Abstract. Background: Tumour-derived microvesicles (TMVs) may interact with cells of the immune system. Our previous observations indicated that TMVs modulate production of cytokines and reactive oxygen species (ROS) by monocytes. This study was designed to determine the role of TMVs in stimulation of chemokine production by human monocytes.

Materials and Methods: Chemokines at the mRNA and protein level were detected by real-time PCR and by Western blot, respectively. Chemokine release and chemotaxis of blood leukocytes were analysed by flow cytometry. Matrigel assay was used to determine angiogenesis in a NOD-SCID mice model. Results: TMVs induced secretion of interleukin-8 (CXCL8), monocyte chemoattractant protein-1 (CCL2), macrophage inflammatory protein-1α (CCL3) and MIP-1β (CCL4), and regulated on activation normal T-cells expressed and secreted (CCL5) chemokines and accumulation of their mRNA in monocytes. Moreover, TMVs enhanced angiogenesis in NOD-SCID mice by delivering chemokines and via stimulation of monocytes. In addition, TMVs may be storage for chemokines thus inducing chemotaxis of blood leukocytes. Conclusion: These results further support the role of TMVs in modulation of monocyte biological activity.

Monocytes are a heterogeneous population of cells with antigen-presenting, phagocytic and antimicrobial activities (1, 2). Their role in cancer is ambiguous as they may produce pro- and anti-tumour factors (3, 4). For example, contact of monocytes with tumour cells in vitro leads to the production by the former of tumour necrosis factor alpha (TNFα), interleukin (IL)-10, IL-12, reactive nitrogen (RNS) and oxygen species (ROS) (5, 6), and chemokines: IL-8 (CXCL8), monocyte chemoattractant protein-1 (CCL2) and regulated on activation normal T-cells expressed and secreted (CCL5) chemokines and accumulation of their mRNA in monocytes. Moreover, TMVs enhanced angiogenesis in NOD-SCID mice by delivering chemokines and via stimulation of monocytes. In addition, TMVs may be storage for chemokines thus inducing chemotaxis of blood leukocytes. Conclusion: These results further support the role of TMVs in modulation of monocyte biological activity.

Correspondence to: Monika Baj-Krzyworzeka, Department of Clinical Immunology, Polish-American Institute of Paediatrics, Jagiellonian University Medical College, Wielicka 265, 30-663 Cracow, Poland. Fax: +48 126581756, e-mail: mibaj@cyf-kr.edu.pl

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specific cytotoxic T-cells, generation of myeloid-derived suppressor cells and by increasing chemoresistance of primary tumours (26). On the other hand, TMVs may activate monocytes to produce cytokines and ROS, which results in the enhancement of their cytotoxic/cytostatic potential both in vitro and in vivo (27). However, little is known of the role of chemokines in the interactions of TMVs with monocytes.

In the present study, we asked whether TMVs may modulate chemokine production by monocytes in a similar manner as tumour cells, and be a source or storage for chemokines.

Materials and Methods

**TMVs isolation.** TMVs were obtained from the following human cell lines: HPC-4 (pancreatic adenocarcinoma, TMV_{HPC}), DeTa (colorectal adenocarcinoma, TMV_{DeTa}) and A549 (lung carcinoma, TMV_{A549}) as previously described (21). Cells were cultured by bi-weekly passages in RPMI-1640 (Sigma, St. Louis, MO, USA) with 5% foetal bovine serum (FBS; PAA Laboratories, Pasching, Germany) centrifuged at 50,000 × g. Cell lines were regularly tested for Mycoplasma sp. contamination by using PCR-ELISA kit according to the manufacturer’s procedure (Roche, Mannheim, Germany). Supernatants from confluent cell cultures were collected, centrifuged at 2,000 × g for 20 min to remove cell debris and then centrifuged again at 50,000 × g (RC28S; Sorvall, Newton, CT, USA) for 1 h at 4˚C. Pellets were washed several times to remove FBS and finally resuspended in serum-free medium. Quantification of TMVs proteins was evaluated by the Bradford method (BioRad, Hercules, CA, USA). TMVs were tested for endotoxin contamination by the Limulus test according to the manufacturer’s instruction (Charles River Laboratories, Inc., Wilmington, MA, USA) and stored at −20˚C. For determination of chemotaxis, TMVs were labelled with PKH26 dye according to the manufacturer’s protocol (Sigma).

**Isolation of blood leukocytes.** Blood leukocytes were isolated from peripheral blood after hypotonic haemolysis of erythrocytes. Lymphocytes, granulocytes and monocytes were distinguished by FSC/SSC parameters during flow cytometric analysis (FACSCalibur). Human peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated blood of healthy donors by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were separated from PBMCs by counter-flow centrifugal elutriation with a JE-6.B elutriation system equipped for 20 min to remove cell debris and then centrifuged again at 50,000 × g (RC28S; Sorvall, Newton, CT, USA) for 1 h at 4˚C. Pellets were washed several times to remove FBS and finally resuspended in serum-free medium. Quantification of TMVs proteins was evaluated by the Bradford method (BioRad, Hercules, CA, USA). TMVs were tested for endotoxin contamination by the Limulus test according to the manufacturer’s instruction (Charles River Laboratories, Inc., Wilmington, MA, USA) and stored at −20˚C. For determination of chemotaxis, TMVs were labelled with PKH26 dye according to the manufacturer’s protocol (Sigma).

**Expression of chemokine mRNA in monocytes and TMVs.** Monocytes (5×10^6) were incubated in low attachment tubes (BD Falcon, Bedford, MA, USA) in serum-free medium alone or with TMVs (30 μg/ml) for 2-4 h at 37˚C. Expression of chemokine mRNA was determined by real-time PCR. The total RNA was extracted from monocytes by the single-step isolation method using TRI-ZOL reagent (Gibco-BRL, Grand Island, NY, USA) according to the manufacturer’s protocol. The first-strand cDNA was obtained from the total RNA samples (1 μg) with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) and oligo-dT (Promega) primer, as specified by the manufacturer’s protocol. The quantitative PCR for CXCL8, CCL2, CCL3, CCL4 and CCL5 (TaqMan Gene Expression Assays; Applied Biosystems, Foster City, CA, USA) was performed using the 7300 Real-Time PCR System (Applied Biosystems). The fluorescent signals generated during the informative log-linear phase were used to calculate the relative amount of mRNA. β-Actin (ACTB) was used as control for each PCR run and the mRNA expression was calculated as a fold difference from that of untreated monocytes normalized by ACTB results (2^(-ΔCT)) Expression of chemokine mRNA in TMVs alone was presented as a threshold cycle (C_T) value.

**Western blot.** TMV samples were lysed with M-Per buffer (Pierce, Rockford, IL, USA) containing inhibitors of proteases, as described elsewhere (21). Samples (50 μg) were then treated for 4 min at 95˚C in sodium dodecyl sulfate (SDS) reducing buffer containing β-mercaptoethanol, followed by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide gels) and transferred onto polyvinylidene difluoride (PVDF) 2 μm membrane (Immune-blot PVDF; BioRad, Hercules, CA, USA). The membranes were blocked for 1 h at room temperature in TBS-0.1% Tween with 1% bovine serum albumin (BSA; Sigma). Next, the membranes were incubated overnight at 4˚C in TBS-Tween-BSA with the following antibodies: rabbit anti-human CXCL8, mouse anti-human CCL5, CCL3 (Santa Cruz Biotech, Santa Cruz, CA, USA), goat anti-human CCL2 (R&D Systems Minneapolis, MN, USA), rabbit anti-human GAPDH (Cell Signaling Systems, Beverly, MA, USA). The membranes were washed in TBS-Tween-BSA and incubated with rabbit anti-goat, rabbit anti-mouse or goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (dilution 1:2,000). All antibodies were purchased from Sigma. The protein bands were visualized with the SuperSignal West Pico Chemiluminescence Substrate kit, as recommended by the manufacturer (Pierce) and analysed with KODAK GEL LOGIC 1500 Digital Imaging System (Kodak, Rochester, NY, USA).

**Determination of chemokine secretion by monocytes.** Monocytes (1×10^6/ml) were cultured with TMVs (30 μg/ml) in serum-free RPMI-1640 medium at 37˚C, 5% CO2 and 95% humidity. After 18 h of culture, supernatants were collected and concentrations of chemokines (CCL2, CCL3, CCL4, CCL5, CXCL8) were measured using FlexSet system (BD Biosciences) followed by flow cytometric analysis (FACSComp; BD Biosciences). Chemokines were also detected in TMVs. For this purpose, 20 μg of intact TMVs and TMVs lysed with M-Per buffer (Pierce, Rockford, IL, USA) were analysed as described previously (21). The FlexSet beads were discriminated in FL-4 and FL-5 channels, while the concentration of specified chemokine was determined by the intensity of FL-2 fluorescence. The amount of chemokines was computed by using the respective standard reference curve and FCAP Array software (BD Biosciences). For all chemokines, the detection level was 10 pg/ml. In some experiments, supernatants were collected, centrifuged (30 min at 50,000× g) and frozen for further chemotaxis experiments. FlexSet results (for CXCL8, CCL5 and CCL3) were confirmed by ELISA method (R&D Systems) according to the manufacturer’s protocol.

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Matrigel matrix implants in NOD-SCID mice. The angiogenesis assay was carried out by injecting 6 to 8 week old NOD-SCID mice (CB-17/IcrCrl) in the abdominal midline with 100 μl of TMVs (30 μg/ml, TMV_HPC, TMV_A549, or TMV_DeTa) mixed with 400 μl of precooled Matrigel matrix (BD Falcon). Matrigel matrix alone was used as background control. Human monocytes (1×10⁶/mouse) stimulated or not with TMVs (30 μg/ml, for 2 h) were implanted into NOD-SCID mice in parallel. After 6 days, mice were euthanased, and the Matrigel matrix was removed and placed in 500 μl BD Recovery Solution (BD Falcon) for the following 7 days. The Matrigel matrix implants were then suspended in Drabkin’s solution (Sigma) and after 30 min centrifuged. The content of haemoglobin was determined by measuring absorbance at 540 nm in U-1800 spectrophotometer (Hitachi, Tokyo, Japan) (28).

Determination of TMV-induced chemotaxis. Leukocytes were placed in the upper chamber (2×10⁵/well) of Transwell 24-well plates with 8 μm pore filter (Costar Corning, Cambridge, MA, USA). PKH26-labelled TMVs (30 μg/ml) were resuspended in the lower chamber in RPMI-1640 medium. In parallel, the same quantity of lysed TMVs were used. Chemotactic assay was performed for 2-4 h, as described elsewhere (21). Cells from the lower chamber were then scored using a FACSCalibur flow cytometer. The cells were gated according to their FSC/SSC parameters and counted during a 20 s acquisition period at the high flow rate. Data are expressed as the percentage of the cell input corrected by the percentage of cells which migrated spontaneously to the medium.

Statistical analysis. Statistical analysis was performed by paired Student’s t-test in all experiments, except mouse experiments, where nonparametric Mann-Whitney test was used. Differences were considered significant at p-values <0.05.

Results

TMVs contain chemokine and mRNA for chemokines. Studies on chemokine content in TMVs by FlexSets revealed that TMV_A549 and TMV_HPC contain CXCL8. The ELISA method showed that intact TMVs contain less CXCL8 than lysed ones (data not shown). As the ELISA method is not designed for TMVs analysis, the presence of CXCL8 in TMVs was confirmed by Western blot (Figure 1). In the case of TMV_DeTa, no chemokines were detected. At the mRNA level, TMV_HPC contained mRNA for ACTB (C_T 28.49), CXCL8 (C_T 33.24), CCL2 (C_T 34.65) and CCL5 (C_T 34.36). HPC-4 cells expressed mRNA for ACTB (C_T 20.09), CXCL8 (C_T 18.93), CCL2 (C_T 28.32), CCL3 (C_T 35.52), CCL4 (C_T 33.98) and CCL5 (C_T 26.32). TMV_A549 had mRNA for ACTB (CT 31.69), CXCL8 (CT 25.31), CCL2 (CT 35.74), and CCL5 (CT 34.93). No mRNA for CCL3 or CCL4 was detected in TMV_HPC and TMV_A549. A549 cells expressed mRNA for ACTB (CT 19.21), CXCL8 (CT 15.69), CCL2 (CT 22.48), CCL3 (CT 35.45), CCL4 (CT 32.18) and CCL5 (CT 32.09). In the case of TMV_DeTa and DeTa cells, no mRNA for chemokines was found.

TMVs induce production of chemokines in monocytes. Monocytes were cultered with TMVs obtained from three different tumour cell lines for 18 h and chemokine secretion was analysed by FlexSets. All TMVs induced secretion of CCL2, CCL3, CCL4, CCL5 and CXCL8 by monocytes (Figure 2). The levels of secreted chemokines were lower than those induced by the appropriate cell lines (not shown). Only low amounts of CXCL8 (less than 20 pg/ml) were found in TMV_HPC and TMV_A549. Since stimulation of monocytes by different TMV was similar, for further experiments, TMV_HPC were chosen as being representative for determination of mRNA expression. Increased expression of mRNA for CXCL8 (21.69 fold; CT 16.55), CCL2 (44.06-fold; CT 17.23), CCL3 (24.7-fold; CT 16.59), CCL4 (54.95-fold; CT 13.17) and CCL5 (10.9-fold; CT 22.95) was found in monocytes after 2 h stimulation with TMV_HPC (Figure 3). Lower C_T values for chemokine mRNA in monocytes stimulated with TMVs in comparison to those in TMVs alone (see above) indicated production of tested chemokines in monocytes and not just a transfer of mRNA by TMVs.

TMVs induce angiogenesis. As CXCL8, which was detected in TMVs, is an inducer of angiogenesis, the proangiogenic potential of TMVs was tested using the Matrigel assay. All tested TMVs induced angiogenesis, with the strongest effect being exerted by TMV_A549 (Figure 4). Monocytes alone showed little angiogenic activity. However monocytes preincubated with TMVs induced stronger angiogenesis than monocytes or TMVs alone (Figure 5). A similar effect on monocytes, as for TMV_A549, was also observed for TMV_HPC and TMV DeTa used in the study.

TMVs induce chemotaxis of leukocytes. Previous observation indicated that TMVs contain CXCL8. Therefore, it was thought that TMVs may induce chemotaxis of blood leukocytes. The chemotactic activity...
of blood leukocytes towards TMVs was evaluated using transwell assays. Cells were collected from the lower transwell chambers, and their number was counted by FACS according to FSC and SSC parameters during a 20 s acquisition. The obtained data show that all tested TMVs induced chemotaxis of leukocytes (Figure 6). However, the strongest chemotaxis of granulocytes, lymphocytes and monocytes was observed using TMV$_{A549}$ as compared to TMV$_{HPC}$ and TMV$_{DeTa}$. Moreover, chemotaxis was twice as strong when lysed TMVs were used in the assay, especially in case of granulocytes and lymphocytes (data not shown).

Figure 2. The secretion of chemokines by monocytes cultured alone (control) or stimulated with TMVs (30 μg/ml) after 18 h culture. The level of chemokines in the culture supernatants was determined by FlexSet method. Data (mean±SD) from four independent experiments are presented. *p<0.05 and **p<0.001 compared to the control. None of these chemokines were detected in TMV alone, except CXCL8.
Discussion

The present study shows that TMVs play an important role in monocyte–tumour cell interactions as they may induce chemokine production by monocytes. In addition, TMVs may act as a ‘storage pool’ for chemokines and chemokine mRNA.

The interactions of monocytes (or TIMs) with tumour cells are still not clear. Our previous data indicated that TMVs induced production of cytokines and ROS by monocytes, activated some signalling pathways that regulate cytokine production, and increased antitumour response (27). This study shows that TMVs may be involved in the chemokine network between monocytes and tumour cells. The present results show that monocytes stimulated with TMVs secreted chemokines of the CXC (CXCL8) and CC (CCL2, CCL3, CCL4 and CCL5) families. Accumulation of chemokine mRNA in monocytes implicates their de novo synthesis. Moreover, we previously showed that TMVs induce TNFα secretion (27), which may, in turn, induce CXCL8 production. It was suggested that hyaluronan or other CD44 ligands carried by TMVs are involved in monocyte–TMV interactions by analogy to monocyte–tumour cell interactions.
(5, 27). This is in keeping with other data that hyaluronan fragments induced secretion of CCL3, CCL4 and CCL5 by macrophages (19) and CCL2, CXCL8 by mesothelial cells (29). Tumour cells stimulate monocytes to secrete greater amounts of chemokines, and the range of chemokines produced was broader as it also included CXCL9 and CXCL10 (data not shown).

The significance of the increased chemokine production during monocyte–tumour interactions may be manifold. First, chemokines secreted by monocytes seem to be biologically active, as stronger angiogenesis was observed when monocytes were preincubated with TMVs. These data may suggest that contact of monocytes with TMVs results in the release of proangiogenic factors, including chemokines (CXCL8, CCL2 and CCL3). We concentrated on the role of chemokines, as the secretion of vascular endothelial growth factor in monocytes stimulated with TMVs is very low (unpublished data), but we cannot exclude the role of other proangiogenic factors (β-fibroblast growth factor, hepatocyte growth factor, etc.) (30). On the other hand, TMVs alone did induce angiogenesis which is in keeping with the findings of others (31, 32). The strongest proangiogenic activity was observed using TMV_{A549}, and the weakest effect with TMV_{DeTa}. This is in keeping with ELISA/Western blot results as TMV_{A549} and TMV_{HPC} were found to contain proangiogenic CXCL8 (18, 33).

As TMVs carry CXCL8 (protein and mRNA), it is not surprising that granulocytes, which express CXCL8 receptors (CXCR1, CXCR2) migrate to them. In contrast, TMV_{DeTa} did not contain any of the tested chemokines and induced the weakest chemotaxis. Surprisingly, chemotaxis of monocytes to TMVs was very weak. This may be because monocytes move at a very low speed in comparison to granulocytes (34), or the amount of chemokines in TMVs is too low to induce stronger response of monocytes, as optimal concentrations of chemokines to induce monocyte chemotaxis are around 20 ng/ml (34).

It is unknown exactly where chemokines are located in TMVs, and whether they are released effectively. Perhaps TMV-related chemokines do not create a concentration gradient, resulting in impaired chemotaxis (23). When lysed TMVs were used for chemotaxis, stronger response of all leukocyte populations was observed (unpublished data), suggesting that chemokines are present in TMVs albeit with limited access. Apart from chemokines, TMVs may carry other chemotactants or proangiogenic factors, e.g. growth factors, tetraspanins or ephrin-B1 (35), which should be taken into consideration as well. It should be stressed that although TMV_{DeTa} did not contain chemokines, they were still able to induce production of chemokines by monocytes, which further supports the view that TMVs not only transfer chemokines but are also inducers of their production.

It was previously mentioned that microvesicles may transfer mRNA from embryonic stem cell and endothelial progenitor cells to different target cells (36, 37). Now, we show that TMVs may also carry chemokines and chemokine mRNA which can probably be transported to target cells.

In summary, evidence was provided that TMVs induce secretion and production of chemokines by monocytes. In addition, TMVs may be important modulators of the chemokine network in the tumour microenvironment. This study investigated TMV interactions with monocytes, but the effect of TMVs on tumour cells should also be taken into consideration. This investigation demonstrated for the first time that TMVs may act as a storage pool for biologically active chemokines and their mRNAs. Further investigations may elucidate the role of TMVs in their interactions with tumour cell.

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