Exogenous Expression of N-Cadherin in Breast Cancer Cells Induces Cell Migration, Invasion, and Metastasis

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Abstract. E- and N-cadherin are calcium-dependent cell adhesion molecules that mediate cell–cell adhesion and also modulate cell migration and tumor invasiveness. The loss of E-cadherin–mediated adhesion has been shown to play an important role in the transition of epithelial tumors from a benign to an invasive state. However, recent evidence indicates that another member of the cadherin family, N-cadherin, is expressed in highly invasive tumor cell lines that lacked E-cadherin expression. These findings have raised the possibility that N-cadherin contributes to the invasive phenotype. To determine whether N-cadherin promotes invasion and metastasis, we transfected a weakly metastatic and E-cadherin–expressing breast cancer cell line, MCF-7, with N-cadherin and analyzed the effects on cell migration, invasion, and metastasis. Transfected cells expressed both E- and N-cadherin and exhibited homotypic cell adhesion from both molecules. In vitro, N-cadherin–expressing cells migrated more efficiently, showed an increased invasion of Matrigel, and adhered more efficiently to monolayers of endothelial cells. All cells produced low levels of the matrix metalloproteinase MMP-9, which was dramatically upregulated by treatment with FGF-2 only in N-cadherin–expressing cells. Migration and invasion of Matrigel were also greatly enhanced by this treatment. When injected into the mammary fat pad of nude mice, N-cadherin–expressing cells, but not control MCF-7 cells, metastasized widely to the liver, pancreas, salivary gland, omentum, lung, lymph nodes, and lumbar spinal muscle. The expression of both E- and N-cadherin was maintained both in the primary tumors and metastatic lesions. These results demonstrate that N-cadherin promotes motility, invasion, and metastasis even in the presence of the normally suppressive E-cadherin. The increase in MMP-9 production by N-cadherin–expressing cells in response to a growth factor may endow them with a greater ability to penetrate matrix protein barriers, while the increase in their adherence to endothelium may improve their ability to enter and exit the vasculature, two properties that may be responsible for metastasis of N-cadherin–expressing cells.

Key words: E-cadherin • motility • FGF-2 • MMP-9

Introduction

To infiltrate host tissues, cancer cells of epithelial origin have to separate from the tumor mass by breaking their cell–cell contacts, known as adherens junctions (Frixen et al., 1991; Vleminckx et al., 1991; Frixen and Nagamine, 1993). Consistent with this hypothesis, the cell adhesion molecule E-cadherin, which is the adhesive component of adherens junctions, is notably absent or dysfunctional in most of the advanced, undifferentiated, and aggressive breast and other epithelial carcinomas (Perl et al., 1998; Christofori and Semb, 1999). These and other findings support the model in which the loss of E-cadherin–based cell adhesion is considered to be an important factor in tumor invasiveness (Behrens et al., 1989; Frixen et al., 1991; Vleminckx et al., 1991; Takeichi, 1993; Birchmeier and Behrens, 1994).

More recent evidence indicates that a gain of expression of another adhesion molecule, N-cadherin, in tumor cells is associated with an increased invasive potential. Our previous study has shown that N-cadherin is upregulated in more invasive and less differentiated breast cancer cell lines that lacked E-cadherin expression (Hazan et al., 1997). N-Cadherin was also reported to induce a mesenchymal-scattered phenotype associated with reduced E- and P-cadherin levels in a squamous cell carcinoma cell

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line (Islam et al., 1996). These findings raised the possibility that N-cadherin contributes directly to the invasive phenotype. Consistent with this idea, E-cadherin has been shown to promote only tight cell–cell adhesion, restricting cell movement, whereas N-cadherin has been postulated to promote both stable and labile cellular interactions that facilitate dynamic processes such as neurite outgrowth and cell migration (Hatta et al., 1987; Bixby and Zhang, 1990; Doherty et al., 1991; Riehl et al., 1996).

In tissues, changes in cancer cell adhesion or locomotion alone are not sufficient for the active penetration of extracellular matrices. To breach biological barriers such as basement membranes, a step crucial for invasion and metastasis, a cancer cell must be able to mobilize or produce proteolytic enzymes (Liotta and Stetler-Stevenson, 1991; Coussens and Werb, 1996). Among the most studied are urokinase plasminogen activator (uPA)[1] generated plasmin and matrix metalloproteinases (MMPs) (Mignatti and Rifkin, 1993). MMPs are a large family of extracellular matrix (ECM)–degrading enzymes, including collagenases, gelatinases, stromelysins, and MT-MMPs (Nagase and Woessner, 1999), most of which are associated with tumor angiogenesis invasion and metastasis (Brooks, 1996; Coussens and Werb, 1996; Kleiner and Stetler-Stevenson, 1999). Several MMPs, including gelatinase B/MMP-9 (Vu et al., 1998), have been shown to be directly involved in cancer invasion and are believed to be predominantly, but not exclusively, produced by stromal cells and sequestered by carcinoma cells (Stetler-Stevenson et al., 1993; Crawford and Matrisian, 1994; Johnsen et al., 1998; Lochter et al., 1998).

The contrasting roles of E- and N-cadherin in cell adhesion and migration and the upregulation of N-cadherin in invasive breast cell lines and squamous tumors (Islam et al., 1996; Hazan et al., 1997) prompted us to directly test the role of N-cadherin in the process of invasion and metastasis, and to investigate the mechanism that may be responsible for this function. We show that the expression of N-cadherin in the weakly invasive and poorly metastatic (Kern et al., 1994), E-cadherin–expressing breast cancer cell line, MCF-7, endows these cells with invasive and metastatic properties. The metastasis promoting effect of N-cadherin is dominant since MCF-7 tumor cells continue to express E-cadherin. Moreover, N-cadherin expression sensitizes the cells to FGF-2–induced increases in cell migration, in vitro invasion, and MMP-9 production.

Materials and Methods

Cell Lines

The MCF-7 and MDA-MB-435 breast cancer cell lines and endothelial HUVEC cells were obtained from the American Type Culture Collection. Mouse L-cells or L-cells transfected with mouse E-cadherin or mouse N-cadherin were provided by Dr. David Colman (Mount Sinai-NYU School of Medicine, New York). Cells were routinely cultured in DME supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere. HUVEC cells were grown in 199 media that included 10% FBS, 0.1 mg/ml heparin and 10 ng/ml FGF-2. All media and serum were from GIBCO BRL.

[1]*Abbreviations used in this paper:* ECM, extracellular matrix; H&E, hematoxylin-eosin staining; MMP, matrix metalloproteinase; uPA, urokinase plasminogen activator.

Reagents

Estrogen pellets (17β-estradiol, 1.7 mg/pellet, 60-d release) were purchased from Innovative Research of America. Collagen and Matrigel were purchased from Collaborative Biomedical products. FGF-2 was purchased from Pepro Tech. Insulin, gelatin, heparin, and crystal violet were obtained from Sigma Chemical Co. Biocoat cell culture inserts (24-well) (Boyden chambers) were obtained from Becton Dickinson & Co.

Antibodies

Monoclonal anti-human E-cadherin and N-cadherin were purchased from Zymed Labs. Polyclonal antibodies directed to the EC1 domain of N-cadherin were a gift from Dr. David Colman (Mount Sinai-NYU School of Medicine, New York). Ascitis-derived monoclonal anti–N-cadherin antibody was purchased from Sigma Chemical Co. and affinity-purified on IgG columns (Pierce Chemical Co.) as indicated by the manufacturer protocol. Affinity-purified IgG_1 immunoglobulins were obtained from Zymed Labs. Monoclonal anticytokeratin (CAM 5.2) either uncoupled or directly coupled to FITC was acquired from Becton Dickinson & Co. Polyclonal antiactin antibodies were obtained from Sigma Chemical Co. Fast diI and Fast diO, FITC, and rhodamine-conjugated secondary antibodies were obtained from Molecular Probes, Inc.

Constructs and Transfections

The cDNA clone for human N-cadherin (pcDNAhN-cad) (Reid and Hemperly, 1990), in the vector pcDNA-neo (Invitrogen Corp.), obtained from Dr. John Hemperly (Becton Dickinson & Co), was transfected into MCF-7 cells using Lipofectamine (GIBCO BRL). Several stable clones as well as a population of stable transfectants (mass cultures) were selected by the addition of (G418) at a concentration of 1–2 mg/ml. Clonal and mass transfectants with empty vector (pcDNA-neo) were also selected with G418. To ensure clonality of the selected clones, subsequent subcloning was performed by limiting dilution.

SDS-PAGE and Immunostaining of N-Cadherin Transfectants

N-cadherin–transfected cells were extracted in solubilization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.2 mM EGTA, 1% Triton X-100) that included 1 mM PMSF and 10 μg/ml aprotinin and leupeptin. 20 μg of soluble protein, as determined by the BCA method (Pierce Chemical Co.), was mixed with sample buffer, boiled for 5 min, loaded on 7.5% SDS–polyacrylamide gels, and transferred onto Immobilon membranes (Millipore). Blots were probed with antibodies to N-cadherin or E-cadherin and developed with chemiluminescence (Amersham Pharmacia Biotech). The subcellular localization of N-cadherin and E-cadherin in transfected MCF-7 cells was determined by immunofluorescence using antibodies to N- and E-cadherin, as previously described (Hazan et al., 1997).

Coaggregation Assays

Single cell suspensions of L-cells, L-E, L-N, MCF-7, and N-cadherin–transfected MCF-7 (N-cad-5) were prepared as described (Hazan et al., 1997), and were labeled with the fluorescent lipophilic dye Fast diI or Fast diO (Molecular Probes, Inc.). Cells (1.5×10^5) of each type were mixed and aggregated for 20 min at 37°C with or without 1 mM $CaCl_2$, as previously described (Hazan et al., 1997). Multiple aliquots from each aggregation experiment were viewed, counted, and photographed under a fluorescence microscope at a magnification of 20.

Adhesion to HUVEC Monolayers

HUVEC cells (10^5) were plated on 1% gelatin-coated glass coverslips and allowed to form a monolayer by overnight incubation. Single cell suspensions from either Neo-5, N-cad-5, and N-cad-17 were labeled with Fast diO, and 10^5 cells were plated over the HUVEC monolayers in 0.5 ml DME, 0.1% BSA, 1 mM $CaCl_2$ in a 12-well plate in triplicate wells for 6 h at 37°C in a humidified 5% CO_2 atmosphere. Unattached cells were washed out by rinsing the cells three times with PBS. Attached cells were fixed in 3.7% paraformaldehyde for 30 min, washed, and visualized under a fluorescent microscope. Each panel is representative of triplicate experiments.

Cell Migration and Invasion through Matrigel

The ability of cells to migrate through control (migration) or invade through Matrigel-coated filters (invasion) was measured in a Boyden chamber. 8-micron Transwell filters, which were coated with or without 50 μg Matrigel (Becton Dickinson & Co.), were used according to standard protocols (Albini et al., 1987). Fibroblast conditioned medium, which was obtained by a 24-h incubation of NIH-3T3 cells with 50 μg/ml ascorbic acid in serum-free DME media, was placed in the lower chamber as a chemoattractant. Single cell suspensions of control or N-cadherin transfectants, incubated overnight in medium alone or in media containing FGF-2 (10 ng/ml) and heparin (0.1 mg/ml), were obtained by treatment with PBS containing 5 mM EDTA. Cells were washed and placed at 10^5 cells per well into the upper chamber in 0.5 ml DME, 0.1% BSA in the presence or absence of FGF-2 (10 ng/ml) and heparin (0.1 mg/ml) for the indicated times. Cells that had not penetrated the filter were wiped out with cotton swabs, and cells that had migrated to the lower surface of the filter were stained with 0.5% crystal violet, examined by bright field microscopy, and photographed. Values for invasion were expressed as the average number of migrated cells bound per microscopic field over four fields per assay and expressed as averages for triplicate experiments.

Substrate Gel Electrophoresis (Zymography)

Secreted metalloproteinases were detected and characterized by zymography (Nakajima et al., 1995). Conditioned media were obtained by a 30-h incubation of N-cadherin and control transfected cells, which were treated overnight with or without FGF-2 (10 ng/ml) and heparin (0.1 mg/ml) in serum-free media. Conditioned media (20 μl) were loaded on 8% SDS-PAGE gels that had been copolymerized with 1 mg/ml gelatin. Electrophoresis was performed under nonreducing conditions at 100 V for 2 h at 4°C. Gels were washed once for 30 min in 2.5% Triton X-100 to remove SDS, and were incubated in collagenase buffer (100 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3) for 40 h at 37°C. Gels were stained with 0.5% Coomassie blue in 30% methanol/10% acetic acid for 30 min at room temperature and destained in 30% methanol/10% acetic acid three times for 15 min. The presence of metalloproteinases was indicated by an unstained proteolytic zone in the substrate. Both active forms and pro-enzymes are revealed by this technique as the exposure of pro-MMPs to SDS during SDS-PAGE leads to activation without proteolytic cleavage. Microdissection and PCR tissue sections (4 μm) from metastatic livers and primary tumors of mice injected with either control (MCF-7) or N-cadherin–transfected cells (N-cad-5 and N-cad-17), were deparafinized as described (Greer et al., 1991) and stained in 2% hematoxylin solution for 1 min. Tumor cells were microdissected from lesions in the liver under a microdissecting scope. Microdissected cells were digested overnight at 55°C with 20 μl proteinase K in digestion buffer (1 mg/ml in 50 mM Tris-HCl buffer, pH 8.0, with 1 mM EDTA, and 0.45% Tween 20). Proteinase K digestion was stopped by incubation at 95°C or 15 min. Cell debris was pelleted out by a centrifugation at 12,000 rpm for 10 min. 2 μl of the resultant crude DNA (supernatant) was used for PCR amplification using the T7 primer that hybridized near the cloning site in pcDNA neo and a downstream primer from human N-cadherin cDNA (5′ CACTGTAAACATCAACAGTGAAATCC 3′). Southern blotting was performed using a nick-translated 32P-dCTP–labeled human N-cadherin cDNA probe.

Nude Mice Experiments

Female athymic nude mice (Taconic Farms) at 6–8 wk of age, preimplanted subcutaneously with 1.7 mg of 17 β-estradiol pellets (60-d release; Innovative Research of America), were simultaneously injected bilaterally into the mammary fat pads with 10^7 of either parental MCF-7 cells, one neomycin-resistant clone (Neo-5), or N-cadherin–transfected clones (N-cad-5 and N-cad-17, N-cad-8 and N-cad-15) as indicated. Mice (8–10 wk after injection) were killed, and the liver, lung, lymph nodes, pancreas, kidney, spleen, omentum, brain, heart, bone, and spinal skeletal muscle were removed. The organs were fixed in 10% buffered formalin, paraffin-embedded, and sectioned at 4 μm. The primary tumors in the mammary fat pads were also removed and weighed.

Hematoxylin-Eosin (H&E) Staining and Histoimmunochemistry

20–50 sections from each organ were deparafinized and stained by H&E. Sections positive for metastases were antigen-retrieved by microwaving in citrate buffer (10 mM citric acid, pH 6.0), blocked in 5% horse serum, and stained for 2 h at room temperature using either of the following antibodies: monoclonal anticytokeratin (CAM 5.2) at a 1:2 dilution either uncoupled or coupled to FITC; monoclonal anti–E-cadherin or N-cadherin at 10 μg/ml; and polyclonal antiactin antibodies at 1:100 dilution. Secondary detection was applied using secondary horse biotinylated anti-mouse antibodies (Vector Labs) at a 1:200 dilution followed by streptavidin-HRP (Zymed Labs) according to the manufacturer's protocol. Sections were developed in DAB solution, counterstained by H&E, and photographed under a microscope. Sections incubated with actin antibodies were incubated with anti-rabbit antibodies coupled to rhodamine, and those incubated with FITC-conjugated anticytokeratin antibodies were washed after the first incubation and mounted for fluorescent microscopy.

Results

Characterization of the Transfectants Expressing N-Cadherin

MCF-7 cells were transfected either with a vector containing the human N-cadherin cDNA (Reid and Hemperly, 1990) (N-cad cells) or with the vector alone (Neo-cells). Mass cultures of G418-selected stable transfectants as well as several clonal cell lines were obtained. The expression of N-cadherin in the cells was evaluated by Western blot (Fig. 1 A) and immunofluorescent staining (Fig. 1 B) using a human N-cadherin–specific mAb. This antibody detected a band of 130 kD in extracts of N-cad cells (Fig. 1 A, bottom panel, lanes 3–7) and in MDA-MB-435 breast cancer cells (Fig. 1 A, bottom panel, lane 8), which are known to express high levels of N-cadherin (Hazan et al., 1997), but not in parental MCF-7 or a clone of vector-transfected cells (Neo-5), (Fig. 1 A, bottom panel, lanes 1 and 2, respectively). The level of N-cadherin in N-cad-mass cells was significantly lower than that in N-cad-5 or N-cad-17 cells (Fig. 1 A, bottom panel, lane 3). Of note, E-cadherin expression was not affected by the presence of N-cadherin (Fig. 1 A, top panel, lanes 1–7). Both E- and N-cadherin expression was readily detectable by immunostaining with specific mAbs, primarily in areas of cell–cell contacts (Fig. 1 B, top and bottom, respectively). Expression of N-cadherin in N-cad-mass cells was heterogeneous, with only ~20% positive cells (Fig. 1 B, bottom panel). The expression of N-cadherin in MCF-7 cells did not alter their epithelial phenotype (Fig. 1 B), and did not induce changes in either cytokeratin or vimentin levels (data not shown).

To determine whether the transfected N-cadherin was functionally active in adhesion and whether it interfered with the adhesive function of E-cadherin, parental MCF-7 cells or the N-cad-5 clone, labeled with Fast diO, were mixed and coaggregated either with parental L-cells or with L-cells transfected with either mouse E- (L-E cells) or N-cadherin (L-N cells), which were labeled with Fast diI (Fig. 2). N-Cad-5 cells formed large coaggregates with both L-E and L-N cells (Fig. 2, E and F). In contrast, MCF-7 cells aggregated with L-E but not with L-N cells (Fig. 2, B and C, respectively). Neither N-cad-5 nor MCF-7 cells aggregated with untransfected L-cells (Fig. 2, A and D), and no aggregation occurred in calcium-free conditions (data not shown). These data demonstrate that both endogenous E- and transfected N-cadherin present on the

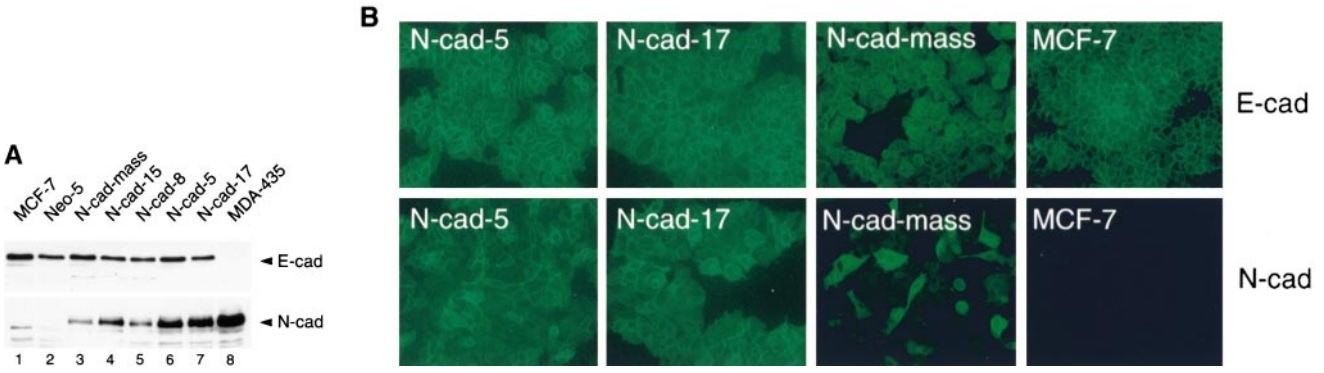

Figure 1. E- and N-cadherin expression and localization in N-cadherin MCF-7 transfectants and control cells. (A) Triton X-100 cell lysates (20 μg of protein) from parental MCF-7 (lane 1), a vector-transfected clone (Neo-5; lane 2), N-cadherin mass cultures (N-cad-mass; lane 3), N-cadherin–transfected clones (N-cad-15, N-cad-8, N-cad-5, and N-cad-17; lanes 4–7, respectively), and MDA-MB-435 (lane 8) were immunoblotted with mAbs to either E- (top panel) or N-cadherin (bottom panel). (B) N-cad-5, N-cad-17, N-cad-mass cells, and parental MCF-7 cells were plated on collagen-coated coverslips, fixed, and stained with monoclonal anti–E- (top) and anti–N-cadherin antibodies (bottom).

surface of MCF-7 cells are capable of mediating calcium-dependent, homotypic cellular interactions.

N-Cadherin Stimulates MCF-7 Tumor Cell Migration and Invasion

Since N-cadherin has been shown to mediate the neurite guidance and cell migration of retinal neurons (Bixby and Zhang, 1990; Doherty et al., 1991), we examined whether the expression of N-cadherin by MCF-7 cells influenced cell motility. The ability of cells to migrate through uncoated porous filters in response to a chemotactic stimulus was examined in a Transwell migration assay (Fig. 3). The two clones with the highest N-cadherin levels (clones 5 and 17) produced the highest numbers of migrating cells, but even the N-cad-mass cells, with only approximately one fifth of the cells expressing N-cadherin, showed a strong increase in migration over the Neo-mass control cells (Fig. 3 A). Similar results were obtained when the different cell types were tested for their ability to invade through Matrigel-coated filters; only N-cadherin–expressing cells invaded into Matrigel, and the two clones with the highest expression of N-cadherin exhibited the highest number of invading cells (Fig. 3 B). Furthermore, treatment of N-cad-5 cells with a purified preparation of antibodies to the NH_2-terminal domain of human N-cadherin (A-CAM) resulted in reduction of cell migration by 50% (Fig. 4), whereas isotype-matched immunoglobulins had a negligible effect on the motility of these cells in vitro. This antibody was shown to disrupt adherens junctions in cultured lens cells (Volk and Geiger, 1984; Volk et al., 1990) and to inhibit cell–cell aggregation in N-cadherin–expressing breast carcinoma cells but not in E-cadherin–expressing cells (Hazan et al., 1997). These results confirm a direct involvement of N-cadherin in the acquisition of a migratory phenotype.

Metastasis of N-Cadherin MCF-7 Transfectants in Nude Mice

We next tested the potential of N-cadherin–transfected MCF-7 cells to metastasize in vivo. In several previous re-

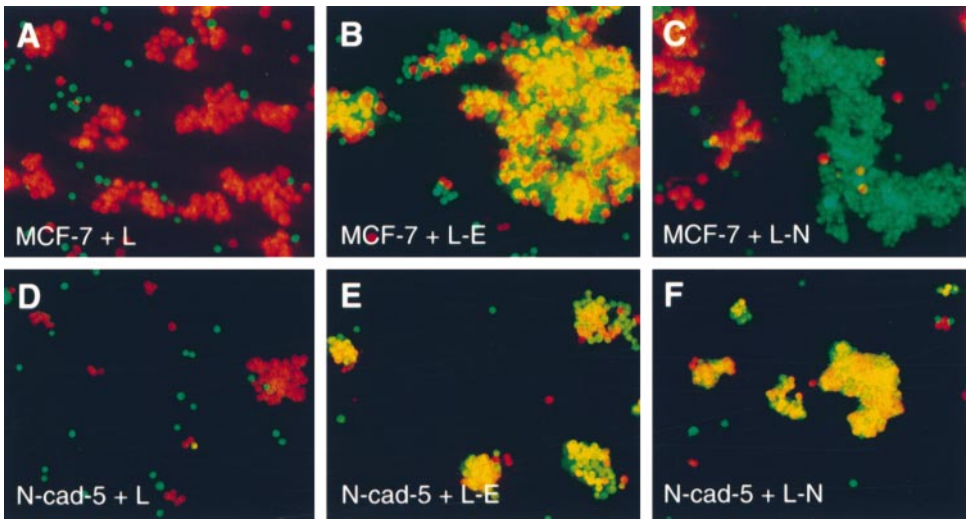

Figure 2. N-cadherin promotes coaggregation of N-cadherin–transfected MCF-7 cells with N-cadherin–transfected L-cells. L-cells and L-E-cadherin and L-N-cadherin cells, labeled with the fluorescent dye diO (green) were coaggregated with diI-labeled (red) MCF-7 cells (A, B, and C, respectively) or N-cad-5 cells (D, E, and F respectively). Yellow appears when green and red are superimposed, indicating coaggregation.

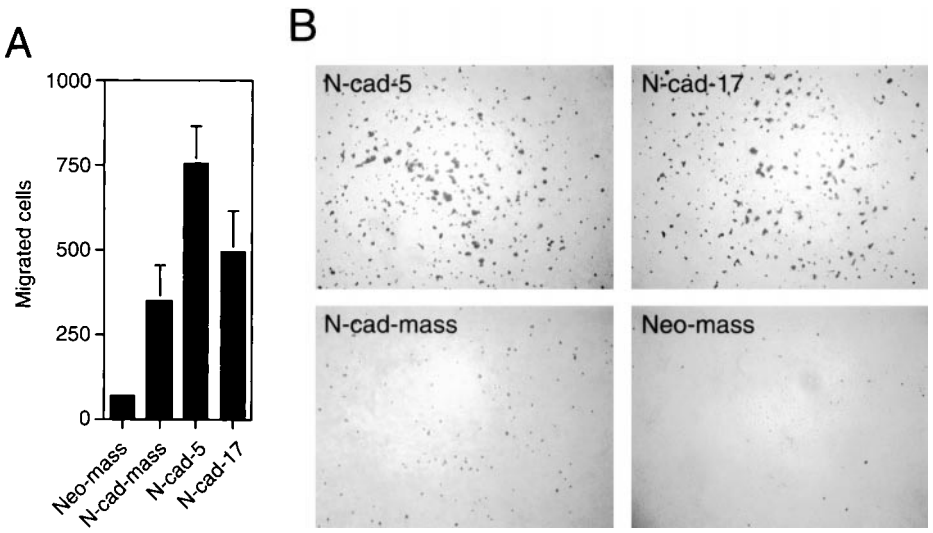

Figure 3. N-cadherin promotes cell migration and invasion. (A) Cell motility through uncoated filters and (B) through Matrigel-coated filters was measured 18 h after plating. The migrating cells were stained (see Materials and Methods), visualized by microscopy, and triplicate filters were counted in three individual experiments. The numbers represent mean ± SD for three experiments. (B) Cells that invaded the Matrigel layer were fixed, stained, and photographed; each panel represents an example of three to six replicates. Because of clustering of migrating cells on the underside of filter, no counting of single cells was possible.

ports, MCF-7 cells displayed weak to undetectable levels of metastasis in this model (Clarke et al., 1993; Kern et al., 1994; Price, 1996). Four independent clones expressing the highest levels of N-cadherin (N-cad-5, N-cad-17, N-cad-8, and N-cad-15), a vector transfected clone (Neo-5), and parental MCF-7 cells were injected into the mammary fat pads of estrogen-treated female nude mice. A total of five to six mice were injected per cell line. 8–10 wk after injection, the primary tumors and distal organs including the liver, lung, pancreas, kidney, brain, lymph nodes, heart, spleen, omentum, salivary glands, and skeletal muscle were resected, fixed, paraffin-embedded, sectioned, and stained either with H&E or with an anti-human cytokeratin-specific antibody (Moll et al., 1982).

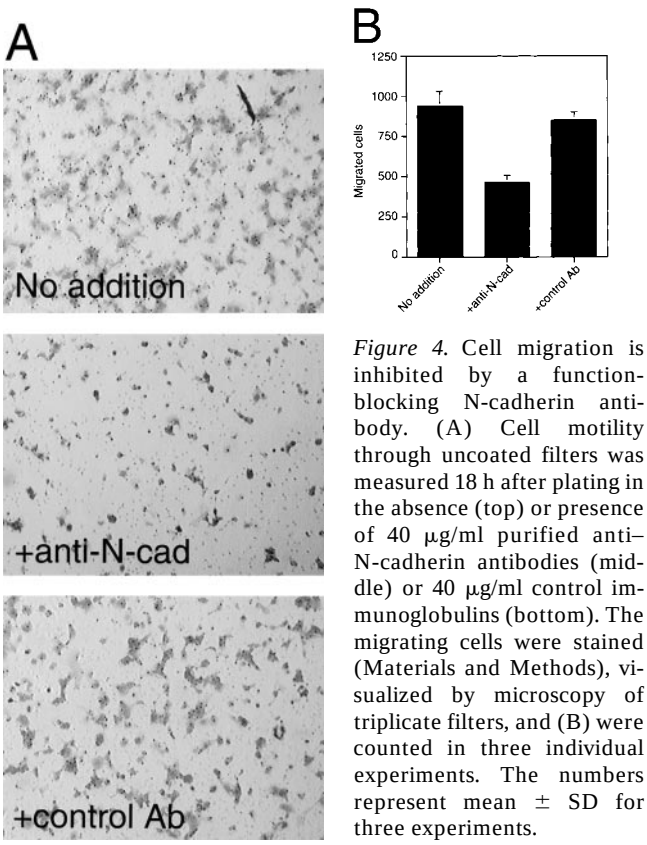

Figure 4. Cell migration is inhibited by a function-blocking N-cadherin antibody. (A) Cell motility through uncoated filters was measured 18 h after plating in the absence (top) or presence of 40 μg/ml purified anti–N-cadherin antibodies (middle) or 40 μg/ml control immunoglobulins (bottom). The migrating cells were stained (Materials and Methods), visualized by microscopy of triplicate filters, and (B) were counted in three individual experiments. The numbers represent mean ± SD for three experiments.

Of a total of 20 mice injected with N-cadherin–expressing cell lines, 13 mice (65%) had primary tumors. Similarly, 10 out of 12 mice (83%) injected with control MCF-7 cells developed primary tumors. N-cadherin–expressing tumors grew slower on average, reaching a weight of ~30% that of control MCF-7 or Neo-5 tumors (Table I). This is consistent with the slower growth rate of the N-cadherin–transfected cells in vitro (data not shown). Most (77%) of the 13 mice with N-cadherin–expressing primary tumors developed metastases in multiple sites, whereas none of the control mice bearing larger tumors had detectable metastases (Table I). Staining with H&E strongly suggested the presence of metastatic cells in the different organs (for examples see Fig. 5, A–G). The human epithelial origin of these cells was confirmed by staining with an anticytokeratin antibody either directly coupled to FITC (Fig. 5, E, F, and I) or followed by secondary HRP detection (Fig. 5, B–D and H). This antibody reacted only with human and not with mouse cytokeratin. The sections of the primary tumors stained equally well, regardless of the method of detection, (peroxidase or FITC) (Fig. 5, C and F). Fig. 5 shows a representative panel of sections from different organs in which metastases were found. In most organs (pancreas, salivary gland, omentum, and muscle, Fig. 5, B, D, E, and I, respectively), large areas of metastatic growth were found, but micrometastases shown in a lung section (Fig. 5, G and H) were also present. In a comparable analysis of tissue sections from mice injected with control MCF-7 or Neo-5 cells, no cytokeratin-reactive cells were ever found in the pancreas, lymph nodes, salivary gland, omentum, liver, lung, and skeletal muscle (Table I), or in other tissues (data not shown). As indicated in Table I, whereas the metastases produced by other clones were more random, the two clones with the highest level of N-cadherin expression produced liver metastases in almost all injected mice.

To verify the presence of the transfected N-cadherin in metastases, DNA was extracted from tumor cells that were microdissected from areas of liver sections deemed

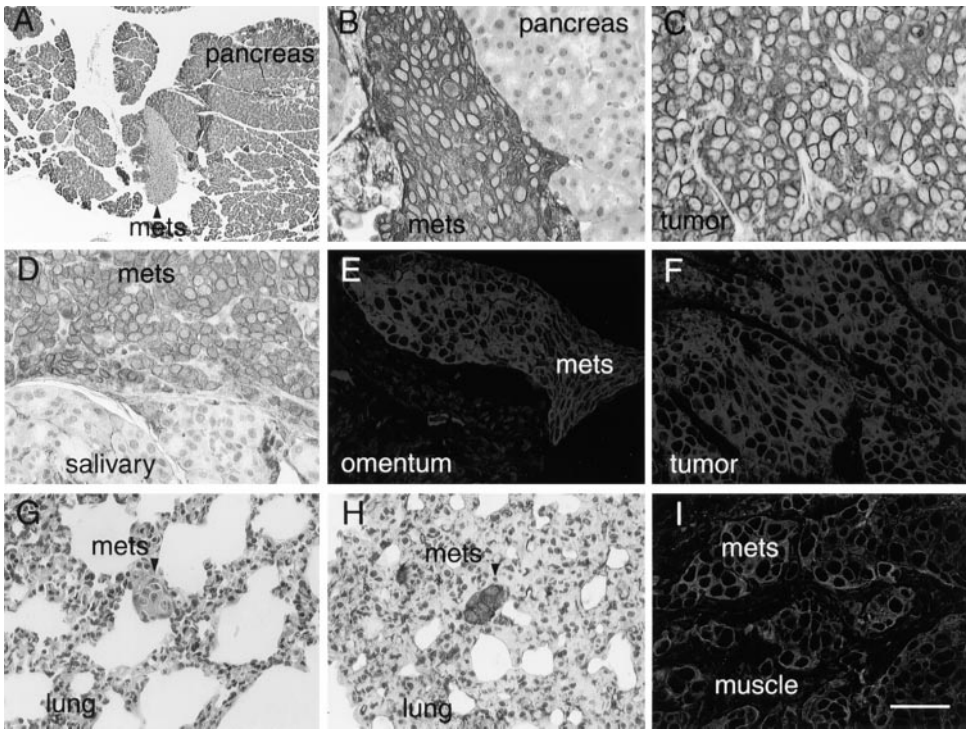

Figure 5. Histopathology of metastatic lesions in nude mice. 5-μm sections of the pancreas of mice injected with N-cad-17 cells were stained with either H&E (A) or cytokeratin antibodies, followed by DAB detection (B). Cytokeratin immunoreactivity of the N-cad-17 primary tumor (C) was compared with the pancreatic lesion produced by this tumor in B. Sections from the salivary gland of mice injected with N-cad-5 cells were stained with anticytokeratin antibodies, followed by DAB detection (D) and sections from the omentum (E) and primary tumor of N-cad-5–injected mice (F) were stained with FITC-conjugated cytokeratin antibodies. N-cad-8 metastatic lesions were detected in lung sections by H&E (G) and cytokeratin/DAB detection (H). N-cad-15 cells were found in the lumbar spinal muscle (I) using double fluorescent staining with antiactin, followed by secondary antibodies coupled to rhodamine (red) and FITC-conjugated anticytokeratin antibodies (green). Bar: (A) 100 μm; (G and H) 50 μm; (B, D, and E) 33 μm; (C, F, and I) 20 μm.

to be positive for metastasis by H&E staining (Fig. 6 A, top and bottom show a section from the liver of an N-cad-17–inoculated mouse) and used in a PCR reaction designed to detect the presence of the transgene (see Materials and Methods). Southern blotting of the reaction products using the human N-cadherin cDNA as a probe confirmed the specificity of the detected band as the transfected N-cadherin (Fig. 6 B, lane 7). DNA isolated from an MCF-7 primary tumor, or from a liver of a MCF-7 tumor bearing mouse did not yield an amplification product (Fig. 6 B, lanes 1 and 2). In contrast, DNA isolated from primary tumors and livers of N-cad-5– and N-cad-17–inoculated mice were positive in the PCR reaction (Fig. 6 B, lanes 3–6).

Coexpression of Cadherins E and N in Metastatic Lesions

The preceding data show that N-cadherin transfection has a striking effect on the metastasis of MCF-7 breast tumor cells. It remained to be determined whether metastatic cells in vivo retained N- and E-cadherin expression. Staining of metastatic lesions in the salivary gland, pancreas (Fig. 7, top) and axillary lymph nodes (bottom right panels) as well as in the lumbar muscle (data not shown), with anti-human E- or N-cadherin antibodies revealed that both cadherins were present in areas of contact between tumor cells. Note the massive infiltration of tumor cells into the salivary gland and pancreas but only marginal invasion of the lymph nodes. In contrast, N-cadherin staining was not detected in primary tumors produced by the nonmetastatic, non–N-cadherin–expressing MCF-7 cells, which continued to express E-cadherin (Fig. 7, bottom left). Since N-cadherin expression was conserved during the metastatic progression of transfected MCF-7 tumor cells, it must play a dominant role over E-cadherin in inducing this phenotype.

FGF-2 Enhances Migration of N-Cadherin–expressing Cells, Upmodulates their MMP-9 Production, and Increases Matrigel Invasion

N-cadherin has been postulated to promote neurite out-

Table I. N-Cadherin Induces Breast Cancer Metastasis in Nude Mice

Cell lines	Primary tumor	*n*	Metastatic organs: Liver	Pan-creas	Salivary gland	Omentum	Lung	Muscle	Lymph nodes
	g								
N-cad-5	0.35 ± 0.1	4	3/3	0/2	3/4	2/3	0/3	0/3	2/4
N-cad-17	0.27 ± 0.09	3	2/3	3/3	0/2	0/2	0/2	0/3	ND
N-cad-8	0.23 ± 0.08	3	0/3	0/3	0/3	0/3	2/3	0/3	0/3
N-cad-15	0.25 ± 0.1	3	0/3	0/3	0/3	0/3	2/3	2/3	0/3
Neo-5	1.20 ± 0.2	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
MCF-7	1.20 ± 0.3	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

Breast cancer metastasis in nude mice injected with either MCF-7, Neo-5 control cells (Neo) or N-cadherin-transfected cell lines (N-cad-5 and N-cad-17, N-cad-8 and N-cad-15) was monitored by H&E and staining for human cytokeratin in at least 20 sections of each indicated organ. The number of mice positive for metastasis over the total number of screened mice is indicated. The number of mice that developed primary tumors at the mammary fat pads (*n*) and their mass is indicated by average weight in grams (g) ± SD. Several additional organs such as the kidney, spleen, bone, and brain, and skeletal muscle were tested and found negative. ND, not determined.

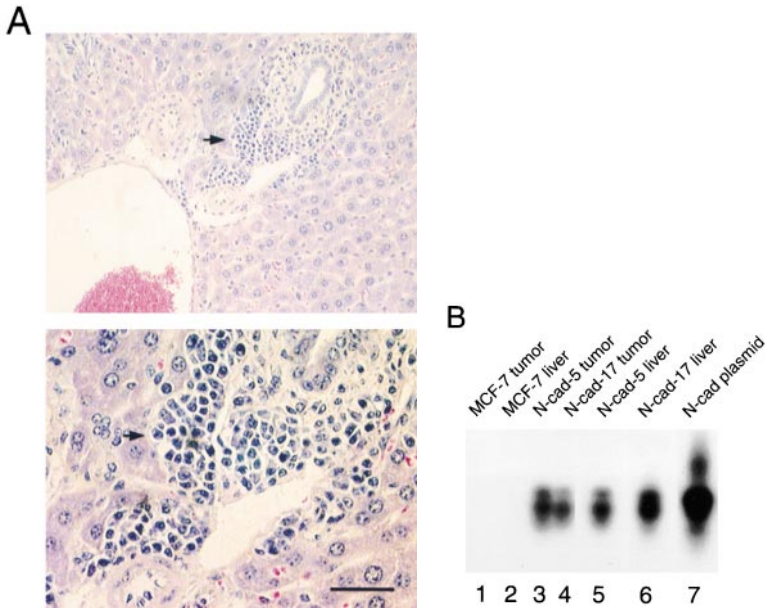

Figure 6. The N-cadherin transgene is present in tumor cells microdissected from liver metastases. (A) Histology of N-cad-17 tumor infiltrates into the mouse liver stained by H &E shown at low (top panel) and high (bottom panel) magnification. (B) The DNA of cells dissected from MCF-7, N-cad-5 and N-cad-17 primary tumors (lanes 1, 3, and 4, respectively) or from liver sections (lanes 2, 5, and 6, respectively) was subjected to PCR and Southern blotting using primers designed to amplify a 200-bp fragment from the transfected human N-cadherin cDNA (see Materials and Methods). Human N-cadherin plasmid DNA was used as a positive control (lane 7). Bar: (A) 100 μm; (B) 33 μm.

growth by a mechanism that involves a cooperative interaction between N-cadherin and FGFR (Williams et al., 1994; Doherty and Walsh, 1996; Saffell et al., 1997; Lom et al., 1998). In an attempt to evaluate whether these two molecules cooperate to promote the metastasis of N-cadherin–expressing MCF-7 cells, we examined the effect of FGF-2 on motility and invasion of control and N-cadherin transfectants. Treatment with FGF-2 did not affect the motility of the Neo-mass cell line, but caused a dramatic increase in motility in all N-cadherin–expressing cell lines (Fig. 8, A and B).

The effect of FGF-2 on the invasive activity of N-cadherin–expressing MCF-7 cells was next assessed in a Matrigel invasion assay after 5 h of incubation (Fig. 9 A). The low basal invasiveness of the N-cad-mass cells and the high invasiveness of the N-cad-5 and -17 clones were all further enhanced by FGF-2 treatment. In contrast, there was no effect on the Neo-mass cell invasion (Fig. 9 A). Of note, we found that the Matrigel-invading N-cadherin–transfected MCF-7 cells appeared on the underside of the filter as cell clusters rather than individual cells. This suggests that the gain of invasive properties by N-cadherin does not involve a switch from an adhesive to a more scattered, mesenchymal phenotype usually observed with invasive carcinomas (Oka et al., 1993; Sommers et al., 1994; Hazan et al., 1997).

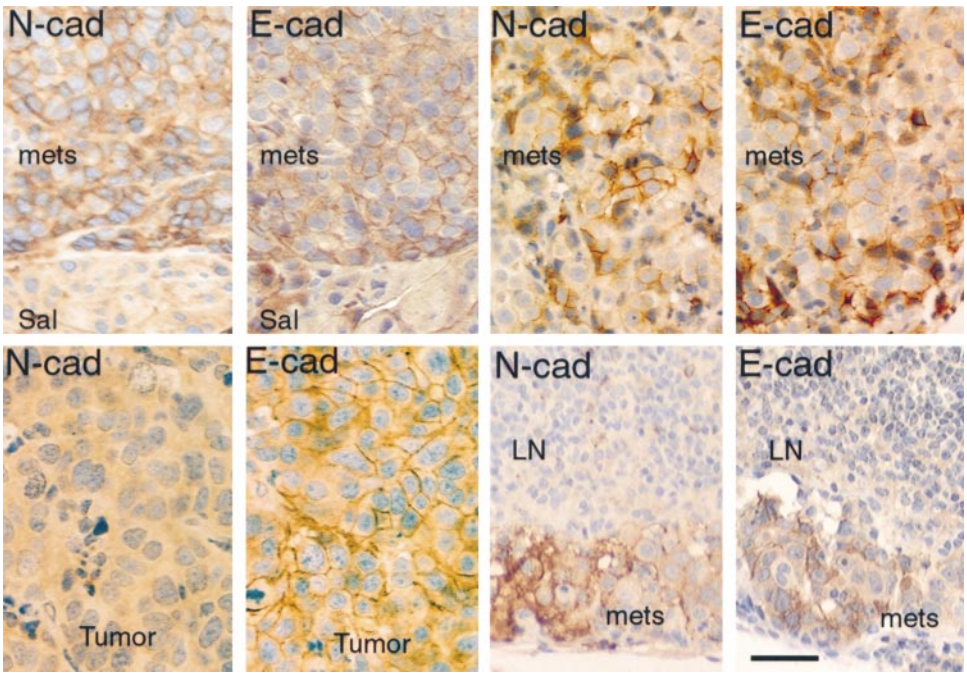

Figure 7. E- and N-cadherin expression in metastatic lesions. Sections of salivary glands (Sal, top left), pancreas (top right); axillary lymph nodes (LN, bottom right), from mice injected with N-cad-5 and N-cad-17 (Table I) were stained with N- and E-cadherin antibodies using a secondary DAB detection. The metastatic cells (mets) express high levels of both N- and E-cadherin. Staining of primary tumors from MCF-7–injected mice (bottom left) shows a positive reaction with E-cadherin but not with N-cadherin antibodies. Bar, 20 μm.

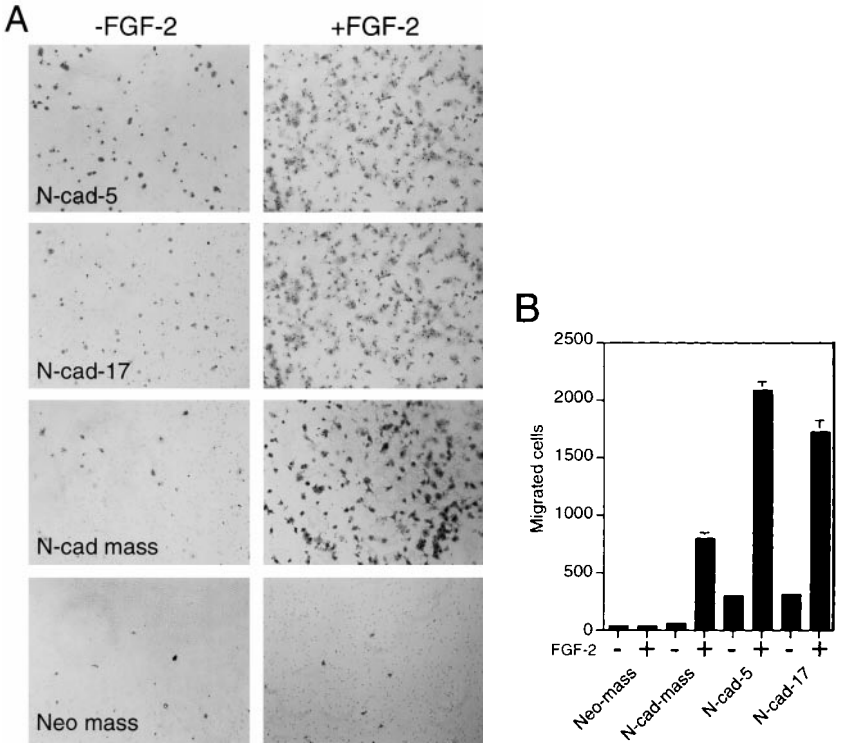

Figure 8. FGF-2 stimulates cell migration of N-cadherin–expressing cells. The motility of control (Neo-mass) and N-cadherin–expressing cells (N-cad-mass, N-cad-5, and N-cad-17), pretreated for 24 h with 10 ng/ml of FGF-2 or left untreated, was measured 5 h after inoculation into the Transwell chambers. (A) The migrating cells were fixed, stained, and photographed; each panel represents an example of 3–6 replicates. (B) Bar graphs represent quantitation of triplicate wells. The experiment was repeated six times.

Since only N-cadherin–expressing cells responded to FGF-2 treatment with increased invasion of Matrigel, we examined whether the response was linked to elevated levels of MMPs, which are well documented ECM-degrading enzymes and whose activity is associated with tumor invasiveness (Coussens and Werb, 1996). MMP activities were measured by a zymography assay of conditioned media from N-cadherin–transfected and control MCF-7 cells, untreated or treated with FGF-2 (Nakajima et al., 1995). Conditioned media collected in the absence of FGF-2 all produced readily detectable small zones of lysis corresponding to MMP-9 and barely detectable zones of lysis corresponding to MMP-2 (Fig. 9 B, lanes 1, 3, 5, and 7). FGF-2 had no effect on MMP production by Neo-mass cells (Fig. 9 B, lane 2). However, the same growth factor dramatically increased the expression of MMP-9 in all N-cadherin transfectants in the absence of detectable effects on MMP-2 production (Fig. 9 B, lanes 4, 6, and 8). The effect of FGF-2 on MMP-9 levels was more pronounced in N-cad-5 and N-cad-17 (Fig. 9 B, lanes 6 and 8) than in N-cad-mass cultures (Fig. 9 B, lane 4), which is consistent with the lower levels of N-cadherin expression in the latter (Fig. 1). Treatment with insulin at a concentration that acts through the IGF-1 receptor produced no effect on MMP levels (Fig. 9 C, lanes 4, 6, and 8) despite the high levels of IGF-1 receptors expressed by MCF-7 cells (Cullen et al., 1990; Surmacz et al., 1998). An additional doublet of 52/46 kD was occasionally observed in conditioned media of FGF-2–treated N-cad-5 and N-cad-17 (Fig. 9 C, lanes 5 and 7), and comigrated with MMP-1 or stromelysin (Herron et al., 1986). The above results imply that N-cadherin expression sensitizes MCF-7 cells to FGF-2, and that this combined effect results in an increased cell migration, enhanced MMP-9 production, and efficient invasion of ECM proteins. Thus, the coordinated action of N-cadherin, FGF-2, and MMP-9 provides a possible mechanism by which N-cadherin may promote cellular invasion and metastasis in vivo.

N-Cadherin Promotes Adhesion to the Endothelium

Additional factors that may critically affect tumor cell metastasis involve interactions between tumor cells and the endothelium. Since endothelial cells are known to express N-cadherin (Salomon et al., 1992), it is possible that the N-cadherin expressed on the surface of tumor cells might promote homophilic interactions with the endothelium. Thus, we tested the ability of control and N-cadherin–expressing MCF-7 cells to adhere to human endothelial monolayers. Whereas N-cad-5 and to a lesser extent, N-cad-17 cells adhered strongly to endothelial cells (Fig. 10, B and C, respectively), control Neo-5 cells exhibited a much weaker adhesion (Fig. 10 A). To control for the relative expression of N-cadherin in these cells, equal amounts of protein extracts from Neo-5 cells, N-cad-5, and HUVEC cells (Fig. 10 D) were immunoblotted with anti–N-cadherin EC1 antibodies. In contrast to control Neo-5 cells, which do not express N-cadherin, HUVEC cells had detectable amounts of N-cadherin, although lesser than that found in N-cad-5 cells. Thus, the ability of N-cadherin–expressing MCF-7 cells to adhere to N-cadherin–expressing endothelial sheets may facilitate their transit through the vasculature and improve their ability to form metastases. In support of these findings, N-cadherin has been shown to mediate the transmigration of melanoma cells through the endothelium (Sandig et al., 1997; Voura et al., 1998).

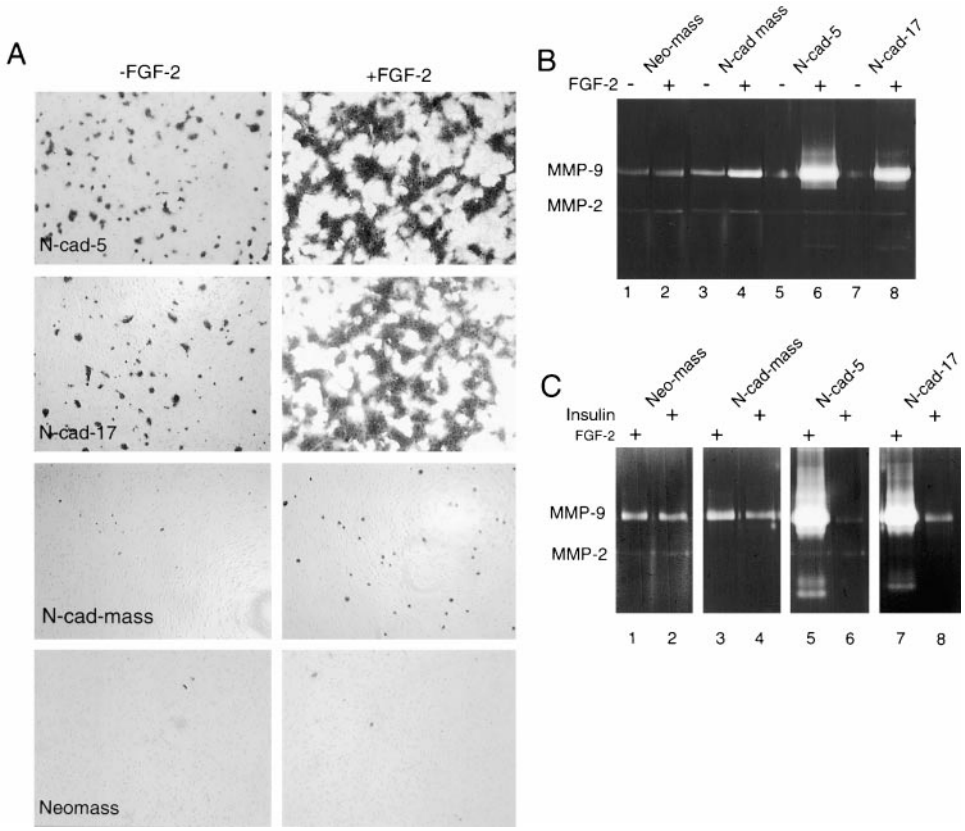

Figure 9. FGF-2 stimulates Matrigel invasion of N-cadherin–expressing cells and expression of MMP-9. (A) Invasion through Matrigel-coated filters of control and N-cadherin–expressing cells, pretreated for 24 h with 10 ng/ml of FGF-2 or left untreated, as measured 5 h after inoculation into Transwell chambers. The invading cells were stained (see Materials and Methods) and photographed using a digital microscope camera. Each panel illustrates a sample representing three to six filters. (B) Gelatinolytic activity of conditioned media of control (Neo-mass) (lanes 1 and 2) or N-cadherin–transfected MCF-7 cells (N-cad-mass, N-cad-5, and N-cad-17; lanes 3–8) that were either treated with FGF-2, or left untreated, as indicated. (C) Gelatinolytic activity of conditioned media of Neo-mass, N-cad-mass, N-cad-5, and N-cad-17 that were pretreated for 24 h either with 10 ng/ml of FGF-2 (lanes 1, 3, 5, and 7, respectively) or 5 μg/ml insulin (lanes 2, 4, 6, and 8, respectively).

Discussion

The present study establishes N-cadherin as a potent inducer of invasion and metastasis. We showed that exogenous expression of N-cadherin in tumorigenic, but weakly metastatic, E-cadherin–expressing MCF-7 cells confers on them the ability to metastasize in vivo. Furthermore, examination of mechanisms that might be responsible for this effect revealed that the presence of N-cadherin resulted in increased adhesion to human endothelial cells, and increased cell migration, and invasion in vitro. Motility and invasion were greatly enhanced by FGF-2, and were accompanied by upmodulation in MMP-9 activity.

Compelling evidence exists to indicate that downregulation of E-cadherin expression or function is a critical factor in the malignant progression of most epithelial tumors (Frixen et al., 1991; Frixen and Nagamine, 1993; Vleminckx et al., 1991; Perl et al., 1998). Transfection of E-cadherin into invasive carcinoma cell lines reduced their ability to invade in vitro, further supporting the role of E-cadherin in maintaining cells in an epithelial state and suppressing the invasive potential of nascent malignant cells (Frixen et al., 1991; Vleminckx et al., 1991). Furthermore, restoration of E-cadherin expression, initially lost in the transition from adenoma to invasive carcinoma in a transgenic mouse model of pancreatic β-cell carcinogenesis resulted in tumor arrest at the adenoma stage (Perl et al., 1998). In the present study, transfection of N-cadherin into MCF-7 cells did not reduce the endogenous expression or the adhesive function of E-cadherin, yet still conferred invasive and metastatic properties. These results suggest

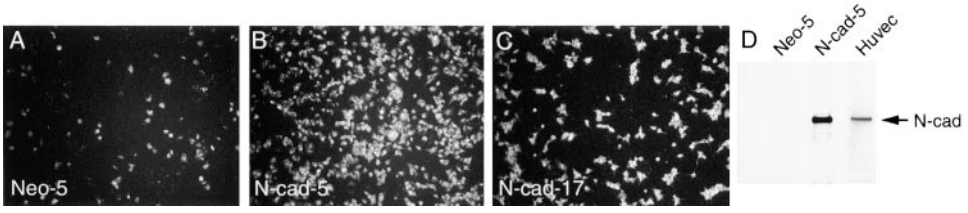

Figure 10. N-cadherin promotes adhesion of MCF-7 cells to HUVEC cells. (A) Each panel shows an unstained (and therefore not visible) monolayer of HUVEC cells incubated with control Neo-5 (A), N-cad-5 (B), or N-cad-17 (C) cells labeled with fluorescent dye (Fast diO) (see Materials and Methods). D represents N-cadherin immunoblotting of cell extracts from HUVEC (right) compared with Neo-5 (left) and N-cad-5 (middle) control cells.

that in this case, N-cadherin has a dominant effect over E-cadherin, which could possibly be due to a higher ratio of N-cadherin relative to E-cadherin in these cells. Alternatively, N-cadherin may activate a metastatic pathway that is coordinated by FGFR and MMP-9 signals in these cells, which can bypass the suppressive signals mediated by E-cadherin. In support of our results, others have shown that exogenous expression of E-cadherin in invasive and N-cadherin–expressing breast cancer cells did not revert their invasive phenotype (Sommers et al., 1991), and that N- and E-cadherin were both colocalized at the invasive edge of squamous tumors (Islam et al., 1996).

The shift in expression from E- to N-cadherin and their mutually exclusive expression pattern in invasive tumor cell lines strongly reflects dedifferentiation from an epithelial to a mesenchymal phenotype often associated with an increased invasive state (Hazan et al., 1997), thus, raising questions as to whether N-cadherin is a cause or a consequence of metastatic progression. In this study, forced expression of N-cadherin in well differentiated MCF-7 cells did not change their epithelial phenotype as indicated by the high levels of cytokeratin and E-cadherin in both the transfected cells and the metastatic lesions, yet these cells metastasized to multiple sites in vivo in spite of forming substantially smaller tumors than parental MCF-7 cells at the primary site. These results suggest an active and dominant role for N-cadherin in an early stage of metastasis, which is independent of the epithelial to mesenchymal transition. Transfected cells also retained their strong adhesive properties in short-term aggregation assays (data not shown) and in the apparently tightly clustered metastases in vivo, suggesting that reduction in cell adhesion is not the mode of action for N-cadherin–induced invasiveness. Rather, it is likely that N-cadherin promotes a state of dynamic adhesion that allows both attachment and detachment of individual cells from the primary tumor and selective association with critical tissues such as the stroma or the endothelium. Consistent with this notion is the fact that individual N-cadherin–expressing cells after detaching from the monolayer can be observed migrating in vitro through the 8-micron pore filter, and then reaggregating on the other side of the filter (data not shown).

In addition to promoting motile and invasive activities of MCF-7 cells in vitro, N-cadherin expression greatly sensitized MCF-7 cells to FGF-2, stimulating marked increases in cell migration and invasion as well as in MMP-9 production. Although, we have not explored the mechanism of the FGF-2 effect and its relationship to N-cadherin, published data provide some insight and support to our findings. For example, N-cadherin has been shown to promote dynamic cellular processes such as neurite outgrowth (Bixby and Zhang, 1990; Doherty et al., 1991), and to require the FGF receptor for this activity (Doherty and Walsh, 1996), whereas a dominant negative FGFR reduced the ability of cerebral neurons to extend neurites on N-cadherin substratum (Lom et al., 1998). These results suggest that N-cadherin cooperates intimately with the FGF receptor to promote its motile activity, probably through a mechanism that involves coclustering of these molecules, which is enhanced by the oligomerization of FGFR by its ligand. In accordance with this idea, we have shown that N-cadherin alone activates a basal level of cell migration that is dramatically enhanced by treatment of cells with FGF-2.

The mechanism by which the combined action of N-cadherin and FGFR leads to increased cell motility and upregulation of MMP-9 remains to be elucidated. However, we speculate that the cooperative action of FGF-2 and N-cadherin in MCF-7 cells involves signal-transducing pathways downstream of FGFR activation (Kouhara et al., 1997). An analogous situation has been described for VE-cadherin, which was shown to be required for the VEGF-A–dependent survival signals in endothelial cells, involving recruitment of β-catenin to the VEGFR/PI3-kinase complex, thus activating AKT signals and cell survival (Carmeliet et al., 1999). Similarly, the increases in MMP-9 expression seen in response to FGF-2 in this study may result from the dynamic interplay between N-cadherin and the FGFR, leading to increased MAPK activation and MMP gene transcription (McCawley et al., 1999; Reddy et al., 1999; Westermarck and Isahari; Zeigler et al., 1998). MMP-9 was further shown to play a predominant role in the process of tumor cell intravasation (Kim et al., 1998), thus, supporting its role in mediating the dissemination of malignant cells. Therefore, we speculate that increased MAPK activity leads to increased MMP-9 levels, which promote degradation of basement membranes and, thus metastasis. Furthermore, treatment of FGF-2–stimulated N-cadherin–transfected cells with nontoxic doses of the zinc-chelator 1,10-phenanthroline, which is known to inhibit MMP catalytic activity (Wang et al., 1999), produced an 85% reduction in invasion of these cells in vitro (data not shown), thus implicating MMP-9, being the only MMP produced by the FGF-2 treated cells, in the invasive process.

In addition to its positive effects on migration and invasion, N-cadherin also facilitated the adhesion of MCF-7 tumor cells to endothelial monolayers, thus promoting what may be a critical step in the breaching of blood vessels by tumor cells (Jahroudi and Greenberger, 1995). Other studies have shown that N-cadherin was enriched at the junction formed between endothelial and melanoma cells during extravasation (Sandig et al., 1997; Voura et al., 1998), suggesting that it actively participates in this process. Thus, the increased metastatic potential of N-cadherin–expressing MCF-7 cells may be due to increased invasion (because of MMP-9) and migration as well as to their ability to enter and exit the circulation by anchoring to the endothelium via N-cadherin.

Altogether, our results suggest that cell adhesion molecules, growth factor–mediated signals, and proteolysis of the ECM converge to enhance cell migration, invasion, and metastasis. This study provides a basis for further characterization of the molecular mechanism of N-cadherin–mediated metastasis, which could yield potential insights for diagnostic or therapeutic applications.

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