$ cells to CCR5$^-$ PBMCs rendered the latter susceptible to infection by M-tropic HIV-1, a mechanism that may account for HIV-infection of CCR5$^-$ cells *in vivo*.

Detection of CCR5 in the supernate of CHO cells and PBMCs

CHO cells stably transfected with human CCR5 and CD4 expressed similar amounts of both molecules, as verified by fluorescence-activated cell sorting (FACS) analysis (Fig. 1*a*). To test for the potential release of CCR5-containing microvesicles, we incubated 1×10^6 CHO cells in PBS for 1 hour at 37 °C. After centrifugation of the samples for 10 minutes at 500*g*, we recovered the cell-free supernate and resuspended the cell pellet in the same volume of PBS. We assessed equal amounts of supernate and resuspended cells by western blot analysis using a previously characterized monoclonal antibody against CCR5 (ref. 11). The supernate from transfected CHO cells contained a substantial amount of CCR5, similar to results obtained with the cell pellet (Fig. 1*b*). In contrast to CCR5, there was little or no CD4 in the supernate from the doubly transfected CHO cells, whereas CD4 was easily detectable in the cell pellet. The differences between cellular shedding of CCR5 and CD4 challenge the idea of a nonspecific release of membrane vesicles or the presence of cell debris in the supernate.

Serial centrifugation (Fig. 1*c*) showed that the CCR5-containing microparticles were partially pelleted from the supernatant fluid after centrifugation at 2,500*g* and almost completely appeared in the pellet after centrifugation at 20,000*g*. To further purify and characterize the microparticles, we then filtered the 500*g* supernate through sterile filters with pores 0.45 and 0.2 µm in size (Fig. 1*d*). Although most of the CCR5$^+$ vesicles passed through the 0.45-µm filter, there was considerable loss of the CCR5 signal after filtration through the 0.2-µm filter. CCR5-containing microparticles were also generated when adherent CHO cells were incubated with culture medium (data not shown). This challenges the possibility that the release of microparticles was due to the detachment of CHO cells from the culture flask by EDTA.

To determine whether the release of CCR5 containing microparticles is a unique feature of CHO cells, we repeated the experiments with a CCR5-transfected rat cell line (rat basophilic leukemia cells) and primary PBMCs. Both cell types released substantial amounts of CCR5 into their supernates. Like CCR5$^+$CD4$^+$ CHO cells, PBMCs released microparticles that contained CCR5 but lacked CD4 (Fig. 1*e*). Electron microscopy showed that the CHO-cell-derived microparticles were small membrane vesicles with a size ranging from 0.2 µm to 1 µm (not shown), which is consistent with the microparticles described before[17] and with our data obtained by filtration through 0.45- and 0.2-µm filters.

Transfer of CCR5$^+$ microparticles to CHO cells and PBMCs

The first indication that CCR5 could be transferred from one cell to another came from the co-incubation of CCR5$^-$ CHO cells with CCR5$^+$ CHO cells. After a short period of co-incubation (15–30 minutes), distinct staining for CCR5 was detectable on most previously CCR5$^-$ cells. Without such co-incubation, there was no binding of CCR5 antibodies (Fig. 2*a*). The intercellular transfer of CCR5 was even greater when CCR5$^-$ PBMCs (Δ32/Δ32) were co-incubated with CCR5$^+$ CHO cells (Fig. 2*b*). As demonstrated by FACS analysis, T cells and monocytes took up CCR5. Co-incubation of CCR5$^-$ PBMCs with CXCR4-containing CHO cells did not result in binding of CCR5 antibodies. The uptake of CCR5 was more substantial in CD4$^+$ T cells than in CD8$^+$ T cells. We obtained essentially identical results with Δ32/Δ32 PBMCs from two additional donors (14.6% and 9.4 % CCR5$^+$CD4$^+$ T cells and 3.7% and 5.6 % CCR5$^+$CD8$^+$ T cells). An association of CCR5 and CD4 has been described[27], which may explain the preferential uptake of CCR5 vesicles by CD4$^+$ T cells and monocytes. In an attempt to block the interaction of CCR5 with membrane-bound CD4, we added 5 µg/ml soluble CD4 to a transfer experiment. Soluble CD4 did not diminish the transfer of CCR5 to

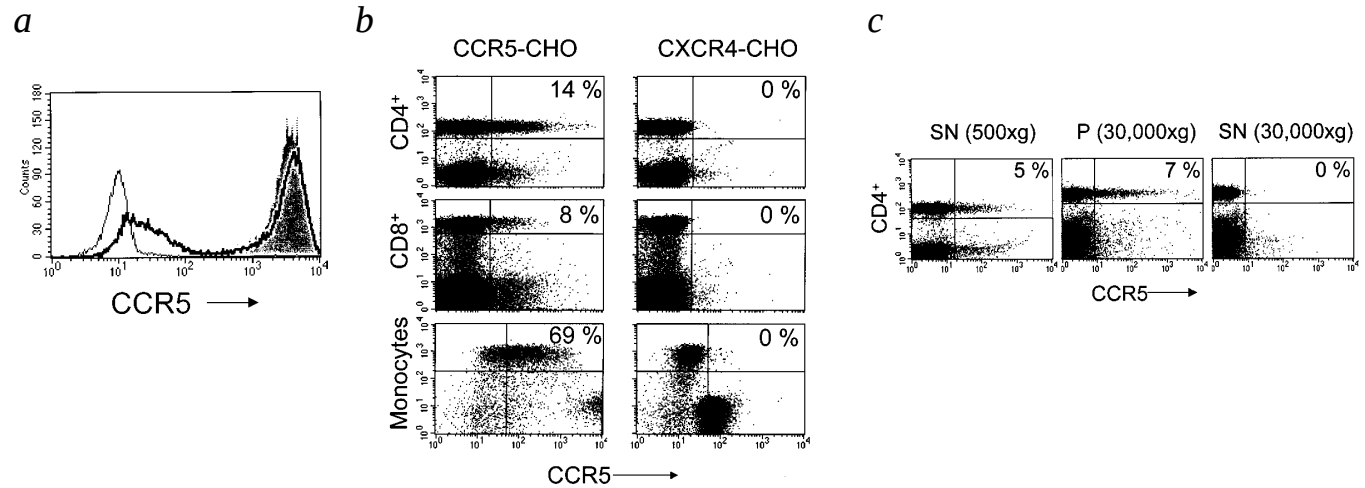

Fig. 2 Transfer of CCR5 from CHO cells to other CCR5⁻ cells. ***a***, CCR5⁺ CHO cells and CCR5⁻ CHO cells were incubated together (thick line) or separately (light or dark shaded areas for CCR5⁻ or CCR5⁺ CHO cells, respectively) and analyzed for the presence of CCR5 by FACS. Transfer of CCR5 to the previously CCR5⁻ CHO cells is evident. ***b***, Co-incubation of CCR5⁻ Δ32/Δ32 PBMCs with CCR5⁺ CHO cells (left) also results in a transfer of CCR5 to T cells and monocytes. Vertical lines, cutoffs according to a staining with an isotype control antibody. Co-incubation with CXCR4⁺ CHO cells (right), negative control. ***c***, Cell-free supernate from adherent CCR5⁺ CHO cells (left) is able to transfer CCR5 to CD4⁺ T cells. Further centrifugation of the supernate shows that the CCR5 containing microparticles are completely pelleted by centrifugation at 30,000*g*. Only the resuspended 30,000*g* pellet can transfer CCR5 (middle); the remaining supernate cannot (right). Numbers in the upper right quadrants, fraction of CCR5⁺ CD4⁺ T cells, CD8⁺ T cells or monocytes.

PBMCs in these experimental conditions (data not shown).

To determine it transfer of CCR5 depends on a close cell-to-cell contact between CHO cells and PBMCs, we repeated the experiments using PBMCs and cell-free supernate from CCR5⁺ CHO cells adhering to the culture flask (Fig. 2*c*). This supernate was also able to deliver CCR5 to PBMCs, although with a lower efficiency. When the supernate was further centrifuged at 30,000*g*, transfer of CCR5 was only possible with the resuspended 30,000*g* pellet and not with the remaining supernate, indicating that most CCR5 microparticles were pelleted by centrifugation at 30,000*g* (Fig. 2*c*).

To determine if the transferred CCR5 could be functionally active, we examined the internalization of the transferred CCR5 receptors after incubation with the chemokines RANTES (regulated upon activation, normal T-cell-expressed and secreted) and amino-oxypentane (AOP)-RANTES. CCR5 is rapidly internalized after exposure to these ligands[28]. Unexpectedly, the internalization of CCR5 occurred only with monocytes, whereas there was little change with CD4⁺ T cells (Fig. 3). This may indicate that on CD4⁺ T cells, the transferred CCR5 receptor is not fully integrated into the plasma membrane or is not connected to the intracellular machinery necessary for internalization.

As phagocytosis may be involved in the transfer of CCR5, we pre-incubated the 'acceptor' PBMCs with cytochalasin D, an inhibitor of actin polymerization and phagocytosis (Fig. 4). After pre-incubation with cytochalasin D, monocytes took up substantially less CCR5, whereas the transfer of CCR5 to T cells was not decreased. The reduction of CCR5 transfer to monocytes by cytochalasin D correlates with their ability to downmodulate CCR5 after incubation with chemokines. Thus, endocytosis of CCR5-containing vesicles may facilitate the correct insertion of CCR5 into the cell membrane of monocytes.

Transfer of CCR5 from PBMCs to endothelial cells

As demonstrated above, PBMCs released CCR5 microparticles from their cell surface (Fig. 1*e*). To study the transfer of CCR5 from PBMCs to other cells, we used the experimental model of transendothelial migration. This model, which mimics events

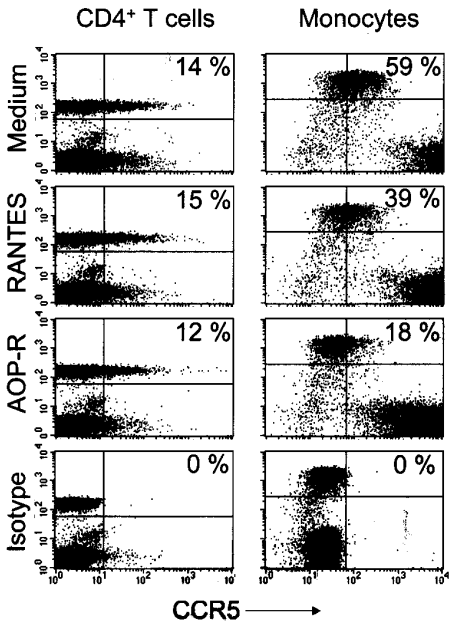

Fig. 3 Transferred CCR5 can be downmodulated from the surface of monocytes (right), whereas there is little downmodulation on CD4⁺ T cells (left). To quantify internalization of CCR5, we incubated the cells with medium or the CCR5 ligands RANTES and AOP-RANTES (AOP-R). Staining with isotype control antibody (bottom), negative control. Numbers in the upper right quadrants, fraction of CCR5⁺ CD4⁺ T cells or monocytes.

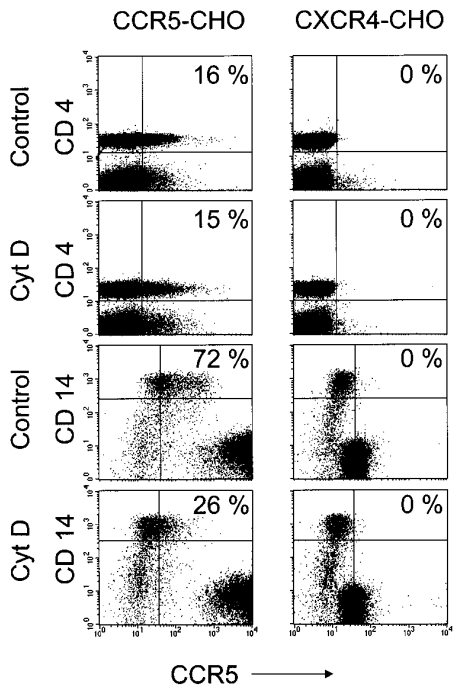

Fig. 4 Effect of cytochalasin D on the transfer of CCR5 to monocytes and CD4+ T cells. CCR5− Δ32/Δ32 PBMCs were pre-incubated with cytochalasin D (Cyt D) or dimethylsulfoxide (Control) and then co-incubated with CCR5+ CHO cells (left) or CXCR4+ CHO cells (right). Cytochalasin D substantially decreases the uptake of CCR5 by monocytes but has no effect on the CCR5 transfer to T cells. Numbers in the upper right quadrants, fraction of CCR5+ CD4+ T cells or monocytes..

during leukocyte recruitment into tissues, requires close contact between PBMCs and endothelial cells. PBMCs were allowed to transmigrate for 2 hours across a monolayer of interleukin-1-stimulated human primary microvascular endothelial cells, following a RANTES gradient. After migration, the PBMCs were washed away and the endothelial cells were removed from the filters. Flow cytometry demonstrated considerable expression of CCR5 on endothelial cells after the transendothelial migration of PBMCs (Fig. 5*a*), whereas no CCR5 was detected in the absence of PBMCs (Fig. 5*c*). Endothelial cells were distinguished by FACS analysis from residual PBMCs by their light-scattering properties and the absence of leukocyte markers such as CD4, CD8 and CD14. The transfer of CCR5 from PBMCs to endothelial cells was further enhanced when 5 ng/ml tumor necrosis factor α was added into the assay (Fig. 5*b*). These results show that endothelial cells obtain certain surface molecules from PBMCs during migration. This phenomenon might also explain the finding that endothelial cells stain positively for CCR5 at sites of chronic inflammation[10].

Transfer of CCR5 enables infection with M-tropic HIV-1

Using CCR5− Δ32/Δ32 PBMCs, we investigated whether these PBMCs could be infected by the M-tropic HIV-1 strain D117/III after transfer of CCR5 microvesicles from CHO cells. We co-incubated CCR5− Δ32/Δ32 PBMCs with CHO cells that stably express either CCR5 or CXCR4. Both types of CHO cells lack CD4 and could not be infected with several M-tropic and T-cell-tropic (T-tropic) HIV-1 strains (data not shown). After co-incubation, the CHO cells were removed from the PBMCs by adhesion to plastic. This procedure also removed most monocytes. We infected the remaining cells with M-tropic and T-tropic HIV-1 strains and quantified virus production by p24 enzyme-linked immunosorbent assay (ELISA). The M-tropic strain D117/III, which depends on CCR5 as a co-receptor, could not infect CCR5− PBMCs that had previously been incubated with CXCR4-containing CHO cells (Fig. 6). In contrast, the M-tropic virus was able to infect CCR5− PBMCs after incubation with CCR5-containing CHO cells (Fig. 6). The kinetics of virus production were similar to those of wild-type PBMCs that endogenously express CCR5, as both cell types showed an increase in p24 of 1,000% from day 4 to day 9. The lower titers of p24 production in the Δ32/Δ32 PBMCs most likely result from the lower density and frequency of CCR5 on Δ32/Δ32 PBMCs after CCR5 transfer. As further control, we infected Δ32/Δ32 PBMCs and wild-type PBMCs with the T-tropic strain HIV-1_{RF}, which requires CXCR4 as co-receptor. As expected, HIV-1_{RF} infected both types of PBMCs at similar levels, independent of a previous incubation with CCR5+ or CXCR4+ CHO cells (data not shown). These experiments provide evidence that CCR5 molecules that are transferred to CCR5− PBMCs by microparticles are able to function as co-receptors for M-tropic HIV-1, allowing productive infection of these cells.

Discussion

Our results have shown that CCR5 was released from the surface of various cell types as microparticles and transferred to previously CCR5− cells. We also provided evidence that transfer of CCR5 occurred from PBMCs onto endothelial cells during transendothelial migration. The transfer of CCR5 to CCR5-deficient Δ32/Δ32 PBMCs then allowed infection of these cells by M-tropic HIV-1. *In vivo*, the transfer of CCR5 may be involved in inflammation and may provide a mechanism of HIV infection of normally CCR5− cells. Beyond HIV, the transfer of receptors through microparticles may represent a hitherto unrecognized

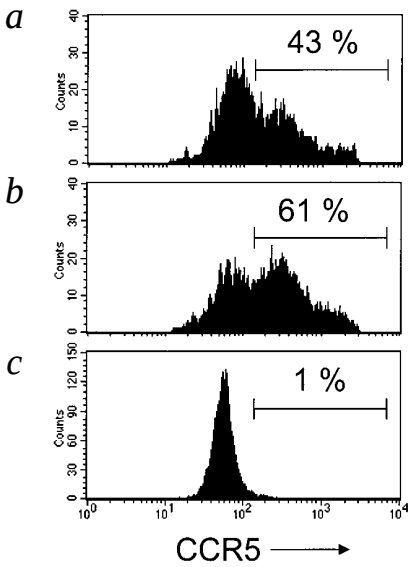

Fig. 5 During transendothelial migration, CCR5 is transferred from PBMCs to endothelial cells. After transmigration of PBMCs, 43% of endothelial cells stain positively for CCR5 ***a***, whereas in the absence of PBMCs no expression of CCR5 is detectable on endothelial cells ***c***. Tumor necrosis factor α (5 ng/ml) further enhances the transfer of CCR5 to endothelial cells ***b***.

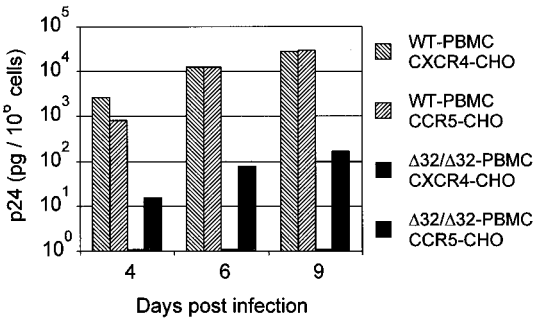

Fig. 6 Transferred CCR5 can function as co-receptor for M-tropic HIV-1 on Δ32/Δ32 PBMCs. Wild-type (WT) PBMCs expressing CCR5 and Δ32/Δ32 PBMCs lacking endogenous CCR5 were co-incubated with CCR5 or CXCR4$^+$ CHO cells. Whereas wild-type PBMCs could be infected independently of a previous incubation with CHO cells, the Δ32/Δ32 PBMCs could only be infected after co-incubation with CCR5$^+$ but not CXCR4$^+$ CHO cells.

mechanism of cell–cell interaction.

It is well established that various cell types, including leukocytes and endothelial cells release small vesicles (microparticles) from their plasma membrane[21–24]. The release of microparticles from eucaryotic cells can occur independently of apoptosis or cell death and seems to be part of a normal cellular function[15]. Until recently, little data were available that indicated a transfer of such vesicles or receptors to other cells. Transfer of arachidonic acid from platelet derived microparticles to other platelets and endothelial cells has been described[19]. In an early report, the adherence of platelet-derived microparticles to monocytes was found to be responsible for 'false-positive' results in flow cytometry[29].

The binding of microparticles to other cells may result in their integration into the cell membrane or could merely represent superficial adhesion of these particles to the cell surface. We therefore used cytochalasin D, an inhibitor of actin polymerization and phagocytosis, to examine its effect on CCR5 transfer by microparticles. Cytochalasin D substantially reduced the uptake of CCR5 by monocytes, but had no effect on the CCR5 transfer to T cells (Fig. 4). This would indicate that incorporation of CCR5 microparticles into monocytes involves an active process that can be inhibited by cytochalasin D, whereas in T cells the microparticles are only passively adsorbed to the cell surface. Complete and active integration of vesicles into the cell membrane of monocytes could lead to a 'correct' insertion of CCR5 into the cell membrane and would enable functional responses like receptor internalization by CCR5 ligands such as RANTES or AOP-RANTES (ref. 28). Indeed, we found substantial ligand-induced downmodulation of transferred CCR5 from the surface of monocytes, supporting the idea of functional integration of CCR5. AOP-RANTES was much more potent than RANTES in this, which resembles the degree of internalization of endogenously expressed receptors by the two ligands[28]. Unexpectedly, there was no downmodulation of CCR5 in the CD4$^+$ T cells. Thus, monocytes and T cells differ in their acceptance of CCR5 microparticles both in terms of their inhibition by cytochalasin D and in terms of the ability to downmodulate the transferred receptor. These differences may relate to different mechanisms of microvesicle incorporation by T cells and monocytes, resulting in different connections to the cytoskeletal apparatus and hence endocytosis.

The potential pathophysiological consequences of microvesicular translocation was emphasized by the substantial transfer of CCR5 from PBMCs to endothelial cells during transendothelial migration. These results indicate that microparticles released from PBMCs are efficiently taken up by endothelial cells during the process of transmigration. Tumor necrosis factor α further increased the transfer of CCR5 from PBMCs to endothelial cells. Activation of monocytes by lipopolysaccharide induces a shedding of microparticles[30]. Tumor necrosis factor α may act in a similar way and result in an enhancement of vesicle formation by monocytes and T cells. In cell culture conditions, endothelial cells lack CCR5. However, *in vivo*, CCR5 is present on endothelial cells during chronic inflammation, where many CCR5$^+$ T cells and monocytes are present[10]. Transcellular delivery of CCR5 to endothelial cells during extravasation of PBMCs could explain the positive staining for CCR5 on endothelium at sites of chronic inflammation. The uptake of CCR5 might then enable endothelial cells to specifically bind and potentially react to chemokine stimulation.

CD4 and CCR5 do not necessarily need to be expressed on the same cell to enable infection with M-tropic HIV-1 strains[31]. The co-incubation of CD4$^+$ cells with CCR5$^+$ cells resulted in a productive infection with HIV; it was concluded that CD4 could also fulfill its receptor function when expressed on separate cells[31]. Given our results, the possibility of a cellular transfer of CCR5 must also be considered. Both mechanisms might enable infection of cells that lack endogenous expression of CCR5 and CD4.

Infection of Δ32/Δ32 PBMCs with M-tropic HIV-1 required pre-incubation of PBMCs with the CCR5$^+$ CHO cells. The CHO cells were subsequently removed by three rounds of adhesion to plastic to prevent overgrowth of the culture. The adhesion also removed most monocytes/macrophages, indicating that the HIV detected was produced by the remaining lymphocytes. Although the transferred CCR5 was able to mediate productive infection of T cells, the receptor could not be downmodulated by chemokines. This is in accordance with reports showing independence of CCR5 signaling and co-receptor function[32–35].

The infection of cells with HIV-1 can be greatly increased at sites of inflammation, especially in the brain and possibly in the kidney[7]. The degree of AIDS dementia correlates with the extent of monocyte/macrophage infiltration into the central nervous system[36]. Once infection has been initiated, ongoing inflammation as well as virally derived proteins such as Tat increase the influx of leukocytes into the effected tissue[37]. Many of the infiltrating leukocytes could serve as source for CCR5, as during HIV infection the percentage of CCR5$^+$ T lymphocytes is substantially increased[38,39]. Some of the infected cells may continuously produce low levels of HIV-1 without undergoing apoptosis[40] and thus represent a reservoir for HIV. In addition, the transfer of CCR5 might only be temporary, such that CCR5 would no longer be detectable on infected cells. The recently described production of antibodies against CCR5 in Δ32-CCR5-homozygous individuals with multiple exposure to HIV-infected CCR5$^+$ cells also raises the question of whether intact cells or CCR5$^+$ microparticles have been transferred from one individual to another[41].

The vesicular transfer of CCR5 from PBMCs to other cells might have considerable consequences for the distribution of HIV-1 *in vivo* and the infection of CCR5$^-$ cells. Infection of cells, independent of endogenous CCR5 expression, might increase the target cell repertoire of HIV-1 and render it more difficult to completely eradicate HIV-1 by highly active anti-retroviral

therapy. Moreover, the transfer of membrane proteins from one cell type to another might have broad implications beyond HIV-1, as intercellular transfer of integral membrane proteins such as receptors by microparticles would represent a previously unrecognized signal mechanism.

Methods

Cell lines and PBMCs. CHO cells were stably transfected with CCR5 as described[28]. Stable transfection with CD4 was subsequently accomplished using the vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Rat basophilic leukemia cells expressing CCR5 were provided by M. Oppermann[42]. PBMCs were isolated from the buffy coats of blood from healthy donors by ficoll density gradient centrifugation and cultured overnight in RPMI medium containing 10% heat-inactivated FCS.

Generation of microparticles and western blot analysis. CHO cells were removed from the culture flasks with PBS containing 1 mM EDTA and were washed twice in cell culture medium without FCS. After centrifugation, the cell pellet was resuspended in PBS at a density of 1×10^6 cells/100 µl PBS and was incubated for 1 h at 37 °C. Cell-free supernate was obtained by centrifugation at 500*g* for 10 min. The supernate containing the membrane vesicles was carefully recovered and the remaining cell pellet was resuspended in 100 µl PBS. Where indicated, the supernate was further centrifuged at 2,500*g* or 20,000*g* or filtered through sterile filters with pores 0.2 µm or 0.45 µm in size. Equal amounts (20 µl) of supernate and resuspended cells were assessed by western blot analysis. Proteins were separated by 12% SDS–PAGE and blotted on Immobilon P membranes by electrotransfer. Membranes were blocked with 5% low-fat milk and stained with MC-5, a monoclonal antibody against CCR5 (ref. 11) and M-T413, an antibody against CD4 (ref. 43), followed by P260, a peroxidase-labeled rabbit polyclonal antibody against mouse (Dako, Carpinteria, California). Signals were visualized by chemoluminescence according to manufacturer's recommendations (Amersham).

CCR5 transfer and FACS analysis. To allow the transfer of CCR5-containing microparticles from one cell to another, 2×10^6 CCR5-transfected donor CHO cells or supernate from 2×10^6 CCR5-transfected CHO cells were incubated for 1 h at 37 °C with 1×10^6 $CCR5^-$ acceptor cells in culture medium containing 10% FCS. Acceptor cells were either CCR5-Δ32/Δ32 PBMCs or untransfected CHO cells. To block the uptake of $CCR5^+$ microparticles, acceptor cells were pre-incubated for 30 min with 10 µg/ml cytochalasin D or control medium containing 1% dimethylsulfoxide. Where indicated, transferred CCR5 was downmodulated from the cell surface by incubation for 30 min at 37 °C with 1 µg/ml RANTES (provided by A. Proudfoot and T. Wells) or 1 µg/ml AOP-RANTES (synthesized by R. Offord and B. Dufour). For infection of PBMCs with HIV-1, the donor CHO cells were removed by several adhesion steps to plastic.

For FACS-analysis, 1×10^6 cells were incubated for 30 min on ice with a monoclonal antibody against CCR5 (MC-1 or MC-5) or the appropriate isotype controls at a concentration of 10 µg/ml. After two washing steps, cells were incubated with a phycoerythrin-conjugated rabbit polyclonal antibody against mouse $F(ab)_2$ fragment (R439; Dako, Carpinteria, California) followed by 10% mouse serum and a combination of antibodies against CD4 (fluorescein-isothiocyanate-conjugated), CD8 (phycoerythrin-cyanin-5-conjugated) and CD14 (allo-phycocyanin-conjugated) (Beckman Coulter, Fullerton, California). Flow cytometry used a FACSCalibur with CellQuest analysis software (Becton-Dickinson, Heidelberg, Germany). Helper T cells, cytotoxic T cells and monocytes were identified by their light-scattering properties, and the expression of CD4, CD8 or CD14. CCR5 expression was calculated after a cut-off according to the signal obtained with isotype control antibodies was defined.

Transendothelial migration. Primary human microvascular endothelial cells (Promocell, Heidelberg, Germany) were grown to full confluence on gelatin-coated filter inserts 3µm pore size; (13 mm in diameter) in endothelial cell growth medium (Promocell, Heidelberg, Germany) and stimulated for 18 h with serum-free medium (Endothelial SFM; Life Technologies) containing 5 ng/ml interleukin-1β. PBMCs (2×10^6) were washed twice with DMEM (Life Technologies) and added in 500 µl assay medium (50% serum-free medium and 50% DMEM) to the filter inserts, which were then transferred to a 12-well plate containing 1.2 ml assay medium with 100 nM RANTES. After incubation for 120 min, PBMCs in the upper well were washed off and the endothelial cells were removed from the filters with PBS containing 1 mM EDTA and were analyzed by flow cytometry as described above.

HIV infection and p24 ELISA. After co-incubation of CHO cells and PBMCs and removal of CHO cells by adhesion to plastic, the non-adherent cells were resuspended in cell culture supernate containing the M-tropic HIV-1 isolate D117/III (provided by H. v. Briesen, Frankfurt, Germany)[44] or the T-tropic isolate HIV-1_{RF} (ref. 45). After cultivation for 16 h at 37 °C, the non-adherent cells were recovered, washed twice with PBS and resuspended in culture medium containing 100 U/ml interleukin-2 (Sigma). One day later, the cells were washed again and counted. Aliquots of virus containing supernates were collected and replaced by fresh culture medium on days 4, 6 and 9. To determine the level of HIV-1 replication, the amount of HIV-1 p24 antigen was measured by a commercially available ELISA (Beckman Coulter, Fullerton, California). Culture supernates were centrifuged and mixed with 5% Triton X-100 for HIV-1 lysis and were stored at –20 °C until the p24 ELISA was done.

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