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EPITHELIAL CELL INVASION OF THE STROMA IN HUMAN SKIN ORGAN CULTURE

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TABLE OF CONTENTS

1. ABSTRACT

Invasion of the surrounding tissue by malignant epithelial cells is a complex process. It involves degradation of basement membrane and stromal connective tissue elements and movement of tumor cells into and through the partially-degraded matrix. While the process of tumor cell invasion has been extensively studied in simple two-dimensional or three-dimensional assay systems, it is difficult to know how the cellular and molecular mechanisms that underlie invasion in these culture systems relate to mechanistic events that drive invasion in intact tissue. To help address this question, our laboratory has developed a human skin organ culture model to study epithelial cell invasion of the stroma in intact tissue. In this model, punch biopsies of human skin are maintained in organ culture under serum-free, growth factor-free conditions or in the same culture medium supplemented with exogenous epidermal growth factor (EGF). In the absence of growth factor stimulation, normal tissue architecture and biochemical function are preserved. However, in the presence of exogenous EGF, the dermal-epidermal juncture is eroded and epithelial cells invade the dermis. Concomitant with dermal invasion is induction of matrix metalloproteinases (MMPs), stimulation of fibronectin synthesis and active epithelial cell migration into the stroma. These observations provide convincing evidence that events thought to be involved in invasion in simple two- and three-dimensional *in vitro* models are also operative during invasion of intact tissue.

2. INTRODUCTION

Normal epithelial cells are separated from the underlying stroma by a layer of specialized connective tissue known as the basement membrane. The major constituents of the basement membrane include laminin, type IV collagen and heparin sulfate proteoglycan (1). Interstitial collagens (types I and III) are the major connective tissue elements of the stroma (1). While normal epithelial cells remain separated from the underlying stroma, a characteristic feature of epithelial malignancies is the invasion of the underlying connective tissue. Invasion of the underlying connective tissue is the first step in the metastasis process. Delineating how invasion occurs, therefore, is critical to understanding and (hopefully) learning to control metastasis formation. Studies conducted over several decades have identified a number of features associated with invasion. These features, which together are referred to as the invasion phenotype, include elaboration of proteolytic enzyme, active cell migration and production of connective tissue elements, all of which are dysregulated in some manner relative to that which occurs in a counterpart, non-invasive tissue.

2.1. Proteolytic enzymes and invasion

Studies conducted over the past years have shown that malignant tumor cells in culture express a number of properties that likely contribute to their capacity for invasion. For example, highly-invasive tumor cells in culture elaborate higher levels of several degradative enzymes than their less-invasive or non-malignant counterpart cells (2). Serine proteolytic enzymes (plasminogen activators), sulfydral enzymes (cathepthin B and H) and several of the matrix metalloproteinases (MMP) have all been implicated in the destruction of stromal tissue accompanying tumor invasion. Interference with proteolytic enzyme function using specific biochemical or molecular approaches has provided convincing evidence that proteolytic enzyme function is critical to invasion (3-5).

2.2. Motility and invasion

In addition to elaborating degradative enzymes, invasive tumor cells also demonstrate increased motility relative to their normal counterpart cells (6, 7). While cell

motility is assumed to be critical to invasion, direct evidence to support this is difficult to obtain. This is due to the nature of motility and the limitations of studying *in vivo* a dynamic process that occurs over hours to days. Our ability to make measurements that unequivocally demonstrate the importance of motility in tumor cell invasion is limited. Thus, we have had to rely on surrogate markers and indirect evidence. While indirect and largely circumstantial, this evidence is, none-the-less, quite convincing. There is, first of all, morphological and ultrastructural evidence. Precise examination of actively moving cells *in vitro* has demonstrated morphological features that are not observed in stationary cells. Specifically, motile cells demonstrate a wide ruffled membrane with multiple focal adhesions at the leading edge and a much narrower ruffling (uropod) at the tail. Cytoskeletal elements are attached at sites of cell-substrate adhesion and oriented in the direction of movement (8-10). These same morphological features are observed in invasive tumor cells at the invasive front *in vivo* (11).

Second, while it is difficult to directly observe motility occurring *in vivo* under most circumstances, there are exceptions. *In vivo* cinematography has demonstrated that circulating tumor cells have the capacity to egress from post-capillary venules by a process that is similar to that used by circulating white blood cells (10, 11). While demonstrating the capacity for diapedesis in tumor cells is clearly not the same as demonstrating motility during tissue invasion, these *in situ* studies provide direct evidence that tumor cell motility does, in fact, occur *in vivo*.

Additional evidence that motility is important for tumor invasion comes from correlative studies. Numerous studies over the past 25 or more years have demonstrated that experimental tumors contain mixtures of cells with different phenotypic characteristics. From many different tumors, subpopulations of cells that differ in their capacity for motility *in vitro* have been selected. When such cells are examined for their capacity to form invasive tumors upon injection into animals, there is virtually a 100% correlation between motility and invasive capacity (6, 7). Among the same populations, there is essentially no correlation between motility and tumorigenicity.

2.3. Adhesion factors and invasion

While a relationship between dysregulation of either proteolytic enzyme expression or motility on the one hand and capacity for invasion on the other is intuitive, it is not so clear how changes in the elaboration of various adhesion-promoting moieties and invasion are related. Very early in the twentieth century, pathologists noted the decreased cohesiveness of most tumor masses relative to that of the surrounding normal tissue (12). While this can be attributed, in part, to the effects of enhanced proteolytic enzyme function, protease activity is only part of the explanation. Altered production of proteins that maintain tissue architecture is also a contributing factor. In stromal tumors, the loss of cell surface fibronectin is a defining phenotypic change (13-15). The loss of fibronectin is also a consistent feature of some epithelial tumors (i.e., those of adenomatous origin), but is not seen in squamous epithelial tumors (16-21). Loss of other cell surface proteins such as E-cadherin is also commonly seen in epithelial tumors, and consistently correlated with decreased epithelial cohesion (22).

Concomitant with altered (cell-cell) cohesive function are alterations in adhesion of tumor cells to their extracellular matrix. The loss of cell surface fibronectin is associated with decreased ability to interact with exogenous fibronectin. Inability to interact with exogenous fibronectin rather than a loss of fibronectin synthetic capacity is assumed, in fact, to be the major reason for the loss of the moiety from the tumor cell surface in most cases (23-28). The problem is how to relate loss of cell-matrix adhesive function with invasive capacity. Several explanations are possible. First, a loss of adhesive function does not mean a total loss. Reduced but not total loss of cellular interaction with fibronectin could promote migration, for example, by reducing the adhesive contacts that hold the cell in place (23, 29-31). Second, a loss of adhesion to one particular matrix component does not mean loss of adhesion to other components of the extracellular matrix. In particular, it has been demonstrated that decreased binding to fibronectin does not prevent adhesive interactions with laminin (32-34), a prominent component of the basement membrane. And finally, as already noted, loss of cell surface fibronectin is not a feature of all tumor cells. In summary, what one can say definitively is that altered expression of several moieties that mediate cell-cell and cell-matrix adhesion functions is in seen in malignant cells of one type or another. Altered expression correlates with changes in cohesive/adhesive functions.

In summary, there is a collection of properties that together define an “invasion phenotype”. At issue is whether this phenotype – based primarily on studies with fully-transformed cells in a non-autochthonous environment – explains how malignant cells developing *in situ* in an autochthonous host acquire over time the capacity to degrade and invade the native basement membrane and adjacent connective tissue. The organ culture invasion model addresses this issue.

3. EPITHELIAL CELL INVASION IN HUMAN SKIN ORGAN CULTURE

Two-mm punch biopsies of healthy human skin can be maintained in organ culture under serum-free, growth factor-free conditions. In the absence of exogenous growth factors, normal histological structure and biochemical function are preserved (35, 36). Alternatively, the same tissue can be maintained in medium supplemented with an exogenous growth factor such as EGF. In the presence of exogenous growth factor, there is a proliferative response in the epidermis. This is accompanied by features of abnormal epidermal differentiation (acanthosis, hyperkeratosis and parakeratosis), by “down-growth” of strands of epidermal cells into dermal space and, eventually, by invasion of epithelial cells into the stroma (37). These histological features are depicted in Figure 1. The normal histological features of skin maintained under growth factor-free conditions

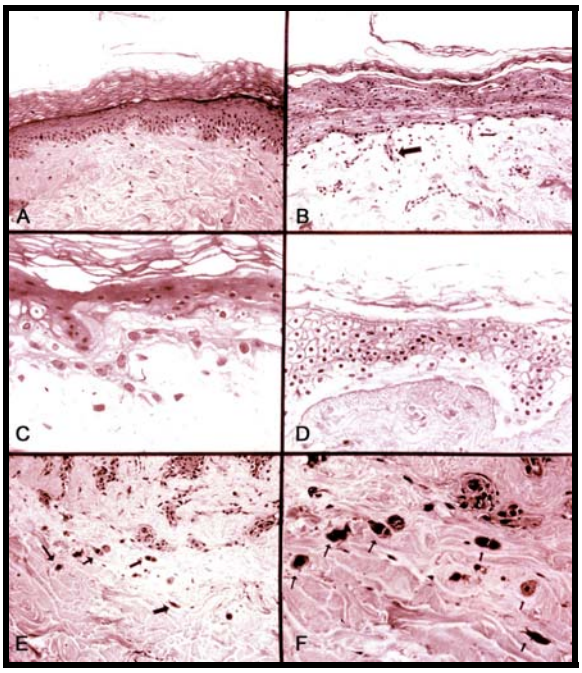

Figure 1. Stromal invasion by human epidermal keratinocytes in organ-cultured human skin: Histological features. A: Histological appearance of human skin after 8 days in organ culture under serum-free, growth factor-free conditions. B - F: Histological appearance of human skin after 8 days in organ culture in the presence of EGF. B and C: Note the strands of epidermal keratinocytes pushing into dermal space (arrows) and the abnormalities in epidermal differentiation. Panels A-C are hematoxylin and eosin-stained. D: Tissue stained with Wilder's reticulum stain to enhance features of the basement membrane. It can be seen that the dermal – epidermal basement membrane is rough and pitted. In places, the basement membrane has been eroded entirely. E and F: Isolated epithelial cells can be seen in the dermis in places. These cells appear to be completely separated from the overlying epidermis (arrows).

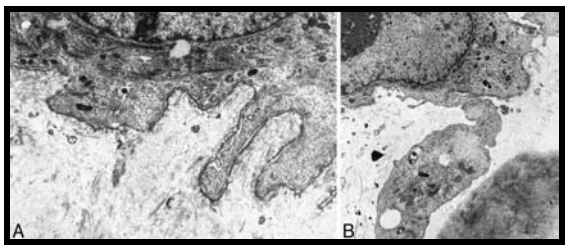

Figure 2. Stromal invasion by human epidermal keratinocytes in organ-cultured human skin: Ultrastructural features. A: Transmission electron microscopic view of a keratinocyte after 8 days in organ culture in the presence of EGF. Note that the basement membrane is intact in most places, but that tiny breaks can be seen in places. Keratinocyte microprocesses are beginning to push down into dermal space, but these are still separated from the dermis by basement membrane. B: Transmission electron microscopic view of a keratinocyte after 8 days in organ culture in the presence of EGF. A pseudopod has formed and is extending into the dermis. The major part of the cell is separated from the dermis by basement membrane, but basement membrane is lacking around the invading pseudopod.

are shown in Figure 1A. The remaining panels of Figure 1 illustrate features seen in growth factor-treated skin. The most common feature is down-growth of epithelial strands into dermal space (Figure 1B and 1C). Erosion of the dermal-epidermal basement membrane occurs, and in some areas, the basement membrane is completely lacking. This can be shown using special staining procedures specific for the basement membrane (Figure 1D). In areas, isolated epithelial cells can be observed completely surrounded by dermal elements (Figure 1E and 1F). Figure 2 depicts the dermal-epidermal juncture as seen at the ultrastructural level. In Figure 2A, a keratinocyte can be seen. It is separated from the stroma by basement membrane, and in places, finger-like projections of the keratinocyte appear to be pushing the basement membrane into dermal space. Tiny breaks in the basement membrane are visible. In Figure 2B, an epithelial cell in the process of invading the stroma is seen. Most of the cell is separated from the stroma by a visible basement membrane. However, at the point of penetration, the basement membrane is missing. These histological features observed in response to stimulation with exogenous growth factors together define an invasive phenotype in organ culture. Since invasion is triggered by exposure of the tissue to an exogenous growth factor, the cellular and molecular phenotype can be characterized in the absence of growth factor and followed for expression of new properties subsequent to growth factor addition. It is, thus, possible to identify changes in cellular and molecular properties that occur (and the sequence in which they occur) as invasive capacity is acquired under *in situ* conditions.

3.1. Phenotypic changes accompanying acquisition of invasive capacity

3.1.1. Proteolytic enzyme expression pattern

Upon exposure of organ-cultured skin to exogenous EGF, there is up-regulation of MMP-1 (interstitial collagenase) and MMP-9 (gelatinase B). MMP-9 is elaborated primarily in epidermal keratinocytes and appears to reflect a direct effect of exogenous growth factor stimulation on keratinocyte function (38). MMP-1 elaboration in EGF-treated skin, on the other hand, occurs primarily in the dermis. Dermal-derived MMP-1 occurs as an indirect consequence of growth factor stimulation of the epidermis via secondary elaboration of interleukin-1 (39). Tissue inhibitor of metalloproteinase-1 (TIMP-1) but not TIMP-2 is present in skin organ culture fluid, but inhibitor levels are comparable under conditions in which invasion does or does not occur (40). Concomitant with the up-regulation of these MMPs in the presence of exogenous growth factor, damage to the underlying connective tissue can be seen at both the light and electron microscopic levels (37, 41 and Figures 1 and 2). MMP activity, matrix degradation and tumor penetration into the dermis are all inhibited in the presence of exogenous TIMP-2 (41). In separate studies, a series of basal cell carcinomas were maintained in organ culture for three days and examined for MMP/MMP-inhibitor expression. Similar to what was observed in normal skin in the presence of EGF, basal cell tumors secreted high levels of MMP-1 and MMP-9 into the culture fluid

without an increase in TIMP-1 elaboration (42).

3.1.2. Fibronectin

There is a substantial induction of fibronectin in organ-cultured skin under conditions in which epithelial cell invasion occurs (37). Resident keratinocytes and fibroblasts are both responsible for production of this matrix component, but given the high ratio of keratinocytes to fibroblasts in growth factor-treated skin (as well as in quiescent tissue), it is likely that the majority of the increase in fibronectin is a result of keratinocyte activity. Studies with keratinocytes in monolayer culture have demonstrated that these cells elaborate high levels of fibronectin upon exposure to EGF (43, 44). Of interest, cell lines derived from squamous epithelial tumors also elaborate large amounts of fibronectin (17). As compared to normal epithelial cells, the maximal production rate does not appear to be significantly different on a per cell basis. However, in normal cells, production falls off dramatically during differentiation (43) whereas differentiation is not seen in the transformed cells.

3.1.3. Motility

Demonstrating that motility occurs in organ-cultured skin (much less, its importance to invasion) is almost as difficult as it is *in situ*. Given the assumed importance of motility to invasion, we have looked for ways to assess motility in organ-cultured skin and to determine if motility is altered in the presence of exogenous, invasion-stimulating growth factors. At the electron microscopic level, one can observe an ultrastructural appearance that is consistent with motility. Where epithelial cells are in the process of penetrating the underlying stromal tissue, filopodia are present at the front of the advancing cells (Figure 2). The morphological and ultrastructural features of the invading cells are indistinguishable from features observed in motile cells in collagen lattice cultures or in other two-dimensional or three-dimensional motility assays. Similar ultrastructural features are also observed at the invasive front in experimental tumors (8). As a second approach, organ-cultured skin was exposed to exogenous EGF in the normal manner. Following this, control or growth factor-treated tissue was minced into tiny fragments and the fragments placed in wells of a 24-well dish under conditions that allowed the tissue fragments to attach. Subsequently, the number of keratinocytes migrating out of the tissue and the kinetics of migration were assessed. By either measure, motility of the growth factor-treated tissue was increased over that of control tissue. While migration out of a tissue fragment is not the same as migration into the dermis, this clearly indicates that the cells are stimulated for motility.

These findings from organ-cultured skin are consistent with what is known concerning keratinocyte migration *in vitro*. Specifically, it has been well-documented that while keratinocyte proliferation occurs in the absence of exogenous growth factors (autocrine production of growth-promoting ligands being sufficient [46]), motility is dependent on exogenous ligands (47). Why this should be is not absolutely clear. It may be a quantitative difference. The amount of exogenous EGF needed to stimulate keratinocyte motility *in vitro* and invasion in organ culture are significantly higher than levels of endogenous EGF-like ligands elaborated in the skin (48). Alternatively, not all EGF receptor ligands behave in identical fashion. For example, amphiregulin appears to be the primary ligand driving keratinocyte proliferation in monolayer culture (46). Amphiregulin also appears to be the major EGF receptor ligand up-regulated in psoriasis (49). In retinoid-induced epidermal hyperplasia, on the other hand, heparin-binding EGF (HB-EGF) appears to be more important (50, 51). HB-EGF is induced in supra-basal keratinocytes following retinoid treatment and acts on cells in the basal layer in a paracrine fashion. This is in contrast to amphiregulin, which is produced primarily in basal cells and acts in an autocrine manner. Such differences may contribute in some way to the phenotypic features that distinguish retinoid hyperplasia from hyperplastic epidermal growth in psoriasis. Likewise, ligand-specific effects may also explain why keratinocyte growth occurs under conditions in which motility is not induced.

In addition to its dependence on an exogenous source of growth factor, keratinocyte motility also requires a suitable substrate over which to move. While a number of extracellular matrix components that make up the provisional or mature matrix will support keratinocyte movement, fibronectin appears to be the most effective (43). Thus, two major requirements for efficient epithelial cell movement (i.e., exogenous growth factor and a suitable matrix) are present during invasion in organ culture.

3.1.4. Summary

Based on these findings, it can be suggested that epithelial cell invasion of the stroma in organ-cultured skin: i) is associated with MMP induction and connective tissue breakdown; ii) depends on elaboration of a motility-supporting matrix (e.g., fibronectin); iii) is associated with induction of motility in the invading epithelial cells; and iv) depends on the presence of epithelial cell growth factors in the culture medium. The extent to which organ culture invasion by growth factor-stimulated normal epithelial cells mimics the invasion process as carried out by malignant tumor cells *in vivo* is not known. Of interest, however, abnormalities in EGF receptor function are among the most common defects in epithelial cell tumors (52, 53). We can hypothesize, therefore, that similar events to those described here are triggered through defects in EGF receptor activation *in vivo*, and contribute to invasion in the intact host.

3.2. Signaling events that underlie acquisition of invasive properties in response to exogenous growth factors

Although our original studies made use of EGF to stimulate invasion, it was subsequently demonstrated that invasion-promoting capacity was not unique to EGF. In a series of experiments, four different growth factors – hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and keratinocyte growth factor (KGF) as well as EGF were compared for effects on human skin in organ culture. When these four growth factors were examined at

concentrations that were equi-potent for stimulating keratinocyte proliferation in monolayer culture, HGF proved to be as effective as EGF in stimulating invasion (41, 54). In contrast, IGF-1 and KGF did not induce invasion under the same conditions. Of interest, HGF was like EGF in that treatment with this factor also induced MMP production and motility in the epithelial cell population. IGF-1 and KGF were less effective in stimulating keratinocyte motility and MMP production (41, 54).

The differential response of human epidermal keratinocytes to these four growth factors has been used to help elucidate the intracellular signaling events that underlie epithelial cell motility and MMP-9 production (55). To summarize, factors that effectively stimulated epithelial cell motility and MMP-9 production as well as proliferation (EGF and HGF) induced sustained signaling through the extracellular signal-related kinase (Erk) and jun-N-terminal kinase (Jnk) MAP kinase pathways, while factors that stimulated proliferation but not motility or MMP production in the same cells induced transient signaling under the same conditions. While sustained signaling through both the Erk and Jnk pathways accompanied induction of motility and MMP-9 production, sustained stimulation of the Erk pathway alone appeared to be sufficient to induce motility. This is based on the finding that transfection of a Jnk-dominant-negative construct into epithelial cells, had no effect on the motility response in spite of profound inhibition of Jnk signaling (55). In contrast, induction of MMP-9 production appeared to depend on both pathways since in either Jnk-dominant-negative transfected epithelial cells or in cells treated with an inhibitor of Erk activation, MMP-9 production was suppressed (55). Based on these findings (and consistent with past observations; references 1-3), we suggest that Erk activation of myosin light chain kinase (MLCK) (a key step in organizing the acto-myosin contractile system) is involved in motility-induction while stimulation of MMP-9 occurs via an effect on AP-1 – mediated gene transcription.

In a similar manner, studies to elucidate the regulation of MMP-1 were carried out. These studies showed that fibroblast responses to exogenous EGF or HGF were weak (at best) (41, 54). In contrast, production of MMP-1 by dermal fibroblasts is known to be strongly induced by interleukin-1 (IL-1) (56). Our studies showed that IL-1 was induced in growth factor-stimulated keratinocytes and that an IL-1 receptor antagonist strongly suppressed keratinocyte stimulation of fibroblast MMP-1 production (39). Subsequent studies demonstrated Erk pathway involvement as well as involvement of the P38 MAP kinase pathway in this process (57). Of interest, Erk-mediated MMP-1 production in fibroblasts accounted for only a small percentage of the total MMP-1 up-regulation. However, this up-regulation was independent of P38. In contrast, P38 played a much bigger role in MMP-1 induction in fibroblasts. P38-mediated MMP-1 elaboration required concomitant functioning of Erk (57). Taken together, these findings demonstrate that in isolated skin, the intracellular signaling events associated with tissue invasion are complex and involve cell-cell interactions among cells within different compartments of the skin.

4. PERSPECTIVE

Two general approaches have accounted for most of what we know about the invasive process. One involves the use of *in vitro* assays to identify and quantify properties thought to be important to invasion. Comparison between malignant and non-malignant cells or between highly-malignant and less-malignant cells in such assays produces a phenotypic profile that distinguishes invasive from non-invasive behavior. The other approach involves taking what is learned from the *in vitro* studies and applying it to the clinical situation. If, for example, *in vitro* studies demonstrate that a certain enzyme is important for local tissue invasion, is that enzyme up-regulated in a specific type of invasive cancer relative to the appropriate control tissue? Such clinical studies are essential for determining if data obtained in experimental systems are relevant to clinical disease. The problem is the difficulty in which invasion can be blocked *in situ*. Without such data, we are left with suggestive evidence but usually no definitive proof. The organ culture model described here is of value because it occupies an intermediary position between the myriad of *in vitro* culture systems and intact tissue. It provides a test system which is much closer to what occurs in intact tissue as cells acquire invasive potential. Yet, like other *in vitro* systems, it is amenable to interventional as well as analytical approaches.

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