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Authors
Tlsty, Thea D
Hein, Patrick W

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Know thy neighbor: stromal cells can contribute oncogenic signals

Thea D Tlsty* and Patrick W Hein

Although the stroma within carcinogenic lesions is known to be supportive and responsive to tumors, new data increasingly show that the stroma also has a more active, oncogenic role in tumorigenesis. Stromal cells and their products can transform adjacent tissues in the absence of pre-existing tumor cells by inciting phenotypic and genomic changes in the epithelial cells. The oncogenic action of distinctive stromal components has been demonstrated through a variety of approaches, which provide clues about the cellular pathways involved.

Addresses
Department of Pathology and UCSF Comprehensive Cancer Center, University of California at San Francisco, San Francisco, California 94143-0506, USA
*e-mail: ttlsty@itsa.ucsf.edu

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Abbreviations
CAF carcinoma-associated fibroblast
HPV human papilloma virus
MMP matrix metalloproteinase
TIMP-1 tissue inhibitor of metalloproteinase 1

Introduction

For many years, most studies of neoplastic transformation have focused on the unit of the cell. Altered signal transduction pathways in neoplastic cells are critical to the perversion of cellular proliferation, death, motility, DNA repair and genomic integrity. Reciprocally, much has been learned from these studies concerning the regulatory circuits that maintain cellular homeostasis. In organs such as mammary gland or skin, however, cellular homeostasis is conjointly dictated by interactions between stromal and epithelial cells [1–4]. These interactions govern glandular size, function, and response to exogenous agents, often through mutual elaboration and modification of the extracellular matrix. During wounding and other pathological conditions, both the stromal and the epithelial cells exhibit fundamental changes in a dynamic molecular dialogue that is important for a proper tissue response [5].

In cancer, the stroma has often been studied in the context of the malignant lesion; rarely has its role before the presence of a tumorigenic growth been considered. Supportive functions, such as angiogenesis, are provided by the stromal components and permit tumor growth. Responsive functions, such as the remodeling of the extracellular matrix during invasion, are elicited by the tumor and are absent without the presence of the lesion. Recent work has begun to illustrate previously unappreciated oncogenic functions of the stroma and to provide new insights into carcinogenesis. These oncogenic functions stimulate the transformation of adjacent cells through transient signaling that results in disrupted genomic integrity. Here we will highlight several recent papers that address the stromal characteristics of neoplastic lesions and how they may contribute to the carcinogenic response. In particular, we will focus on the oncogenic effect of stromal cells on non-transformed, as well as transformed cells.

Stromal changes in cancer: altered morphology and gene expression

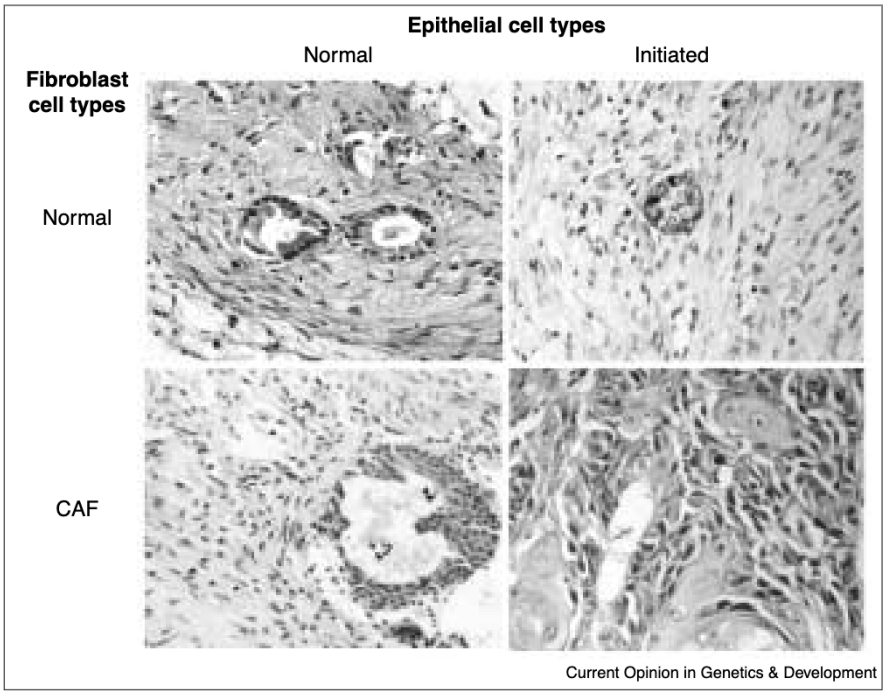
As the predominant cell in the stroma, the fibroblast is responsible for the elaboration of most of the components of connective tissue, such as the different collagens, proteolytic enzymes, proteolytic inhibitors, growth factors and structural proteoglycans [5]. Each organ has specialized requirements, and hence fibroblasts from different organs demonstrate organ-specific variations of the classes of basic molecules listed above. Furthermore, in response to different physiologic signals — be they normal or pathologic — the fibroblasts of the stroma change accordingly.

The first indication that fibroblasts and their surrounding milieu could change during cancer came from the observations of pathologists. The proliferation of fibroblasts, the increased presence of collagen in the vicinity of cancer cells (both creating dense masses) and the novel expression of -smooth-muscle actin indicated a change had occurred in the resting state, which was termed desmoplasia [5,6]. The desmoplastic reaction is a common aspect of many solid tumors including those of the breast, prostate, colon and lung, and in some cases is accompanied by the recruitment of inflammatory cells. The fibroblasts that comprise the tumor stroma have been termed myofibroblasts, peritumoral fibroblasts, reactive stroma and carcinoma-associated fibroblasts (CAFs). For the purposes of this review we refer to them as CAFs, indicating their origin but avoiding mechanistic attributes.

Ultrastructural studies, immunohistochemistry and biochemical analyses have each contributed to the appreciation that the stroma is altered in critical aspects during the neoplastic process [7,8]. Early studies documented a change in the expression of proteins with an acquired expression of -smooth-muscle actin, vimentin, smooth muscle myosin, calponin, tenascin and desmin [7,9]. This activation of a partial smooth muscle differentiation program and the excessive elaboration of collagen types III and V in the area of a neoplastic lesion led to labeling the desmoplastic cells as a type of myofibroblast. The above described proteins are also often expressed as a response to wound healing or inflammation, as myofibroblasts orchestrate the repair response (reviewed in [5]). In addition, the distribution of laminin, a molecule critical for the architectural integrity of undisturbed tissue, is reduced and altered in fibroblasts found associated with malignant

Figure 1

Histological appearance of tissue recombinants. Histology of tissue recombinants demonstrates the functional role of CAFs in the stimulation of tumorigenesis. Normal and carcinoma-associated prostatic fibroblasts were combined with both normal and initiated (non-tumorigenic, Simian virus-40 large T-antigen-expressing, immortalized) prostatic epithelial cells in a recombinant graft placed under the renal capsule of athymic nude mice. Normal fibroblasts promoted normal or hypoplastic epithelial structures in normal and initiated epithelia, respectively, but CAFs stimulated the formation of abnormal proliferative structures. When combined with normal epithelia, CAFs promote a piling up of epithelia, indicative of a hyperplastic response associated with prostatic intraepithelial neoplasia. When CAFs are combined with initiated epithelia, large tumor masses form with a disrupted architecture marked by streaming epithelia with enlarged nuclei.



cells [7]. More recent studies documented alterations in dipeptidyl peptidase IV, matrix metalloproteinases (MMPs), inhibitors of metalloproteinases, growth factors and collagens [10–14].

Stromal changes in cancer: altered phenotypes in vitro

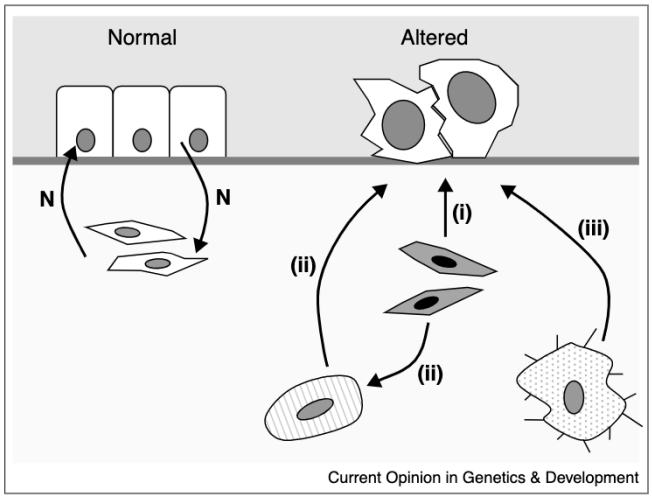
When removed from malignant lesions, CAFs are often found to exhibit altered phenotypes by many different assays. Enhanced collagen production and stimulation of hyaluronate synthesis were observed in fibroblasts isolated from a variety of human tumors [15,16]. Disorganized growth patterns, uncontrolled growth and altered proliferation potential of these fibroblasts were also reported (see [7] and references therein). Phenotypes that may aid in tumor progression, such as the increased elaboration of growth factors that may stimulate proliferation of epithelial cells, are often noted in cultured CAF cells [14].

Expression of 'fetal phenotypes' (increased fibroblast migration) has been documented in motility assays [17]. Intriguingly, some of these phenotypes have also been detected in fibroblasts taken from distal, non-tumorigenic sites from patients with cancer. Schor and co-workers [17,18] found altered invasive properties in skin fibroblasts from patients with various cancers and noted that they were more prominent in fibroblasts from patients with hereditary predisposition to cancer. Likewise, skin fibroblasts from patients with hereditary cancers such as hereditary non-polyposis colorectal cancer exhibit disorganized actin and growth patterns and reduced requirements for serum during *in vitro* growth ([19]; reviewed in [20]). Schor *et al.* [18] postulated that fibroblast abnormalities may influence the appearance of epithelial tumors and that congenital defects can affect stromal–epithelial interactions and promote tumor formation. These studies provide evidence that CAFs display an altered phenotype, and thus invite the question of whether this has functional consequences on tumorigenesis.

Does the abnormal stroma play a functional role in the carcinogenic process?

To address whether stromal fibroblasts have a functional role in carcinogenesis, researchers have performed experiments in which various fibroblasts are grafted in combination with epithelial cells into rodents (see Figure 6 in [21] for references). Early studies, by Chung and co-workers [22,23], measured stromal effects on tumor progression. They analyzed recombinant grafts that combined tumorigenic epithelial cells with fibroblasts (usually of murine origin) that were normal, immortalized, transformed by viral or chemical carcinogens, or tumorigenic. Depending on the characteristics of the epithelial tumor cell, the fibroblasts exerted either a positive or negative effect on tumor production. Three-dimensional skin raft cultures have also been used to examine the effect of fibroblasts on malignant epithelial cells *in vitro* [24]. These studies demonstrated the stimulation of malignant phenotypes in the transformed epithelial cells when cultured with CAFs. Taken in concert, these studies show that tumorigenic epithelial cells within the recombinant graft respond to signals from the fibroblasts and that the

Figure 2



Stromal influences on tumorigenesis. Stromal components can influence tumorigenesis in many different ways. Normal communication (N) between the stroma and epithelial cells is important for normal tissue organogenesis and homeostasis. (i) Cells within the stroma can directly influence tumor processes (for examples, see [21 ,28]). (ii) Stromal cells (fibroblasts) can recruit additional cells, such as mast cells, to the tumor site where they influence tumor processes (for examples see [27,28]). (iii) Stromal cells can produce viral products that stimulate tumorigenesis.

response depends on the molecular changes present in the epithelial cell.

In an alternative approach using human tissues, Olumi *et al.* [21] examined the effect of fibroblasts on 'non-tumorigenic' epithelial cells. The aim of this study was to determine whether carcinoma-associated fibroblasts send signals that either initiate abnormal epithelial growth or enhance progression of a non-tumorigenic cell to a tumorigenic one. The combination of normal human prostatic epithelial cells with CAFs (obtained from prostatic adenocarcinoma) showed an interaction that was limited in growth but produced ductal structures that resembled prostatic intraepithelial neoplasia (Figure 1). Strikingly, when CAFs were grafted with immortalized human prostatic epithelial cells, a dramatic interaction resulted in tumors that could exceed 5 grams in wet weight, and often eclipsed the weight of control grafts by 200-fold (Figure 1). Histological examination of these tumors revealed their malignant nature compared with that observed with the control recombinants (Figure 1).

Remarkably, isolation of pure human epithelial cell populations from these tumors and the subsequent grafting into host animals showed that these epithelial cells could now form tumors by themselves (in other words, contributing activity from CAFs was no longer necessary for production of a malignant tumor; P Hein, unpublished data). In addition, the transformation of these cells was accompanied by non-random chromosomal changes (P Hein, unpublished data). Hence, oncogenic signals from CAFs can stimulate the transformation of non-tumorigenic cell populations to tumorigenic ones. When grafted with CAFs, but not with normal fibroblasts, the immortalized epithelial cells demonstrated altered cell proliferation, cell death, angiogenesis, adhesion and genomic instability. Many of these phenotypes could be recapitulated when the identical pairs of cells were co-cultured *in vitro* [21]. These studies show that stromal cells can stimulate a non-tumorigenic cell to a malignant state through a time-limited interaction of stromal and epithelial cells, thus verifying its active role in tumorigenesis.

The stroma can acquire oncogenic activity by many processes

The previous studies obtained fibroblasts from an existing tumor and demonstrated their ability to stimulate oncogenesis. Can the stroma develop oncogenic signals in the absence of a tumor? If stromal cells could acquire the properties that stimulate tumor initiation and progression through independent means, it would open considerable insights into the risk factors for tumorigenicity. Recent experiments suggest that this mechanism of tumor generation is feasible and have begun to identify the processes involved.

Exposure to carcinogens

Decades ago, using skin [25] and bladder [26,27] tissues, investigators observed enhanced tumor formation when carcinogen-treated stroma was heterotypically grafted with untreated epithelial cells. Most recently, the effects of carcinogen-treatment on stromal cells have been examined in murine mammary tissues [28]. In the latter study, irradiation of epithelial cell-free mammary stroma (cleared fat pads) facilitated tumor progression of transformed epithelial cells that were subsequently introduced into the treated stroma. In the irradiated stroma, the mammary epithelial cells developed tumors faster, more often, and reached a greater size than the same cells transplanted into unirradiated stroma. These data indicate that carcinogens can affect the neoplastic process not only by inducing genetic changes in the epithelial cell, but also by altering the stromal cells in such a way that they stimulate tumor progression.

Manipulation of MMPs

A rapidly growing body of work is providing evidence that, early in the neoplastic process, alterations in the balance of matrix remodeling enzymes can influence stroma so that it modulates the carcinogenic potential of the adjacent epithelial cells. In general, the lack of MMP activity at these early stages can suppress tumorigenesis [29], whereas the hyperactivity of the same family of enzymes can enhance both spontaneous and carcinogen-induced tumorigenesis [30–33,34 ,35]. Several different members of the MMP family and their inhibitors have been found to influence the early stages of carcinogenesis at various organ sites.

The joint studies of Sympson, Bissell and Werb [31] demonstrated that overexpression of auto-activating

stromelysin-1 in epithelial cells of a transgenic mouse leads to altered stromal–epithelial interactions and tumorigenesis. The molecular basis for this MMP action on tumorigenesis was examined more recently using two genetic approaches [34]. In the first approach, auto-activating stromelysin-1 was expressed in immortal murine mammary epithelial cells under a regulated promoter. When the stromelysin-1-transfected cells were injected into mammary fat pads previously cleared of epithelial glands, they grew into duct-like pseudo-glandular structures. Induction of stromelysin-1 expression resulted in the formation of small tumors within 6 weeks. A pulsed expression of stromelysin-1 either *in vivo* or *in vitro* still resulted in tumors, although at a lower rate. These data suggest that, once initiated, the tumors become independent of the continued expression of stromelysin-1.

The second approach examined transgenic mice expressing auto-activating stromelysin-1 under the control of the whey acidic protein promoter, which therefore targeted expression to the milk-producing epithelial cells. Transgenic mice exhibited the hallmarks of 'reactive' stroma and lesions consistent with multistage neoplastic progression. Non-transgenic littermate controls did not develop dysplasias and carcinomas. Postulating that the induction of neoplasia by stromelysin-1 was due to its proteolytic activity, Sternlicht *et al.* [34] engineered a mouse that concomitantly overexpressed TIMP-1 (tissue inhibitor of metalloproteinases-1). The tumorigenic phenotype of the double transgenic was greatly reduced, indicating that active stromelysin-1 is required for mammary lesions to develop. The tumors from the stromelysin-1 transgenic mice displayed non-random chromosomal changes that were not detected in non-neoplastic tissue from the same mouse. These studies make two important points. First, in the absence of a previously formed tumor, altered MMP expression is causative of the neoplastic phenotype; second, aberrant expression of a mutated MMP can generate genetically altered cells.

As noted by Wilson *et al.* [29], the removal of MMP function has also been shown to have an effect on tumorigenicity. Masson *et al.* [36] generated stromelysin-3-deficient mice by homologous recombination and found them to be viable and fertile, indicating that stromelysin activity is dispensable for normal organogenesis and tissue homeostasis. When challenged with a chemical carcinogen, however, the stromelysin-3-deficient animals were found to have reduced tumorigenesis. Furthermore, cells from these animals failed to promote the growth of tumorigenic human cells in a nude mouse assay [36]. A more careful examination of these latter results provided evidence that stromelysin-3 was acting through an MMP-induced release of growth factors. To demonstrate this interaction, an *in vitro* assay was carried out in which tumor cells and fibroblasts overexpressing stromelysin-3 were co-cultured in a reconstituted basement membrane. In the absence of growth factors, neither wild-type nor stromelysin over-expressing fibroblasts were able to stimulate the epithelial component. When embedded in reconstituted basement membrane that contained standard growth factors, however, the tumor cells provided a differential response to the fibroblasts. Fibroblasts that overexpressed stromelysin-3 produced tumors, whereas control wild-type fibroblasts and stromelysin-3$^{-/-}$ fibroblasts failed to do so [36]. These experiments highlight the causative relationship between fibroblast expression of MMPs and tumor cell growth, providing clues as to critical phenotypes effected by stromelysin-3.

The recruitment of inflammatory cells to the stroma of the tumor site can provide oncogenic stimuli

During the course of a normal response to tissue disruption, fibroblasts often recruit inflammatory cells. Using a transgenic mouse model that expresses human papilloma virus (HPV)-16 genes in basal keratinocytes and examining the ensuing pre-malignant lesions, Coussens *et al.* [37 ,38] found that carcinogenesis was accompanied by the infiltration of mast cells. The mast cells were documented to activate MMP-9 by the release of serine proteases. Pre-malignant angiogenesis, an important marker for tumorigenesis, was ablated in mast cell deficient HPV16 transgenic mice.

Thus, in this model system, inflammatory cells are recruited to reorganize the stromal architecture, which leads partly to the stimulation of angiogenesis, probably by releasing sequestered angiogenic activators. Eventually the angiogenic process becomes independent of mast cell stimulation, as the tumor cell itself directly upregulates angiogenic growth factor gene expression. Although inflammatory processes are usually evoked to destroy pathogens or initiate repair, they have also been reported to be in close association with the invading edges of aggressive neoplasias, contributing to the idea that tumors are wounds that do not heal [39]. Whereas the studies discussed in 'Manipulation of MMPs' demonstrate an oncogenic effect of MMPs by the direct alteration of MMP expression using genetic manipulation, the studies discussed in this section show that the activation of MMPs can be achieved by the recruitment of inflammatory cells to the tumor site.

Viral alteration of stromal signals

An intriguing additional possibility for stromal involvement in human neoplasia comes from the study of viral-associated cancers. A member of the cytokine family, interleukin-6, is known to have growth-promoting properties in myeloma. Rettig *et al.* [40], alerted by the report that human herpes virus codes for interleukin-6, examined myeloma samples for viral sequences. These authors reported that viral sequences were detected in stromal (dendritic) cells but not in the malignant myeloma cells themselves. Because dendritic cells normally mediate growth control of hematopoietic cells through a paracrine pathway, Rettig *et al.* [40] postulated that abnormal regulation of interleukin-6 expression in the non-malignant stromal cells stimulated carcinogenesis in the cells that ultimately gave rise to multiple myeloma.

A similar scheme has been proposed by McGrath *et al.* [41] in neoplasms associated with auto-immune deficiency syndrome. These authors speculated that the production of growth factors (such as interleukin-6) by virus-infected macrophages drives the initial proliferation of the future malignant cells. In both of these reports [40,41], although the authors failed to detect virus in the malignant cells, they found viral expression localized to tumor-associated stromal cells, dendritic cells and macrophages. The combined data suggest that, in these examples of viral pathogenesis, tumorigenesis may be initiated by cytokines generated by virally infected stromal cells and that, after an initial stimulation of premalignant cells, continuous stimulation may no longer be necessary. Early lesions, initially stimulated by exogenous cytokines, would later outgrow their need for continuous paracrine stimulation by conversion to an autocrine mechanism. The correlation of viral-induced cytokine expression in stromal cells with the appearance of transformed cells suggests a direction for future studies.

Taking these results together, stromal alterations have tremendous potential to influence the oncogenic process (Figure 2). Equally remarkable is the realization that the initial stimuli need only be applied in a transient manner to trigger the formation of the lesion. In the examples described, the need for altered stroma to contribute oncogenic signals for the generation of a tumorigenic lesion was transient (the time-limited interaction of human CAFs with epithelial cells [21], the pulse of carcinogen administered to the stroma [28], the pulsed expression of MMPs [34], the transient association of mast cells or the virally induced 'hit-and-run' production of cytokines [40,41]). In each example, the transformed cell ultimately becomes independent of the stromal signals and, by altering its phenotype, assumes these important duties.

How do altered stromal–epithelial interactions contribute to the carcinogenic process?

Disruption of stromal–epithelial interactions and cell adhesion alters cellular signaling, which influences proliferation, death, angiogenesis, differentiation, motility, genomic integrity and other phenotypes [42]. Indeed, altered adhesion in epithelial cells, but not fibroblasts, can modulate levels of the tumor suppressor and cell cycle regulatory protein p53 [43], suggesting an important link between the cell surface and internal regulatory circuits.

Translation of three-dimensional structure into a correct readout for cellular signaling is in its infancy [44,45], and is beyond the scope of this review. The investigation of these processes will provide opportunities for the identification of novel targets for prevention and therapy.

Conclusions

The importance of the stromal tissue in regulating the physiological processes of the body is undeniable. Likewise, the roles of the stroma in supporting the tumorigenic process and responding to the oncogenic lesion are also recognized [46]. This review articulates a perspective that differs from the conventional view in that it marshals the accumulating evidence that the stroma may also actively generate the transformed lesion (Figure 2). As such, the oncogenic signals can be dependent or independent of genetic mutations in the fibroblasts. As a supportive and responsive agent in tumorigenesis, the stroma is induced by tumor cells to express critical signals that drive proliferation, angiogenesis, and motility while suppressing cell death. As an oncogenic agent in tumorigenesis, the stroma can provoke tumorigenicity in adjacent cells in the absence of pre-existing tumor cells and with the acquisition of genomic changes. Investigating the mechanism by which the oncogenic signals of the stroma facilitate the generation of malignant epithelial cells will provide insights into the generation of cancer cells.

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