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E-Cadherin loss associated with EMT promotes radioresistance in human tumor cells

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Abstract

Background and purpose—Hypoxia is a hallmark of solid cancers and associated with metastases and treatment failure. During tumor progression epithelial cells often acquire mesenchymal features, a phenomenon known as epithelial-to-mesenchymal transition (EMT). Intratumoral hypoxia has been linked to EMT induction. We hypothesized that signals from the tumor microenvironment such as growth factors and tumor oxygenation collaborate to promote EMT and thereby contribute to radioresistance.

Materials and methods—Gene expression changes under hypoxia were analyzed using microarray and validated by qRT-PCR. Conversion of epithelial phenotype upon hypoxic exposure, TGF β addition or oncogene activation was investigated by Western blot and immunofluorescence. Cell survival following ionizing radiation was assayed using clonogenic survival.

Results—Upon hypoxia, TGF β addition or EGFRvIII expression, MCF7, A549 and NMuMG epithelial cells acquired a spindle shape and lost cell–cell contacts. Expression of epithelial markers such as E-cadherin decreased, whereas mesenchymal markers such as vimentin and N-cadherin increased. Combining hypoxia with TGF β or EGFRvIII expression, lead to more rapid and pronounced EMT-like phenotype. Interestingly, E-cadherin expression and the mesenchymal appearance were reversible upon reoxygenation. Mesenchymal conversion and E-cadherin loss were associated with radioresistance.

Conclusions—Our findings describe a mechanism by which the tumor microenvironment may contribute to tumor radioresistance via E-cadherin loss and EMT.

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Keywords

Tumor hypoxia; Microenvironment; E-Cadherin; Radioresistance; EMT

Epithelial-mesenchymal transition (EMT) is an essential process during embryonic development where epithelial cells convert to mesenchymal cells. During EMT, epithelial cells lose cell–cell contacts and apico-basal polarity and acquire migratory properties. EMT is a complex program accompanied by the loss of epithelial markers such as adherens proteins E-cadherin and the acquisition of mesenchymal markers such as vimentin and fibronectin. Importantly, EMT can be usurped by transformed cells and has been implicated in the initiation of and progression toward more invasive and metastatic phenotypes [1]. More recently EMT has been associated with cancer stem cells and resistance to oncogene and chemotherapy-induced apoptosis [2]. Many pathways can activate EMT in normal and cancer cells such as receptor tyrosine kinase signaling, TGF β , Wnt- β -catenin and Notch signaling [1]. The constitutively active EGFRvIII variant has also been linked to increased malignancy and potentially EMT [3,4]. The role of TGF β in inducing EMT is complex. Whereas it is a homeostasis-guarding factor that restricts growth of normal epithelium, its function is converted to promote tumor growth and metastasis in advanced malignancies [5]. TGF β is also an important mediator of pathological fibrosis upon radiation [6]. In recent years, additional signals have emerged that induce EMT in mammals, including poor tissue oxygenation [7].

The microenvironment of solid tumors is a highly heterogeneous milieu, characterized by low pH, low nutrient levels, and both chronic and fluctuating levels of oxygen [8]. Hypoxia is a hallmark of solid cancers and associated with treatment failure and poor prognosis. It can activate programs that induce cell survival, invasion and metastases and can induce chemo- and radioresistance [9,10]. Constitutive activation of the hypoxia inducible transcription factor (HIF) pathway leads to downregulation of E-cadherin expression [11], a common feature of epithelial cells undergoing EMT [12]. We and others have previously shown that transcriptional induction of the EMT regulator Twist1 is part of a conserved response to hypoxia in normal and cancer cells [13,14]. Twist1 is a bHLH transcription factor part of a family of EMT regulators including the zinc-finger transcription factors Snail, Slug, ZEB1/2, all potent repressors of E-cadherin [15]. Furthermore, downregulation of E-cadherin via Snail can occur directly via hypoxia induced gene expression and indirectly via induction of lysyl oxidase (lox), a positive regulator of Snail activity [15].

Expression of these EMT regulators is often deregulated in cancer and correlated with loss of E-cadherin and metastasis free survival [16]. In addition to its role in cancer progression, EMT has also been linked with increased drug resistance [16,17] and a growing body of literature now suggests that changes in tumor cell differentiation, including EMT, contribute to drug resistance [18]. Much less data are available that link EMT and the response to ionizing radiation [19,20].

In the present study, we therefore investigated the contribution of microenvironment-induced EMT-like phenotypic changes to radiation response in cancer cells and found that E-cadherin plays a central role in response to radiation.

Materials and methods

Cell lines and reagents

MCF7, MDA-MB 231 (breast carcinoma), A549 (lung carcinoma) and NMuMG (non-transformed mouse mammary gland epithelial cells) cell lines (ATCC, Manassas, VA, USA) were incubated at 37 °C with 5% CO₂ and 95% air. For hypoxia exposure, cells were cultured in a hypoxic chamber (MACS VA500 workstation; Don Whitley Scientific) for the desired time periods. Culture media were obtained from Invitrogen and fetal bovine serum from Hyclone (Perbio; Thermo Scientific). Isogenic MCF7 cells were generated using the Flp-In T-Rex Core Kit from Invitrogen, and doxycycline (1 µg/ml) used to induce gene expression. For infection of MDA-MB 231 cells, viral particles were produced in 293FT cells (Invitrogen) by transfecting pEF-neo-hEcad (kind gift of Dr. P. Derksen), pvpack-gp (Stratagene) and VSV-G (Addgene) plasmids with P-PEI (Polysciences Inc.). MDA-MB 231 cells were infected for four times every other day, of which the second day polybrene was added (1 µg/ml). The fifth day cells were trypsinized, selected with G418 (500 µg/ml) and expanded. MDA-MB 231 cells stably expressing E-cadherin were designated MDA-MB 231/RevCdh1. TGF-β experiments were performed by incubating cells with recombinant human TGFβ1 (5 ng/ml) for the indicated time periods.

Antibodies, Western blotting and immunofluorescence

Western blotting and immunofluorescence was done according to standard protocols as described [21]. Antibodies used were mouse anti-human E-cadherin (1:1000; Becton Dickinson), mouse anti-human vimentin (1:1000; Neomarkers), mouse monoclonal anti-N-cadherin (1:100; Becton Dickinson), mouse anti-human β-actin (1:100,000; MP Biomedicals), anti-mouse secondary horseradish peroxidase conjugated antibody (1:2500) and goat anti-mouse Alexa 488 (1:500; Cell Signaling). Quantification of blots was performed using ImageJ. Chamber slides were analyzed using a Zeiss Axioskop fluorescence microscope. Western blots are representative for 2–4 independent experiments.

Micro-array analysis and quantitative real-time PCR

MCF7 breast cancer cells were exposed to anoxia and total RNA was isolated using RNeasy (Qiagen). RNA quantity/quality was determined using an ND-1000 spectrometer and RNA Nano LabChip kit on the 2100 BioAnalyzer. Equal RNA amounts from three independent experiments were pooled for each time-point. Twenty nanograms of pooled RNA were processed according to the manufacturer's protocols (Affymetrix, Santa Clara, CA) for 2-cycle amplification and samples were hybridized to Affymetrix HG-U133 Plus 2.0 GeneChips. All analyses were performed in R (v2.12.2). Data processing was performed using RMA (affy package, v1.28.0) for pre-processing and updated Entrez GeneID annotation (hgu133plus2hsentrezgcdf package, v14.0.0). More detailed information on the micro-array analysis can be found in Starmans et al. (Radiotherapy and Oncology, this issue).

RNA extraction for qRT-PCR was performed using the NucleoSpin RNA II isolation kit (Machery-Nagel) and qRT-PCR was performed as previously described [22]. The primer sequences used are

E-cadherin	Forward 5'-TGCCCAGAAAATGAAAAGG-3'
	Reverse 5'-GTGTATGTGGCAATGCGTTC-3'
Vimentin	Forward 5'-GAGAACTTTGCCGTTGAAGC-3'
	Reverse 5'-GCTTCCTGTAGGTGGCAATC-3'
N-cadherin	Forward 5'-ACAGTGGCCACCTACAAAGG-3'
	Reverse 5'-CCGAGATGGGGTTGATAATG-3'
CAIX	Forward 5'-CATCCTAGCCCTGGTTTTTGG-3'
	Reverse 5'-GCTCACACCCCTTTGGTT-3'

Survival assays

Clonogenic assays were performed as previously described [21].

Statistical analysis

Statistical analysis was carried out using the program GraphPad Prism version 5.01 for Windows (GraphPad Software, 2007, California, USA). Two-way ANOVA analysis was performed to determine differences in survival assays.

Results

Gene expression analysis upon hypoxia exposure

We and others have shown previously that activation of the bHLH protein and EMT regulator Twist1 is a part of conserved response of animal cells to hypoxia [13,23]. To explore whether Twist1 induction by hypoxic tumor cells was part of a common EMT response in epithelial cancer cells, we analyzed gene-expression changes at different time-points after hypoxia by microarray analysis in MCF7 cells. Among the hypoxia-induced genes were a number of signature genes associated with mesenchymal conversion, such as α -smooth muscle actin (α SMA) and fibroblast specific protein1 (FSP-1). Twist1 was also up-regulated, thereby confirming our previous observations [13]. Transcription of genes important for epithelial cell–cell contacts and for maintenance of apico-basal polarity such as Syndecan1, Plakoglobin, or Matrix metalloproteinase (MMP) inhibitors, like TIMP-2, was down-regulated (Fig. 1).

To extend these findings, we analyzed mRNA levels of a set of signature EMT markers by qRT-PCR in both MCF7 and A549 cells upon hypoxia. In line with the micro-array data, E-cadherin levels remained rather stable upon hypoxic incubation. Mesenchymal markers were more highly expressed upon hypoxia in these cancer cells (Supplementary Fig. 1).

A mesenchymal phenotype can be induced by hypoxia and is reversible

To investigate if the molecular changes that occurred upon hypoxia result in a more mesenchymal appearance, we cultured cells in hypoxic conditions and monitored their appearance by microscopy. Fig. 2a shows that transformed breast epithelial MCF7 cells and A549 lung adenocarcinoma cells display epithelial characteristics and diminished cell–cell

contacts under normoxic conditions (left panel), induce scattering and acquire a spindle-shape phenotype after 96 h in 0.2% oxygen. Next we investigated whether the morphological conversion and gene expression changes associated with EMT were also reflected by changes in protein levels. With increasing hypoxic exposure time, MCF7 cells exhibited downregulation of total E-cadherin levels in cell lysates (Fig. 2b) and by immunofluorescence (Fig. 2c). Since E-cadherin mRNA levels did not so much change under hypoxia, these data indicate changes at the post-transcriptional level. Vimentin levels in MCF7 cells were too low to be detectable. In A549 cells, the most predominant feature was the gain of mesenchymal markers, exemplified by the increase in vimentin expression (Fig. 2b) and the more intense membranous N-cadherin expression (Fig. 2c). In addition, E-cadherin downregulation could be observed upon 96 h of hypoxic exposure (Fig. 2b, lower panel). Also in non-transformed breast epithelial NMuMG cells, hypoxia reduced E-cadherin expression and cells adopted a more mesenchymal appearance, reminiscent of EMT (Supplementary Fig. 2A). Although the degree of gene expression and protein levels varied between different cell lines, our data indicate that exposure to hypoxia resulted in morphological transformation indicative of EMT.

Next we investigated whether the hypoxia-induced EMT-like phenotype was reversible. Cells were incubated for 96 h under hypoxia, and then transferred back to normoxia followed by vimentin and E-cadherin expression analysis. As shown in Fig. 2d, in both A549 and MCF7 cells, E-cadherin levels decreased upon hypoxia but fully returned after reoxygenation. Vimentin protein levels in A549 cells increased upon hypoxic exposure and stabilized for at least 48 h after reoxygenation before decreasing to basal levels after 3–7 days (Fig. 2d). Thus the molecular features associated with hypoxia-induced EMT-like phenotypes in these cells are at least partly reversible by oxygen.

Induction of EMT is more pronounced upon combination of various stimuli

Within the tumor microenvironment, a plethora of signaling factors in conjunction with hypoxia may influence EMT induction. We therefore investigated whether we could recapitulate the observed hypoxia-induced phenotype by other tumor or microenvironment-related stimuli such as TGF β addition or by expression of the constitutive EGFRvIII mutant, stimuli known to influence EMT.

Treatment of MCF7 and A549 cells with TGF β resulted in down-regulation of E-cadherin and/or up-regulation of vimentin (Fig. 3a). This was also observed in NMuMG cells (Supplementary Fig. 2B). MCF7-VIII cells, treated with doxycycline to induce EGFRvIII expression (shown in Supplementary Fig. 3), showed a time-dependent downregulation of E-cadherin (Fig. 3b).

Since all these stimuli result in EMT-like phenotypes and may occur simultaneously in the tumor microenvironment, we addressed whether combining different stimuli resulted in a stronger EMT-like phenotype. Combining hypoxia with TGF β or EGFRvIII expression resulted in a more pronounced EMT, as shown by a further reduction in E-cadherin and a gain of vimentin protein (Supplementary Fig. 4). Addition of EGF did not result in a similar more pronounced switch.

E-Cadherin modulates radioresistance in breast cancer cells

To address the importance of EMT-induced transdifferentiation on radiosensitivity, we compared the radiation response of cells modified to adopt either a more mesenchymal or epithelial phenotype by modulating the levels of E-cadherin.

In a first approach, MCF7 cells were seeded at increasing densities. As expected E-cadherin was predominantly membrane-associated in the dense MCF7 cultures but was virtually absent at the membrane in sparsely seeded cultures where it accumulated in the cytoplasm (Fig. 4c). We confirmed that total levels of E-cadherin did not change at different densities and that only its subcellular localization was different (see Western blot in Fig. 4b and immunofluorescent staining in Fig. 4c). When MCF7 cells at different density were exposed to ionizing radiation, we found that cells seeded at low density were significantly more resistant ($p < 0.05$, two-way Anova) than cells seeded at high density at all doses tested (Fig. 4a).

We reasoned that if cells with low cell surface E-cadherin are more resistant to radiation, reversion to the epithelial phenotype by reintroduction of E-cadherin may sensitize cells. To test this hypothesis, we compared the radiation response of the mesenchymal MDA-MB 231 metastatic breast cancer cells with an epithelial revertant, generated by expressing E-cadherin in MDA-MB 231 (RevCdh1) cells. Parental MDA-MB 231 cells show undetectable E-cadherin expression (Fig. 4e) and appear with a mesenchymal morphology. Expression of E-cadherin induced a marked conversion to a more epithelial morphology (compare left and right panels in Fig. 4f). Next we tested the radiation sensitivity of two independently generated MDA-MB 231 cell lines in which we reconstituted E-cadherin expression. MDA-MB 231 cells expressing E-cadherin were significantly more sensitive than their parental E-cadherin negative MDA-MB 231 cells as determined by their clonogenic survival after irradiation at increasing doses of γ -irradiation ($p < 0.001$, two-way Anova) (Fig. 4d).

Discussion

In the present study, we have shown that various stimuli emanating from tumor cells or their microenvironment can induce mesenchymal conversion of normal and cancerous epithelial cells. Interestingly, our data indicate that combining these signals results in a more pronounced EMT-like induction. Moreover, EMT-like transformation by the microenvironment is reversible upon reoxygenation. We have correlated these findings with changes in the expression of E-cadherin, a cell adhesion protein implicated in EMT and associated with tumor progression in many human cancers. We found that breast cancer cells that express E-cadherin were more sensitive to radiation than their counterparts without E-cadherin. Thus changes in E-cadherin expression in tumors induced by changes in the microenvironment such as hypoxia and reoxygenation may contribute to the intrinsic sensitivity of tumor cells to radiotherapy.

Our data are in line with previous studies that highlight the contribution of hypoxia in inducing phenotypic changes in cells. This induction has been linked to various mechanisms including the involvement of uPAR [24], activation of the PI3K/Akt pathway [7], inactivation of GSK3 β or the production of ROS [25]. Other reports have indicated a role for

Snail [4], and we and others have shown Twist1 to be induced upon hypoxia [13,23]. Here we confirm that hypoxia attenuates E-cadherin expression in epithelial cells as previously observed by others [11,26].

Since the tumor microenvironment is highly complex [10], we reasoned that other factors inherently present in solid tumors would also contribute to this phenotypic switch. Some of these signals might function as autocrine loops in cancer cells, such as expression of EGFRvIII, shown to be expressed in malignant brain, lung and breast tumors [27,28]. We have previously shown that EGFRvIII contributes to increased malignancy and survival under hypoxic conditions [22]. Others have reported that EGFRvIII expression disrupts adherens junctions [3] and that silencing EGFRvIII reduces expression of factors involved in EMT [29]. Our present data are consistent with both these observations and show additionally that the effect is even more pronounced under hypoxic conditions.

Another key signaling molecule in human tumors that might promote EMT is TGF β . Synergism between TGF β and hypoxia pathways has been observed in VEGF gene expression [30] and in the context of EMT, both hypoxia and TGF β are emerging as potentially important signals affecting the activity of E-cadherin repressors [15]. We observed cooperativity between hypoxia and TGF β in our experimental settings and hypothesized that cell–cell adhesion, and more specifically E-cadherin expression, could be a major factor influencing radiation response.

Many studies report a role for EMT and resistance to chemotherapy in human tumors (for a review see [18]). In contrast, less is known about the involvement of EMT in radioresistance. Snail has been shown to be involved in radioresistance of hematological progenitor cells *in vivo* [31]. Radioprotection can also be conferred by cell–matrix interactions and signaling through integrin receptors [32]. More direct evidence was provided by a study showing that radioresistant endometrial carcinoma cells exhibit phenotypic changes associated with EMT [33]. We noted that sparsely seeded MCF7 cells, showing less membrane-associated E-cadherin, were more radioresistant. In addition, mesenchymal MDA-MB 231 cells could be radiosensitized by reconstitution of E-cadherin expression. Both model systems suggest an important role for E-cadherin in radioresponse. Interestingly, the EMT transition accompanied by E-cadherin loss has recently been associated with cancer stem cells [34]. These cells have also been associated with tumor relapse and resistance to radiation [35]. Thus we propose that hypoxia may attenuate E-cadherin loss and induce EMT-like phenotypes that may promote increased invasion and metastases, increased cancer stem cell renewal but may also contribute to acquired radioresistance. Whether the effect of E-cadherin loss in radioresistance is direct or indirect as a consequence of deregulation of the DNA repair and cell cycle checkpoints by EMT [20] or by the acquisition of stem cell like properties remains to be investigated.

Further experiments will be necessary to determine how crucial E-cadherin downregulation is in inducing radioresistance in the context of hypoxia and the tumor microenvironment. Although the contribution of EMT to radioresistance *in vivo* remains unexplored, our data warrant further investigation into the impact of EMT on cancer treatment. As cancers are heterogeneous, for future treatments the plasticity of cancer cells of which EMT may only

be a transient but a decisive factor in tumor growth and response, will undoubtedly need to be considered.

Appendix A. Supplementary material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Micro-array mRNA expression levels of EMT markers during hypoxia. mRNA expression levels of mesenchymal (FSP-1, αSMA), epithelial (Syndecan-1, Plakoglobin, TIMP-1, Scribble, Discs large) and hypoxia markers (LOX, CAIX) measured by microarray analysis as a function of time under anoxia.

Fig. 2.

Switch to an EMT-like phenotype can be induced by hypoxia and is reversible. (a) Phase contrast microscopy images of MCF7 (upper panel) and A549 (lower panel) cells cultured in normoxic conditions or after incubation for 96 h in 0.2% oxygen. (b) Representative and quantified Western blots of MCF7 and A549 cells after culturing cells in normoxia or for different times in 0.2% oxygen showing E-cadherin and vimentin levels. (c) Immunofluorescent staining of E-cadherin in MCF7 cells and N-cadherin in A549 cells upon incubation under normoxia or 96 h of 0.2% hypoxia. Nuclei are stained with Hoechst. (d) Western blot of A549 and MCF7 lysates showing E-cadherin and vimentin levels upon exposure for 96 h under 0.2% oxygen(H), followed by different times of reoxygenation as indicated.

Fig. 3. Induction of EMT-like phenotype by expression of EGFRvIII and TGF β treatment. (a) Western blots for E-cadherin and vimentin from lysates of MCF7 and A549 cells treated with or without TGF β (5 ng/ml) for 48 h. (b) Representative anti-E-cadherin Western blot from lysates of MCF7-VIII cells treated with doxycycline (1 μ g/ml) for indicated time points. Densitometric quantification for the different blots is shown.

Fig. 4.

Cells with mesenchymal phenotype show increased radioresistance (a) Clonogenic survival of MCF7 cells seeded at different densities. (b) Western blot for E-cadherin of MCF7 lysates made from cell cultures grown at different densities. (c) Immunofluorescent E-cadherin staining (green) of densely seeded (left panel) and sparsely seeded (right panel) MCF7 cells. Nuclei are stained with Hoechst. (d) Clonogenic survival of MDA-MB 231 and two independent MDA-MB 231 (RevCdh1) clones with reconstituted E-cadherin expression. (e) Western blot showing E-cadherin levels of parental MDA-MB 231 cells and two (c1 and c2) independent MDA-MB 231 (RevCdh1) clones. (f) Phase contrast microscopy pictures of parental MDA-MB 231 cells and MDA-MB 231 (RevCdh1) c1. For clonogenic assays, cells were seeded, allowed to attach overnight and the next morning irradiated with the indicated doses. Shown are average survival $\pm$ SEM ($n = 3$).