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Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression

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SUMMARY

Reduced gap junction activity has long been implicated in tumorigenesis. To elucidate the potential role of intercellular communication in melanoma development, we examined gap junctional capability of melanocytic cells from various stages of tumor progression in coculture models using dye transfer assays. Normal melanocytes coupled with keratinocytes by gap junctional formation, whereas melanoma cells did not. Instead, melanoma cells communicated among themselves and with fibroblasts. This switch in communication partners coincided with a shift from E-cadherin to N-cadherin expression during melanoma development. Forced expression of E-cadherin by adenoviral gene transfer in N-cadherin-expressing melanoma cells restored gap junctional compatibility with keratinocytes. Our data suggest that (1) melanocyte transformation is associated with loss of the pre-existing gap junctional activity with keratinocytes but a concomitant gain of communication with a newly juxtaposed cell type, the fibroblasts, (2) the specificity of gap junctional formation during melanoma development is determined by the cadherin profile on the melanocytic cells and (3) the overall gap junctional activity of melanocytic cells is not reduced with transformation.

Key words: Keratinocyte, Fibroblast, Melanocyte, Connexin, Adenoviral gene transfer

INTRODUCTION

Human skin is a multi-layered, cohesive tissue with a unique functional architecture affording the primary barrier to the outside environment. In the epidermis, melanocytes residing at the basement membrane are interspersed among basal keratinocytes. Melanocytes synthesize melanin, an optically dense pigment, for protection from the deleterious effects of ultraviolet light. Under normal circumstances, they undergo controlled self-replication, resulting in a stable, life-long ratio with basal keratinocytes of 1:5-6. During melanoma development and progression, profound histological alterations take place: aberrant cell growth and migration lead to cluster formation, dermal invasion and distant metastasis (Clark, 1991). Compelling evidence suggests that the characteristic, intimate spatial relationship of cells comprising the skin is fundamental for the maintenance of a symbiotic equilibrium of cell growth and differentiation (DeLuca et al., 1988; Scott and Haake, 1991; Shih et al., 1994, Valyi-Nagy et al., 1990, 1993). However, the exact mechanism by which this local control occurs has not been defined. Using various coculture models, we and others have demonstrated that neither soluble factors nor extracellular matrix derived from keratinocytes are able to recapitulate the multiplicity of control (Valyi-Nagy et al., 1990, 1993). Direct cell-cell communication via gap junctions appears to be a good candidate for a role in conserving epidermal homeostasis, which is disturbed during melanocyte transformation.

Gap junctions are clusters of integral membrane proteins, called connexin (Cx). Six Cx subunits are radially arranged around a central 2 nm pore, thereby forming a connexon (hemi-channel) assembly. The half-channels align and interact with complementary connexons present in the plasma membrane of neighboring cells (Holder et al., 1993; Evans, 1994; Ruch, 1994). This provides a direct pathway for small molecules (<2 kDa), which may include ions (Ca^{2+}, H^{+}), secondary messengers (phosphatidyl inositides, cAMP) and metabolic products (amino acids), between the coupled cells (Bennett and Goodenough, 1978). Specificity of gap junction allows formation of communication compartments essential for tissue function and homeostasis. Reduced or loss of gap junctional activity has been implicated in various human cancers such as squamous cell carcinoma (Tada and Hashimoto, 1997), lung cancer (Jinn et al., 1998), gastric carcinoma (Uchida et al., 1995), hepatocellular carcinoma (Krutovskikh et al., 1994; Yamaoka et al., 1995), glioblastoma (Huang et al., 1999) and prostate cancer (Tsai et al., 1996). Exogenous expression of Cxs in Cx- and gap junction-deficient cell lines was shown to restore functional communication, which in turn retarded tumor growth both in vitro and in vivo (Eghbali et al., 1991;

Hirschi et al., 1996; Zhang et al., 1998; Huang et al., 1998). More recent studies aiming at functional abrogation of Cxs by antisense or dominant negative mutant approaches have also demonstrated a promoting effect towards a more malignant phenotype in several cell types (Duflot-Dancer et al., 1997; Omori and Yamasaki, 1998; Krutovskikh et al., 1998). In addition, Cx32 knock-out mice displayed a high incidence of spontaneous and chemically induced liver tumors suggesting that gap junction genes may act as tumor suppressors (Temme et al., 1997).

To investigate the potential involvement of gap junctional perturbation in the pathogenesis of human melanoma, we studied intercellular communication in mono- and cocultures of melanocytic and stromal cells using dye transfer assays. We found that melanocytes and melanoma cells have vastly different communication partner preferences. While melanocytes were compatible with their natural neighbors, the keratinocytes, melanoma cells exhibited communication capabilities among themselves and with dermal fibroblasts. The selective formation of heterotypic gap junctions between skin cells appeared to be linked to cell sorting by specific cadherins. Loss of E-cadherin and upregulation of N-cadherin expression during melanoma progression (Hsu et al., 1996) may endow melanoma cells with new adhesive properties facilitating gap junction formation with N-cadherin-expressing neighboring melanoma cells or dermal fibroblasts. Restoration of E-cadherin expression in melanoma cells by adenoviral gene transfer resulted in re-establishment of gap junctional communication with keratinocytes. Our data suggested that cadherin-mediated cell sorting and locus-specific membrane docking dictate gap junctional specificity in melanocytic cells during tumor development. This selective intercellular communication may contribute to the regulation of growth and survival of melanocytic cells in both physiological and pathological conditions.

MATERIALS AND METHODS

Cell culture

The isolation and culture of normal human melanocytes, and primary and metastatic melanoma cells was performed as previously described (Hsu and Herlyn, 1996; Hsu et al., 1999). Briefly, melanocytes were cultured in MCDB153/L15 medium (v/v: 4/1) supplemented with $CaCl_2$ (2 mM), insulin (5 μg/ml), epidermal growth factor (EGF, 5 ng/ml), 12-O-tetradecanoyl phorbol-13-acetate (10^{-7} M), bovine pituitary extract (40 μg/ml) and 2% fetal bovine serum (FBS). Melanoma cells were cultured in melanocyte growth medium in the absence of EGF, phorbol ester and pituitary extract. Keratinocytes were grown in serum-free keratinocyte growth medium (KGM) containing modified MCDB 153 supplemented with bovine pituitary extract (140 μg/ml), EGF (10 ng/ml), ethanolamine (0.1 mM), hydrocortisone (5×10^{-7} M), insulin (5 μg/ml) and O-phosphoryl ethanolamine (0.1 mM). Cocultures of melanocytic cells and keratinocytes were maintained in KGM. In the presence of keratinocytes, melanocytes have been shown to survive and proliferate without protein kinase C activators such as phorbol ester (DeLuca et al., 1988; Halaban et al., 1988; Valyi-Nagy et al., 1993). In contrast, keratinocytes undergo senescence in melanocyte growth medium where cell growth is inhibited and terminal differentiation is induced by phorbol ester, FBS and high calcium concentration in the medium (Boyce and Ham, 1985; Wille et al., 1985). Primary human dermal fibroblasts were initiated as explant cultures from trypsin-treated and epidermis-stripped neonatal foreskin. These cells were passaged in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Cocultures composed of melanocytic cells and fibroblasts were also grown in DMEM containing 10% FBS. The transcomplementing 293 cells (Graham et al., 1977), a cell line immortalized and transformed by adenovirus E1a and E1b, respectively, were obtained from the Vector Core of the Institute for Human Gene Therapy (University of Pennsylvania, Philadelphia, PA, USA) and grown in DMEM with 10% FBS. All tissue culture reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) except for EGF (Collaborative Biochemical Products, Bedford, MA, USA) and DMEM (Gibco BRL, Gaithersburg, MD, USA).

Antibodies and streptavidin conjugates

The mouse monoclonal anti-human E-cadherin antibody, HECD-1 (IgG_1), was purchased from Zymed Laboratories (San Francisco, CA, USA). The mouse anti-chicken N-cadherin (IgG_1, Clone GC-4) monoclonal antibody (mAb) with cross-reactivity to human tissue was obtained from Sigma. Mel-5, also known as TA99, is a mouse mAb of the IgG_{2a} subclass against tyrosinase-related protein-1 (TRP-1, a melanocytic marker) and was purchased from Signet (Dedham, MA, USA). Monoclonal mouse anti-Cx 43 (IgG_1, catalog no. C13720) was obtained from Transduction Laboratories (Lexington, KY, USA). Mouse mAbs SAP (IgG_1) directed to β3 integrin subunit (Van Belle et al., 1999) and A32 (IgG_1) against MelCAM/MUC-18 (Shih et al., 1994) were generated as previously described. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, FITC- and Cy3-conjugated streptavidin and aminomethylcoumarin (AMCA)-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Biotinylated goat anti-mouse IgG_1 (γ_1 heavy chain specific) was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA).

Immunofluorescence

5 days after coculture, melanocytic cells and keratinocytes were fixed with 3% paraformaldehyde for 12 minutes, and permeabilized by treatment with 0.5% NP-40 in phosphate-buffered saline (PBS) for 1 minute. After incubation with blocking solution (5% BSA in PBS), cells were exposed sequentially to mAb Mel-5 (10 μg/ml), FITC-conjugated goat anti-mouse IgG, anti-Cx43 mAb (monoclonal mouse IgG_1, 10 μg/ml), biotinylated goat anti-mouse IgG_1 (γ_1 heavy chain specific), and Cy3-conjugated streptavidin. All incubations were performed at room temperature for 1 hour.

Dye transfer assay following microinjection for the detection of gap junctional communication

2 days after coculture on glass coverslips with either keratinocytes or fibroblasts (at a 1:5 ratio), melanocytic cells (identified by their multi-dendritic morphology) were microinjected with a mixture of gap junction-permeable neurobiotin tracer (8%; 323 Da; Vector Laboratories, Inc., Burlingame, CA, USA) and gap junction-impermeable rabbit IgG marker (7 mg/ml; 95 kDa; Jackson ImmunoResearch Laboratories, Inc.) in 0.5× PBS. 30 minutes after injection, cells were fixed with 3% paraformaldehyde for 10 minutes at room temperature followed by permeabilization with 0.5% NP-40. The injected melanocytic cells were visualized by the immunoreactivity of AMCA-conjugated goat anti-rabbit IgG (blue). The neurobiotin tracer was stained with FITC-conjugated streptavidin (green).

Semi-quantitative dye transfer assay utilizing fluorescence-activated cell sorting (FACS) analysis

A modified protocol was employed based on methods described by Kiang et al. (1994). Briefly, the donor and recipient cell populations were differentially labeled for 1 hour with calcein AM (0.02 μM; Molecular Probes, Eugene, OR, USA) and CellTracker Orange (0.25 μM; Molecular Probes) in serum-free DMEM, respectively. After washings, the cells were incubated in their optimal growth medium for another hour before use to allow intracellular conversion of calcein AM to gap junction-permeable calcein (623 Da), which emits green

fluorescence. Labeled cells were then seeded and cocultured overnight in triplicate wells of 24-well plates at 1:1 ratio at subconfluence (4×10^5 cells/well). The cocultures were harvested by trypsinization, washed with DMEM containing 10% FBS, resuspended in PBS, and subjected to FACS analysis. In the presence of gap junctional communication, calcein dye was transferred from the donor cells (green) to the recipient cells (red). The percentage of communicating cells was determined by the following formula: (no. of double stained cells/total no. of red cells) × 100.

Flow cytometry

Cultured cells were detached with 10 mM EDTA in PBS, washed once with 0.1% bovine serum albumin (BSA) in PBS, and stained for 40 minutes with 10 μg/ml of primary antibodies at 4°C. After final incubation with FITC-conjugated goat anti-mouse IgG, cells were analyzed by FACS using an Ortho Cytofluorograf 50H connected to a 2150 Data Handling System (Ortho Diagnostics, Inc., Westwood, MA, USA). As a negative control, isotype-matched, non-binding mouse IgG was used.

Construction of replication-defective E-cad/Ad5 adenoviral vector

Full-length human E-cadherin cDNA was a kind gift from Dr David L. Rimm, Yale University, New Haven, CT, USA (Rimm and Morrow, 1994). The adenoviral vector was constructed according to methods described previously (Hsu et al., 2000). Briefly, the 5′ *Xba*I-*Kpn*I fragment of human E-cadherin cDNA was cloned sequentially into the multiple cloning sites of pUC BM20 (Boehringer Mannheim, Indianapolis, IN, USA) and pAd.cytomegalovirus (CMV)-Link.1 (Kozarsky et al., 1993; obtained from Vector Core, Institute for Human Gene Therapy, University of Pennsylvania, Philadelphia, PA, USA) using *Eco*RV and *Kpn*I restriction enzymes. The 3′ *Kpn*I fragment of E-cadherin was then introduced. The resulting shuttle vector (E-cad/pAd.CMV-Link.1) was linearized and cotransfected with the *Cla*I-digested, E1-E3-deleted human adenoviral DNA dl7001 (Ranheim et al., 1993) into 293 cells using calcium phosphate precipitation. After overnight incubation, the cells were overlaid with 0.8% agar in MEM (Gibco BRL) supplemented with 2% FBS and then refed every 4 days. When cytopathic effects were evident, individual plaques were picked and screened by Southern blotting for incorporation of the E-cadherin sequence. Positive plaques were repurified three times in 293 cells to eliminate contamination of wild-type virus or virus without the transgene sequence. Plaque-purified virus propagated in 293 cells was purified by ultracentrifugation in a cesium chloride gradient. Viral titer was assayed by plaque formation in permissive 293 cells. Control adenoviral vector containing the lacZ cDNA (lacZ/Ad5) was purchased from the Vector Core.

Viral infection of melanocytic cells

Optimal viral titer was defined as the minimum amount of virus required to yield the highest overall gene transfer efficiency without apparent alteration in cellular phenotype. In melanocytes and melanoma cells, this is equivalent to 20 plaque forming units (pfu)/cell, which results in 100% gene transfer efficiency. Subconfluent melanocytic cells were infected with 20 pfu/cell of replication-deficient adenoviruses for 2 hours at 37°C in the minimum amount of serum-free DMEM sufficient to cover the culture vessels. Viral suspension was then replaced with regular medium. Cells were allowed to recover for 24 hours before use.

RESULTS

Normal melanocytes but not melanoma cells exhibit heterotypic gap junctional intercellular communication with keratinocytes

Dye transfer experiments designed to detect gap junctional intercellular communication between melanocytic cells from different stages of tumor progression and keratinocytes in monolayer coculture were performed by microinjecting a mixture of the gap junction-permeable tracer Neurobiotin and the gap junction-impermeable rabbit IgG as control. The seeding ratio of 1:5 (melanocytes/keratinocytes) not only was physiological but also created an experimental setting favoring heterotypic dye transfer, since the injected individual donor melanocytic cells were completely surrounded by keratinocytes. Fig. 1A shows that normal melanocytes (FM997) communicated with keratinocytes (FK997) via heterotypic gap junction formation, as indicated by extensive dye coupling. Conversely, the control rabbit IgG remained restricted to the injected melanocytes. Similar results were obtained in cocultures of FM1005 and FK1005 (data not

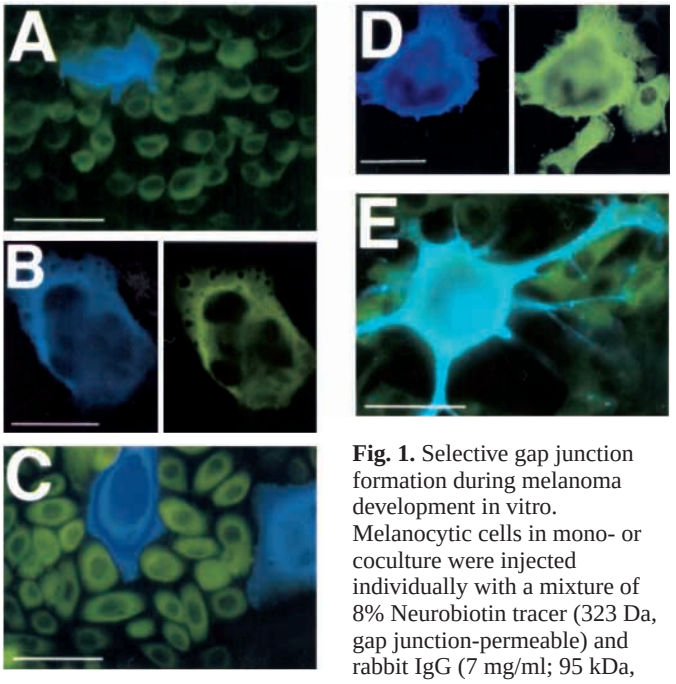


Fig. 1. Selective gap junction formation during melanoma development in vitro. Melanocytic cells in mono- or coculture were injected individually with a mixture of 8% Neurobiotin tracer (323 Da, gap junction-permeable) and rabbit IgG (7 mg/ml; 95 kDa, gap junction-impermeable) in 0.5× PBS. 30 minutes after injection, cells were fixed, permeabilized and stained with FITC-conjugated streptavidin (green staining for Neurobiotin detection) and AMCA-goat anti-rabbit IgG (blue staining). A non-injected culture stained with FITC-streptavidin alone served as a negative control. (A) Normal melanocytes in coculture with keratinocytes. Heterotypic gap junctional communication is indicated by the extensive dye coupling between the injected melanocyte (blue) and surrounding keratinocytes (green). (B) Melanoma cells (WM164) in coculture with keratinocytes. The gap junction tracer (green) remained restricted in the injected melanoma cell (blue), indicating no detectable heterotypic gap junction activity. (C) Microinjected WM1232 melanoma cells (blue) in coculture with keratinocytes. The exceptional WM1232 melanoma cells, which retained endogenous E-cadherin expression, were gap junction-compatible with keratinocytes. (D) Melanoma cells (WM164) in mono-culture. Homotypic gap junction formation led to transfer of the gap junction tracer (green) from the injected melanoma cell (blue) to the neighboring melanoma cells. (E) Melanoma cells (WM164) in coculture with fibroblasts (FF1072). Heterotypic junctional formation was revealed by the dye transfer from the injected melanoma cell (blue) to the adjacent fibroblasts (green). Bars, 35 μm (A); 11 μm (B); 35 μm (C-E).

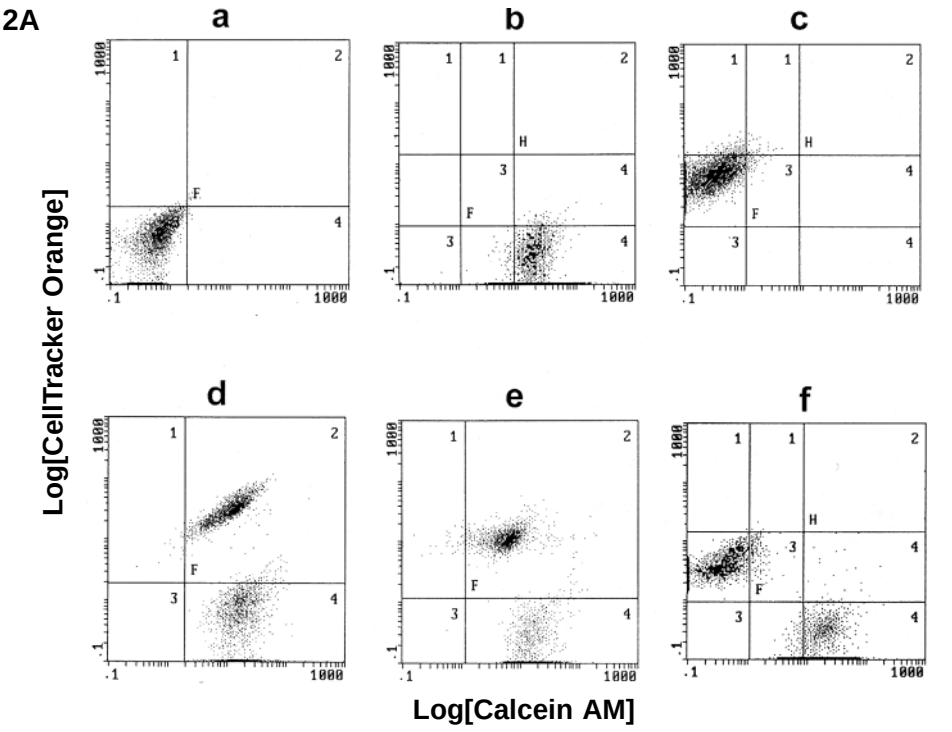
shown). Three out of four melanoma cell lines tested (WM115, WM164 and 1205Lu), exhibited no detectable gap junctional activity with keratinocytes. Representative results from WM164 were shown in Fig. 1B. One single exceptional melanoma cell line, WM1232, however, did couple with keratinocytes (Fig. 1C).

Melanoma cells acquire new communication skills for interactions among themselves and with dermal fibroblasts

Dye transfer assays using the microinjection technique in monocultures of melanoma cells (WM115, WM164 and 1205Lu) and cocultures with fibroblasts (FF1004 and FF1072) revealed formation of both homo- (Fig. 1D) and hetero-typic gap junctions (Fig. 1E). There was extensive dye transfer from the injected donor melanoma cells to the surrounding recipient melanoma cells and fibroblasts. In contrast, normal melanocytes and dermal fibroblasts were not compatible (data not shown). Gap junctional capability of melanocytic cells was further studied in detail using a modified semi-quantitative dye transfer assay based on FACS analyses. We confirmed the switch in communication partners during melanoma progression as demonstrated in Fig. 1. Melanocytes displayed gap junctional communication with keratinocytes (Fig. 2Ae), whereas melanoma cells exhibited gap junctional compatibility only with fibroblasts (Fig. 2Ad,f). Fig. 2B shows the result of a large-scale screening. Almost all melanoma cell lines tested (10 out of 11) displayed gap junctional activity among themselves (Fig. 2B, solid bars). In addition, using fibroblasts as donor cells, our FACS data also demonstrated transfer of calcein dye to the recipient melanoma cells (Fig. 2B, open bars).

Cx expression in melanocytic cells

Gap junction formation requires Cx molecules aligning in hemi-channels. It is known that keratinocytes express multiple Cx isoforms including Cx26, Cx31 and Cx43 (Brissette et al., 1994; Fitzgerald et al., 1994; Salomon et al., 1994; Wenzel et al., 1998) whereas dermal fibroblasts synthesize Cx43



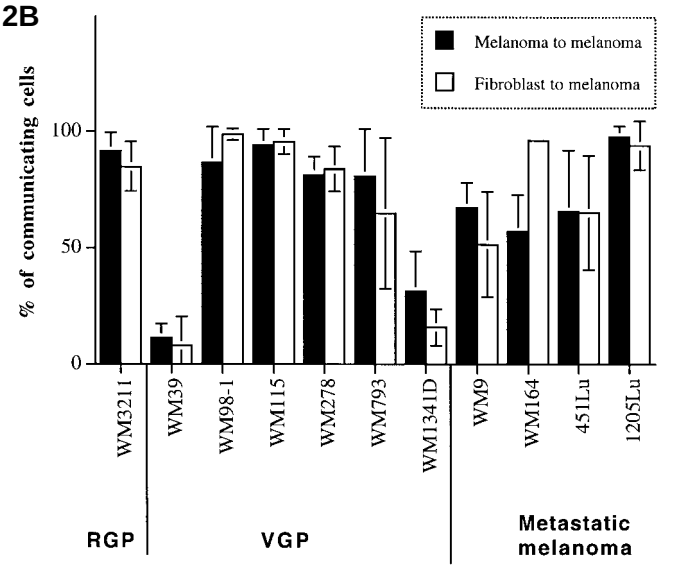


Fig. 2. FACS analyses of heterotypic gap junctional activity in melanocytic cells. (A) FACS distribution of selective heterotypic gap junctional communication during melanoma progression. *y* axes represent CellTracker Orange fluorescence intensity, *x* axes the calcein fluorescence intensity. (a) Cells without preloading of either fluorescence dye, as negative control. (b) Cells pre-labeled with calcein AM only. (c) Cells prelabeled with CellTracker Orange only. (d) Heterotypic dye transfer using melanoma cells (WM164) as donor and dermal fibroblasts (FF2221) as recipient. Heterotypic gap junction was indicated by the migration of CellTracker Orange-labeled recipient fibroblasts away from the *y* axis after receiving calcein dye from donor melanoma cells. (e) Heterotypic dye transfer using keratinocytes as donor and melanocytes as recipient. (f) Coculture of keratinocytes (prelabeled with calcein AM) with WM164 melanoma cells (prelabeled with CellTracker Orange). No substantial dye transfer occurred. The degree of dye transfer between all these heterotypic cell populations is independent of the direction of dye transfer since switch of the dye labeling did not affect the outcome. (B) FACS analyses of homotypic and heterotypic gap junctional activity in melanoma cells from three stages of tumor progression: radial growth phase (RGP) primary, vertical growth phase (VGP) primary, and metastatic melanoma. Differentially labeled cells were mixed and cocultured overnight. Transfer of calcein dye from melanoma to melanoma cells (solid bars) and from fibroblasts to melanoma cells (open bars) was detected by FACS analyses indicating homotypic or heterotypic gap junctional activity, respectively. Values are means ± s.d. of the percentage of communicating cells in triplicate samples.

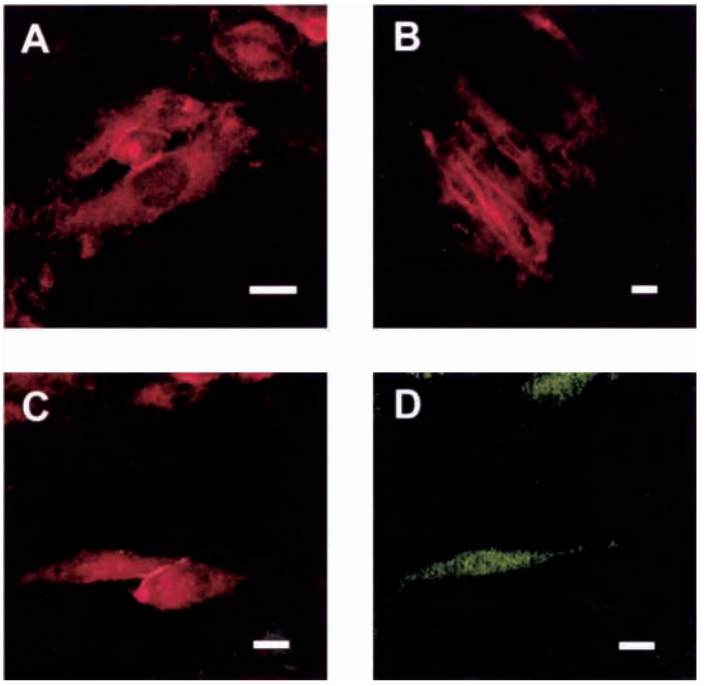
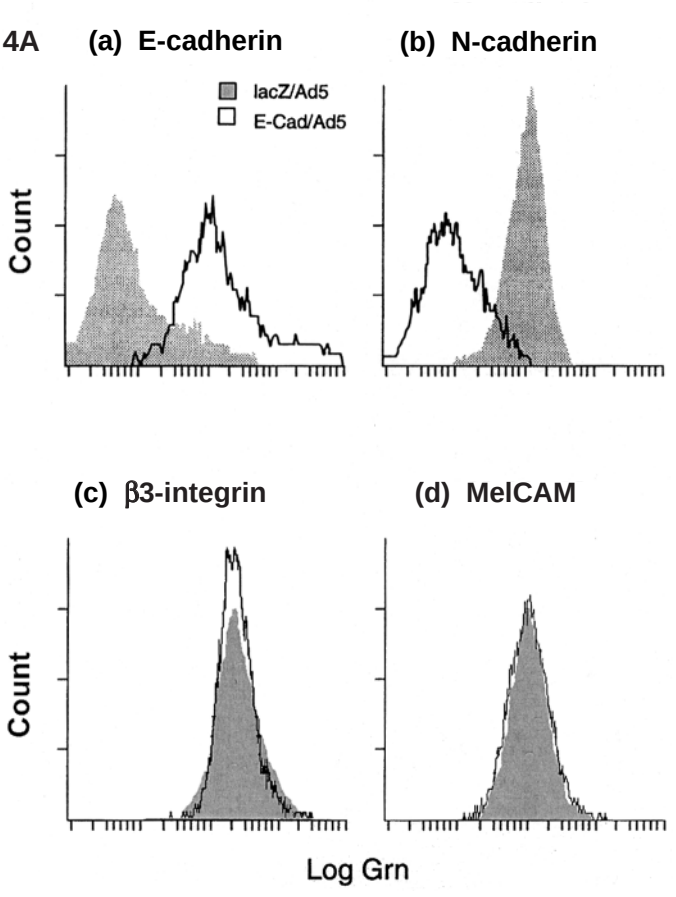


Fig. 3. Expression of Cx43 in melanocytes and keratinocytes in mono- or coculture by immunofluorescence. (A) Cx43 staining of keratinocytes in monoculture. (B) Cx43 staining of melanocytes in monoculture. (C,D) Cx43 staining of cocultured melanocytes and keratinocytes. Melanocytes in coculture were identified by green fluorescence using mAb Mel-5, which recognized pigment cell-specific TRP-1. The expression of Cx43 isotype was detected with specific Ab followed by biotin-conjugated subtype-specific secondary Abs and Cy3-conjugated streptavidin (red). Isotype-matched irrelevant Ab was used as a negative control. Both melanocytes and keratinocytes in coculture reacted with mAb against Cx43 at compatible levels to those in monoculture. Bars, 5 μm.



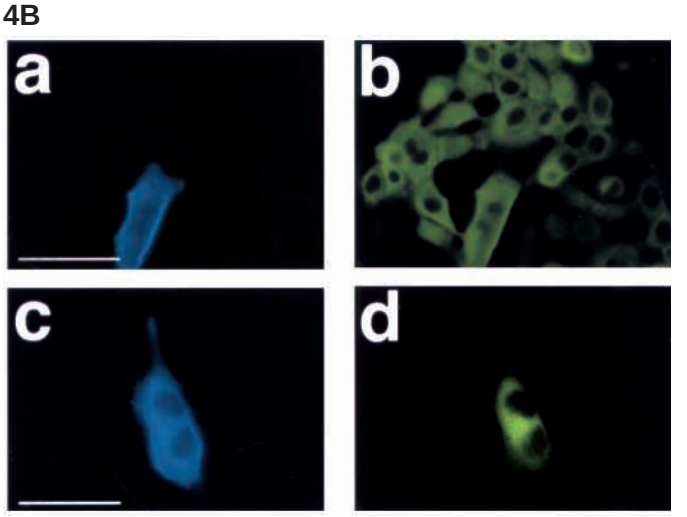


Fig. 4. Induced expression of E-cadherin using an adenoviral vector (E-cad/Ad5) for gene transfer in melanoma cells downregulated N-cadherin and restored gap junctional compatibility with keratinocytes. (A) FACS analyses of transduced melanoma cells. E-cadherin-negative melanoma cells were transduced with E-cadherin or lacZ. 24 hours after transduction, cells were detached, washed and analyzed for the expression of E-cadherin (a), N-cadherin (b), β3 integrin subunit (c), and MelCAM/MUC-18 (d) using isotype-matched irrelevant Ab as negative control. Representative results from WM164 melanoma cells are shown. x axes represent the intensity of fluorescence and *y* axes the cell number. (a) Ectopic E-cadherin expression in E-cadherin-transduced cells (solid line) with lacZ-transduced cells (shaded peak) as control. (b) The concomitant downregulation of N-cadherin in E-cadherin-expressing melanoma cells (solid line). N-cadherin expression in lacZ-transduced cells remained unchanged (shaded peak). In contrast, the expression of other surface molecules such as β3 integrin subunit and MelCAM/MUC-18 (c,d) was not altered after transduction of E-cadherin. (B) Gap junctional communication between melanoma cells and keratinocytes following restoration of E-cadherin expression. Melanoma cells transduced with E-cadherin (a,b) and control lacZ (c,d) were seeded at a 1:5 ratio with keratinocytes. 30 minutes after injection of melanoma cells, cells were fixed and permeabilized. The injected neurobiotin tracer and rabbit IgG marker were visualized with FITC-conjugated streptavidin (green) and AMCA-goat anti-rabbit IgG (blue), respectively. E-cadherin-expressing melanoma cells exhibit heterotypic gap junctions with keratinocytes (a,b), whereas the control lacZ-transduced cells remain gap junction-incompetent (c,d). Bars, 35 μm.

(Clairmont et al., 1996; Zhang et al., 1995), and that both cell types exhibit homotypic gap junctional activity (Salomon et al., 1988; Clairmont et al., 1996; Zhang et al., 1995). Heterotypic gap junction formation between melanocytes and keratinocytes has also been described in mouse cells (Hunter and Pitts, 1981); however, murine melanocytes are topographically different from their human counterparts. Melanocytes in mouse skin predominantly reside deep in the hair shafts and not in the epidermis. To test whether human melanocytic cells express Cx, double immunofluorescence was performed on cocultures using an antibody raised against Cx43. Melanocytes in cocultures were identified by their immunoreactivity with mAb Mel-5 (Fig. 3D). As shown in Fig. 3C, both melanocytes and keratinocytes express Cx43 in coculture. In monocultures, the levels of Cx43 expression in either keratinocytes (Fig. 3A) or melanocytes (Fig. 3B) were similar to their counterparts in coculture (Fig. 3C). In addition, regardless of gap junction-compatibility either with keratinocytes or fibroblasts, melanocytic cells from all stages of tumor progression examined expressed Cx43 by western blotting (data not shown). As a control study, expression levels of E-cadherin in melanocytic cells in mono- or cocultures were also examined and a significant difference was not observed (data not shown).

Ectopic expression of E-cadherin by adenoviral gene transfer restores heterotypic gap junctional communication between melanoma cells and keratinocytes

The lack of correlation between gap junction competence with keratinocytes and the expression of Cx43 in melanocytic cells indicated that the basis for partner selection may rest on other components of the gap junction-forming pathway. Several reports have demonstrated that cadherin-mediated homophilic binding enhanced efficiency of gap junction formation, probably by facilitating the initial recognition of cells and subsequent docking of opposing connexons (Kanno et al., 1984; Jansen et al., 1996). The fact that only the exceptional melanoma cell line (WM1232), capable of forming heterotypic gap junctions with keratinocytes, displayed functional E-cadherin/catenin complexes (Hsu et al., 1996) further supported this notion. Thus, shifts in cadherin profile during melanoma progression from the E- to the N-isotype (Hsu et al., 1996) may be responsible for the observed communication partner switch from E-cadherin-expressing keratinocytes to dermal fibroblasts and melanoma cells, both of which possess functional N-cadherin (Matsuyoshi and Imamura, 1997; Matsuyoshi et al., 1997).

To study the role of cadherin expression in determining the appropriate communication partner during melanoma development, we re-expressed E-cadherin in E-cadherin-deficient melanoma cells using adenoviral gene transfer. Surprisingly, E-cadherin transduction inhibited N-cadherin expression in melanoma cells (Fig. 4A), although the degrees of N-cadherin downregulation varied among the four cell lines tested (WM115, WM164, WM852 and 1205Lu). The best result was obtained in WM164; ectopic expression of E-cadherin nearly abolished endogenous N-cadherin expression with a positive staining percentage dropping from 96.8% (in lacZ-transduced cells) to 13% (Fig. 4A). This E-cadherin-induced reciprocal regulation appeared to be specific to N-cadherin, since the expression of cell surface molecules of the integrin (β3 subunit of the vitronectin receptor) and immunoglobulin gene superfamily (MelCAM/MUC-18) was not affected (Fig. 4A). In addition, the expression level of Cx43 was compatible before and after transduction by immunofluorescence (data not shown). The transduced cells were then tested for junctional competence with keratinocytes in cocultures. The same seeding ratio (melanoma cells:keratinocytes = 1:5) as employed previously was used to demonstrate heterotypic transfer. The coupling between E-cadherin-expressing melanoma cells and keratinocytes through gap junctions was evident by the dye transfer from the injected melanoma cells to the surrounding keratinocytes (Fig. 4Ba,b) whereas the lacZ/Ad5-transduced cells remained gap junction-deficient (Fig. 4Bc,d).

DISCUSSION

Impaired or absence of gap junctional communication is known to associate with carcinogenesis (Yamasaki et al., 1995). Here, we provide evidence for the first time that tumorigenesis in melanocytic cells involves not only the alleviation of pre-existing gap junctional communication with epidermal keratinocytes but also the establishment of new interactions between adjacent cancer cells and stromal fibroblasts as the melanoma cells escape from the epidermis, and invade and colonize the dermis. Consistent with results from studies using murine, rat or avian cells (Matsuzaki et al., 1990; Musil et al., 1990; Holden et al., 1997; Jansen et al., 1997; Prowse et al., 1997; Rundus et al., 1998), our transfection data also indicate that gap junction assembly depends on cadherin-mediated specific cell-cell recognition. Other divergent effects of exogenous cadherins on gap junctional communication have been reported, including inhibition (Wang and Rose, 1997) and no changes (Woodward et al., 1998). These may reflect fundamental differences of species and cell types studied.

In the normal epidermis, E-cadherin serves as an intercellular glue between melanocytes and keratinocytes, enabling gap junctional communication. During melanoma development and progression, loss of E-cadherin and upregulation of N-cadherin expression resulted in a switch of communication partners to N-cadherin-expressing adjacent melanoma cells and dermal fibroblasts. One melanoma cell line, WM1232, which displayed functional E-cadherin complexes and retained gap junctional competence with keratinocytes, was an exception to the rule. Interestingly, this line was derived from a familial melanoma patient (Hsu et al., 1999). We speculate that melanomagenesis in this case might involve a pathway differing from that commonly seen in sporadic tumors. Our dye transfer assays using FACS-based techniques demonstrated that all but one melanoma cell lines examined displayed gap junction compatibility among themselves and with fibroblasts. This communication-deficient, VGP melanoma cell line, WM39, was previously shown to be negative for N-cadherin expression (Hsu et al., 1996).

Using an adenoviral gene transfer technique, we achieved a high efficiency of E-cadherin expression in melanoma cells. Interestingly, overexpression of E-cadherin downregulated endogenous N-cadherin in melanoma cells. Similar findings

have previously been reported by Li et al. (1998) in SCC9 cells. Conversely, inhibition of N-cadherin by means of antisense transfection in human squamous carcinoma cells was shown to upregulate E-cadherin expression (Islam et al., 1996). Taken together, these observations reflect a global, intrinsic reciprocal regulation of cadherin expression.

Gap junction formation between different cell types producing communication compartments within a given organ is not unique to the skin. Similar heterotypic gap junctions have been identified between endothelial cells and smooth muscle cells within the arteriolar wall (Little et al., 1995), and between astrocytes and pinealocytes in the brain (Cieciura and Krakowski, 1991). Like the skin, these are areas where multiple cell populations in close vicinity are functionally required to maintain domains of homeostasis. In addition to the stromal fibroblasts, we were able to demonstrate that N-cadherin-expressing endothelial cells establish gap junctions with melanoma cells (data not shown). This further confirms that gap junctional communication with melanoma cells is N-cadherin-dependent but not cell type-specific.

The mechanistic details of how the different heterotypic gap junctions serve to coordinate epidermal morphogenesis and melanocytic transformation remain at present unclear. The properties of gap junctions, such as size permeability and ionic conductivity, are dependent upon the molecular make-up of Cx isoform(s). Complicating the situation further, the current of message transmissions was shown to be either uni- or bidirectional in heterotypic channels (Robinson et al., 1993; Harris and Bevans, 1998). Using two different dye transfer assays based on microinjection and FACS techniques, we demonstrated bidirectional coupling between melanoma cells and fibroblasts as well as between melanocytes and keratinocytes. It is conceivable that alterations in gap junction signaling may directly stimulate melanomagenesis or simply allow tumor progression towards a more malignant phenotype through the loss of normal homeostatic growth regulation. Further elucidation of the molecular components of the partner-specific gap junctional signaling pathways in skin cells may provide new insights into the pathogenesis of human melanoma.

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