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Stromal cells can contribute oncogenic signals

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The majority of studies of neoplastic transformation have focused attention on events that occur within transformed cells. These cell autonomous events result in the disruption of molecular pathways that regulate basic activities of the cells such as proliferation, death, movement and genomic integrity. Other studies have addressed the microenvironment of tumor cells and documented its importance in supporting tumor progression. Recent work has begun to expand on these initial studies of tumor microenvironment and now provide novel insights into the possible initiation and progression of malignant cells. This review will address the transforming effect of stromal cells on epithelial components.

Key words: carcinogenesis / genomic instability / stromal–epithelial interactions / carcinoma-associated fibroblasts / reactive stroma

Introduction

In studies of developmental biology the importance of cellular interactions is well known.[1–4] These interactions drive organogenesis and provide the homeostatic balance in adult tissues. The stromal components of normal tissues govern size, function and response to exogenous agents through elaboration and modification of the extracellular matrix (ECM), which, in turn, conveys signals to the adjacent epithelial cells. During wounding and other pathological conditions the stroma exhibits fundamental changes that are important in proper tissue response.[5]

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As the predominant cell in the stroma, the fibroblast is responsible for the elaboration of most of the connective tissue components such as the different collagens, proteolytic enzymes and their inhibitors, growth factors and determinants of intercellular adhesions.[5] Each tissue has specialized requirements and hence fibroblasts from different organs demonstrate specific variations of the classes of basic molecules mentioned above. Furthermore, in response to different physiologic signals, whether they are normal or pathologic, the fibroblasts of the stroma change accordingly. Of particular interest in this review are the stromal characteristics of neoplastic lesions and how stroma may contribute to carcinogenic processes.

Stromal changes in cancer: altered morphology and gene expression

Pathologists were the first to note the stromal changes that accompany cancer formation. The firm nature of the carcinogenic lesion was attributed to the increase in collagen and fibroblasts that were often found in the vicinity of the neoplastic growth. This proliferation of fibroblasts, the novel expression of α-smooth muscle actin, and the increased presence of collagen in the vicinity of cancer cells indicated a change from the resting state and was termed desmoplasia.[6] Figure 1 illustrates the desmoplastic response seen in prostatic adenocarcinoma. 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 (CAF). For the purposes of this review we refer to them as CAF indicating their origin but avoiding mechanistic attributes. Ultrastructural studies, immunohistochemistry and biochemical analysis have each contributed to the appreciation that the stroma

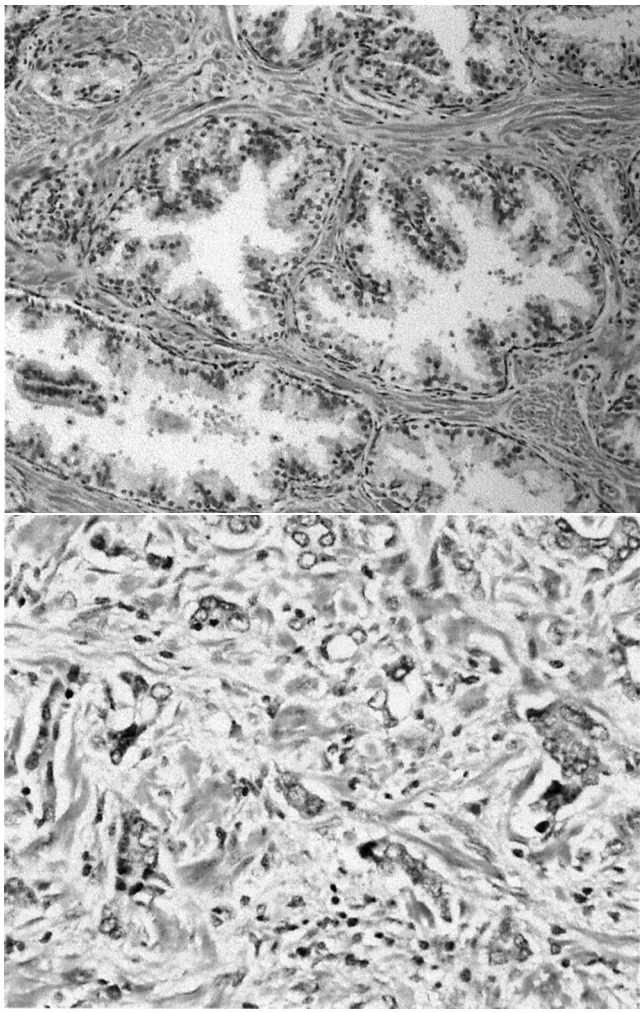

Figure 1. Histology of a prostate adenocarcinoma showing the desmoplastic changes in the stromal compartment of both differentiated (a) and undifferentiated (b) tumors.

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 α-smooth muscle actin, vimentin, smooth muscle myosin, calponin, tenescin and desmin.[7,9] The above described proteins are often expressed as a response to would healing or inflammation as myofibroblasts orchestrate the repair response.[5] Additionally, the distribution of laminin, a molecule critical for the architectural integrity of undisturbed tissue, is reduced and altered in fibroblasts found associated with malignant cells. More recent studies documented the alteration in additional molecules including dipeptidyl peptidase IV, matrix metalloproteinases (MMP), inhibitors of metalloproteinases, growth factors and collagens.[10–14]

Stromal changes in cancer: carcinoma-associated fibroblasts have unusual phenotypic properties

The fibroblasts in the vicinity of tumors have been reported to have several altered properties. Enhanced collagen production and stimulation of hyaluronate synthesis was observed in fibroblasts isolated from human tumors.[15,16] Likewise, several *in vitro* studies demonstrated disorganized growth patterns, uncontrolled growth and altered proliferation potential (immortality) of the fibroblasts isolated from within tumorigenic lesions.[7] Intriguingly, phenotypic changes in fibroblasts have also been found outside the immediate vicinity of the lesion. Schor and co-workers found altered invasive properties in skin fibroblasts from patients with various cancers and noted that they were more prominent in fibroblasts from patients with a hereditary predisposition to cancer.[17,18] Other studies found aberrant *in vitro* behavior in skin fibroblasts obtained from patients with hereditary cancers such as familial polyposis which included disorganized actin and growth patterns and reduced requirements for serum during *in vitro* growth.[19] Schor postulated that fibroblast abnormalities may influence the appearance of epithelial tumors[17] and that congenital defects can affect stromal–epithelial interactions and promote tumor formation.

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

To directly address a functional role for stromal fibroblasts in the carcinogenic process, experiments that grafted various fibroblasts in combination with epithelial cells have been performed.[20] Early studies, by Chung and co-workers[21,22] 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 graft cultures have also been used to examine the effect of fibroblasts on malignant epithelial cells *in vitro*[23] and demonstrated the stimulation of malignant phenotypes in the transformed epithelial cells when cultured with CAF.

In an alternative approach, a recent study has examined the effect of fibroblasts on *non-tumorigenic cells.*[20] Human prostatic cells were used in all cases. Biopsies from normal human prostates yielded normal prostatic epithelial cells and prostatic fibroblasts. Initiated (immortalized) epithelial cells were obtained by the expression of SV40 TAg in normal prostatic epithelial cells. Finally, carcinoma-associated fibroblasts (CAF) were obtained from lesions of prostatic adenocarcinoma as identified by pathologists. Each of the four populations of human cells was non-tumorigenic when grafted alone into host animals. However, when carcinoma-associated fibroblasts were grafted with human prostatic epithelial cells it was obvious that specific phenotypes of the epithelial cells were altered. The combination of normal human prostatic epithelial cells with CAF led to limited growth, but of ductal structures that resembled prostatic intraepithelial neoplasia (PIN). These grafts indicate that the normal prostatic epithelial cells receive signals from the CAF but the cell–cell interaction does not result in full-scale tumorigenicity. The most dramatic effect was seen when initiated (immortalized) human epithelial cells were grafted with CAF. The resulting tumors could exceed 5 g in wet weight, easily eclipsing the weight of control grafts by 500-fold. The histological examination of these tumors demonstrated their malignant nature. Remarkably, isolation of pure human epithelial cell populations from these tumors and the subsequent grafting into host animals demonstrated that the epithelial cells could now form tumors by themselves (i.e. growth with CAF was no longer necessary for production of a malignant tumor) (P.H. in preparation). Hence, oncogenic signals from the CAF had stimulated the progression of a non-tumorigenic cell population to a tumorigenic one. The transformation of these cells was accompanied by non-random chromosomal changes (P.H. in preparation). CAF stimulated the epithelial cells to display an increase in cell proliferation, a decrease in cell death, an increase in angiogenesis, an alteration of adhesion properties of the epithelial cells and finally, an increase in genomic instability. Many of these phenotypes could be recapitulated when the identical pairs of cells were co-cultured *in vitro.*[20]

Oncogenic stromas are experimentally generated by different conditions

While these studies demonstrated that fibroblasts from a malignant lesion could generate oncogenic signals, they did not address the possibility that the oncogenic signals could evolve 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 begin to identify the processes involved.

Exposure to carcinogens

Decades ago investigators observed enhanced tumor formation when carcinogen-treated stroma was heterotypically grafted with untreated epithelial cells. Studies using skin[24] and bladder[25,26] each showed increased tumorigenicity when non-treated epithelial cells were grafted with carcinogen-treated stroma. Most recently the effects of carcinogen-treatment on stromal cells have been examined in murine mammary tissues.[27] In this study, irradiation of epithelial cell-free mammary stroma (cleared fat pads) facilitated tumorigenesis in 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 effect 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. The stromal effect in this study aided a weakly tumorigenic cell population to become more tumorigenic.

Manipulation of matrix metalloproteinases (MMP)

A rapidly growing body of work is providing evidence that alterations in the balance of matrix remodeling enzymes can produce stroma that modulates the carcinogenic potential of the overlying epithelial cells. In general, the lack of MMP expression can suppress tumorigenesis,[28] while the overexpression of the same family of enzymes can enhance both spontaneous and carcinogen-induced tumorigenesis.[29–33] The joint work of Bissell and Werb has demonstrated that overexpression of stromelysin-1 in the epithelial cells of a transgenic mouse leads to altered stromal–epithelial interactions and tumorigenesis.[30] These studies targeted expression of an autoactivating mutant of stromelysin-1 to mammary epithelium. Expression resulted in reduced mammary function during

pregnancy and development of pre-neoplastic and neoplastic lesions. Early in development, expression of the transgene leads to increased expression of endogenous stromelysin-1 in stromal fibroblasts, an up-regulation of other MMPs, and the development of a 'reactive' stroma. The altered stroma contained increased collagen and vascularization, a pattern also seen in stroma surrounding breast cancer lesions. These alterations preceded the appearance of pre-neoplastic and neoplastic lesions.

The molecular basis for these observations was examined in a recent report[33] using two genetic approaches. In the first approach, stromelysin-1 was expressed in immortal murine mammary epithelial cells under a tetracyclin-regulated promoter. When stromelysin-1 was activated, the epithelial cells mimicked the characteristics usually displayed by epithelial cells responding to fibroblasts expressing stromelysin-1, namely a conversion from an epithelial morphology to a mesenchymal morphology. The cells lost E-cadherin-based cell–cell contacts, displayed a scattered morphology, down-regulated cytokeratins, and up-regulated vimentin. 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. Removal of tetracyclin from the drinking water and induction of stromelysin-1 expression resulted in the formation of small tumors within 6 weeks. A pulsed expression of stromelysin-1 for 12 d after implantation to the cleared fat pad, still resulted in tumors although at a lower rate. Remarkably, even induction of the cells during growth in tissue culture produced cells that had tumorigenic potential and produced large tumors when injected into the host animal. These data suggest that, once initiated, the tumors become independent of continued stromelysin-1 expression.

The second approach examined transgenic mice that expressed stromelysin-1 under the control of the whey acidic protein (WAP) promoter and hence targeted expression of the milk-producing epithelial cells.[33] Transgenic mice exhibited the hallmarks of 'reactive' stroma and lesions consistent with multistage neoplastic progression appeared at 6–24 months of age. The majority of animals (77%) had moderate to severe fibrosis, fewer animals (64%) exhibited severe hyperplasias, 20% had dysplasias or ductal carcinoma *in situ*, and a minority (7%) developed carcinomas. Non-transgenic littermate controls were devoid of dysplasias and carcinomas. Postulating that the induction of neoplasia by stromelysin-1 was due to its proteolytic activity, Sternlicht and co-workers 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 involved murine chromosomes 4, 6, 7, and 15 that were not detected in non-neoplastic tissue from the same mouse.

As noted above, the removal of MMP function has also been shown to have an effect on tumorigenicity. Stromelysin-3 deficient mice were generated by homologous recombination and found to be viable and fertile, indicating that this activity is dispensable for normal organogenesis and tissue homeostasis.[34] When challenged with a chemical carcinogen the stromelysin-3 deficient cells were found to have reduced tumorigenesis. Furthermore, cells from these animals failed to promote the growth of tumorigenic human mammary epithelial cells (MCF-7) in a nude mouse assay. A more careful examination of these latter results provided evidence that the stromelysin-3 was acting through a paracrine-induced release of growth factors. MCF-7 cells and mouse fibroblasts induced to overexpress stromelysin-3 were placed in Matrigel with and without growth factors. The stromelysin-3 producing cells generated tumors when grafted in growth factor-containing Matrigel but failed to do so when the Matrigel was devoid of growth factors. Control fibroblasts and stromelysin-3 −/− fibroblasts failed to promote tumor growth regardless of the presence or absence of growth factors in the Matrigel. These experiments provide clues as to critical phenotypes effected by stromelysin-3.

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

While the above studies implicate MMPs by direct alteration of MMP expression using genetic manipulation, studies by Coussens *et al.*[35,36] implicate MMP activity in a novel fashion. Using an experimental model that expresses Human Papilloma Virus (HPV) 16 genes in basal keratinocytes of transgenic mice and examines the ensuing pre-malignant lesions, the authors found that carcinogenesis was stimulated by the infiltration of mast cells. The mast cells were documented to activate MMP-9 by the release of serine proteases. Pre-malignant angiogenesis was ablated in mast cell-deficient (KITw/KITwv) HPV16 transgenic mice. Thus, in this model system, inflammatory cells

are recruited to reorganize the stromal architecture and lead in part to the stimulation of angiogenesis. While 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.[37] Eventually, the angiogenic process becomes independent of mast cell stimulation as the tumor cell itself directly up regulates angiogenic growth factor gene expression.

Viral alteration of stromal signals

An intriguing additional possibility for stromal involvement in human neoplasia comes from the study of viral-associated cancers. Rettig and co-workers, alerted by the report that interleukin-6 is coded in a human herpes virus and familiar with the growth-promoting role of interleukin-6 in myeloma, examined myeloma samples for viral sequences.[38] The authors reported that viral sequences were detected in stromal (dendritic) cells, but not in the malignant myeloma cells themselves. Since the dendritic cells normally mediate growth control of hematopoietic cells via a paracrine fashion, they 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 scenario was hypothesized by McGrath and colleagues[39] in auto-immune deficiency syndrome-associated neoplasms. These authors speculate 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, while the authors failed to detect virus in the malignant cells, they found viral expression localized to tumor-associated stromal cells, dendritic cells and macrophages, respectively. 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. Since the viral-expressing cell alteration may result in the stimulation of the ultimate malignant cell population, this mechanism was termed sequential neoplasia[39] or hit-and-run carcinogenesis.

How may stroma develop oncogenic signals?

Events that have the potential to create 'reactive' stroma or stroma with altered homeostatic balance could contribute to the risk of malignant disease. The studies cited in this review provide clues as to how altered stroma may be generated.

Direct induction by tumorigenic cells

Experimental evidence indicates that fibroblasts grown in association with tumorigenic cells can acquire at least some of the phenotypes identified *in vivo*. Elegant studies by Ronnov-Jessen and co-workers[40] have provided evidence that tumor cells may alter the phenotypes of fibroblasts when the two are grown in co-culture. Isolation of stromal cells and subsequent co-culture with mammary tumor cells showed that the majority of phenotypic and biochemical changes associated with desmoplasia could be induced in fibroblasts and to a much lesser degree with vascular smooth muscle cells. Conversion of pericytes to the desmoplastic phenotype was very limited. The finding that normal fibroblasts, placed in association with tumor cells, readily convert to α-smooth muscle actin-expressing fibroblasts suggests that factors elaborated from tumor cells modulate this response. Transforming growth factor-β (TGF-β), often produced by tumor cells and present in a latent form in the ECM, has been shown to induce α-smooth muscle actin and collagen production in cultured fibroblasts[8] and is a reasonable candidate to participate in the induction of the desmoplastic response. The complex cellular signals that induce the desmoplastic response in neoplastic lesions are under investigation.

Wound healing

It has already been noted that the stroma at the site of wound healing and neoplastic lesions share many characteristics. Indeed, experimental evidence indicates that wounding has a tumor-promoting effect and TGF-β has been postulated as the effector.[41] The increased incidence of tumor formation at the sites of scar tissue and in areas of chronic damage is consistent with the idea that stromal changes that accompany wounding can enhance tumorigenicity.

Carcinogen exposure

Physical carcinogens, such as ultraviolet light,[27] chemical carcinogens, such as nitrosamines[26] and

viral carcinogens[38,39] each have demonstrated potential to alter the cells of the stroma with a resulting stimulation of tumorigenicity. The mechanisms responsible for this stimulation are presently unknown. One could envision a direct mechanism in which cellular responses to these agents generate 'reactive stroma' and the elaboration of phenotypes described in this review. In addition, one could also envision a more indirect mechanism whereby the various carcinogenic agents elicit a mutational change within the stromal cells. It is attractive to speculate that activating mutations in genes that produce MMPs or growth factors and inactivating mutations in genes that inhibit these actions could each contribute to a tissue phenotype conductive to tumorigenesis.

Aging or induction of senescence

Increased collagen and altered expression of MMPs are part of a gene expression program that is activated when fibroblasts enter senescence and when tissues become aged.[42] This expression program can be activated either by the prolonged growth of fibroblasts in tissue culture or by aberrant expression of oncogenes.[43] Future studies will determine whether these cells contribute to the alteration of stromal–epithelial interactions that modulate carcinogenesis as previously suggested.[44,45]

Hormone imbalance

Extensive epidemiological data implicate hormonal imbalance in the generation of some tumors and experimental manipulation of hormone levels in animals verifies this observation. In the Noble rat model exposure to hormones was shown to dramatically alter stromal–epithelial interactions and generate adenocarcinomas.[46] Critical regulators, such as TGF-β, insulin-like growth factor-1 and vascular endothelial growth factor are altered upon administration of oncogenic doses of sex hormones. The authors postulate that alterations in these paracrine regulators of stromal–epithelial interactions are responsible for the hormonally-induced disease.[46] These molecules are often stored in the ECM and are liberated and activated following the appropriate signals.

Congenital or acquired mutations that alter stromal–epithelial signals

Fibroblasts containing congenital mutations that predispose to various cancers (e.g. APC or Adenomatous Polyposis Coli) have been reported to have abnormal phenotypes.[17–19] If these mutations alter stromal–epithelial interactions, they may explain some aspects of tissue specificity in the resulting tumor spectra. Such a situation has been postulated for juvenile polyposis of the colon.[47] In these polyps, deletions in chromosomes 10 and 18 have been detected in stromal cells, but not epithelial cells. These data strongly suggest that inherited mutations in the stroma may predispose to carcinogenic conditions.

In addition to congenital mutations, acquired mutations in stromal cells may contribute to carcinogenesis. New molecular tools have provided the means to examine tumor components individually. The combination of microdissection (using laser capture microscopes) and PCR has provided evidence that fibroblasts can acquire mutations independent of those detected in epithelial cells.[48] Examination of stromal and epithelial cells from patients with ductal carcinoma *in situ*, or infiltrating ductal carcinoma of the breast, uncovered a group of mutations that can occur in both types of cells or each independently. The high frequency of fibroblasts with acquired mutations distal to carcinomatous tissue in contrast to their absence in normal mammary tissue suggests that, in some cases, stromal alterations may precede mutations in the epithelia.

Taken together, the above events have tremendous potential to influence the oncogenic process. 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 tumorigenic lesion was transient (i.e. the growth of human CAF with epithelial cells,[20] the pulse of carcinogen administered to the stroma,[27] the overexpression of MMPs,[33] the recruitment of mast cells or the virally-induced production of cytokines.[38,39]

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

The elaboration of ECM components by fibroblasts not only provides structural support for the surrounding cells but also influences the growth and differentiation programs of these cells. Disruption of the ECM alters cellular signaling influencing proliferation, death, angiogenesis, differentiation, motility, genomic integrity and other phenotypes.[49] Pioneering studies of the translation of the three-dimensional structure into a correct readout for cellular signaling are in their infancy and are beyond the scope of the

current review.[50,51] The investigation of these processes will provide opportunities for the identification of novel targets for prevention and therapy.

Conclusions

Although the stromal cells of the carcinogenic lesion have long been known to be supportive and responsive, new data demonstrate that they also have a more active role in the tumorigenic process. The oncogenic action of distinctive stromal components has been demonstrated through a variety of approaches and provides clues about the cellular pathways involved. Key evidence demonstrates that stromal components may modulate the initiation and progression of cancer. 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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