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Review

The Epithelial-Mesenchymal Transition (EMT) and Colorectal Cancer Progression

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KEY WORDS

colon cancer, epithelial-mesenchymal transition, integrins, cytokines, tumor progression

ABBREVIATIONS

CRC	colorectal cancer
HNPCC	hereditary non-polyposis colon cancer
EMT	epithelial-mesenchymal transition
TNF-α	tumor necrosis factor-alpha
TGF-β	transforming growth factor beta
VEGF	vascular endothelial growth factor

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ABSTRACT

During embryonic development, epithelial cells must escape the structural constraints imposed by tissue architecture and adopt a phenotype more amenable to cell movement, a process known as the epithelial-mesenchymal transition (EMT). The progression of carcinomas to invasive and metastatic disease may also involve localized occurrences of EMT. However, data that support the actual occurrence of EMT in specific carcinomas and the relevance of this process to the progression of these tumors had been scant. This review highlights recent studies that substantiate the importance of the EMT to colorectal carcinoma. Specifically, a novel model for studying the EMT of colorectal carcinoma has been used to gain insight into the nature of the EMT itself and to identify molecular events that contribute to disease progression. Although loss of E-cadherin function is a primal event for the EMT, the expression of specific integrins such as αvβ6 as a consequence of the EMT enables invasive cells to interact with interstitial matrices and to sustain activation of TGF-β. Of note, αvβ6 expression in tumors is a marker of cells that have undergone an EMT and it is prognostic for tumors that will progress more rapidly to terminal disease. The EMT also induces autocrine signaling involving VEGF and Flt-1 that enable invasive cells to become 'self-sufficient' for survival. Thus, the EMT appears to be an integral component of colorectal cancer progression and its analysis can yield novel targets for prognosis and therapy.

INTRODUCTION

Despite welcome declines in the mortality rate over the past decade, colorectal cancer (CRC) remains the second leading cause of cancer incidence and death among adult Americans.[1] In fact, the American Cancer Society estimates that in 2005, almost 150,000 new cases and 60,000 deaths will result from this disease. Similarly, CRC is also the second most common cause of cancer death in Europe, where comparable numbers will account for approximately one-tenth of all tumor-related deaths.[2] Fewer than 10% of these cases will be in individuals who have an inherited predisposition to the disease, arising from germline mutations in specific genes. In the case of familial adenomatous polyposis (FAP), mutations of the *APC* gene result in the development of hundreds to thousands of colonic polyps, a condition that virtually assures the individual of developing CRC during their lifetime.[3,4] Germline mutations in DNA Mismatch Repair (MMR) genes underlie the genetic basis for hereditary nonpolyposis colon cancer (HNPCC),[4,5] in which affected persons develop far fewer polyps, however these polyps are very likely to transition to cancer, and with a rapid rate of progression.[6] Other familial colon cancers will account for up to 25% of all CRC cases, wherein affected families show incidences of disease that are too frequent to be considered sporadic but not in a pattern consistent with an inherited syndrome.[6]

The vast majority of CRC (approximately 70%) develop in the population as a sporadic disease. Indeed, it has been the extensive study of sporadic colon cancer over the last two decades that has led to this disease representing a paradigm for the development of other solid tumors. Our understanding of the molecular basis for tumor development has been strongly influenced by the seminal contributions of Vogelstein and colleagues, who characterized the multistep model of carcinogenesis.[7,8] This concept describes an accumulation of genetic events (somatic mutations), each conferring a selective growth advantage to the colon cell, which eventually result in uncontrolled cellular proliferation, clonal tumor development, and progression from adenoma to invasive carcinoma. The role of critical oncogenes (such as *ras* and *myc*) and the inactivation of tumor suppressors (such as *p53*), as well as other modulators (e.g., COX-2, EGF-R) have subsequently been identified and characterized.

Despite our knowledge of the molecular basis for CRC development, the translation of this information into effective therapies and treatments has been limited. It is true that more rigorous screening and prevention have contributed to the declining death rates, and that several novel agents offer promise for adjuvant therapies.[9] The persistent high mortality and morbidity of this disease, however, reflect the fact that diagnosis frequently occurs after the primary tumor has developed. Although surgical resection is highly effective for early stage tumors (with cure rates of over 90% in Stage I and 75% in Stage II disease),[1] treatment of advanced colon cancer remains essentially palliative. Moreover, these later stage malignancies tend to respond poorly to current chemotherapeutic regimens, and consequently the prognosis for these patients is often grave. Thus, in a field where our understanding of the genetics exceeds our understanding of the cell biology, it is essential to acquire a better understanding of the cell biological events that contribute to progression, especially those processes that facilitate the switch to invasive and metastatic disease.

EMT—A PARADIGM FOR INVASIVE CARCINOMA

During key biological processes such as embryonic development, tissue remodeling, restitution and wound repair, there is a requirement for epithelial cells to escape from the rigid structural constraints provided by the tissue architecture and adopt a phenotype more amenable to cell migration and movement. The highly conserved and fundamental process that achieves this morphogenetic transformation is known as the epithelial-mesenchymal transition (EMT).[10-12] Essentially, during this event, epithelial cells actively downregulate cell-cell adhesion systems, lose their polarity, and acquire a mesenchymal phenotype with reduced intercellular interactions and increased migratory capacity. Of particular to interest to cancer biologists is the emerging realization that the progression of epithelial-derived tumors (carcinomas) may also involve spatial or temporal occurrences of EMT. Therefore, and not unlike other physiological processes that become coopted by tumor cells, the EMT can be considered a pathological process that contributes to cancer progression, particularly as it relates invasion and metastasis.[13] De-differentiation, loss of adhesive constraints, and enhanced motility and invasion, are all hallmarks of increased malignancy, and the EMT provides a mechanism for carcinoma cells to acquire this more aggressive phenotype.

The strongest evidence for the importance of the EMT relates to the cell adhesion molecule Epithelial (E)-cadherin. E-cadherin is a member of the cadherin family of homophilic cell adhesion molecules, and is essential for the maintenance of adherens junctions that confer physical integrity and polarization to epithelial cells. Notably, targeted disruption of this molecule during tumor progression resulting in decreased intercellular adhesiveness is one of the most common alterations in human cancers.[14,15] Further, abolishing E-cadherin function in vitro confers invasive properties to noninvasive cells and conversely, introduction of E-cadherin into invasive epithelial cell lines abrogates their invasive potential. In this regard, E-cadherin is considered a broadly acting suppressor of invasion and metastasis, and its functional inactivation represents a critical step in the acquisition of this capability.[15] Not surprisingly, then, loss of E-cadherin expression is a defining characteristic of EMT.[16] Although a subset of tumors, including some lobular breast carcinomas and gastric cancers,[17-20] exhibit mutations in the E-cadherin gene, multiple mechanisms contribute to the inactivation of E-cadherin including promoter methylation, phosphorylation and transcriptional repression.[21] Indeed, the discovery that transcriptional repressors of E-cadherin such as Snail, Slug and Twist contribute to invasion and metastasis has strengthened the evidence for the importance of the EMT in carcinoma progression.[22-24]

THE TGF-β PARADOX

The EMT event is not a cell-autonomous process and requires, at the very least, initiating signals to drive the transition. Moreover, the tumor microenvironment, in particular the reactive stroma, is an active participant in tumor progression, with complex interactions between tumor and stromal cells enhancing tumorigenesis by supporting cancer cell proliferation, survival and migration.[25,26] These facts prompt the question of whether signals from this source may modulate EMT sensitivity.

Enter the growth regulatory proteins of the transforming growth factor-beta (TGF-β) family. The three highly homologous members of this family play complex roles in a vast array of biological processes, and represent one of the few classes of endogenous inhibitors of cell growth.[27] In fact, the potent ability of TGF-β to inhibit epithelial cell proliferation led to its initial description as a tumor suppressor.[28] Paradoxically, TGF-β was subsequently found to be abundantly expressed in many epithelial tumors where it acts in both an autocrine manner on the tumor cells themselves and as a paracrine modulator of the stroma (reviewed in refs. 27, 29 and 30). In colon carcinoma, TGF-β also acts differently depending on the differentiation stage of the tumor, in general by switching from an early inhibitor of proliferation to a stimulator of growth and invasion during tumor progression.[31,32] In addition, while loss of the receptors for TGF-β are relatively rare oncogenic events, the locus for the type II TGF-β receptor is frequently mutated in HNPCC patients. Ironically, the complete genetic inactivation of TGF-β signaling in these tumors is associated with a better prognostic outcome.[33]

Although beyond the scope of this review, it is now clear that TGF-β unequivocally demonstrates both tumor suppressor and oncogenic activities.[34] Of particular interest, it appears that when the growth inhibitory responses become selectively lost in colon tumors, concomitant enhancements of tumorigenic properties become uncovered, including sensitivity to EMT.[35] TGF-β has been implicated as a key inducer of EMT,[36-39] and it has now apparent that the transition requires cooperativity between continuous TGF-β signaling and an activated Ras pathway.[40-42] It is perhaps not coincidental, then, that activating *ras* mutations are a common and early event in colorectal carcinogenesis. It is tempting to speculate that oncogenic activation of this canonical signaling pathway not only provides a continuous growth stimulus for these cancers,[6] but also potentially increases the propensity of this tumor type to undergo EMT.

EMT IN COLON CARCINOMA—THE LIM 1863 SPHEROID MODEL

Unfortunately, it is difficult to follow EMT in human tumors either temporally or spatially because of the great diversity of cellular organization displayed by neoplasms in vivo.[16] Consequently, the importance of this process in carcinoma progression has taken longer to establish, and there remains speculation as to its significance, particularly in "well-differentiated" solid tumors, such as most colon carcinomas. However, routine pathological analysis is unlikely to

detect EMT because this process probably occurs transiently in discrete areas within tumors.[43] Therefore, much of our understanding of the mechanisms and pathways that regulate EMT derives from the use of cellular models. Although such models are subject to criticism (e.g., they reflect the tumor situation in vivo poorly, they lack stromal interactions), they have proven to be highly relevant for unraveling the contribution of specific proteins to tumor progression.[43] Indeed, much of the current knowledge of the mechanisms involved in the EMT has been obtained from the use of a handful of immortalized cell lines, such as EpH4/EpRas, NMuMG, MDCK and MCF-10.[37,38,40,44,45] There are some significant drawbacks to use of these cell lines, however. The genesis of an epithelial phenotype often requires culture in three-dimensional matrix, EMT usually takes several days to complete, and few common carcinoma cell types with a well-defined epithelial phenotype (such as colon cancer) can undergo EMT in vitro.[16]

The multi-cellular nature of the EMT implies that its analysis in vitro requires cell systems such as spheroids that reiterate the epithelial phenotype. Indeed, in contrast to most traditional cell culture systems, spheroids provide a unique opportunity to recapitulate aspects of cell homeostasis and as such reflect in vivo tumor biology better.[46] Recently, we developed a novel tumor model of EMT by using a three-dimensional spheroid system comprised of highly differentiated colon carcinoma cells that undergo EMT in response to cytokine stimulation.[47] LIM 1863 cells are well-differentiated colon carcinoma cells that grow as structured spheroids, termed organoids, around a central lumen.[48] Tight junctional complexes and epithelial polarity are hallmarks of this line that grows in suspension.[49,50] This remarkable degree of organization is intrinsic to the cells and does not depend on either exogenous basement membrane or culture within a three-dimensional matrix, unlike other spheroid tumor models.[51,52] The LIM 1863 organoids will undergo an EMT conversion from this well-differentiated spheroid structure to a migratory monolayer phenotype in response to TGF-β. Moreover, we found that the EMT process was accelerated dramatically by costimulation with the inflammatory cytokine TNF-α.[47] In fact, exposure to TNF-α results in the establishment of an autocrine loop of TNF-α stimulation, that sensitizes the cells for the subsequent modifying effects of TGF-β.[47] We have also shown that the exogenous application of TGF-β to these cells also elicits the autotropic secretion of TGF-β itself,[53] a response that has important biological implications for tumor progression, as discussed below. Overall, ongoing research from our laboratory has demonstrated clear advantages in the use of the LIM 1863 cell line for studies on EMT, particularly as these organoids are capable of undergoing an EMT that mimics the progression to invasive colon carcinoma. Significantly, not only has this model provided conceptual correlates to CRC progression in humans, it has identified and validated novel molecular targets with relevance to human disease.

THE INTEGRIN αVβ6—A NOVEL MARKER OF THE EMT AND PROGNOSTIC FACTOR FOR AGGRESSIVE COLORECTAL CARCINOMA

While the loss of E-cadherin may be a primal event for EMT, alterations in other adhesion mechanisms must occur to generate a mesenchymal phenotype. In this respect, the paradigm of an EMT is apt because an invasive carcinoma cell must acquire the ability to interact with distinct interstitial matrices subsequent to its transgression of the basement membrane. Accordingly, the integrin family of adhesion receptors molecules represent the major receptors that mediate attachment to the ECM, with ligand occupancy triggering critical intracellular signaling pathways.[54,55] While there is little doubt that these molecules play critical roles in the capability of tumor cells for tissue invasion and metastasis,[15] surprisingly little is known about how integrin expression and function are regulated during EMT. To investigate this possibility, we analyzed integrin dynamics in our colon carcinoma model as and discovered that EMT induced a marked increase in the expression of the αvβ6 integrin, a receptor for fibronectin and tenascin.[53] Importantly, the consequences of this elevated αvβ6 expression are directly linked to both the function of mesenchymal cells and to the mechanism of EMT itself.

The αvβ6 integrin is expressed primarily during development, and high αvβ6 expression in adults is restricted to only a few epithelial tissues.[56,57] However, αvβ6 can be reexpressed in parallel with specific morphogenetic events such as inflammation and wound healing. The latter, in particular, involves the EMT process, and the induction of αvβ6 implies a central role for this receptor.[57-59] Interestingly, studies on the phenotypic changes induced by EMT have traditionally focused on the repression of epithelial markers that maintain polarity, and the functional consequences of induced mesenchymal markers. Our discovery that an epithelial integrin is dynamically regulated during the EMT of colon carcinoma, and specifically one that is upregulated during development, is consistent with the long-standing hypothesis that colorectal carcinogenesis more accurately reflects a dedifferentiation of epithelia to a more embryonic phenotype.

But what of the selective advantages to increased αvβ6 expression, and how might EMT promote a more malignant phenotype in colon tumors? Firstly, since the colonic epithelium resides upon a basement membrane comprised mainly of laminins, collagen IV and proteoglycans, expression of a specific fibronectin receptor would facilitate the escape and dissemination of invading carcinoma cells into the lamina propria. The structural composition of this zone differs in its high fibrillar collagen content, and also in its enrichment with fibronectin and tenascin, both ligands for αvβ6.[60,61] Moreover, an increased propensity to adhere to fibronectin via this receptor would promote colon carcinoma metastasis to the liver by aiding extravasation. Given the absence of a basement membrane under the endothelium of liver microvessels, it has been suggested that adhesion to fibronectin may be of importance, since there is abundant expression of this protein on the surface of hepatocytes.[62] Thus, in addition to the anatomical considerations of hepatic blood flow, elevated expression of the αvβ6 integrin may add another dimension to the colonization of this organ. Consistent with this, we observed high expression of this receptor in liver metastases from colorectal patients.[53]

Secondly, we have gained insights to the complexity and relevance of the interplay between this integrin and the EMT-inducing cytokine TGF-β. As discussed above, TGF-β has been proposed to act as a cell autonomous promoter of late stage tumor development because of its ability to regulate EMT,[35] and LIM 1863 organoids undergoing EMT exhibit autocrine production and secretion of this cytokine.[53] TGF-β family members are secreted as latent complexes that require activation in order to bind their receptors, and an essential role for αvβ6 to bind and activate TGF-β has emerged.[63] In assessing the ability of αvβ6 to activate TGF-β during EMT in our colon model, we established that post-EMT cells acquired the capacity to activate latent TGF-β, and that this effect was attributable to the increase in the levels of this integrin.[53] Thus, in the context of an in situ tumor, autocrine TGF-β production would provide the means

to stabilize and sustain the EMT by continuous TGF-β receptor signaling in a cell-autonomous fashion. Moreover, induced expression of αvβ6 in colon carcinoma would provide a mechanism to regulate TGF-β function locally in vivo, to provide a feedback loop to perpetuate the EMT process and, in turn, to create a tumor microenvironment more amenable to progression.

One of the limitations to studying the EMT in vivo that was alluded to above is that it is difficult to detect by routine methods. It appears, however, that αvβ6 may be a useful marker for the detection of the EMT in vivo based on our analysis of the LIM 1863 cells grown as xenografts in mice. As expected, the organoid-derived tumors stained strongly for E-cadherin and weakly for αvβ6 (Fig. 1). However, a dramatic induction of β6 expression was evident in localized regions of the tumor and, in particular, on tumor cells that were invading into the stroma. Significantly, these invading cells had lost expression of E-cadherin.[53] These observations provide strong evidence for relevance of the EMT to disease progression. Moreover, the findings are consistent with a functional role for αvβ6 in facilitating the invasion and dissemination of colon carcinoma cells.

Clearly, an increased understanding of EMT mechanisms contributes greatly to our knowledge of tumor progression, yet it could be argued that it is ultimately of little consequence if the biology that is uncovered fails to impact human disease. To this end, we defined αvβ6 expression as a novel, independent prognostic indicator for aggressive colorectal cancer.[53] This significance is based, in part, on its frequency of expression in a subset of malignant tumors, and the finding that elevated receptor expression is associated with a significantly reduced survival time of patients in comparison with tumors displaying little or no expression of the molecule.[53] Importantly, the elevated β6 expression did not merely reflect increasing tumor stage, and its prognostic value was in fact more significant for patients with early-stage disease. Thus, it is tempting to extrapolate from our cellular model and speculate that EMT is directly contributing to the development of human metastatic disease. Along these lines, several studies have shown neo-expression of this integrin in oral squamous cell carcinomas,[57,64,65] although a link between expression and patient outcome has not been established. Overall, establishing αvβ6 as a novel prognostic marker for human CRC validates the EMT model as a valuable tool for the identification of clinically relevant markers. The αvβ6 integrin also represents an attractive new therapeutic candidate for CRC for two reasons: (i) it raises the possibility of selectively targeting invading and metastasizing cells and (ii) it may represent a feasible option for earlier intervention because elevated expression is predictive of outcome in early-stage disease.

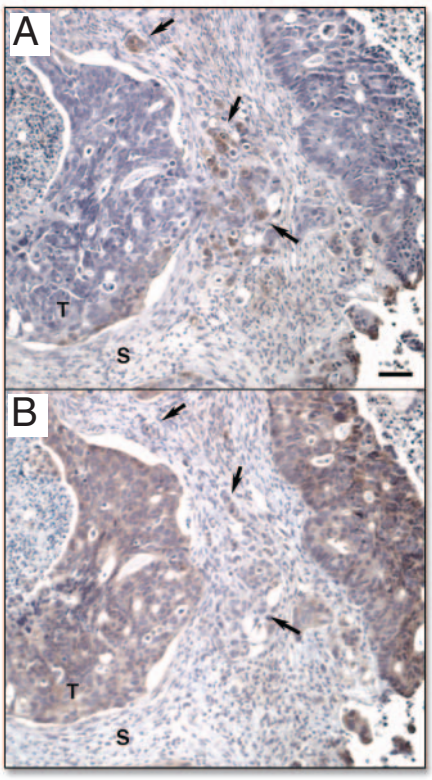

Figure 1. Evidence that αvβ6 is a marker of EMT in vivo. Integrin β6 (A) or E-cadherin (B) immunostaining of sequential tumor sections derived from LIM 1863 xenografts. E-cadherin expression is prominent in the tumor tissue (*T*) and absent from the stroma (*S*). β6 immunoreactivity is relatively weak in the tumor masses, but is strongly upregulated in tumor cells invading the stroma, which have lost E-cadherin expression (arrows). Scale bar: 50 μm. (From Bates et al., 2005. Used with permission).[53]

A ROLE FOR EMT IN ABERRANT TUMOR CELL SURVIVAL

Aberrant cell survival and resistance to apoptosis are also hallmarks of tumor invasion and progression to metastatic disease,[66] and while the molecular mechanisms underlying the EMT are currently being elucidated, little attention has been given to the possibility that anomalous cell survival mechanisms are an essential component of the process. To this end, we have previously shown that the degree of morphological specialization exhibited by LIM 1863 cells is critical for their survival, with the intercellular contact provided by the organoid structure necessary to maintain viability.[50] However, following the EMT, individual cells migrate and survive in the absence of any cell-cell contact, indicating that this phenotypic transition results in an escape from the constraints of adhesion-dependent survival. VEGF was identified as the candidate survival factor whose expression is increased in response to EMT,[67] and a concomitant upregulation of Flt-1, a tyrosine kinase VEGF receptor, accompanied the increase in VEGF production. Importantly, we characterized this VEGF/Flt-1 autocrine interaction as necessary for survival of the colon carcinoma cells, since perturbation of Flt-1 function caused massive apoptosis only in cells that underwent EMT. Significantly, this finding defined the acquisition of the self-regulated survival pathway as an essential component of the EMT process itself.[67] In addition, this VEGF pathway is critical for the survival of other invasive colon carcinoma cell lines, and we observed a correlative upregulation of Flt-1 expression linked to in vivo human cancer progression, as predicted by the model.

The fact that a VEGF autocrine loop is elaborated as a consequence of EMT is consistent with the notion that autocrine signaling of survival is manifested during the later stages of carcinoma progression.[68] Clearly, the acquisition of an inherent ability to suppress apoptosis, in an autocrine manner, would play a vital role in colorectal tumorigenesis and points to the selective advantages of Flt-1 expression by colon carcinomas. Indeed, these findings are substantiated by other reports of both VEGF and Flt-1 gene expression as being upregulated during neoplastic progression of the colonic mucosa,[69] as well as the increased detection of Flt-1 mRNA in hepatic metastases.[70]

The identification of VEGF/Flt-1-mediated survival may have important therapeutic implications for CRC, substantiating the clinical usefulness of the EMT. In a number of mouse models, gene transfer of soluble Flt-1, which acts in a dominant-negative fashion

to the endogenously expressed receptor,[71] has been effective for a variety of cancer types, including colon, ovarian, pancreatic, and lung.[72-76] These studies concluded that the suppression of primary tumor growth and prolonged animal survival were based on the ability of the soluble receptor to block angiogenesis, presumably by antagonizing VEGF signaling responses. Thus, anti-angiogenic gene therapy using soluble Flt-1 is considered to be a potential avenue for therapeutic exploitation. However, as mentioned in the Introduction, CRC diagnosis is often a late occurrence, and effective late-stage chemotherapeutic options remain limited. For these reasons, it would appear that anti-angiogenic approaches, which target earlier biological processes critical for progression, might be less effective for this disease. Yet, the aberrant survival function data we obtained suggest that it may be possible to selectively target invading and metastasizing cells, strengthening the conclusions of the integrin findings discussed above. In this regard, it is interesting to note that VEGF inhibitors represent a new class of novel therapeutic agents for the treatment of CRC.[9] One in particular, known as bevacizumab (Avastin, Genentech), has shown considerable promise when used in combination with standard therapy regimes,[77] and is presently in Phase III clinical trials for metastatic CRC.[78] This humanized antibody binds to VEGF and neutralizes VEGF function by steric hindrance, therefore its effects in vivo have been interpreted as acting via an inherent ability to inhibit angiogenesis. However, since these trials are limited to patients who have advanced metastatic disease, and in light of the results we are presenting here, it is not mutually exclusive that this drug might also be affecting VEGF-mediated survival pathways in the tumor cells, pathways that are elaborated as a consequence of the EMT.

CONCLUDING REMARKS

In spite of rapid advances in our understanding of the molecular genetics of CRC, the burden of disease remains high and the outlook for patients with advanced cancer is still poor. For this tumor type in particular, where the switch to the malignant phenotype is critical for outcome, it is of paramount importance to elucidate the molecular biology that underlies the metastatic evolution of this disease. As has been shown, the EMT provides a powerful paradigm for investigating both genetic and epigenetic aspects of late stage tumor progression. In addition, since over 80% of human cancers arise from the epithelium as carcinomas, the benefits of EMT research will not be restricted to CRC, and should impact the bulk of solid tumors. Compelling insights to the biochemical framework by which EMT promotes invasion and metastasis have already been discovered, and further functional analyses using well-defined models and more elaborate approaches will contribute to the development of novel diagnostic and therapeutic strategies for this insidious killer.

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