Hypoxia/reoxygenation induction of monocyte chemoattractant protein-1 in melanoma cells: involvement of nuclear factor-κB, stimulatory protein-1 transcription factors and mitogen-activated protein kinase pathways

Manfred KUNZ*[1], Gisela BLOSS*, Reinhard GILLITZER†, Gerd GROSS*, Matthias GOEBELER†, Ulf. R. RAPP‡ and Stephan LUDWIG‡

*Department of Dermatology, University of Rostock, Augustenstr. 80-84, 18055 Rostock, Germany, †Department of Dermatology, University of Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany, and ‡Institut für Medizinische Strahlenkunde und Zellforschung, Versbacherstr. 5, 97078 Würzburg, Germany

Monocyte chemoattractant protein-1 (MCP-1) expression is found in malignant melanoma and melanoma metastases. Since areas of hypoxia/reoxygenation (H/R) are a common feature of malignant tumours and metastases, we addressed the question whether melanoma cells produce MCP-1 upon exposure to H/R. In the present study, we show that melanoma cells up-regulate MCP-1 mRNA and protein under H/R. By means of reporter gene analysis, we further demonstrate that H/R induces transcriptional activation of the MCP-1 promoter carrying a stimulatory protein-1 (SP1) and two nuclear factor-κB (NF-κB) binding motifs. Accordingly, H/R-stimulated melanoma cells showed enhanced binding activity of both transcription factors NF-κB and SP1 in electrophoretic mobility-shift assay. A common upstream activator of NF-κB, inhibitory κBα kinase, was not significantly activated under H/R conditions. Further analysis of upstream signalling events revealed that members of the mitogen-activated protein kinases family, namely extracellular signal-regulated protein kinase, c-Jun N-terminal kinase/stress-activated protein kinase and p38 stress kinase, may be involved in MCP-1 transcriptional regulation under H/R. In summary, we conclude that H/R induces MCP-1 production in melanoma cells via the co-operative action of both transcription factors NF-κB and SP1, and involves mitogen-activated protein kinase signalling pathways. Functionally, H/R-induced MCP-1 production may contribute to tumour progression by committing selective pressure on tumour cells via chemoattraction and activation of tumour-infiltrating monocytes/macrophages.

Key words: intracellular signalling, monocyte chemotactic protein-1, tumour progression.

INTRODUCTION

Human malignant melanoma is a highly metastatic tumour of steadily increasing incidence and poor prognosis after metastasis [1]. There is increasing evidence arguing for a central role of the immune system in the control of local tumour spread and metastasis (reviewed in [2,3]). Although many immunogenic molecules and peptides as well as mechanisms of the T-cell immune response have been identified, treatment methods based on these findings have failed so far to improve the overall prognosis of melanoma patients [4]. Thus the precise function of the inflammatory infiltrate in primary and metastatic melanoma is still a matter of debate. It is still controversial whether the extent of the inflammatory infiltrate surrounding primary melanomas and metastases contributes to a better prognosis or not. In this respect, it is of particular importance that patients with regressive melanomas, i.e. tumours that are heavily infiltrated by immune cells, have the same statistical overall prognosis compared with patients suffering from non-regressive melanomas [5]. A reasonable explanation for these unexpected findings might be the presence of immune escape mechanisms of tumour cells. Tumours might even use the immune system to select for highly aggressive, metastatic phenotypes [6]. Therefore factors that interfere with the immune reaction of T-cells and monocytes or macrophages are of central importance for the understanding of local tumour growth and metastasis.

The expanding family of CC and CXC chemokines play a prominent role in the chemoattraction of immune cells under various inflammatory conditions (see for reviews [7,8]). It may also control the recruitment of immune cells to tumour sites. The chemotactic and activating factors promoting infiltration of immune cells into malignant melanoma lesions have been defined poorly so far. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine involved in the recruitment of monocytes, lymphocytes and natural killer cells under a variety of inflammatory conditions [7,8]. Recent studies [9–11] demonstrated that MCP-1 is also expressed in several malignant tumours and metastases. It has been shown that in ovarian cancer patients there is a positive correlation between MCP-1 expression and the histological grade of the tumour [9]. In contrast, non-tumourigenic cervical carcinoma cell lines displayed high MCP-1 expression, whereas tumourigenic cells lines showed no MCP-1 expression [12,13]. Thus MCP-1 expression by tumour cells appears to be cell type- and stimulus-dependent. Evidence was provided in *in vivo* studies that chemokines, including MCP-1, might be of importance for the recruitment and control of the local immune response in malignant melanoma [14,15]. More recent results [16] indicated that the role of MCP-1 in melanoma progression is dependent on its expression level. In the latter report, high levels of MCP-1 lead to tumour rejection, whereas low levels supported tumour growth.

Abbreviations used: AP-1, activator protein 1; DMRIE-C™, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated protein kinase; GST, glutathione S-transferase; H/R, hypoxia/reoxygenation; IκBα, inhibitory κBα; IKK, IκBα kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1, NF-κB, nuclear factor-κB; SAPK, stress-activated protein kinase; SP1, stimulatory protein-1; STAT3, signal transducer of transcription 3; TNF-α, tumour necrosis factor-α.

[1] To whom correspondence should be addressed (e-mail manfred.kunz@med.uni-rostock.de).

In the present study, we addressed the question of molecular mechanisms that may contribute to MCP-1 production in malignant melanoma cells. Since areas of hypoxia/reoxygenation (H/R) are a typical feature of rapidly growing and metastasizing tumours [17,18], we investigated whether H/R is an appropriate stimulus to induce MCP-1 in malignant melanoma. We show that melanoma cells are responsive to H/R treatment *in vitro* and the enhanced MCP-1 production may be attributed to a transcriptional activation of the MCP-1 promoter. Moreover, signalling events involving members of the mitogen-activated protein kinase (MAPK) family, namely extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 stress kinase, exert an influence on MCP-1 transcriptional regulation under H/R.

MATERIALS AND METHODS

Reagents and antibodies

The specific chemical inhibitor PD98058 (Calbiochem, Bad Soden, Germany) for inhibition of MAPK/ERK kinase activation was used at a concentration of 20 μM, and the specific chemical p38 inhibitor SB203580 (Calbiochem) was used at a concentration of 10 μM. Immunoprecipitation and immunoblotting were performed using antibodies raised against ERK-2 (sc-154), JNK-1 (sc-474) and p38 (sc-535) respectively, all purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The rabbit anti-inhibitory κBα (anti-IκBα) antibody was purchased from New England Biolabs (Schwalbach, Germany).

Cell lines and culture conditions

The human melanoma cell lines 1F6, 530, MV3 and BLM [19,20] were kindly provided by G. N. P. van Muijen (Department of Pathology, University of Nijmegen, The Netherlands) and maintained in RPMI 1640 medium (Linaris, Bettingen, Germany), supplemented with 10% foetal calf serum (Linaris), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 1% non-essential amino acids. 1F6 and 530 represent low metastatic, but MV3 and BLM represent highly metastatic cell lines. For H/R stimulation, cells were first put in an anaerobic culture chamber Anaerocult A (Merck, Darmstadt, Germany) and then re-exposed to normal oxygen tension (H/R) for the indicated time period.

Cytokine-ELISA

Cell-free supernatants were harvested at appropriate times and stored at −80 °C before the analysis. A commercial sandwich-ELISA kit for human MCP-1 (Quantikine, R&D Systems, Bad Nauheim, Germany) was used. Assays were performed according to the manufacturer's instructions.

RNA extraction and Northern-blot analysis

Total cytoplasmic RNA was isolated from melanoma cells using the total RNeasy kit (Qiagen, Hilden, Germany). RNA samples (15 μg) were run on an agarose gel in formamide gel running buffer and blotted as described previously [21]. A cDNA probe of human MCP-1 [22] was labelled with [^{32}P]dATP using a random primer labelling system (Roche Molecular Biochemicals, Mannheim, Germany). After hybridization and washing, membranes were exposed to Hyperfilm™ (Amersham) with intensifying screens at −80 °C. mRNA loading was controlled by a glyceraldehyde-3-phosphate dehydrogenase mRNA blot.

cDNA constructs

The following cDNA constructs were used for transient transfection studies in reporter gene analyses: wild-type ERK-2, SAPK-β54 and p38 kinase respectively and dominant-negative mutants of ERK-2 (ERK-2B3), SAPK-β54 (SAPK Lys → Arg) and p38 (p38AF) respectively [23,24]. Kinases had been subcloned into the eukaryotic expression vector pKRSPA. Melanoma cells were transiently transfected with the vector constructs or empty vector (pKRSPA) as control.

Transfection and luciferase assay

Firefly luciferase expression vectors, harbouring the distal MCP-1 enhancer directly linked to the 5′-flanking region of the MCP-1 minimal promoter, were kindly provided by T. Yoshimura (Laboratory of Immunobiology, National Cancer Institute, Frederick, MD, U.S.A.) [25,26]. They carry the nuclear factor-κB-like (NF-κB-like) binding sites fused to the stimulatory protein-1 (SP1) binding site immediately upstream of the MCP-1 gene. The following promoter–luciferase constructs were used: MCP–PRM (basal promoter containing an SP1 site), MCP–ENH (distal enhancer containing two NF-κB sites fused to the basal SP1 containing promoter) and MCP–mA1A2 construct (MCP–ENH construct with mutated binding motifs for NF-κB). Melanoma cells were transiently transfected with 2 μg of the appropriate plasmid DNA using the 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE-C™) reagent (Gibco, Life Technologies, Eggenstein, Germany) in accordance with the manufacturer's instructions. For studies on the influence of MAPK signalling on MCP-1 promoter activity, co-transfections were performed using plasmid constructs of wild-type and dominant-negative kinases of ERK-2, JNK/SAPK and p38 respectively (as described above). Total cell extracts were prepared and luciferase assays were performed as described previously [21] by using a Berthold luminometer for measurement of luciferase activity (Berthold, Bad Wildbach, Germany). The luciferase activities were normalized on the basis of protein content as well as on β-galactosidase activity of co-transfected RSV-β-gal vector. The β-galactosidase assay was performed with 20 μl of precleared cell lysate according to a standard method as described previously [24]. Data are given as means ± S.D. of relative light units (RLU) of luciferase activity for four independent experiments.

Preparation of nuclear extracts and electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were prepared according to the Dignam's procedure [27]. Cells were washed with ice-cold PBS and pelleted. Supernatants were removed and the cells resuspended in 500 μl of buffer A [10 mM Hepes (pH 7.9)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol (DTT)/0.5 mM PMSF] and allowed to swell for 10 min. Cells were pulled 10–15 times by a 26G3/8 needle for cell membrane disruption and nuclei were pelleted in a microcentrifuge. Nuclei were washed twice in buffer A and resuspended in 50 μl of buffer C (20 mM Hepes/20% glycerol/0.4 M NaCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.2 mM PMSF) and incubated on ice for 45 min with occasional shaking. After centrifugation, supernatants were harvested, frozen and stored at −70 °C until use. The following oligonucleotides were used: NF-κB A2 probe (derived from MCP-1 promoter, according to Ueda et al. [26]), 5′-AGAGTGGGAATTTCCACTCA-3′; and SP1 probe, 5′-ATTCGATCGGGGCGGGGCGAGC-3′. Both were annealed to the corresponding antisense oligonucleotide. For EMSA, 3 μg of nuclear proteins

were incubated at room temperature for 20 min in 20 μl of binding reaction mixture containing 20 mM Hepes, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5 % glycerol, 2 μg of poly(dI/dC) and 50000–100000 c.p.m. of ^{32}P-end-labelled probe. The protein–DNA complexes were subjected to gel electrophoresis on a 5 % non-denaturing polyacrylamide gel in 0.5 % Tris/borate/EDTA buffer. The antibodies α-p50 and α-p65 for supershift analyses of NF-κB complexes and α-SP1 and α-SP3 for SP1 complexes were purchased from Santa Cruz Biotechnology. The anti-signal transducer of transcription 3 (STAT3) antibody from Santa Cruz Biotechnology served as a negative control in supershift analyses. Gels were dried at 80 °C for 1 h and exposed to Hyperfilm™ (Amersham) at −80 °C.

IκBα kinase (IKK) and MAPK *in vitro* kinase assays and immunoblots

IKK and MAPK *in vitro* kinase assays were performed as described recently [23,24,28]. Melanoma cells (cell line 1F6) were lysed in 20 mM Tris (pH 7.4), 137 mM NaCl, 10 % glycerol, 1 % (v/v) Triton X-100, 2 mM EDTA, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM Pefabloc (Merck), 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 mM benzamidine (TLB buffer) at 4 °C for 30 min. Cell lysates were incubated with Protein A–agarose (Roche Molecular Biochemicals) and rabbit antiserum against IKKβ (H-470, Santa Cruz Biotechnology) for 2 h at 4 °C. Immunoprecipitation of MAPK was performed using antibodies raised against ERK-2 (sc-154), JNK-1 (sc-474) and p38 (sc-535).

Immunoprecipitates were washed twice in TLB buffer supplemented with 500 mM NaCl and three times in a kinase buffer [25 mM Hepes (pH 7.5)/10 mM $MgCl_2$/25 mM sodium β-glycerophosphate supplemented with 5 mM benzamidine/1 mM sodium orthovanadate/0.5 mM DTT] containing 3 M urea before samples were equilibrated by two washes in kinase buffer. Urea was omitted in the kinase buffer for MAPK. Immune complexes were then incubated with recombinant glutathione S-transferase–IκBα (GST–IκBα) as a substrate for IKK, with myelin basic protein (Sigma, Deisenhofen, Germany) as a substrate for ERK, with GST–c-Jun as a substrate for JNK/SAPK and 3pK (K/M) [an ATP-binding site mutant of chromosome 3p kinase; K/M stands for the replacement of lysine with methionine] as a substrate for p38 [23,24]. Incubations were performed in the presence of 100 μM unlabelled ATP, 5 μCi [γ-^{32}P]ATP and kinase buffer for 15 min at 30 °C as described recently [23,24,28]. Samples were subsequently subjected to SDS/PAGE, blotted and revealed by autoradiography or detected by a BioImaging Analyzer BAS 2000 (Fuji; via Raytest, Straubenhardt, Germany). Immunoblot analysis was performed to confirm equal loading of IKKβ, ERK-2, JNK/SAPK and p38 proteins respectively. IκBα expression was detected in crude Laemmli lysates with rabbit anti-IκBα.

Statistical analysis

MCP-1 levels in culture supernatants and firefly luciferase activities in reporter gene assays were given as means ± S.D. Student's *t*-test was used for statistical analysis and $P < 0.05$ was regarded as statistically significant.

RESULTS

H/R induces MCP-1 mRNA and protein synthesis in melanoma cells

We performed Northern-blot hybridizations after 8, 12, 24 and 36 h of exposure of melanoma cells to hypoxia and 12 h of hypoxia followed by 8, 12, 24 and 36 h of reoxygenation (designated H/R; Figure 1A). 1F6 melanoma cells showed a strong up-regulation of MCP-1 mRNA after 12 h of H/R, which decreased over time. After 36 h of H/R, however, the MCP-1

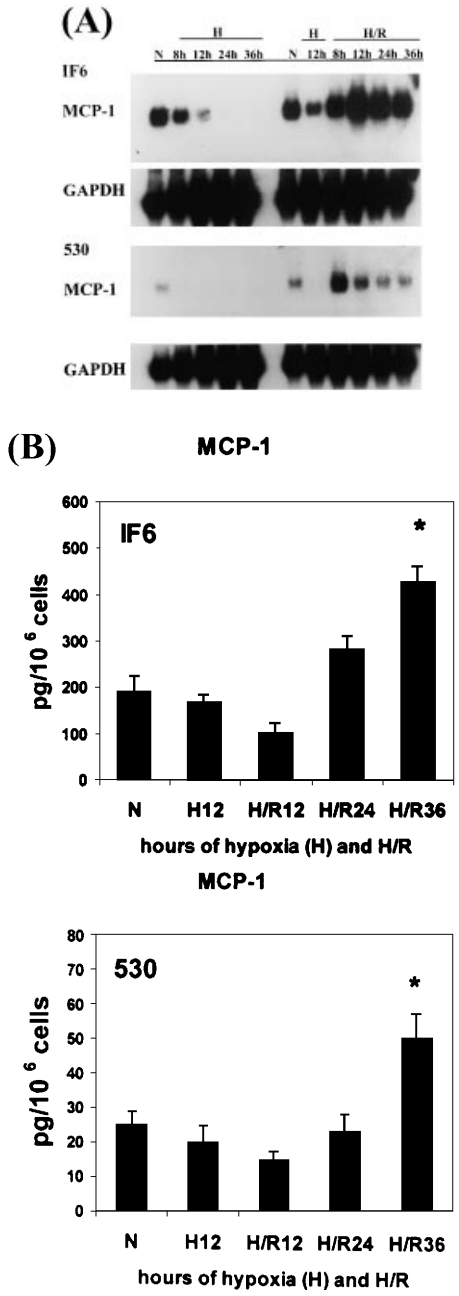

Figure 1 Northern-blot analysis of MCP-1 mRNA expression and ELISA of protein secretion into culture supernatants from malignant melanoma cells

(**A**) 1F6 and 530 melanoma cells were incubated under normoxic condition (N), 8, 12, 24 or 36 h of hypoxia (H), and 8, 12, 24 or 36 h of H/R, following 12 h of hypoxia. Total RNA (15 μg/lane) was analysed using a MCP-1 cDNA fragment as a probe (upper panel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in the corresponding lower panel. (**B**) Melanoma cell lines 1F6 and 530 were kept under normoxic condition (N) or exposed to hypoxia (H) for 12 h, and thereafter, to H/R for 12, 24 and 36 h respectively. MCP-1 production (pg/10^6 cells) was determined by a commercial solid-phase ELISA in culture supernatants and data are given as means ± S.D. for four independent experiments. *Statistical significance of the differences between baseline and H/R-induced expression; $P < 0.05$.

Table 1 Transcriptional activity of MCP-1 promoter constructs after H/R stimulation in melanoma cells measured by firefly luciferase assay

To investigate the influence of H/R on the transcriptional regulation of the MCP-1 promoter, firefly luciferase assays were performed. Melanoma cells (cell line 1F6) were transfected with MCP–ENH, MCP–mA1A2 (mutated NF-κB binding sites) and MCP–PRM constructs using the DMRIE-C^{TM} transfection reagent. Cells were exposed to 12 h of hypoxia, or 12 and 16 h of H/R. The luciferase activities of the MCP promoter constructs were measured in cell lysates. Luciferase activities were normalized on the basis of protein content and β-galactosidase activity of co-transfected RSV-β-gal vector. Data are given as means $\pm$ S.D. of RLU measured by a Berthold luminometer in four independent experiments.

	Normoxic condition	Hypoxia	H/R 12 h	H/R 16 h
MCP–ENH	5179 $\pm$ 796*	2827 $\pm$ 569	11 239 $\pm$ 760*	18 490 $\pm$ 2223*
MCP–mA1A2	2571 $\pm$ 331	2494 $\pm$ 427	2666 $\pm$ 351	4257 $\pm$ 366*
MCP–PRM	4341 $\pm$ 210	3209 $\pm$ 537	2992 $\pm$ 283	6469 $\pm$ 834*

* Statistical significance of differences between baseline and H/R-induced promoter activity; $P < 0.05$.

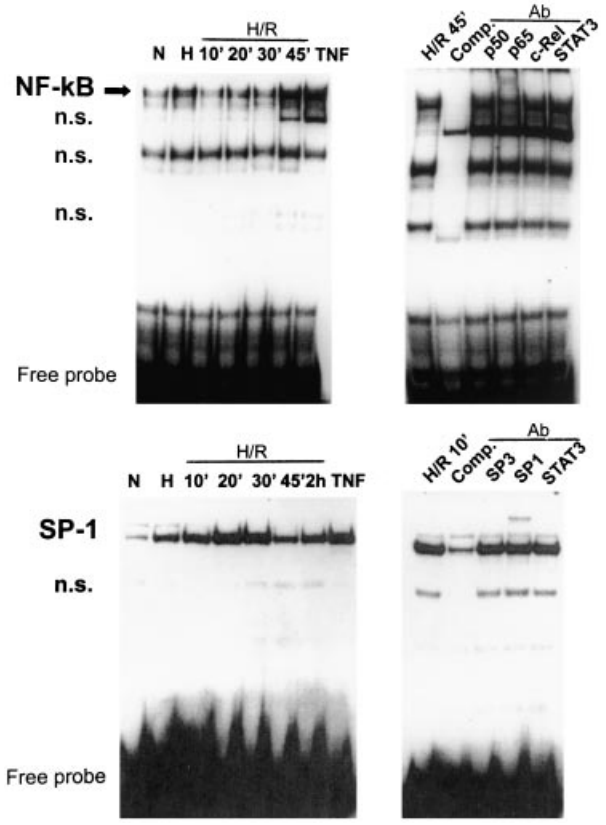

Figure 2 EMSA for NF-κB and SP1 in melanoma cells under hypoxia and H/R

1F6 melanoma cells were exposed to 12 h of hypoxia (H) and thereafter to H/R for another 10, 20, 30 and 45 min and 2 h. Control cultures were kept under normoxic condition (N). For NF-κB-EMSA, the following ^{32}P-labelled double-stranded oligonucleotide was used (A2 motif derived from MCP-1 promoter region): 5′-AGAGTGGGAATTTCCACTCA-3′, hybridized to its antisense oligonucleotide. For SP1-EMSA, a commercially available probe (see the Materials and methods section) harbouring the common SP1 binding motif present in the MCP-1 promoter was used. Supershift analyses of NF-κB protein–DNA complexes were performed using α-p50, α-p65, α-cRel, and for control purposes, anti-STAT3 antibody. Supershift analyses of SP1 protein–DNA complexes were performed using α-SP1, and for control purposes α-SP3 and anti-STAT3 antibody. Competition experiments (Comp.) were performed using a 100 times molar excess of unlabelled probe. Free probe is indicated. n.s., non-specific.

mRNA expression was still above the baseline. Essentially the same results were obtained in 530 cells. In these cells, H/R induction of MCP-1 mRNA appeared earlier (after 8 h) and mRNA signals were weaker. In contrast with the induced mRNA expression under H/R, under hypoxia we observed a constant decrease in both cell lines, finally leading to undetectable signals after 36 h. Again, both cell lines showed similar time courses. Two further cell lines were tested (BLM and MV3; both highly metastatic human melanoma cell lines) and no mRNA transcripts could be detected by Northern-blot hybridization under hypoxia or H/R (results not shown). MCP-1 protein secretion was analysed in culture supernatants from melanoma cell lines 1F6 and 530 after exposure to hypoxia and H/R. As shown in Figure 1(B), hypoxia as well as early H/R (12 h) did not induce MCP-1 protein expression. At later time periods (24 and 36 h), MCP-1 protein expression increased up to 2.5 times over baseline expression.

H/R induces transcriptional activation of the MCP-1 promoter

To analyse further the underlying transcriptional mechanisms of H/R-induced mRNA expression, we performed reporter gene analyses in 1F6 cells using different MCP-1 promoter constructs [25,26]. No induction of transcriptional activity was observed on hypoxic treatment (12 h) in all the three promoter constructs, MCP–ENH, MCP–mA1A2 and MCP–PRM (Table 1). However, after exposure to H/R for 16 h a 3.5-fold up-regulation of reporter gene activity was observed in the experiments with the MCP–ENH construct carrying the distal enhancer and proximal promoter of the MCP-1 gene. This induction was dramatically reduced when the MCP–mA1A2 promoter construct was used, where both NF-κB binding sites are mutated. Accordingly, a strongly reduced gene induction after 16 h of H/R was observed in the experiments using the MCP–PRM construct carrying the proximal MCP-1 promoter only. Taken together, up-regulation of MCP-1 mRNA correlates with an enhanced transcriptional activity of the MCP-1 promoter in 1F6 melanoma cells. NF-κB appears to be of central importance for H/R induction of MCP-1. The proximal promoter region carrying an SP-1 binding motif may contribute to H/R induction of MCP-1 promoter activity.

H/R induces enhanced binding of NF-κB and SP1 transcription factors

It has been shown that induction of MCP-1 gene expression is mainly dependent on the activation of the transcription factors NF-κB and SP1 [25,26]. Two NF-κB binding sites are present in the distal enhancer region of the MCP-1 gene and an SP1 consensus motif is present in the proximal promoter (for details, see the Materials and methods section). To address further the question whether both NF-κB and SP1 are activated in melanoma cells under H/R, EMSA was performed. Specific oligonucleotides of MCP-1 promoter-derived consensus motifs for NF-κB (NF-κB A2) [26] and SP1 were used as probes. As shown in Figure 2, low basal constitutive NF-κB binding activity was observed in 1F6 melanoma cells under normoxic condition. A slight induction of NF-κB binding activity was observed after 12 h of hypoxia. On exposure to H/R, however, a strong induction of NF-κB binding was observed after 45 min. This returned to baseline activity after further exposure to H/R (results not shown). Competition and supershift analyses revealed that the binding activity under H/R was due to specific NF-κB complexes consisting of p65/RelA proteins (Figure 2). The anti-STAT3

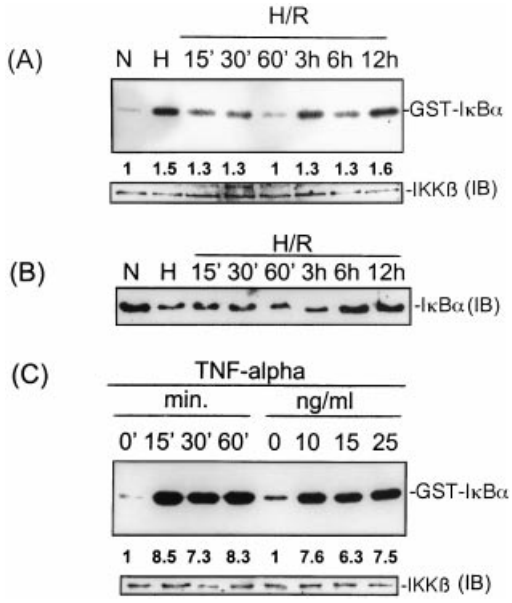

Figure 3 Immune complex kinase assays for IKK in melanoma cells under H/R

(**A**) Immune complex kinase assays for IKKβ (upper panel). Immunoblot (IB) analysis of IKKβ to confirm equal loading of the immunoprecipitated protein (lower panel). (**B**) Immunoblot (IB) of IκBα from total cell lysates for detection of specific protein degradation. (**C**) Immune complex kinase assays for IKKβ after stimulation with TNF-α. TNF-α stimulation served as a positive control for IKKβ activity. For immune complex kinase assays of 1F6 melanoma cells, total cell lysates were prepared after exposure to hypoxia (H) and H/R or after TNF-α treatment. Control cultures were kept under normoxic condition (N). Cell lysates were immunoprecipitated with a rabbit antiserum raised against IKKβ. Immunoprecipitated IKKβ was incubated with radioactive labelled [γ-^{32}P]ATP and recombinant GST–IκBα as kinase substrate. Numbers given under the kinase assays indicate the fold induction of kinase activity compared with baseline activity under normoxic condition or before TNF-α stimulation.

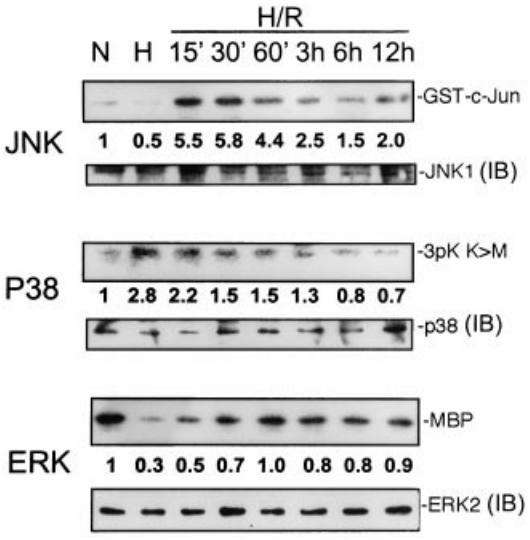

Figure 4 Immune complex kinase assays for MAPK in melanoma cells under H/R

Immune complex kinase assays and immunoblots for JNK/SAPK, p38 kinase and ERK. For immune complex kinase assays of 1F6 melanoma cells, total cell lysates were prepared after exposure to hypoxia (H) and H/R. Control cultures were kept under normoxic condition (N). Cell lysates were immunoprecipitated with rabbit antisera raised against JNK/SAPK, p38 and ERK-2. Immunoprecipitated kinases were incubated with radioactive-labelled [γ-^{32}P]ATP and recombinant GST–c-Jun for JNK/SAPK, 3pK (K/M) for p38 and myelin basic protein (MBP) for ERK respectively as kinase substrates. Immunoblot (IB) analyses were performed to confirm equal loading of immunoprecipitated kinases. Numbers given under the kinase assays indicate the fold induction of kinase activity compared with baseline activity under normoxic condition.

antibody, which was used as a negative control, α-cRel and α-p50 did not supershift the protein–DNA complexes (Figure 2). Essentially the same binding pattern was observed for SP1. Hypoxia only slightly induced SP1 binding. However, strong complexes were formed on H/R after 20 and 30 min. Competition and supershift analyses revealed that the shifted complexes were specific for SP1. Tumour necrosis factor-α (TNF-α; 10 ng/ml for 1 h) was used as a positive control and induced strong binding activity for both NF-κB and SP1 (Figure 2). In summary, these results suggest that both transcription factors NF-κB and SP1 may be involved in the induction of MCP-1 under H/R.

IKK activity is not induced by H/R in melanoma cells

Upon stimulation with cytokines, such as TNF-α and IL-1, NF-κB is activated by phosphorylation and degradation of IκBα and release of transcriptionally active NF-κB factors to the nucleus [29]. Therefore we analysed whether H/R might induce IKK activity in melanoma cells. As shown in Figure 3(A), there was no significant up-regulation of IKK activity on hypoxia or H/R as measured by *in vitro* kinase assays. Accordingly, IκBα protein degradation could not be detected upon hypoxia and H/R (Figure 3B). In contrast, IKK activity was strongly induced after TNF-α stimulation (Figure 3C). Taken together, these results demonstrate that, probably, IKK activation does not contribute to NF-κB-dependent activation of the MCP-1 gene under H/R.

MAPK activity of JNK/SAPK and p38 is induced by H/R in melanoma cells

Immune complex kinase assays were performed for MAPK pathways to address the question whether other upstream signalling pathways might be involved in NF-κB activation under H/R. Upon exposure to 30 min of H/R, a strong activation of JNK/SAPK activity was observed (up to 5.8-fold; Figure 4). In contrast, hypoxia led to a down-regulation of JNK/SAPK activity. H/R induced p38 kinase activity; however, hypoxia was also able to induce the p38 kinase. Finally, ERK did not show enhanced activity under H/R. However, after down-regulation under hypoxia, ERK was re-activated and rapidly reached its strong baseline kinase activity. Taken together, H/R strongly induces JNK/SAPK and p38 activity. It leads to a rapid reconstitution of ERK activity after down-regulation under hypoxia.

Transcriptional activation of the MCP-1 promoter under H/R is influenced by three MAPK pathways

It is well known that members of the MAPK pathways, e.g. p38 stress kinase, modulate NF-κB-dependent gene transcription (see [30] for a review). These pathways had been clearly dissected from IKK-induced NF-κB activation. We then analysed the influence of MAPK pathways on the transcriptional activation of the MCP-1 promoter under H/R. In these experiments, the MCP–ENH construct was used. By means of co-transfection

Table 2 Transcriptional activity of the MCP-1 promoter after H/R stimulation measured by firefly luciferase assays: influence of MAPK pathways

To investigate the influence of MAPK pathways on the transcriptional regulation of the MCP-1 promoter, firefly luciferase assays were performed with 1F6 melanoma cells transfected with the MCP–ENH construct in the presence of wild-type and dominant-negative kinase constructs or pharmacological inhibitors of kinases. For this purpose, 1F6 melanoma cells were transfected with 2 μg of MCP–ENH luciferase constructs and co-transfected with indicated MAPK constructs using the DMRIE-C^{TM} transfection reagent. Cells were kept under normoxic conditions or exposed to 16 h of H/R. Luciferase activities were normalized on the basis of protein content and β-galactosidase activity of co-transfected RSV-β-gal vector. Data are given as means $\pm$ S.D. of RLU measured by a Berthold luminometer in four independent experiments.

	Mock	ERK-2	ERK-2B3	PD98058	p38	p38AF	SB203580	SAPKβ54	SAPKK-R
N	5702 $\pm$ 411	6606 $\pm$ 674	3776 $\pm$ 301*	3655 $\pm$ 278*	5526 $\pm$ 483	4983 $\pm$ 573	5215 $\pm$ 611	6038 $\pm$ 632	4127 $\pm$ 372*
H/R 16 h	17177 $\pm$ 1501	16296 $\pm$ 1367	8304 $\pm$ 691*	4624 $\pm$ 566*	16932 $\pm$ 1152	8779 $\pm$ 1095*	5330 $\pm$ 909*	18696 $\pm$ 2102	9153 $\pm$ 1236*

* Statistical significance of differences between baseline/induced expression and reduction of promoter activity by dominant-negative kinases or pharmacological inhibitors; $P < 0.05$.

with wild-type or dominant-negative interfering kinases of members of the MAPK pathways, it could be shown that dominant-negative ERK-2 (ERK-2B3) and JNK/SAPK (SAPK Lys → Arg) inhibited baseline transcription under normoxic condition (Table 2). However, all three dominant-negative kinases inhibited the transcriptional activation of the MCP–ENH construct under H/R (Table 2). No effects of the co-transfected kinases were observed under hypoxia (results not shown). Essentially the same results were obtained for the pharmacological inhibitors of ERK, PD98058, and p38 kinase, SB203580. The observed effects were even more impressive since both pharmacological inhibitors were able to reduce kinase activity to baseline values. Taken together, these results indicate that H/R-induced transcription of MCP-1 may be modulated by all three different MAPK pathways.

DISCUSSION

In the present study, we show that the CC chemokine MCP-1 is inducible by oxidative stress (H/R) in human malignant melanoma cells. We also show that NF-κB and SP1 transcription factors are active under these conditions and may contribute to enhance MCP-1 promoter activity. These results suggest that MCP-1 production under H/R is at least partly due to enhanced transcription of the MCP-1 gene under these conditions. Furthermore, we could show that members of the MAPK pathways interfere with MCP-1 regulation under H/R in melanoma cells.

It has been previously shown that melanoma cells express MCP-1 mRNA *in situ* in primary malignant melanoma lesions and metastases [14,15]. However, the expression was mainly focal, suggestive of a particular influence of the local tissue environment [14,15]. Since metastases of rapidly growing tumours are exposed to low and varying oxygen pressure, areas of hypoxia and H/R are a typical feature of these tumours [17,18]. Indeed, we could demonstrate that MCP-1 mRNA and protein expression in melanoma cells are up-regulated *in vitro* under H/R. Interestingly, hypoxia alone did not induce MCP-1, or even down-regulate its expression. Similar findings of hypoxia-induced down-regulation of MCP-1 expression have been described for ovarian cancer cells exposed to both TNF-α and hypoxia [31].

The regulatory mechanisms underlying enhanced expression of MCP-1 under H/R were further analysed. It could be demonstrated that H/R strongly induced MCP-1 promoter activity, involving NF-κB distal enhancer motifs and the proximal SP1 binding site. Full activation of the MCP-1 promoter was achieved when both NF-κB and SP1 binding sites were present. Accordingly, as shown by EMSA analysis, enhanced binding of both NF-κB and SP1 was observed under H/R conditions. Together, our results imply a co-operative role of both transcription factors for MCP-1 expression in melanoma cells under H/R. A slight induction of SP1 binding was observed under hypoxic conditions. Since hypoxic induction of SP1 binding in the present EMSA analyses had no effect on the transcriptional activation of the MCP-1 reporter gene, this activation of transcription factor binding appears not to be sufficient for gene induction. It may be speculated that a certain cut-off of transcription factor binding might be necessary for MCP-1 promoter activation. Interestingly, in contrast with our findings, it has been recently shown that hypoxic activation of SP1 may contribute to gene transcription [32].

It has been shown that NF-κB transcriptional activation is influenced by protein phosphorylation, especially involving the p38 kinase (see [30] for a review). In accordance with these findings, we could further show that dominant-negative interfering members of the MAPK family of protein kinases and pharmacological inhibitors of MAPK pathways inhibited H/R activation of the MCP-1 promoter. Thus NF-κB phosphorylation under H/R might contribute to the enhanced transcriptional activity observed in our study. The role of NF-κB for MCP-1 expression had extensively been studied [25,26]. Although initially regarded as a regulator of baseline gene expression for MCP-1, SP1 has gained recent interest for its active role in cytokine-induced MCP-1 expression, in co-operation with NF-κB [33]. In a recent report, Finzer et al. [13] showed that a distant activator protein 1 (AP-1) binding site might control MCP-1 expression in cervical carcinoma cell hybrids after TNF-α stimulation. Formally, this latter mechanism cannot be excluded by our findings. However, the strong activation of the promoter constructs used in our study lacking a distant AP-1 motif argues against a prominent role of AP-1 in H/R activation of the MCP-1 promoter.

Our results indicate that H/R-induced NF-κB activation does not involve IKK activation or IκB degradation and, therefore, differs from cytokine-induced activation of the transcription factor. Thus the classical activation pathway of NF-κB via IκBα degradation appears to be not active under H/R. This is not due to disturbed IKK signalling in melanoma cells, since IKK was readily inducible after TNF-α stimulation. Similar findings of NF-κB activation without proteolytical degradation of IκBα have been described previously [34,35].

At present, it is still a matter of debate whether monocyte/macrophage infiltrates of tumours support tumour growth or are growth inhibitory [36]. On the one hand, available experimental evidence suggests that MCP-1 expression may negatively influence tumour growth in certain tumours [37,38]. Retardation

of tumour growth due to MCP-1 production of tumour cells has also been reported in a mouse model of malignant melanoma [39]. On the other hand, the anti-tumour effects of MCP-1 might induce a selective pressure on melanoma cells, finally leading to enhanced tumour growth. Recent studies pointed out that the effects of MCP-1 on melanoma growth are dependent on the expression level [16]. It could be shown that low levels support tumour growth, whereas high levels lead to tumour rejection. Thus aggressive tumour cells might select non- or low producers for MCP-1. Indeed, it has been shown that late-stage, highly aggressive melanoma cells do not produce significant amounts of MCP-1 [40]. Accordingly, two highly aggressive, late-stage melanoma cell lines tested in our experiments did not express MCP-1 (results not shown).

Since it is well accepted that hypoxia and H/R lead to enhanced aggressiveness of tumours [41,42], one might speculate that H/R-induced MCP-1 production might also contribute to this phenomenon. Inhibition of locally occurring oxidative stress might therefore have beneficial effects on the overall prognosis of melanoma patients.

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