Stimulation by Monocyte Chemoattractant Protein-1 Modulates the *Ex-vivo* Colony Formation by Head and Neck Squamous Cell Carcinoma Cells

GUNNAR WICHMANN*, CAROLIN KÖRNER*, ANDREAS BOEHM, CHRISTIAN MOZET and ANDREAS DIETZ

Department of Otolaryngology, Head and Neck Surgery, University of Leipzig, Leipzig, Germany

Abstract. *Background: The outcome of patients with head and neck squamous cell carcinoma (HNSCC) is still poor. To improve therapy of HNSCC, biomarkers indicating progression of the disease or modifiers with potential as therapeutic targets and therapy need to be investigated. Since monocyte chemoattractant protein (MCP1) is potentially involved in tumorigenesis of HNSCC, we aimed to clarify its role in HNSCC and investigated the influence of stimulation by MCP1 and its depletion using antibodies against MCP1 (anti-MCP1) on colony formation by HNSCC cells. Materials and Methods: Biopsies of HNSCC were treated according to the protocol of the FLAVINO assay with cisplatin, docetaxel, temsirolimus or cilengitide alone, or combined with MCP1 or anti-MCP1. After a 72-h incubation, ethanol-fixed and fluoresceine-isothiocyanate (FITC)-labeled epithelial colonies were counted. Results: Colony formation was significantly suppressed by MCP1 and 3.3 μM cisplatin, while docetaxel, cilengitide and temsirolimus at concentrations of 0.275, 10 and 0.50 μM caused insignificant effects. Addition of MCP1 to cisplatin, docetaxel and cilengitide increased efficacy of cytostatics in inhibition of colony formation, whereas those with temsirolimus were increased by anti-MCP1 that when applied alone failed to modulate colony formation. Overall regarding facilitated chemosensitivity, there was a statistical trend in favor of MCP1 stimulation over depletion. Conclusion: Our ex vivo results show context-dependent effects of MCP1 in HNSCC cells. An increase of MCP1 level or its addition to cisplatin, docetaxel and cilengitide reduce colony formation but the efficacy of temsirolimus is augmented by MCP1 depletion. These context-dependently opposite outcomes call for further translational investigations in HNSCC.*

Malignancies of the head and neck, particularly in the mouth and throat were the fifth most common types of cancer in Germany in 2008. These tumors were mainly squamous cell carcinonomas (HNSCC) (1). Although therapies of advanced HNSCC have changed to increased use of multi-modal strategies combining surgery and radiotherapy or radiochemotherapy, the outcome regarding 5-year survival has not improved significantly (2). To improve treatment outcome and earlier detection of relapse, the detection of new biomarkers or even targets of therapy is needed therefore we started to search for biomarkers that might be of value if they are associated with chemoresponse or progression of disease, or an increasing potential for metastatic spreading.

There is a huge body of evidence that cytokines play an important role in the tumor environment (3) and are involved in tumor progression and metastasis. Consequently, this investigation focused on monocyte chemoattractant protein-1 (MCP1) purported to contribute to tumor growth and angiogenesis, and metastatic spread of HNSCC. MCP1 is a pleiotropic CC-chemokine and a central chemoattractor for monocytes and macrophages. In colonic carcinomas, MCP1 mediates metastatic spread *via* increasing vascular permeablility (4). In patients with squamous cell carcinomas of the esophagus, increased MCP1 level predicts poor prognosis (5). HNSCC cells also show production of MCP1 (6) and increased levels of MCP1 are detected in sera of patients with advanced stages of HNSCC and in particular in those with metastases (7). Through modulation of pro-survival signaling in HNSCC, MCP1 may promote progression of cancer (8). Investigations concerning MCP1 in HNSCC are still rare and studies about the effects of MCP1 on response to chemotherapy to the best of our knowledge are lacking.

*These Authors contributed equally to this study.

Correspondence to: Dr. Gunnar Wichmann, Ph.D., ENT-Research Laboratory, Department of Otolaryngology, Head and Neck Surgery, University of Leipzig, Germany, Liebigstrasse 21, 04103 Leipzig, Germany. Tel: +49 3419721926, Fax: +49 3419721909, e-mail: gunnar.wichmann@medizin.uni-leipzig.de, http://www.uni-leipzig.de/~hno/

Key Words: MCP1, anti-MCP1, HNSCC, FLAVINO assay, *ex-vivo* chemoresponse.

Table I. *Characteristics of patients included in this investigation.*

Patient ID	Age (years)	Gender	Location	T	N	M	Stage (UICC)	Grading	Alcohol (0=no, 1=yes)	Tobacco (0=no, 1=yes)
1	72	Male	Larynx	2	0	0	II	2	0	1
2	65	Male	Larynx	2	2b	0	IVA	3	1	1
3	74	Male	Larynx	4a	1	0	IVA	2	0	0
4	50	Male	Oropharynx	y1	y2b	y0	IVA	3	0	1
5	82	Male	Oropharynx	3	2b	0	IVA	*	0	1
6	50	Male	Hypopharynx	2	1	0	III	2	0	1
7	50	Female	Nasopharynx	4a	0	0	IVA	3	1	0
8	42	Male	Oral cavitiy	4a	0	0	IVA	3	0	0
9	50	Male	Oropharynx	3	0	0	III	3	1	1
10	73	Male	Oropharynx	3	2b	0	IVA	2	0	0
11	56	Male	Oropharynx	4a	2b	0	IVA	3	1	1
12	50	Female	Larynx	3	2c	0	IVA	2	*	1
13	58	Male	Oropharynx	4a	2c	0	IVA	3	0	1
14	70	Female	Oropharynx	4a	0	0	IVA	3	0	0

*There was no information concerning grading of cancer of patient number 5 and alcohol consumption of patient number 12. UICC: Unified International Classification of Cancer (14).

MCP1 is reported to play a key role in the tumor microenvironment *via* activating monocytes and their production of growth factors such as vascular endothelial growth factor (VEGF), thus promoting angiogenesis and tumor progression. On the other hand, MCP1 may play an important role in the tumor defense of the host and in processes of tumor development *via* inducing monocytes to produce a number of other growth factors (9). Therefore, it can be hypothesized that the concentration of MCP1 is critically involved in the decision of whether the neoplasm is combated by monocytes/macrophages or whether growth factors produced by tumor-associated macrophages contribute to angiogenesis and tumor growth. Low concentrations of MCP1 may preferentially lead to tumor growth, whereas high amounts of MCP1 might be able to attract a huge number of monocytes/macrophages and further their interaction with tumor-infiltrating T-cells, resulting in destruction of the tumor (10). We aimed to clarify the role of MCP1 utilizing the FLAVINO assay, an *ex vivo* clonogenic colony-formation assay, comparing the impact not only of stimulation of HNSCC by MCP1 but also its depletion by an antibody against MCP1 (anti-MCP1) on the *ex vivo* chemoresponse of HNSCC cells. The HNSCC cells were treated with MCP1 or anti-MCP1 alone or combined with cytostatics namely cisplatin and docetaxel, which are widely used in clinical practice, and cilengitide and temsirolimus, as promising new agents. Cilengitide is a novel integrin inhibitor (11). Temsirolimus is an inhibitor of mammalian target of rapamycin (mTOR) (12) which has an anti-angiogenic potential, particularly in hypoxic solid tumors (13).

Materials and Methods

Patients. After receiving each patient's informed consent, biopsies of HNSCC were taken during panendoscopy or definitive surgical treatment procedures under general anesthesia. After collecting the specimens in tubes containing tumor medium supplemented with antimycotics and antibiotics (see below), the specimens were immediately transferred to the ENT Research Laboratory of the Department of Otolaryngology, Head and Neck Surgery, University of Leipzig, where the FLAVINO assay, an *ex vivo* colony-formation assay was performed. A total of 32 histopathologically confirmed HNSCCs were included in this investigation. Table I shows the characteristics of 14 out of the 32 patients with HNSCC fulfilling the inclusion criteria regarding sufficient colony formation and which were therefore included in this study. Information about age, sex, location of the primary tumor, tumor stage according to the TNM and UICC classification (14), risk factors in the patient's lifestyle regarding alcohol consumption and tobacco smoking, as well as the type of specimen are shown.

Materials. A combination of flavin-free RPMI-1640 medium, 1% glutamine (200 mM; Biochrom, Berlin, Germany), 10% fetal calf serum (Invitrogen, Darmstadt, Germany), 2% amikacin (Fresenius Kabi, Bad Homburg, Germany), 2% nystatin (Sigma-Aldrich, Steinheim, Germany) and 1% penstrep (penicillin 10,000 U/ml, streptomycin 10,000 μg/ml; Invitrogen) was used as tumor medium for cell culture and for preparing dilutions of cytostatics. Flavin-free RPMI-1640 medium, 1% glutamine and 10% fetal calf serum was used for KB cell culture. Cisplatin was purchased from Sigma-Aldrich (Steinheim, Germany). Docetaxel (Taxotere) was acquired from Sanofi Aventis (Frankfurt, Germany) as pharmaceutical preparation. Cilengitide (EMD 121974) was made available by Merck (Darmstadt, Germany). Temsirolimus (Torisel) was from Wyeth® (Münster, Germany). The anti-MCP1 (rabbit polyclonal antibody) and recombinant human MCP1 (rh-MCP1) were from

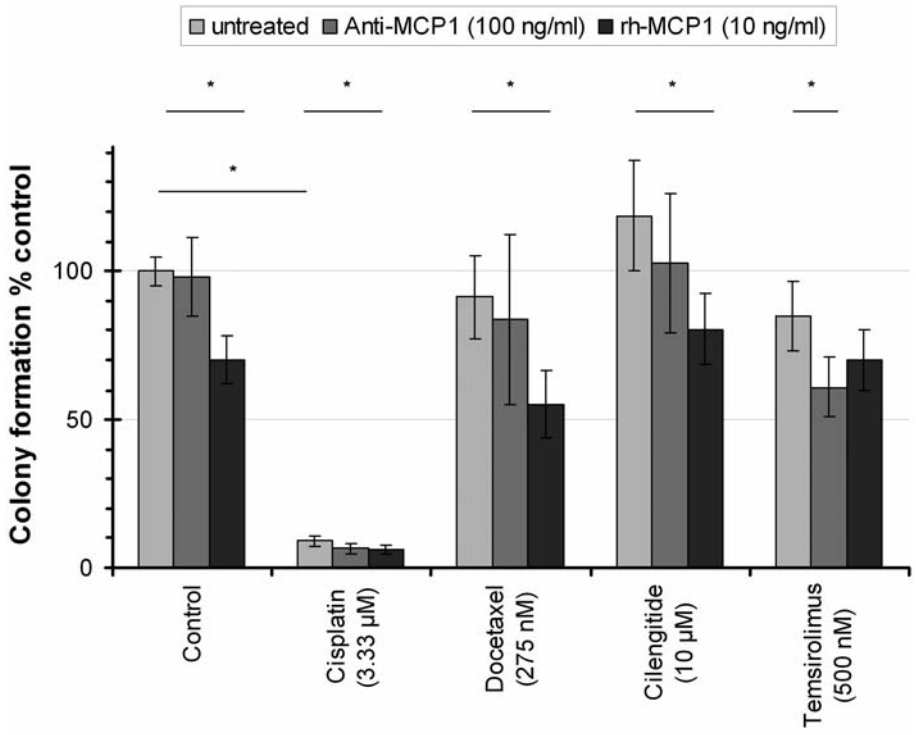

Figure 1. *Effect of rh-MCP1 and anti-MCP1 on colony formation of primary head and neck squamous cell carcinoma (HNSCC) cells. Cisplatin ($p=5.1\times10^{-36}$) and MCP1 (p=0.0020) significantly suppressed colony formation compared to untreated controls. Docetaxel (p=0.5699), cilengitide (p=0.3507) and temsirolimus (p=0.2445) modulated colony formation insignificantly. There was only a statistical trend between the efficacy of MCP1 stimulation and inhibition (p=0.0798). Asterisks indicate significant (p<0.05) reduced relative colony formation in the comparison between treatments indicated.*

Relia Tech (Wolfenbüttel, Germany). Cytostatics and antibodies were diluted with tumor medium to achieve final drug dilutions (3.33 μM cisplatin, 0.275 μM docetaxel, 10 μM cilengitide, 0.5 μM temsirolimus).

FLAVINO assay. The FLAVINO assay is a quality-controlled clonogenic colony-forming assay carried out under flavin-protecting conditions (lamps with wavelength λ=589 nm) to assess the response of HNSCC cells to a treatment to be tested (15). For quality control, KB cells were tested simultaneously with HNSCC cells from each patient under the same conditions and using the same dilutions applied to the primary HNSCC cells. The cell line KB originates from an epidermoid cancer of a Caucasian that was overgrown by an epithelial cell line originating from a cervical adenocarcinoma, known as HeLa (16, 17).

At the laboratory, the HNSCC specimens were weighed and minced into pieces of about 1 mm^3. Collagenase type IV (230 mU/ml) was added to the pre-incubated specimen before further incubation for 16 h under standard conditions (36.5˚C, 3.5% CO_2, humidified air). The digestion was stopped by centrifugation for 10 min at 300 ×g, the pellet was carefully re-solved in tumor medium. Assays were carried out in 48-well cell culture plates coated with collagen I, fibronectin, and laminin (all from Roche, Roche Diagnostics GmbH, Mannheim, Germany). Collagenase-IV digested HNSCC sample (2 mg) were added into wells already containing the indicated concentrations of cisplatin, docetaxel, cilengitide or temsirolimus alone, or combined with rh-MCP1, or anti-MCP1, or remained untreated as controls. After incubation for 72 h under standard conditions, 400 μl of supernatants for ELISA measurements were aspirated and frozen at −20˚C. Plates were carefully washed with phosphate-buffered saline and fixed with ethanol at 40% and 90% before air drying. Epithelial cells were stained using a pan-cytokeratin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and a Cy2-labeled anti-murine antibody (Jackson ImmunoResearch, Newmarket, Suffolk, UK). Fluorescent colonies (at least a conglomeration of six tumor cells) were counted using a Zeiss Axiovert 200M (Zeiss, Jena, Germany) and HNSCC were judged to be evaluable if at least four colonies were counted in each well of the respective untreated controls. Due to large heterogeneity in colony formation of individual HNSCCs, all colony counts were normalized to the mean colony formation in untreated controls and hence are expressed as relative values (% of controls).

Statistical investigations. For statistical analysis, Microsoft EXCEL 2003 (Microsoft Germany, Unterschleißheim, Germany), WinSTAT® (R. Fitch Software, Bad Krozingen, Germany), SPSS version 20 (IBM Corporation, Armonk, New York), and the *t*-test for paired samples were used. Differences were regarded significant at $p<0.05$. A modified probability sum test was used to investigate the mode of interaction of MCP1 stimulation and depletion in their combinations with the chemotherapeutics and to assess if their effects on the chemotherapeutics were best described as antagonism, additivity or

synergism. This was carried out as described earlier (18, 19) by use of the formula (Equation 1) to calculate the interaction quotient *q*:

$$q=(P_{AB})/(P_A+P_B - P_A \times P_B) \quad \text{(Eq. 1)}$$

in which P_{AB} is the observed effect of both compounds A and B, and P_A and P_B are the effects exerted by each of the compounds alone when applied at the same concentration as in combination. According to the model of independent action, additive effects are present whenever q lies within the interval between 0.85 and 1.15, while significant deviation from this model can be judged as synergism when q has higher values (q>1.15) or antagonism if q<0.85 (18, 19).

Results

Patient characteristics. Table I provides information concerning 14 patients and their carcinomas included in this investigation showing sufficient and homogeneous colony formation. Specimens of three female (21.4%) and 11 male (78.6%) patients were evaluable. The mean±SD age of patients was 60.1±12.2 years. HNSCCs were mainly localized in the oropharynx (n=7, 50%), four tumors had their origin in the larynx (28.6%), whereas one HNSCC each was located at the hypopharynx, nasopharynx and oral cavity (n=1, 7.1%). Thirteen HNSCCs were locally advanced tumors staged as UICC III (n=2, 14.3%) and UICC IVA (n=11, 78.6%), whereas only one HNSCC was early-stage (UICC II: n=1, 7.1%). There was no patient with HNSCC with distant metastasis (all M0), whereas nine had lymph node metastases (N1: n=2, 14.3%; N2b: n=4, 28.6%; yN2b: n=1, 7.1%; N2c: n=2, 14.3%). Consumption of alcohol (χ^2=0.133, p=0.715) and tobacco (χ^2=2.715, p=0.099) did not differ significantly between male and female patients.

Chemoresponse tests. A total of 16 out of the 32 tested HNSCC showed sufficient and homogeneous colony formation (≥4 colonies in untreated controls). One evaluable test was infected by *Candida* spp. and a first histopathologically-confirmed case of HNSCC was later found to be concomitant with non-Hodgkin's lymphoma and hence excluded from further analyses. Therefore only 14 out of 16 sufficiently colony-forming tests were included in the statistical analyses. KB cell tests gave homogeneous results regarding the half-maximal inhibitory concentration (IC_{50}) of the compounds tested (data not shown), consequently all of the 14 remaining *ex vivo* chemoresponse tests were carried out correctly. Figure 1 shows the mean and 95% confidence intervals of colony formation data, while Table II shows additionally p and q values for comparisons in the FLAVINO assay.

Cisplatin. At 3.33 μM, half the maximum tolerable plasma level (20), cisplatin reduced colony formation significantly (mean relative formation=9.14%, $p=5.1\times10^{-36}$). Addition of rh-MCP1 and anti-MCP1 led to further suppression of colony formation (Table II).

Table II. *Combinatory effects of cisplatin, docetaxel, cilengitide and temsirolimus with recombinant human MCP1 (rh-MCP1, 10 ng/ml) and with anti-MCP1 (100 ng/ml) compared to solely applied treatments and untreated controls (TM) of primary head and neck squamous cell carcinoma (HNSCC) cells from 14 patients.*

n=14	TM	Mean (%)	Colony formation *p*-Value	*q*
	TM	100.00	–	–
	Anti-MCP1	98.10	0.8954	–
	rh-MCP1	70.16	0.0020	–
Cisplatin (3.33 μM)[#]	Control	9.14	5.1×10^{-36}	–
	Anti-MCP1	6.40	2.5×10^{-8}	1.028[b]
	rh-MCP1	6.24	5.3×10^{-10}	1.002[b]
Docetaxel (275 nM)[$]	Control	91.31	0.5699	–
	Anti-MCP1	83.88	0.8227	1.546[c]
	rh-MCP1	55.30	0.0563	1.244[c]
Cilengitide (10 μM)	Control	118.66	0.3507	–
	Anti-MCP1	102.59	0.6060	0.158[a]
	rh-MCP1	80.33	0.0975	1.175[a]
Temsirolimus (500 nM)	Control	84.76	0.2445	–
	Anti-MCP1	60.84	0.1352	2.323[c]
	rh-MCP1	70.13	0.3642	0.737[a]

Mean relative to individual untreated control of epithelial colonies formed within 3 days, p-values in the t-test for paired samples, and assessment of the mode of interaction of binary mixtures as estimated using the quotient q according to Mozet et al. (18) and Stöhr et al. (19). Half-maximum tolerable plasma level according to [#]Desoize *et al*. (20); [$]Bissett *et al*. (21); [a]antagonism (q<0.85) or adverse effects of the tested substances; [b]additivity (0.85≤q≤1.15); [c]synergism (q>1.15).

Docetaxel. At 275 nM, half the maximum tolerable plasma level (21), docetaxel reduced colony formation only slightly (mean relative formation=91.3%, p=0.5699). rh-MCP1 and anti-MCP1 reduced colony formation significantly further (Table II).

Cilengitide. Under the influence of cilengitide, an insignificant stimulation of colony formation (mean relative formation= 118.66%, p=0.3507) was observed. Addition of either rh-MCP1 or anti-MCP1 acted antagonistically with these effects and led to suppression of colony formation (Table II).

Temsirolimus. Colony formation was insignificantly reduced by temsirolimus (mean relative formation=84.76%, p=0.2445). When rh-MCP1 or anti-MCP1 were added to temsirolimus, the colony formation was more strongly reduced than by temsirolimus alone (Table II).

MCP1 treatment. Significant suppressive effects of rh-MCP1 on colony formation (mean relative formation=70.16%, p=0.0020) were detected. When rh-MCP1 was combined with cisplatin, it additively increased the suppression of colony

Table III. *p-Values in the t-test for paired samples for differences in colony formation of primary head and neck squamous cell carcinoma (HNSCC) treated with recombinant human MCP1 (rh-MCP1, 10 ng/ml) versus those treated with anti-MCP1 (100 ng/ml) in combination with chemotherapeutics.*

Agent	*p*-Value
TM	0.0798
Cisplatin (3.33 μM)[#]	0.9542
Docetaxel (275 nM)[$]	0.3762
Cilengitide (10 μM)	0.4193
Temsirolimus (500 nM)	0.5324

TM: Untreated control; half-maximum tolerable plasma level according to [#]Desoize *et al*. (20); [$]Bissett *et al*. (21).

formation (mean relative formation=6.24%; $p=5.287\times10^{-10}$; $q=1.002$). Synergy in reducing colony formation was observed for the combination of rh-MCP1 and docetaxel (mean relative formation=55.30%; $p=0.0563$; $q=1.244$), whereas antagonism was seen for rh-MCP1 and cilengitide (mean relative formation=80.33%; $p=0.0975$) and temsirolimus (mean relative formation=70.13%; $p=0.3642$) in colony formation (since cilengitide or temsirolimus at the applied concentrations actually stimulated colony formation).

Anti-MCP1 treatment. The treatment with anti-MCP1 alone failed to influence colony formation significantly (mean relative formation 98.10%, $p=0.8954$). Additive effects regarding reduction of colony formation were observed for anti-MCP1 combined with cisplatin (mean relative formation=6.40%; $p=2.524\times10^{-8}$; $q=1.028$). The combination of anti-MCP1 and docetaxel (mean relative formation= 83.88%; $p=0.8227$; $q=1.546$) and temsirolimus (mean relative formation=60.84%; $p=0.1352$; $q=2.323$) was synergistic regarding suppression of colony formation, whereas antagonism was detected for anti-MCP1 combined with cilengitide (mean relative formation=102.59%; $p=0.6060$). The increased colony formation that was seen under cilengitide alone was abrogated by combinations of cilengitide and anti-MCP1.

The effects of MCP1 and anti-MCP1 on colony formation did not differ significantly ($p=0.0798$; Table III). This outcome might be related not only to the heterogeneity of HNSCC but also to the very slightly different effects of MCP1 and its depletion, and to the small number of samples. However, there was a trend for stronger suppression of colony formation by rh-MCP1 in otherwise untreated HNSCC cells and in its combination with cisplatin, docetaxel and cilengitide (Figure 1). Of particular interest was the impact on colony formation of HNSCC cells of the combination of anti-MCP1 and temsirolimus (mean relative formation=60.84%; $p=0.1352$; $q=2.323$). This combination led to higher efficacy regarding colony suppression than did the combination of rh-MCP1 and temsirolimus (mean relative formation=70.13%; $p=0.3642$; $q=0.737$).

Chemoresponse of HNSCC differs by individual. At the individual level, there were differences in chemoresponse of the tested HNSCCs regarding their chemoresponse and the impact of treatment with either rh-MCP1 or anti-MCP1. Contrary to the nearly uniform response of HNSCC cells to 3.33 μM cisplatin, the impact of other treatments was found to be much more heterogeneous and this heterogeneity increased when agents were in combination with rh-MCP1 or anti-MCP1. This implies the existence of HNSCC subgroups reacting to either rh-MCP1 or anti-MCP1 in different ways. Therefore an analysis of the impact of rh-MCP1 and of anti-MCP1 on the chemoresponse of HNSCC at the individual level was undertaken. Table IV shows that the chemosensitivity of individual HNSCCs often differs from the presented mean values and suggests that not all of the observations regarding combinatory effects may be true in general. For instance, a significant impact of rh-MCP1 on colony formation was observed in 5/14 HNSCCs whose colony formation was reduced. In 1/14 HNSCC, colony formation was significantly

Table IV. *Results of individually tested chemoresponses of 14 specimens. t-Test for paired samples was used for statistical analysis. Only significant differences (p-values <0.05) in stimulation (↑) and inhibition (↓) of colony formation are shown.*

	Patient ID													
Agent	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Anti-MCP1 (100 ng/ml)										↑				
rh-MCP1 (10 ng/ml)	↓			↓	↓			↓	↓					
Cisplatin (3.33 μM)[#]	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Docetaxel (275 nM)[$]		↓			↓			↓		↓	↓	↓		
Cilengitide (10 μM)					↓					↓				
Temsirolimus (500 nM)										↓				

rh-MCP1: recombinant human MCP1; half-maximum tolerable plasma level according to [#]Desoize *et al*. (20); [$]Bissett *et al*. (21).

stimulated by anti-MCP1. Cisplatin led to reduced colony formation in all tested HNSCCs, whereas significant colony suppression by docetaxel was observed in only six out of 14 specimens. Cilengitide reduced colony formation in two out of 14 HNSCCs, while temsirolimus reduced it in one of 14 specimens.

Discussion

The so far mostly unexplored role of MCP1 in HNSCC could not be clarified here in a general way, due to the large heterogeneity of primary HNSCCs in their response to rh-MCP1 stimulation or MCP1 depletion. On the one hand, we showed that MCP1 can improve the efficacy of standard pharmaceuticals such as cisplatin and docetaxel. On the other, we showed that MCP1 depletion can also improve the efficacy of cytostatics. This was significant for the combination of anti-MCP1 and temsirolimus, arguing for a role of MCP1 in the resistance of HNSCC to temsirolimus. The interaction of MCP1 and cytostatics was not uniform; it remains to be clarified if MCP1 expression might be a biomarker or a target in future therapies.

In general, cisplatin was the strongest inhibitor of colony formation of HNSCC *ex vivo*. This is due to the cytotoxic effects of cisplatin. Our results fit to experiences in clinical settings. Cisplatin is a standard pharmaceutical in chemotherapy of HNSCC. In locally advanced HNSCC, a local failure rate of 30% under cisplatin monotherapy is observed. The addition of 5-fluorouracil as PF (combination of cisplatin and 5-fluorouracil)-based therapy reduces the local failure rate (22). In our investigation, docetaxel insignificantly reduced colony formation. This is why we believe that docetaxel does not lead to benefit in monotherapy of HNSCC. Therefore, therapies for locally advanced HNSCC combine docetaxel with standard pharmaceuticals such as cisplatin and 5-fluorouracil. Addition of the taxane increases the 3-year survival rate in patients with locally advanced HNSCC compared to PF-based chemotherapy (23). A promising new agent seemed to be cilengitide, which is a selective antagonist of $\alpha_v\beta_3$-and $\alpha_v\beta_5$-integrins (11) since it contains the amino acid sequence Arg-Gly-Asp (RGD sequence) able to block the binding site required for their ligands and hence abrogates consecutive signaling (24). Integrin inhibitors such as cilengitide disrupt cell adhesion of tumor cells and tumor angiogenesis (25). The randomized, controlled phase I/II ADVANTAGE study was initiated to evaluate the efficacy and safety of cilengitide in recurrent/metastatic HNSCC in combination with cisplatin, 5-fluoruracil and cetuximab. In phase I, cilengitide combined with cisplatin and cetuximab was well tolerated (26). Reynolds *et al*. discussed integrin inhibitors such as cilengitide on the one hand as being promising new therapy options, but also noted that low concentrations of cilengitide can trigger angiogenesis *via* stimulation of VEGF release and thereby add to tumor growth and progression of the disease. These effects occur at concentrations of 0.2 to 20 nM (27). There might exist hormetic effects on cancer cells treated with low concentrations of cilengitide. Hormesis is an effect that is widely known in biology (28). Biological systems, and also tumor cells, exhibit biphasic dose relation curves, with increased proliferation at low doses and decreased growth at high doses. The adaptation of cells to their environment might be a possible reason for developing resistance towards treatment (29). We found that cilengitide failed to reduce colony formation; it even stimulated colony formation of HNSCC cells. It might, therefore, have a hormetic effect on HNSCC cells. We used 10 μM for the chemoresponse tests, a dose at which stimulatory effects (as Reynolds *et al*. mentioned) should not occur. Perhaps cilengitide was underdosed, but KB cell tests gave homogeneous results. In general, the efficacy of cilengitide should be considered critically. In other studies such as the phase III CENTRIC study in which cilengitide was tested together with temozolomide and radiation in patients suffering from glioblastoma (30, 31), cilengitide failed to improve overall survival of patients suffering from glioblastoma when added to standard therapy of temozolomide and radiation (32). Results of the phase II ADVANTAGE study also did not lead to any benefit in progression-free survival in patients with recurrent/metastatic HNSCC when cilengitide was combined with cisplatin, 5-fluorouracil and cetuximab compared with cisplatin, 5-fluoruracil and cetuximab alone (33).

Another promising drug is temsirolimus. Temsirolimus is an inhibitor of mTOR able to trigger apoptosis (12). It seems to be promising in recurrent/relapsed HNSCCs that were refractory to platin-based therapies. The primary end-point of a 12-week progression-free survival rate of 20% in the TEMHEAD study, a phase II single-arm study, was exceeded with 40% progression-free survival with temsirolimus (34, 35). In our study, temsirolimus reduced colony formation insignificantly. But synergy was observed when anti-MCP1 was added to temsirolimus (Figure 1). This effect might be caused by blocking signaling pathways. The inhibition of MCP1 signaling by a neutralizing antibody leads to inhibition of activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/ protein kinase B (Akt) (PI3K/AKT) pathway (36, 37). Downstream of this is mammalian target of rapamycin (mTOR), which is a serine/threonine kinase regulating many functions in the cell cycle and acting as a stimulator of transcription and translation. Temsirolimus and anti-MCP1 can act in a synergistic way because they lead to simultaneous inhibition of different steps in signaling. On the contrary, addition of rh-MCP1 to temsirolimus caused antagonism. Stimulation by rh-MCP1 significantly reduced colony formation of HNSCC *ex vivo*, which is unexpected since the effect of rh-MCP1 was stronger than that of docetaxel, cilengitide and temsirolimus alone. rh-MCP1 was the most powerful agent after cisplatin in reduction of colony formation by HNSCC cells in the FLAVINO assay (Figure 1).

In literature, there are contradictory opinions on the role of MCP1 in tumor defense and tumor growth. On the one hand, high amounts of MCP1 lead to tumor defense *via* activating tumor-associated macrophages. On the other hand, low concentrations of MCP1 lead to attraction of monocytes/macrophages, producing growth factors such as VEGF, promoting angiogenesis and tumor growth (10). MCP1 overexpression in HNSCC may promote tumor progression through up-regulation of pro-survival signaling pathways. Ji *et al*. describe a relationship between high cellular MCP1 expression and poor overall survival rate in patients with HNSCC. Therefore MCP1 might be a prognostic marker (8). MCP1 also seems to be a potential growth factor for stem cells *via* activating hypoxia-related genes and enhancing expression of pluripotent marker genes (38). However, Rollins and Sunday, and Manome *et al*. discussed the possibility of vaccination by MCP1 or MCP1-expressing tumor cells in cancer therapy (39, 40). Our observations show context-dependent tumor suppressive effects of MCP1. If added to cisplatin and docetaxel, MCP1 supported suppression of colony formation in an additive way. Therefore MCP1 might act as a supportive agent for cisplatin and docetaxel in chemotherapy.

In our investigation anti-MCP1 applied alone failed to significantly modulate colony formation *ex vivo*. This, however, is somewhat contradictory to findings of Salcedo *et al*. who described anticancer effects of an anti-MCP1 towards xenotransplanted human breast cancer cell lines in immunodeficient mice (41). But addition of anti-MCP1 to cisplatin, docetaxel and temsirolimus improved their colony-formation suppressive effects in an additive or even synergistic way. Therefore the depletion of MCP1 might also support chemotherapy. However, and since the biology of HNSCC is very heterogeneous, further studies concerning the role of MCP1 in HNSCC and its influence on cytokine production in HNSCC are needed.

Conclusion

Our data show that MCP1 can act as an effective drug in tumor defense, *ex vivo* improving the efficacy of standard pharmaceuticals used in therapy against HNSCC. However, we also showed that the inhibition of MCP1 can also improve the efficacy of cytostatics. It remains to be clarified if MCP1 expression might be a target in future therapies. We showed differences in chemoresponse of individual HNSCCs. Therefore, predictive chemoresponse tests are needed to decide which therapy is favorable for each patient individually. We believe that further investigations on MCP1 as chemoattractant of tumor-associated monocytes are needed to understand its role in tumorigenesis of HNSCC. Studies concerning modulation of cytokine expression, *e.g*. interleukin-6 and interleukin-8, by MCP1 and anti-MCP1 should follow.

References

1 Robert-Koch-Institut: Krebs in Deutschland 2007/2008. 8th ed. Berlin, 2012.

2 Guntinas-Lichius O, Wendt T, Buentzel J, Esser D, Lochner P, Mueller A, Schultze-Mosgau S and Altendorf-Hofmann A: Head and neck cancer in Germany: a site-specific analysis of survival of the Thuringian cancer registration database. J Cancer Res Clin Oncol *136(1)*: 55-63, 2010.

3 Mann EA, Spiro JD, Chen LL and Kreutzer DL: Cytokine Expression by Head and Neck Squamous Cell Carcinomas. Am J Surgery *164*: 567-573, 1992.

4 Wolf MJ, Hoos A, Bauer J, Boettcher S, Knust M, Weber A, Simonavicius N, Schneider C, Lang M, Stürzl M, Croner RS, Konrad A, Manz MG, Moch H, Aguzzi A, van Loo G, Pasparakis M, Prinz M, Borsig L and Heikenwalder M: Endothelial CCR2 Signaling Induced by Colon Carcinoma Cells Enables Extravasation *via* the JAK2-Stat5 and p38MAPK Pathway. Cancer Cell *22(1)*: 91-105, 2012.

5 Koide N, Nishio A, Sato TI, Sugiyama A and Miyagawa SI: Significance of Macrophage Chemoattractant Protein-1 Expression and Macrophage Infiltration in Squamous Cell Carcinoma of the Esophagus. Am J Gastroenterology *99(9)*: 1667–1674, 2004.

6 Heimdal JH, Olsnes C, Olofsson J and Aarstad HJ: Monocyte and monocyte-derived macrophage secretion of MCP-1 in co-culture with autologous malignant and benign control fragment spheroids. Cancer Immunol Immunothr *50*: 300-306, 2001.

7 Körner C, Wichmann G, Boehm A, Reiche A, Kehlen A, Hoffmann T, Herrmann K, Meilke L and Wichmann G: Monocyte chemoattractant protein-1 (MCP-1) in head and neck squamous cell carcinoma: evidence for its involvement in tumorigenenic processes? Eur Arch Otorhinolaryngol *269(4)*: 1368, 2012.

8 Ji WT, Chen HR, Lin CH, Lee JW and Lee CC: Monocyte Chemotactic Protein 1 (MCP1) Modulates Pro-Survival Signaling to Promote Progression of Head and Neck Squamous Cell Carcinoma. PLoS ONE *9(2)*: e88952, 2004 doi:10.1371/journal.pone.0088952.

9 Kirchner H, Kruse A, Neustock P and Rink L: *In*: Cytokine und Interferone. Botenstoffe des Immunsystems. Spektrum Akad. Verl. Heidelberg, 1994.

10 Nesbit M, Schaider H, Miller TH and Herlyn M: Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells. J Immunol *166(11)*: 6483-6490, 2001.

11 Alghisi G C, Ponsonnet L and Rüegg C: The integrin antagonist cilengitide activates alphaVbeta3, disrupts VE-cadherin localization at cell junctions and enhances permeability in endothelial cells. PLoS ONE *4(2)*: e4449, 2009.

12 Bjornsti MA, Houghton PJ: The TOR pathway: a target for cancer therapy. Nat Rev Cancer *4(5)*: 335-348, 2004.

13 Del Bufalo D, Ciuffreda L and Trisciuoglio D: Antiangiogenic Potential of the Mammalian Target of Rapamycin Inhibitor Temsirolimus. Cancer Res *66(11)*: 5549-5554, 2006.

14 Sobin LH, Gospodarowicz MK and Wittekind C: TNM classification of malignant tumours, 7th edn. Wiley-Blackwell, Oxford, 2009.

15 Dietz A, Tschöp K, Wichmann G and Granzow C: Method and kit for the *ex vivo* evaluation of the response of a tumor to conditions to be tested. Patent Cooperation Treaty (PCT) WO 2009/124997 A1, 2009.

16 Masters JRW: Human cancer cell lines: fact and fantasy. Nat Rev Mol Cell Biol *1*: 233-236, 2000.
17 Wichmann G, Horn IS, Boehm A, Mozet C, Tschöp K, Dollner R and Dietz A: Single tissue samples from head and neck squamous cell carcinomas are representative regarding the entire tumor's chemosensitivity to cisplatin and docetaxel. Onkologie *32(5)*: 7, 2009.
18 Mozet C, Stoehr M, Dimitrova K, Dietz A and Wichmann G: Hedgehog targeting by cyclopamine suppresses head and neck squamous cell carcinoma and enhances chemotherapeutic effects. Anticancer Res *33(6)*: 2415-2424, 2013.
19 Stoehr M, Mozet C, Boehm A, Aigner A, Dietz A and Wichmann G: Simvastatin suppresses head and neck squamous cell carcinoma *ex vivo* and enhances the cytostatic effects of chemotherapeutics. Cancer Chemother Pharmacol *73(4)*: 827-837, 2014.
20 Desoize B, Berthiot G, Manot L, Coninx P and Dumont P: Evaluation of a prediction model of cisplatin dose based on total platinum plasma concentration. Eur J Cancer *32A*: 1734-1738, 1996.
21 Bissett D, Setanoians A, Cassidy J, Graham MA, Chadwick GA, Wilson P, Auzannet V, Le Bail N, Kaye SB and Kerr DJ: Phase I and pharmacokinetic study of taxotere (RP 56976) administered as a 24-hour infusion. Cancer Res *53*: 523-527, 1993.
22 Seiwert TY and Cohen EEW: State-of-the-art management of locally advanced head and neck cancer. Br J Cancer *92(8)*: 1341-1348, 2005.
23 Haddad R, Tishler RB, Norris CM, Mahadevan A, Busse P, Wirth L, Goguen LA, Sullivan CA, Costello R, Case MA and Posner MR: Docetaxel, cisplatin, 5-fluorouracil (TPF)-based induction chemotherapy for head and neck cancer and the case for sequential, combined-modality treatment. Oncologist *8(1)*: 35-44, 2003.
24 Goodman SL, Hölzemann G, Sulyok GAG and Kessler H: Nanomolar small molecule inhibitors for alphav(beta)6, alphav(beta)5, and alphav(beta)3 integrins. J Med Chem *45(5)*: 1045-1051, 2002.
25 Weis SM, Cheresh DA (2011): v Integrins in Angiogenesis and Cancer. Cold Spring Harb Perspect Med *1(1)*: a006478, 2011. doi10.1101/cshperspect.a006478.
26 Vermorken JB, Guigay J, Mesia R, Trigo JM, Keilholz U, Kerber A, Bethe U, Picard M and Brummendorf TH: Phase I/II trial of cilengitide with cetuximab, cisplatin and 5-fluorouracil in recurrent and/or metastatic squamous cell cancer of the head and neck: findings of the phase I part. Br J Cancer *104(11)*: 1691-1696, 2011.
27 Reynolds AR, Hart IR, Watson AR, Welti JC, Silva RG, Robinson SD, Da Violante G, Gourlaouen M, Salih M, Jones MC, Jones DT, Saunders G, Kostourou V, Perron-Sierra V, Norman JC, Tucker GC and Hodivala-Dilke KM: Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. Nat Med *15(4)*: 392-400, 2009.
28 Mattson MP: Hormesis defined. Ageing Res Rev *7(1)*: 1-7, 2008.
29 Calabrese EJ and Nascarella MA: Tumor resistance explained by hormesis. Dose Resp *8(1)*: 80-82, 2010.
30 Stupp R, Hegi ME, Neyns B, Goldbrunner R, Schlegel U, Clement PM, Grabenbauer GG, Ochsenbein AF, Simon M, Dietrich PY, Pietsch T, Hicking C, Tonn JC, Diserens AC, Pica A, Hermisson M, Krueger S, Picard M and Weller M: Phase I/IIa study of cilengitide and temozolomide with concomitant radiotherapy followed by cilengitide and temozolomide maintenance therapy in patients with newly diagnosed glioblastoma. J Clin Oncol *28(16)*: 2712-2718, 2010.
31 Reardon DA and Cheresh D: Cilengitide: A Prototypic Integrin Inhibitor for the Treatment of Glioblastoma and Other Malignancies. Genes & Cancer *2(12):* 1159-1165, 2012.
32 Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, Aldape KD, Lhermitte B, Pietsch T, Grujicic D, Steinbach JP, Wick W, Tarnawski R, Nam DH, Hau P, Weyerbrock A, Taphoorn MJ, Shen CC, Rao N, Thurzo L, Herrlinger U, Gupta T, Kortmann RD, Adamska K, McBain C, Brandes AA, Tonn JC, Schnell O, Wiegel T, Kim CY, Nabors LB, Reardon DA, van den Bent MJ, Hicking C, Markivskyy A, Picard M and Weller M: Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071-22072 study): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol *15(10)*: 1100-1108, 2014.
33 Vermorken JB, Peyrade F, Krauss J, Mesía R, Remenar E, Gauler TC, Keilholz U, Delord JP, Schafhausen P, Erfán J, Brümmendorf TH, Iglesias L, Bethe U, Hicking C and Clement PM: Cisplatin, 5-fluorouracil, and cetuximab (PFE) with or without cilengitide in recurrent/metastatic squamous cell carcinoma of the head and neck: results of the randomized phase I/II ADVANTAGE trial (phase II part). Ann Oncol *25(3)*: 682-688, 2014.
34 ClinicalTrials.gov: Efficacy Study of Temsirolimus to Treat Head and Neck Cancer (TEMHEAD). NCT01172769. http://clinicaltrials.gov/show/NCT01172769, Last accessed on 24/04/2015.
35 Grünwald V, Keilholz U, Boehm A, Guntinas-Lichius O, Hennemann B, Schmoll HJ, Ivanyi P, Abbas M, Lehmann U, Koch A, Karch A, Zörner A and Gauler TC: TEMHEAD: a single-arm multicentre phase II study of temsirolimus in platin- and cetuximab refractory recurrent and/or metastatic squamous cell carcinoma of the head and neck (SCCHN) of the German SCCHN Group (AIO). Ann Oncol *26(3)*: 561-567, 2015.
36 Mellado M, Rodríguez-Frade JM, Vila-Coro AJ, Fernández S, de Ana AM, Jones DR, Torán JL and Martínez-AC: Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. EMBO J *20(10)*: 2497-2507, 2001.
37 Mellado M, Rodríguez-Frade JM, Aragay A, del Real G, Martín AM, Vila-Coro AJ, Serrano A, Mayor F and Martínez AC Jr.: The Chemokine Monocyte Chemotactic Protein 1 Triggers Janus Kinase 2 Activation and Tyrosine Phosphorylation of the CCR2B Receptor. J Immunol *161*: 805-813, 1998.
38 Hasegawa Y, Tang D, Takahashi N, Hayashizaki Y and Forrest ARR: CCL2 enhances pluripotency of human induced pluripotent stem cells by activating hypoxia related genes. Sci Rep *4*: 5228, 2004.
39 Rollins BJ and Sunday ME: Suppression of tumor formation *in vivo* by expression of the JE gene in malignant cells. Mol Cell Biol *11(6)*: 3125-3131, 1991.
40 Manome Y, Wen PY, Hershowitz A, Tanaka T, Rollins BJ, Kufe DW and Fine HA: Monocyte chemoattractant protein-1 (MCP1) gene transduction: an effective tumor vaccine strategy for non-intracranial tumors. Cancer Immunol Immunother *41(4)*: 227-235, 1995.
41 Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ and Murphy WJ: Human endothelial cells express CCR2 and respond to MCP1: direct role of MCP1 in angiogenesis and tumor progression. Blood *96(1)*: 34-40, 2000.

Received April 1, 2015
Revised April 25, 2015
Accepted April 28, 2015