Integrin and Cadherin Synergy Regulates Contact Inhibition of Migration and Motile Activity

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Abstract. Integrin receptors play a central role in cell migration through their roles as adhesive receptors for both other cells and extracellular matrix components. In this study, we demonstrate that integrin and cadherin receptors coordinately regulate contact-mediated inhibition of cell migration. In addition to promoting proliferation (Sastry, S., M. Lakonishok, D. Thomas, J. Muschler, and A. Horwitz. 1996. *J. Cell Biol.* 133:169–184), ectopic expression of the α5 integrin in cultures of primary quail myoblasts promotes a striking contact-mediated inhibition of cell migration. Myoblasts ectopically expressing α5 integrin (α5 myoblasts) move normally when not in contact, but upon contact, they show inhibition of migration and motile activity (i.e., extension and retraction of membrane protrusions). As a consequence, these cells tend to grow in aggregates and do not migrate to close a wound. This phenotype is also seen with ectopic expression of β1 integrin, paxillin, or activated FAK (CD2 FAK) and therefore appears to result from enhanced integrin-mediated signaling. The contact inhibition observed in the α5 myoblasts is mediated by N-cadherin, whose expression is upregulated more than fivefold. Perturbation studies using low calcium conditions, antibody inhibition, and ectopic expression of wild-type and mutant N-cadherins all implicate N-cadherin in the contact inhibition of migration. Ectopic expression of N-cadherin also produces cells that show inhibited migration upon contact; however, they do not show suppressed motile activity, suggesting that integrins and cadherins coordinately regulate motile activity. These observations have potential importance to normal and pathologic processes during embryonic development and tumor metastasis.

C ELL migration plays a central role in diverse processes, including embryonic development, wound healing, inflammation, and tumor metastasis. Directional cell migration requires an integrated response to multiple external cues and therefore is likely to require the participation of different families of cell surface receptors (Huttenlocher et al., 1995). However, the mechanism by which these signals integrate to form a coordinated migratory response is poorly understood. Cell surface adhesion receptors, including integrins and cadherins, mediate cell–extracellular matrix (ECM)[1] and cell–cell interactions that play an important role during cell migration. Differential expression of both integrins and cadherins has been associated with changes in the migratory phenotype of cells during both development and other processes, including tumor invasion and metastasis (Hynes and Lander, 1992; Takeichi, 1993; Gumbiner, 1996; Varner and Cheresh, 1996).

Integrin receptors are αβ heterodimers that recognize and bind to components of the extracellular matrix as well as counter-receptors on the surface of cells (Hynes, 1992). In addition to providing a link between the ECM and actin cytoskeleton, integrin receptors serve as signaling receptors that transduce information from the ECM to affect cell behavior and gene expression (Damsky and Werb, 1992; Juliano and Haskill, 1993; Clark and Brugge, 1995). They play an important role during cell migration by linking the extracellular matrix and the actin cytoskeleton and by transmitting the forces required for migration (Lauffenburger and Horwitz, 1996). In addition, signaling through integrin receptors can affect migration independent of their adhesive role (Bauer et al., 1992).

Cadherins are transmembrane glycoproteins that promote calcium-dependent homophilic cell–cell adhesion (Takeichi, 1988, 1995; Gumbiner, 1996). Like integrins, cadherins serve both a structural function, linking to the

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; FN, fibronectin; IL2β1, interleukin-2 β1 cytoplasmic domain; UT, untransfected myoblast.

actin cytoskeleton, and as signaling receptors that affect cell behavior, including cell proliferation (Watabe et al., 1994; Caveda et al., 1996) and differentiation (Larue et al., 1996; George-Weinstein et al., 1997; Redfield et al., 1997). Cadherins promote strong intercellular adhesions, and their expression is associated with decreased tumor cell invasiveness and metastasis in vivo. (Takeichi, 1993). Studies in vitro suggest two probable mechanisms for this inhibition: increased cell–cell adhesion and effects on cell motility (Chen and Obrink, 1991; Chen et al., 1997).

Since both integrins and cadherins play central roles in regulating diverse processes such as differentiation and cell migration, it is likely that these two families of cell surface adhesion receptors act coordinately to regulate these processes. An example of such cross talk between cadherin and integrin receptors has been demonstrated in keratinocytes, where cadherins downregulate integrin expression during keratinocyte differentiation (Hodivala and Watt, 1994). It is likely that integrin expression also alters cadherin expression or function, although this has not been shown previously.

In this study, we show that integrin and cadherin receptors coordinately regulate contact-mediated inhibition of cell migration. Our previous studies have shown that ectopic expression of the α5 integrin in primary myoblasts (α5 myoblasts) promotes cell proliferation and inhibits differentiation through enhanced adhesive signaling (Sastry, S., and M. Lakonishok, unpublished results). Here we show that ectopic expression of either the α5 or β1 integrin subunit or putative downstream effectors of integrin signaling promotes a striking contact-mediated inhibition of cell migration. α5 myoblasts, for example, move normally when not in contact, but upon contact they exhibit inhibition of both cell migration and motile activity (membrane ruffling and lamellipodial activity). This contact-mediated inhibition of migration is mediated by N-cadherin, which is markedly upregulated in the α5 myoblasts. Cells expressing ectopic N-cadherin also remain in contact; however, they do not show inhibited motile activity like the α5 myoblasts. Taken together, our results with primary myoblasts suggest that contact inhibition of migration and motile activity are regulated by a synergy between integrin and cadherin receptors.

Materials and Methods

Primary Cell Culture and Transfection

Myoblasts were isolated from the pectoralis muscle of 9-d-old Japanese quail embryos as previously described (Sastry et al., 1996). Cells were dissociated from muscle tissue with 0.1% dispase (Boehringer-Mannheim Corp., Indianapolis, IN) in PBS. The cells were filtered through a Sweeney filter and plated on 0.1% gelatin-coated tissue culture plates. The cells were grown in myoblast media containing DME (Sigma Chemical Co., St. Louis, MO) with 15% horse serum, 5% chicken embryo extract, 1% pen-strep, and 1.25 mg/ml fungizone (GIBCO BRL, Gaithersburg, MD). Myoblasts were used between passages 1 and 8.

Myoblasts between passages 1–3 were plated on 60-mm tissue culture plates coated with gelatin (0.1%) in myoblast media for 12–24 h (Sastry et al., 1996). For transfections, 8 μg of plasmid DNA and 50 μg of Lipofectamine™ (GIBCO BRL) in 0.3 ml of DME (Sigma Chemical Co.) were added to the cultures for 12–18 h as described. Transient transfection efficiency is ∼40%. Cells were selected by using selection media 0.5 mg/ml of G418 (GIBCO BRL) or 0.25 mg/ml Zeocin (Invitrogen, Carlsbad, CA). Myoblasts were selected for 4–7 d and then maintained in myoblast medium containing either 0.2 mg/ml of G418 or 0.125 mg/ml of Zeocin. Results represent the findings from multiple separate transfections of α5 integrin, N-cadherin, and control plasmids alone, i.e., pRSVneo (Sastry et al., 1996) or pcDNA 3.1/zeo (Invitrogen).

Vector Construction

The eukaryotic expression vector pRSVneo and retroviral plasmid 1654 containing hα5 cDNA were prepared as described previously (Sastry et al., 1996). The chicken N-cadherin (Hatta et al., 1988) and mutant Δ390, which lacks most of the extracellular domain (Fujimori and Takeichi, 1993), were provided by M. Takeichi (Kyoto University, Kyoto, Japan) in Bluescript and were subcloned into the eukaryotic expression vector pcDNA3.1/Zeo (Invitrogen). All restriction enzymes were purchased from GIBCO BRL. The N-cadherin and mutant Δ390 were both excised using SalI and Sma. The pcDNA3.1/Zeo was cut using XbaI, converted to a blunt end, and then cut with Xho. The 3.0-kb N-cadherin cDNA and 2.0-kb Δ390 cDNA were then subcloned into pcDNA 3.1/Zeo. The CH8 epitope–tagged chicken β1 integrin in pBJ-1 vector was provided by Yoshikazu Takada (Scripps Research Institute, La Jolla, CA) (Takada and Puzon, 1993); the chimeric receptor, interleukin-2 β1 cytoplasmic domain (IL2β1) in pCMV-IL2R/β1 cyto, was provided by S. LaFlamme (Albany Medical College, Albany, NY) (LaFlamme et al., 1992); CD2FAK was provided by A. Aruffo (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA) (Chan et al., 1994), and pcDNA3 paxillin was provided by C. Turner (Albany Medical College) (Turner and Miller, 1994). The β1 integrin and IL2β1 were both subcloned into the eukaryotic expression vector pRSVneo (Sastry et al., 1996). A 1-kb hindIII fragment from the CH8β1 pBJ-1 construct containing the CH8 epitope tag was subcloned into β1pRN to produce full-length CH8 β1 cDNA in pRSVneo. IL2β1 was excised from pCMV at NheI and XbaI and ligated in pRSVneo at XbaI.

Flow Cytometry

Flow cytometry was performed as described previously (Sastry et al., 1996). Briefly, cells were washed with PBS and detached using 0.02% EDTA in calcium-magnesium–free Hepes-Hanks buffer (CMF-HH). The cells transfected with human α5 integrin were stained with a specific human α5 antibody (VIF4) from R. Isberg (Tufts University, Boston, MA) at a dilution of 1:5 of hybridoma supernatant in CMF-HH containing 5% goat serum (Sastry et al., 1996). The cells were then stained with an FITC-labeled sheep anti–mouse IgG (Cappell, Durham, NC). N-cadherin expression was determined by staining with the chicken N-cadherin mAb, 6B3, at 20 μg/ml (George-Weinstein et al., 1997). Flow cytometry was performed using an EPICS™ cell sorter (Coulter Electronics Inc., Miami Lakes, FL) equipped with Cyclops software for data analysis (Cytomation, Fort Collins, CO). The human α5–transfected myoblasts were sorted to enrich for a population that was >80% positive for human α5 expression as described previously (Sastry et al., 1996). Cells expressing IL2β1 integrin and β1 integrin also were sorted to enrich a population that was >80% positive for surface expression. Surface expression of α5 integrin, N-cadherin, β1 integrin, CD2FAK, and IL2-β1 integrin were documented by flow cytometry.

Antibodies and Reagents

The rat NCD-2 cadherin function perturbing antibody was purchased from Zymed (S. San Francisco, CA) and the mAb VIF4, which recognizes the human α5 integrin extracellular domain, was a gift of R. Isberg. The β1 integrin antibody W1B10 was purified as described previously (Hayashi et al., 1990) and used at a concentration of 20 μg/ml. The monoclonal antibody against human β1, TS2/16, was from M. Hemler (Dana Farber Cancer Institute, Boston, MA) and used as ascites at a dilution of 1:2000 (Hemler et al., 1984). The N-cadherin mAb, 6B3, was prepared as described (George-Weinstein et al., 1997) and used at a concentration of 20 μg/ml. The α-catenin antibody IG5 and the β-catenin antibody 15B8 were prepared as described (Johnson et al., 1993). The plakoglobin antibody PG5.1 was purchased from IBL Research Products Corp. (Cambridge, MA). A control antibody P3/X63-Ag8 was prepared as described (Kohler and Milstein, 1976). The fibronectin was purified from human plasma by affinity chromatography (Ruoslahti et al., 1982).

Immunofluorescence

Coverslips were coated with fibronectin after being acid washed and etha-

nol treated as described previously (Huttenlocher et al., 1996). The glass coverslips were coated with fibronectin at 10 μg/ml, blocked with 2% BSA, and washed with PBS before plating the cells. After 3 h, the cells (plated at 1×10^4 cells per well in a 24-well plate) were fixed in PBS containing 3% formaldehyde (Ted Pella, Inc., Irvine, CA) for 15 min, treated with 1% Triton X-100 in PBS for 10 min, and blocked in PBS containing 5% goat serum for 30 min. For staining with 6B3, cells were quick-fixed in cold methanol for 5 min. After incubating with the primary mAbs, N-cadherin (6B3), or β1 integrin antibody (W1B10), the cells were washed with PBS three times and then incubated with the FITC sheep anti–mouse at 1:200 in blocking buffer (Cappell). The coverslips were mounted and observed using a fluorescence microscope at 63× (model Axioplan; Carl Zeiss, Inc., Thornwood, NY). Pictures were taken using TMAX 400 film (Eastman Kodak Co., Rochester, NY).

Cell Extraction and Immunoblot Analysis

Cells were prepared for extraction by plating untransfected myoblasts and α5 myoblasts at a density of 2×10^6 cells/60-mm tissue culture plate coated with fibronectin (10 μg/ml) and culturing for 24 h in myoblast medium. The medium was aspirated, the cells were washed with cold PBS, and then 200 μl of cold modified RIPA extraction buffer was added to the plates. The modified RIPA buffer contained 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1.0% Triton X-100, 0.25% sodium deoxycholate, 2 mM EGTA. The following protease and phosphatase inhibitors were added before extraction: 20 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1 mM phenanthroline, 2 mM phenyl-methyl-sulfonyl-chloride, 0.05 U of aprotinin and 30 mM sodium pyrophosphate, 40 mM NaF, and 1 mM sodium orthovanadate. Cells were scraped using a rubber spatula, incubated on ice, and then centrifuged at 14,000 rpm at 4°C for 5 min. Lysates were frozen in liquid N_2 and stored at −80°C. Protein concentration was determined using the Pierce BCA assay (Rockford, IL) with BSA as the standard.

Western blotting was performed by using 5–10 μg of protein, first boiled in Laemmli sample buffer containing 5% β-mercaptoethanol, then separated on 10% SDS-PAGE gels, and finally transferred to nitrocellulose membranes as described (Towbin et al., 1979). Membranes were blocked in 1% heat-denatured BSA in TST buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) overnight at 4°C. The blots were incubated with primary antibody (20 μg/ml of 6B3 or W1B10) for 30 min, washed with TST, and then incubated with secondary anti–mouse antibody conjugated with HRP diluted 1:20,000 in blocking buffer (Jackson ImmunoResearch Labs, West Grove, PA). Blots were visualized using chemiluminescence (Super Signal™; Pierce Chemical Co.). After incubating the membrane with substrate for 5 min, the membranes were exposed to x-ray film. Expression of CD2FAK and paxillin were also documented by Western immunoblotting. Expression of CD2FAK was at times found to be in a cleaved rather than an intact form. The phenotype promoting aggregate growth was only seen when the CD2FAK was expressed in its intact form (data not shown).

Cell Migration Assays

Time lapse video microscopy was performed as previously described (Schmidt et al., 1993). Non–tissue culture plates were coated with fibronectin (10 μg/ml) for 1 h at 37°C and blocked with 2% BSA for 1 h at 37°C. The cells were plated on a 35-mm plate at a density of 0.5×10^5 cells for low-density cell tracking and 5×10^5 cells for high-density cell tracking. The cells were tracked and video images processed using an image processing system (Biological Detection Systems, Inc., Pittsburgh, PA). Transwell assays were performed as described previously (Huttenlocher et al., 1996). Briefly, membranes were coated with fibronectin (10 μg/ml), and cells were plated in myoblast medium and then fixed and stained after 3 h. Wound healing assays were performed by plating cells on a fibronectin substratum (10 μg/ml). After 12–24 h, when the cells were confluent, a wound was made using a pipet tip to scrape off the cells. The wound was observed 24 h later by blinded observers. Number of individual cells in the wound was quantified at 24 h from pictures of multiple fields (three to five) for each experiment. The results of all experiments represent the average of two to five experiments from separate transfections. Transfected cells were compared with untransfected cells and myoblasts transfected with control plasmids for all studies.

Motile activity was studied by observing cells using time lapse video microscopy 10 h after plating the cells. Cells were scored for membrane ruffling at areas of cell–cell contact by observing the cells for 5 min. Cells with visible membrane activity (protrusions and retractions in the range of 2–3 μm) at contact sites were defined as active (similar to changes demonstrated in Fig. 10). A minimum of 50 cells were studied in three separate experiments for each condition.

Results

Ectopic Expression of α5 Integrin in Myoblasts Promotes Aggregate Growth and Contact-mediated Inhibition of Cell Migration

To address the role that integrin expression plays in cell migration, we ectopically expressed α5 integrin subunit in primary quail myoblasts. Myoblasts were transfected with the expression plasmids 1654 and pRSVneo containing human α5 cDNA as previously described (Sastry et al., 1996). Cells expressing pRSVneo or pcDNA3.1/zeo alone were used as controls. After selection in G418, transfected cells were analyzed for surface expression of the human α5 integrin and then enriched by FACS®. All experiments were
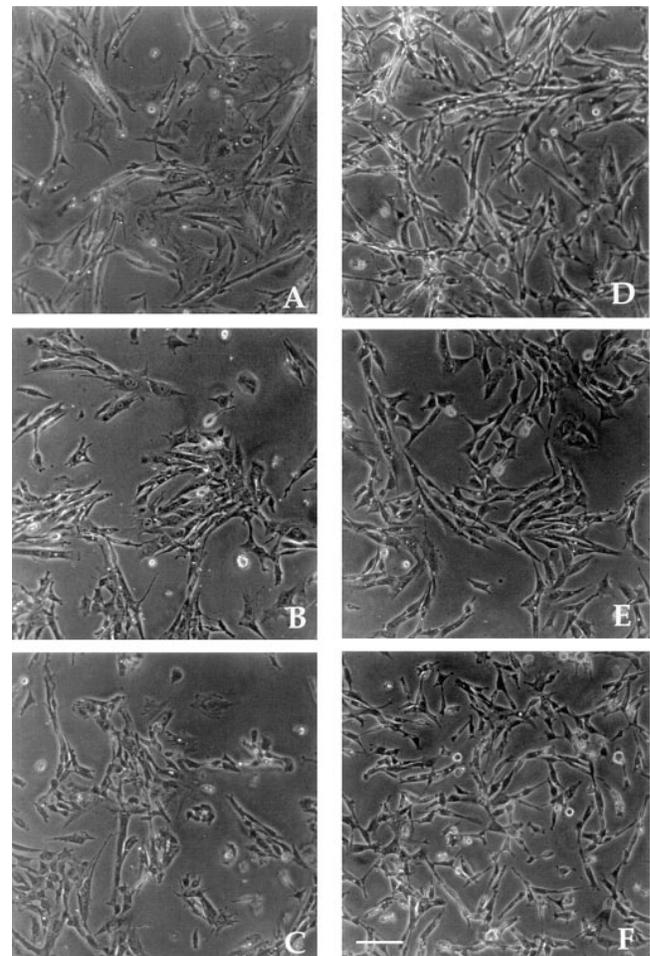

Figure 1. Myoblasts ectopically expressing α5 or β1 integrin grow in aggregates. Cells were plated on a fibronectin substratum and pictures were taken after 24 h. Myoblasts transfected with the α5 integrin (*B*) tend to grow in tight aggregates surrounded by relatively cell-free areas, whereas control myoblasts (*A* and *D*) or myoblasts transfected with control plasmids are more evenly distributed on the substratum and show fewer cell–cell contacts. Ectopic expression of paxillin or β1 integrin also promotes growth in aggregates (*C* and *E*), while ectopic expression of IL2β1 does not (*F*). Bar, 20 μm.
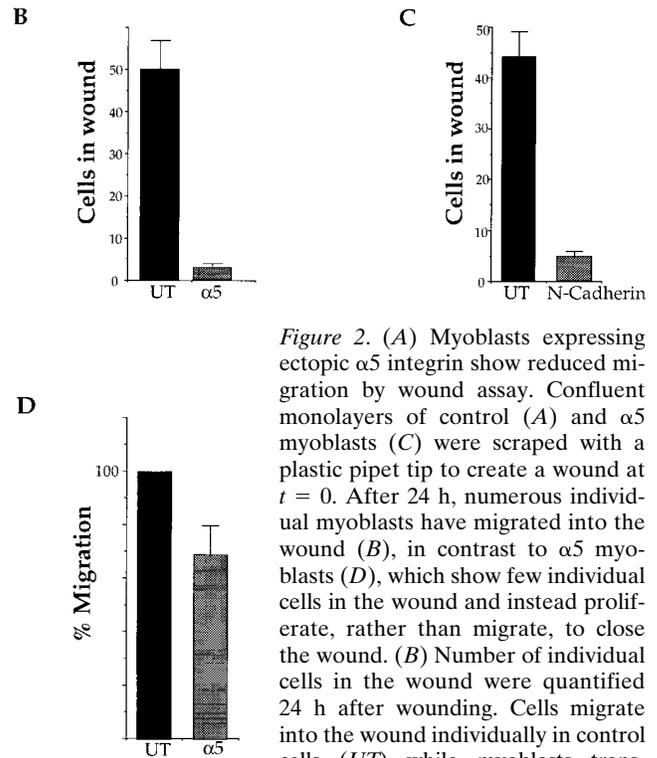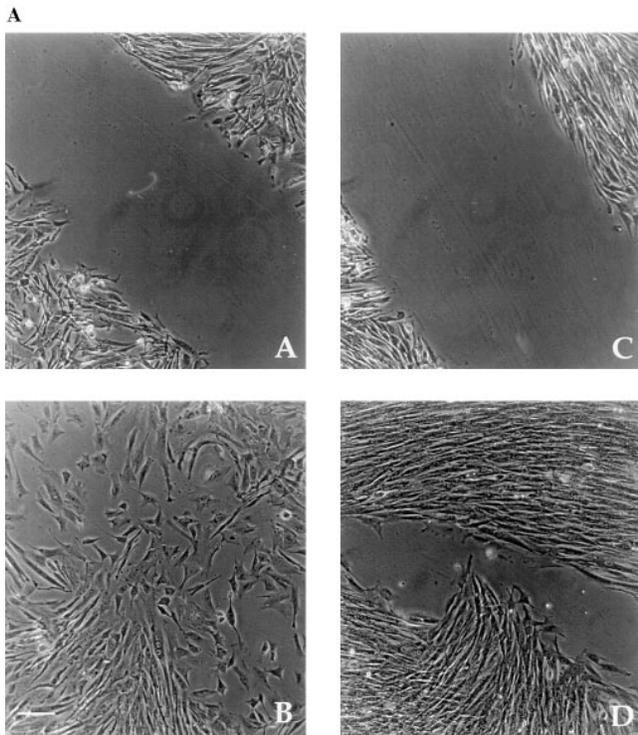
Figure 2. (*A*) Myoblasts expressing ectopic α5 integrin show reduced migration by wound assay. Confluent monolayers of control (*A*) and α5 myoblasts (*C*) were scraped with a plastic pipet tip to create a wound at *t* = 0. After 24 h, numerous individual myoblasts have migrated into the wound (*B*), in contrast to α5 myoblasts (*D*), which show few individual cells in the wound and instead proliferate, rather than migrate, to close the wound. (*B*) Number of individual cells in the wound were quantified 24 h after wounding. Cells migrate into the wound individually in control cells (*UT*) while myoblasts transfected with α5 integrin do not migrate into the wound individually. (*C*) Number of individual cells in the wound were quantified 24 h after wounding in N-cadherin–transfected and control myoblasts. Similar to α5 myoblasts, N-cadherin–transfected myoblasts (*N-Cadherin*) do not migrate into the wound individually (*D*). Migration by Transwell assay is similar in control and α5 myoblasts. Random Transwell migration was assayed by coating the upper and lower surfaces of the membranes with 10 μg/ml of fibronectin. Cells were plated on the upper surface of the membrane, and the number of migrated cells was quantified 3 h later as described in Materials and Methods. The results represent the average of multiple fields from at least three separate experiments and error bars show standard deviation.

performed on a population that was 80–90% positive for α5 integrin expression (Sastry et al., 1996). Cells transfected with hα5 integrin show a three- to fivefold increase in cell surface α5 integrin expression and a twofold increase in expression of β1 integrin with little apparent change in the expression of other integrins (Sastry et al., 1996).

Ectopic expression of α5 integrin in primary myoblasts was shown previously to promote proliferation and inhibit differentiation (Sastry et al., 1996). Myoblasts ectopically expressing α5 integrin (α5 myoblasts) also grow in tight aggregates (i.e., clusters) rather than dispersed like control cells (Fig. 1, *A* and *B*). To determine if the growth in aggregates reflects a change in migration of the α5 myoblasts, wound assays were performed. Migration of cells during wound closure was studied after scraping cells from an area in a confluent monolayer of cells. Closure is inhibited in the α5 myoblasts when assayed 24 h after wounding (Fig. 2 *A*). In comparison to untransfected myoblasts or myoblasts transfected with control plasmids, which migrate into the wound as individual cells, the α5 myoblasts do not migrate into the wound individually. Quantification of the number of individual cells within the wound show that few if any individual α5 myoblasts are found within the wound, in contrast to the control cells (Fig. 2 *B*). Observations by time lapse video microscopy demonstrate that α5 myoblasts eventually close the wound by cell proliferation rather than migration.

In contrast to the wound assay, which examines cells migrating away from a confluent monolayer, migration of α5 myoblasts is not significantly altered in assays that measure migration rates of individual cells. For example, using a short-term Transwell assay (3 h), there is only a small difference in the migration of control and α5 myoblasts (Fig. 2 *D*). Furthermore, there is no significant difference in the migration rates of α5 myoblasts and untransfected myoblasts (or those transfected with control plasmids), as assayed by video microscopy, when the cells are not in contact. The average migration speed of untransfected myoblasts on fibronectin was 17 μm/h with 70% (33/47) of the cells defined as motile (cell speed greater than 5 μm/h), as compared with 15 μm/h in α5 myoblasts, with 60% (16/27) of the cells defined as motile. In contrast, time-lapse video microscopy of higher-density cultures show that migration of α5 myoblasts is greatly inhibited when the cells come into contact, whereas control cells are not (Fig. 3). Thus, the α5 myoblasts show a contact-mediated inhibition of migration that results in a phenotype promoting aggregate growth and delayed wound closure.

Enhanced Integrin-mediated Signaling Promotes Aggregate Growth and Contact-mediated Inhibition of Migration

The α5 phenotype is not specific to the α5 integrin but rather appears to result from an increase in integrin-mediated signaling. Ectopic expression of either the β1 integrin
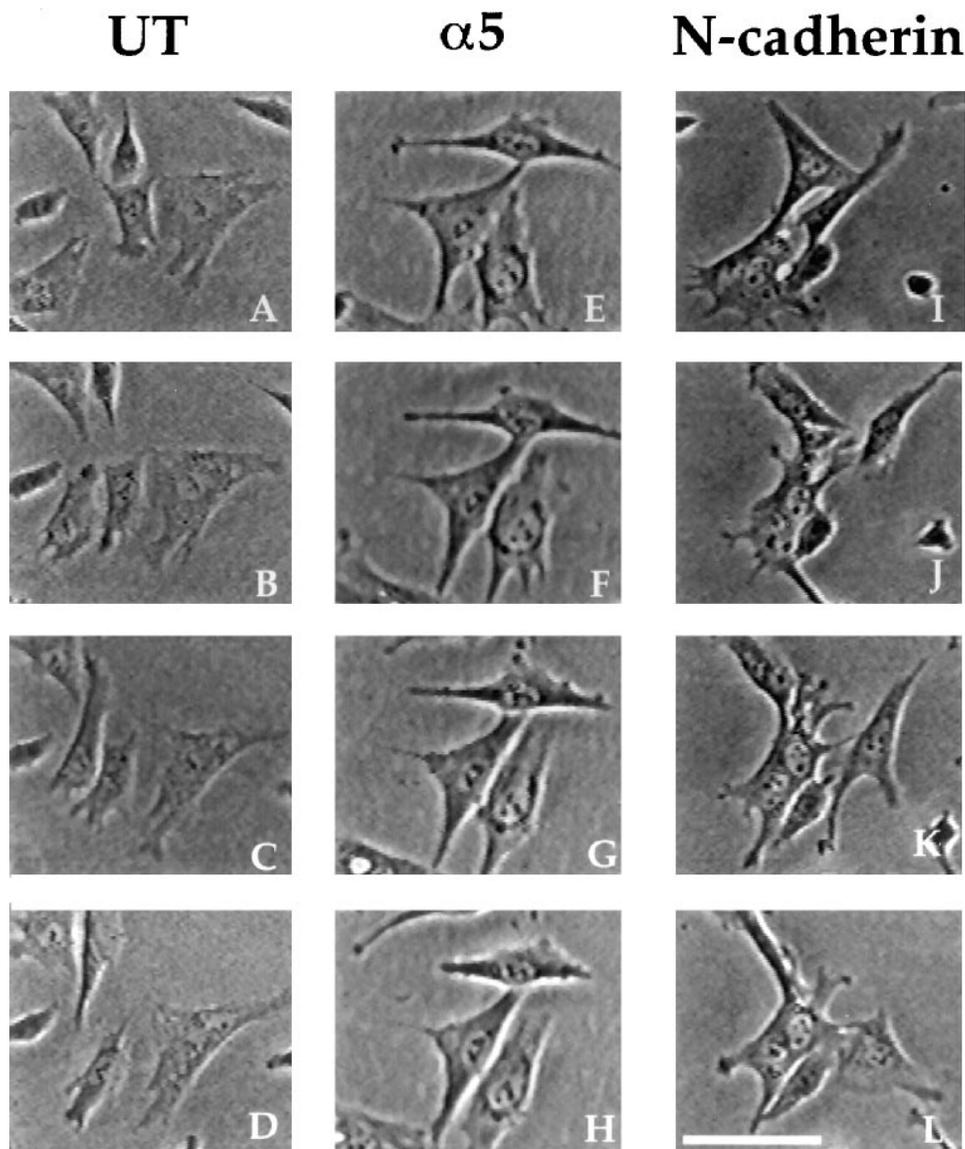
UT   α5   N-cadherin

Figure 3. Effect of ectopic α5 integrin and N-cadherin expression on the motile activity and migration of primary quail myoblasts. Cells were plated on 10 μg/ml of fibronectin, and migration was observed by time-lapse video microscopy. The images represent time-lapse images at 30-min intervals in UT (untransfected; *A–D*), α5-transfected (*E–H*), and N-cadherin–transfected (*I–L*) cells. Control cells (*A–D*) that interact often separate from each other after making cell–cell contacts and do not remain in aggregates. Cells expressing ectopic α5 integrin (*E–H*) tend to reside in aggregates, and when in contact they exhibit reduced membrane protrusion and retraction locally (*E* and *F*) and subsequently globally (*G* and *H*). *E* shows α5 myoblasts after an initial contact; note the protrusive activity on the lower right cell at its edge distal to the contact area. This activity is not seen in *G* and *H*. In contrast, cells ectopically expressing N-cadherin (*I–L*) also do not separate after contact and tend to grow in aggregates. However, they exhibit persistent membrane activity and migration along each other while in contact (*K* and *L*). Note for example, the persistent membrane activity seen in the lower left cell. The time-lapse pictures are representative of pictures of cells observed in multiple experiments. Bar, 20 μm.

or putative downstream signaling molecules results in growth in clusters as well as promoting proliferation and inhibiting differentiation (Sastry, S., and M. Lakonishok, unpublished) (summarized in Table I). Ectopic expression of chicken β1 integrin, for example, promotes aggregate growth (Fig. 1 *E*), delays wound closure, and results in contact-mediated inhibition of migration as measured by time-lapse video microscopy. We find that the level of β1 integrin expressed on the surface of these cells is increased twofold over controls and is similar to the increase in β1 integrin expression in the α5 myoblasts (data not shown).

Downstream integrin-signaling molecules also promote growth in aggregates (Fig. 1, summarized in Table I). Ectopic expression of the integrin-associated protein paxillin promotes growth in aggregates similar to α5 myoblasts (Fig. 1 *C*). Ectopic expression of a membrane-bound constitutively active form of FAK, CD2-FAK, also promotes growth in aggregates when expressed at high levels (data not shown). These data support the idea that it is enhanced integrin signaling rather than increased α5 integrin expression per se that produces these phenotypic changes.

However, the signaling pathways regulating cell proliferation and contact inhibition of migration are separable. The chimeric integrin, IL2β1, has previously been shown to localize to focal contacts and promote the activation of certain integrin-mediated signaling pathways (LaFlamme et al., 1992). We find that ectopic expression of IL2β1 in myoblasts promotes cell proliferation but not contact-inhibition of migration. Cells transfected with IL2β1 grow dispersed (Fig. 1 *F*), and studies by time-lapse video microscopy show that they are not contact-inhibited in their migration (data not shown). In contrast, cells expressing the chimeric receptor IL2α5 cytoplasmic domain show normal differentiation and a dispersed growth pattern comparable to untransfected myoblasts (data not shown). These results support a separation of the signaling pathways regulating these two processes.

Myoblasts Ectopically Expressing the α5 or β1 Integrins Show Increased Cadherin Expression

To characterize the mechanism of the aggregate growth

Table I. Summary of Phenotypes

Cell type	Enhanced cell–cell adhesion	Enhanced proliferation	Upregulated cadherin
Myoblasts	−	−	−
Control plasmid	−	−	−
N-cadherin	+	−	+
α5 integrin	+	+	+
β1 integrin	+	+	+
IL2-β1	−*	+	−
CD2FAK	+	+	+**
Paxillin	+	+	+**

Proliferative data is by Sastry, S., and M. Lakonishok (unpublished results).
*Cells were more dispersed than control cells.
**Cadherin upregulation was only approximately twofold as compared to 5–10-fold for myoblasts expressing the α5 and β1 integrin.

and contact-mediated inhibition of cell migration mediated by the α5 integrin, we determined whether there were any changes in expression of cell surface adhesion receptors due to ectopic α5 integrin expression. Candidate cell–cell adhesion receptors include integrins, cadherins, and members of the immunoglobulin superfamily. Staining with the N-cadherin antibody 6B3 shows increased surface staining of N-cadherin, particularly at the areas of cell–cell contact in α5 myoblasts (Fig. 4). In contrast, staining with neural cell adhesion molecule (N-CAM) antibodies shows no detectable difference in staining patterns between the α5 myoblasts and untransfected cells (data not shown). Staining with the β1 integrin antibody, W1B10, shows an increase in β1 integrin in focal adhesions in α5 myoblasts but no significant difference in the staining patterns at sites of cell–cell contact (Fig. 4).

FACS® analysis confirms the increase in surface expression of N-cadherin in α5 myoblasts when compared with untransfected cells (Fig. 5 *A*). 24 h after plating, α5 myoblasts express approximately five times more N-cadherin

on their surface. Although a small increase in N-cadherin is seen during normal differentiation (1.5–2-fold at 48 vs. 24 h), this is significantly less than that seen with ectopic α5 integrin expression. We also found a small increase (1.5-fold) in N-CAM surface expression in the α5 myoblasts in comparison to control cells (data not shown).

Western immunoblot analyses confirm the increase in N-cadherin expression in α5 myoblasts (Fig. 5, *B* and *C*). Densitometry shows that β1 integrin expression increases approximately twofold in α5 myoblasts (Fig. 5 *C*), whereas N-cadherin expression increases more than fivefold. Myoblasts transfected with control plasmids show no increase in N-cadherin by immunoblotting (data not shown). Ectopic expression of β1 integrin also upregulates N-cadherin more than fivefold in contrast to expression of IL2β1 integrin, in which there is no upregulation of N-cadherin. The results presented in Table I demonstrate a direct correlation between upregulation of N-cadherin expression and enhanced growth in clusters. In summary, enhanced integrin-mediated signaling upregulates N-cadherin expression, demonstrating a cross talk between integrin and cadherin receptor expression.

Cadherin function is mediated, at least in part, by its association with multiple cytoplasmic proteins including α-catenin, β-catenin, and plakoglobin. Cadherin function and signaling may be altered through changes in the levels or functions of these cytoplasmic proteins (Gumbiner, 1995). We therefore characterized the expression of these proteins in myoblasts ectopically expressing the α5 integrin. Significant increases (twofold) in α-catenin and plakoglobin expression are seen in α5 myoblasts when compared with untransfected cells (Fig. 6 *A*). In contrast, there is no significant change in β-catenin expression in α5 myoblasts. Similar changes in expression of the cytoplasmic proteins are seen with ectopic expression of N-cadherin alone (Fig. 6 *B*). These studies demonstrate that in addi-
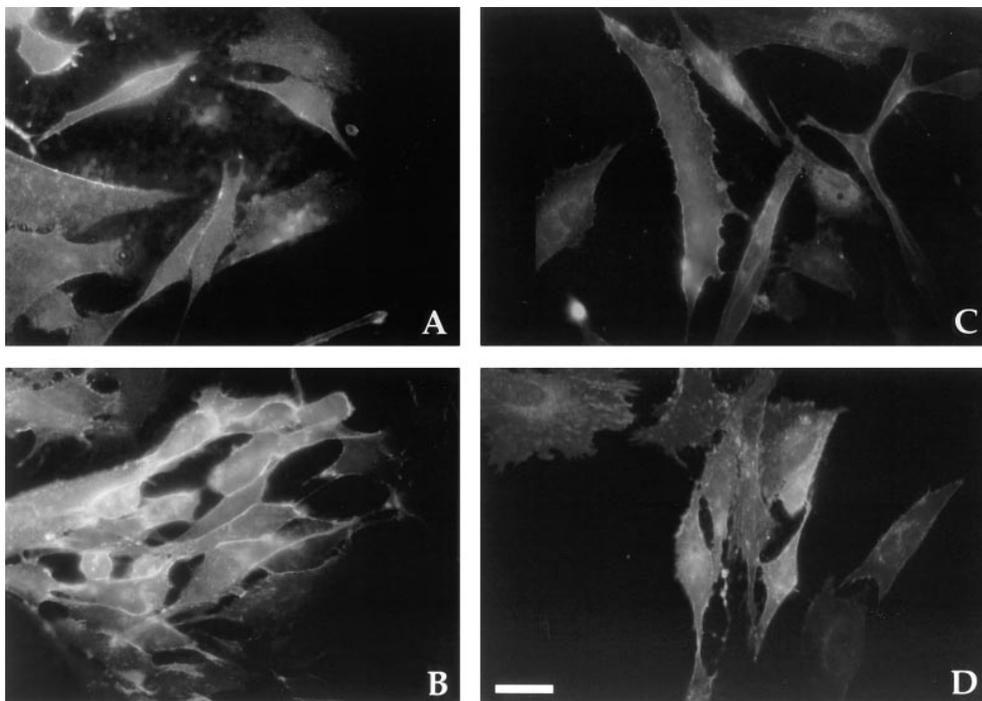

Figure 4. Myoblasts ectopically expressing α5 integrin show increased surface staining for N-cadherin. Myoblasts expressing the α5 integrin and untransfected myoblasts were plated on fibronectin-coated coverslips in myomedium overnight. Cells were stained with antibodies against N-cadherin (*A* and *B*) and β1 integrin (*C* and *D*). α5 myoblasts show markedly increased surface staining for N-cadherin (*B*) when compared with control cells (*A*). Note the high intensity of staining in contact regions. Staining with β1 integrin shows that the α5 myoblasts (*C*) show some increased staining in the focal contacts but not at areas of cell–cell contact (*D*). Bar, 20 μm.
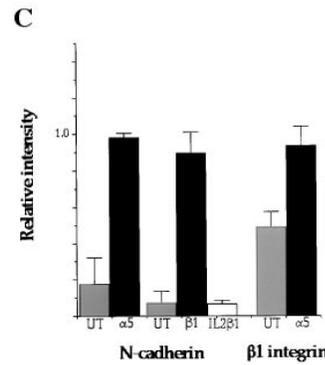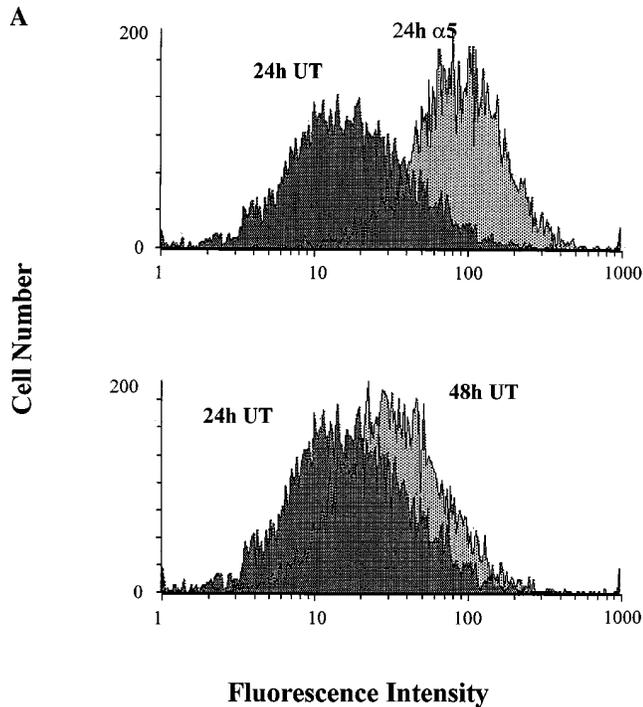
A

B

C

Figure 5. Ectopic expression of α5 integrin in myoblasts results in increased expression of N-cadherin as analyzed by FACS® and immunoblot analysis. (*A*) Analysis by FACS® using the N-cadherin antibody 6B3 shows over a fivefold increase in surface expression of N-cadherin in α5 myoblasts (*24h α5*) when compared with control cells (*24 h UT*). Myoblasts show a small, roughly twofold increase in their N-cadherin surface expression during normal differentiation at 48 h (*48 h UT*) when compared with 24 h (*24h UT*) after plating. (*B*) α5 and β1 myoblasts show increased N-cadherin expression by Western immunoblot analysis using the N-cadherin antibody 6B3. Extracts of untransfected myoblasts (*UT*) and myoblasts expressing α5, β1, and IL2β1 were collected 24 h after plating cells on fibronectin. Densitometry (C) shows a 5–10-fold increase in cadherin expression in α5 and β1 myoblasts at 24 h in comparison to control myoblasts or those transfected with IL2β1. β1 integrin expression increases only twofold at this time in α5 myoblasts as compared with control cells. The densitometry is an average of three separate experiments with the error bars showing standard deviation.

tion to upregulating N-cadherin, ectopic expression of α5 integrin results in increased expression of some cadherin-associated cytoplasmic proteins.

Aggregate Growth and Contact-mediated Inhibition of Migration in the α5 Myoblasts Is Mediated by N-cadherin

To implicate N-cadherin directly in contact-mediated inhibition of cell migration, we determined if perturbing N-cadherin function disrupts the α5 phenotype. Since cadherins are calcium-dependent adhesion molecules, we plated α5 myoblasts in a low-calcium medium that inhibits cadherin function (growth in aggregates) but not cell–substratum adhesion. α5 myoblasts grown in low-calcium medium adhere normally to the substratum but demonstrate a more dispersed pattern of growth than α5 myoblasts grown in calcium-containing medium (Fig. 7). In addition, by time-lapse video microscopy these cells do not show the contact inhibition of migration that characterizes α5-expressing cells grown in normal media (data not shown). These observations suggest that the α5 integrin-mediated contact inhibition is calcium-dependent at concentrations consistent with cadherin function, implicating cadherin receptors in this process.

Function-perturbing antibodies further implicate N-cadherin in the α5 integrin–mediated contact inhibition of cell migration. Pretreatment of α5 myoblasts with NCD-2, a function-perturbing mAb directed against N-cadherin, disrupts the α5 integrin–mediated aggregate growth at concentrations (100 μg/ml) that inhibit cadherin-mediated ad-

hesion (Fig. 8). α5 myoblasts pretreated with this antibody demonstrate a dispersed pattern of growth, similar to that of untransfected myoblasts.

Experiments using the dominant-negative cadherin Δ390 further support a role for N-cadherin in the contact-mediated inhibition of migration. This construct is a nonfunctional cadherin with an intact cytoplasmic domain and a truncated extracellular domain (Fujimori and Takeichi, 1993). α5 myoblasts were transfected with either a control plasmid or the N-cadherin mutant Δ390. Unlike the control plasmid, we were unable to establish stable transfectants expressing the Δ390 mutant. This is consistent with a recent study showing that Δ390 inhibits proliferation and promotes differentiation in developing keratinocytes (Zhu and Watt, 1996). We therefore used transient transfections and studied the effects on wound closure 48 h after transfection. The dominant-negative cadherin partially reverses the inhibitory effect of ectopic α5 integrin expression on wound closure (Fig. 9). The α5 cells expressing the Δ390 N-cadherin display wound closure with cells moving individually into the wound. The number of single cells migrating into the wound was quantified. An average of 31 ± 6.2 (SD) cells in the Δ390-transfected α5 myoblasts as compared with 7 ± 3.8 in α5 myoblasts transfected with control plasmid migrate into the wound.

Ectopic N-cadherin expression in myoblasts also promotes contact-mediated inhibition of migration (Fig. 3). Myoblasts were transfected with chicken N-cadherin (N-cadherin myoblasts), and a stable population was selected. The N-cadherin myoblasts show a level of N-cadherin expression similar to that of α5 myoblasts by both

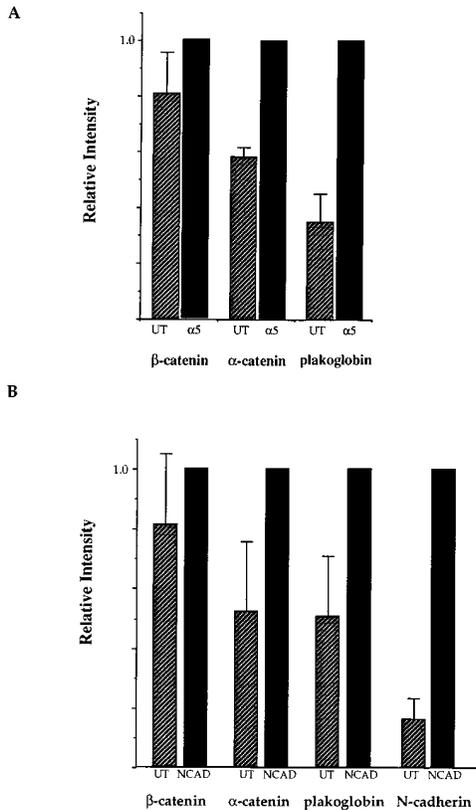*Figure 6.* α5 myoblasts (*A*) and N-cadherin (*B*) transfected myoblasts show increased expression of α-catenin and plakoglobin, but not β-catenin. Densitometry of immunoblots using mAbs against α-catenin, β-catenin, and plakoglobin shows that the α5 myoblasts express an approximately twofold increase in α-catenin and a threefold increase in plakoglobin, but no significant difference in β-catenin when compared with control cells (*UT*). Similar changes are seen in myoblasts transfected with N-cadherin (*B*). The densitometry represents the average of three separate experiments with the error bars showing standard deviation.

Figure 7. Low calcium disrupts the increase in cell–cell clustering of α5 myoblasts. α5 myoblasts were grown in normal medium (*A*) and low calcium (calcium-free media with 300 µM EGTA) medium (*B*) on a fibronectin substratum. α5 myoblasts in low calcium medium display a dispersed phenotype, whereas α5 myoblasts in normal medium grow in aggregates. Observations by time-lapse video microscopy (not shown) show that the α5 myoblasts in low-calcium environment do not display contact inhibited cell migration. Bar, 20 µm.

Western immunoblotting and FACS® analysis (Fig. 6 *B*). The changes in expression of the cadherin-associated signaling molecules, including the catenins and plakoglobin, are similar to the α5 myoblasts (Fig. 6 *B*), although there was no significant change in the expression of β1 integrin in the N-cadherin myoblasts (data not shown). Like the α5 myoblasts, myoblasts expressing ectopic N-cadherin grow as aggregates (Fig. 3, *I–L*) and show a delay in wound closure (Fig. 2 *C*). Interestingly, and in contrast to α5 myoblasts, ectopic N-cadherin expression alone promotes differentiation but not proliferation (data not shown). Taken together, all of these observations provide strong evidence for the participation of N-cadherin in contact-mediated inhibition of migration of the α5 myoblasts.

Contact-mediated Inhibition of Motile Activity in α5 Myoblasts Differs from that of Myoblasts Expressing Ectopic N-cadherin

Two possibilities can be offered to explain the contact-mediated inhibition of migration seen in α5 myoblasts. One is that upregulation of N-cadherin increases cell–cell adhesion, and as a result the cells simply cannot separate from on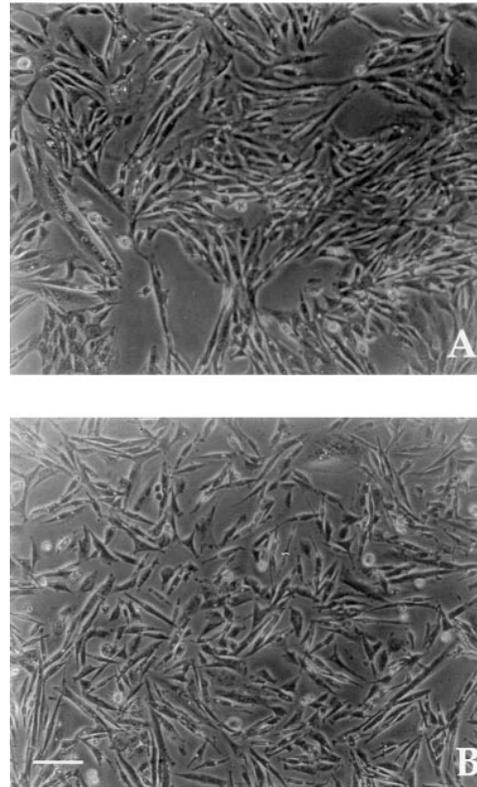e another once in contact. Alternatively, the cell–cell contact may serve a signaling function that inhibits cell movement by negatively regulating motile activity, i.e., membrane protrusions and retractions. To distinguish between these possibilities, the migration of myoblasts transfected with α5 integrin or N-cadherin was observed by time-lapse video microscopy (Fig. 3). In general, when control myoblasts come into contact with one another, their movement and motile activity, both locally (i.e., at the site of contact) and globally, are not significantly inhibited. In contrast, when myoblasts expressing the α5 integrin come into contact, they show a striking inhibition of cell migration and cessation of motile activity first locally and subsequently globally (Figs. 3, *E–H*, and 9). Once in contact, α5 myoblasts generally do not separate from one another. Video microscopic analyses show that upon initial contact (first 30–60 min), the α5 myoblasts tend to have decreased motile activity locally (i.e., membrane protrusions and retractions), and with more prolonged contact (hours), the cells tend to demonstrate global quiescence. An exception to this is seen when cells divide. After a division cells show increased motile activity despite being in contact with other cells; but as these cells subsequently spread, this motile activity decreases. Furthermore, we
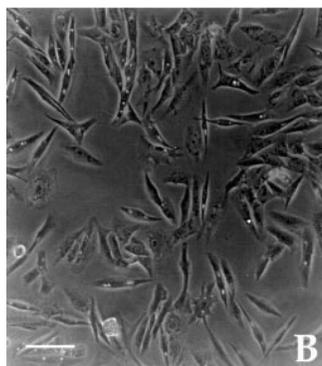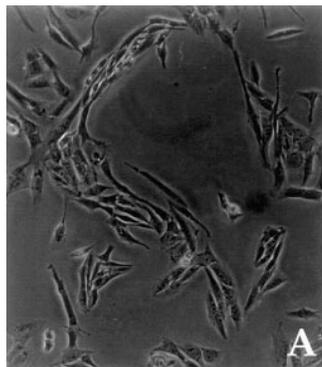
Figure 8. N-cadherin adhesion-perturbing antibodies disrupt the aggregate growth of α5 myoblasts. Cells were pretreated with 100 μg/ml of the adhesion-perturbing antibody NCD-2 and then plated on fibronectin-coated plates. α5 myoblasts treated with NCD-2 at 100 μg/ml (*B*) grow in a dispersed pattern at 24 h in comparison to α5 myoblasts, treated with a control antibody (*A*). Bar, 20 μm.
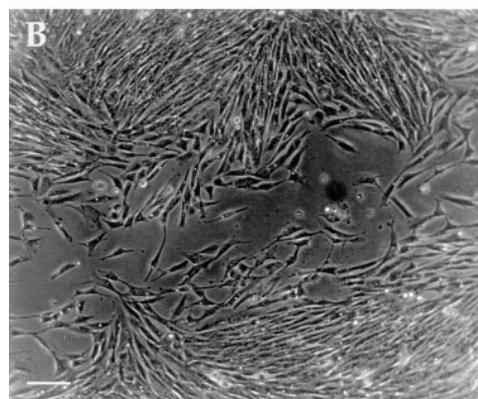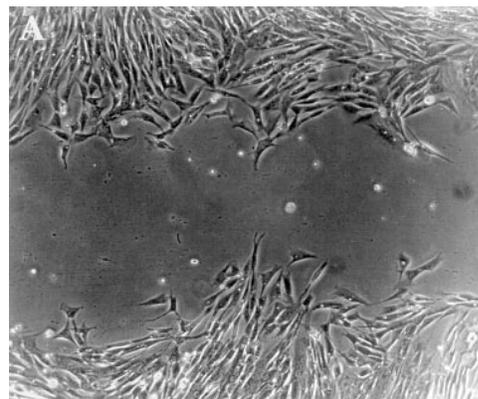

Figure 9. Transient expression of a dominant-negative mutant of N-cadherin disrupts the contact inhibition of migration in α5 myoblasts. Myoblasts ectopically expressing the α5 integrin were transiently transfected with the N-cadherin extracellular domain truncation mutant Δ390. 48 h after transfection, the cells were plated at high density on a fibronectin substratum. A wound was scored, and 24 h later, migration of cells into the wound was assayed. α5 myoblasts expressing the Δ390 mutant (*B*) migrate as individual cells into the wound, whereas few cells are observed in the wound in α5 myoblasts transiently transfected with plasmid DNA containing no insert (*A*). Bar, 20 μm.

generally observed that the less spread cells within an aggregate have increased motile activity. Consistent with these observations are preliminary studies showing that the shutdown in motile activity observed in the α5 myoblasts is most marked at higher substrate concentrations where the cells are more spread. Taken together, these observations suggest that N-cadherin likely mediates a signaling process that inhibits motile activity in α5 myoblasts.

To determine if increased expression of N-cadherin alone is sufficient to induce the cessation of motile activity, we also studied the effects of ectopic N-cadherin expression on the contact-mediated inhibition of motile activity. Myoblasts ectopically expressing N-cadherin display growth in clusters and generally do not separate from one another after making contact. In contrast to the α5 myoblasts, however, N-cadherin myoblasts do not exhibit the striking contact-mediated cessation of motile activity, either locally or globally. The cells continue active membrane protrusions and retractions throughout prolonged contact (Figs. 3, *I–L*, and 10) and frequently migrate along each other within an aggregate. As a result, the clusters of N-cadherin myoblasts are not as tight as those of α5 myoblasts. Therefore, the shutdown in motile activity (i.e., quiescence) seen in the α5 myoblasts seems to require an integrin-mediated event, most likely enhanced signaling and/or cytoskeletal organization, and not just increased cell–cell adhesion.

Discussion

In this study, we show that ectopic expression of the α5 integrin upregulates N-cadherin expression on the cell surface and together with N-cadherin produces a contact-mediated inhibition of cell migration and cessation of mo-

tile activity in contacting cells. This phenotype is not unique to ectopic expression of α5 integrin but appears to reflect enhanced signaling through integrin receptors. The N-cadherin specifically mediates the contact inhibition of migration. Perturbation studies using low calcium conditions, antibody inhibition, and ectopic expression of wild-type and mutant N-cadherins all directly implicate N-cadherin. However, cessation of motile activity requires, in addition to N-cadherin, an increase in expression of either α5 or β1 integrins, or integrin-related molecules, since cessation is not reproduced in cells with only increased N-cadherin. This suggests that while cadherins are necessary for the cessation of motile activity, they are not sufficient. These observations demonstrate that integrin signaling not only regulates cadherin expression in skeletal muscle cells but also acts coordinately with N-cadherin to produce a highly aggregated, nonmotile phenotype. This novel observation has potential importance to normal and pathological phenomena including embryonic development, tumor invasion, and metastasis.

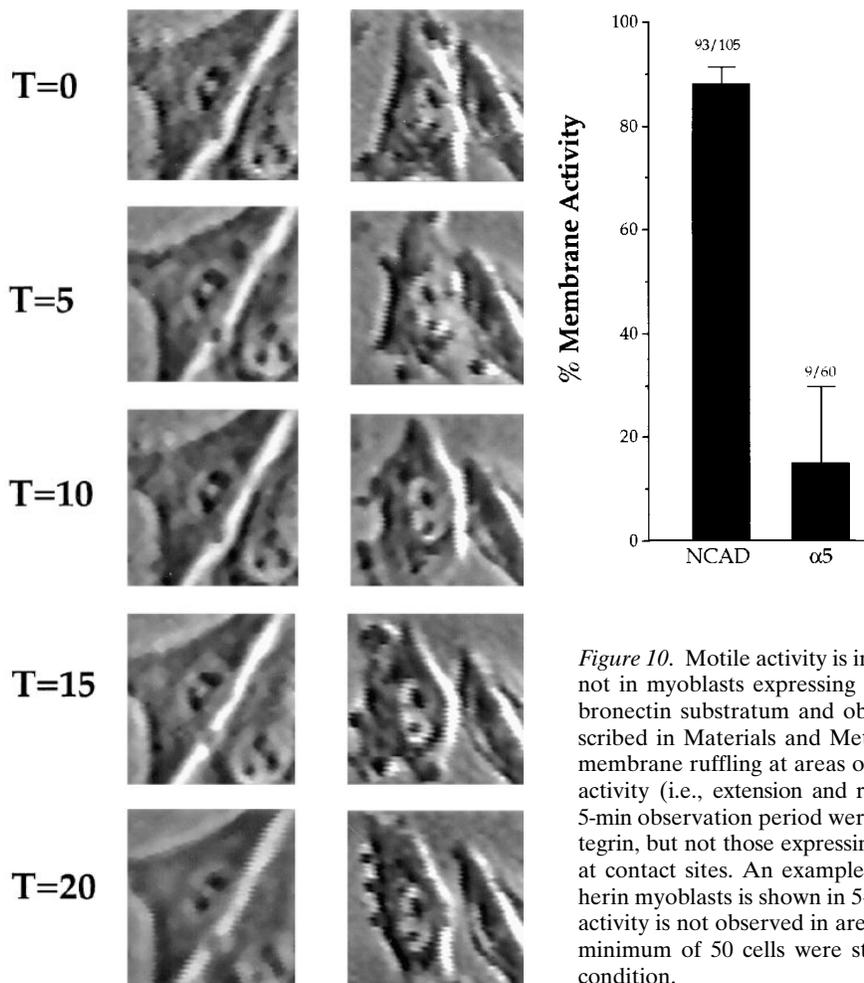Contact inhibition of cell migration was originally de-

Figure 10. Motile activity is inhibited in myoblasts expressing α5 integrin but not in myoblasts expressing ectopic N-cadherin. Cells were plated on a fibronectin substratum and observed by time-lapse video microscopy as described in Materials and Methods. 10 h after plating, cells were scored for membrane ruffling at areas of cell–cell contact. Cells with visible membrane activity (i.e., extension and retraction of protrusions) at contact sites over a 5-min observation period were defined as active. Myoblasts expressing α5 integrin, but not those expressing N-cadherin, show inhibition of motile activity at contact sites. An example of the membrane activity observed in N-cadherin myoblasts is shown in 5-min interval time-lapse sequences; comparable activity is not observed in areas of cell–cell contact with the α5 myoblasts. A minimum of 50 cells were studied in three separate experiments for each condition.

scribed in fibroblasts by Abercrombie and Heaysman (1953), who observed that motile activity, including ruffling and lamellipodium formation, is inhibited locally when fibroblasts make contact. With prolonged contact, the fibroblasts frequently separate and move away from the region of direct cell–cell contact. The contact inhibition of migration in the α5 myoblasts is more dramatic than that described for fibroblasts. After making contact, the α5 myoblasts do not generally separate from one another and instead exhibit an initial local cessation of motile activity followed by global cessation. Ectopic expression of the β1 integrin subunit or a downstream target of integrin signaling, paxillin, produce a similar aggregated phenotype. Thus, the effects on cell aggregation and migration are not unique to the α5 integrin but rather appear to reflect an increase in integrin-mediated signaling. Together, these results suggest that increased integrin-mediated signaling promotes an increase in cell–cell aggregation and reduces migration through an increase in cadherin expression and a coordinate regulation of motile activity. While the role of integrin-mediated adhesion in this contact inhibition of migration is seen most clearly in these ectopic expression experiments, it is likely that it reflects a continuum. In this view, the degree of contact inhibition would depend on the specific levels of cadherin and integrin signaling.

Although ectopic expression of both α5 integrin and N-cadherin both produce highly aggregated cells that do not tend to separate once in contact, they have very different effects on motile activity. Ectopic N-cadherin expression alone does not result in the contact-mediated suppression of motile activity that is seen in ectopic α5-expressing cells. When myoblasts overexpressing N-cadherin come into contact, they do not separate from each other; however, they do continue to move relative to each other and remain active with membrane protrusions and retractions. This difference does not likely arise from differences in N-cadherin, catenin, or plakoglobin expression. The levels of these molecules in the N-cadherin–transfected cells are similar to those in the α5-transfected cells. A more likely hypothesis is that increased integrin and cadherin signaling together promote the shutdown in motile activity, suggesting that these two signaling systems are synergistic. In fact, the data suggest that cadherins are necessary but not sufficient for the shutdown in motile activity. In any case, these results show that the regulation of cadherin-mediated adhesion and motile activity are separable. This is supported by a recent study showing a separation in the regions of the E-cadherin cytoplasmic domain implicated in adhesion and in suppressing motile activity (Chen et al., 1997).

The signaling pathways that promote a cessation of motile activity with cell–cell contact have not been elucidated.

Likely downstream candidates include members of the rho family of small GTP-binding proteins, including rho, rac, and cdc42, since they regulate the actin cytoskeleton (Hall, 1994). Rac, in particular, may play an important role since it stimulates lamellipodial formation and ruffling (Hall, 1994), and its expression is associated with increased invasiveness in vivo (Michiels et al., 1995). Therefore, regulators of rac, such as GTPase-activating proteins or GAPs (Hall, 1994), are likely candidates to be downstream effectors that may negatively regulate motile activity.

In addition to having distinct effects on migration, ectopic α5 integrin and N-cadherin expression also have different effects on proliferation and differentiation. Myoblasts expressing ectopic N-cadherin differentiate but show inhibited proliferation. In contrast, despite comparable levels of N-cadherin expression, the α5-transfected myoblasts continue to proliferate but do not differentiate under normal conditions (Sastry et al., 1996). It is intriguing that ectopic α5 integrin expression promotes contact-mediated inhibition of cell migration but does not inhibit cell proliferation. Interestingly, despite their tight cell–cell contacts, the α5 myoblasts show increased proliferation, suggesting a separation in the pathways regulating contact-mediated inhibition of cell migration and proliferation.

Complex processes such as cell migration and differentiation likely require an integrated response to multiple external stimuli. Evidence for cross-talk between integrin and growth factor receptors has been shown to play an important role in the regulation of cell proliferation, differentiation (Roskelley et al., 1995; Sastry and Horwitz, 1996), and migration (Huttenlocher et al., 1995). Similar integration or cross regulation also occurs between cell surface adhesion receptors. For example, changes in cadherin expression alter integrin expression and thereby affect differentiation (Hodivala and Watt, 1994). Our observations are the first to demonstrate that alterations in integrin expression can result in changes in both cadherin expression and its function (i.e., in promoting a cessation of motile activity with cell–cell contact). We demonstrate a fivefold increase in N-cadherin expression by ectopic α5 or β1 integrin expression in myoblasts. However, ectopic N-cadherin expression does not result in a change in β1 integrin expression in this system (data not shown). Thus, it appears that it is the level or alternatively the ratio of integrin and cadherin signaling that determines the phenotype of migrating cells that come into contact.

Our observations clearly show that the level of integrins and other adhesion-related molecules must be carefully regulated since two- to threefold changes in integrin expression have dramatic effects on cadherin expression and function. Such a highly poised system, in the context of the dynamic patterns of integrin expression during development and other processes, is likely to have significant effects on cell behavior. Examples include cadherin-mediated processes in which cells need to sort out and reposition with respect to each other versus those in which stable, long-term associations form. Myogenic precursors in the somite, for example, interact via cadherins but are highly adherent and quiescent, while in contrast, myoblasts in the limb sort out, through cadherin-mediated interactions involving a more motile type of adhesion.

While our studies focus on myoblasts over-expressing the α5 and β1 integrins, their implications are likely more general and pertain to related phenomena in other cell types. The increase in proliferation seen in α5 myoblasts is also seen in myoblasts ectopically expressing some other integrin subunits, e.g., β1 and β3, but not α6 integrin (Truong, T., unpublished), and β3 (Blaschuk et al., 1997) but not α6 (Sastry et al., 1996). Recent studies on the enhancing effects of α5 integrin and inhibiting effects of the α6 integrin on proliferation point to modulation of β1 integrin signaling as the likely origin (Sastry et al., 1996; Sastry, S., and M. Lakonishok, manuscript in preparation). Homology in the cytoplasmic domains of the β1 and β3 integrins suggest that the phenotype of ectopic β3 arises from similar signaling. It is interesting that expression of the β3 subunit changes as myoblasts initiate terminal differentiation (Blaschuk et al., 1997). It is possible, therefore, that the integrin cadherin synergy functions to facilitate the sorting and alignment of myoblasts in nascent muscle tissue. Our observations are not unique to skeletal muscle, however. It has previously been demonstrated that increased α5 integrin expression is associated with decreased tumorigenicity and may function as a tumor suppressor (Giancotti and Ruoslahti, 1990; Schreiner et al., 1991). We have preliminary evidence suggesting that ectopic α5 integrin expression in colon carcinomas promotes contact-mediated cessation of motile activity (Huttenlocher, A., unpublished), suggesting that these findings may have direct implications for tumor invasion and metastasis.

In summary, our results provide evidence that integrin and cadherin receptors act coordinately to regulate cell migration through a contact-mediated inhibition of migration and motile activity. They demonstrate that enhanced adhesive signaling through ectopic α5 or β1 integrin expression in this case leads to the upregulation of N-cadherin expression and inhibition of cell migration through enhanced cell–cell aggregation and cessation of motile activity. These studies suggest a novel role for integrin-mediated adhesive signaling in promoting contact inhibition of migration through a synergy with cadherin signaling. A coordination of cell–cell and cell–matrix interactions is likely critical to cell migration and cell sorting during embryogenesis and during the invasion of tumor cells in cancer.

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