Altered Microenvironment Promotes Progression of Preinvasive Breast Cancer: Myoepithelial Expression of αvβ6 Integrin in DCIS Identifies High-risk Patients and Predicts Recurrence

Michael D. Allen1, Gareth J. Thomas3, Sarah Clark1, Marwa M. Dawoud1, Sabarinath Vallath1, Sarah J. Payne1, Jennifer J. Gomm1, Sally A. Dreger1, Sarah Dickinson1, Dylan R. Edwards4, Caroline J. Pennington4, Ivana Sestak2, Jack Cuzick2, John F. Marshall1, Ian R. Hart1, and J. Louise Jones1

The London School of Medicine and Dentistry, London,3Cancer Research Pathology Group, Southampton General Hospital, Southampton; and UK Clinical Centre, Somers Cancer Research Building, Experimental Vane Science Centre;2Wolfson Institute of Preventive Medicine, Barts and lence, Centre for Tumour Biology, Queen Mary University of London, John V./C211

doi: 10.1158/1078-0432.CCR-13-1504
Fax: 44 0 20 7882 3884. E-mail: l.j.jones@qmul.ac.uk
London EC1M 6BQ, United Kingdom. Phone: 44 0 20 7882 3577; University of London John Vane Science Centre, Charterhouse Square, Corresponding Author:

Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Abstract

Purpose: This study investigated the functional and clinical significance of integrin αvβ6 upregulation in myoepithelial cells of ductal carcinoma in situ (DCIS).

Experimental Design: Archival samples of DCIS and DCIS with associated invasion (n = 532) were analyzed for expression of αvβ6 by immunohistochemistry and ability to predict recurrence and progression assessed in an independent, unique cohort of DCIS cases with long-term follow-up. Primary myoepithelial cells and myoepithelial cell lines, with and without αvβ6 expression, were used to measure the effect of αvβ6 on growth and invasion of tumor cell lines in vitro and in a xenograft mouse model. Involvement of TGFβ signaling was established using mink lung epithelial cell (MLEC) assay and antibody inhibition, and expression and activation of matrix metalloproteinase (MMP)-9 established by Real Time-PCR and zymography.

Results: Expression of αvβ6 is significantly associated with progression to invasive cancer (P < 0.006) and with recurrence over a median follow-up of 114 months in a series of matched DCIS cases treated with local excision. We show that expression of αvβ6 drives myoepithelial cells to promote tumor cell invasion in vitro and enhances mammary tumor growth in vivo. The tumor-promoting effect of αvβ6-positive myoepithelial cells is dependent on TGFβ-driven upregulation of MMP9 and can be abrogated by inhibiting this pathway.

Conclusion: These findings indicate that altered myoepithelial cells in DCIS predict disease progression and recurrence and show that upregulation of αvβ6 on myoepithelial cells generates a tumor promoter function through TGFβ upregulation of MMP-9. These data suggest that expression of αvβ6 may be used to stratify patients with DCIS. Clin Cancer Res; 20(2); 344–57. ©2013 AACR.

Introduction

The majority of invasive breast cancers arise from the precursor lesion ductal carcinoma in situ (DCIS; ref. 1), whereby neoplastic cells proliferate and fill the breast ductal tree but are separated from the surrounding stroma by the intact myoepithelial–basement membrane wall of the ducts (Fig. 1A). At some point during progression, the tumor cells penetrate the myoepithelial–basement membrane interface and move into the interstitial matrix becoming an established invasive carcinoma with metastatic potential (Fig. 1A). However, DCIS does not invariably progress to invasive breast cancer: an early study of untreated low-grade DCIS indicated that 39% (11 of 28 patients) progressed to invasive breast cancer after a median follow-up of 31 years (2). High-grade DCIS is considered to progress more rapidly to invasive disease, but overall it is estimated that between one-third and half of DCIS diagnosed will progress to invasive breast cancer during a woman’s lifetime (3). The frequency of DCIS has increased dramatically with the widespread use of mammographic screening, accounting now for 1 in 4 breast cancer diagnoses (3), contributing to recent concerns surrounding overdiagnosis and overtreatment within the breast screening setting (4, 5). Indeed, the need to understand more about the biologic and molecular mechanisms underlying progression of DCIS to invasive disease and to generate robust risk stratification methods for...
Altered Myoepithelial Cells in DCIS

Translational Relevance

Ductal carcinoma in situ (DCIS) is the main precursor of invasive breast cancer and now accounts for 25% to 30% of all breast cancers detected; however, only half will progress to invasive disease during a woman’s lifetime. Currently, there are no robust markers to distinguish between those that will progress and those that will not. In this study, we show that the tumor-suppressive myoepithelial cells are altered in a subset of DCIS with upregulation of the integrin αvβ6, which is almost universal in DCIS that is associated with an invasive component, and the presence of αvβ6 is significantly associated with recurrence and progression. In vitro studies show that αvβ6-positive myoepithelial cells promote tumor invasion through a TGFβ- and MMP9-dependent mechanism. This suggests that detection of αvβ6 on myoepithelial cells may be of prognostic importance and provide new therapeutic avenues.

Materials and Methods

Human tissue specimens

Clinical samples were obtained from surgical specimens from patients undergoing breast surgery between 2004 and 2009 at Barts Health NHS Trust London. Further details are provided in the Supplementary Methods.

The study was conducted following patient consent and approval from the local research ethics committee (ref: 05/Q0403/199 and 09/H075/39). Further analysis was conducted on a series of cases derived from the UK-DCIS study (22, 23) to determine relationship between αvβ6 and progression/recurrence. The UK/ANZ DCIS trial was a randomized 2 × 2 factorial trial recruiting women between May 1990 and August 1998. Inclusion criteria were diagnosis of DCIS, undergoing breast conserving surgery, and with confirmed clear margins. Patients were randomized to receive radiotherapy, tamoxifen, or both (22). Clinicopathologic details are provided in Supplementary Methods.

Immunohistochemistry

Sections were dewaxed in xylene and antigen retrieved in pepsin solution followed by incubation with 0.5 mg/mL αvβ6 mouse monoclonal antibody (6.2G Stromedix; refs. 24, 25), MMP9 (My Biosource, MBS 240189), p63 (Dako, M7247), SMA (Dako, M0851), or CK5/6 (Dako, M7237) overnight. Sections subsequently were incubated with rabbit anti-mouse biotinylated F(ab′)2 for 30 minutes, developed using ABC reagent and superDAB (Dako), and then counterstained with hematoxylin. Details of scoring method are given in Supplementary Methods.

Cell lines and tissue culture conditions

T47D, MCF-7, MDA-MB-231, and HFFF2 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) with 10% fetal calf serum (FCS) and 2 mmol/L glutamine, AM12 cells transfected with pBABE puro β6 were maintained in DMEM with 10% FCS and 2 mmol/L glutamine with puromycin (1 μg/mL, Sigma). Mink lung epithelial cells (MLEC; a kind gift from D. Rifkin, New York University) were maintained in DMEM 10% FCS and 2 mmol/L glutamine with puromycin (1 μg/mL). Primary myoepithelial cells were isolated as described previously (26). An immortalized myoepithelial cell line (1089) (provided by M. O’Hare and P. Jat, Institute of Neurology, UCL) was generated from normal myoepithelial cells isolated from reduction mammaplasty material (27). Cells were sorted using integrin β4–labeled sheep anti-mouse magnetic beads (Invitrogen), to purify the myoepithelial cell population (1089) and maintained in Ham’s/F12 media with 10% FCS, 2 mmol/L glutamine, hydrocortisone (1 μg/mL), EGF (10 ng/mL), and insulin (5 μg/mL). All cell lines were verified by STR profiling (LGC Standards, tracking number 710081047). The 1089 cell line showed no similarity to any other cell line in the ATCC database (LGC Standards, tracking number U10010392). Frozen stocks were banked, and cell lines were grown for no longer than 6 months then...
discarded and fresh cells were then recovered from liquid nitrogen. Transient transfection of primary myoepithelial cells

Primary myoepithelial cells were recovered from liquid nitrogen and plated onto a 6-well plate precoated with collagen (10 mg/mL) at a density of $2 \times 10^4$ per well. Cells were transfected 72 hours later using the standard JetPrime protocol (PolyPlus) and 2 μg of integrin β6 pcDNA1 neo (Addgene plasmid 13580; ref. 28) or pcDNA1 empty vector. After 24 hours, media were changed for serum-free Ham’s/F12 and conditioned for 48 hours.

Stable expression of β6 in 1089 myoepithelial cells

Conditioned medium containing retrovirus from AM12/pBABE-β6 or AM12/pBABE-Puro cells, was incubated with 1089 cells (29). β6-positive 1089 and puromycin control cells were selected using puromycin (1 μg/mL, Sigma). Control, puromycin-resistant 1089 cells were designated normal (N-) 1089; β6-transduced cells were further enriched using integrin αvβ6-labeled anti-mouse IgG magnetic beads (Invitrogen) and designated β6-1089.

Immunofluorescence

A total of $2 \times 10^4$ cells were plated onto glass coverslips and grown for 48 hours before fixation with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS before staining. All antibodies were diluted in 20% normal rabbit serum in PBS, except for the αvβ6 antibody (MAB2077Z, Clone 10D5; Millipore), which was diluted in 0.1% bovine serum albumin (BSA) in DMEM. Slides then were stained with a secondary rabbit anti-mouse

Figure 1. Characterization of normal and αvβ6-overexpressing myoepithelial cell line and primary cells. A, normal breast duct comprises an inner luminal epithelial cell layer surrounded by a myoepithelial cell layer (stained for CK5/6), separating the epithelial compartment from the surrounding duct. In DCIS, the lumen of the duct becomes filled with neoplastic cells but still surrounded by an intact myoepithelial cell layer. With progression to invasive cancer, the tumor cells breach the myoepithelial cell layer (arrow) and invade the surrounding stromal compartment. B, normal breast ducts, ducts showing epithelial hyperplasia and examples of low-grade and high-grade DCIS show an intact myoepithelial cell layer, as shown by smooth muscle actin (SMA) immunoreactivity. No staining for αvβ6 is seen in the normal or hyperplastic ducts or in this example of low-grade DCIS, but myoepithelial expression of αvβ6 is seen in the duct showing high-grade DCIS. Magnification, ×100 and ×400. Scale bar, 100 and 20 μm, respectively. C, immunofluorescent staining montage; N-1089, β6-1089, and primary myoepithelial cells stained for a panel of markers identifying myoepithelial cells: Integrin β4 (β4), P-cadherin (P-Cad), CK17, and desmoglein 3 (Dsg3). All cell types exhibited positive staining for these markers; however, staining for αvβ6 was only detectable in β6-1089. These images are representative of at least 3 different experiments. Magnification ×630. Scale bar, 20 μm. D, flow cytometric profile of N-1089 and β6-1089 stained for the αvβ6 integrin (Millipore, clone 10D5), the dotted line indicates N-1089 (mean fluorescence intensity (MFI) = 3.23), and the dashed line indicates β6-1089 (MFI = 40). The black line indicates the IgG isotype control staining (MFI = 3.48). These data are representative of at least 3 independent experiments. E, Western blotting: the upper band shows expression of integrin αvβ6 (Santa Cruz, clone I098, 106 kDa) in β6-1089 and N-1089 cell lines, markedly higher levels of αvβ6 were detected in the β6-1089 lane. The lower band shows HSC70 (Santa Cruz, 70 kDa) to indicate equal loading. Representative of 3 independent Western blots is shown.
488 (Invitrogen, A11059) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were viewed on a Zeiss LSM 510 Meta microscope.

Flow cytometry
A total of $3 \times 10^5$ cells were incubated with primary antibody diluted in 0.1% BSA in PBS, except for the anti-\(\alpha\)v\(\beta\)6 antibody (10D5), which was diluted in 0.1% BSA in DMEM. Cells subsequently were stained with secondary rabbit anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Dako, F0313) before reading on a Flow Cytometer (BD LSR II).

Western blotting
Cells were trypsinized, centrifuged at 1,200 rpm, re-suspended in radioimmunoprecipitation (RIPA) lysis buffer (Millipore) with "Complete" protease inhibitors (Roche). Protein concentrations were determined using the DC Protein Assay (Bio-Rad), and 50 \(\mu\)g of protein was loaded on to an 8% Tris/Glycine gel, electrophoresed and transferred on to nitrocellulose membranes that were blotted with \(\alpha\)v\(\beta\)6 antibody (1:1,000, SC-6632, Santa Cruz), \(\beta\)-actin (1:10,000, Santa Cruz, SC47778), or HSC70 (1:10,000, SC-53935, Santa Cruz,) and donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary (1:2,000, Dako) or goat anti-mouse HRP-conjugated secondary (1:2,000, Dako) diluted in 5% milk in TBS/T.

TGF\(\beta\) quantitation assay
MLECs transfected with a PAI-1 promoter fused to a luciferase reporter gene were used to determine the amount of active TGF\(\beta\) (30). Briefly, 4 \(\times\) \(10^5\) MLECs were seeded in a 96-well plate and incubated overnight then serum starved for 4 hours before 4 \(\times\) 10^4 test cells were plated on top of the MLECs and cocultured for 16 hours. Medium was removed, lysis buffer (Promega) added, and the plate incubated at \(-80^\circ\)C for 1 hour before thawing at room temperature. Cells were scraped to ensure lysis and the luciferase substrate added according to manufacturer’s instructions (Promega). Luminescence was recorded on a Victor microplate reader.

Adhesion to latency-associated peptide
Latency-associated peptide (LAP, Sigma) was diluted to 0.5 \(\mu\)g/mL, aliquots were added to the wells of a 96-well plate and incubated for 1 hour at room temperature. A total of 2 \(\times\) 10^4 cells were incubated with IgG isotype control antibody or \(\alpha\)v\(\beta\)6-blocking antibody (10D5) at 10 \(\mu\)g/mL in serum-free media (SFM) for 20 minutes at 4°C before plating onto LAP. Cells were incubated at 37°C for 45 minutes before fixing with 30% methanol and staining with 0.1% crystal violet. Stained cells were dissolved with 30% acetic acid and absorbance read at 550 nm.

Migration assays
Twenty-four-well Transwells (8-\(\mu\)m pore, BD Bioscience) were coated on the underside with 0.5 \(\mu\)g/mL LAP, for 1 hour at room temperature, and then placed in a 24-well plate with DMEM, lacking serum, in the lower chamber. A total of 3 \(\times\) \(10^4\) cells were incubated with either an IgG isotype antibody or an \(\alpha\)v\(\beta\)6-blocking antibody (10D5) diluted in SFM (20 minutes at 4°C). Cells then were placed in the upper chamber and incubated for 24 hours. Migrating cells were quantified by applying trypsin to the underside of the Transwell and counting the detached cells on a CASY counter.

Invasion assays
Conditioned medium was generated from N-1089 or \(\beta\)-1089 by culturing in serum-free Ham’s/F12 for 48 hours and was stored at \(-80^\circ\)C. The upper surface of 24-well Transwell was coated with 70 \(\mu\)L growth factor-reduced Matrigel (diluted 1:3 in Ham’s/F12 without serum) and incubated for 1 hour at room temperature. Five hundred microliters conditioned medium from either N-1089 or \(\beta\)-1089 was then placed in the lower well. For inhibitor experiments, either dimethyl sulfoxide (DMSO) or MMP9 inhibitor (Millipore, 50 nmol/L, 444278) was added to conditioned media. Two hundred microliters of 3 \(\times\) \(10^4\) tumor cells was added to each well and incubated for 24 to 48 hours. Invading cells were quantified by applying trypsin to the bottom of the Transwell and counting the detached cells on a CASY counter.

Organotypic invasion assays
The skin organotypic method developed by Fusenig and colleagues (31), was adapted by Nystrom and colleagues (32), and further modified. Matrigel/collagen gels were prepared as follows: 3.5 volumes of collagen type 1 (BD Bioscience) and 3.5 volumes of Matrigel were mixed on ice to achieve a 1:1 ratio. The gel was polymerized at 37°C for 30 minutes. The upper surface of 24-well plates was coated with 1 mL of DMEM, lacking serum, in the lower chamber. A total of 10^4 cells were incubated with either an IgG isotype antibody or an \(\alpha\)v\(\beta\)6-blocking antibody (10D5) diluted in SFM. Cells then were placed in the upper chamber and incubated for 24 hours. Migrating cells were quantified by applying trypsin to the underside of the Transwell and counting the detached cells on a CASY counter.

Mice
Female 6-week-old CB17 SCID mice were purchased from Harlan and were maintained in IVC cages in a pathogen-free, temperature-controlled, 12-hour light and dark cycle environment. All animals were used in accordance...
with United Kingdom Home Office regulations and ethical approval by the local ethics committee. Tumor volumes based on caliper measurements were calculated by the modified ellipsoidal formula: tumor volume = 1/2(length × width²) (33). Further information on the mouse experiments are in the Supplementary Methods.

**Zymography**

Cells (1 × 10⁵) were preincubated with IgG antibody, β6-blocking antibody (10 µg/mL, 10D5), or TGFβRII-blocking antibody (10 µg/mL, AF-241-NA, R&D Systems) in SFM before they were plated on coverslips coated with fibronectin (1 µg/mL) or LAP (0.5 µg/mL). Media were collected after 24 hours, and MMP9 activity was analyzed using SDS-PAGE substrate gels. Gelatin (Bloom 300, Sigma) was added to a 10% acrylamide separating gel at a final concentration of 1 mg/mL. The intensity of the bands was measured by densitometric analysis. Direct comparisons between separate gels were not made, as the intensity of background staining was variable. Experiments were repeated a minimum of 3 times in triplicate. Further information on the zymogram protocol is in the Supplementary Methods.

**Real-time PCR**

RNA was isolated from N-1089 and β6-1089 cell pellets using a kit (Qiagen, 74104). Analysis was conducted on RNA from 3 separate experiments. TaqMan real-time PCR for MMP9 (Invitrogen, Hs00234579_m1) was carried out as previously described (34).

**Statistical analysis**

We determined statistical significance of the differences between experimental groups by the 2-tailed Student t test with PRISM software (GraphPad Software). P < 0.05 was considered significant. The relationship between staining patterns and DCIS grade, between MMP9 and αvβ6, and between αvβ6 and outcome was analyzed using Pearson χ² test using SPSS software (SPSS Inc.) with P < 0.05 considered as significant. Cases from UK-DCIS were matched on treatment received, age and time of follow-up and analyzed by logistical regression, adjusted and unadjusted for tumor grade and size. Animal studies were assessed for statistical significance between 2 medians by Mann–Whitney U test.

**Results**

**Myoepithelial expression of αvβ6 in DCIS is associated with progression to invasive cancer and predicts disease recurrence**

A total of 583 clinical breast samples comprising normal breast tissue, epithelial hyperplasia of usual type, "pure" DCIS and DCIS with invasion were analyzed for myoepithelial-associated expression of αvβ6. This revealed no significant staining for αvβ6 integrin in normal or hyperplastic cases (Fig. 1B), whereas 69% of high-grade and 52% of non–high-grade DCIS cases without invasion showed myoepithelial staining for αvβ6 (Table 1), with a significantly higher frequency of positivity in high-grade cases (P < 0.001). The frequency of myoepithelial cell αvβ6 positivity in DCIS with associated invasion is significantly higher than in pure DCIS (P < 0.001) with 96% of high-grade and 87% of non–high-grade DCIS exhibiting staining.

To assess the predictive value of αvβ6, we examined expression in a unique series of DCIS with long-term follow-up, derived from the UK/ANZ DCIS study, the endpoint assessed was the appearance of any new breast cancer, either DCIS or invasive cancer, as the numbers are insufficient to assess progression to invasion only. Cases and controls were matched for treatment protocol. The median follow-up was 114 months (22, 23). Staining was evaluated in 52 case–control pairs (details of cases shown in Supplementary Table S1), with cases scored using an H score to take account of the extent and intensity of myoepithelial-associated αvβ6 positivity, according to the criteria described by Allred and colleagues (35). Analysis showed a significant association between extent of αvβ6 expression (evaluated as an H score; ref. 35) and disease recurrence or progression (P = 0.006). This association was maintained following adjustment for DCIS grade and size (P = 0.02, H score; Table 2). All cases were confirmed as having clear margins as part of the inclusion criteria to the trial. We found that those cases positive for αvβ6 recurred more quickly than those lacking the integrin, with the αvβ6-positive cases showing a median time to recurrence of 2.3 years and those negative for αvβ6 having a median of 11.4 years to recurrence.

| Table 1. Expression of αvβ6 in pure DCIS and DCIS associated with invasive disease |
|---------------------------------|----------------|----------------|
|                                 | Positive | Negative | Total |
| Normal                          | 0       | 28       | 28    |
| Hyperplasia                     | 0       | 23       | 23    |
| Pure DCIS                       |          |          |       |
| High grade                      | 104 (69%) | 47 (31%) | 151   |
| Non–high grade                  | 44 (52%) | 40 (48%) | 87    |
| DCIS from invasive              |          |          |       |
| High grade                      | 189 (96%) | 8 (4%)   | 197   |
| Non–high grade                  | 84 (87%) | 13 (13%) | 97    |

NOTE: Formalin-fixed, paraffin-embedded sections of normal/benign tissue, pure DCIS, and DCIS with associated invasion were stained for αvβ6 integrin, and the absence or presence of myoepithelial cell staining was recorded, the number and percentage of cases in each category is indicated. In 189 of 197 (96%) of high-grade DCIS with invasive cancer cases, the myoepithelial cell compartment stained positively for αvβ6, whereas 104 of 157 (62%) of high-grade pure DCIS cases exhibit αvβ6 expression. There is no detectable staining of αvβ6 in normal or hyperplastic breast tissue.
LAP, which is part of the trimeric complex that forms...
Figure 2. $\alpha v\beta 6$ functional assays in the $\beta 6-1089$ cell line using blocking antibodies. A, adhesion assay; $N-1089$ and $\beta 6-1089$ cells were incubated with either an IgG isotype antibody (IgG) or an anti-$\alpha v\beta 6$ antibody (10D5) then plated onto TGF-$\beta$ LAP (0.5 $\mu$g/mL), PDL (1 $\mu$g/mL), or BSA (0.1%) for 45 minutes at 37°C. The cells were fixed and stained with crystal violet, which was solubilized and the absorbance read at 550 nm. The background binding to BSA was subtracted from the PDL and LAP values, and adhesion was normalized to IgG controls. (Continued on the following page.)
transfected with pcDNA1 or pcDNA1-β6 was placed in the lower well. After varying incubation times, invading cells were quantified (see Materials and Methods). MDA-MB-231 and MCF7 tumor cells showed a significant increase in invasion \( (P < 0.0001) \) in the presence of CM from primary myoepithelial cells expressing ovβ6 (Fig. 3A). The same assays were conducted with MDA-MB-231, MCF7, and T47D cell lines, using N-1089 or β6-1089 CM in the lower well. Invasion of MCF-7, T47D, and MDA-MB-231 cells was increased significantly \( (P = 0.0007, \ P = 0.02, \ P = 0.006, \text{respectively}) \) when exposed to CM from β6-1089 cells (Fig. 3B) compared with CM from N-1089 cells.

We have modified the organotypic skin model, developed by Fusieng and colleagues (31), to establish a more physiologically relevant model of breast cancer invasion. Fibroblasts (HFFF2) were embedded in a 1:1 collagen:Matrigel mixture to which the N-1089 or β6-1089 cells were plated with MDA-MB-231 cells layered on top. After 10 days, culture gels were processed and embedded in paraffin and sections stained with hematoxylin and eosin (H&E; Fig. 3C), p63 (to label myoepithelial cells), pan-Cytokeratin (to show myoepithelial cells and MDA-MB-231), and ovβ6 (Supplementary Fig. S3 and data not shown). The "invasion index" was determined as described in Materials and Methods. Gel analysis showed a significant increase in the invasion index of MDA-MB-231 cells when cocultured with β6-1089 compared with N-1089 cells (Fig. 3D, \( P < 0.0001 \)).

**Myoepithelial cells expressing ovβ6 promote breast tumor growth in vivo**

The mammary fat pads of female severe combined immunodeficient mice (SCID) mice were injected with 3 million cells comprising a 50%/50% mixture of the breast cancer cell line MDA-MB-231 and either N-1089 or β6-1089. Tumor measurements were taken 3 times a week and the subcutaneous volume determined. It was observed that the MDA-MB-231 cells mixed with β6-1089 cells formed established tumors more rapidly than did those tumor cells injected with N-1089 cells (data not shown). Those tumors produced in the presence of β6-1089 were significantly larger in volume (β6-1089 median; 174 mm\(^3\)) than those produced when mixed with N-1089 (N-1089 median; 75 mm\(^3\), \( P = 0.018 \); Fig. 3E and F). These data indicate that a distinct growth advantage was conferred upon the MDA-MB-231 mixed with β6-1089; possibly due to increased MMP secretion allowing more unrestricted growth and invasion.

\[ \text{ovβ6-expressing myoepithelial cells promote invasion in a TGFβ- and MMP9-dependent manner} \]

Previously, we showed that ovβ6 expression by keratinocytes results in upregulation of MMP9 and a concomitant increase in invasive capacity (29). Normal myoepithelial cells have been shown to downregulate MMP9 in breast cancer cell lines (17). Therefore, we investigated whether ovβ6 expression by myoepithelial cells modulates MMP9 levels. Conditioned media from N-1089, β6-1089, MDA-MB-231, and MCF-7 cells were generated over 24 hours, after which medium was collected and run on a gelatin zymogram. A clear band at 92 kDa, corresponding to MMP9, was identified in the CM from β6-1089 cells but not in CM from breast cancer cells (Fig. 4A). This was confirmed by quantitative PCR (Fig. 4B), which showed significant upregulation of MMP9 RNA in β6-1089 cells, versus their normal counterparts (\( P = 0.01 \)).

The β6-1089 cells were preincubated with a β6-blocking antibody (10D5) or IgG isotype control before plating and incubating for 24 hours. The resultant CM was collected and run on a gelatin zymogram. A clear band, corresponding with the recombinant MMP9, was detected from β6-1089 treated with IgG antibody, but not with β6-blocking antibody. To determine whether ovβ6 upregulated MMP9 through TGFβ activation, the β6-1089 cells were preincubated with a TGFβRII-blocking antibody before plating. The CM was run on a gelatin zymogram and a clear band corresponding with the recombinant MMP9 was detected from the β6-1089 cells treated with IgG antibody but not TGFβRII antibody-treated cells (Fig. 4C).

Transwell invasion assays were conducted using N-1089 or β6-1089 CM in the presence of an MMP9 inhibitor (50 nmol/L, Millipore, 444278) or DMSO control (Fig. 4D). MDA-MB-231 cell invasion was decreased significantly, to control levels, in the presence of β6-1089 CM plus MMP9 inhibitor (\( P = 0.005 \)), whereas the presence of the MMP9 inhibitor had no significant effect on invasion mediated by N-1089 CM. Thus, upregulation of myoepithelial cell ovβ6 expression caused a TGFβ-dependent increase in MMP9 secretion and a concomitant increase in tumor cell invasion.

The clinical relevance of these findings was verified by staining serial sections of pure DCIS and DCIS with invasion \( (n = 108) \) for MMP9 and ovβ6. Staining for MMP9 was heterogeneous, with some tumor cell...
Figure 3. Effect of myoepithelial cell expression of αvβ6 on in vitro and in vivo tumor cell behavior. A, Transwell invasion assay with primary myoepithelial cells: MCF-7 and MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in SFM). CM from primary myoepithelial cells transfected with pcDNA1 or pcDNA1-αvβ6 grown in SFM for 24 hours was placed in the lower wells. The plates were incubated for 48 hours (or 24 hours for MDA-MB-231), and the number of invading cells was quantified by counting the cells on the underside of the Transwell. Results are plotted as a ratio where the invasion of the tumor cells in the presence of CM from primary myoepithelial cells transfected with pcDNA1 is set as 100% and the invasion of tumors cells with CM from primary myoepithelial cells transfected with pcDNA1-αvβ6 CM is determined against this. Significantly higher invasion is seen in MCF-7 and MDA-MB-231 cells exposed to CM from primary myoepithelial cells transfected with pcDNA1-αvβ6 cells. Error bars show SD, the graph is a pool of at least 3 independent experiments. Statistical analysis by the Student t test, *P < 0.05 considered significant; **P < 0.01; ***P < 0.001. B, Transwell invasion assay with 1089 myoepithelial cell line: T47D, MCF-7 and MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in SFM). CM from N-1089 and β6-1089 cells grown in SFM for 48 hours was placed in the lower wells. The plates were incubated for 48 hours (or 24 hours for MDA-MB-231) and the number of invading cells was quantified by counting the cells on the underside of the Transwell. Results are plotted as a ratio where the invasion of the tumor cells in the presence of N-1089 CM is set as 100%, and the invasion of tumors cells with β6-1089 CM is determined against this. Significantly higher invasion is seen in MCF-7, T47D, and MDA-MB-231 cells exposed to CM from β6-1089 cells. Error bars show SD, the graph is a pool of at least 3 independent experiments. (Continued on the following page.)
cytoplasmic staining as well as stromal and inflammatory cell positivity, whereas those frequently was enhanced staining at the myoepithelial–basement membrane interface (Fig. 4E). Strong staining for αvβ6 was restricted to myoepithelial cells (Fig. 4E). Overall, 64% (n = 101) of DCIS ducts exhibited myoepithelial positivity for MMP9 whereas 59% (n = 93) showed αvβ6 staining, with a highly significant association expression of both molecules (P < 0.0001; Fig. 4F and Table 3).

Discussion

Breast cancer develops through defined clinical and pathologic stages, from hyperplasia to DCIS and finally invasive disease and metastasis. Since the introduction of mammographic screening programs, the incidence of detection of DCIS has increased 7-fold (3), and recent reviews suggest that only one-third to half of these cases will progress to invasive breast cancer during a woman’s lifetime (3, 4). Current management strategies treat all DCIS lesions as potentially invasive as there are no robust markers to distinguish between those cases with the potential to progress and those unlikely to. In DCIS, neoplastic cells fill and distend ducts but are separated from the surrounding stroma by an intact myoepithelial–basement membrane interface. Myoepithelial cells have been shown to express several tumor suppressor proteins (e.g., p63, p73, 14-3-3-σ, Maspin; refs. 43–45), consistent with their established tumor suppressor function (14). However, it has been shown that tumor-derived myoepithelial cells differ from normal and lose the ability to polarize luminal epithelial cells (20). Further studies have shown differences in gene expression between normal and DCIS-associated myoepithelial cells as increased lysoxidase (LOX; ref. 46) and neurofilament 1 (47). Allinen and colleagues examined the microenvironment of normal and tumor breast tissue and found that myoepithelial cells exhibited the most numerous changes in gene expression of all the microenvironmental cell types in DCIS (19), although the predictive and functional relevance of such changes has not been established.

In this study, we show that myoepithelial cells exhibit functionally relevant changes in their phenotype and behavior in a subset of DCIS cases, leading to acquisition of invasion-promoting properties. We have shown upregulation of integrin αvβ6 by myoepithelial cells in 52% to 69% of non–high-grade and high-grade DCIS, respectively, but it is present in almost 100% of DCIS associated with invasive disease. These data suggest that αvβ6 might be a marker of DCIS that is more likely to progress to invasive disease. It already has been established that high-grade DCIS progresses to invasion and local recurrence more quickly than low-grade (48, 49). To more directly address whether expression of αvβ6 can predict recurrence and progression, we assessed expression in a cohort of DCIS cases treated with local excision, confirmed margins free of disease and with long-term follow-up as part of the UK/ANZ DCIS trial (22), and showed a significant correlation between αvβ6 expression in myoepithelial cells and recurrence of breast cancer either as in situ or invasive disease, independent of patient age, disease grade, or extent. Furthermore, those with αvβ6 positivity developed recurrence more quickly than those lacking αvβ6, with median time to recurrence of 2.3 versus 11.4 years, respectively. This work supports the proposal that expression of αvβ6 in the DCIS-associated myoepithelial cells is a marker of DCIS cases more likely to progress to invasion and recurrence.

The integrin αvβ6 is restricted to epithelial cells and is usually expressed only in embryogenesis and wound healing in adult humans. αvβ6 primarily binds to the RGD motif of LAP of pro-TGF-β leading to activation of the protein. The downstream consequence of αvβ6 activation has so far been shown to lead to activation of MMPs (e.g., MMP3 and MMP9; refs. 50–52). Activation of MMPs plays an important role in the remodeling of the ECM and deregulation of these proteins in cancer leads to destruction of basement membrane that would otherwise constrain tumor cells from invading.

Previous studies have identified αvβ6 expression in invasive carcinomas, and these indicate that expression is associated with aggressive tumor behavior and reduced survival

(Continued.) Statistical analysis by the Student t test, P < 0.05 considered significant; *, <0.05; **, <0.01; ††, <0.001. C, organotypic assay for invasion; fibroblasts were mixed into collagen Matrigel (50:50), (i) N-1089 or (ii) β6-1089 cells were plated on top of the gel and allowed to attach before MDA-MB-231 cells were overlaid. Organotypics were then raised onto a metal grid and fed from below with media. These were cultured for 10 days, changing the media every 2 days; gels were then fixed in formal saline and embedded in paraffin. Five-micrometer serial sections were stained with H&E, p63, pan-cytokeratin, and αvβ6 (not shown). The p63- and αvβ6-positive cells remained on the gel surface. Magnification, ×100. Scale bar, 100 μm. D, invasion of MDA-MB-231 cells in the presence of either N-1089 or β6-1089 cells was quantitated by taking images of 3 random fields of view from triplicate gels and analyzing the number of invasive particles, the percentage area of the field the particles occupied and the depth the particles penetrated the gel (determined by 3 measurements per field of view using Image J (NIH). The product of the 3 values gave an overall “invasive index” and shows significantly more invasion by MDA-MB-231 in the presence of β6-1089 compared with N-1089. Error bars show SD, the graph is a pool of at least 3 independent experiments. Statistical analysis by the Student t test, P < 0.05 considered significant; ††, <0.001. E, MDA-MB-231 cells mixed with N-1089 (total cell number 3 × 10⁶ /mouse) were injected into the mammary fat pad of 10 SCID mice. A further 10 SCID mice were injected with MDA-MB-231 cells mixed with β6-1089 cells (total cell number 3 × 10⁶ /mouse). After 13 weeks, tumor measurements were made and the longest (L) and the shortest (W) were used to calculate the tumor volume using the standard formula: 1/2(L × W²). MDA-MB-231 cells injected with β6-1089 cells formed significantly larger tumors then those injected with N-1089 (P = 0.0185). Statistical analysis by Mann–Whitney U test, P < 0.05 considered significant; ††, <0.005. F, MDA-MB-231 cells mixed with N-1089 (total cell number 3 × 10⁶ /mouse) were injected into the mammary fat pad of 10 SCID mice. A further 10 SCID mice were injected with MDA-MB-231 cells mixed with β6-1089 cells (total cell number 3 × 10⁶ /mouse). After 13 weeks, tumor measurements were made and the longest (L) and the shortest (W) were used to calculate the tumor volume using the standard formula: 1/2(L × W²). MDA-MB-231 cells injected with β6-1089 cells (dashed and grey line) developed larger tumors more quickly than those injected with N-1089 (black lines). Thin lines show individual tumor sizes and thick lines show average tumor sizes.
mRNA. to a blocked by an antibody and migration to the a.

Figure 4. αvβ6 induces MMP9 production through TGFβ activation in j6-1089 cells. A, gelatin zymogram on CM from MDA-MB-231, MCF-7, j6-1089, N-1089, and T47D cells cultured for 24 hours. Medium was run on a 10% gelatin zymogram and incubated for 24 hours at 37°C. The gel was stained with Coomassie blue and washed; a band detected at 92 kDa indicates the source of the MMP9 is the j6-1089 cell line. rMMP9 = recombinant MMP9 (1 μg/mL). B, real-time PCR on RNA isolated from N-1089 or j6-1089 cells shows increased MMP9 RNA in j6-1089 samples. Data, from a pool of 3 separate experiments, show a significant increase in expression of MMP9 in j6-1089 cells relative to N-1089, which was set as 100%. Statistical analysis by the Student t test, P < 0.05 considered significant; *, <0.05. C, gelatin zymogram on CM from j6-1089 preincubated with either IgG isotype antibody, anti-αvβ6 antibody (10D5), or TGFβRII-blocking antibody, plated on TGF-β LAP (0.5 μg/mL), and cultured for 24 hours. Medium was run on a 10% gelatin zymogram and incubated for 24 hours at 37°C. The gel was stained with Coomassie blue and washed; a band was detected at 92 kDa with control antibody but not in the presence of blocking antibodies indicating the secretion of MMP9 from j6-1089 cells is dependent on activation of TGFβRII as well as binding of αvβ6; rMMP9 = recombinant MMP9 (100 μg/mL). D, Transwell invasion assay; MDA-MB-231 cells were plated in Transwells coated with Matrigel (diluted 1:3 in SFM), CM from N-1089 and j6-1089 cells, grown in SFM for 48 hours, were placed in the bottom wells with either DMSO or MMP9 inhibitor (50 nmol/L). Plates were incubated for 24 hours, and the number of invading cells quantitated by counting the cells on underside of the Transwell. Results are plotted as a ratio where the invasion of the tumor cells in the presence of N-1089 CM is set as 100% and the invasion of tumor cells with j6-1089 CM is determined against this. Significantly higher invasion is observed where MDA-MB-231s were exposed to CM from N-1089 or j6-1089 cells with DMSO, which was ablated when the MMP9 inhibitor was added to the j6-1089 CM. Error bars show SD, the graph is a pool of at least 3 independent experiments. Statistical analysis by the Student t test, P < 0.05 considered significant; *, <0.05; **, <0.01; ***, <0.001. E, myoepithelial cells exhibit coincident expression of MMP9 and αvβ6. Serial sections from 104 DCIS cases were stained and representative images are shown for (i) MMP9 and (ii) αvβ6. Staining for αvβ6 is limited to the myoepithelial cell layer in the DCIS duct and there is colocalized strong staining for MMP9. Top, taken at ×200 and bottom are the same section at ×400. F, graph shows the percentage of cases stained positive or negative for αvβ6 and MMP9 on serial sections, this was scored and the correlation was determined by Pearson χ² test, P < 0.05 considered significant; *, <0.05; **, <0.01; ***, <0.001.

(53–55). However, this is the first study to describe upregulation of αvβ6 in the nonmalignant microenvironment of a tumor.

We used both primary human myoepithelial cells and a myoepithelial cell line (N-1089) engineered to overexpress αvβ6 as a model of DCIS-associated myoepithelial cells. The functionality of αvβ6 was confirmed by enhanced attachment and migration to the αvβ6 ligand LAP, which is blocked by an αvβ6-blocking antibody (10D5) and siRNA to αvβ6. Furthermore, we show that αvβ6 activates a PAI-1 TGFβ luciferase reporter, which is also blocked by 10D5 and siRNA.

Using our model system in Transwell invasion assays of several breast cancer cell lines, we have shown that CM from the β6-1089 cell line promotes invasion of MDA-MB-231 (basal-like), T47D [estrogen receptor (ER+)] and MCF-7 (ER−) when compared with the model of normal myoepithelial cells (N-1089), with a similar activity shown by primary myoepithelial cells. The breast cancer cell lines studied represent a cross-section of different breast cancer cell types indicating the broad relevance of the effect of αvβ6-positive myoepithelial cells in breast cancer. We further show enhanced invasion in the presence of αvβ6-positive myoepithelial cells in a modified organotypic assay.
used previously in our laboratory (32). In this assay, MDA-MB-231 cells exhibited significantly increased invasion into a Matrigel:collagen:fibroblast mixture when cocultured with β6-1089 compared with N-1089 cells. To show that this effect was not simply an in vitro phenomenon, we injected the mammary fat pad of 20 SCID mice with a 50:50 mixture of MDA-MB-231 and either β6-1089 or N-1089. The tumors that formed in the β6-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Fig. 3F and e, respectively).

As αvβ6 activates TGFB-β in a localized manner and does not release it from the matrix, and therefore we postulated that this effect was not simply an in vitro phenomenon, we injected the mammary fat pad of 20 SCID mice with a 50:50 mixture of MDA-MB-231 and either β6-1089 or N-1089. The tumors that formed in the β6-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Fig. 3F and e, respectively).

To show that this effect was not simply an in vitro phenomenon, we injected the mammary fat pad of 20 SCID mice with a 50:50 mixture of MDA-MB-231 and either β6-1089 or N-1089. The tumors that formed in the β6-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Fig. 3F and e, respectively).

As αvβ6 activates TGFB-β in a localized manner and does not release it from the matrix, and therefore we postulated that this effect was not simply an in vitro phenomenon, we injected the mammary fat pad of 20 SCID mice with a 50:50 mixture of MDA-MB-231 and either β6-1089 or N-1089. The tumors that formed in the β6-1089 cohort grew with faster kinetics and the median tumor size was significantly increased compared with the N-1089 group after 13 weeks (Fig. 3F and e, respectively).
The authors thank Dr. Paul Weinreb (BioGen IDEC) and Dr. Sheila Violette (Stromedix) for supplying the 6.2G2 antibody; Prof. Dean Sheppard for providing the integrin pdCA1 neo via Addgene; and Prof. D. Rifkin for providing the MLEC TCG-β reporter cell line.

Grant Support
M.D. Allen was funded by the Breast Cancer Campaign.

References
Altered Microenvironment Promotes Progression of Preinvasive Breast Cancer: Myoepithelial Expression of αvβ6 Integrin in DCIS Identifies High-risk Patients and Predicts Recurrence


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1504

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/10/22/1078-0432.CCR-13-1504.DC1

Cited articles
This article cites 61 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/2/344.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/2/344.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/20/2/344.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.