NEOPLASIA
www.neoplasia.com

Volume 19 Number 12 December 2017 pp. 1033–1042 1033

Tumor Acidosis and Hypoxia Differently Modulate the Inflammatory Program: Measurements *In Vitro* and *In Vivo*

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Abstract

Inflammatory mediators produced by the tumor cells are of importance for immune response but also for malignant progression. The aim of the study was to analyze the expression of monocyte chemoattractant protein-1, interleukin-6 (IL-6), tumor necrosis factor-α, inducible isoform of nitric oxide synthase (iNOS), cyclooxygenase-2, and osteopontin *in vitro* in two different tumor cell lines under hypoxia ($pO_2 \approx 1.5$ mmHg) and/or acidosis (pH = 6.6) for up to 24 hours since hypoxia and acidosis are common characteristics of solid tumors. Additionally, the same tumor cell lines implanted *in vivo* were made hypoxic and acidotic artificially for 24 hours, after which the cytokine expression was measured. Finally, the activation of ERK1/2 and p38 by acidosis/hypoxia and their impact on cytokine expression were studied. The results indicate that acidosis and hypoxia have fundamentally different (often opposing) effects on cytokine expression. In addition, these effects were tumor cell line specific. When combining hypoxia and acidosis, the overall changes reflect an additive effect of both conditions alone, indicating that hypoxia and acidosis act by independent mechanisms. The *in vivo* changes corresponded well with the results obtained in the isolated tumor cells. Only iNOS expression was downregulated *in vivo* but increased in cell culture. For IL-6 expression, the acidosis-induced changes were dependent on ERK1/2 activation. In conclusion, it was demonstrated that the environmental pO_2 and pH strongly affect the expression of inflammatory mediators in tumor cells. *In vivo*, most of the inflammatory mediators were downregulated, which could limit the activation of immune cells and by this foster the immune escape of tumors.

Neoplasia (2017) 19, 1033–1042

Introduction

Inflammation is a critical process during tumor progression. Various cytokines which are an important component of the inflammatory tumor microenvironment have been attributed to initiate and promote different steps of malignant behavior like proliferation, invasiveness, metastasis, or angiogenesis [1–3]. Inflammatory mediators like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), or TGF-β can foster proliferation, epithelial-mesenchymal transition (EMT), tumor cell migration, and metastasis [2]. IL-6, cyclooxygenase-2 (COX-2), NO, monocyte chemoattractant protein-1 (MCP-1), and many others participate in angiogenesis, which is an essential step for tumor growth, but also promote the metastatic potential of malignancies [1,2]. Osteopontin (OPN) activates several intracellular signaling cascades (e.g., NF-κB or STAT3) and thereby can induce tumor progression, angiogenesis, and metastasis [4].

Within the tumor tissue, several sources of inflammatory mediators have to be taken into account. First of all, immune cells like tumor-associated macrophages, neutrophils, mast cells, or dendritic cells are secreting a broad spectrum of cytokines into the tumor tissue microenvironment [3]. In addition, stromal cells like tumor-associated fibroblasts are also producing inflammatory cytokines either as a result of other mediators in the tumor interstitial space or by direct contact with tumor cells [3,5]. Finally, the tumor cells themselves are known to produce various inflammatory mediators [1,3] which then act on other cancer cells and on inflammatory or matrix cells. In return, these cytokines can modulate the anticancer inflammatory response of the

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Received 17 July 2017; Revised 29 September 2017; Accepted 29 September 2017

1476-5586
https://doi.org/10.1016/j.neo.2017.09.005

organism and by this affect tumor progression [6]. The initial step for the secretion of inflammatory mediators by tumor cells is the intrinsic stimulation of immune cells and their cytokine secretion [3]. But also the expression of oncogenes (e.g., RAS) can induce the cytokine production in tumor cells [7,8].

Tumor metabolism is fundamentally different from that in normal tissues. Hypoxia and intensified glycolytic metabolism are characteristic features of solid tumors. Oxygen deficiency is a consequence from an insufficient vascular network leading to an inadequate O_2 supply, whereas glycolysis results either from anaerobic glucose breakdown due to hypoxia or from aerobic glycolysis (Warburg effect) [9]. For this reason, tumor tissue often shows quite low pO_2 values down to 1 or 2 mmHg and an acidic extracellular pH (pH 6.0-6.8) [10]. Hypoxia has been shown to induce the cytokine production in immune and cancer cells among others *via* the HIF-1–mediated activation of the NF-κB cascade [11,12]. But also other HIF-independent mechanisms have been described for the regulation of cytokine expression in tumor cells [4,13]. However, the role of cancer-associated normal cells for the hypoxia-induced secretion of inflammatory cytokines (e.g, TNF-α, IL-6) is unclear. Some studies revealed that the extracellular acidosis, typically found in inflammatory tissues or tumors, affects the expression of immunomodulatory cytokines in monocytes or macrophages [14,15]. These effects varied profoundly between monocytes and differentially polarized macrophages and showed a time-dependent increase in COX-2, IL-1β, and inducible isoform of nitric oxide synthase (iNOS) activity over a period of 24 hours [15]. For normal fibroblasts, only sparse data are available which show a time-dependent change in cytokine expression (such as IL-8, COX-2, or iNOS) [16,17]. However, the expression of inflammatory cytokines by the tumor cells themselves under acidic conditions has not been studied systematically.

For this reason, the aim of the present study was to analyze the expression of relevant inflammatory mediators in two different cancer cells lines under different pathophysiological conditions for up to 24 hours. The set of inflammatory markers analyzed included MCP-1/CCL2, IL-6, TNF-α, iNOS/NOS2, COX-2/PTGS2, and OPN/SPP1. The pathophysiological conditions comprised hypoxia ($pO_2 \approx 1.5$ mmHg), extracellular acidosis (pH 6.6), as well as the combination of hypoxia and acidosis (hypoxia + acidosis), which is the effective situation in the tumor. In order to assess whether the changes seen in cell culture reflect the situation in a solid growing tumor, the expression of these cytokines was measured in experimental tumors *in vivo* under severe hypoxic + acidic conditions. Finally, it was studied whether the MAP kinases p38 and ERK1/2, which have been described to be phosphorylated during acidosis in tumor cells [18], are involved in the signaling cascade inducing changes in cytokine expression under adverse environmental conditions.

Material and methods

Cell culture and experimental setup

The subline AT1 of the rat R-3327 Dunning prostate carcinoma (CLS, Eppelheim, Germany) was grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and subcultivated twice per week. Walker-256 rat breast carcinoma cell line (CLS, Eppelheim, Germany) was grown in RPMI medium supplemented with 10% FCS, 10 mM HEPES, 10 mM L-glutamine, and 1.5 g/l $NaHCO_3$ and subcultivated twice per week. Normal rat kidney fibroblasts (NRK-49F, ATCC CRL-1570) were grown in DMEM supplemented with 5% FCS and 1.5 g/l $NaHCO_3$ at 37°C under a humidified 5% CO_2 atmosphere and subcultivated once a week.

For all experiments, cells were grown in Petri dishes and incubated without FCS supplementation for an overall period of time of 24 hours, which did not lead to major changes in pH_e or induction of necrotic or apoptotic cell death. For 3- or 6-hour incubation periods, cells were serum-starved in medium devoid of FCS for 21 or 18 hours, respectively. Subsequently, the cells were transferred to bicarbonate HEPES-buffered Ringer solution adjusted to pH 7.4 (control) or bicarbonate morpholinoethanesulfonic acid–buffered Ringer solution with pH adjusted to 6.6 (acidosis). For oxygenation experiments cells were grown either at room air (normoxia) or at 0.1% $O_2 \approx 1.5$ mmHg pO_2 (hypoxia). MAP kinase inhibitors (10 μM U0126, 10 μM SB203580) were added during the entire incubation period of 3 hours.

Animal Experiments

Both tumor cell lines were used for *in vivo* experiments growing subcutaneously in male Wistar rats for Walker-256 tumors or Copenhagen rats for AT1 tumors (body weight 130-180 g). The rats were housed in the animal care facility of the University Halle-Wittenberg. All experiments had previously been approved by the regional animal ethics committee and were conducted in accordance with the German law for animal protection and the UKCCCR Guidelines [19]. Animals were allowed access to food and water *ad libitum*. Solid tumors were heterotopically implanted by injection of a cell suspension (0.4 ml, approx. 10^4 cells/μl) subcutaneously into the dorsum of the hind foot. Tumors grew as flat, spherical segments and replaced the subcutis and corium completely. Volumes were determined by measuring the three orthogonal diameters (d) of the tumors and using an ellipsoid approximation with the formula: $V = d_1 \times d_2 \times d_3 \times \pi/6$. Tumors were used when they reached a volume between 0.5 to 1.5 ml approx. 7 to 10 days after tumor cell inoculation.

In order to induce a pronounced extracellular acidosis in the solid growing tumors, animals were treated with a combination of inspiratory hypoxia and meta-iodobenzylguanidine (MIBG) in order to force anaerobic glycolysis in the tumor cells [20]. Therefore, tumor-bearing animals received an MIBG injection (20 mg/kg b.w., i.v. dissolved in isotonic saline) and were then housed in a hypoxic atmosphere containing 8% O_2 and 92% N_2 for 24 hours. This procedure has been shown to reduce the extracellular pH in these tumors from 7.05 to 6.65 [21]. Animals housed in room air receiving an equivalent injection of the solvent served as control. After 24 hours in the respective atmosphere, the animals were sacrificed, the tumors were surgically removed, and the expression of the inflammatory mediators was analyzed by quantitative polymerase chain reaction (qPCR).

qPCR

Total RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. One microgram of RNA was subjected to reverse transcription with SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) and analyzed by qPCR using the Platinum SYBR Green qPCR Supermix (Thermo Fisher Scientific, Waltham, MA, USA), each step according to the manufacturer's instructions. The obtained data were normalized against *Rn18S* and related to the respective control. The obtained ΔΔCt values for each experiment

were averaged and subjected to statistical analysis. Table 1 shows the primers used.

Western Blot

Western blotting was performed according to standard protocols. Cells were lysed (0.5 M Tris–HCl pH 6.8; 10% SDS; 10% 2-mercaptoethanol; 20% glycerol; 0.01% bromophenol blue), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Subsequently, membranes were incubated with antibodies specific for ERK1/2, p38, phospho-ERK1/2, and phospho-p38 (Cell Signaling Technology, Danvers, MA). The bound primary antibody was visualized using horseradish peroxidase (HRP)–conjugated secondary antibodies and Serva chemoluminescence reagent for HRP (Serva, Heidelberg, Germany) with the Molecular Imager ChemiDoc XRS System (Biorad, Munich, Germany). Quantitative analysis was performed with Quantity One software (Biorad, Munich, Germany). For quantification, the ratio of the phosphorylated ERK1/2 or p38 to total ERK1/2 or p38, respectively, was calculated.

Data Analysis

Data are presented as mean ± SEM. For all experiments, n equals the number of cell passages, and three different passages were performed at least. Statistical significance was determined by unpaired Student's t test or analysis of variance, as appropriate. Differences were considered statistically significant when $P < .05$.

Results

Expression of Inflammatory Mediators in Tumor Cells

Figures 1 and 2 show the changes in the expression of inflammatory mediators in the two tumor cell lines (AT1, Walker-256) after incubating the cells for up to 24 hours under acidic conditions alone (pO_2 = 150 mmHg; pH 6.6), under hypoxia (pO_2 = 1.5 mmHg; pH 7.4), or combined acidosis + hypoxia (pO_2 = 1.5 mmHg; pH 6.6). The results indicate that acidosis and hypoxia have fundamentally different (often opposing) effects on cytokine expression. In addition, these effects were tumor cell line specific. In AT1 tumor cells, acidosis increased the expression of IL-6, iNOS, and OPN, whereas hypoxia significantly reduced the expression of all inflammatory mediators. For most of the mediators, the combined acidosis + hypoxia reflects an additive effect of both conditions alone, indicating that hypoxia and acidosis affect the expression by independent mechanisms. Only for OPN (Figure 2) did the combination result in a stronger reduction of the expression than would be expected by an independent influence of each environmental parameter. Interestingly, the impact of acidosis on gene expression was seen at short incubation times of 3 to 6 hours (except for OPN), whereas regulation by hypoxia occurred both at short-term (3 and 6 hours) as well as long-term incubation (24 hours) and was sometimes even opposing, for instance, the iNOS expression.

The expression changes in Walker-256 cells were similar to those seen in AT1 cells for iNOS and OPN (Figure 2). Acidosis increased the level of iNOS and OPN, whereas hypoxia led to a decrease in OPN (at least after 24 hours) and a mild decrease in iNOS expression after 3 and 6 hours and a strong increase after 24 hours. For MCP-1 and IL-6, Walker-256 carcinoma cells showed a fundamentally different behavior in comparison to AT1 carcinoma cells (Figure 1). The expression decreased under acidic conditions (at least after 24 hours of incubation), whereas hypoxia for 24 hours markedly increased the expression of these cytokines. The expression of TNF-α was temporarily increased by acidosis but decreased by hypoxia, while after 24 hours, acidosis reduced and hypoxia raised the TNF-α level, which resulted in no regulation in total when looking at the combination of acidosis + hypoxia. Walker-256 cells displayed a distinct time response for the regulation by hypoxia. Short-term hypoxic incubation decreased the level of inflammatory mediators (MCP-1, IL-6, TNF-α, iNOS), while long-term incubation strongly increased the level. Once again, combining acidosis with hypoxia led to a change in expression of all inflammatory mediators, which more or less reflects an additive impact of both parameters alone.

Since in some tumors up to 80% of the cell mass consists of fibroblasts [22] and these cells are also exposed to the acidic and/or hypoxic environment, the impact of the microenvironment on the expression of inflammatory mediators was analyzed in fibroblasts (Supplementary Figure S1). In normal rat fibroblasts (NRKF), an acidic microenvironment for 3 to 6 hours led to a marked increase of TNF-α, iNOS, and COX-2 [17], whereas MCP-1 and OPN were unaffected. After 24 hours at pH 6.6, the expression was either not changed (iNOS, COX-2, OPN) or reduced (MCP-1, TNF-α). Hypoxia alone had no impact on the mediators with the exception of COX-2 (increase after 3 and 24 hours) as well as TNF-α (decrease after 6 hours). Therefore, these cells showed a markedly different behavior when compared with the tumor cell lines. The combination of both environmental parameters resulted in changes similar to those seen with acidosis alone, even for TNF-α and COX-2, where a weak regulation by hypoxia was observed. Thus, pH and not pO_2 seems to be the critical parameter for the regulation of these inflammatory mediators in fibroblasts.

Expression of Inflammatory Mediators in Solid Tumors

In order to verify whether these changes in mediator expression can also be found in solid tumors *in vivo*, AT1 and Walker-256 tumors were made artificially acidotic by inspiratory hypoxia (8% O_2) in combination with inhibition of the respiratory chain by MIBG. This treatment has been shown to acidify the tumor pH by 0.4 unit, leading to an intratumoral pH of about 6.6 [21] and a pO_2 of about 1 to 3 mmHg [23]. Since the animals were housed in a hypoxic

Table 1. Primers Used for qPCR

Target	Forward Primer	Reverse Primer
Ccl2/MCP1	CCCAGAAACCAGCCAACT	TGCTGCTGGTGATTCTCTTG
Il6	AGCCAGAGTCATTCAGAGCA	AGCATTGGAAGTTGGGGTAGG
SPP1/OPN	CCTCTGAAGAAACGGATGACT	CTGGGCAACTGGGATGACCTT
Nos2/iNos	GCAGGTTGAGGATTACTTCTTCCA	GCCCTTTTTTGCTCCATAGGAAA
Tnfα	CCACCACGCTCTTCTGTCTACTGAACT	CCATTGGCCAGGAGGGCGTT
Ptgs2/COX2	TACAAGCAGTGGCAAAGGCC	CAGTATTGAGGAGAACAGATGGG
Rn18s	CTGAGAAACGGCTACCACATC	CCCAAGATCCAACTACGAGC

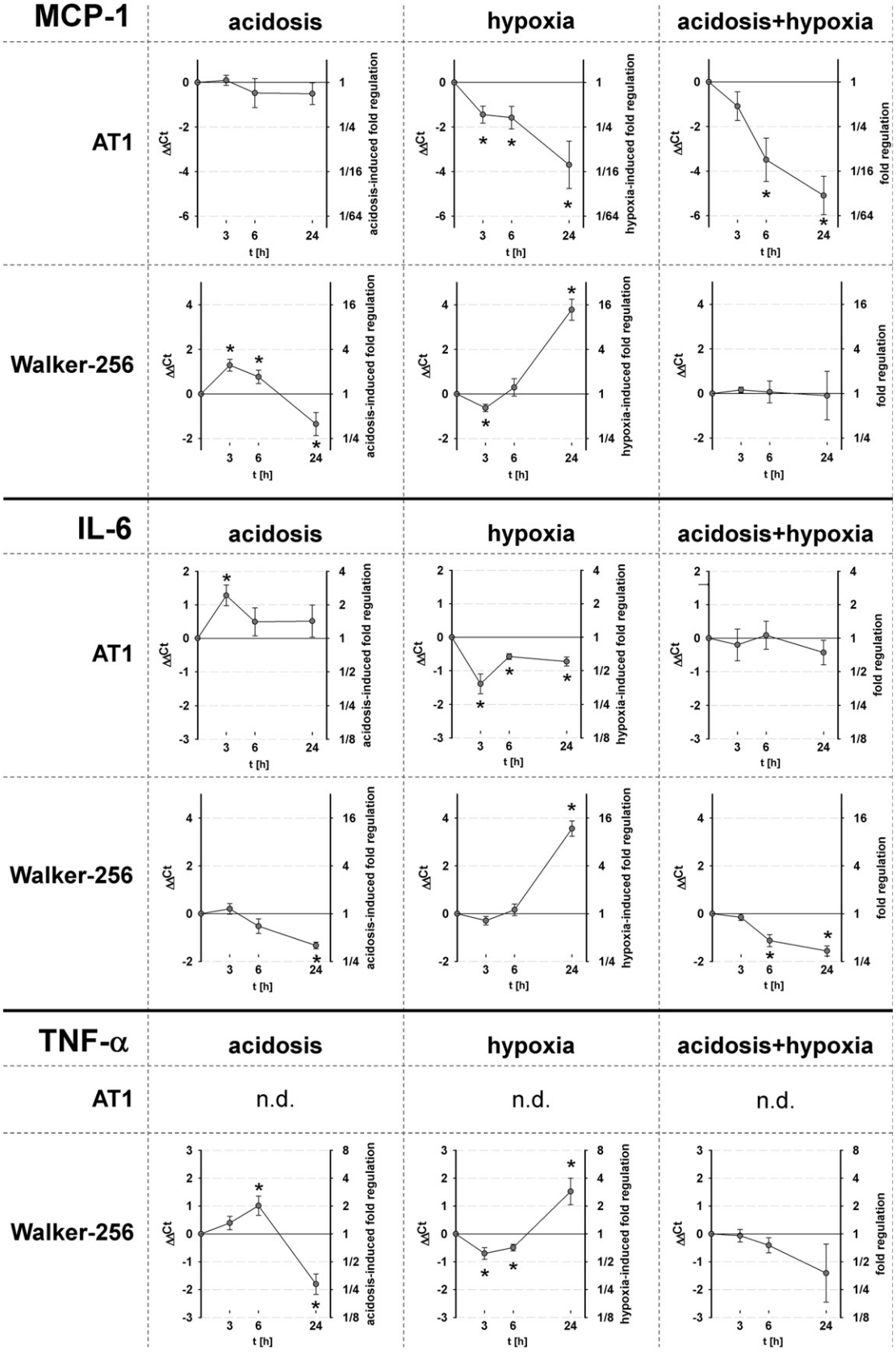

Figure 1. Change in MCP-1, IL-6, and TNF-α expression in AT1 and Walker-256 cells during extracellular acidosis (pH 6.6), hypoxia (pO_2 = 1.5 mmHg), or combined acidosis + hypoxia. n = 5-17 for AT1, n = 4-11 for Walker-256; *P < .05; n.d., not detectable.

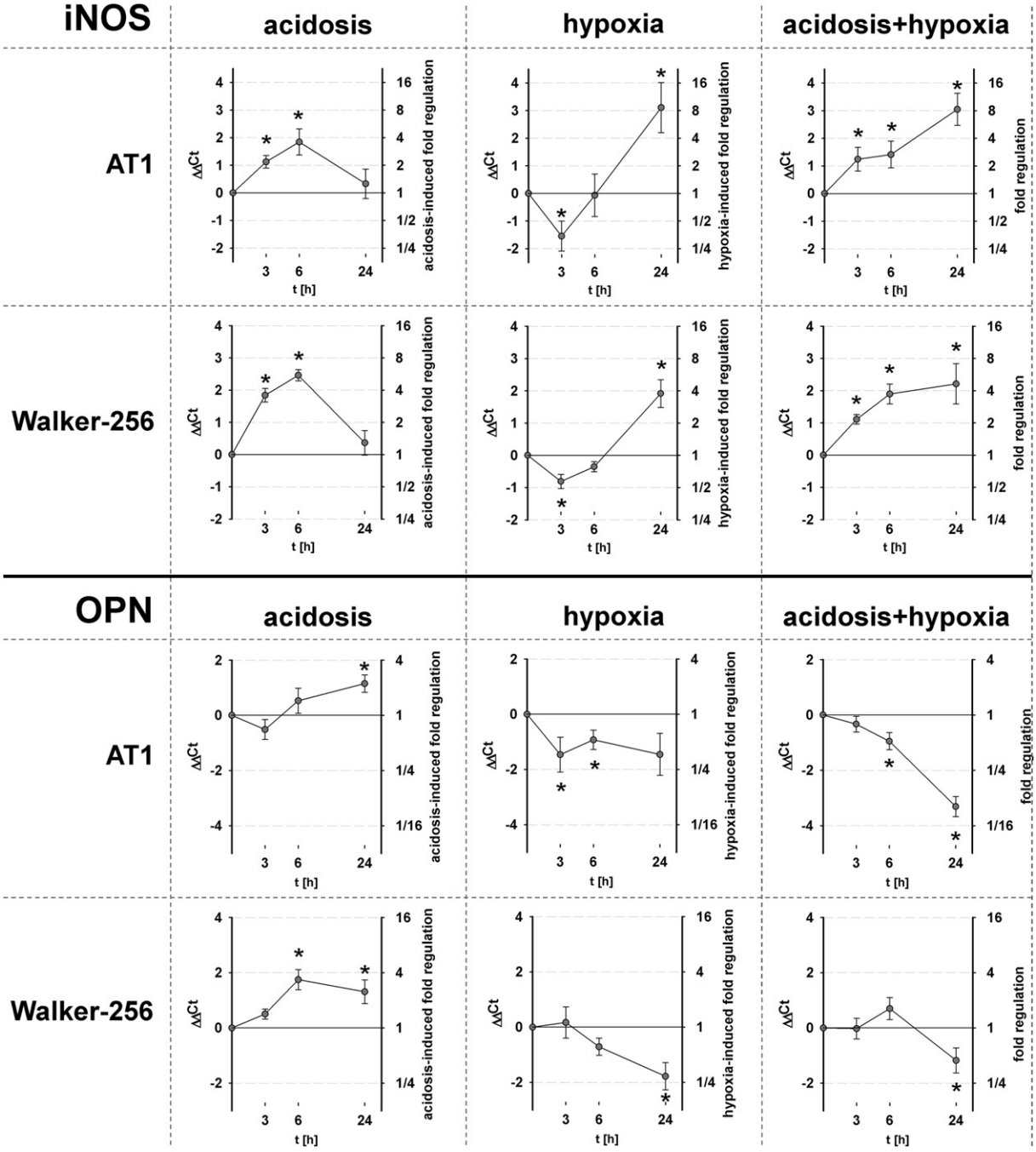

Figure 2. Changes in iNOS and OPN expression in AT1 and Walker-256 cells during extracellular acidosis (pH 6.6), hypoxia (pO_2 = 1.5 mmHg), or combined acidosis + hypoxia. n = 4-17 for AT1, n = 5-13 for Walker-256; *P < .05.

environment (8% O_2) leading to a decrease in tumor pO_2, this procedure corresponds to the cell experiments with combined acidosis and hypoxia. Figure 3 (black bars) shows the changes in the expression of MCP-1, IL-6, TNF-α, iNOS, COX-2, and OPN in solid tumors after forcing the glycolytic metabolism (hypoxia + acidosis) compared to the cell culture data for the respective tumor cells (dark gray bars) or fibroblasts (light gray bars) since these represent a relevant part of the tumor mass. The graphs indicate that, *in vivo*, almost all inflammatory mediators were downregulated.

In AT1 tumors, the expression changes of MCP-1 and IL-6 corresponded well with the data of the AT1 cells. OPN expression was considerably low and could only be detected in two tumors. Therefore, no definitive conclusion on statistical significance can be drawn. In Walker-256 tumors, the regulation was also comparable to that seen in the isolated cells. Only iNOS expression was markedly different in both AT1 and Walker-256 tumors where it was significantly decreased, whereas it was upregulated in cell culture experiments. In NRKF fibroblasts, the expression of several inflammatory mediators showed changes which were not related to the data observed in tumors (e.g., IL-6, iNOS, OPN).

MAPK Signaling and Its Role for Cytokine Regulation

In order to analyze possible signaling mechanisms by which the extracellular environment may affect the cytokine expression, the activation of MAP kinase pathways was measured. Figure 4 shows the time course of MAPK phosphorylation in the different cell lines. The

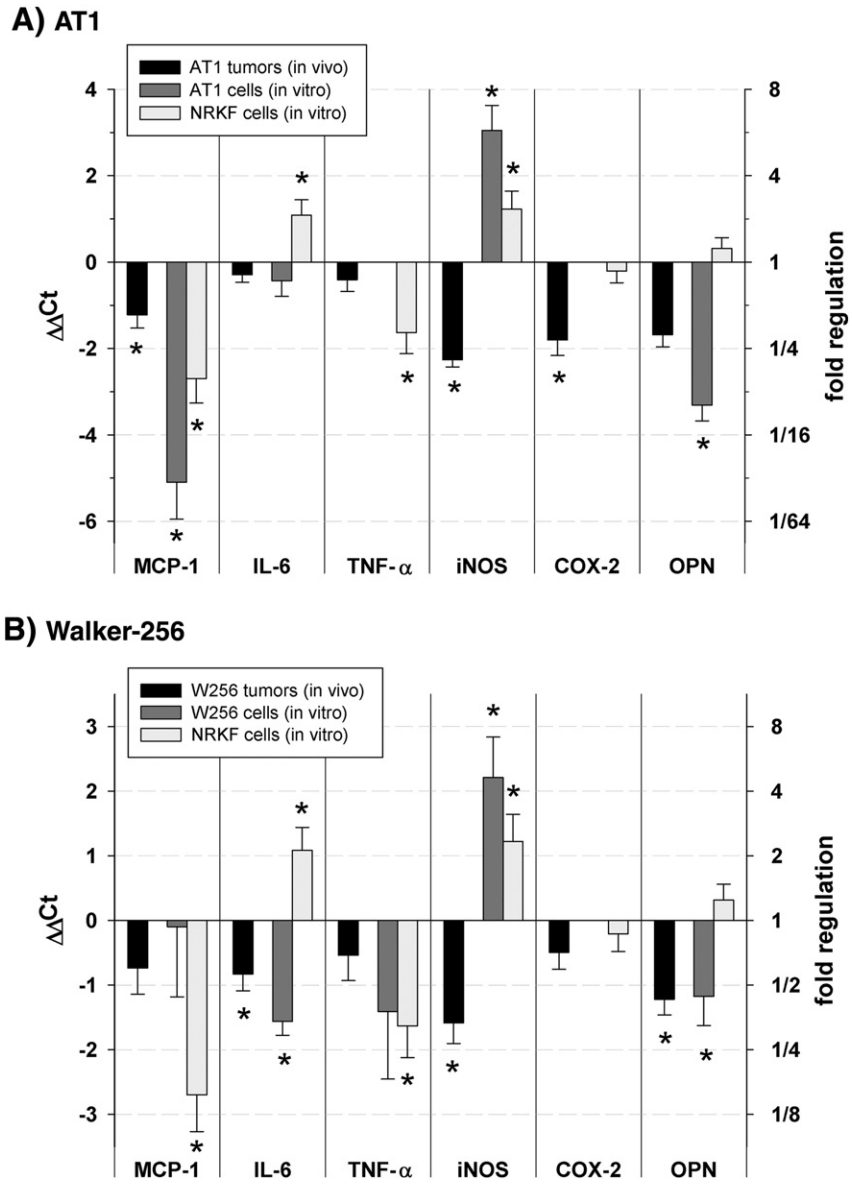

Figure 3. Modulation of the expression of inflammatory mediators in solid (A) AT1 and (B) Walker-256 tumors (black bars) by combined acidosis and hypoxia for 24 hours. For comparison, the *in vitro* data of the respective tumor cells (dark gray) and normal fibroblasts (NRKF, light gray) after the same incubation conditions are shown. $n = 5$ AT1 tumors, $n = 6$ Walker-256 tumors; *$P < .05$ versus control.

figure displays the ratio of the phosphorylated to total MAPK amount normalized to control conditions under normoxia at pH 7.4. The data clearly indicate that extracellular acidosis for 3 hours led to an activation of both MAP kinases in almost all cells. After 24 hours, the phosphorylation returned to the control level. Hypoxia had almost no impact on ERK1/2 or p38 either at short intervals (3 hours) or after 24 hours. The combination of hypoxia + acidosis corresponded more or less to the effects seen with acidosis alone, indicating that the impact of acidosis and hypoxia on the MAPK phosphorylation seems to be independent of each other.

In order to test whether the changes in MAPK phosphorylation are responsible for the observed changes in expression of inflammatory mediators, cells were incubated with inhibitors of the respective MAPK pathway (U0126 for ERK1/2, SB203580 for p38) under acidic conditions for 3 hours. Figure 5*A* shows the phosphorylation of ERK1/2 and p38, respectively, after 3 hours at pH 6.6. ERK1/2 activation was markedly upregulated in all cell lines, whereas p38 phosphorylation increased only in AT1 and NRKF cells but not in Walker-256 cells. Blocking ERK1/2 by U0126 disrupts the acidosis-induced upregulation of IL-6 in AT1 and NRKF cells (Figure 5*C*). These data indicate that acidosis-induced IL-6 expression is under control of ERK1/2, and this might be a common mechanism. Inhibition of p38 had no impact on IL-6 expression. For all other inflammatory mediators studied, blocking the MAP kinases had no (TNF-α and OPN, data not shown) or cell line–specific effects, such as MCP-1 expression which was downregulated by ERK1/2 inhibition in AT1 cells (Figure 5*B*) and iNOS expression that depended on p38 in NRKF cells [17] (Figure 5*D*).

Discussion

The results of the present study clearly reveal that the hypoxic and/or acidic environment of tumors affects the expression of inflammatory mediators in tumor cells. However, acidosis and hypoxia showed differing effects for most mediators studied. During acidosis, MCP-1,

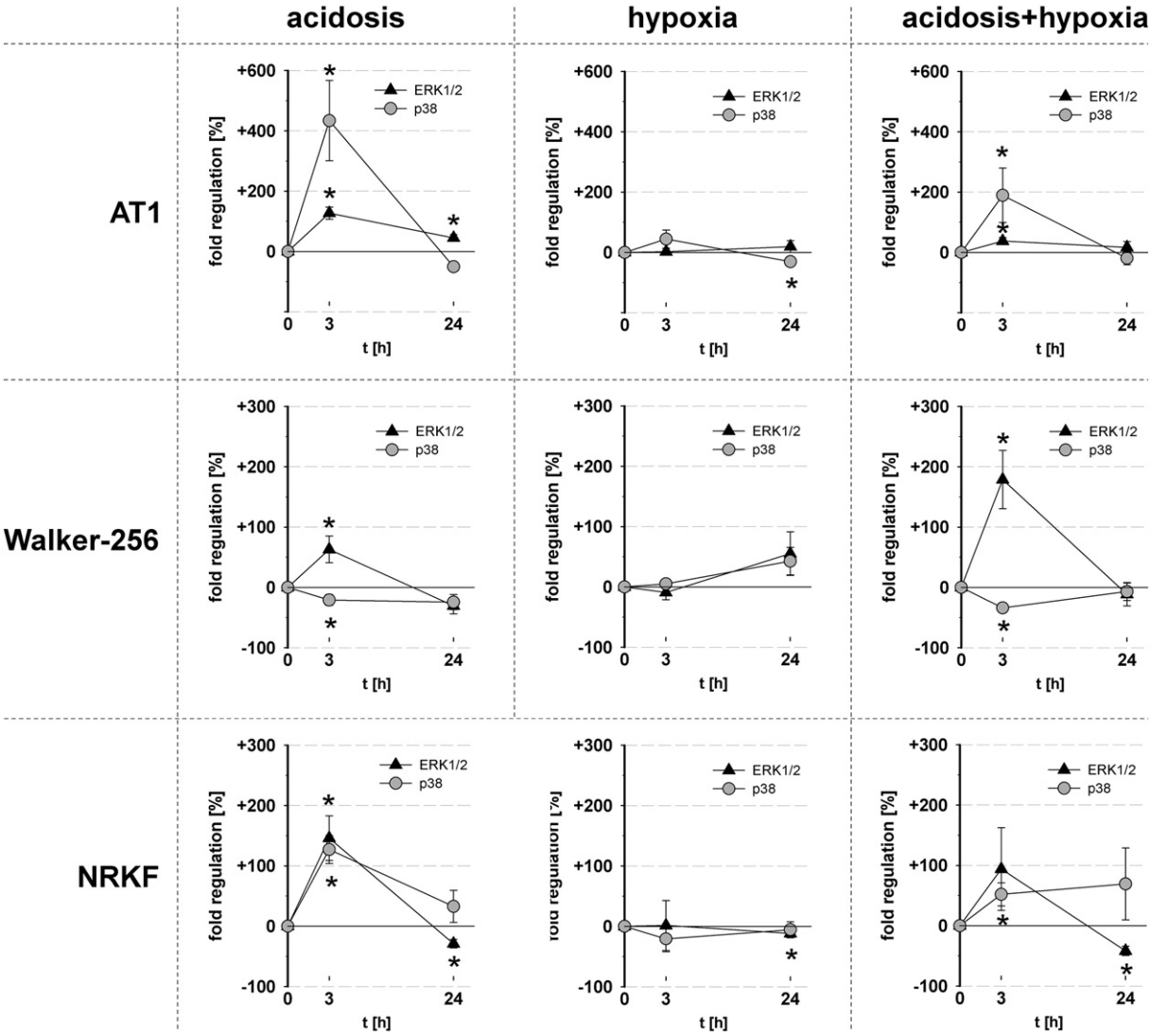

Figure 4. Change in ERK1/2 and p38 phosphorylation in AT1, Walker-256, and NRKF cells during extracellular acidosis (pH 6.6), hypoxia (pO_2 = 1.5 mmHg), or combined acidosis + hypoxia. The graphs show the change in the ratio of phosphorylated to total MAPK level, normalized to the control (normoxia, pH 7.4). n = 5-11 for AT1, n = 5-6 for Walker-256, n = 5-8 for NRKF; *$P < .05$ versus control (normoxia, pH 7.4).

IL-6, TNF-α, and iNOS expression slightly increased for 3 to 6 hours, after which the expression returned to or was even below control level after long-term acidosis (24 hours). In contrast, hypoxia had a strong impact, which was most prominent after 24 hours of O_2 deficiency. However, for MCP-1 and IL-6, the direction of this effect was cell line specific. In AT1 cells, the expression was significantly reduced, whereas in Walker-256 cells, the expression of these cytokines was considerably increased. The results of the Walker-256 cells are in accordance with previous studies in cancers showing a positive correlation between HIF-1α and MCP-1 expression [24] as well as in skeletal muscle where a strong increase in MCP-1 by acute hypoxia was described [25]. The decrease of MCP-1 by hypoxia, as seen in the AT1 cells in the present study (Figure 1), has not been described before.

Expression of iNOS is known to be induced by hypoxia in cancer and normal tissues [26–28], which is in good accordance with the present study (Figure 2). The present results show that also acidosis for 3 to 6 hours significantly induces iNOS expression in tumor cells, which may affect tumor cell survival and proliferation.

Hypoxia had almost no or a slightly depressing effect on the expression of OPN (Figure 2), which is in contrast to some findings where hypoxia induced OPN expression [13]. However, other studies clearly showed that hypoxia-induced expression is cell line dependent and a function of the magnitude of hypoxia [29]. In the study of Wohlleben et al., OPN expression was increased only during moderate hypoxia (1% O_2) but not with severe hypoxia (0.1% O_2) as used in the present experiments. It has been proposed that OPN expression is not regulated directly by hypoxia but indirectly *via* changes in the expression of other cytokines [4]. Acidosis for 6 to 24 hours had a slightly increasing effect on OPN expression. After 3 hours of acidosis, the expression stayed at the control level, a finding which has also been described for monocytes and macrophages [15].

The effects stated above are induced by either acidosis or hypoxia alone. However, the actual situation in a tumor is the simultaneous combination of hypoxia and acidosis. For most of the inflammatory mediators, the results obtained under combined hypoxia + acidosis reflect an additive effect (as compared to the respective environmental factor alone). These data indicate that hypoxia and acidosis modulate the cytokine expression by separate

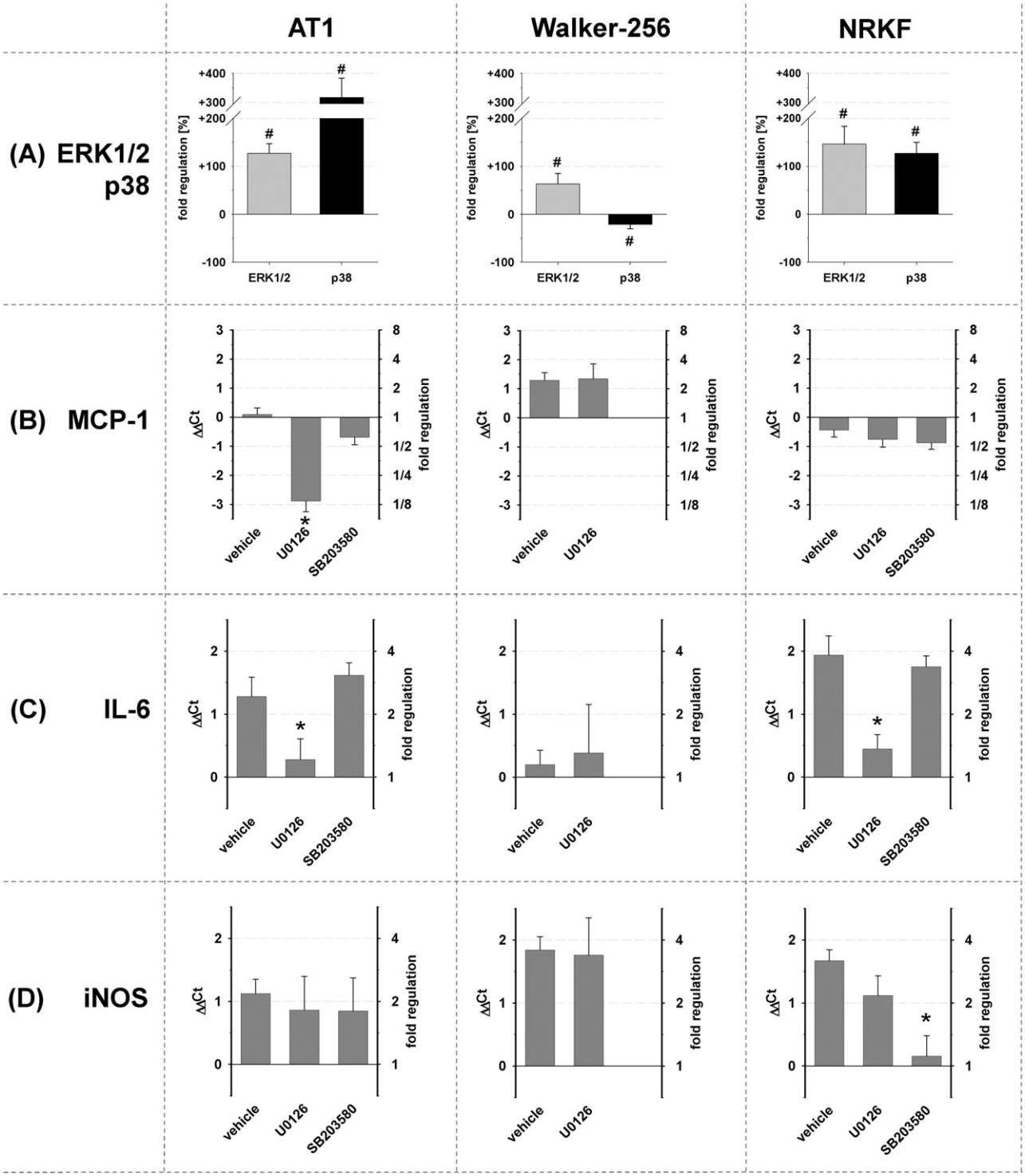

Figure 5. (A) Change in ERK1/2 and p38 phosphorylation in AT1, Walker-256, and NRKF cells during extracellular acidosis (pH 6.6) for 3 hours; #$P < .05$ versus pH 7.4. Modulation of (B) MCP-1, (C) IL-6, and (D) iNOS expression in these cells during extracellular acidosis (pH 6.6) for 3 hours (vehicle) and during acidosis as well as incubation with an inhibitor of the ERK1/2 pathway (U0126) or the p38 pathway (SB203580); *$P < .05$ versus vehicle. $n = 5$-11 for AT1, $n = 5$-6 for Walker-256, $n = 5$-12 for NRKF.

mechanisms. For some mediators (e.g., MCP-1), the changes induced by combined hypoxia + acidosis are more pronounced than by each factor alone since both factors changed the expression in the same direction.

The question arises whether these effects seen in cell culture experiments can also be found in solid growing tumors *in vivo*. Since the tumor mass consists not only of tumor cells but also of fibroblasts (and miscellaneous immune cells), the changes in expression during combined hypoxia + acidosis found in homogenized tumor tissue (Figure 3 *black bars*) resulted not only from tumor cells. For this reason, we contrasted these data with the expression results found in isolated tumor cells (*dark gray bars*) or fibroblasts (*light gray bars*) *in vitro* under the same conditions (hypoxia + acidosis for 24 hours). Figure 3 clearly shows that, for most of the cytokines, the changes found in the tumor tissue correspond well with the *in vitro* results from the respective tumor

cell line but seldom with the fibroblasts, indicating that the changes in the solid tumors predominantly result from the tumor cells. The only exception was iNOS expression that was decreased in the solid tumor while increased in tumor cells and fibroblasts *in vitro*. Therefore, other sources of iNOS within the tumor have to be taken into account. A substantial amount of NO in tumors is produced from iNOS strongly expressed in macrophages [30]. It was shown that, in unstimulated macrophages, hypoxia had only a negligible effect on iNOS expression [31], whereas acidosis reduced iNOS in M2 macrophages [15]. For this reason, the observed iNOS reduction *in vivo* may be attributable to acidosis-induced changes in the macrophage population. These findings were concordant in both tumor lines investigated.

As shown in Figure 3, most of the inflammatory mediators (MCP-1, TNF-α, iNOS, OPN) were significantly downregulated *in vivo* by combined hypoxia + acidosis. These cytokines predominantly trigger the immune response. They can attract and activate immune cells like monocytes, macrophages, or T lymphocytes; induce the production of other proinflammatory mediators; or foster the phagocytic activity. If under hypoxic/acidic conditions the expression of these cytokines is downregulated, the physiological immune response against the tumor may be restricted [32]. Additionally, the efficacy of a therapeutic immune modulation of cancers (immune therapy), which relies on an adequate cytokine production [33], may be limited under hypoxic/acidotic conditions due to a suppression of cytokine secretion. On the other hand, several inflammatory factors are known to contribute to the malignant progression of tumors by enhancing proliferation, EMT, invasiveness, or metastasis formation [1–3]. For these processes, a reduced cytokine expression may have a beneficial impact on tumor control. Due to these opposing effects on antitumoral immune response and malignant progression, the net effect of the hypoxia/acidosis-induced downregulation of inflammatory mediators on tumor growth cannot be predicted definitively.

The complexity of the interaction between hypoxia/acidosis and the immune response is even further increased by the impact of the fibroblasts on tumor immunity. The tumor microenvironment leads to activation of fibroblasts which are highly heterogeneous and impact on cancer progression, metastasis, and total immune response [34–36]. Cancer-associated fibroblasts primarily foster the immunosuppressive tumor microenvironment either directly or indirectly by the regulation of angiogenesis, by extracellular matrix remodeling, and by their secretory phenotype leading to the modulation of immune cell recruitment and activation [34]. Among the secreted immunomodulatory mediators in fibroblasts are IL-6, TNF-α, MCP-1, PGE2, and NO [37,38], which were also regulated by combined hypoxia + acidosis in NRKF cells (Figure S1). IL-6 signaling for instance was shown to restrict maturation of dendritic cells and induces the differentiation of macrophages, blocks T-cell activation, and recruits as well as activates mast cells [39–41]. Interestingly, our results suggest that the expression of inflammatory mediators of NRKF fibroblast is mainly affected by extracellular acidosis and not by hypoxia alone. Since the induction of an immunosuppressive microenvironment involves the complex interaction between cancer cells, fibroblasts, and immune cells and might even comprise partly overlapping secretomes (as seen in the present study for iNOS expression in fibroblasts and cancer cells), the overall impact on total immune response and tumor progression needs further investigation.

In the present study, also the role of MAP kinases in the signal transduction of environmental parameters on the cytokine expression was analyzed. As shown in Figure 4, extracellular acidosis led to a marked phosphorylation of both MAPKs in almost all cell lines. The activation of MAPK by the extracellular pH has already been shown for many tumor and normal tissue cell lines [15,18,42]. The results reveal that this activation is only transient since, after 24 hours of acidosis incubation, the phosphorylation level returned to control level. Hypoxia did not induce MAPK phosphorylation in the cell lines studied (Figure 4). Only in Walker-256 cells was there a slight trend towards ERK1/2 and p38 activation after 24 hours of severe hypoxia (Figure 4). These data are in accordance with the very few studies on this topic in which an increased p38 or ERK1/2 phosphorylation was seen in cells after 24 hours of anoxia [43,44]. Accordingly, the activation during combined acidosis + hypoxia reflects more or less the impact of acidosis alone.

To test whether the acidosis-induced increase in MAPK phosphorylation might be responsible for the observed changes in cytokine expression, cells were incubated with inhibitors of the respective MAP kinase pathways. For IL-6 expression, the acidosis-induced increase was reversed if the ERK1/2 cascade was blocked. These results indicate that the ERK1/2 pathway, which is activated by acidosis, is involved in IL-6 expression, a finding which has also been described for normal tissue and other cancer cells [45–47]. The impact of hypoxia on cytokine expression seems to be mediated by other mechanisms, for instance, the HIF pathways [11,12].

In conclusion, the present study clearly demonstrates that the pathophysiological parameters hypoxia and acidosis affect the expression of inflammatory mediators like MCP-1, IL-6, or iNOS in tumor cells. The impact induced by O_2 deficiency and low pH seems to be independent of each other, indicating distinct mechanisms by which the cytokine expression is modulated (e.g., MAP kinase activation by acidosis, HIF pathway by hypoxia). In the situation of simultaneous hypoxia and acidosis, which is the situation at hand in many tumors, the changes in the expression of the mediators reflect an additive effect of each parameter alone. In solid tumors *in vivo*, the observed changes correspond preferentially to those seen in the tumor cells, indicating that fibroblasts incorporated into the tumor mass are of less importance as a source of cytokines. *In vivo*, most of the inflammatory mediators were downregulated, which could limit the activation of immune cells and by this foster the immune escape of tumors.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2017.09.005.

Acknowledgements and Funding

This study was supported by the Wilhelm-Roux program of the medical faculty, University of Halle (FKZ 29/12) and the DFG (grant TH-482/6-1). We acknowledge the financial support of the Open Access Publication Fund of the Martin-Luther-University Halle-Wittenberg.

Disclosure Statement

The authors declare that they have no conflict of interest.

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