β3 Integrin Expression Increases Breast Carcinoma Cell Responsiveness to the Malignancy-Enhancing Effects of Osteopontin

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Abstract

Osteopontin (OPN) is a secreted phosphoprotein that has been associated with malignancy of breast and other cancers. OPN binds to several cell surface integrins including αvβ3, αvβ5, and αvβ1. Although the relative contribution of these integrins to breast cancer cell malignancy is uncertain, correlative studies suggest that αvβ3 may be particularly associated with increased tumor aggressiveness. Previously, we reported that tumorigenic, nonmetastatic 21NT mammary carcinoma cells respond to OPN through αvβ3 and αvβ1 but not αvβ5. Here, we determined that 21NT cells lack β3 expression, and we asked whether expression of αvβ3 could enhance the ability of breast cancer cells to respond to the malignancy-promoting effects of OPN both in vitro and in vivo. 21NT cells stably transfected with β3 showed significantly increased adhesion, migration, and invasion to OPN in vitro compared with vector control. To determine if β3 could also enhance the response of breast epithelial cells to OPN in vivo, cells stably transfected with both β3 and OPN (NT/Oβ3) were injected into the mammary fat pad of female nude mice and primary tumor growth was assessed relative to controls. Mice injected with NT/Oβ3 cells demonstrated a significantly increased primary tumor take (75% of mice) compared with controls (0–12.5% of mice) as well as a decreased tumor doubling time and a decreased tumor latency period. These results suggest that increased expression of the αvβ3 integrin during breast cancer progression can make tumor cells more responsive to malignancy-promoting ligands such as OPN and result in increased tumor cell aggressiveness.

Introduction

Osteopontin (OPN) is a secreted glycoprotein that is overexpressed in a number of different carcinomas, including breast cancer (1, 2). OPN levels have been found to be elevated in primary tumors or blood of patients with breast cancer and in some cases have been reported to be associated with poor prognosis (3–7). OPN may also be a useful clinical marker for ovarian, colon, lung, liver, prostate, and perhaps other cancers (8–12).

Elevated OPN expression has also been associated with increased malignancy and metastasis in experimental models of breast cancer (13–16). Functional studies have shown that breast epithelial cells in culture can adhere to OPN (17), migrate toward OPN (18), and invade through basement membrane in response to OPN (15). Associated with these behavioral changes are OPN-mediated changes in gene expression, including increased expression of urokinase-type plasminogen activator as well as increased expression and activation of the hepatocyte growth factor receptor (Met) and the epidermal growth factor receptor (18–20). Cells of higher in vivo malignancy not only tend to express more OPN but also may be more responsive to OPN (15). OPN binding has been found to involve both integrin and nonintegrin receptors in different cell types (17, 18, 21–27). The primary integrin receptors for OPN are αvβ3, αvβ1, and αvβ5 (26, 27). Correlative evidence has shown that the αvβ3 integrin receptor appears to be preferentially used by more malignant breast epithelial cell lines in binding and migrating toward OPN or vitronectin (18, 20). However, it is not known whether experimentally altered levels of αvβ3 can lead to changes in cellular behavior in response to OPN.

Our previous studies (18) have shown that tumorigenic, nonmetastatic 21NT cells (29) differ from tumorigenic, metastatic MDA-MB-435 cells (30) in terms of the specific integrin receptors involved in their response to OPN. Although both cell lines respond to OPN with increased adhesion, migration, and invasion, blocking antibody studies show that 21NT cells respond to OPN via αvβ3 and αvβ5 integrins, whereas MDA-MB-435 cells preferentially use αvβ5 (18). Our goal in the present study was to determine if αvβ3 expression could confer on 21NT cells a more aggressive phenotype by altering their responsiveness to OPN. We first characterized the integrin expression profiles of the two cell lines by flow cytometry and found that both NT/C (control vector-transfected 21NT cells) and MDA-MB-435 cells express αvβ3 and αvβ1 integrins, but only MDA-MB-435 cells express αvβ5. To
determine whether increased α$_3$β$_3$ expression in 21NT cells can increase OPN responsiveness, we generated stable transfectants of 21NT cells that overexpress the β$_3$ integrin subunit (NT/C$\beta_3$). In vitro, the NT/C$\beta_3$ cells were found to adhere better, migrate better, and invade through basement membrane better in response to OPN than the corresponding vector controls. For the in vivo studies, we stably transfected 21NT cells to express both β$_3$ and OPN (NT/O$\beta_3$). After having verified high levels of $\beta_3$ and OPN in the cotransfectants, we assessed the ability of these cells to form primary mammary tumors relative to cells that express β$_3$ alone (NT/C$\beta_3$), OPN alone (NT/O), or the vector control (NT/C). Following mammary fat pad injection, mice injected with the NT/O$\beta_3$ cells demonstrated a significantly increased primary tumor take, a decreased tumor doubling time, and a decreased tumor latency period relative to controls. Our findings suggest that expression of the α$_3$β$_3$ integrin may alter how a tumor cell interacts with its environment, at least in part by increasing sensitivity to malignancy-enhancing ligands such as OPN.

Results

Metastatic MDA-MB-435 and Nonmetastatic 21NT Breast Carcinoma Cells Show Different Integrin Expression Patterns

Flow cytometry was used to test for surface expression of the α$_3$β$_3$, α$_3$β$_1$, and α$_3$β$_3$ integrins in MDA-MB-435 and NT/C cells. Both MDA-MB-435 and NT/C cells expressed α$_3$β$_3$ and α$_3$β$_1$ integrins (Fig. 1A). When both cell lines were also tested for β$_3$ integrin expression, MDA-MB-435 cells were found to have high levels of surface β$_3$, while NT/C cells were negative for β$_3$ (Fig. 1B). These results thus explain our previous findings (18) that the migration of 21NT cells to OPN was not reduced by blocking α$_3$β$_3$ due to their lack of expression of the β$_3$ subunit.

Transfection and Characterization of 21NT Cells Overexpressing the β$_3$ Integrin Subunit

Following determination that the NT/C cells did not express the β$_3$ integrin subunit, we stably transfected them with full-length human β$_3$ integrin cDNA or with the vector only as a control. Clonal and pooled cell populations were isolated and characterized for their β$_3$ protein and mRNA expression levels using flow cytometry and Northern analysis, respectively. Three NT/C$\beta_3$-transfected cell populations, representing a clone and two pools, were characterized in addition to a pooled population of control vector-transfected NT/C cells (Fig. 1C). Flow cytometry analysis demonstrated that the NT/C$\beta_3$ clone A4 cell population had high surface expression levels of β$_3$ (98% of cells), while NT/C$\beta_3$ pools A and C were more heterogeneous (as expected for pooled populations), with 62% and 80%, respectively, of cells expressing β$_3$ integrin. Consistent with the results shown in Fig. 1B, vector control NT/C cells showed undetectable levels of β$_3$ integrin (Fig. 1C). The transfected cells were also characterized for β$_3$ mRNA levels using Northern analysis (Fig. 2). The NT/C$\beta_3$ clone A4 and pools A and C were found to express high levels of β$_3$mRNA compared with the NT/C control transfected cells. These three β$_3$-transfected cell populations were used in the subsequent studies to assess the abilities of the cells to respond to OPN relative to NT/C vector control cells.

Expression of β$_3$ Integrin in 21NT Breast Carcinoma Cells Increases Cell Migration in Response to OPN

In transwell migration assays, all three of the NT/C$\beta_3$ cell populations showed significantly increased migration in response to OPN as compared with control vector-transfected cells ($P < 0.008$ for all; Fig. 4). For each cell line, migration in the presence of OPN was also significantly greater than that with BSA alone ($P < 0.04$ for all). We also observed that the NT/C$\beta_3$ cells showed some increase in basal migration even in the absence of exogenously added OPN. The NT/C$\beta_3$ cells not only migrated better to the undersurface of the membrane in response to OPN but also appeared to spread better over the undersurface of the membrane.

Expression of β$_3$ Integrin in 21NT Breast Carcinoma Cells Increases Cell Invasiveness in Response to OPN

In transwell invasion assays through Matrigel (Becton Dickinson, Bedford, MA), all three of the NT/C$\beta_3$ cell populations showed significantly increased invasion in response to OPN as compared with control vector-transfected cells ($P < 0.001$ for all; Fig. 5). For each cell line, invasion in the presence of OPN was significantly greater than that with BSA alone ($P < 0.004$ for all). In contrast to the migration assays, we did not observe an increase in basal invasion (i.e., in the absence of OPN) in the NT/C$\beta_3$ cell populations relative to vector control.

Expression of β$_3$ Integrin Enhances the Response of 21NT Breast Carcinoma Cells to OPN in Vivo and Results in Increased Tumorigenicity

To assess the effect of β$_3$ integrin expression on the
responsiveness of the 21NT cells to OPN in vivo, it was necessary to provide the \( \beta_3 \)-transfected cells with a source of OPN in the tumor microenvironment. We therefore generated 21NT stable transfectants, which overexpressed both \( \beta_3 \) and OPN (NT/O\( \beta_3 \)), and used these cells to determine if \( \beta_3 \) could enhance the response of breast epithelial cells to OPN in vivo relative to cells that express \( \beta_3 \) alone (NT/C\( \beta_3 \) pool A), OPN alone (NT/O), or the vector control (NT/C). The mRNA expression levels of \( \beta_3 \) and OPN in pooled populations of transfected cells were first confirmed by Northern blot analysis (Fig. 6). Relative to the NT/C vector control cell population, the NT/C\( \beta_3 \) and NT/O\( \beta_3 \) cell populations were found to express increased levels of \( \beta_3 \) mRNA (12.5- and 12.2-fold, respectively). In contrast, the NT/O cell population expressed very low levels of \( \beta_3 \) (2.7-fold increase relative to NT/C control). The NT/O and NT/O\( \beta_3 \) cell populations were confirmed to

![Flow cytometry analysis](https://example.com/flow_cytometry_analysis.png)

**FIGURE 1.** Flow cytometry analysis of the integrin expression profiles of MDA-MB-435, NT/C, and NT/C\( \beta_3 \) cells. Cells were incubated with anti-integrin antibodies, as indicated, or with nonspecific isotype control primary antibodies (blue), and surface expression of specific integrins was assessed by flow cytometry, as described in “Materials and Methods.” A. Surface expression of \( \alpha_v \( \beta_5 \) (yellow) and \( \beta_3 \) (green) by MDA-MB-435 and NT/C cells. B. Surface expression of \( \beta_3 \) (red) by MDA-MB-435 and NT/C cells. C. Surface expression of \( \beta_3 \) (red) by control vector NT/C cells and NT/C\( \beta_3 \) transfectants (\( \beta_3 \) clone A4, \( \beta_3 \) \( \beta_3 \) pool A, and \( \beta_3 \) pool C).
overexpress OPN mRNA (7.2- and 6.6-fold, respectively, relative to the NT/C vector control). The NT/C3 cell population expressed levels of OPN comparable with the NT/C control cells.

These cell populations were then injected into the mammary fat pad of female nude mice, and various aspects of primary tumor growth were assessed (Table 1). Primary mammary tumors were first observed between days 62 and 69 in five of eight mice injected with cells expressing both β3 and OPN (NT/ O3), and one additional mouse in this group developed a primary tumor by day 105. These mice (six of eight, 75%) were euthanized between days 107 and 167 based on tumor burden. In contrast, a primary tumor was observed in only one of eight (12.5%) mice in the NT/C group, and none of the mice injected with cells expressing β3 alone (NT/C33) or OPN alone (NT/O3) formed palpable primary tumors. Primary tumors, which formed from the NT/O33 cells, also demonstrated a decreased doubling time and a decreased latency period compared with vector control (Table 1). Of the mice in the NT/O33 group that formed primary tumors, 50% demonstrated lymphatic invasion at the site of the primary tumor and 33% demonstrated lymph node metastasis. No lymphatic involvement was observed in the control tumor (Table 1).

Discussion

Elevated levels of OPN in both tumor tissue and blood have been identified in patients with breast and other cancers and have been shown to be associated with poor prognosis (3–12, reviewed in 2, 31). Recent microarray gene expression studies have also implicated OPN in the progression of various tumor types (10, 12, 32, 33), and several other studies have demonstrated that OPN can contribute functionally to cancer progression (reviewed in 2, 31). Potential mechanisms of OPN-mediated malignancy include induction of expression and activity of proteases, such as urokinase-type plasminogen activator, as well as growth factor receptors, such as hepatocyte growth factor receptor (Met) and epidermal growth factor receptor, which are important in tumor motility, invasion, and metastasis (15, 18–20). Other possible mechanisms include protection from apoptosis or host immune-mediated destruction (34–37). The effects of OPN on cancer cells are thought to involve integrin signaling and cross-talk with growth factor receptors, such as Met, as well as interactions with other nonintegrin surface receptors such as CD44 (reviewed in 2, 21, 31, 38, 39).

OPN can bind to a number of cell surface integrins, including αvβ3, αvβ1, and αvβ5 (26, 27). High levels of the αvβ3 integrin combined with low levels of αvβ1 and αvβ3 have, in some cases, been associated with malignancy and progression in breast cancer, melanoma, and prostate cancer (28, 40–45). However, it has not previously been shown whether experimentally altered levels of cell surface integrin expression can lead to changes in cellular behavior in response to OPN. The current study provides, for the first time, direct experimental evidence that changing the pattern of integrin expression (in this case, expression of the αvβ3 integrin) can contribute functionally to increased malignancy of breast cancer cells in response to OPN both in vitro and in vivo.

We previously showed that tumorigenic, nonmetastatic 21NT cells and metastatic MDA-MB-435 human breast cancer cells both responded to exogenous OPN or transfected, endogenous OPN with increased aggressive behavior in vitro, including cell migration and invasion, indicating that OPN can contribute functionally to the malignant behavior of breast cancer cells (15, 18–20). These effects could be functionally blocked in MDA-MB-435 cells with antibodies to the αvβ3 integrin but not with antibodies to αvβ1 or αvβ5. Conversely, in 21NT cells, anti-αvβ3 antibodies had no effect, but invasion and migration were blocked by antibodies to αvβ1 or αvβ5 (18). These findings suggested that different patterns of integrin expression might determine how cells responded to a ligand such as OPN in the tumor microenvironment.

In the present study, we first characterized the integrin expression profile of 21NT cells and found that they were positive for surface expression of both αvβ3 and αvβ5 but were negative for expression of the αvβ1 integrin subunit both on the cell surface and at the RNA level. This finding provides an explanation for the failure of an anti-β3 blocking antibody to inhibit their migration or invasion in response to OPN, as previously reported (18). In contrast, MDA-MB-435 cells showed cell surface expression of all three integrins, although only αvβ3 and not anti-αvβ1 or anti-αvβ5 antibodies were effective at blocking their migration and invasion in response to OPN (18), indicating that these more aggressive cells preferentially use αvβ3 to respond to OPN. We then experimentally altered the integrin expression pattern of the 21NT cells by transfecting them to overexpress the β3 integrin subunit. Relative to the vector control transfecants, the β3-expressing 21NT cells were found to be more aggressive in terms of in vitro adhesion, migration, and invasion in response to OPN. Furthermore, in vivo studies demonstrated that mammary fat pad injection of 21NT cells, which expressed β3 in combination with OPN (NT/O3), resulted in an increased tumor take, a decreased tumor doubling time, and a decreased tumor latency period relative to controls. Parental 21NT cells typically take up 1 year to develop primary tumors and only in 10–20% of mice (29 and A.B. Tuck, unpublished data). Thus, the observation that the NT/O3 cells can form tumors in 75%
of mice as early as 62 days postinjection is a novel and significant finding. In addition, the primary tumors that do form from parental 21NT cells have never been observed to demonstrate either lymphatic invasion at the site of the primary tumor or lymph node metastasis. The observation that tumors, which develop from NT/O\(\beta_3\) cells, show lymph node invasion in three of eight mice and lymph node metastasis in two of eight mice therefore indicates that, in addition to affecting tumorigenicity, cooperative expression of both \(\alpha_v\beta_3\) and OPN in 21NT cells can also lead to increased tumor aggressiveness in terms of lymphatic involvement.

OPN present in tumor tissue can be produced both by tumor cells and by host infiltrating inflammatory cells (1, 5, 46), although it remains to be elucidated whether the specific functional consequences of OPN on tumor cell malignancy differ depending on the cellular source of OPN. However, it is clear that OPN produced by various cell types in the tumor microenvironment can influence cell behavior in either an autocrine or a paracrine manner. The model system described here indicates that the \(\alpha_v\beta_3\) integrin may be important for tumor cell response to both exogenously produced OPN (as demonstrated by the in vitro studies) and endogenously produced OPN (as demonstrated by the in vivo studies). The present study provides evidence that increased \(\beta_3\) integrin expression may contribute to breast cancer progression by making the cancer cells more responsive to the malignancy-enhancing effects of microenvironmental ligands such as OPN.

\[1\] A.B. Tuck, unpublished data.

**FIGURE 3.** Expression of the \(\beta_3\) integrin increases adhesion of NT/C cells to OPN. **A.** Adhesion of NT/C\(\beta_3\) and NT/C control cells to OPN (10 \(\mu\)g/ml) or BSA. Cells (1 \(\times\) 10\(^4\)) were added to a 96-well plate coated with either OPN or BSA, and the assay was carried out for 3 h at 37\(^\circ\)C. For antibody blocking experiments, cells were preincubated with a mouse monoclonal antibody against human \(\alpha_v\beta_3\) for 15 min at 37\(^\circ\)C before plating. Columns, mean number of cells from five counts of triplicate wells; bars, SEM. NT/C\(\beta_3\) cells showed significantly increased adhesion to OPN versus NT/C control cells (P < 0.002 for all). Adhesion of NT/C\(\beta_3\) cells but not NT/C cells could be significantly blocked by an anti-\(\alpha_v\beta_3\) antibody (P < 0.002 for all). **B.** Cell morphology of NT/C\(\beta_3\) clone A4 and NT/C control cells adhered to BSA, OPN, or OPN in the presence of anti-\(\alpha_v\beta_3\). NT/C\(\beta_3\) pools A and C showed similar morphology as the NT/C\(\beta_3\) clone A4 cell population (data not shown).
Materials and Methods

Cell Lines and Growth in Tissue Culture

The tumorigenic, nonmetastatic 21NT human mammary carcinoma cell line was used (a gift from Dr. Vimla Band, Dana-Farber Cancer Institute, Boston, MA; 29). 21NT cells were previously stably transfected with an unmodified pcDNA3 mammalian expression vector (NT/C cells) or with a full-length human OPN cDNA contained within the pcDNA3 vector (NT/O cells; 15). Transfected 21NT cells were grown in αHE medium with 10% fetal bovine serum, as previously described (15), with 200 μg/ml active G418 (Life Technologies, Inc., Burlington, Ontario) to maintain expression of the transfected plasmids. The highly metastatic MDA-MB-435 human breast carcinoma cell line (a gift from Dr. Janet Price, Anderson Cancer Center, Houston, TX; 30) was also used and was grown in αMEM medium supplemented with 10% fetal bovine serum.

Flow Cytometry

Primary antibodies used for flow cytometry were mouse antihuman antibodies directed against the α5β1 integrin (clone P1F6, Life Technologies), the αvβ3 integrin (clone P4C10, Life Technologies), and the αvβ3 integrin (clone PM6/13, Chemicon International, Inc., Temecula, CA). A negative immunoglobulin G isotype primary mouse antibody (Cedarlane Biological Laboratory, Burlington, Canada) was used as a control.

FIGURE 4. Expression of the β3 integrin increases migration of NT/C cells to OPN. Cell migration assay was performed using gelatin-coated transwells (6 μg per well). Wells contained 4 × 10^4 cells in the upper chambers and 50-μg/ml OPN (diluted in αHE + 0.1% BSA media) or αHE + 0.1% BSA media alone in the lower chambers. The assay was carried out for 4.5 h at 37°C. Columns, mean number of cells from five counts of triplicate wells; bars, SEM. NT/Cβ3 cells showed significantly increased migration to OPN versus NT/C control cells (P < 0.008 for all).

FIGURE 5. Expression of the β3 integrin increases invasion of NT/C cells to OPN. Cell invasion assay was performed using Matrigel-coated transwells. Wells contained 4 × 10^4 cells in the upper chambers and 100-μg/ml OPN (diluted in αHE + 0.1% BSA media) or αHE + 0.1% BSA media alone in the lower chambers. The assay was carried out for 72 h at 37°C. Columns, mean number of cells from five counts of triplicate wells; bars, SEM. NT/Cβ3 cells showed significantly increased cell invasion to OPN versus NT/C control cells (P < 0.001 for all).
Cloning of the pcHygro-β3 Plasmid

Full-length human β3 integrin cDNA, contained within the pcDNA3 expression vector (Invitrogen, San Diego, CA; pcDNA3-β3), was obtained as a gift from Dr. David Wilcox (Medical College of Wisconsin, Milwaukee, WI). Because the recipient NT/C cells already contained the pcDNA3 vector, the β3 integrin cDNA was cloned into the pcDNA3.1/Hygro+ mammalian expression vector (Invitrogen) using the HindIII and XhoI sites to generate the pcHygro-β3 plasmid. Correct orientation and integrity of the β3 gene was confirmed with restriction digests and verified by sequencing (Genbank accession no. NM 000212).

Transfections

Two sets of transfections (with NT/C cells and NT/O cells) were performed using the pcHygro-β3 and the control vector pcDNA3.1/Hygro+ plasmids in both cases. The Lipofectin reagent (Life Technologies) was used for the transfections, according to the manufacturer’s guidelines. Triplicate 60-mm tissue culture dishes (Becton Dickinson) were seeded with 2.5 × 10^5 cells and incubated for 24 h at 37°C in a CO_2 incubator. After 24 h, cells had reached 50% confluency and were transfected as per the manufacturer’s guidelines. For each plate, 1 μg of DNA was mixed with 10 μl of Lipofectin reagent and the transfection was carried out overnight at 37°C in a CO_2 incubator. In the morning, the transfection medium was removed and replaced with normal growth medium and the cells were allowed to recover and grow for an additional 48 h at 37°C. The cells were then trypsinized, replated, and grown again for 48 h. Normal growth medium containing hygromycin B (500 μg/ml), a concentration previously determined to give 100% death of parental NT/C and NT/O cells) was added to the cells and colonies were allowed to develop. Clonal cell populations were generated from a single colony, whereas pooled cell populations represent a collection of colonies. The specific cell populations isolated were as follows: three β3-transfected NT/C populations were named NT/C_β3 pool A, NT/C_β3 pool B, and NT/C_β3 pool C. NT/O_β3, NT/O, and NT/O_β3 OPN cells were allowed to recover and grow for an additional 48 h at 37°C. The cells were then trypsinized, washed, and resuspended in 1 ml of flow buffer before being scanned on an EPICS XL-MCL flow cytometer (Beckman-Coulter Canada Inc., Mississauga, ON, Canada).

Table 1. Summary of in Vivo Data

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor Take (No. of mice)</th>
<th>Tumor Latency Period (days to 500 mm^3)</th>
<th>Doubling Time (days)</th>
<th>Lymphatic Invasion (No. of Mice with Lymphatic Invasion/No. of Mice With Primary Tumors)</th>
<th>Lymph Node Metastasis (No. of Mice With Lymphatic Metastasis/No. of Mice With Primary Tumors)</th>
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<tr>
<td>NT/C</td>
<td>1/8</td>
<td>233</td>
<td>6.4</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>NT/C_β3 (vector control)</td>
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<td>N/A</td>
<td>N/A</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>NT/O_β3 (β3 alone)</td>
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<td>N/A</td>
<td>N/A</td>
<td>0/0</td>
<td>0/0</td>
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<tr>
<td>NT/O (OPN alone)</td>
<td>6/8^a</td>
<td>109.3 ± 11.7^b</td>
<td>4.9 ± 0.42^b</td>
<td>3/6^c</td>
<td>2/6^c</td>
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</tbody>
</table>

Note: Pooled populations of transfected cells were injected into the mammary fat pad of female nude mice using 1 × 10^7 cells per mouse and eight mice per treatment group. The end point was set at 1 year postinjection.
^aTumor take was significantly higher in the NT/O_β3 group relative to the other groups (P < 0.001 using Fisher’s exact test).
^bData are presented as the means ± SE.
^cLymphatic invasion at the site of the primary tumor and lymph node metastasis was determined using standard H&E staining as assessed by a pathologist (A.B.T.).
pooled population was named NT/O (consisting of 5 colonies). One β3-transfected NT/O pooled population was named NT/Oβ3 (consisting of 15 colonies) and one control vector-transfected pooled population was named NT/C (consisting of 8 colonies).

Northern Analysis

RNA was isolated using TRIZol reagent (Life Technologies) from subconfluent cells according to the manufacturer’s recommendations. RNA (10 μg per sample) was run on a 1.1% agarose gel with 6.8% formaldehyde and was capillary transferred to a nylon GeneScreen Plus membrane (NEN Life Science Products, Boston, MA). Blots were probed with denatured, oligolabeled 32P-dCTP cDNA probes using an oligolabeling kit (Amersham Pharmacia Biotech, Inc., Baie d’Urfé, Quebec) according to the manufacturer’s recommendations. The β3 integrin probe used was a 551 bp, KpnI-digested fragment of the pcHygro-β3 plasmid containing the first 500 bp of the human β3 cDNA. The OPN probe used was a full-length human OPN cDNA (1493 bp) generated using EcoRI digestion of the OP-10 plasmid (15). To confirm even loading of lanes, human 18S rRNA from the p100D9 plasmid (a gift from Dr. David T. Denhardt) was also used. Densitometric analysis was carried out using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Cell Adhesion Assay

On the day prior to the experiment, sterile 96-well nontissue culture plates (Titertek, Flow Laboratories, McLean, VA) were treated in triplicate with either 10 μg/ml of human recombinant OPN (17) or 0.2% BSA (Sigma Chemical Co., St. Louis, MO) and incubated overnight at 4°C. The wells were then rinsed and blocked with αHE + 0.2% BSA medium. The cells were resuspended in αHE + 0.2% BSA, and 1 × 104 cells were added per well. For antibody blocking experiments, cells were preincubated on a rotating plate with saturating concentrations (as determined by preliminary titration experiments) of mouse monoclonal antibodies against human αsβ3 (Chemicon International) or a control negative immunoglobulin G isotype primary mouse antibody (Cedarlane Laboratories) for 15 min at 37°C before plating. Plates were incubated for 3 h at 37°C, and the medium was then removed and nonadhered cells were rinsed away with three gentle washes of PBS. Adhered cells were fixed in fresh 2% gluteraldehyde for 20 min then rinsed with three gentle washes of PBS. Adhered cells were fixed using fresh 2% gluteraldehyde for 20 min then washed with PBS and stained using Harris’ hematoxylin for 15 min. The staining was then intensified using a 1% solution of ammonia water. Nonmigrated cells remaining on the upper side of the membrane were removed with a cotton swab. The blue-stained cells on the lower surface were then counted using a light microscope. Five high-power fields of cells were counted for each well, and the mean number of cells per field was calculated. This assay was performed twice and gave similar results.

Cell Migration Assay

Cell migration was assessed as previously described (15). Briefly, on the day prior to the experiment, triplicate 6.5-mm transwells with 8-μm pores (Costar Corp., Cambridge, MA) were coated with 6 μg of gelatin (denatured collagen; Sigma Chemical) per well and dried overnight in a tissue culture hood. On the following morning, the gelatin was reconstituted with αHE medium for 90 min. Subconfluent cells were gently trypsinized and washed twice with αHE + 0.1% BSA. The cells were resuspended in αHE + 0.1% BSA and 4 × 106 cells were added to each upper well. Each lower chamber contained either αHE + 0.1% BSA (negative control) or 50-μg/ml recombinant human OPN diluted in αHE + 0.1% BSA. The plates were then incubated for 4.5 h at 37°C. The upper well was removed and fixed in fresh 2% gluteraldehyde for 20 min then washed with PBS and stained using Harris’ hematoxylin for 15 min. The staining was then intensified using a 1% solution of ammonia water. Nonmigrated cells remaining on the upper side of the membrane were removed with a cotton swab. The blue-stained cells on the lower surface were then counted using a light microscope. Five high-power fields of cells were counted for each well, and the mean number of cells per field was calculated. This assay was performed twice and gave similar results.

Cell Invasion Assay

Cell invasion through Matrigel was assessed as previously described (15). Briefly, on the day prior to the experiment, triplicate 6.5-mm transwells with 8-μm pores were coated with 20 μg of Matrigel per well and dried overnight in a tissue culture hood. On the following morning, the Matrigel was reconstituted by adding αHE medium and the plate was agitated for 90 min. Subconfluent cells were prepared by gentle trypsinization followed by two washes with αHE + 0.1% BSA medium. The cells were resuspended in αHE + 0.1% BSA and 4 × 104 cells were added to each upper well. Each lower chamber contained either αHE + 0.1% BSA (negative control) or 100-μg/ml human recombinant OPN diluted in αHE + 0.1% BSA medium. The plates were then incubated for 72 h at 37°C to allow for invasion of the cells through the Matrigel to the underside of the transwell membrane. The wells were then fixed, stained, and counted as described above. This assay was performed thrice and gave similar results.

Mammary Fat Pad Injections

Female athymic NCr nude mice (nu/nu) were housed and cared for in accordance with the recommendations of the Canadian Council on Animal Care under a protocol approved by the University of Western Ontario Council on Animal Care. Cell lines were grown in 150-mm tissue culture dishes to ~80% confluency (log phase of growth). The cells were gently trypsinized, washed twice with sterile PBS, and resuspended in serum-free αHE media at a concentration of 1 × 107 cells per 100 μl. Pooled populations of 21NT cells stably transfected with the control vector (NT/C), β3 alone (NT/Cβ3 pool A), OPN alone (NT/O), or both OPN and β3 (NT/Oβ3) were injected into the second thoracic mammary fat pad of 8–9-week-old female nude mice, as described elsewhere (30), using 1 × 106 cells per mouse and eight mice per treatment group. Animals were routinely monitored for health and primary tumors were measured every 7–14 days. Animals were
enuanished early if the tumor burden became too great or at the end point of the experiment (1 year postinjection). Animals were necropsied, and tissues and organs (including primary mammary tumor and/or mammary fat pad, lymph nodes, lungs, liver, and spleen) were fixed in 10% neutral-buffered formalin before processing. Tissues were embedded in paraffin wax, sectioned (4 μm thick), and subjected to standard H&E staining to observe histopathological characteristics.

Statistical Analysis

For the in vitro studies, statistical differences in cell adhesion, migration, and invasion between groups were determined by Student’s t test. For the in vivo studies, statistical differences in tumor take between groups were determined using Fisher’s exact test. In all cases, values of P < 0.05 were considered to be significant.

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References


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