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Extracellular Acidosis Modulates the Expression of Epithelial-Mesenchymal Transition (EMT) Markers and Adhesion of Epithelial and Tumor Cells

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Abstract

Epithelial-to-mesenchymal transition (EMT) is an important process of tumor progression associated with increased metastatic potential. EMT can be activated by external triggers such as cytokines or metabolic parameters (e.g. hypoxia). Since extracellular acidosis is a common finding in tumors, the aim of the study is to analyze its impact on the expression of EMT markers *in vitro* and *in vivo* as well as the functional impact on cell adhesion. Therefore, three tumor and two normal epithelial cell lines were incubated for 24 h at pH 6.6 and the expression of EMT markers was studied. In addition, mRNA expression of transcription and metabolic factors related to EMT was measured as well as the functional impact on cell adhesion, either during acidic incubation or after priming cells in an acidic milieu. E-cadherin and N-cadherin were down-regulated in all tumor and normal cell lines studied, whereas vimentin expression increased in only two tumor and one normal cell line. Down-regulation of the cadherins was seen in total protein and to a lesser extent in surface protein. *In vivo* an increase in N-cadherin and vimentin expression was found. Acidosis up-regulated Twist1 and Acsl1 but down-regulated fumarate hydratase (Fh). Cell adhesion during acidic incubation decreased in AT1 prostate carcinoma cells whereas preceding acidic priming increased their subsequent adhesion. Low tumor pH is able to modulate the expression EMT-related proteins and by this may affect the stability of the tissue structure.

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Introduction

Epithelial-to-mesenchymal transition (EMT), a process during which cells lose their epithelial properties gaining mesenchymal features, has shown to be a relevant step in cancer progression. During EMT epithelial cells lose their junctions and their apico-basal polarity, change the cytoskeletal structure and increase their motility, all resulting in an invasive phenotype [1–3]. Tumor cells undergoing EMT can leave the tumor mass and migrate into the surrounding normal tissue leading to local progression of these tumors [3,4]. Also the invasion of blood circulation, as well as the transmigration of the epithelial lining in a new tissue, increases by EMT leading to a higher rate of far distant metastases resulting in a worse prognosis of tumor patients [5]. EMT is characterized by a reduced expression of epithelial markers in combination with a higher expression of mesenchymal-related proteins. A typical pattern is the down-regulation of E-cadherin and an up-regulation of N-cadherin and/or vimentin [3,4,6].

Several triggers have been identified to foster the change from an epithelial to a mesenchymal cancer cell phenotype. The effect of

Abbreviations: Acsl1, acyl-CoA synthetase long-chain family member 1; Fh, fumarate hydratase; Gpi, glucose-6-phosphate isomerase; Idh2, isocitrate dehydrogenase 2; MIBG, meta-iodobenzylguanidine; NGS, next-generation sequencing; Scd1, stearoyl-CoA desaturase 1; Scd2, stearoyl-CoA desaturase 2; Snai1, snail family transcriptional repressor 1; Twist1, twist family bHLH transcription factor 1; Zeb1, zinc finger E-box binding homeobox 1; Zeb2, zinc finger E-box binding homeobox 2.

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transforming growth factor β (TGF-β) which induces, after binding to the TGF receptor II, E-cadherin loss and smooth muscle actin up-regulation is well studied [7]. The signaling cascades responsible for cellular responses to TGF-β have been studied extensively, showing a central role of SMAD family members and several transcription factors such as ZEB1 and 2, TWIST1 and 2, SNAIL1 and 2 as well as various members of the FOX family [2–4,8]. But also other extracellular signals such as TNF-α, IL-6 or components of the extracellular matrix (e.g. collagen I) have been shown to modulate these signaling pathways and by this induce EMT [9].

Finally, also metabolic parameters of the tissue can directly or indirectly induce EMT and by this may foster tumor progression and worsen the patient's prognosis. The metabolic microenvironment of solid tumors differs fundamentally from normal tissues. Hypoxia, intensified glycolytic metabolism and the formation of reactive oxygen species are common features of malignancies [10]. Oxygen deficiency results from an insufficient vascular network, from a reduced oxygen transport capacity (e.g. by anemia) or from an increased diffusion distance and leads to an inadequate oxygen supply of the tumor cells [11]. The insufficient O_2 supply or intrinsic changes of the energy metabolism (the so called "Warburg" effect) cause the glycolytic metabolism of tumors [12]. Lactic acid formation from glycolysis leads to an extracellular acidosis with pH values even below 6.0 [10]. Especially hypoxia has been shown to induce EMT. The mechanisms include either hypoxia-inducible factor 1 (HIF-1)-regulated EMT-related signaling pathways (e.g. SNAIL, NOTCH, ZEB) [9,13,14]) or hypoxia/HIF-dependent growth factors (e.g. VEGF) that foster EMT [14,15]. Also, an increased formation in ROS has been proposed to induce EMT [14,15]. Finally, several processes of energy metabolism have been associated with the activation of EMT. Enzymes of glycolysis (e.g., glucose-6-phosphate isomerase), lipid metabolism (e.g., acyl-CoA synthetase) or glutaminolysis (e.g. glutaminase 1) have been associated with EMT [6]. However, only very few data on the impact of extracellular acidosis on the induction of EMT have been published.

For this reason, the aim of the present study was to analyze the expression of EMT markers in different tumor lines and normal epithelial cells on mRNA and protein level. The two epithelial cell lines as well as the tumor cell line NCI-H358 are model systems for EMT induction. In contrast, the other two tumor cell lines are metastasizing carcinoma models. AT-1 tumor cells are already anaplastic and Walker-256 tumor cells grow in suspension and lack epithelial properties. In order to investigate the functional impact, adhesion of cells directly exposed to low pH as well as in cells pre-treated in an acidic environment was measured. In parallel, the effect of acidosis on experimental tumors of the same cancer cells was studied *in vivo*. Finally, the expression of several EMT-related transcription factors was analyzed in two of the tumor cell lines.

Materials and Methods

Cell Lines

The *in vitro* experiments were performed in two normal epithelial cell lines: (1) normal rat kidney epithelial cells (NRK-52E, ATCC #CRL-1571) and (2) the subline C7 of MDCK (Madin-Darby canine kidney) cells [16]. For comparison three tumor cell lines were used: (1) subline AT1 of the Dunning rat prostate carcinoma R3327 (CLS # 500121, CLS GmbH, Eppelheim, Germany), (2) Walker-256 mammary carcinoma of the rat (ATCC # CCL-38, LGC Standards GmbH, Wesel, Germany) and (3) human NCI-H358 bronchioalveolar carcinoma cells (ATCC #CRL-5807). AT1, NCI-H358, NRK-52E and MDCK are adherent whereas Walker-256 are non-adherent cells. The Walker-256 cell line consists of two distinct populations (undifferentiated, differentiated) and is lacking epithelial cell markers. The AT1 line is undifferentiated whereas NCI-H358 cells are weakly differentiated with glandular features and were described as suitable model for EMT [17,18]. AT1, Walker-256 and NCI-H358 cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and for Walker-256 cells additionally with 10 mM L-glutamine, 20 mM HEPES and 0.15% $NaHCO_3$. NRK-52E and MDCK cells were cultivated in DMEM medium supplemented with 5% (NRK-52E) or 10% (MDCK) FCS, respectively. Cells were kept at 37 °C in a humidified 5% CO_2 atmosphere and were sub-cultivated twice per week. For the experiments cells were kept in FCS-lacking medium for 24 h to 48 h at normal pH (pH 7.4) or at pH 6.6. The control pH of 7.4 and extracellular acidosis (pH 6.6) were obtained by buffering medium with $NaHCO_3$, 10 mM HEPES and 10 mM MES (morpholinoethanesulfonic acid), pH adjustment with 1 N NaOH.

In Vivo Tumor Models

The impact of the extracellular micromilieu on gene expression in solid growing tumors *in vivo* was analyzed using AT1 and Walker-256 cell lines. Solid AT1 tumors were studied *in vivo* in male Copenhagen rats (body weight 180–250 g) and Walker-256 tumors in Wistar rats (body weight 200–250 g), housed in the animal care facility of the University of Halle. 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 before the investigation. Solid tumors were induced heterotopically by injection of cell suspension (4×10^7 cells/0.4 ml isotonic saline) subcutaneously into the dorsum of the hind foot. Tumors grew as flat, spherical segments and replaced the subcutis and corium completely. Tumor volumes were determined by measuring the three orthogonal diameters with a caliper and using an ellipsoid approximation with the formula: $V = d_1 \cdot d_2 \cdot d_3 \cdot \pi/6$. Tumors were investigated when they reached a volume of 0.5–1.5 mL.

In order to study the impact of acidosis on gene expression *in vivo*, two different approaches to reduce the intratumoral pH were used. In the first set of experiments tumor-bearing animals were treated with a combination of inspiratory hypoxia and meta-iodobenzylguanidine (MIBG) which forces glycolytic metabolism [20]. Therefore, animals received a MIBG injection (20 mg/kg b.w., i.p. dissolved in isotonic saline) and were then housed in a hypoxic atmosphere containing 8% O_2 and 92% N_2 for 24 h. Animals kept in room air receiving only the solvent served as control. This procedure reduces the extracellular tumor pH in AT1 tumors from 7.02 ± 0.04 (control) to 6.48 ± 0.08 (acidosis) and in Walker-256 tumors from 7.16 ± 0.03 (control) to 6.65 ± 0.07 (acidosis) [20]. In the second set of experiments the extracellular space of tumors was acidified by direct intratumoral injection of a small amount of lactic acid. Therefore, 50 µl of a 0.222 mM solution of lactic acid (in H_2O) was injected into the tumor tissue at a depth of 2–3 mm. The same amount of a 0.222 mM sodium lactate solution was applied in the contralateral tumor and this tumor served as intra-individual control. After 24 h after starting the respective acidification procedure animals were sacrificed, the

tumors were surgically removed, minced and total RNA was extracted using TRIzol reagent.

qPCR

After 24 h incubation under pH 7.4 or pH 6.6, cells were harvested and total RNA was isolated using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. MRNA expression of E-cadherin (Cdh1), N-cadherin (Cdh2) and vimentin (Vim) were analyzed by quantitative PCR. Therefore 1 μg RNA was subjected to reverse transcription with SuperScript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) 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. Table S1 (Suppl. Mat.) shows the primers used. In addition, for AT1 and Walker-256 tumor cells mRNA analyses were also performed by NGS using Illumina HiScan SQ (Illumina, San Diego, USA). In this approach 21477 genes of the rat were detectable. Low abundant genes were excluded from further analysis and for the remaining genes the expression ratio at pH 6.6 to pH 7.4 was calculated.

Western Blot and Flow Cytometry

Western blotting was performed according to standard protocols. In brief, 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 E-cadherin, N-cadherin and vimentin (Cell Signaling, Danvers, USA). 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). The protein level during acidic condition was normalized to control values at pH 7.4.

E- and N-cadherin expression on the tumor cell surface was measured by flow cytometry. In brief, after incubating the cells at pH 6.6 or pH 7.4, respectively, for 24 h 10^6 cells were washed in PBS and incubated with primary antibodies (CD324/CD325/mouse IgG1 isotype control, BD Bioscience, Franklin NJ, USA) for 30 min. After washing, a FITC-labeled secondary antibody (Rockland, Limerick, USA) was added. Cell-bound fluorescence was then measured with a LSR Fortessa flow cytometer (BD Bioscience). The fluorescence level of acidic incubated cells was normalized to control values at pH 7.4.

Cell Adhesion

Cell adhesion was measured by continuous impedance measurements of monolayer cells (xCELLigence DP; OLS OMNI Life Science, Bremen, Germany) in accordance to the manufacturer's instructions. Cells were growing in special 16-well plates which contain gold biosensors embedded in the bottom of each well. The impedance between these sensors depends on the adherence of the cell and increases with stronger adhesion. In order to analyze the impact of the acidic environment on cellular anchorage two different experimental settings were used. In the first series it was tested whether cells lose their adherence if they are exposed to low pH. Therefore, cells were plated on 16-well plates in normal growth medium for 48 h to establish a tight contact between cells and plastic. After that the medium was changed to either pH 7.4 or pH 6.6 and the impedance was measured over a period of 50 h. For analysis the impedance values at 24 and 48 h after switching to the acidic conditions were used. In the second series it was tested whether priming the cells at low pH for 24 h will affect the ability to adhere on the plastic surface. For this purpose cells were kept at pH 6.6 or 7.4 in normal petri dishes for 24 h. Subsequently, cells were mechanically detached and the cell suspensions were then transferred to 16-well plates in which the impedance was measured during the next 12 h.

Immunofluorescence Imaging

The cells were grown on microscope slides. Afterwards, they were incubated in buffer at pH 7.4 or 6.6 for 48 h following paraformaldehyde fixation and permeabilization (Triton X100). After blocking the cells were incubated with the primary (anti-E-cadherin, clone CD324, BD Bioscience, dilution 1:200; anti-N-cadherin, clone CD325, Cell Signaling, dilution 1:200; anti-vimentin, clone D21H3, Cell Signaling, dilution 1:100) and the respective secondary antibody (Oregon Green 488 IgG, Invitrogen, Carlsbad CA, USA, dilution 1:1000). The staining was analyzed on an inverse microscope (BZ-8100E, Keyence, Osaka, Japan).

Statistical Analysis

Results are expressed as means ± SEM. Differences between groups were assessed by the two-tailed t-test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all comparisons.

Results

Acidosis-Induced Changes of Cadherins and Vimentin in Cells

The expression of the EMT markers E-cadherin, N-cadherin and vimentin was studied on mRNA and on protein level. Figure 1 shows the changes of mRNA of these markers after 24 and 48 h at pH of 6.6. The characteristic pattern for EMT (decrease of E-cadherin, increase of N-cadherin and vimentin) was seen only in NCI-H358 bronchial carcinoma cells (a cell line that has been described to show typical EMT changes after TGF-β1 incubation [8]). In both other tumor lines E-cadherin mRNA was not detectable as expected. In Walker-256 mammary carcinomas vimentin increased significantly after 48 h at low pH whereas in AT1 cells vimentin expression was independent from the pH. On protein level a pH-induced decrease of E- and N-cadherin was seen in tumor cell lines (Figure 2). In NCI-H358 cells, N-cadherin protein decreased even though the mRNA level showed a significant increase at low pH. In Walker-256 suspension cells neither E- nor N-cadherin protein was detectable. Vimentin protein was upregulated by acidosis by trend in all tumor and epithelial cell lines studied. Cadherins are responsible for the adherence of cells among each other and are therefore responsible for the stability of the tumor tissue structure. Since for cell–cell interaction only cadherins on the cell surface are of importance, the expression of membrane-bound E- and N-cadherins on NCI-H358 cells (the only tumor line on which both cadherins were detectable) was analyzed by flow cytometry. As shown in Figure 3, surface expression was also reduced after 48 h at low pH, however, this reduction was less pronounced than modulation of total protein level. These results were confirmed by

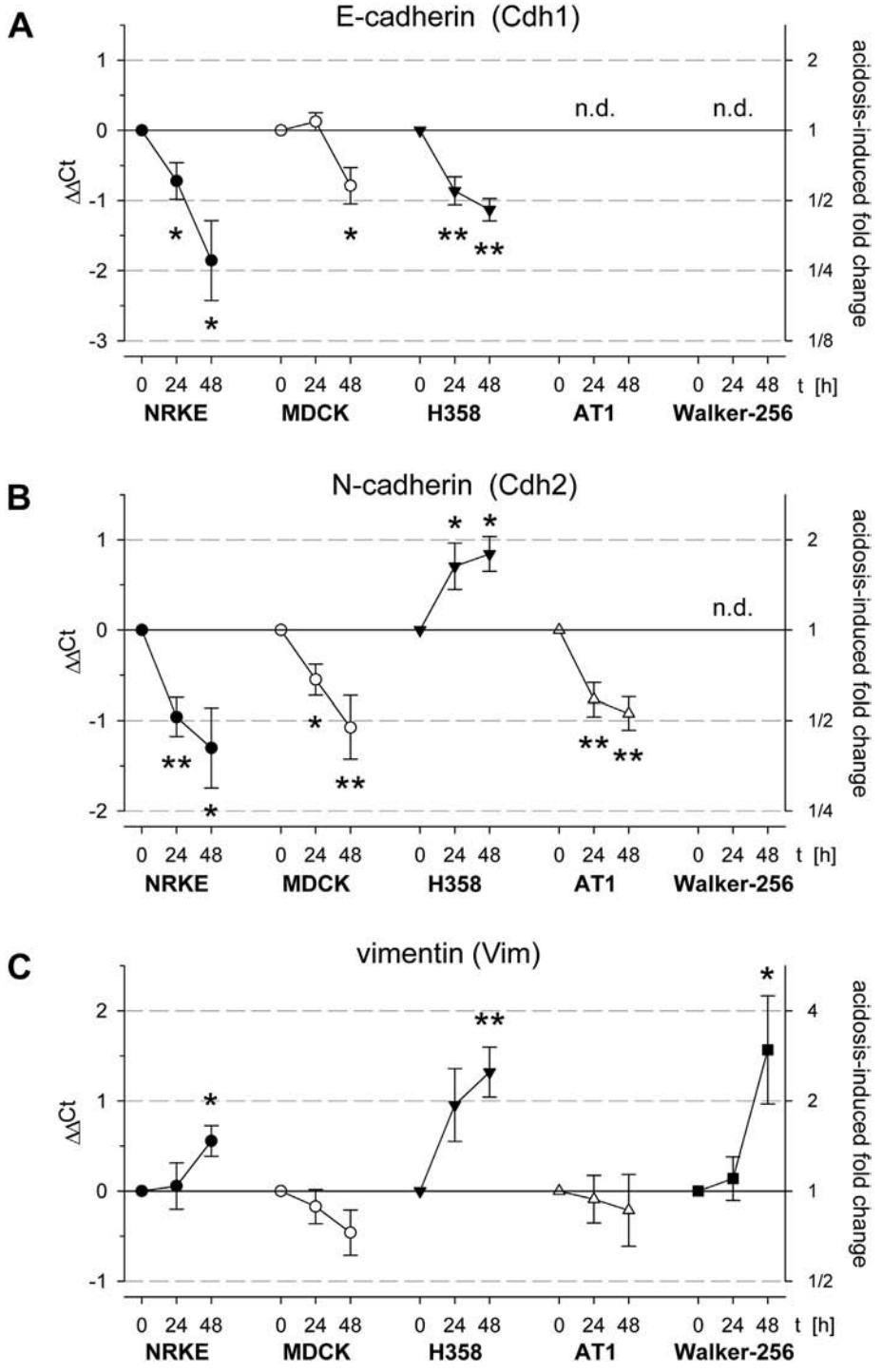

Figure 1. Expression changes of (**A**) E-cadherin, (**B**) N-cadherin and (**C**) vimentin genes in normal (NRK-52E, MDCK) and tumor (NCI-H358, AT1, Walker-256) cells during incubation at pH 6.6 for up to 48 h. Values are normalized to control conditions at pH 7.4. Mean ± SEM, n.d. not detectable; n = 6–13, (*) $P < .05$, (**) $P < .01$ vs. 0 h.

immunofluorescence images of E- and N-cadherin expression in NCI-H358 cells indicating a pH-induced reduction of the surface expression of these proteins (Suppl. Figs. S1, *A* and *B*). The vimentin protein expression was found to be more or less evenly distributed in the cytoplasm and was increased at low pH (Suppl. Fig. S1*C*).

In both normal cell lines (NRK-52E, MDCK) E- and N-cadherin were significantly downregulated on mRNA and on protein level (Figures 1 and 2). The effect on the vimentin mRNA expression, however, was non-uniform. NRK-52E cells showed a slight increase of vimentin mRNA after 48 h whereas in MDCK the level was more or less unaffected (Figure 1). Vimentin protein expression was increased by 25% in both cell lines (Figure 2). Thus, when looking at protein concordant results for acidosis-induced up-regulation of vimentin and down-regulation of E-cadherin and N-cadherin was found in all cell types studied, irrespective of their origin (tumor vs. non-tumor) or differentiation state.

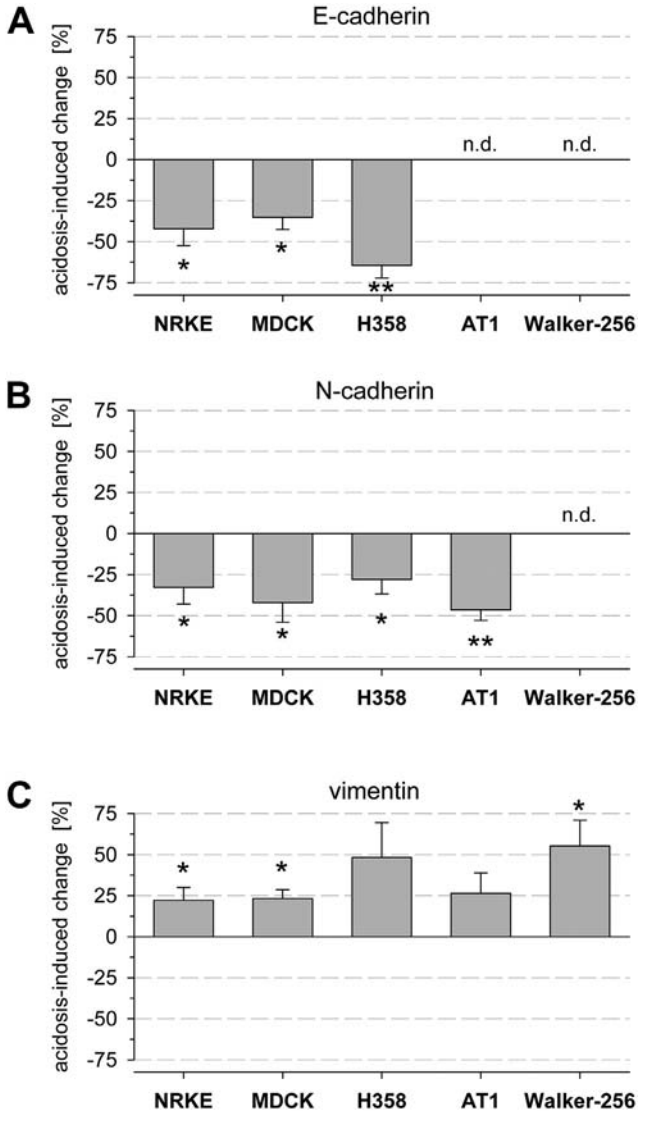

Figure 2. Expression changes of (**A**) E-cadherin, (**B**) N-cadherin and (**C**) vimentin protein in normal (NRK-52E, MDCK) and tumor (NCI-H358, AT1, Walker-256) cells after incubation at pH 6.6 for 48 h. Values are normalized to β-actin as housekeeper and subsequently related to control conditions at pH 7.4. Mean ± SEM, n.d. not detectable; n = 4–9, (*) $P < .05$, (**) $P < .01$ vs. pH 7.4.

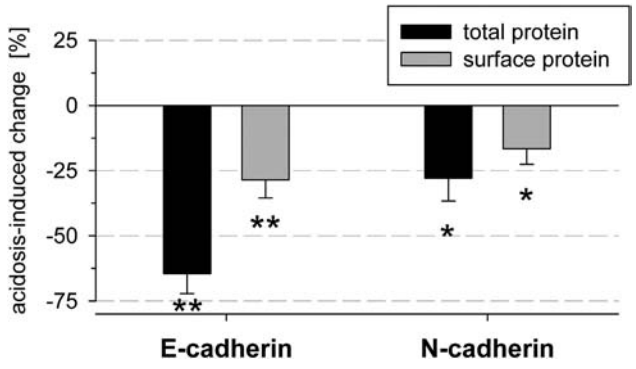

Figure 3. Changes of E- and N-cadherin protein expression in NCI-H358 tumor cells after 48 h at pH 6.6. Shown are the total protein amount (Western blot) and the protein on the cell surface (flow cytometry). Values are normalized to control conditions at pH 7.4. Mean ± SEM; n = 6–9, (*) $P < .05$, (**) $P < .01$ vs. pH 7.4.

Acidosis-Induced Changes of N-Cadherin and Vimentin in Solid Tumors

In order to analyze the impact of acidosis on the expression of EMT-related markers in solid experimental tumors, both rat tumor cell lines were implanted in animals subcutaneously. Acidification of the tumor was achieved either by intensifying the glycolytic metabolism (by inspiratory hypoxia and uncoupling of the respiratory chain resulting in a reduction of the tumor pH by about 0.5) or by direct intratumoral injection of small amounts of lactic acid. After 24 h, only the expression of N-cadherin and vimentin mRNA was analyzed in lysates of AT1 and Walker-256 tumors since the cell experiments (Figures 1 and 2) already showed that E-cadherin was not detectable in both cell lines and N-Cadherin was absent in Walker-256 *in vitro*. In AT1 tumors, both EMT parameters increased significantly with both acidification strategies by about 50% (Figure 4). In solid Walker-256 mammary carcinomas metabolic acidification (inspiratory hypoxia+MIBG) increased vimentin expression also approximately by 50%, however, direct lactic acid injection had only a minor impact (Figure 4).

Impact of Extracellular Acidosis on Cell Adhesion

Adhesion molecules play an important role for the stability of the tissue structure but also for adherence of circulating cells to the endothelial lining as a first step of transmigration through the endothelial barrier. Changes in the expression of cadherins but also of vimentin controlling integrin function [21] may therefore affect the ability of cell adherence. For this reason, the stability of cell binding to an artificial surface and the development of a stable cellular layer was measured under acidic conditions. In the present study two different processes were analyzed. In the first series, the impact of extracellular acidosis on already adherent cells was tested. This experiment simulates the loosening of cell–cell structure as an initial step of metastasis. In the second series, tumor cells were primed in an acidic environment and subsequently the adherence of these cells was measured. This experiment simulates the situation that tumor cells, which leave the acidic tumor environment and circulate in the blood, adhere in another tissue forming distant metastasis.

Figure 5*A* shows the impact of the extracellular pH on already adherent cells. In tumor cells the reduction of the pH down to 6.6 led to a significant decrease of cell adherence (at least after 48 h). This effect was most prominent in AT1 cells, but was also detectable in NCI-H358 cells. Normal epithelial cells (NRK-52E, MDCK) showed no significant effect. Figure 5*B* illustrates the adherence behavior of acidicly primed cells after 12 h. Here the impact of acidosis on tumor cells was non-uniform. NCI-H358 cells showed a reduced impedance, indicating that cells did not get firm contact to the surface. In contrast, AT1 cells which were primed at low pH showed a significantly stronger adherence. In both normal cell lines acidic priming had no impact on the re-adherence of the cells.

Transcription and Metabolic Factors During Extracellular Acidosis

Numerous transcription factors (e.g. Zeb, Twist, Snail) but also genes related to metabolism (e.g., Gpi, Idh2, Scd) have been described to activate epithelial-to-mesenchymal transition. In AT1 and Walker-256 tumor cells the expression of such genes was

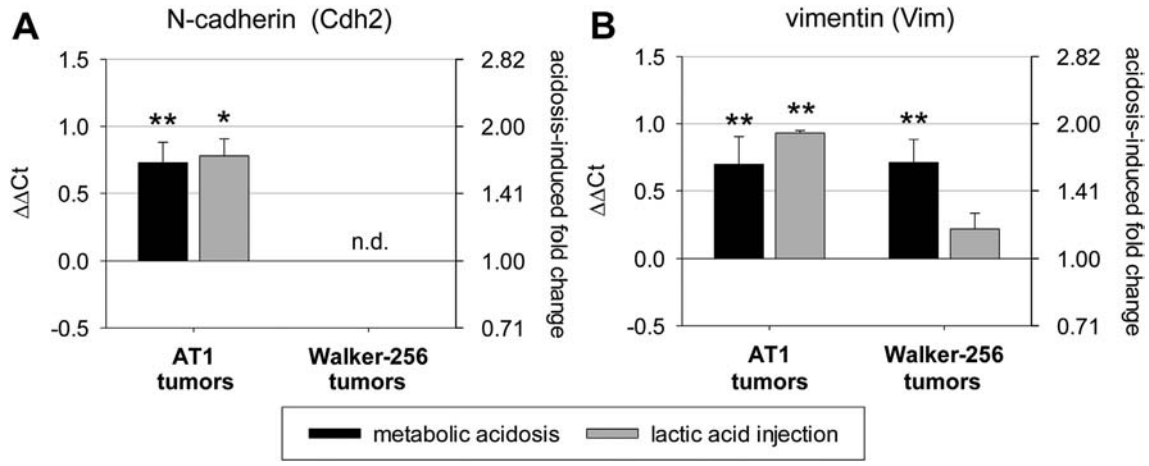

Figure 4. Changes of (**A**) N-cadherin and (**B**) vimentin gene expression of experimental AT1 and Walker-256 tumors in animals which underwent an intensified tumor acidification either by forcing glycolytic metabolism or by direct injection of lactic acid. Values are expressed compared to tumors of animals kept under control conditions. Mean ± SEM; n.d. not detectable; n = 2–11, (*) $P < .05$, (**) $P < .01$ vs. control conditions.

analyzed after 24 h of incubation at pH 6.6. Figure 6 shows the acidosis-induced changes in the expression. Some of these factors such as Twist1 or fumarate hydrase (Fh) were regulated consistently in both cell lines. However, some factors (e.g., Snai1, glucose-6-phosphate isomerase Gpi) were differentially affected in both tumor entities by low pH.

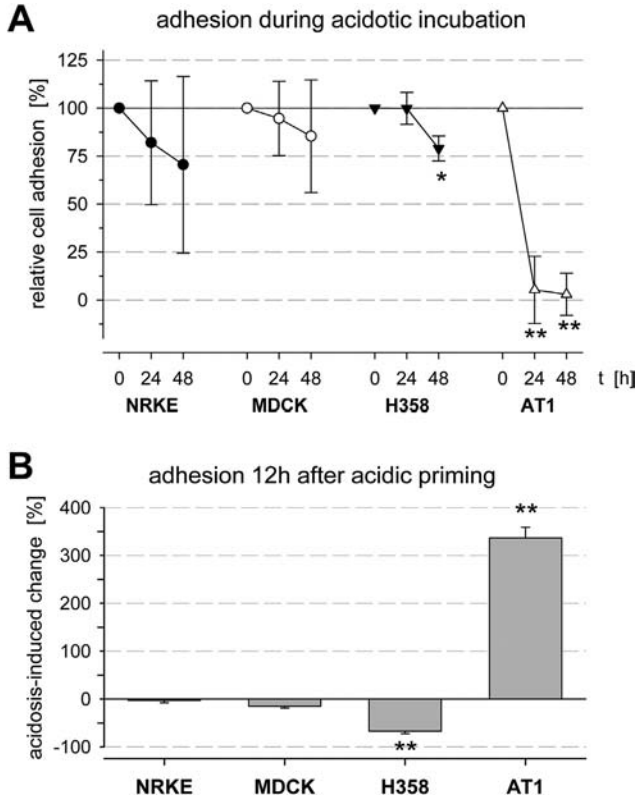

Figure 5. Impact of extracellular acidosis on adhesion of normal (NRK-52E, MDCK) and tumor (NCI-H358, AT1) cells measured by impedance of the cell layer. (**A**) Initially cells were grown at normal pH after which (t = 0 h) the medium was changed to pH 6.6. The change of impedance was followed up to 48 h. (**B**) Cells were initially primed at low pH and then plated at normal pH. The results show the changes of impedance after 12 h. Values are normalized to control conditions at pH 7.4. Mean ± SEM; n = 3–6, (*) $P < .05$, (**) $P < .01$ vs. pH 7.4.

Discussion

The present study clearly demonstrates that extracellular acidosis changes significantly the expression of EMT-related cell markers. Two of three tumor lines studied were rat cancers which could be subcutaneously implanted in animals to compare the changes *in vitro* with the *in vivo* situation. The third tumor line (NCI-H358 bronchioalveolar carcinoma) was chosen because this cell line is well known to show EMT-related changes after stimulation [17,22]. The effects in tumor cells were compared to normal epithelial cells in order to analyze whether the changes are tumor-specific or whether acidosis modulates the epithelial properties of normal epithelial cells to more mesenchymal-like. E-cadherin was found to be downregulated (on mRNA as well as on protein level) not only in NCI-H358 tumor cells, but also in both normal cell lines. A similar acidosis-dependent downregulation of the E-cadherin expression has also been described for melanoma cells [23] but also for normal human kidney epithelial cells [24]. For this reason, the loss of E-cadherin expression by low pH seems to be a typical cellular response and not a tumor-specific property. It might be a result of a generally reduced protein synthesis as seen for several other genes under acidotic conditions [25,26]. On the other hand, it has been shown that acidosis can induce inflammatory cytokines in tumor cells [25] and it was discussed that EMT-promoting factors (e.g. TGF-β) are secreted by the tumor cells in a pH-dependent manner [27]. Also, carbonic anhydrase IX could be involved, since it is up-regulated by an acidic microenvironment in AT1 prostate carcinoma cells [28] and melanoma, breast and colorectal cancer cells [29]. However, AT1 tumor cells displayed comparable changes in the expression of EMT marker as did Walker-256 tumor cells, where no CA IX protein was detectable (data not shown) and the normal cell lines lacking the tumor-specific CA IX activity. Therefore, an involvement of CA IX for the EMT marker expression in the cell lines described in this study seems to be of less importance. Since for cell adherence the expression of E-cadherin on the cell surface is essential [30], pH-dependent changes of the membrane bound protein were analyzed. As

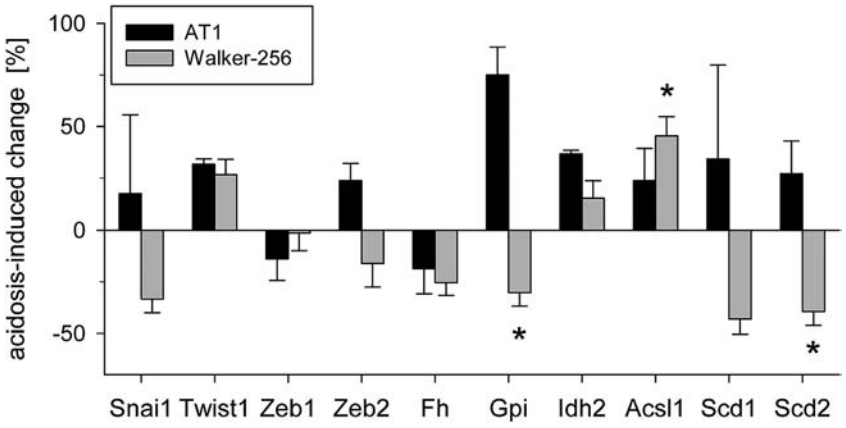

Figure 6. Expression changes of EMT-related genes in AT1 and Walker-256 tumor cells during incubation at pH 6.6 for 24 h measured by NGS. Values are normalized to control conditions at pH 7.4. Mean ± SEM; n = 3, (*) $P < .05$ vs. pH 7.4. (Snai1: snail family transcriptional repressor 1, Twist1: twist family bHLH transcription factor 1, Zeb1: zinc finger E-box binding homeobox 1, Zeb2: zinc finger E-box binding homeobox 2, Fh: fumarate hydratase, Gpi: glucose-6-phosphate isomerase, Idh2: isocitrate dehydrogenase 2, Acsl1: acyl-CoA synthetase long-chain family member 1, Scd1: stearoyl-CoA desaturase 1, Scd2: stearoyl-CoA desaturase 2).

shown in Figure 3 a decrease of surface E-cadherin was observed, which could be an indication of a pH-dependent loss of cell adherence.

Concerning vimentin expression during acidosis, Peppicelli et al. [23] showed an increase of protein expression by almost 100% after 24 h at pH 6.7 in melanoma cells. In the present study vimentin protein expression was significantly increased only in Walker-256 mammary carcinoma cells and in both normal cell lines. The increase in Walker-256 cells was less pronounced than in the study of Peppicelli et al. (Figure 2). In both other tumor lines an increase elevation was seen, which was, however, not statistically significant. On the level of mRNA, significant higher expression was found in NCI-H358 bronchial carcinoma cells. However, studies comparing EMT marker expression at various stages of malignancy have also found profound differences in the expression level in general and in the magnitude of the observed effects [31]. The three tumor lines used in the present study represent different stages of differentiation (NCI-H358: weakly differentiated, epithelial properties still present; AT1: undifferentiated and metastatic; Walker-256: metastatic, nonadherent). However, the impact of acidosis on vimentin expression was comparable independent from their stage. An increase of vimentin mRNA was also detected in solid tumors, even in AT1 tumors although the isolated tumor cells *in vitro* showed almost no regulation of vimentin mRNA. The difference might be explained by additional cell types (e.g. fibroblasts, immune cells) which are part of the tumor stroma and may affect the gene expression in tumor cells. Taking these results together, a reduced extracellular pH leads to an increase in vimentin expression in tumor as well as in normal cells.

N-cadherin protein expression was down-regulated in all tumor and normal cell lines. These results are in contrast to the findings in melanoma cells which showed an increase by 100% after incubation at pH 6.7 for 24 h [23]. The reason for this difference remains unclear. A discrepancy in N-cadherin expression has also been described in patients with pancreatic carcinoma or cholangiocarcinoma [32,33]. From the present results, it seems to be obvious that the pH-induced reduction of N-cadherin expression is not a tumor-specific property but could be part of the cellular stress response. However, for the functional role of N-cadherin for cell adhesion another aspect could be of interest. Several studies revealed that the functional adherence properties of the N-cadherin molecule are pH-dependent. It has been shown that low pH can either promote adhesion by N-cadherin [34] but also weaken N-cadherin-mediated adhesion [35].

In the present study the down-regulation of E- and N-cadherin was also reflected in a marked reduced adhesion during acidosis. In the first series of experiments (Figure 5*A*) the impact of extracellular acidosis on already adherent cells was tested. This is the situation in solid tumors when cells may lose their contact to neighbors and extracellular matrix as a first step of metastasis [36]. These experiments revealed a strongly reduced adherence of AT1 tumor cells and -to a lesser extent- of NCI-H358 cells, which was also seen in other cell lines in the literature [37]. However, functional changes of adhesion which are independent from the expression of the cadherins may also play a role. In the second experimental series, tumor cells were primed in an acidic environment and then adherence to the surface at normal pH was measured. This setting is similar to tumor cells circulating in the blood stream after evading an acidic tumor and then adhering in another host tissue. In this experimental setting, AT1 cells showed strongly increased adherence after acidic priming. These results are in good accordance with *in vivo* experiments with the same cell line, in which cells primed at low pH and then injected i.v. showed a significantly higher rate of lung metastasis formation [38]. However, in the present study adherence of NCI-H358 cells was reduced after acidic priming. These results indicate that the functional effects of acidosis on tumor cell adhesion are not only mediated solely by the expression of EMT-related marker.

Finally, the impact of acidosis on EMT-regulating transcription factors (e.g. Zeb, Twist, Snail) was analyzed (Figure 6). Whereas Snai1 and Zeb1 were regulated by acidosis inconsistently in both tumor lines, Twist1 was slightly elevated. Similar results were seen in melanoma cells at pH 6.7 in which the Twist expression was even stronger elevated by 100% [23]. Sciacovelli and Frezza [6] listed numerous genes of the cell metabolism which can activate EMT in tumors. Since extracellular acidosis is known to modulate the expression of metabolic factors [39] it might be possible that low pH activates EMT via factors of glucose or lipid metabolism. Most of the factors analyzed were differentially regulated in both tumor lines.

Only down-regulation of the fumarate hydratase (Fh) and up-regulation of the acyl-CoA synthetase long-chain family member 1 (Acsl1) by acidosis were consistent in both tumor cell lines and the direction of this change is known to activate EMT [6]. From these results it might be possible that low pH modulates the expression of metabolic enzymes and by this affects the process of EMT.

In conclusion, the present study illustrates that extracellular acidosis modulates the expression of EMT-related markers in tumor and normal cells, however, the impact can be cell line-specific. Forcing glycolytic metabolism in solid tumors *in vivo* and acidifying the extracellular space also leads to comparable changes of the EMT markers. Functional changes of cell adhesion under acidic conditions (either changes during continuous acidotic conditions or adhesion after acidic priming) varied between tumor cell lines and could lead either to reduced cell adherence in the tumor tissue or an increased attachment of circulating tumor cells in a new host tissue during metastasis. Therefore low tumor pH is able to modulate the expression of EMT-related proteins and by this affects the stability of the tissue structure. Targeting of the tumor pH, e.g. by neutralizing extracellular acidosis with systemic buffers such as sodium bicarbonate, TRIS (tris(hydroxymethyl)aminomethane) or IEPA (2-imidazole-1-yl-3-ethoxycarbonylpropionic acid), might be a promising therapeutic approach and could counteract the acidosis-induced changes in EMT marker expression and functional implications concerning adhesion if the tumor tissue is markedly acidic compared to the surrounding normal tissue [40]. But also modulating the cellular H^+ homeostasis (e.g., Na^+/H^+-exchanger, proton pumps) or mechanisms of pH-dependent intracellular signaling pathways could have an impact on the expression of the EMT markers and by this influence the invasive and metastatic potential of tumors [39].

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Conflict of Interest

The authors declare no competing conflicts of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.03.004.

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