Vol. 10, 1901–1910, March 15, 2004

Featured Article

Transendothelial Migration of Myeloma Cells Is Increased by Tumor Necrosis Factor (TNF)-α *via* TNF Receptor 2 and Autocrine Up-Regulation of MCP-1

Karin Jöhrer,[1] Katrin Janke,[1] Jens Krugmann,[3] Michael Fiegl,[2] and Richard Greil[1,2]

[1]Tyrolean Cancer Research Institute at the University of Innsbruck; [2]Laboratory of Molecular Cytology, Department of Internal Medicine, Division of Hematology and Oncology, University Hospital Innsbruck; and [3]Institute of Pathology, University of Innsbruck, Innsbruck, Austria

Abstract

The proinflammatory cytokine tumor necrosis factor (TNF)-α has been shown to facilitate leukocyte transendothelial migration. In multiple myeloma, TNF-α is an important factor in the promotion of growth and survival of the malignant cells. Studies have shown that enhanced TNF-α levels in myeloma patients correlated with aggressive disease. Therefore, we investigated the effect of recombinant human TNF-α on the migrational behavior of myeloma cells across the physiological barrier of the major disease compartment, *i.e.,* human bone marrow endothelial cells. In the presence of TNF-α, we observed significantly increased migration both in established myeloma cell lines and in plasma cells from myeloma patients. Expression of TNF-receptor 2 (TNF-R2) but not TNF-receptor 1 (TNF-R1) was detected in myeloma cell lines. Myeloma cells of patients also showed expression of TNF-R2 but not TNF-R1. The effect of TNF-α could not be explained by altered expression of adhesion molecules or metalloproteases. Instead, we found an up-regulation of monocyte chemoattractant protein (MCP)-1 and confirmed that myeloma cells express the relevant receptor C-C chemokine receptor 2. Preincubation of myeloma cells with recombinant human MCP-1 also enhanced cell migration, and this effect, as well as the effect of TNF-α, was abolished by treatment with anti-MCP-1 antibody. In contrast, migration of myeloma cells in the direction of an MCP-1 gradient, *i.e.,* chemotaxis, could not be observed in the cell lines investigated. Additionally, the mRNA level of TNF-α was up-regulated by the cytokine treatment, which points to an autocrine loop augmenting and/or stabilizing the TNF-α–MCP-1 pathway. In summary, our data clearly support additional investigations using anti-MCP-1 antibodies in myeloma progression.

Introduction

Malignant cells of the hematological system usually originate from normal cells with the inherent capacity to circulate through the bloodstream. In multiple myeloma, the cells constituting the tumor burden in the bone marrow are generally assumed to originate from a B-cell clone that has been antigen-selected in the germinal center of the lymph nodes and that homes predominantly to, and spreads within, the bone marrow (1). This implies that myeloma cells and/or their precursor cells traverse the bone marrow endothelial barrier for entry as well as for subsequent extravasation to distant bone marrow sites.

The fact that nonmalignant, activated B cells are capable of migrating across the endothelial layer in response to proinflammatory cytokines such as tumor necrosis factor (TNF)-α (2, 3) provides a basis for the assumption that this cytokine might also be important for the transmigration process in myeloma. Under physiological conditions, TNF-α is a potent chemoattractant for neutrophils and increases their adherence to the endothelium. It induces the synthesis of a number of chemoattractants and modulates the chemoattractant properties of other chemotactic reagents for neutrophils (2). In addition to chemotaxis, TNF-α has also been shown to induce chemokinesis in several cell types including memory and naïve B cells (4). The cytokine has been shown to regulate expression of chemokines and chemokine receptors via TNF receptor 2 (TNF-R2) in a cell-type and tissue-specific manner (5). Such a role for TNF-α, however, has not yet been investigated in multiple myeloma.

Within the major compartment of myeloma disease, *i.e.,* the bone marrow, TNF-α is readily produced not only by cells of the microenvironment but also by myeloma cells themselves (6). This cytokine, important in myeloma establishment and progression, has been shown to act as a survival factor for myeloma cells (7) and, in combination with interleukin 4, it has also been implicated in myeloma precursor cell differentiation (8). Furthermore, TNF-α is able to trigger the secretion of interleukin 6 from bone marrow stromal cells, which constitutes a major growth factor for the tumor cells (9, 10), and it displays proangiogenic properties *in vivo* (11). In terms of clinical relevance, it has been shown that freshly explanted bone marrow cells from patients produced higher amounts of TNF-α than the comparable controls. The extent of production correlates with the stages of the disease, with the presence of extensive osteolytic bone disease (12), and with the percentage of plasma cells in the bone marrow (13). These results point to a central role for

Received 7/17/03; revised 11/14/03; accepted 12/10/03.

Grant support: Grant SFB-FO2 from the Tyrolean Cancer Research Institute (K. Jöhrer, K. Janke, and R. Greil), the Cancer Aid of Tyrol, the Verein für Klinische Malignom- und Zytokinforschung, and the Raiffeisenbank Tyrol.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Dr. Greil is currently at the IIIrd Medical Department of Internal Medicine, Private Medical Paracelsus-University of Salzburg, Salzburg, Austria.

Requests for reprints: Richard Greil, Tyrolean Cancer Research Institute at the University of Innsbruck, Innrain 66, A-6020 Innsbruck, Austria. E-mail: richard.greil@uibk.ac.at.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

TNF-α in growth promotion, survival, and expansion of the malignant clone.

TNF-α, either as soluble protein or in its membrane-bound form, signals via two distinct TNF receptors, TNF receptor 1 (TNF-R1) and TNF-R2 and their respective signaling cascades (14). Ligand binding to TNF-receptors can result in cell activation, migration, proliferation, apoptosis, or even inhibition of apoptosis (15). The biological consequence of ligand binding depends on the cell lineage, metabolic state of the cell, kinetics of ligand binding, and which isoform of TNF-receptor is expressed. TNF-R1, which belongs to the death-receptor domain family, is found on a number of cell types, particularly on cells susceptible to the cytotoxic action of TNF-α, *i.e.,* induction of apoptosis, and binds soluble TNF-α with higher affinity than does TNF-R2 (16). TNF-R2 is present on cell types of lymphoid origin, *i.e.,* on stimulated T cells and B cells. In contrast to TNF-R1, signaling via TNF-R2 is not linked to a death receptor domain.

TNF-α has been shown to play an essential role in the differentiation and survival of myeloma cells. However, according to its function in other cell systems, TNF-α might also be involved in the migration of myeloma cells, thus contributing to the spread of the disease. We here investigated the impact of TNF-α on the migrational capacity of myeloma cells.

Patients, Materials, and Methods

Cell Culture. Myeloma cell lines RPMI-8226, U266, and NCI-H929 were obtained from American Type Culture Collection (Rockville MD), and grown in RPMI-1640 (Life Technologies, Inc. Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS (PAA, Linz, Austria), L-glutamine 100 μg/ml (Life Technologies, Inc., NY), and penicillin-streptomycin 100 units/ml (Life Technologies, Inc.). The human bone marrow endothelial cell line HBMEC-60 was kindly provided by Dr. C. E. van der Schoot (CLB, Amsterdam, the Netherlands; Ref. 17). Endothelial cells were cultured in M199 (PAA, Linz, Austria) supplemented with HEPES 25 mM (MERCK, Darmstadt, Germany), 10% heat-inactivated human serum (Biowhittaker, Belgium), 10% heat-inactivated FCS (PAA), bFGF 1 ng/ml (Clonetics Corporation, San Diego, CA), heparin 5 units/ml (Biochrom Ltd, Cambridge, United Kingdom), L-glutamine 0.3 mg/ml, penicillin-streptomycin 100 units/ml, and geneticin 100 μg/ml (all Life Technologies, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Recombinant human TNF-α and recombinant human monocyte chemoattractant protein 1 (MCP-1) were purchased from Sigma (Germany) and from R&D systems (Minneapolis, MN), respectively.

Native Myeloma Cells. Bone marrow samples from myeloma patients were obtained after informed consent. Myeloma cells were purified using magnetic cell sorting CD138 Microbeads (clone B-B4; Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturers' instructions. Briefly, bone marrow mononuclear cells were diluted in HEPES-buffered RPMI medium and separated by Ficoll-Paque; cells were then labeled with CD138 magnetic cell-sorting beads and were positively selected over a magnetic column with ~95–99% purity. CD138-positive cells and the remaining CD138-depleted cell fraction were immediately used for transmigration assays. Relevant patients' information is summarized in Table 1. All of the patients were in stage IIIa of myeloma and patients MM1–4 were untreated before bone marrow aspirates were taken. Patient MM5 was recently treated and patient MM6 was in relapse 3 years after the initial therapy. Percentage of plasma cells in the bone marrow of patients was assessed by cytological (patient MM1–4) or histological methods (patients MM5–6; see Table 1).

Table 1 Characteristics of myeloma patients

Patients	Sex	Age	% plasma cells in bone marrow	Stage
MM1	M	69	36.5	IIIa
MM2	F	69	37.5	IIIa
MM3	F	62	63.0	IIIa
MM4	M	74	72.5	IIIa
MM5	M	61	60.0	IIIa
MM6	M	72	65.0	IIIa

Transmigration Assay. Endothelial cells were seeded onto fibronectin-coated (BD Bioscience, Bedford, MA) transwells (5 μm pores; Costar, Corning, Inc., Corning, NY) and were allowed to grow to confluence. Per transwell, $\sim 1 \times 10^5$ myeloma cells at a concentration of 0.5×10^6 cells/ml [unstimulated or prestimulated for 4 h with human recombinant TNF-α (50 ng/ml) and human recombinant MCP-1 (100 ng/ml), respectively] were washed in PBS to eliminate residual TNF-α, were seeded on the endothelial monolayer, and were allowed to migrate for 4–10 h. Migrated cells at the bottom of the wells were counted using a Coulter Counter (Coulter Electronics LTD, Harpenden Herts, England) and the percentage of migrated cells was calculated. Plasma cells from patients were seeded onto uncoated transwells after incubation with TNF-α as above and were allowed to migrate for 4–6 h. Blocking experiments were performed by incubating the cells with 10 μg/ml blocking antibody (anti-MCP-1; R&D Systems) for 10 min at room temperature or with the corresponding isotype-matched control antibody before incubation with TNF-α and MCP-1, respectively.

Flow Cytometry Analysis. Myeloma cells were either stimulated with TNF-α or not. Flow cytometric analyses were performed on a FACScan (Becton Dickinson). The cells were incubated with the specific monoclonal antibodies or an unspecific, isotype-matched murine antibody as a control for 30 min at 4°C, washed in PBS/0.2% BSA and, in case of unconjugated specific monoclonal antibodies, were subsequently incubated with a secondary FITC-conjugated rabbit antimouse-immunoglobulin monoclonal antibodies for 30 min at 4°C. Cells were again washed in PBS/0.2% BSA and analyzed. Monoclonal antibodies and matched control antibodies were purchased from Serotech (CD120a/TNF-R1, CD120b/TNF-R2), Ancell (CD49d, CD106, CD44, CD54) PharMingen (CD11a, CD50), and R&D Systems (CCR2, clone 48607.2).

Apoptosis Assay and Proliferation Assay. To measure the impact of TNF-α and MCP-1 stimulation on apoptosis of myeloma cells, 0.5×10^6 cells/ml were seeded into 96-wells and incubated for 24 h and 48 h with or without cytokine (50–100 ng/ml) or chemokine (100 ng/ml), respectively. Apo-

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

ptosis was detected by fluorescence-activated cell sorting analysis of the binding of AnnexinV/FITC (Molecular Probes, Leiden, the Netherlands) and propidium iodide (Sigma). Experiments were performed in quadruplicates. Proliferation was measured in the same setting using the Proliferation Assay CellTiter 96 (Aqueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI). Briefly, 0.5×10^6 cells/ml cells were seeded in 96-well plates and incubated for 24 h or 48 h with or without recombinant human TNF-α or recombinant human MCP-1. Twenty μl of the cell titer solution were added for the last 4 h of the incubation period, and absorbance was measured at 490 nm using a microplate reader (model Benchmark; BIO-RAD Laboratories, Hertfordshire, United Kingdom).

Adhesion Assay. Adhesion assays were performed as described previously (18). Endothelial cells were grown to confluence in fibronectin-coated 24-well tissue culture dishes (Costar) and were washed with phenol red-free medium (M199 as above, except for 2% FCS). Myeloma cells (1×10^6/ml) were either stimulated or not with recombinant human TNF-α (50 ng/ml, 4 h), labeled with 10 μg/ml Calcein-AM for 30 min (Molecular Probes), washed in PBS, resuspended in phenol red-free medium, and allowed to bind to the endothelial monolayer for 30 min at 37°C. The nonadherent cells were subsequently discarded by washing, and adherent cells were lysed with lysis medium [PBS supplemented with 0.1% (v/v) Tween 20, 0.2% (w/v) hexadecyl-trimethyl-ammonium-bromide, 0.2% (w/v) BSA (Sigma), and 20 mM EDTA (Merck, Munich, Germany)]. The fluorescence of the lysed cells was measured by a spectrofluorometer (Model Fluoroscan Ascent, λ_{ex} 485 nm, λ_{em} 525 nm).

Gelatin Zymography Assay. Gelatinolytic activities in conditioned medium of myeloma cell lines U266 and RPMI-8226 were determined as described previously (19). Briefly, 1×10^6 cells/ml were grown in RPMI 1640 (Life Technologies, Inc.), supplemented with L-glutamine, antibiotics, and 1% heat-inactivated FCS, for 60 h. During the last 12 h, TNF-α (50 ng/ml) was added as indicated. The supernatants were collected and concentrated using Centriprep columns (Millipore, Bedford, MA). Protein content was measured by Bradford assay (20), and 10 μg of protein were mixed with SDS sample buffer without reducing reagent and were subjected to SDS-PAGE (10%) copolymerized with gelatin (1 mg/ml; Sigma). After electrophoresis, SDS was removed from gels by incubation in renaturing buffer containing 2.5% Triton X (two times for 20 min). Reactivation of the metalloproteases was achieved by incubating the gels over night in activation buffer [10 mM Tris-HCl (pH 7.5), containing 1.25% Triton X-100, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$] at 37°C. Gels were stained with Coomassie Blue thereafter and destained with 50% methanol/10% acetic acid/40% water. The proteolytic activity was visualized as clear bands (zones of gelatin degradation) against the blue background of stained gelatin. Semiquantitative analysis was done by densitometry using a computer-assisted scanner (HEIDELBERG Linoscan1400) and standard software (ADOBE-Photoshop).

MCP-1 ELISA. ELISA plates coated with anti-MCP-1 monoclonal antibodies were purchased from R&D systems and assays were performed according to the manufacturer's instructions. We analyzed supernatants collected from myeloma cell cultures (1×10^6 cells/ml) after 12-h growth in complete RPMI 1640 with or without the addition of recombinant human TNF-α (50 ng/ml). Chemokine content was quantified at 450 nm with a reference wavelength of 570 nm (Bio-Rad Benchmark Microplate Reader).

Microarray. Total RNA from U266 and U266 stimulated for 2 h and 4 h, respectively, with TNF-α was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA), and mRNA was prepared using the Oligotex mRNA Purification System (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Messenger RNA (600 ng) was sent to Incyte Genomics Corporation, and the company performed mRNA labeling and hybridizations to the Human Foundation 4 microarray, which contains more than 9700 sequences. Data analysis was performed using the GEMTools 2.5 analysis program.

NF-κB ELISA. The DNA-binding activity of NF-κB in U266 cells was quantified by ELISA by the *trans*-AM NF-κB p65 Transcription Factor Assay kit (Active Motif North America, Carlsbad, CA) according to the manufacturer's instructions. In brief, RPMI-8226 and U266 cells were treated with TNF-α between 5 and 240 min or were left untreated. Whole cell extracts were prepared as described above, and 15 μg of protein/sample were incubated in 96-well plates coated with immobilized oligonucleotides containing the consensus binding site for the p65 subunit of NF-κB, according to instructions. Quantification was obtained at 450 nm with a reference wavelength of 655 nm using the Bio-Rad Benchmark Microplate Reader.

Reverse Transcription-PCR. Total RNA from myeloma cells was prepared using TRIzol Reagent (Invitrogen Corp.) and following the producer's instructions. Transcription of cDNA was performed using a mixture containing 1 μg of RNA, 500 ng of oligo(dT) primer, 10 mM dNTP, 1× reverse transcription buffer, and 200 units of moloney murine leukemia virus-reverse transcriptase and incubated for 90 min at 37°C. PCR conditions for each set of primers were 10 mM dNTP, 25 mM $MgCl_2$, 10 μM each primer, and 1 unit of *Taq*DNA polymerase (Promega, Mannheim, Germany). PCR cycles started with 95°C for 3 min and continued for 30 cycles with 95°C for 30 s/specific annealing temperature for 1 min/70°C for 1 min. The following primers were used (5′–3′): TNF-α sense (sense), GCA CTG AAA GCA TGA TCC GGG, and TNF-α antisense GTG ACA AGC CTG TAG CCC ATG, annealing temperature 57°C, which resulted in a 270-bp fragment; matrix metalloprotease (MMP)-2 (sense) GGC CCT GTC ACT CCT GAG AT, and MMP-2 (antisense) TCC CCG ATA ACC TGG ATG CC (57°C, 574 bp); MMP-9 (sense) ACC GCT ATG GTT ACA CTC G, and MMP-9 (antisense) ACA ACA TCA CCT ATT GGA TCC (52°C, 227 bp); tissue inhibitors of metalloproteases (TIMP)-1 (sense) CCA AGT TCG TGG GGA CAC, and TIMP-1 (antisense) TGC AGT TTT CCA GCA ATG AG (52°C, 207 bp); TIMP-2 (sense) GGC GTT TTG CAA TGC AGA TGT AG, and TIMP-2 (antisense) ACA GGA GCC GTC ACT TCT CTT G (57°C, 496 bp); MCP-1 (sense) CTC ATA GCA GCC ACC TTC AT, and MCP-1 (antisense), GCT TTT CCT CTT GAA CCA CA (52°C, 495 bp fragment); and housekeeping gene *L32* (sense) CAT TGA CAA CAG GGT TCG TAG, and L32 (antisense) GCC AGG CTG CGC AGT GAA G (59°C, 278-bp fragment).

Real-Time PCR. Total RNA was isolated using TRIzol reagent and following the manufacturer's instructions. cDNA

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

probes were generated using random hexamer primers and using Superscript II Reverse Transcriptase (Life Technologies, Inc.). Real-time PCR was performed using a PCR kit (Brilliant Quantitative PCR Core Reagent kit, Stratagene Europe (Amsterdam, the Netherlands) and analysis was performed on an AbiPrism 7700 sequence detector (Applied Biosystems). Expression of C-C chemokine receptor 2 (CCR2) was normalized against the amount of *18S* RNA. Primers and probes (5′FAM/3′TAMRA label) were selected using the Primer Express software (Applied Biosystems), and sequences were as follows: CCR2 (sense) GAC CAG GAA AGA ATG TGA AAG TGA, and CCR2 (antisense) GCT CTG CCA ATT GAC TTT CCT T, and TaqMan Probe CCR2 CAC AAG GAC TCC TCG ATG GTC GTG G; 18S (sense) CCA TTC GAA CGT CTG CCC TAT, and 18S (antisense) TCA CCC GTG GTC ACC ATG, and TaqMan Probe 18S ACT TTC GAT GGT AGT CGC CGT GCC T.

Immunohistochemistry. For immunohistochemical analysis, 5-μm serial sections from bone marrow of myeloma patients were cut, deparaffinized, and subsequently rehydrated. Using Ventana nexus module (Ventana Medical Systems, Tucson, AZ), we analyzed the expression of TNFR2 (clone 22221.311; R&D Systems) using the antibody in a dilution of 1:10.

Results

TNF-α Prestimulation Enhances Transmigration of Myeloma Cell Lines through HBMEC-60. To investigate the migrational behavior of myeloma cells resulting from TNF-α stimulation, we preincubated the cells with TNF-α for 4 h and seeded them onto transwells coated with fibronectin and covered by a human bone marrow endothelial cell line monolayer (HBMEC-60). Myeloma cells showed a spontaneous migration in the range between 2 and 8% [RPMI-8226 (migration ± SD), 2.3 ± 1.1%; U266 (migration ± SD), 4 ± 1.8%; and NCI-H929 (migration ± SD), 8.8 ± 1%); and this migration was significantly increased (RPMI-8226, $P = 0.029$; U266, $P = 0.0003$) after preincubation with TNF-α in two of three cell lines after 10 h (Fig. 1). Spontaneous migration rate was cut in approximately one-half when we used fibronectin-coated or uncoated transwells without an endothelial monolayer, but the extent and pattern of TNF-α-induced migration remained the same (results not shown). This suggests that the TNF-α-induced migration was due to alterations of the innate migrational behavior of the myeloma cells which do not interfere with the transendothelial migration process. In contrast to myeloma cell lines, other cell lines of B-cell origin (Raji, B-cell lymphoma cell line) or T-cell origin (CEM-C7H2, T-ALL cell line) did not demonstrate an enhanced migrational response to TNF-α stimulation (Raji +/− TNF-α, $P = 0.1$; CEM-C7H2 +/− TNF-α, $P = 0.16$). Analysis of expression of TNF-receptors showed that two of three myeloma cell lines expressed TNF-R2 and hardly any of them expressed TNF-R1 (Fig. 2). A positive correlation ($r = 0.99$, $P = 0.04$) was found between the level of TNF-R2 expression and the percentage of myeloma cells migrating after TNF-α prestimulation (data not shown). The cell line NCI-H929, which expressed neither TNF-R1 nor TNF-R2, did not show enhanced migrational behavior in response to the TNF-α stimulus. Cell lines of other hematopoietic tumors without altered migration levels in response to TNF-α (CEM-C7H2, Raji) expressed various amounts of TNF-R2 and TNF-R1 [CEM-C7H2: TNF-R2, mean fluorescence intensity = 1.4; TNF-R1, mean fluorescence intensity = 3.2; Raji: TNF-R2, mean fluorescence intensity = 2.5; TNF-R1 mean fluorescence intensity = 2.1). There was no significant influence of TNF-α on apoptosis and proliferation of myeloma cells after 48 h of stimulation (data not shown).

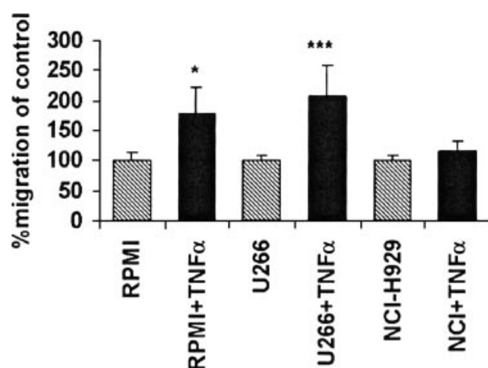

Fig. 1 Transmigration of myeloma cell lines across a human bone marrow endothelial cell line monolayer (HBMEC-60) with/without tumor necrosis factor α (TNF-α) prestimulation. Two of three myeloma cell lines [RPMI-8226 ($n = 4$; *, $P = 0.029$), U266 ($n = 6$; ***, $P = 0.0003$), and NCI-H929 ($n = 6$; $P = 0.09$)] showed significantly increased migration through 5-μm transwells coated with fibronectin and covered by an endothelial monolayer after TNF-α preincubation (*RPMI-8226+TNF*α and *U266+TNF*α); migration is presented as increase in percentage compared with untreated control (*RPMI,* 100%); *bars,* SE; statistical analysis was performed using paired *t* test.

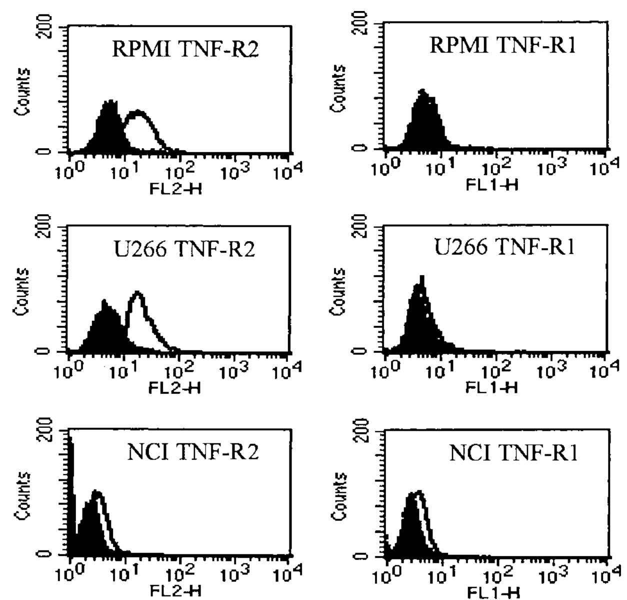

Fig. 2 Tumor necrosis factor receptor expression on myeloma cell lines. Flow cytometry analysis showed that TNF receptor 2 (*TNF-R2*) is expressed on all of the cell lines, whereas TNF receptor 1 (*TNF-R1*) is not. TNF-R2 expression correlates with enhanced migration after TNF-α stimulation in the cell lines RPMI-8226 and U266. Cell line NCI-H929 does not show expression of TNF-R2 or TNF-R1 (*filled profile,* isotype; *black line,* specific signal).

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

Adhesion Molecules and Matrix-Metalloproteases Are Not Involved in TNF-α-Induced Migration. Activation of NF-κB was previously described to be involved in TNF-α-induced up-regulation of adhesion molecules leading to enhanced adhesion of myeloma cells to bone marrow stromal cells (10). We, therefore, analyzed the activation of NF-κB in our cell lines and found relatively high levels of constitutive activation in U266 but no impact on activation by TNF-α stimulation. Activation was enhanced in RPMI-8226 starting after 2 h cytokine treatment of the cells (Fig. 3*A*). We then performed flow cytometry analysis of several adhesion molecules known to be expressed and suggested as playing a role in the adhesion process of myeloma cells. We found no significant quantitative change in the expression of CD11a, CD44, CD49d, CD50, CD54, or CD106 after preincubation with TNF-α (Fig. 3*B*) in myeloma cell lines RPMI-8226 and U266. Expression of adhesion molecules was analyzed at different time points and using different concentrations of TNF-α to evaluate the expression in our migrational set-up and to compare our data with the previously published ones (10). Adhesion assays of myeloma cells on HBMEC-60 supported our fluorescence-activated cell sorting analysis data because no significant change in the extent of adhesion due to cytokine stimulation was found [RPMI-8226 (mean ± SE), 104.4 ± 8.9% adhesion of control; U266 (mean ± SE), 89.75 ± 14.4% adhesion of control]. We next tested the possible involvement of matrix-metalloproteases-2 (MMP-2) and -9 (MMP-9). In gelatin zymography assays, we found no change in protease activity due to TNF-α treatment for 12 h (Fig. 3*C*). Additionally, the amounts of mRNA of metalloproteases (MMP-2 and MMP-9) and their inhibitors (TIMP-1 and TIMP-2) were not changed by the TNF-α-treatment (50 ng/ml), either, as was shown by microarray-based expression profiling and semiquantitative reverse transcription-PCR (data not shown).

Up-Regulation of MCP-1 and TNF-α by TNF-α. Because we did not find significant changes in the expression of molecules generally involved in transmigration (namely, adhesion molecules and matrix-metalloproteases), we performed microarray analysis of U266 with or without TNF-α stimulation for 2 and 4 h in search of potential candidate genes. A surprisingly low number of genes (*i.e.,* 7 genes of 9700) matched the criteria of at least a 2-fold regulation. In addition to expressed sequences of unknown function (EST) and genes regulating TNF-α-induced signaling pathways, we found a significant rise in *MCP-1* mRNA (4.8-fold induction after 2 h and 3.9-fold after 4 h). All responsive myeloma cell lines displayed basal mRNA

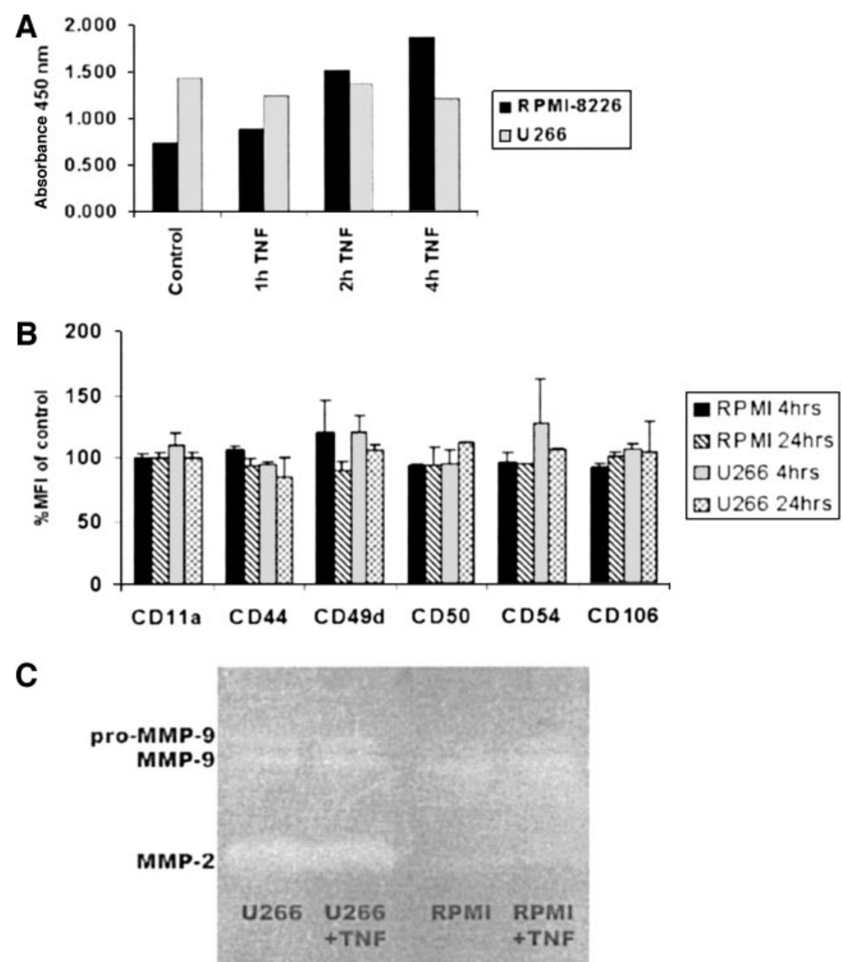

Fig. 3 Nuclear factor-κB (NF-κB) activation, expression of adhesion molecules, and activity of metalloproteases in response to tumor necrosis factor α (TNF-α) stimulation. *A,* NF-κB activation was analyzed at 0–4 h of cytokine treatment using an ELISA assay. Absorbance at 450 nm was measured and cell line RPMI-8226 showed a 2.5-fold activation after cytokine treatment for 4 h. *B,* cell lines RPMI-8226 and U266 were incubated with different concentrations of TNF-α (10 and 50 ng/ml, respectively) and the mean fluorescence intensity (*MFI*) of various adhesion molecules known to be involved in myeloma cell adhesion was measured by fluorescence-activated cell sorting analysis at two time points (4 h and 24 h) to investigate our settings and to compare with previously described data (Ref. 10; $n = 4$; *bars,* SD). *C,* gelatin zymography assay was used to show that activity of metalloproteases (*MMP-2* and *MMP-9*) was not changed by 12 h TNF-α incubation (+*TNF*). Expression of both metalloproteases was higher in U266 than in RPMI-8226.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

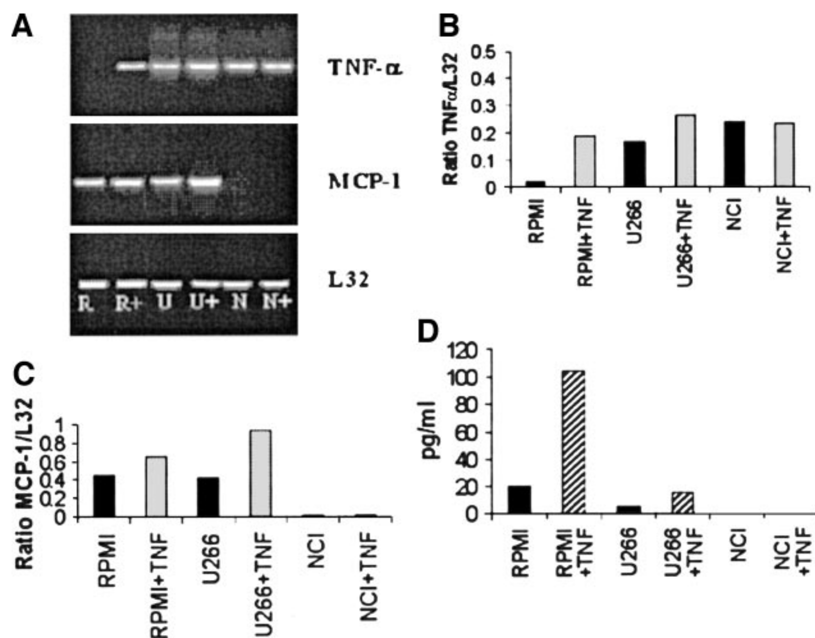

Fig. 4 Monocyte chemoattractant protein 1 (*MCP-1*) and tumor necrosis factor α (*TNF*-α) are induced by treatment with *TNF*-α. *A,* expression of *MCP-1* and TNF-α and the regulation after *TNF*-α incubation (4 h) was confirmed by reverse transcription-PCR. A representative experiment of three is shown. *R,* RPMI; *U,* U266; *N,* NCI-H929; +, incubation with *TNF*-α. *B* and *C,* densitometric analysis is presented as the ratio of *TNF*-α:*L32* housekeeping gene (*B*) and as the ratio of MCP-1:*L32* (*C*). *D,* expression of *MCP-1* at the protein level was confirmed by ELISA. Increase of *MCP-1* protein is calculated in percentage of control, cytokine treatment is indicated by +TNF. Cell line NCI-H929, which did not migrate in response to TNF-α stimulation, neither expressed *MCP-1* nor showed up-regulation of *TNF*-α mRNA.

expression levels and up-regulation of MCP-1-mRNA after TNF-α stimulation. In contrast, the unresponsive cell line NCI-H929 neither expressed basal levels of MCP-1 nor showed a TNF-α-induced up-regulation of this chemokine (Fig. 4, *A–C*). The production of MCP-1 protein and the TNF-α induced up-regulation of MCP-1 were confirmed by ELISA assay (Fig. 4*D*). Additionally, TNF-α mRNA was up-regulated in all of the cell lines except NCI-H929 in response to the cytokine treatment, although basal expression levels of TNF-α mRNA were detectable also in this cell line (Fig. 4, *A* and *B*). The high-affinity receptor for MCP-1, *i.e.,* chemokine receptor CCR2 was expressed by all of the myeloma cells as shown by real-time PCR experiments (Fig. 5*A*) and flow cytometry analysis (Fig. 5*B*) and was not significantly regulated by the TNF-α treatment either at mRNA or at the protein level in fluorescence-activated cell sorting analysis (data not shown). Preincubation of the myeloma cells with recombinant human MCP-1 enhanced migration to an extent similar to that after TNF-α treatment. These chemokinetic effects of MCP-1 and of TNF-α preincubation were almost completely abolished by incubation with anti-MCP-1 antibody in both cell lines, U266 and RPMI-8226 (Fig. 6). In contrast to this clear chemokinetic effect, we could not confirm previously described chemotactic effects of MCP-1 on the myeloma cell lines (Ref. 21; data not shown).

Native Myeloma Cells Express TNF-R2 and Show Enhanced Migration after TNF-α Preincubation. To underline the possible *in vivo* relevance of the cytokine-induced migration that we found in myeloma cell lines, we tested native myeloma cells (CD138-positive cells purified from bone marrow aspirates) of six myeloma patients on their migrational behavior after TNF-α preincubation. Migration of myeloma cells was investigated across transwells for 4–6 h and migrated cells were counted as described above. We compared the number of migrated CD138+ myeloma cells and CD138-depleted bone marrow cells in response to TNF-α (Fig. 7). The rate of spontaneous migration of myeloma cells varied depending on the patient (rate of migration ± SD, 4.3 ± 3.3%). Significantly enhanced migration (from 215 to 450%, depending on patient

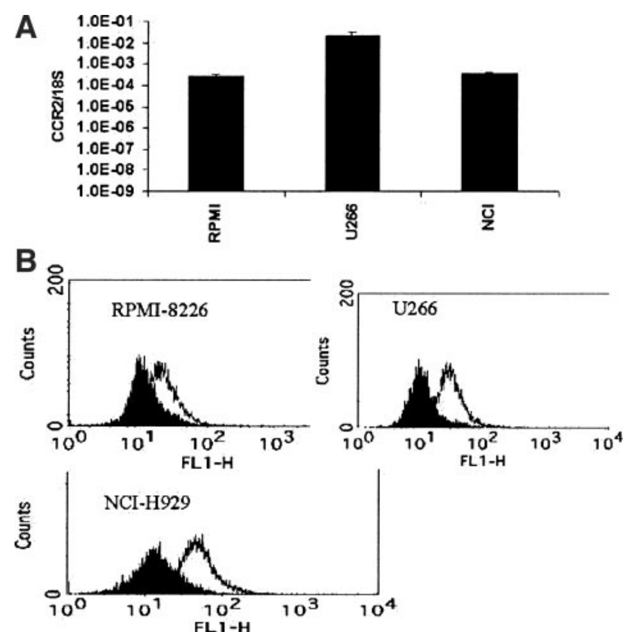

Fig. 5 Myeloma cell lines express C-C chemokine receptor 2 (CCR2). *A,* the expression of the chemokine receptor CCR2 is shown by real-time PCR correlating the RNA amount of the *18S* housekeeping gene with the mRNA amount measured for the CCR2-specific PCR. *B,* flow cytometric analysis (*filled profile,* isotype; *black line,* CCR2) of the cell lines confirmed the expression on protein level.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

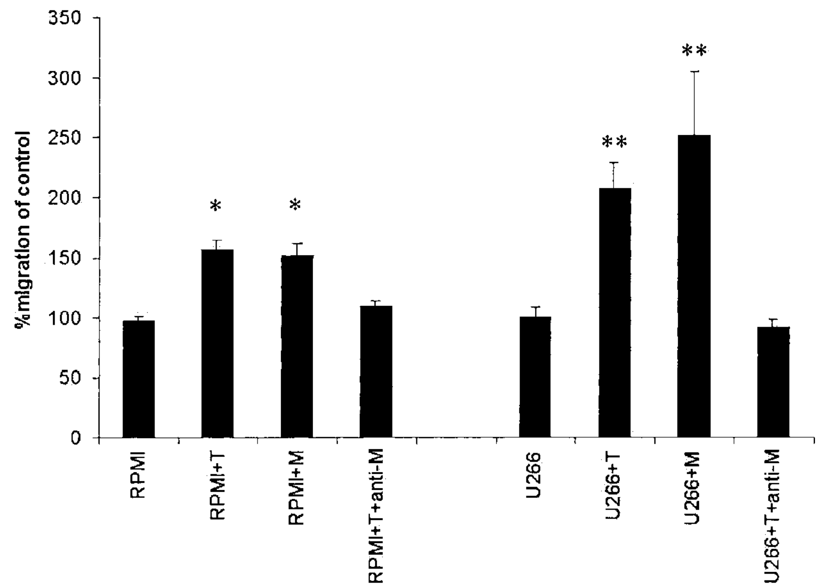

Fig. 6 Migration and inhibition of migration of myeloma cell lines. Preincubation with tumor necrosis factor α (TNF-α; *T*) and monocyte chemoattractant protein 1 (MCP-1; *M*) enhanced migration of RPMI-8226 and U266, and migration was blocked by anti-MCP-1 monoclonal antibody (*anti-M*). Migration is calculated as increase in comparison with unstimulated cells (100%); *bars,* SE. Statistical analysis was performed using paired *t* test (*, $P < 0.01$; **, $P < 0.001$).

samples) due to cytokine-treatment was found in CD138+ cells in four of six samples, whereas the cell fraction depleted of CD138+ cells did not show any change in migration. Myeloma cells of patient MM4 showed only weak enhancement (126%), and migration of myeloma cells of patient MM6 were even blocked by TNF-α treatment. These results show that the mechanism of migrational enhancement due to TNF-α could also be operative in a subset of native myeloma cells.

Because myeloma cell lines expressed TNF-R2, we performed immunohistochemical analysis of TNF-R2 on slides of bone marrow from the six myeloma patients tested above. Myeloma cells infiltrating the bone marrow of these patients showed a positive immunostaining. This finding was supported by the results of flow cytometry analysis of magnetic cell-sorting CD138+ selected myeloma cells from additional patients. We found that these cells expressed TNF-R2 and CCR2 but not TNF-R1 (data not shown).

Discussion

Multiple myeloma is characterized by a massive infiltration of the bone marrow by end-stage plasma cells and many but not all clinical symptoms are caused by this infiltration. It is a matter of discussion whether these cells by themselves can account for the malignancy and spread of the disease, or whether a pool of less mature cells that are able to circulate, disseminate, and differentiate into plasma cells are responsible for disease establishment and progression (22, 23). Experiments in mice models suggest that disease establishment and progression might be attributed to tumor cells at various differentiation stages (22,

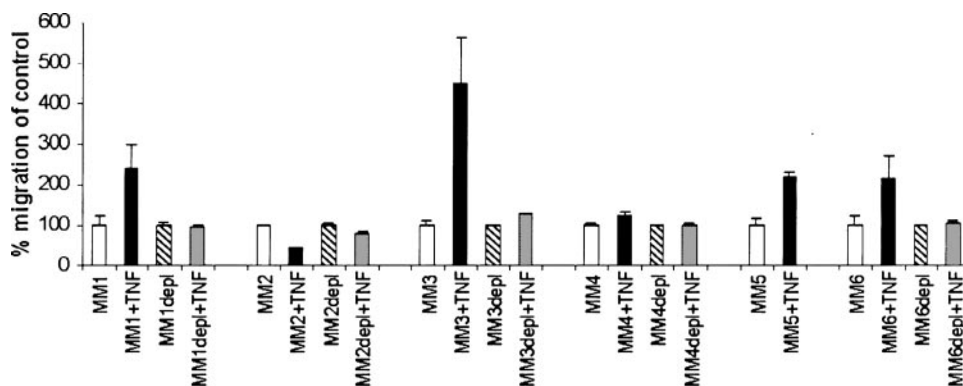

Fig. 7 Migration of CD138+ myeloma cells from the bone marrow of patients. We compared the migrational behavior of CD138+ selected cells without (*0, white columns*) and with (*+TNF, black columns*) tumor necrosis factor α (TNF-α) stimulation from six myeloma patients (*MM1–MM6*). Additionally, the CD138 fraction of the same bone marrow cells were tested without (*hatched columns*) and with TNF-α stimulation (*+TNF, gray columns*) for all of the patients except patient MM5. Percentage of migration is shown in comparison with migration of unstimulated cells (*numbers on the vertical axis,* percentage of migration of control; *bars,* ±SD). Analysis was performed in duplicate.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

24–26). In patients, the number of peripheral monoclonal plasma cells has been shown to predict survival (27). Furthermore, the number of malignant precursor cells in mobilized peripheral blood stem cell harvests has also been inversely correlated with the duration of disease-free intervals (28). However, it cannot be excluded that in these patients, disease progression might also be due to a small fraction of therapy-resistant plasma cells below detection levels. In this case, mechanisms boosting plasma cell growth and migration might have great impact on disease progression and spread.

In our experiments, we could show that myeloma cell lines as well as native end-stage plasma cells, *i.e.,* CD138+ cells from the bone marrow of patients, were able to migrate through a human bone marrow endothelial cell monolayer and that this migration was enhanced in response to TNF-α. The primary signal is probably delivered via TNF-R2 because myeloma cell lines did not express TNF-R1, the second and higher-affinity receptor for TNF-α. Moreover, the extent of migration correlated with the extent of surface TNF-R2 expression. Native myeloma cells infiltrating the bone marrow were also shown to express TNF-R2. In additional experiments, we found that there was no enhanced migration of tumor cells of other hematopoietic origin that displayed both receptors on their surface in response to TNF-α. This points to a possible interference of the two TNF-receptor pathways, with TNF-R1 dominating or even suppressing TNF-R2 pathways. Thus, it appears that lack of TNF-R1 in myeloma cells would allow migrational response. In fact, native myeloma cells exclusively expressed TNF-R2, and migration of native cells was clearly enhanced by cytokine stimulation in four of six patients and to a lesser degree in one patient. All in all, we found that enhanced migration after TNF-α treatment correlated with the rate of spontaneous migration, suggesting a predisposition of the patients' cells to migrate by, *e.g.,* the existence of an autocrine TNF-α loop. In addition, microenvironmental factors might contribute to such a predisposition. This might explain the complete unresponsiveness of myeloma cells of patient MM2; in fact in this patient, there was decreased migration of cells after cytokine treatment. The tumor-supporting interplay between the bone marrow microenvironment and myeloma cells awaits further elucidation.

To investigate the molecules involved in TNF-α-induced migration, we analyzed expression of adhesion molecules and MMPs that has been reported to play a role in myeloma cell migration. At the RNA level as well as in functional experiments, we could not find an involvement of these molecules in the enhanced migration of myeloma cells. It has been observed previously that murine myeloma cells, isolated from a mouse model, acquired MMP-9 activity (29). However, because in our experiments, the induction of migration via TNF-α stimulation was not dependent on the availability of an endothelial cell monolayer or the presence of fibronectin, the regulation of adhesion molecules and/or activity of metalloproteases might not play an essential role in this process. Using microarray analysis, we found that TNF-α-induced up-regulation of MCP-1 in myeloma cells correlated with migration, and blocking MCP-1 abrogated the enhanced migration induced by TNF-α stimulation. Migration of myeloma cells has previously been correlated with MCP-1, -2, and -3 in a mouse model and also in an *in vitro* model (21, 30). In these investigations, the authors showed that MCP-1, MCP-2, and MCP-3, produced by endothelial cells and/or bone marrow stromal cells, promoted the directed migration of myeloma cells in response to chemotactic gradients, which would explain the restricted localization of the tumor. In our experiments, using RPMI-8226 and U266 cell lines, we could not confirm these observations regarding chemotactic properties. This might point to differences in the chemotactic responses between murine and human myeloma cells or to the different chemotactic responsiveness of various cell lines. Although MCP-1 was clearly not chemotactic in our experimental set-up, transmigration induced by it was apparently caused by chemokinesis of the cells, a finding that has not been described previously. Different from chemotaxis, *i.e.,* migration in the direction of a chemokine gradient, this induction of MCP-1 enhances a migrational capacity of the myeloma cells that is not necessarily directed. Autocrine chemokine-loops in myeloma cells themselves might allow the cells to interfere with the chemokine gradients presented in the microenvironment. It has been reported that bone marrow stromal cells produce MCP-1, -2, and –3 (21). Our microarray data showed that U266 did not express MCP-2 and did not regulate MCP-3 and MCP-4 in response to TNF-α, and that only MCP-1 was under the regulation of this cytokine. This points to a pivotal role of autocrine MCP-1 and the TNF-α/MCP-1 loop in regulating the migrational activity of myeloma cells. *In vivo*, additional mechanisms might play a role and might potentiate the effect that we observed *in vitro*. For example, MCP-1-induced up-regulation of MMP-9 as a result of TNF-α stimulation in peripheral blood monocytes has been reported previously (31). A prerequisite for this effect was the autocrine up-regulation and production of TNF-α. We could show that myeloma cells can, indeed, up-regulate autocrine cytokine production, and, although TNF-α did not influence metalloprotease production in the time frame examined, the long-term *in vivo* stimulation might in fact stimulate metalloprotease expression and, thus, modulate the migratory capacity of the cells. Previous examinations of sdf-1-induced migration on high-grade non-Hodgkin lymphoma in a xenograft mouse model have shown that antibody strategies targeting the migrational process may delay disease progression and prolong overall survival of the animals (32). In theory, the TNF-α/MCP-1 loop described here suggests a hierarchical model favoring the targeting of TNF-α. In fact, thalidomide, which dramatically down-regulates TNF-α, has been shown to simultaneously reduce tumor burden and provide clinical benefit to myeloma patients (33). It remains to be determined whether, at least in part, thalidomide acts via the suppression of migration via the mechanisms described in this article. Although anti-TNF-R2 targeting strategies might be feasible from a therapeutic point of view, our preliminary experiments showed an enhancement rather than an inhibition of transmigration. This might be caused by a signaling capacity of the anti-TNF-R2 antibody. In fact, in a recent study, anti-TNF-R2 antibodies in myeloma patients failed to show tumor-suppressing effects but were associated with elevated levels of TNF-α (34). It will be interesting to investigate whether the mechanisms involved include the regulation of MCP-1 also. Although our data seem to suggest that successful suppression of TNF-α would also decrease MCP-1 levels and effects, it must be kept in mind that MCP-1 and TNF-α might be produced by different cell types,

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

are differentially regulated by the cytokine network, and also exert pleiotropic biological effects. MCP-1 activity has also been shown to contribute to neovascularization (35, 36) and immune suppression (37) in other types of malignant disease. Furthermore, in some of these tumor types, high MCP-1 levels have been correlated with advanced disease (38). A promising avenue for research for the future, therefore, is investigation of the net effect on growth, expansion, and/or immunological control of myeloma cells exerted by differential or combined blocking of TNF-α and/or MCP-1. The clear-cut inhibitory effects of anti-MCP-1 antibodies on the suppression of myeloma cell transmigration support the potential power of antibody-strategies.

Acknowledgments

We thank Dr. C. E. van der Schoot (Central Laboratory, Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) for providing the cell line HBMEC-60.

References

1. Van Riet I, Vanderkerken K, De Greef C, Van Camp B. Homing behaviour of the malignant cell clone in multiple myeloma. Med Oncol 1998;15:154–64.

2. Drost EM, MacNee W. Potential role of IL-8, platelet-activating factor and TNF-α in the sequestration of neutrophils in the lung: effects on neutrophil deformability, adhesion receptor expression, and chemotaxis. Eur J Immunol 2002;32:393–403.

3. Ebert EC. Tumour necrosis factor-α enhances intraepithelial lymphocyte proliferation and migration. Gut 1998;42:650–5.

4. Corcione A, Ottonello L, Tortolina G, et al. Recombinant tumor necrosis factor enhances the locomotion of memory and naive B lymphocytes from human tonsils through the selective engagement of the type II receptor. Blood 1997;90:4493–501.

5. Hornung F, Scala G, Lenardo MJ. TNF-α-induced secretion of C-C chemokines modulates C-C chemokine receptor 5 expression on peripheral blood lymphocytes. J Immunol 2000;164:6180–7.

6. Sati HI, Greaves M, Apperley JF, Russell RG, Croucher PI. Expression of interleukin-1β and tumour necrosis factor-α in plasma cells from patients with multiple myeloma. Br J Haematol 1999;104:350–7.

7. Jourdan M, Tarte K, Legouffe E, Brochier J, Rossi JF, Klein B. Tumor necrosis factor is a survival and proliferation factor for human myeloma cells. Eur Cytokine Netw 1999;10:65–7.

8. Sawamura M, Murakami H, Tsuchiya J. Tumor necrosis factor-α and interleukin 4 in myeloma cell precursor differentiation. Leuk Lymphoma 1996;21:31–6.

9. Thomas X, Anglaret B, Magaud JP, Epstein J, Archimbaud E. Interdependence between cytokines and cell adhesion molecules to induce interleukin-6 production by stromal cells in myeloma. Leuk Lymphoma 1998;32:107–19.

10. Hideshima T, Chauhan D, Schlossman R, Richardson P, Anderson KC. The role of tumor necrosis factor α in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene 2001;20: 4519–27.

11. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P. Tumor necrosis factor type α, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. Proc Natl Acad Sci USA 1987; 84:5277–81.

12. Lichtenstein A, Berenson J, Norman D, Chang MP, Carlile A. Production of cytokines by bone marrow cells obtained from patients with multiple myeloma. Blood 1989;74:1266–73.

13. Jurisic V, Colovic M. Correlation of sera TNF-α with percentage of bone marrow plasma cells, LDH, β2-microglobulin, and clinical stage in multiple myeloma. Med Oncol 2002;19:133–9.

14. Wallach D. TNF ligand and TNF/NGF receptor families. *In:* Oppenheim JJ, Feldmann M, editors. Cytokine reference. Orlando, FL: Academic Press; 2000. p. 377–412.

15. Aggarwal B, Samanta A, Feldmann M. The TNF family, *In:* Oppenheim J, Feldmann M, editors. Cytokine reference. Orlando, FL: Academic Press; 2000. p. 413–34.

16. Grell M, Douni E, Wajant H, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell 1995;83:793–802.

17. Rood PM, Calafat J, von dem B, Gerritsen WR, van der Schoot CE. Immortalisation of human bone marrow endothelial cells: characterisation of new cell lines. Eur J Clin Invest 2000;30:618–29.

18. Rood PM, Gerritsen WR, Kramer D, Ranzijn C, von dem B, van der Schoot CE. Adhesion of hematopoietic progenitor cells to human bone marrow or umbilical vein derived endothelial cell lines: a comparison. Exp Hematol 1999;27:1306–14.

19. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem 1980;102:196–202.

20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

21. Broek IV, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, Van R, I. Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3. Br. J Cancer, 2003;88:855–62.

22. Pilarski LM, Hipperson G, Seeberger K, Pruski E, Coupland RW, Belch AR. Myeloma progenitors in the blood of patients with aggressive or minimal disease: engraftment and self-renewal of primary human myeloma in the bone marrow of NOD SCID mice. Blood 2000;95: 1056–65.

23. Yaccoby S, Barlogie B, Epstein J. Primary myeloma cells growing in SCID-hu mice: a model for studying the biology and treatment of myeloma and its manifestations. Blood 1998;92:2908–13.

24. Urashima M, Chen BP, Chen S, et al. The development of a model for the homing of multiple myeloma cells to human bone marrow. Blood 1997;90:754–65.

25. Yaccoby S, Epstein J. The proliferative potential of myeloma plasma cells manifest in the SCID-hu host. Blood 1999;94:3576–82.

26. Pilarski LM, Belch AR. Clonotypic myeloma cells able to xenograft myeloma to nonobese diabetic severe combined immunodeficient mice copurify with CD34 (+) hematopoietic progenitors. Clin Cancer Res 2002;8:3198–204.

27. Witzig TE, Gertz MA, Lust JA, Kyle RA, O'Fallon WM, Greipp PR. Peripheral blood monoclonal plasma cells as a predictor of survival in patients with multiple myeloma [see comments]. Blood 1996;88: 1780–7.

28. Mitterer M, Oduncu F, Lanthaler AJ, et al. The relationship between monoclonal myeloma precursor B cells in the peripheral blood stem cell harvests and the clinical response of multiple myeloma patients. Br J Haematol 1999;106:737–43.

29. Van Valckenborgh E, Bakkus M, Munaut C, et al. Upregulation of matrix metalloproteinase-9 in murine 5T33 multiple myeloma cells by interaction with bone marrow endothelial cells. Int. J Cancer 2002;101: 512–8.

30. Vanderkerken K, Van de Broek I, Eizirik DL, et al. Monocyte chemoattractant protein-1 (MCP-1), secreted by bone marrow endothelial cells, induces chemoattraction of 5T multiple myeloma cells. Clin Exp Metastasis 2002;19:87–90.

31. Robinson SC, Scott KA, Balkwill FR. Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-α. Eur J Immunol 2002;32:404–12.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023

32. Bertolini F, Dell'Agnola C, Mancuso P, et al. CXCR4 neutralization, a novel therapeutic approach for non-Hodgkin's lymphoma. Cancer Res 2002;62:3106–12.
33. Kumar S, Gertz MA, Dispenzieri A, et al. Response rate, durability of response, and survival after thalidomide therapy for relapsed multiple myeloma. Mayo Clin Proc 2003;78:34–9.
34. Tsimberidou AM, Waddelow T, Kantarjian HM, Albitar M, Giles FJ. Pilot study of recombinant human soluble tumor necrosis factor (TNF) receptor (p75) fusion protein (TNFR:Fc; Enbrel) in patients with refractory multiple myeloma: increase in plasma TNF α levels during treatment. Leuk Res 2003;27:375–80.
35. Ohta M, Kitadai Y, Tanaka S, et al. Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Cancer 2002;102:220–4.
36. Salcedo R, Ponce ML, Young HA, et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 2000;96:34–40.
37. Karpus WJ, Kennedy KJ, Kunkel SL, Lukacs NW. Monocyte chemotactic protein 1 regulates oral tolerance induction by inhibition of T helper cell 1-related cytokines. J Exp Med 1998;187: 733–41.
38. Hefler L, Tempfer C, Heinze G, et al. Monocyte chemoattractant protein-1 serum levels in ovarian cancer patients. Br. J Cancer 1999; 81:855–9.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/10/6/1901/1956007/1901.pdf by guest on 30 November 2023