Motility Induction in Breast Carcinoma by Mammary Epithelial Laminin 332 (Laminin 5)

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Abstract
Host interactions with tumor cells contribute to tumor progression by several means. This study was done to determine whether mammary epithelium could interact with breast carcinoma by producing substances capable of inducing motility in the cancer cells. Conditioned medium of immortalized 184A1 mammary epithelium collected in serum-free conditions induced dose-dependent motility in the MCF-7 breast carcinoma cell line by both a semiquantitative scattering assay and a Boyden chamber assay. Purification of the motility factor revealed that it was laminin 332 (formerly laminin 5) by mass spectrometry. A Western blot of the 184A1 conditioned medium using a polyclonal antibody confirmed the presence of laminin 332 in the conditioned medium. Blockage of the motility with antibodies to the laminin 332 and its receptor components, α3 and β1 integrins, provided further evidence that tumor cell motility was caused by the laminin 332 in the conditioned medium. Invasion of MCF-7, BT-20, and MDA-MB-435S was induced by purified laminin 332 and 184A1 conditioned medium and blocked by an anti-α3 integrin antibody. Staining of carcinoma in situ from breast cancer specimens revealed that laminin 332 in the myoepithelium adjacent to the preinvasive cells provided a source of laminin 332 that could potentially encourage the earliest steps of stromal invasion. In metaplastic breast carcinomas, the presence of laminin 332-producing cells coexpressing α3 integrin and the greater metastatic potential of tumors with higher laminin 332 levels suggest that laminin 332 expression is associated with aggressive features in these human breast cancers. (Mol Cancer Res 2009;7(4):462–75)

Introduction
A series of steps must occur for a tumor cell to leave its primary site and invade the surrounding tissue (reviewed in refs. 1-5). In order for cells to invade, they must adhere to and actively migrate through the basement membrane into the adjacent stroma. This migratory activity, or motility, in a cell is often initiated or enhanced by interaction with motility factors, which include cytokines (5) and extracellular matrix components (6, 7). The requirement for tumor cell motility for the ultimate development of invasion and metastasis has been shown in several model systems (3, 8-10).

There is growing evidence that the tumor microenvironment and interaction of tumor cells with the surrounding stromal cells and extracellular matrix is a major contributor to tumor progression (10-18). During the progression of breast neoplasia from hyperplasia to in situ carcinoma to invasive carcinoma, the neoplastic cells may encounter ductal or lobular epithelium, myoepithelium, basement membrane components, fibroblasts, extracellular matrix, inflammatory cells, vascular elements, and other cell types. Any of these cell types, or substances produced by them, have the potential to influence the course of neoplastic progression. Among the ways that this might occur is the enhancement of breast tumor cell motility, and subsequently invasion and metastasis, by substances in the microenvironment. Evidence to support this possibility includes the observations that breast epithelium (11), fibroblasts (12, 17), macrophages (10, 13-15), and lymphocytes (14) produce motility factors for breast cancer cells.

Among the microenvironmental proteins that could be implicated in paracrine interactions with breast cancer cells is laminin 5, a 400 kDa heterotrimeric protein consisting of the α3, β1, and γ2 chains of laminin. It is designated laminin 332 using a recently introduced simplified nomenclature (19). The major functions of laminin 332 include binding of epithelial cells to the basement membrane through the formation of hemidesmosomes (20) and the migration of epithelial cells during wound repair (21-23). The former interaction is mediated through binding to α3β4 integrins (20) on the epithelial cell surface and the latter is mediated by laminin 332 binding to α3β1 integrin (21, 24, 25), although the α3β4 integrin may also be involved in adhesion (26). Laminin 332 interaction with α3β4 integrin may also sustain anchorage independence in tumorigenic breast cell lines (27). The migratory activity by laminin 332 requires processing of the α3 and γ2 chains by proteinases, including matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase and others (reviewed in refs. 24, 28, 29).
In addition, laminin 332 has been implicated in tumor progression (28-31). In this report, we suggest a role for laminin 332 secreted by normal breast epithelium in the motility and invasion of breast cancer cells and the increased aggressiveness of breast cancer cells that express laminin 332.

Results

Induction of Motility in MCF-7 Breast Cancer Cells by Conditioned Medium of Mammary Epithelial Cells

We have previously reported our preliminary observations that coculture of MCF-7 breast cancer cells with normal cultured human mammary epithelial cells or the immortalized human mammary epithelial cell clone 184A1 induces motility in MCF-7 cells (11). Further, this activity appeared to be due to a component of the conditioned medium secreted by the human mammary epithelial cells. Because 184A1 is immortal, it is a useful continuous source of conditioned medium. These cells are able to survive in the absence of serum or other exogenous protein components for ~2 days, which is useful for producing conditioned medium with only the secreted products of the 184A1 cells present. Although they are an established cell line, they share many similarities with the parental primary mammary epithelial cells (32) and elicit motility in MCF-7 cells to the same degree as human mammary epithelial cells (11). Using time-lapse video microscopy, we have shown previously that MCF-7 cell scattering is due to their migration away from cell clusters in the presence of 184A1 conditioned medium.

This scattering response in the presence of 184A1 conditioned medium provided a quantitative and reproducible high-throughput scattering assay validated by time-lapse video microscopy that has been described previously (11, 14). Briefly, time-lapse video recordings of MCF-7 cells exposed to 184A1 conditioned medium exhibited continuous movement radially away from cell clusters at a rate of ~25 μm/h. Cells that migrated always exhibited a separation from their cluster of origin, characterized by a gap between the migrating cell and the nonmigrating cells in the cluster. Furthermore, they displayed pseudopodia or lamellipodia along the leading migrating edges of the cells, which was usually opposite from the cluster. After prolonged continuous scattering, cells were distributed along the plate as individual cells, no longer adherent to one another in clusters. An example of complete MCF-7 cell scattering is shown in Fig. 1.

Dose-Response and Time Course of MCF-7 Scattering Response to Conditioned Medium

To determine whether motility induction by 184A1 conditioned medium was dose-dependent, two different types of measurements of MCF-7 cell motility were done at different concentrations of 184A1 conditioned medium. These experiments allowed comparison of MCF-7 cell scattering and motility by the Boyden chamber assay and provided an estimation of the amount of motility-inducing activity produced by different numbers of 184A1 cells. This was achieved by measuring the volume of the conditioned medium, the number of cells in the plate at the time of conditioned medium collection, and the length of time that cells were exposed to the medium. From these measurements, the degree of motility provided by the secretion products of a given number of cells in a 24 h period was estimated.

For the scattering assay, conditioned medium produced by increasing numbers of 184A1 cells ranging from $10^4$ to $5 \times 10^5$ cells was added to wells of $10^4$ MCF-7 cells. The MCF-7 cells were stained with crystal violet after 48 h and assessed for a scattering response by determining the percentage of cells, which migrated out from the main cluster of groups of 20 to 80 cells. Increasing concentrations of conditioned medium induced dose-dependent scattering in the breast cancer cells (Fig. 2A).

As a further test of the dose-dependent motility induction by 184A1 conditioned medium, $10^5$ MCF-7 cells were placed in Boyden chamber inserts with 12 μm pores on the bottom membrane. A range of concentrations of conditioned medium were then added to the wells. After 48 h, the number of cells/mm² migrating to the other side of the membrane was counted. These experiments showed dose-dependent migration of MCF-7 cells through the Boyden chamber membrane in response to the 184A1 conditioned medium (Fig. 2B). The degree of motility induced by the conditioned medium of any

![Figure 1](image-url)
given number of 184A1 cells was almost identical between the two assays, which further validates the semiquantitative scattering assay as a measurement of motility comparable with the Boyden chamber assay.

It is possible that the motility factor of the 184A1 conditioned medium was only a product of confluent cells and that the dose response measured in this experiment was that of the serially diluted conditioned medium of the confluent cells. To rule this out and to further show that motility of MCF-7 was dependent on the number of 184A1 cells, a dose-response experiment was done using conditioned medium from different numbers of 184A1 cells/mL. Wells of 184A1 cells in 1 mL medium, with increasing numbers of 184A1 cells per well, were incubated for 2 days, and the conditioned medium from each well was transferred to wells of MCF-7 cells. Because the medium was conditioned for 2 days, the motility factor present was equivalent to that produced by twice the number of cells per well. This was done because 1 mL wells were confluent at 100,000 184A1 cells per well. Scattering of the MCF-7 cells increased with the number of 184A1 cells in a dose-dependent manner, as shown in Fig. 2C.

Because the conditioned medium was collected using RPMI 1640 without additives, serum or other growth factor components normally present within tissue culture medium were not the source of the motility-inducing effects of the conditioned medium. Conditioned medium from 10⁵ or 10⁶ MCF-7 cells previously grown in mammary epithelial growth medium (MEGM) did not induce scattering in MCF-7 cells (data not shown), ruling out the possibility that the process of conditioned medium collection or carryover of MEGM nonspecifically induced motility in MCF-7 cells.

Previously, we have observed movement of MCF-7 cells at the 24 h time point, indicating that motility persists with continuous exposure to 184A1 conditioned medium (11). To extend this observation, and to determine the time it takes for scattering to become first apparent and to establish the duration of the scattering response in the presence of conditioned medium, a time-course experiment was done. MCF-7 cells were plated and exposed to 5% 25× concentrated conditioned medium or an equal volume of RPMI 1640 after allowing them to adhere to the plates for 2 h. Medium was removed from wells of cells and fixed for 1 min with 70% ethanol at time points up to 100 h. Scattering was measurable within 1 h and continued to increase with time. No scattering was observed in untreated cells throughout the entire experiment.

FIGURE 2. Dose dependence and time course of motility induced by 184A1 conditioned medium. Using the scattering assay (A), 2 × 10⁴ MCF-7 cells in growth medium were incubated with the diluted conditioned medium equivalent to the products secreted in 1 day by increasing numbers of 184A1 cells ranging from 10⁴ to 5 × 10⁵ cells. After 48 h, the average percentage of MCF-7 cells displaying a scattering response was determined for each condition. Boyden chamber assays (B) were done using 10⁵ MCF-7 cells incubated with the conditioned medium products secreted in 1 d by increasing numbers of 184A1 cells ranging from 10⁴ to 4 × 10⁵ cells. The average number of MCF-7 cells/mm² migrating through the membrane in 48 h was calculated for each condition. The number of scattered cells per cluster (A) and the average number of MCF-7 cells migrating in the Boyden chamber assay (B) increased with the dose of conditioned medium in an almost identical fashion. C, 3 × 10⁵ MCF-7 cells/mL in growth medium were incubated with the conditioned medium from individual wells of increasing numbers of 184A1 cells ranging from 10⁴ to 2 × 10⁵ cells. Increasing numbers of 184A1 cells per well also induced dose-dependent scattering in MCF-7 cells. To measure the effect of incubation time on the scattering response (D), 3 × 10⁵ MCF-7 cells/mL in growth medium were incubated with 184A1 conditioned medium, and the scattering measured over a 96 h interval. Scattering was measurable within 1 h and continued to increase with time. No scattering was observed in untreated cells throughout the entire experiment.
to 96 h. Cells were stained and scattering was quantified. Scattering was observed within 1 h, was complete within 24 h, and persisted throughout the 96 h experiment (Fig. 2D). No motility was apparent among untreated cells. Furthermore, motile MCF-7 cells in response to the conditioned medium removed from the plate and transferred to another plate no longer exhibited motility, providing evidence that the effect of the conditioned medium is not mainly driven by the cancer cells themselves but requires the presence of the motility factor in the conditioned medium.

Preliminary Molecular Weight Estimation

Our next goal was to identify the motility factor. Because excluding known motility factors individually was impractical and would be unsuccessful for an uncharacterized protein, we chose the strategy of purification of the active components followed by molecular characterization of them. Initial attempts began with a rough estimate of the molecular weight range of the motility factor of the 184A1 cells. A preliminary molecular weight determination could offer two potential benefits. First, knowing the size of the motility factor might provide a hint of which category of substances the motility factor belonged. Second, the information might be useful for determining a purification strategy. The 184A1 conditioned medium was sequentially passed through filters designed to selectively exclude molecules of 100, 50, 30, and 10 kDa, respectively.

The retenates from each filter were used for motility assays in a 96-well plate. At least 50% of the motility-inducing activity of the conditioned medium was retained by a YM100 filter designed to exclude proteins of \( \geq 100 \) kDa, and at least 80% was retained by a YM50 filter for \( \geq 60 \) kDa proteins (Fig. 3). These results suggested that a larger molecular weight molecule, such as an extracellular matrix component, more likely provided the majority of the motility-inducing activity, compared with a small molecule such as a chemokine, which are 8 to 14 kDa (33).

Purification of the Motility Factor

To determine the nature of the motility factor, the active component of concentrated conditioned medium was purified with a three-step process, which included gel filtration with Sephacryl 200 HR, concanavalin A affinity chromatography, and reactive blue 4 agarose affinity chromatography. Fractions from each step were monitored by absorbance at 280 nm and the ability of the fraction to induce scattering in MCF-7 cells. Activity was retained by the concanavalin A and reactive blue columns but appeared in the void volume of the Sephacryl column. Because the latter column excludes very high molecular weight proteins, this result confirmed that the motility induction of MCF-7 was due to a substance of \( >100 \) kDa, such as an extracellular matrix component, rather than a smaller protein such as a chemokine or cytokine. The final purification process yielded three bands on an 8% SDS-polyacrylamide gel (Fig. 4) with molecular weights of 165, 135, and 105 kDa. Matrix-assisted laser desorption/ionization time of flight mass spectroscopic analysis of bands cut from the gel showed that the three bands consisted only of the \( \alpha_3 \), \( \beta_3 \), and \( \gamma_2 \) chains of laminin, respectively. The combination of these chains is unique to laminin 332 (34), providing evidence that the motility-inducing component of the conditioned medium was laminin 332. There were no visible bands of lower molecular weight than 100 kDa on the gel of the final purification of the motility factor (Fig. 4). The identification of laminin 332, a 400 kDa protein, is consistent with the apparent large molecular weight of the motility factor noted during the purification.

The identity of the bands was confirmed by Western analysis of unpurified conditioned medium using a polyclonal rabbit antibody against the entire laminin 332 heterotrimer (35). The pattern of bands on the Western was almost identical to the pattern seen on the SDS gel (Fig. 4). An additional band just below the \( \gamma_2 \) chain band was present on the Western blot. Although this exact band could not be distinguished from other proteins on a gel of the crude conditioned medium, an 85 kDa band from partially purified conditioned medium was used for matrix-assisted laser desorption/ionization time of flight analysis, which revealed fragments of the \( \alpha_3 \) and \( \beta_3 \) chains. This suggests that the lower band on the Western blot was a processed or partially proteolyzed form of the protein that would have been present in the unpurified conditioned medium that was used for Western blotting but removed from the conditioned medium during purification.

Antibody Inhibition

To further test whether laminin 332 was the motility factor of 184A1 conditioned medium, antibody inhibition of laminin 332-induced motility was done using antibodies to laminin 332 and its receptor. Initial experiments indicated that relatively high concentrations of commercially available antibodies were necessary to block laminin 332 motility. To best achieve blocking, antibodies were incubated with tissue culture plates.
in which the motility-inducing activity was bound to the bottom of the plates. As expected for an extracellular matrix protein such as laminin 332, most of the motility-inducing effect of 184A1 conditioned medium bound to the plastic of the tissue culture plates. The laminin 332 blocking antibody P3H9 (100 μg/mL) or the same concentration of mouse immunoglobulin (IgG) was incubated in wells of a 96-well plate in which the motility-inducing activity was adherent to the bottom of the wells. The antibody was removed and MCF-7 cells were added, and the scattering was assessed after overnight incubation. Figure 5 shows that the anti-laminin 332 antibody significantly blocked motility (P = 0.002, Student’s unpaired t test) compared with mouse antibody.

Because an antibody against laminin 332 was able to inhibit motility, it is expected that antibodies against its receptor may also inhibit laminin 332-induced motility. Earlier studies have shown that motility on laminin 332 is mediated by the α3β1 integrin (21, 24, 25). Thus, we tested the ability of blocking antibodies of α3 and β1 integrins to inhibit motility in the presence of 184A1 conditioned medium. As shown in Fig. 6A, antibodies against both integrins inhibited scattering in a dose-dependent manner. To rule out simple inhibition of binding to the tissue culture plate, or nonspecific inhibition of motility, the same antibodies were tested for their effect on tumor necrosis factor (TNF)-α-associated scattering (Fig. 6B), which is mediated by the TNF receptor (14). No inhibition of TNF-mediated scattering was observed.

Invasion Induction by 184A1 Conditioned Medium

Because tumor cell motility is necessary for cancer invasion, we investigated the ability of 184A1 conditioned medium to induce invasion in tumor cell lines. The membranes of migration chamber tissue culture inserts were coated with Matrigel diluted with an equal volume of 10× concentrated conditioned medium, resulting in a final concentration of Matrigel of 200 μg/mL and 100,000 MCF-7, BT-20, or MDA-MB-435S cells were added to the upper chamber. Invasion to the lower chamber was measured after overnight incubation in the presence or absence of 184A1 conditioned medium. Figure 7 shows a marked statistically significant increase in invasion in MCF-7, BT-20, and MDA-MB-435S cells exposed to 184A1 conditioned medium. It is likely that MDA-MB-435S is of melanoma rather than breast carcinoma origin (36). If this is the case, then this result is consistent with previous reports that laminin 332 induces melanoma invasion (37). These results are additional evidence that paracrine motility factors originating from mammary epithelium could contribute to the progression of other tumor cell lines, including those originating from breast carcinomas. Furthermore, almost all the invasion was inhibited by a blocking antibody to α3 integrin. This is consistent with the observation that antibodies to α3 integrin block motility induced by 184A1 conditioned medium and suggests that invasion is mediated by laminin 332. The α3 integrin antibody was used because α3 is more specific component of the laminin 332 binding integrin than the β1 component, which combines with a wide variety of α integrins to bind to several extracellular matrix proteins (38).

Motility and Invasion by Purified Laminin 332

As a final test of whether laminin 332 was responsible for the motility-inducing effects of 184 A1 conditioned medium, scattering and invasion assays were done using purified laminin 332. For these and subsequent experiments, laminin 332 could be purified with a higher yield by passing 184A1 conditioned MEGM over a reactive blue affinity column, eluting with a gradient of 0.15 to 0.75 mol/L NaCl, and isolating the laminin 332 by gel filtration. Wells of MCF-7 cells were incubated with concentrations of laminin 332 ranging from 1 to 25 μg/mL in the presence or absence of 184A1 conditioned medium.
100 μg/mL, and scattering was assessed after overnight incubation. Figure 8A shows a dose-dependent motility response to laminin 332, similar to what was observed with serial dilutions of 184 A1 conditioned medium (Fig. 2A) or increasing numbers of 184 A1 cells (Fig. 2C). Similarly, an invasion assay with 50 μg/mL laminin 332 in the opposite chamber from the cells showed increased invasion of MCF-7 cells in the presence of laminin 332 compared with controls (Fig. 8B).

Immunohistochemistry of Ductal Carcinoma In situ

The study of a motility factor is most likely to be potentially clinically relevant if its source can be determined and if the source of the motility factor and the tumor are in a position that allows direct communication between them in a pathologic state. To test whether mammary epithelial laminin 332 exists in the tumor microenvironment where it would have the opportunity to interact with tumor cells, we examined laminin 332 expression in ductal carcinoma in situ (DCIS). Because the tumor cells of DCIS reside within breast ducts, we sought to determine whether laminin 332 was expressed in the residual basal epithelial cells of DCIS. Of the 25 DCIS breast cancer cases stained with laminin 332 antibody, 16 were cases of DCIS alone and 9 cases showed both DCIS and invasive breast cancer. Of the in situ cases, 9 were low grade, 6 were intermediate grade, and 10 were high grade. Laminin 332 staining was observed in the basement membrane and myoepithelial cell layer in ≥50% of ducts and lobules involved by DCIS in 14 of 25 (56%) of cases. Similar laminin 332 staining was noted in high-grade DCIS using an antibody against the γ2 lamin 332 chain. An example of basal laminin 332 staining is shown in Fig. 9.

Immunohistochemistry of Invasive Breast Carcinoma

To determine whether a role for laminin 332 in invasive breast carcinomas was possible, we examined 25 samples of the metaplastic carcinoma subtype of invasive breast carcinoma, which expresses laminin 332 (39). The clinical and histopathologic features of these cases are shown in Table 1. All tumors were intermediate to high grade, and 18 of the tumors were ≥2 cm. One to 9 lymph node metastases had occurred in 6 patients, 18 patients were free of metastases at the time of surgery, and 1 patient refused lymph node sampling. Among these cases, 24 of 25 expressed laminin 332 in >5% of cells, and the staining was strong in all the cases in which laminin 332 expression was observed. Metaplastic carcinomas generally behave more aggressively than the usual type of infiltrative ductal carcinoma of the breast (40-42), which normally do not express laminin 332 (43). This observation raised the possibility that...
laminin 332 expression in breast carcinomas might confer a more aggressive phenotype in these tumors. Microscopic examination revealed that in 23 of the 24 positively staining tumors; the greatest concentration of laminin 332 was present at the tumor-stroma interface, in infiltrative nests of tumor, and at the invasive front of tumor nodules. These are the cells that are positioned to invade into the stroma and potentially into vascular spaces, leading to metastases. An example of staining at the tumor border is illustrated in Fig. 10A.

Because the antibody inhibition experiments and earlier studies favor the \( \alpha_3\beta_1 \) integrin as the ligand involved in laminin 332-induced motility, the 25 metaplastic carcinomas were immunoperoxidase-stained with an anti-\( \alpha_3 \) integrin antibody. All 25 tumors showed strong staining in the majority of tumor cells. An example of the tumor staining is illustrated in Fig. 10B using a photomicrograph of the same case depicted in Fig. 10A. As with laminin 332, increased staining at the tumor stroma interface also occurred with the \( \alpha_3 \) integrin antibody.

The percentage of tumor cells staining with laminin 332 was estimated by microscopic observation of each case, and the positively tumors were designated as having 1+, 2+, or 3+ staining based on 5% to 29%, 30% to 69%, or 70% to 100% of cells staining, respectively. Eight tumors showed 1+ staining, 11 showed 2+ staining, and 5 showed 3+ staining. For the 6 patients with lymph node metastases, laminin 332 staining was significantly greater than the laminin 332 expression of the tumors that did not metastasize (Fig. 11A). Traditional predictors of tumor aggressiveness, such as Nottingham score (Fig. 11B) and primary tumor size (Fig. 11C), were not different between the patients with and without lymph node metastases for this tumor type in this small sample. There was no significant difference between laminin 332 expression in large or small tumors or between intermediate-grade and high-grade carcinomas.

**Discussion**

In this study, we show that conditioned medium from 184 A1 mammary epithelial cells promoted dose-dependent scattering and motility in MCF-7 breast carcinoma cells. Laminin 332 purified from the conditioned medium induced scattering and invasion, and a Western blot of the conditioned medium confirmed the presence of laminin 332. Inhibition of motility by antibodies to \( \alpha_3 \) and \( \beta_1 \) integrins is consistent with previous observations that laminin 332-associated motility is mediated by \( \alpha_3\beta_1 \) integrin (21, 24, 25).

The results from this study are consistent with previous observations that laminin 332 enhances the migratory phenotype of several cancer cell types, including glioma, melanoma, and prostate carcinoma cells (25, 37, 44-46), and that cultured mammary epithelial cells such as MCF-10A are among the cell types that produce laminin 332 (25, 47). In several tumor types, laminin 332 expression is associated with tumor invasion (reviewed in refs. 28, 30, 31). Laminin 332 deposition by melanoma cells may also promote tumorigenesis by providing a matrix through which tumor cells can line their own blood vessels, a process known as vasculogenic mimicry (48). Prior work, however, has not provided a clear role for laminin 332 in breast tumor progression. On one hand, laminin 332 was found at the invasive front of a few breast carcinomas (49), and the \( \gamma_2 \) chain was noted to be expressed at greater levels in the highly metastatic MTLn3 breast carcinoma cells compared with the low metastatic MTC variant of these cells (50). Using cDNA array analysis of human breast carcinomas, transcripts of laminin 332 chains such as \( \alpha_3 \) and \( \gamma_2 \) were...
detected in a subset of ~15% of the tumors, which were characterized as having a basal-like epithelial phenotype (51). Similarly, we have detected laminin 332 expression in the rare but aggressive metaplastic subtype of breast cancer. In contrast, other studies indicate that laminin 332 is downregulated in breast cancer cell lines (47, 52, 53) or absent from malignant cells in invasive breast cancers (43).

The present study addresses this question of whether laminin 332 has a role in the progression of breast carcinoma and extends previous work in two ways. First, we showed that mammary carcinoma cell lines are more invasive into Matrigel containing laminin 332 than Matrigel diluted with an equal amount of RPMI 1640 alone. Thus, we show that laminin 332 is incapable of inducing invasion in breast cancer cells. Similarly, laminin 332 increases the invasiveness of glioma (45) and melanoma cell lines (37). In contrast, inhibition of laminin 332 with small interfering RNA diminishes the invasiveness of one oral cavity squamous carcinoma cell line (54). It is uncertain whether these studies done in squamous cells are relevant to breast cancer. Second, we noted that laminin 332 was present in the myoepithelial layer of DCIS and in 96% of metaplastic breast carcinomas. Similarly, laminin 5 has been noted previously in the myoepithelium of DCIS (55).

In DCIS, the proximity of neoplastic cells to the laminin 332-expressing myoepithelium suggests a mechanism of how these cells could interact with the tumor cells to encourage their progression. In this situation, the outer layer of DCIS cells is exposed to the greatest concentration of laminin 332. These cells are the nearest to the basement membrane and are expected to be the first to undergo microinvasion. This raises the possibility that laminin 332 promotes the earliest step in the transition from in situ to invasive cancer, that is, the microinvasion of individual cells through the basement membrane into the stroma. A similar mechanism has been proposed for the transition from prostatic intraepithelial neoplasia to early invasive prostatic adenocarcinoma (44). The presence of laminin 332 in the myoepithelial layer surrounding DCIS, however, is not sufficient evidence that laminin 332 causes invasion in this situation, only that if the interaction in vivo is similar to what we have observed in vitro, then the opportunity for induction of invasion exists and cannot be ruled out. Further study will be needed to better define the role of laminin 332 in the interaction of DCIS with its environment. Alternatively, laminin 332 from myoepithelium could induce migration in the DCIS cells in the form of intraductal spread. Morphologic observations suggest that mammary DCIS migrates into apparently normal ducts (56). Laminin 332 may provide a stimulus that encourages migration of the in situ carcinoma cells into the ducts, suggesting another mechanism by which motility factors originating from mammary epithelium might contribute to human disease.

Together, these in vitro studies provide a rationale for a role of breast epithelium as one of the factors in the tumor micro-environment that could lead to tumor progression. The presence of benign cells expressing laminin 332 adjacent to malignant cells seems analogous to the observation that nontransformed 184A1 cells produce laminin 332 that affects the motility of malignant breast cancer cells in vitro. In support of this hypothesis, several other studies have proposed a role for normal breast cells in breast cancer progression. For example, when either human or animal breast carcinoma cells are implanted

### Table 1. Tumor Information for Cases of Metaplastic Breast Carcinoma

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###FIGURE 10. Laminin 332 β3 chain (A) and α3 integrin (B) expression in invasive metaplastic carcinoma of the breast. Immunoperoxidase staining reveals expression of both laminin 332 and α3 integrin in cells at the tumor-stroma interface. Original magnification, ×200 (A) and ×400 (B).
in the mouse mammary fat pad, the cells are usually more tumorigenic, and the tumors grow larger and are more likely to metastasize than if they were implanted in the subcutis (57-60). The mechanism for this orthotopic site preference of mammary carcinoma metastasis is unknown, but for mouse mammary carcinoma cells the presence of benign mammary epithelial cells appears to exert a positive effect on the cancer cells.

Other studies have provided evidence that myoepithelial cells contribute to tumor progression (61-64). For example, Allinen et al. found that the chemokine CXCL14 expressed in tumor myoepithelial cells could bind to receptors on breast cancer epithelial cells and enhance their proliferative and invasive properties. They used Ki-67 expression levels to provide evidence that the DCIS cells adjacent to the myoepithelium showed greater proliferation than tumor cells in other parts of the ducts and that gene expression in the myoepithelial cells in DCIS is different than the gene expression of normal myoepithelial cells (61). Similarly, Zhu et al. showed that in DCIS the expression of several genes, including those potentially involved in invasion and metastasis, varied depending on location of tumor cells in the ducts (62). Furthermore, genetic and morphologic alterations of the ductal cells occur at sites of myoepithelial cell disruptions in DCIS (63). These and other studies have been reviewed (64).

Complete understanding of mammary epithelial-tumor interactions is complicated because mammary-derived growth inhibitors of breast cancer such as mammastatin (65) and motility and invasion inhibitors such as maspin (66) have been identified. In some situations, it appears that myoepithelial cells inhibit breast cancer progression (67-70). For example, myoepithelial cells from salivary gland and breast myoepithelial tumors induce apoptosis (67), inhibit invasion (68), and inhibit proteinases implicated in tumor invasion (69) in breast cancer cell lines, suggesting that myoepithelium provides a barrier to breast cancer spread. These apparently contrasting observations suggest that in the initial stages, such as atypical hyperplasia and low-grade DCIS, the myoepithelium may act as a barrier, containing the proliferating cells in the ducts. As myoepithelial cells become associated with tumor cells they may become altered (61) or lose their ability to express some proteins, such as laminin 1, which is required for normal breast cell polarity (71). Next, as the neoplasm acquires additional genetic alterations, it may respond more aggressively to signals secreted by the myoepithelium, including increased invasive ability in response to laminin 332. Next, myoepithelial cell alterations may make them more receptive to allowing invasion, whereas further myoepithelial layer disruptions encourage additional proinvasive features of the tumor (66) followed by tumor cell release or escape past the basement membrane (67). Finally, additional microenvironmental signals can act on the tumor cells as they enter the extracellular matrix and stroma (16-18, 61).

Laminin 332 may also play a role in the aggressive properties of invasive breast carcinomas, and examination of its expression in human tumors can provide circumstantial evidence in support of this hypothesis. We examined a group of invasive mammary carcinomas that express laminin 332, the metaplastic carcinomas. Several features of the laminin 332 staining of these tumors are associated with tumor aggressiveness. First, metaplastic carcinomas usually present at a higher stage than most breast carcinomas and tend to have a poor prognosis (40-42). In all but one of the metaplastic carcinomas examined, the strongest expression of laminin 332 was greater at the tumor stroma interface, including in cells at the invasive tumor front. This finding is similar to the observations of Pyke et al., who observed that laminin 332 is often localized at the invasive front of aggressive tumors, including a few examples of breast carcinoma (49). The increased staining of laminin 332 at this location is of uncertain significance and further study is needed to determine whether preferential expression in this
location aids in the invasive process. Furthermore, an antibody to α3 integrin stained all metaplastic carcinoma cases examined, but additional studies are needed to determine whether autocrine or paracrine stimulation by laminin 332 occurs in these cells and, if so, whether it contributes to motility and invasion. In one series of breast cancers, coexpression of laminin 332 and β3 integrin, as a component of the α3β4 heterodimer, resulted in a trend toward decreased survival in breast cancer patients, but the sample size was small in that study (55).

Metaplastic carcinomas that had metastasized to the lymph nodes had significantly higher levels of laminin 332. In contrast, traditional markers of tumor aggressiveness such as primary tumor size and Nottingham score did not correlate with lymph node metastasis. Although metaplastic carcinomas are rare, laminin 332 is also present in estrogen receptor and HER-2-negative carcinomas, which appear to have a basal-like phenotype, have a poor prognosis, and represent 15% of invasive carcinomas (51). Additional studies will be needed to determine whether the expression of laminin 332 causes the tumor cells to behave more aggressively or whether it can predict prognosis in breast carcinoma. Further, additional genes also likely contribute to the aggressive properties of these tumors.

The relationships between breast cancer cells and the mammary epithelium are clearly complex. Motility of cancer cells is clearly a prominent component of their ability to invade and metastasize, and many of the mechanisms influencing cell motility have yet to be elucidated. Clinically invasive breast cancer reflects a predominance of the inherent and host-induced invasive and growth properties of the cancer cell over the host defenses inhibiting growth and invasion. Because in untreated cancer the aggressive components of the equation predominate, it is critical that they are understood so that they can be targeted during the development of novel therapeutic strategies.

Materials and Methods

Tissue Culture

Tissue culture medium components and other chemicals were obtained from Sigma, except where otherwise indicated. MCF-7 cells were a gift from Dr. Dan Mercola. The human tumor cell lines BT-20 and MDA-MB-435S and the immortalized mammary cell line 184A1 were obtained from the American Type Culture Collection. 184A1 cells have been well characterized and have many characteristics of the parental normal cells including anchorage dependence, nontumorigenicity, and epidermal growth factor-dependent growth (32). MCF-7 cell medium consisted of RPMI 1640 supplemented with 4 mmol/L glutamine, 0.2 units/mL bovine insulin, 10 units/mL penicillin, 10 μg/mL streptomycin, 5% heat-inactivated newborn calf serum (Irvine Scientific), and 10 nmol/L estradiol. BT-20 and MDA-MB-435S were maintained in RPMI 1640 with 4 mmol/L glutamine, 10 units/mL penicillin, 10 μg/mL streptomycin, and 10% heat-inactivated FCS (Gemini Scientific). 184A1 cells were routinely grown in MEGM (Cambrex). MEGM consists of modified MCDB 170 medium supplemented with ~52 μg/mL bovine pituitary extract, 10 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 50 μg/mL gentamicin sulfate, and 50 ng/mL amphotericin B.

Production of Conditioned Medium

Conditioned medium was collected from 70% to 100% confluent plates of 184A1 cells. To minimize the amount of exogenous protein in the conditioned medium, the MEGM used to establish the plate was discarded, the plate was rinsed with 0.14 mol/L NaCl and 10 mmol/L sodium phosphate (pH 7.4; PBS), and the medium was replaced with RPMI 1640 without serum. In these conditions, the cells remained viable and secreted proteins into the medium. The daily secretion products of a given number of cells could be determined for each batch of conditioned medium. To estimate the quantity of the secretion products of the 184A1 cells, the volume of the conditioned medium was measured after either 24 or 48 h, and the conditioned medium was passed through a filter with 0.2 μm pores to remove any cells or debris. The adherent cells remaining in the plate were trypsinized, and the number of viable cells producing the conditioned medium was counted with a hemocytometer. The number of cells divided by the medium volume provided the number of cells secreting motility factor per milliliter of conditioned medium. The medium typically contained the products secreted by 5 × 10^5 cells in 1 day per milliliter. Large volumes of several hundred milliliters or more were concentrated by ultrafiltration through a YM10 filter in a Millipore-10 bioconcentrator apparatus (Millipore), which retained proteins with molecular weights of ≥10 kDa.

MCF-7 Scattering Assay

Scattering of MCF-7 cells is due to the separation and migration of individual cells from clusters of cells, as we have shown previously using time-lapse video microscopy (11), which then provided validation of an in vitro scattering assay that could be done in multi-well tissue culture plates. The effect of 184A1 conditioned medium on MCF-7 cell scattering was measured by adding conditioned medium to a well of a 24- or 48-well tissue culture plate containing 10^4 MCF-7 cells/mL. For the negative controls, the same volume of RPMI 1640 was added in place of the conditioned medium. Experiments were terminated after overnight incubation by rinsing the cells briefly with PBS and staining with 2% crystal violet in 40% ethanol. The percentage of motile cells was determined by a semiquantitative method (11, 14, 72). Briefly, wells of crystal violet stained MCF-7 cells were divided into four quadrants, and the total number of cells in a single cluster of 20 to 80 cells in predetermined coordinates was counted for each quadrant. The number of cells that have separated from the main group and exhibited lamellipodia or pseudopodia, features confirmed as MCF-7 cell motility by time-lapse video microscopy, were counted in each of these clusters to determine the number of motile cells per colony. The average percentage of motile cells per condition and the SE were calculated from these counts.

Dose-Response Analysis of 184A1 Conditioned Medium, 184A1 Cells, and Laminin 332

To determine the dose response of 184A1 conditioned medium-induced motility of MCF-7 cells, concentrated conditioned medium was serially diluted such that the equivalent
of protein produced by $2 \times 10^4$ to $5 \times 10^5$ cells was present in 1 mL MCF-7 growth medium. Each dilution of conditioned medium was then used in a modified Boyden chamber assay or scattering assay as described below. To determine the response of MCF-7 to increasing numbers of 184A1 cells, wells containing from $10^4$ to $2 \times 10^5$ 184A1 cells were allowed to establish themselves overnight in MEGM, which was then removed and replaced with RPMI 1640 for 2 days. The conditioned medium was removed from each well and transferred to new wells with MCF-7 cells and a scattering assay was done. For the laminin 332 dose-response determination, the scattering assay was done using duplicate wells of each concentration of laminin 332, with concentrations ranging from 1 to 100 μg/mL, or an equal volume of column buffer [0.15 mol/L NaCl and 50 mmol/L Tris-HCl (pH 7.4)] as a control.

**Time-Course Assay**

MCF-7 cells seeded in 96-well plates at 3,000 per well were allowed to adhere to the wells for 2 h, after which time 5% 25× concentrated 184A1 conditioned medium was added to the half the wells, and an equal volume of RPMI 1640 was added to an equal number of wells. At the time of conditioned medium or RPMI 1640 addition, medium was removed from duplicate wells of cells and fixed for 1 min with 70% ethanol. Ethanol was removed and the plates were returned to the incubator, and the process of medium removal and ethanol fixation was repeated in additional wells at several time points up to 96 h. Cells were stained and scattering quantified in two random clusters of each well.

**Boyden Chamber Assay**

In wells of a 24-well tissue culture plate, 10^5 MCF-7 cells were placed on one side of a Millicell polycarbonate membrane (Millipore) with pores measuring 12 μm in diameter. The total volume of each well was 1 mL culture medium with either 184A1 conditioned medium or an equal volume of RPMI 1640 addition, medium was removed from duplicate wells of cells and fixed for 1 min with 70% ethanol. Ethanol was removed and the plates were returned to the incubator, and the process of medium removal and ethanol fixation was repeated in additional wells at several time points up to 96 h. Cells were stained and scattering quantified in two random clusters of each well.

**Purification of the Motility Factor**

Concentrated 184A1 conditioned medium was passed over a Sephacryl 200 HR gel filtration column eluted with 0.15 mol/L NaCl and 10 mmol/L Tris-HCl (pH 7.4; TBS), and fractions were evaluated for their ability to scatter MCF-7 cells. Pooled fractions with the greatest activity were added to a concanavalin A agarose (Vector Laboratories) column and eluted with 0.15 mol/L NaCl, 10 mmol/L Tris-HCl, and 100 mmol/L α-methyl mannoside. The eluted active fractions were placed on a reactive blue 4 agarose affinity chromatography column, which was eluted with 0.5 mol/L NaCl and 50 mmol/L Tris-HCl. Active fractions concentrated with Centricon 30 filters (Millipore) and boiled in reducing Laemmli buffer underwent SDS-PAGE in a 10% gel, which was then washed with distilled water and stained with GelCode blue (Pierce). Bands cut from the gel were sent to Protana, where they were digested with trypsin and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectroscopy (73). The molecular weights of the peptide fragments were used to predict the identity of the protein in the band.

For subsequent experiments requiring greater amounts of laminin 332, 1 L 184A1 conditioned MEGM was passed directly over a reactive blue column overnight, and active fractions were eluted with a gradient of 0.15 to 0.75 mol/L NaCl and 50 mmol/L Tris (pH 7.4). The volume was reduced to 4 mL by ultrafiltration and passed over a Sephacryl 400 HR column. Fractions were assayed for scattering activity, and $A_{280}$, and tested for purity by SDS-PAGE. Active fractions with only laminin 332 on Coomassie blue-stained gels were pooled and concentrated, and the final concentration was determined by $A_{280}$ against a bovine albumin standard curve. Typical yields using this procedure were 3 to 5 mg laminin 332.

**Western Blot**

Crude concentrated 184A1 conditioned medium was boiled in a reducing Laemmli buffer and applied to an 8% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and incubated with rabbit polyclonal antilaminin 332 antibody 421 diluted 2,000 times in blotto buffer. Antibody 421, kindly provided by Dr. Kim Yancey has been previously characterized (35). After application of a biotinylated anti-rabbit secondary antibody diluted 10,000 times, the bands were developed with the Pico SuperSignal West chemiluminescent detection system (Pierce) according to the manufacture’s instructions and visualized by exposure to Kodak XAR film.

**Neutralizing Antibody Assay**

Incubation of 50 μL of $5 \times$ 184A1 conditioned medium in wells of a 96-well plate for 4 h at 4°C allowed binding of the motility-inducing activity of the conditioned medium to the bottom of each well. Medium was removed, the cells were rinsed with PBS, and either 100 μg/mL anti-laminin 5 blocking antibody P3H9 (Chemicon) or an equal concentration of purified mouse IgG (Chemicon) was added each to duplicate wells. After 4 h at 4°C, the antibody was removed, the wells were rinsed, and 3,000 MCF-7 cells in growth medium were added. After an overnight incubation, scattering assays were done.
To examine the effect of inhibition of the laminin 332 receptor on motility, scattering assays were done in 96-well plate with 3,000 MCF-7 cells in 100 μL in the presence of 16.7% concentrated conditioned medium, and concentrations of 0.1, 0.5, 1, 5, and 10 μg/mL of mouse IgG1, anti-α3 integrin (clone P1B5; Chemicon), or anti-β3 integrin (clone 6SC; Chemicon). To rule out inhibition of scattering as a result of blocking of cells to the tissue culture plastic, the experiment was repeated with 100 ng/mL TNF, kindly provided by Genentech, and the same concentrations of immunoglobulins. The results were expressed as fraction of motile cells.

**Invasion Assay**

A potential marker of tumor aggressiveness is the ability of the malignant cells to invade through basement membrane material in vitro (74). For this reason, the role of the mammary epithelial motility factor in tumor invasion was investigated. Invasion assays were done in the same manner as the modified Boyden chamber assays, except that the membrane of the 12 mm Millicel inserts were coated with 20 μL reconstituted Engelbreth-Holm-Swarm basement membrane material (Matrigel; Collaborative BioSciences) diluted with an equal volume of 10× concentrated 184A1 conditioned medium or RPMI 1640 opposite the cellson the bottom of the membrane. In half of the wells with the conditioned medium in the lower chamber, 5 μg/mL of either anti-α3 integrin neutralizing antibody or mouse IgG was added. After overnight incubation, the cells and Matrigel were removed from the upper surface, and the cells migrating through the membrane were stained and counted as for the Boyden chamber assay. For the laminin 332 invasion assay, the Matrigel on the membrane had a final concentration of 100 μg/mL laminin 332, and the concentration of laminin 332 in the lower chamber was 50 μg/mL. For the negative control, the same volume of TBS column buffer was used in place of the laminin 332. As in the previous experiment, 103 MCF-7 cells were placed in the upper chamber and the migration was measured as described above.

**Breast Carcinoma Cases**

The surgical pathology archives of the University of California-Irvine Medical Center, the University of California-San Diego Medical Center, and the City of Hope Medical Center were searched for DCIS and metaplastic breast carcinomas, carcinomas, spindle cell malignancies, and squamous carcinomas. Spindle cell tumors that displayed cytokeratin staining, a clear epithelial component, or DCIS were categorized as metaplastic carcinomas for this study (75). Cases were included in the study if diagnostic criteria were met and sufficient material was available in the paraffin blocks for study. The carcinoma component was graded using the method of Elston and Ellis (76). Tumor size and lymph node status were obtained from the surgical pathology reports of the cases. The study was approved by the institutional review boards of the University of California-Irvine, the University of California-San Diego, and the City of Hope.

**Immunohistochemistry**

Sections of breast surgical specimens with a diagnosis of either DCIS or metaplastic carcinoma alone were stained for laminin 332. Each 4-μm-thick section on capillary gap slides was deparaffinized with Histoclear (National Diagnostics). Sections were then rehydrated through decreasing concentrations of isopropanol alcohol. Sections for immunoperoxidase staining were steam pretreated for 20 min in Antigen Retrieval Citra Buffer (Biogenex). After blockage of endogenous peroxidase with 3% hydrogen peroxide, avidin-biotin complex immunoperoxidase reactions were done using the anti-kalinin B1 monoclonal antibody (Transduction Laboratories), which reacts with the β3 laminin chain, diluted 1:250 (1 μg/mL) in PBS. Selected slides were stained with anti-laminin (Chemicon), which is specific for the γ2 chain, at a dilution of 1:20 (50 μg/mL). Staining for α3 integrin was done using clone P1B5 (Chemicon) diluted 1:50 (20 μg/mL). The reactions were followed by a biotinylated goat anti-mouse IgG secondary antibody and then an avidin-biotin peroxidase complex. The chromogen was diaminobenzidine for all reactions. Cells were counterstained with hematoxylin. Negative controls were done in the same fashion, except that the primary antibody was substituted with mouse immunoglobulin. A section of normal breast provided the positive controls.

After staining, the sections were examined by light microscopy by a surgical pathologist with experience in diagnostic breast pathology. On the sections, the laminin 332 staining of ducts with DCIS was noted. Ducts involved by DCIS were determined to exhibit a positive reaction if the majority of myoepithelial cells surrounding the DCIS stained with the laminin 332 antibody. The percentage of ducts exhibiting a positive reaction was determined for each case. For invasive carcinomas, the percentage of malignant cells exhibiting cytoplasmic staining for laminin 332 was estimated. For metaplastic carcinomas, the percentage of cells staining and the intensity of staining were estimated independently by two pathologists. Discrepancies were resolved by examining the cases together with a double-headed microscope. The percentage of moderate to strongly staining cells was assessed, and staining was scored based on the following percentages of positive cells: 0 (no staining to <5% of cells staining), 1+ (5-29% of cells staining), 2+ (30-69% of cells staining), and 3 (70-100% of cells staining). Statistical significance of differences in staining between groups of tumors was determined using Student’s t test.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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**References**


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Molecular Cancer Research

Motility Induction in Breast Carcinoma by Mammary Epithelial Laminin 332 (Laminin 5)

Philip M. Carpenter, Anh V. Dao, Zahida S. Arain, et al.

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