A Transient, EMT-Linked Loss of Basement Membranes Indicates Metastasis and Poor Survival in Colorectal Cancer

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Background & Aims: Loss of the basement membrane (BM) is considered an important step toward tumor malignancy. However, the BM is still expressed in most typical colorectal adenocarcinomas; nevertheless, these tumors can invade and develop metastases. The aim of this study was to investigate the role, mechanisms, and clinical relevance of BM turnover in malignant colorectal cancer (CRC) progression. **Methods:** Expression of BM components and their transcriptional regulation and clinical relevance were investigated in human CRCs and cell lines. **Results:** Our data show new aspects in BM turnover in CRCs with impact on malignant tumor progression: (1) The BM is still expressed in the main tumor mass of most colorectal adenocarcinomas, but selectively lost at invasive regions of the tumor in many cases. (2) Selective loss of the BM at the invasive front has high clinical and tumor biologic relevance for distant metastasis and survival. (3) The BM is reexpressed in metastases, indicating that its loss is transient and regulated by environmental factors. (4) This transient loss is not only due to proteolytic breakdown but to a downregulated synthesis and linked to an epithelial-mesenchymal transition (EMT) in tumor cells, and, thereby, zinc-finger-enhancer protein 1 (ZEB1) is the crucial transcriptional repressor of BM components in CRCs. **Conclusions:** A transient BM loss at the invasive front is correlated with increased distant metastasis and poor patient survival, indicating its tumor biologic relevance and usefulness as a prognostic marker. Targeting ZEB1 might be a promising therapeutic option to prevent metastasis.

Typically, colorectal carcinomas (CRCs) are well to moderately differentiated and have a good clinical prognosis if detected early. Nevertheless, a fraction of these tumors develop metachronous distant metastases, often leading to the patient's death. Therefore, it is important to understand the underlying biologic processes, allowing a clinically relevant prediction of metastasis in these tumors with usually good prognosis.

In many cases, a breakdown of the basement membrane (BM) is considered to be a crucial step toward malignancy, allowing tumor cell detachment, migration, and, finally, dissemination through blood or lymphatic vessels.[1] Important structural components of the BM are laminins, type IV collagen, nidogens, and proteoglycans, which are synthesized both by epithelial and stromal cells.[2] In particular, laminin-5, built up by laminin α3 (Lama3), β3 (Lamb3), and γ2 (Lamc2) chains, is an important epithelial cell-derived component of BMs.[3] Disturbance in the stoichiometry of the 3 laminin-5 chains has strong consequences because, in the absence of α-chains, laminin β-chains are not secreted, and, thus, laminin-5 and subsequently BMs can not be built up.[4] In general, a destruction of an existing BM by proteolytic enzymes is made responsible for its loss in invading tumors. However, some reports already described a reduced production of important BM components; for instance, the Lama3 chain[5] is reduced during invasion, whereas other components, such as the Lamc2 chain, are overexpressed and even support invasion.[6–8]

During the last few years, another process became accepted as an important mechanism of tumor progression, in particular in CRCs: Invasion of many CRCs is associated with a dedifferentiation of the neoplastic tumor cells in invasive regions, at which the tumor contacts invaded tissue (subsequently called "invasive front") from a polarized, epithelial to a mesenchymal-like phenotype, resembling an epithelial-mesenchymal transition (EMT).[9,10] EMT is a basic embryonic mechanism involved in many developmental processes during tissue formation and organogenesis and is considered to be aberrantly reactivated in tumor progression.[11] Thereby, EMT is triggered by the inductive environment and mediated intracellularly through different transcriptional repressors. These repressors are aberrantly expressed in various types of carcinomas and typically suppress transcription of the E-cadherin gene, an important marker of epithelial differentiation. They include members of the snail family (Snail[12,13] and Slug[14]), Twist,[15] the E2A gene product E12/47,[16] and members of the Zincfinger-Homeobox (ZFH)-family of repressors (ZFHX1a, also called zinc-finger-enhancer binding protein 1 [ZEB1] or delta E-box factor (δEF)1[17] and ZFHX1b, also called Smad interacting protein 1 [SIP1] or ZEB2[18]). We described an EMT at the invasive front of colorectal adenocarcinomas but strikingly detected a mesenchymal to epithelial retransition (MET) in metastases, which in most cases shows again the same differentiated phenotype as the primary tumor.[9,19] This indicates that the EMT at the invasive front is a transient process and dynamically regulated by tumor environmental factors.

In addition to its mechanical barrier functions, the BM acts as an inducer of epithelial cell polarity and differentiation,

Abbreviations used in this paper: BM, basement membrane; CRC, colorectal carcinoma; EMT, epithelial mesenchymal transition; MET, mesenchymal to epithelial retransition; ZEB1, zinc-finger-enhancer binding protein 1.

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Table 1. Patients and Tumor Characteristics

	n	%
Sex		
Male	71	56.8
Female	54	42.2
Tumor site		
Upper third	35	28.0
Middle third	47	37.6
Lower third	43	34.4
Depth of invasion		
pT3a ≤5 mm	65	52.0
pT3b >5 mm[a]	60	48.0
Grading		
Low to medium	115	92.0
High	10	8.0
Venous invasion		
None (V0)	105	92.0
Microscopic (V1)	10	8.0
Lymph node metastasis		
N0	73	58.4
N1	30	24.0
N2	22	17.6
5 year metachronous distant metastasis		
M0	96	76.8
M1	29	23.2
Treatment		
Anterior resection	109	87.2
Abdominoperineal excision	16	12.8

NOTE. Inclusion criteria: invasive solitary rectal carcinoma, low to medium grading; pT3, no synchronic distant metastases; R0-resection, no additional treatment.
Mean cancer-related 5-year survival rate, 76.8%; mean age, 64 years (range, 36–91), mean follow-up time, 75 months.
[a]Invasion beyond muscularis propria.[33]

thereby regulating development and homeostasis of epithelial tissues.[20] In this context, the importance of BM loss for malignancy is not only the loss of a barrier but the disturbance of tissue architecture and the epithelial phenotype resulting in an EMT-like process. We here describe a transient, selective loss of the BM in invasive tumor regions in a part of well-differentiated CRCs, which still express BMs in the main tumor mass. Based on this initial finding, we assumed a down-regulation of BM-component synthesis in selected regions of the tumor host interface, which is regulated by the tumor environment in a coordinated manner with EMT. Therefore, we further analyzed BM expression in human primary CRCs and corresponding metastases as well as a potential regulation of BM-component expression by EMT-associated transcriptional repressors and correlated our findings with patients follow-up data.

Materials and Methods

Tissue Specimens, Patient Data, and Statistics

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas and corresponding metastases from patients who underwent surgery without additional treatments were retrieved from the archives of the Department of Pathology, University of Erlangen-Nürnberg. For clinical correlation with metachronous distant metastasis and survival, we used a collection of 125 pT3 M0 R0 rectal carcinomas with long-time clinical follow-up (patients' and tumor characteristics are shown in Table 1). The Kaplan–Meier method was used to calculate the rates of distant metastases and cancer-related survival (only deaths attributable to initial cancer were counted as events). The log-rank test was used for calculation of significance of loss of BM/EMT at the invasive front. The Cox regression analysis was used to identify variables with independent influence on prognosis.[21]

Immunohistochemistry

Flourescence immunohistochemistry and immunocytochemistry on human tumors and on cultured cells was done as previously described.[22] Only frozen sections were used without antigen retrieval because the monoclonal antibody anti-laminin $\alpha 3$ did not work on formalin-fixed material. Antibodies and dilutions used are described in Table 2.

DNA Constructs

For sequence comparison to detect homologies and conserved repressor binding sites in the mouse and human gene, we used the BLAST N algorithm (Pubmed). Sites in the human (−454 to +1) lama3a promoter/luciferase reporter construct were mutated using the Quick Change site-directed mutagenesis kit (Stratagene, LaJolla, Ca). All oligonucleotide sequences and plasmids used for this study are listed in Table 2.

Cell Culture and Assays

All cell lines were purchased from ATCC (ATCC, Manassas, VA). Cell culture, transient transfections, reporter assays, electromobility shift assays (EMSAs), immunoblots, transient siRNA-mediated knockdown, and real-time reverse-transcription polymerase chain reaction (RT-PCR) for quantification of messenger RNA (mRNA) expression were done as previously described.[8,22,23] For immunoblot of secreted laminin-5, the extracellular matrix was lysated in lysis buffer directly on the floor of tissue culture wells. All oligonucleotide sequences, plasmids, and antibodies used are listed in Table 2. All experiments were done at least 3 times. The doxycyclin-inducible cell line DLD-1TR21 for expression of Snail was described previously.[24] In addition, stable clones for hSlug were obtained with sequence verified pcDNA4/TO-hSlugMyc/His plasmids. Expression of Snail or Slug was induced using doxycycline (1 μg/mL, Sigma-Aldrich, St. Louis, MO).

Results

Selective Loss of the BM at the Invasive Front Associated With EMT

Loss of the BM is generally thought to be an important hallmark for the transition from benign to malignant tumor growth. However, when analyzing colorectal adenocarcinomas, we found that all well- to moderately differentiated tumors expressed BMs (Figure 1), which is in line with previous observations in differentiated types of gastrointestinal cancers.[2] BMs, detected by staining of Lama3, surrounded tubular differentiated tumor glands built up by polarized tumor cells in the main tumor mass (Figure 1*B*). Nevertheless, many of the differentiated tumors expressing BMs had distant metastases. However, in a part (52.5%) of the 50 analyzed cases, a selective loss of the BM, demonstrated by a loss of the epithelial BM component Lama3, was found at the invasive front. This loss of the BM correlated with a dedifferentiation/EMT of the tumor cells (Figure 1*C*), further indicated by aberrant expression of mesen-