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Inactivation of the Integrin β6 Subunit Gene Reveals a Role of Epithelial Integrins in Regulating Inflammation in the Lungs and Skin

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Abstract. The integrin αvβ6 is only expressed in epithelial cells. In healthy adult epithelia, this receptor is barely detectable, but expression is rapidly induced following epithelial injury. Mice homozygous for a null mutation in the gene encoding the β6 subunit had juvenile baldness associated with infiltration of macrophages into the skin, and accumulated activated lymphocytes around conducting airways in the lungs. $\beta6^{-/-}$ mice also demonstrated airway hyperresponsiveness to acetylcholine, a hallmark feature of asthma. These results suggest that the epithelial integrin αvβ6 participates in the modulation of epithelial inflammation. Genetic or acquired alterations in this integrin could thus contribute to the development of inflammatory diseases of epithelial organs, such as the lungs and skin.

INTEGRINS are heterodimeric receptors for extracellular matrix and cell surface ligands that have been suggested to play important roles in development, inflammation, wound healing, and tumorigenesis (Hynes, 1987, 1992; Ruoslahti and Pierschbacher, 1987). In vitro, integrins have been shown to contribute to cell adhesion, spreading and migration, and to more complex processes including cell proliferation, apoptosis, and the regulation of gene expression (Hynes, 1992; Juliano and Haskill, 1993). In vivo, the biological importance of integrins has been most clearly demonstrated for control of leukocyte migration and platelet aggregation. At least some integrins play critical roles in development, as demonstrated by the findings that the inactivation of the genes encoding two different integrin α subunits, α4 (Yang et al., 1995) and α5 (Yang et al., 1993), are embryonic lethal mutations. However, although epithelial cells express several different integrins, the roles epithelial integrins play in health and disease remain largely unknown.

The integrin β6 subunit is expressed exclusively in epithelial cells, and only in a single integrin heterodimer, αvβ6, a receptor for the extracellular matrix proteins fibronectin (Busk et al., 1992; Weinacker et al., 1994) and tenascin (Prieto et al., 1993). αvβ6 is highly expressed in the lung, skin, and kidney during organogenesis (Breuss et al., 1995). In epithelia of healthy adults, this receptor is expressed at very low levels, except in the endometrium, where αvβ6 is highly expressed during the secretory phase of the menstrual cycle, and in the lung and kidney, where αvβ6 is often expressed in a patchy distribution associated with subclinical inflammation (Breuss et al., 1995). αvβ6 is highly expressed in response to injury or inflammation, and in malignant epithelial neoplasms (Breuss et al., 1995). For example, in experimental skin wounds, αvβ6 is highly expressed in the keratinocytes at the wound edge within a few days of wounding, where it remains expressed until soon after wound closure is complete. In the respiratory epithelium, αvβ6 mRNA expression is induced within 5 h of acute injury, and αvβ6 protein can be detected in the epithelium of patients with a variety of inflammatory lung diseases (Breuss et al., 1995; Weinacker et al., 1995). These findings suggest a role for this receptor in the response of epithelia to injury.

To examine the role(s) that αvβ6 plays in vivo, we have generated mice lacking β6 expression using homologous recombination in embryonic stem cells. These mice develop and reproduce normally, but develop functionally significant infiltration of their skin and lungs with inflammatory cells. In the conducting airways of the lung, these morphologic changes are associated with enhanced bronchoconstrictor sensitivity to acetylcholine, the central physiologic abnormality in human asthma.

Materials and Methods

Inactivation of the β6 Subunit Gene in Mouse Embryonic Stem Cells

We initially amplified a 240-bp fragment of mouse β6 cDNA by polymerase chain reaction (PCR) with degenerate mixtures of oligonucleotides based on the human and guinea pig β6 sequences (Sheppard et al.,

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1990). We then used the resultant fragment as a probe to screen a genomic mouse 129 strain library, and obtained a 15-kb clone containing two exons and portions of three large introns. The most 5′ exon extended to within 50 amino acids of the predicted 5′ end of the mature protein, predicted on the basis of the human sequence. We used a 7-kb EcoRI fragment of this clone to construct a replacement vector that contained a neomycin resistance gene inserted into the second of the two exons in our clone, and a thymidine kinase gene at the 5′ end (see Fig. 1). We introduced this vector into 129 strain mouse embryonic stem (ES)[1] cells and identified targeted clones by Southern blotting with two different probes and by PCR. The ES cell line used, RF8, was derived from agouti 129/terSV mice (a gift from Dennis Huszar, GenPharm International, Mountain View, CA) and cultured on SNL76/7 mitotically inactive feeder cells (a gift from Allan Bradley, Baylor College of Medicine, Houston, TX). Only targeted clones resulting from a single integration event (as judged by Southern blotting using a neo probe) were used for blastocyst injection. Targeted clones were injected into C57Bl/6 blastocysts, and one of these clones produced two 90% chimeric male offspring that transmitted the inactivated gene through the germline, as determined by both Southern blotting and PCR. Heterozygous offspring of crosses between these high percentage chimeras and pure C57Bl/6 females were crossed to produce mice that were homozygous for the null mutation, and homozygous wild-type litter mates that served as controls in subsequent experiments. The 240-bp fragment used for library screening was originally amplified by PCR with β subunit primers B1AF (5′-CCIA(G)TIGAC(T)C(AT)TTAC(T)T(A)T(A)IC-(T)TIATGGA -3′) and B2AR (5′-GGICTT(C)CCACCIA(G)AICTA-(G)CGG(T)TAITACG-3′). The resultant fragment was reamplified with degenerate β6 primers β63F (5′-GA(TC)GA(TC)CTIAA(CT)ACIAT-(CAT)AA(GA)GA-3′) and β64R (5′-TC(GA)TT(GA)AA(CT)CT(CT)-TCIGC(GA)TC(GA)TT-3′) designed based on human and guinea pig β6 sequences (Sheppard et al., 1990).

Detection of Recombinant Clones by PCR and Southern Blot

RF8 embryonic stem cells were grown in embryonic stem cell complete media. The targeting vector was linearized at a unique SacII site and transfected into RF8 ES cells by electroporation. Selection medium containing the neomycin analogue G418 (0.15 mg/ml) and FIAU (0.2 μm) was used to obtain resistant clones. Individual colonies were screened by PCR and Southern blotting. PCR was performed with a forward primer from within the introduced neomycin resistance gene (neoF-5′CAGTAAATCGTTGTC-AACAG) and a reverse primer from the mouse β6 gene 3′ of the targeting vector (Kmβ6R-5′GTGGATCTGCTAAGTTAACC). For Southern blotting, genomic DNA digested with BamHI was blotted with two different probes, one specific for mouse β6 and the other for the inserted neomycin resistance gene, and only clones with a single integration were used for blastocyst injection.

Generation of Germline Chimeras

Chimeras were generated as described by Bradley (1987). ES cells were injected into C57Bl/6 blastocysts and the injected embryos were transferred into the uteri of pseudopregnant recipients. Chimeras identified by the presence of an agouti coat color were test-mated with C57Bl/6J females. Offspring were tested for the targeted β6 gene by PCR and Southern blotting.

Keratinocyte Culture and Immunoprecipitation

Mouse skin was removed and placed in 0.1% protease (Sigma Chem. Co., St. Louis, MO) at 4°C overnight. The following day the skin was transferred to 0.05% trypsin and the epithelial layer was scraped off with a surgical blade. After 30 min incubation in trypsin, cells were disaggregated by pipetting, and passed through a 150-μm nylon mesh to remove residual hair. The cells were suspended in keratinocyte growth medium (Clonetics, San Diego, CA), plated onto dishes coated with collagen, and grown to confluence. After overnight labeling with [^{35}S]methionine, cells were lysed in immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 0.1% NP-40, 300 mM NaCl) and immunoprecipitated with Ab 206 which was raised against the cytoplasmic domain of human β6. Samples were analyzed by SDS-PAGE on 7.5% acrylamide gels and exposed to film at −80°C.

Reverse Transcriptase-PCR

Total RNA was harvested from lung and kidney tissue using Trizol reagent (BRL, Grand Island, NY) and cDNA was synthesized using Superscript reverse transcriptase (BRL). A 360-bp fragment of murine β6 flanking the neomycin resistance gene insertion site using primers m$\beta_6$6F (5′ CAGTTCTGACATTGTTCAGA 3′) and m$\beta_6$5R (5′ TGTTAATGG-CAAAATGTGCT 3′).

Collection of Lung Cells and Flow Cytometry

Mouse lungs were perfused with PBS via the main pulmonary artery to remove intravascular cells. The lungs were removed and minced into fine fragments that were gently dispersed into RPMI medium (GIBCO BRL) using a syringe plunger and passed through a 0.75-μm nylon mesh filter. Cells from minced mouse lungs were stained with phycoerythrin-, fluorescein isothiocyanate-, or biotin-conjugated antibodies against CD4, CD8, B220, and CD25 (Caltag, South San Francisco, CA) and analyzed for surface expression using a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA).

Histology and Immunohistochemistry

Freshly isolated organs were embedded in OCT and quick frozen in liquid nitrogen. Serial 5-μm sections were prepared and fixed in Histochoice (Fisher Scientific, Pittsburgh, PA) for hematoxylin and eosin staining. For immunohistochemistry, frozen sections were fixed in cold acetone for 5–10 min and air-dried. Sections were blocked for endogenous peroxidase and biotin activities with Peroxoblock solution (Zymed Labs, S. San Francisco, CA) and Avidin/Biotin Blocking Kit (Vector Labs, Inc., Burlingame, CA) at room temperature. After rinsing, sections were blocked with 0.25% casein/0.025% thimerosal in PBS for 15 min and then incubated overnight at 4°C in biotin-labeled primary antibodies against CD3 (T cells), B220 (B cells), and F4/80 (macrophages) (all from Caltag). After washing, sections were incubated in ABC avidin/peroxidase reagent (Vector) for 1 h at room temperature. Chromagen was developed using the DAB Plus Kit (Zymed). Finally, sections were dehydrated and mounted with permount onto clean slides.

Measurement of Airway Resistance

Mice were anesthetized with pentobarbital (50 mg/kg, i.p.), the chest was opened, and a tracheotomy tube was inserted. The mice were paralyzed with pancuronium bromide (0.1 mg/kg), and then ventilated with 100% oxygen by a Harvard small animal ventilator at a rate of 150 breaths/minute and a tidal volume of 9 μl/gm. In pilot experiments, we determined that these settings result in near normal values of arterial (left ventricular) pH and pCO_2. A heparinized, indwelling catheter was placed in the tail vein, and the animal was placed in an airtight plexiglass plethysmograph, with the venous catheter threaded through a small hole in the plethysmograph. Airway pressure and plethysmograph pressure were continuously measured by differential pressure transducers and recorded on a Hewlett-Packard chart recorder. Pulmonary resistance (RL), tidal volume, flow, and dynamic lung compliance were continuously calculated by a Buxco Pulmonary Mechanics analyzer. RL was calculated on each inspired breath as the ratio of driving pressure to airflow at 70% of inspired tidal volume. Increasing concentrations of acetylcholine were administered through the tail vein catheter at 2-min intervals, and peak pulmonary resistance in response to each concentration was determined.

Elispot Assay

Elispot assay were performed as described (Corry et al., 1996). This assay is a modification of a sandwich ELISA that allows the identification of individual cells that are secreting specific antigens. Briefly, wells of 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with either mAb against IFN-γ (R46A2) or IL-4 (BVD4-1D11.2) at 4°C overnight and blocked with 10% FBS. Pooled cells from minced lungs were plated and incubated at 37°C overnight. Plates were incubated for 1 h with biotinylated secondary antibodies XMG-1.2 to IFN-γ and BVD6-24G.2 to IL-4, and then with streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) for another hour. Between incubations, plates were washed with phosphate buffered saline supplemented with

1. *Abbreviations used in this paper*: ES, embryonic stem; IL-4, interleukin-4; RL, pulmonary resistance.

0.05% Tween-20. Final color development was obtained by incubating plates with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 2-amino-2-methyl-1-propanol buffer (Sigma) suspended in 0.6% agarose low-melt gel. After solidification of the agarose, individual blue spots were counted under an inverted microscope.

Results

Mice Homozygous for a Null Mutation in the β6 Subunit Develop Juvenile Baldness

To assess whether $\beta6^{-/-}$ mice produced β6 mRNA, we amplified cDNA obtained from the lung and kidney of $\beta6^{-/-}$ and $\beta6^{+/+}$ mice by PCR using oligonucleotide primers flanking the exon disrupted by our targeting vector (Fig. 1). The expected 360-bp fragment could be amplified from the cDNA of $\beta6^{+/+}$ mice, but no amplification product was detectable from the cDNA of $\beta6^{-/-}$ mice (Fig. 2 *A*). To confirm that $\beta6^{-/-}$ mice were not capable of making β6 protein, we attempted to immunoprecipitate β6 from metabolically labeled lysates of cultured keratinocytes with an antibody raised against a peptide based on the cytoplasmic domain sequence of human β6 (Ab 206 [Busk et al., 1992]). The anti-β6 antibody immunoprecipitated two bands of the appropriate apparent molecular masses to be αv and β6, from $\beta6^{+/+}$ keratinocytes, but no bands were immunoprecipitated from $\beta6^{-/-}$ keratinocytes (Fig. 2 *B*).

Mice homozygous for the null mutation were born at approximately the expected Mendelian frequency from heterozygous intercrosses (30% +/+, 48% +/−, and 22% −/− of 234 offspring analyzed), demonstrating that this integrin subunit, in contrast to α4 (Yang et al., 1995) and α5 (Yang et al., 1993), is not absolutely required for embryonic development. However, $\beta6^{-/-}$ mice were not completely normal. All $\beta6^{-/-}$ mice failed to develop hair normally over the tops of their heads, the backs of their necks, and the inner surface of their thighs (Fig. 3 *A*). These abnormalities were visually apparent by postnatal day 5, and persisted through day 20–30. After this time, hair growth resumed over the head and neck but remained sparse over the inner surface of the thighs. No gross abnormalities in any other organs have been apparent in mice followed for up to 6 mo. Furthermore, $\beta6^{-/-}$ mice gained weight normally and were fertile.

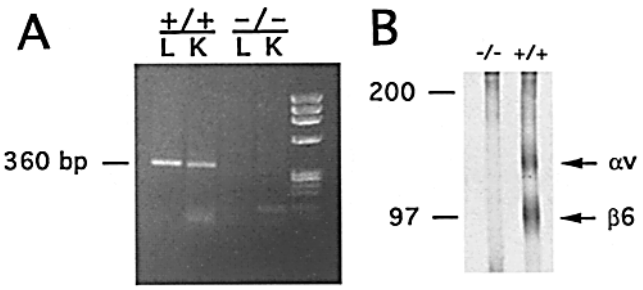
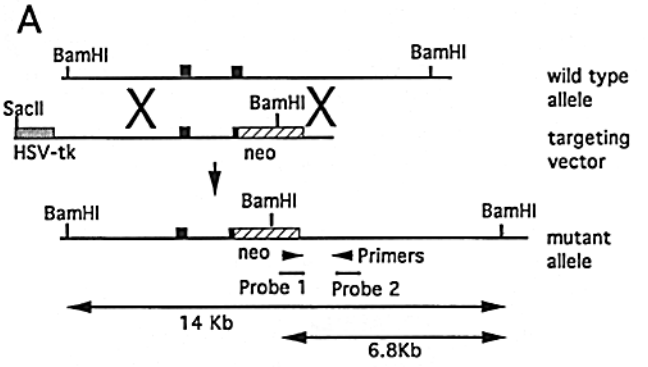


Figure 2. (*A*) RT-PCR analysis of mRNA from $\beta6^{+/+}$ and $\beta6^{-/-}$ mice. Total RNA was extracted from mouse lung and kidney and transcribed to complementary DNA (cDNA). A 360-bp amplification fragment was detectable in tissues of $\beta6^{+/+}$ mice but not of $\beta6^{-/-}$ mice with primers specific for wild-type β6 cDNA. (*B*) Immunoprecipitation of αvβ6. Primary cultures of keratinocytes from $\beta6^{+/+}$ and $\beta6^{-/-}$ were labeled overnight with [^{35}S]methionine and cell lysates were immunoprecipitated with Ab 206, raised against the human β6 cytoplasmic domain. Immunoprecipitated proteins were analyzed by 7.5% SDS-PAGE under nonreducing conditions. The positions of molecular size markers (in kD) are shown to the left.

The Dermis in Bald Areas of $\beta6^{-/-}$ Mice Is Infiltrated with Macrophages

The hair loss seen in $\beta6^{-/-}$ mice was associated with morphologic abnormalities in the dermis of affected areas. In comparison to $\beta6^{+/+}$ mice, $\beta6^{-/-}$ mice had fewer hair follicles, and numerous degenerating hair follicles surrounded by foci of mononuclear cells (Fig. 3 *C*). The mononuclear cells resembled tissue macrophages and stained with the monocyte/macrophage marker F4/80. Furthermore, staining with F4/80 demonstrated increased numbers of monocytes/macrophages throughout the dermis of the bald areas in $\beta6^{-/-}$ mice (Fig. 3 *E*). Staining with antibodies specific for mouse T cells and B cells did not demonstrate



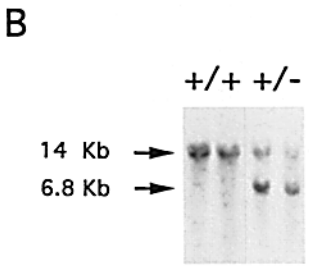


Figure 1. (*A*) Replacement vector for inactivation of the β6 gene in mouse embryonic stem (ES) cells. Top panel shows the structure of a 15-kb fragment of the mouse β6 gene isolated from a 129 strain genomic library. The two exons in this fragment are shown as solid boxes. Middle panel shows the targeting plasmid constructed in pBluescript (Stratagene, La Jolla, CA) and linearized at a unique SacII site. A neomycin-resistance gene under the control of the RNA polymerase II promoter (neo) and herpes simplex virus thymidine kinase (HSV-tk) gene (gifts from Kirk Thomas, University of Utah, Salt Lake City, UT) are shown as shaded boxes. Bottom panel shows the expected structure of the β6 gene after homologous recombination. The arrowheads represent PCR primers used to identify homologous recombination events in ES cell colonies. The forward primer is located in the neo gene and the reverse primer is 3′ of the targeting vector. These primers generated a 1.2-kb fragment in targeted clones. The locations of the two probes used for Southern blotting are shown as lines. Hybridization of either probe with genomic DNA digested with BamHI yielded a new 6.8-kb band in targeted clones. (*B*) Southern blot analysis of wild-type (*first two lanes*) and targeted (*last two lanes*) ES clones digested with BamHI and hybridized with the external probe (probe 2).

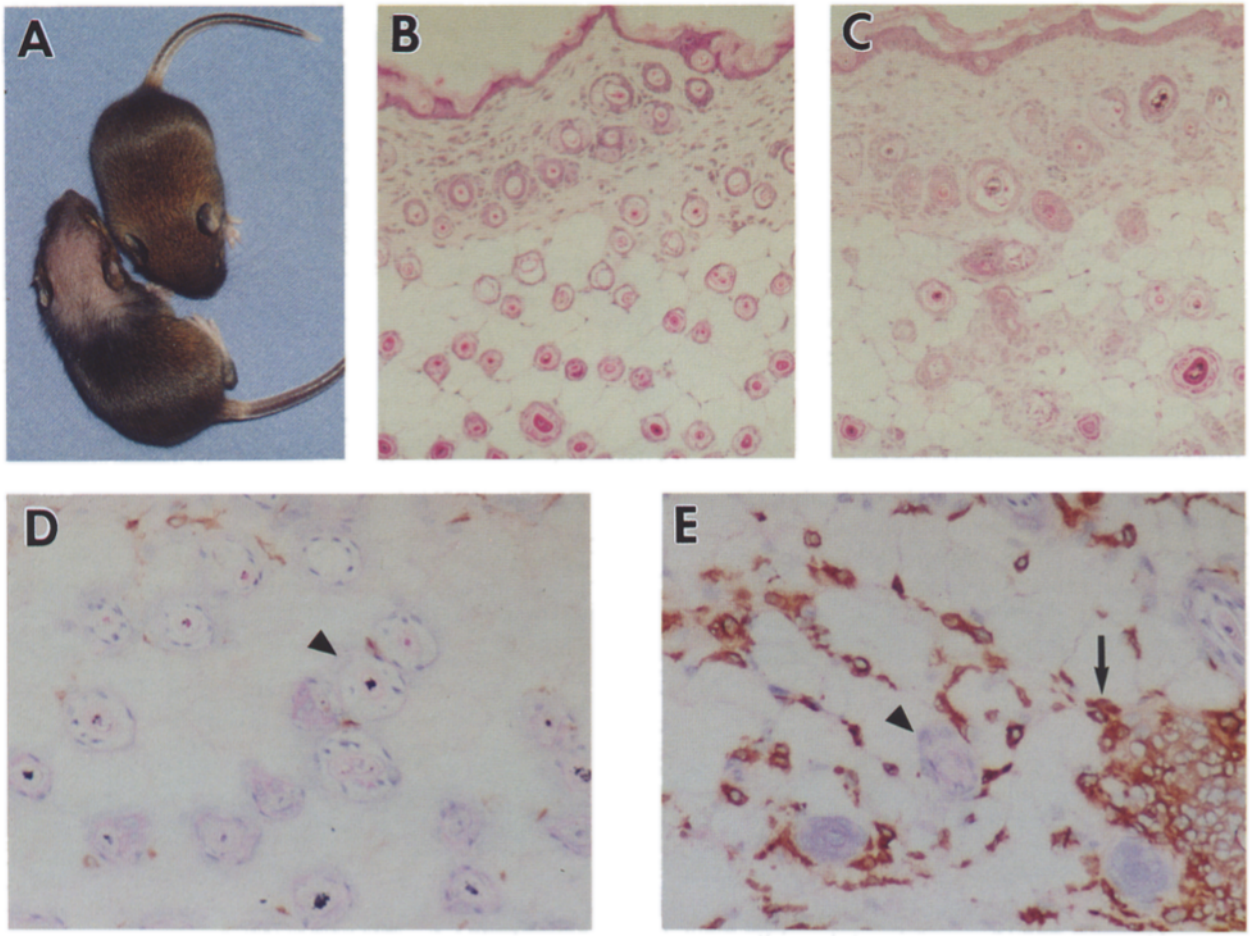


Figure 3. (*A*) Photograph of a $\beta6^{-/-}$ mouse (*left*) and a $\beta6^{+/+}$ littermate (*right*) at 10 d of age. In contrast to the $\beta6^{+/+}$ mouse, the $\beta6^{-/-}$ mouse demonstrates the typical pattern of juvenile baldness over the head and neck. $\beta6^{-/-}$ mice also lack hair over the inside of their thighs. *B* and *C* are low power photomicrographs of sections of frozen inner thigh skin from a $\beta6^{+/+}$ (*B*) and a $\beta6^{-/-}$ mouse (*C*) stained with hematoxylin and eosin (H and E), and demonstrate loss of hair follicles and infiltration of the dermis in the $\beta6^{-/-}$ animal. *D* and *E* are higher power photomicrographs of frozen sections of skin from the same areas of the same $\beta6^{+/+}$ (*D*) or $\beta6^{-/-}$ mouse (*E*) stained with the monocyte/macrophage marker F4/80, and lightly counterstained with H and E. Hair follicles are marked with arrows. Infiltrating macrophages are marked with arrowheads. Note the marked increase in F4/80 positive cells in the $\beta6^{-/-}$ mouse.

any increase in lymphocytes in the skin lesions (data not shown). No morphologic abnormalities were seen in skin taken from unaffected areas.

Expression of αvβ6 is induced within a few days of cutaneous wounding, is expressed only in the keratinocytes at the wound edge, and remains expressed until wounds are completely closed (Breuss et al., 1995). The two known ligands for αvβ6, fibronectin (Busk et al., 1992; Weinacker et al., 1994), and tenascin (Prieto et al., 1993), are components of the provisional matrix across which keratinocytes must migrate during wound repair. Therefore, we hypothesized that this integrin might be critical for normal cutaneous wound healing. However, when either incisional or excisional wounds (2 or 4 mm in diameter punch biopsy wounds) were made in the backs or necks of $\beta6^{-/-}$ or $\beta6^{+/+}$ mice, the rate of healing and the morphology of healing wounds was similar in both groups for mice examined 2, 4, 6, or 12 d after wounding. In both groups, all incisional wounds were completely healed by day 6, and all excisional wounds were healed by day 12. These data indicate that αvβ6 is not required for normal wound healing. This result could be explained by the expression of other keratinocyte fibronectin and tenascin receptors. For example, the fibronectin receptor, α5β1, was found to be increased at the wound edge in both $\beta6^{-/-}$ and $\beta6^{+/+}$ mice, and the tenascin receptor, α9β1 (Palmer et al., 1993; Yokosaki et al., 1994), was constitutively expressed on keratinocytes from both types of mice (data not shown).

$\beta6^{-/-}$ Mice Demonstrate Infiltration of the Conducting Airways of the Lung by Activated Lymphocytes

The other significant pathologic finding observed in $\beta6^{-/-}$ mice was in the lungs. Lung morphology was normal in mice examined up to 13 d after birth. However, beginning at ~21 d, $\beta6^{-/-}$ mice developed infiltration of the walls of the conducting airways with mononuclear cells (Fig. 4). These cells morphologically resembled lymphocytes. In contrast to the skin lesions, the lung lesions did not contain increased numbers of F4/80 + cells, but did contain a mixture of cells that stained with the B cell marker, B220, or the T cell marker CD3 (data not shown). This pathology

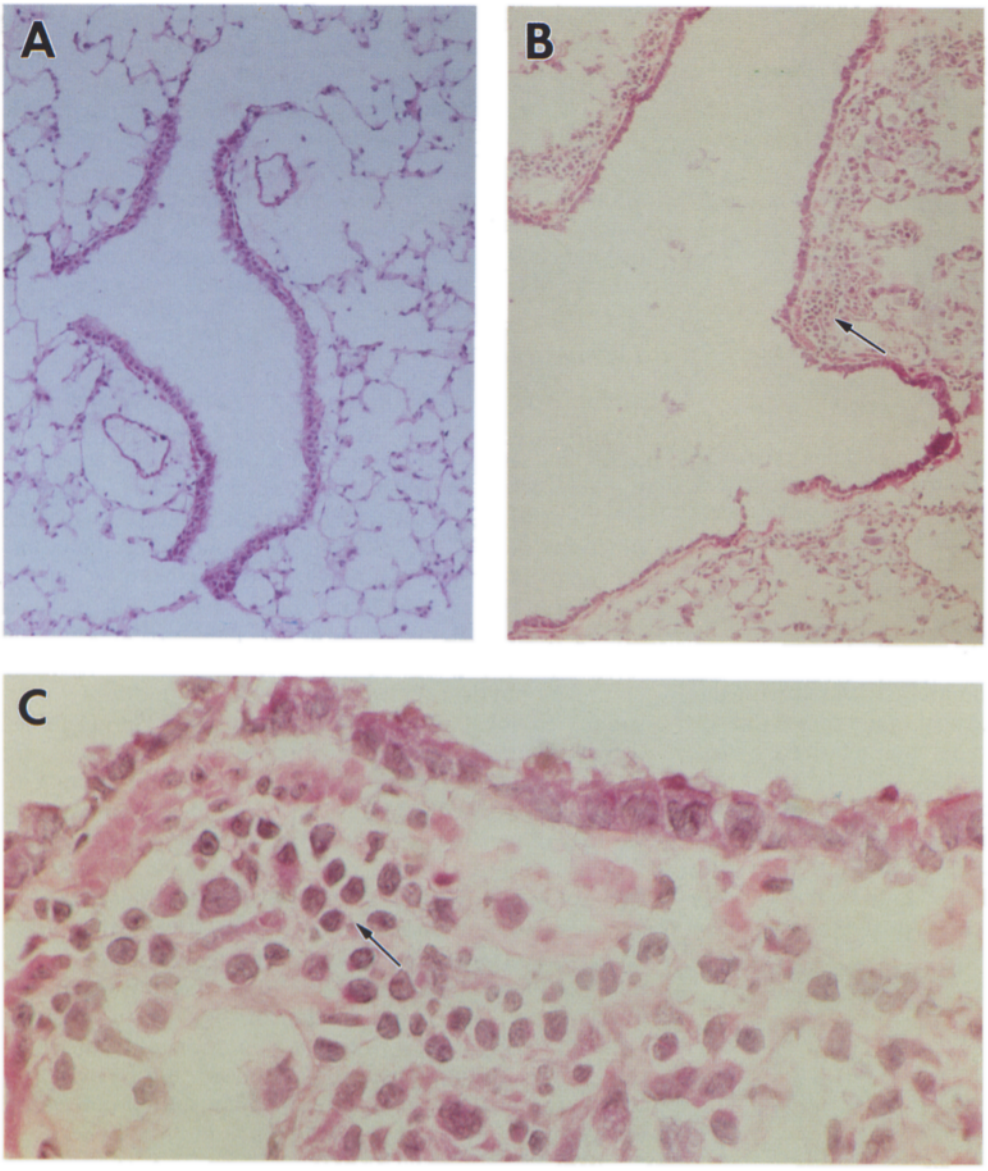


Figure 4. Lung histology. Low power photomicrograph of H and E stained frozen section of a conducting airway from a $\beta6^{+/+}$ (*A*) and a $\beta6^{-/-}$ mouse (*B*) demonstrate accumulation of mononuclear cells (*arrowheads*) diffusely around a large conducting airway in the $\beta6^{-/-}$ mouse. This pathology was seen in all 11 $\beta6^{-/-}$ mice examined, but in none of 16 $\beta6^{+/-}$ or $\beta6^{+/+}$ mice. (*C*) Higher power photomicrograph of the same airway shown in *B*.

was seen in all 11 $\beta6^{-/-}$ mice analyzed after 21 d of age. No similar lesions were observed in 16 $\beta6^{+/+}$ or $\beta6^{+/-}$ mice. Flow cytometry of cells obtained from minced lungs demonstrated approximately threefold increases in the percentages of B cells and CD4+ and CD8+ T cells in $\beta6^{-/-}$ mice. These mice also demonstrated a marked increase in the number of CD4+ T cells that expressed the activation marker CD25 (Fig. 5). These abnormalities in lung morphology and cellularity were not likely to be due to lung infection, since all animals were housed together in a barrier facility. Sentinel mice were examined weekly and found to be free of specific pathogenic viruses.

$\beta6^{-/-}$ Mice Demonstrate Increased Airway Responsiveness

Despite the progressive abnormalities in lung morphology noted in the $\beta6^{-/-}$ mice, these mice appeared healthy, without noticeable respiratory distress. However, because activated T cells have been implicated in the development of asthma (Gavett et al., 1994), we sought to determine whether these animals would demonstrate one of the hallmark features of asthma, airway hyperresponsiveness to bronchoconstrictor agents such as acetylcholine (Boushey et al., 1980). Groups of $\beta6^{-/-}$ and $\beta6^{+/+}$ mice were anesthetized, tracheostomized, and ventilated in a whole body plethysmograph, and pulmonary resistance (RL) was measured at baseline and after intravenous administration of increasing concentrations of acetylcholine. Baseline RL was the same in both groups of mice (Fig. 6). The $\beta6^{+/+}$ mice developed only small increases in RL, even after administration of the highest concentrations of acetylcholine used, whereas the $\beta6^{-/-}$ mice demonstrated markedly larger responses to each of the two highest concentrations ($P < 0.001$).

Cells Obtained from the Lungs of $\beta6^{-/-}$ Mice Express the Th2-associated Cytokine IL-4, but Not the Th_1-associated Cytokine Interferon-γ

In the most widely studied experimental model of asthma, antigen-induced airway hyperresponsiveness, increased

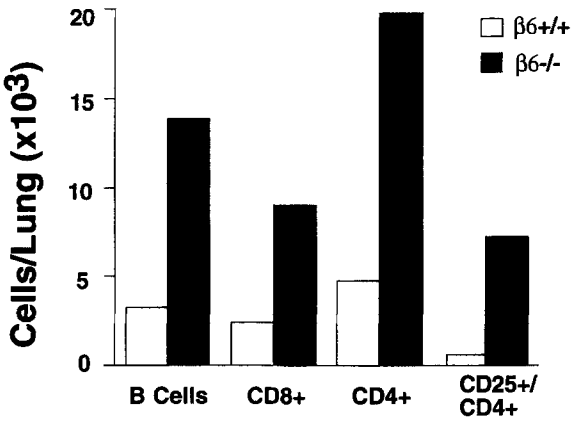


Figure 5. Lymphocyte subsets in mouse lungs. Lung cells were obtained by mincing lungs pooled from groups of $\beta6^{+/+}$ (*open bars*) and $\beta6^{-/-}$ mice (*shaded bars*). Cells were stained with antibodies specific for mouse B cells (B220), or T cell markers CD4 or CD8, and analyzed by flow cytometry. To identify activated CD4+ T cells, cells were simultaneously stained with antibodies to CD4 and to the lymphocyte activation antigen CD25.

airway responses to acetylcholine are associated with the presence of T cells in the airway wall that express the so-called Th2 (T helper 2) phenotype, characterized by expression of the cytokines interleukin-4 (IL-4), IL-5, and IL-10. In contrast, Th1 cells, thought to be important for cytotoxic T cell responses, express the cytokine Interferon-γ (IFN-γ), but not IL-4, IL-5, or IL-10. Recent data using antibodies against IL-4 and IL-4 knockout mice, suggest that IL-4, in particular, is crucial to the development

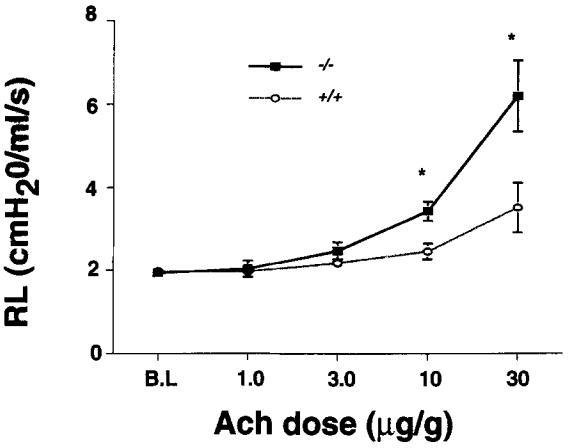


Figure 6. Airway responsiveness to acetylcholine in $\beta6^{+/+}$ and $\beta6^{-/-}$ mice. Total pulmonary resistance (RL) was measured in a whole body plethysmograph in seven $\beta6^{+/+}$ mice (*open circles*) and in six $\beta6^{-/-}$ mice (*closed squares*) from matched litters, at baseline (B.L.) and after the administration of successively increasing doses of acetylcholine into an indwelling tail vein catheter. Data are plotted as the mean (±SD). Pulmonary resistance was calculated using an analogue computer (model 6, Buxco, Sharon, CT) (22) in mice that were anesthetized, paralyzed, and ventilated breathing 100% oxygen with a rodent ventilator (Harvard-Ealing, Millis, MA) at a rate of 150 breaths/min and a tidal volume of 9 μl/g, settings that resulted in normal arterial blood gases in pilot experiments. $*P < 0.0001$ as determined by Student's *t* test for unpaired data, adjusted for multiple comparisons.

of airway hyperresponsiveness in this model (Brusselle et al., 1995; Corry et al., 1996). To determine whether the lymphocytes in the lungs of $\beta6^{-/-}$ mice were expressing either Th1 or Th2 cytokines, we performed Elispot assays to detect IL-4 or IFN-γ secretion from cells obtained from minced lungs of $\beta6^{-/-}$ or $\beta6^{+/+}$ mice. These assays, which allow us to count individual lung cells secreting each cytokine, demonstrated a fourfold increase in the percentage of lung cells that secrete IL-4 with no increase in the percentage of cells that secrete IFN-γ in $\beta6^{-/-}$ mice (Fig. 7).

Discussion

Multiple members of the integrin family are expressed in vivo in epithelial cells, including α2β1, α3β1, α9β1, α6β4, αvβ5, and αvβ6. Of these, α6β4 is a critical component of the hemidesmosomes that basal epithelial cells use for attachment to the underlying basement membrane, but the functions each of the other integrins play in epithelial tissues remain largely unknown. αvβ6 is the only known member of the integrin family that is restricted in its distribution to epithelial cells. However, unlike most epithelial integrins, αvβ6 is not constitutively expressed in healthy epithelia, but is rapidly and transiently induced in response to local injury or inflammation. The principal ligands identified for αvβ6, fibronectin and tenascin, are also absent from healthy epithelia, but present in most injured and inflamed epithelia. Based on these observations, we and others hypothesized that αvβ6 might play some critical role in the repair of injured epithelial tissues. Because of the known in vitro roles played by integrins in cell spreading, migration and proliferation, we presumed that any in vivo role of αvβ6 would probably involve one or more of these functions. Such effects of αvβ6 would also be consistent with the induction of αvβ6 during organogenesis, its high level expression in secretory phase endometrium, and its expression in tumors derived from epithelia; since cell migration, spreading, and proliferation are important in organ development, endometrial regeneration, and tumorigenesis.

The results of the present study are therefore somewhat surprising. $\beta6^{-/-}$ mice develop and reproduce normally, and are fully capable of healing cutaneous wounds. Although these observations by no means prove that αvβ6

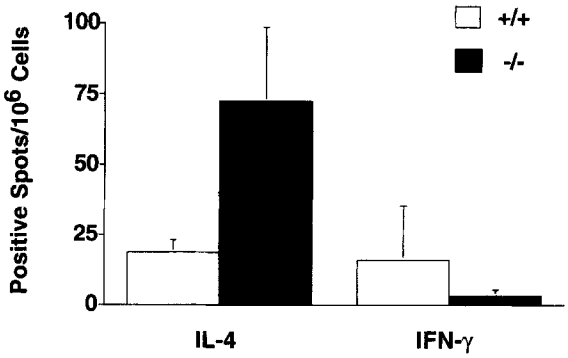


Figure 7. Cytokine production by lung cells. Purified lung cells were assessed for secretion of IL-4 and IFN-γ using an Elispot assay to detect cytokine secretion from individual cells. Data are expressed as the mean (±SD) number of positive spots/10^6 cells.

does not normally contribute to development reproduction, and/or wound healing, they do suggest that other receptors can serve the same functions. However, our observation that $\beta6^{-/-}$ mice all develop inflammatory cell infiltrates in the skin and lungs suggest a previously unexpected role for this integrin in modulating inflammation in these epithelial organs.

It is unclear why the pathology seen in $\beta6^{-/-}$ mice has thus far been confined to the lungs and skin. In addition to these two organs, αvβ6 can be expressed on epithelial cells in a variety of other organs, including the kidney, uterus, testes, ovary, salivary glands, and gall bladder (Breuss et al., 1993). At least in the kidney, as in the lung and skin, αvβ6 expression is dramatically upregulated by injury or inflammation. In the present study, the mice we describe were housed continuously in a specific pathogenic virus free barrier facility, so they were largely protected from environmental insults. However, the conducting airways of healthy mammals are repeatedly exposed to aspirated gastric contents, and we hypothesize that focal areas of injury or inflammation might be routine events, even in animals maintained in an environmentally controlled barrier facility. In addition, even in a barrier facility, mice are potentially exposed to inhaled antigens from their food and bedding. Similarly, the pattern of abnormality seen in the skin of $\beta6^{-/-}$ mice might be explained by repeated low-level injury. Baldness and macrophage infiltration of the skin was most prominent in the head and neck skin of mice that had not yet been weaned, and resolved by ~30 d of age. This pattern corresponds to the area used by mothers to lift and move mouse pups. The inside of the thighs is also an area that could be subjected to continuous low-level irritation. In this region, hair loss persists even in adult $\beta6^{-/-}$ mice.

One explanation for our findings is that αvβ6 participates in the regulation of local factors that are responsible for activation, recruitment and/or proliferation of lymphocytes and/or monocytes. For example, expression of αvβ6 following epithelial injury could modulate the synthesis of inflammatory cytokines by epithelial cells, contributing to the termination of the local inflammatory response. Inactivation of αvβ6, especially in sites such as the airways and skin that are repeatedly exposed to injurious stimuli, would thus lead to persistent inflammation. A role for integrins in regulation of cytokine gene expression has been demonstrated in mononuclear cells, where the regulation of expression of several cytokine genes can be dramatically altered by plating cells on different extracellular matrix substrates, an effect that is mediated by integrins (Haskill et al., 1988; Juliano and Haskill, 1993; Miyake et al., 1993). It is now well-recognized that epithelial cell-derived chemokines and other cytokines play important roles in initiating and modulating inflammatory responses in epithelial organs, including the lungs and skin (Becker et al., 1994; Bellini et al., 1993; DiCosmo et al., 1994; Elias et al., 1994; Sousa et al., 1994). For example, one of the cytokines synthesized by airway epithelial cells, IL-6, induces the proliferation of both B lymphocytes and T lymphocytes, and also induces lymphocyte activation, as detected by expression of the interleukin-2 receptor, CD-25 (Lotz et al., 1988; Tosato et al., 1988; Tosato and Pike, 1988). Overexpression of IL-6 in the airway epithelium of transgenic mice induced a very similar pathology to that seen in the $\beta6^{-/-}$ mice we describe, including large accumulations of B cells and T cells adjacent to conducting airways (DiCosmo et al., 1994).

One surprising feature of our results is that the character of the inflammatory cells in the skin and lungs of $\beta6^{-/-}$ mice is different. The cells in the skin are principally macrophages, whereas the cells in the lung are principally B and T lymphocytes. This difference suggests that either the effects of αvβ6 ligation on keratinocytes and airway epithelial cells are different, or that the subsequent cellular responses to any effects of αvβ6 ligation are different in the microenvironments of the skin and lungs. Differential effects of cytokines in different tissues are well described. For example, transgenic mice overexpressing interleukin-6 in airway epithelial cells develop foci of lymphocytes surrounding conducting airways in a pattern quite similar to that seen in the $\beta6^{-/-}$ mice we describe (DiCosmo et al., 1994). However, mice overexpressing IL-6 in keratinocytes do not develop lymphocytic infiltrates in the skin (Turksen et al., 1992).

The $\beta6^{-/-}$ mice we describe demonstrate dramatic increases in bronchomotor responsiveness to acetylcholine, the hallmark feature of asthma in humans (Boushey et al., 1980). Lymphocytes are thought to play an important role in induction of airway hyperresponsiveness in human asthma (Robinson et al., 1992; Walker et al., 1991) and are the principal cell type recruited to the airways in these mice. Furthermore, as in human asthma, the CD4+ T cells in the airways of $\beta6^{-/-}$ mice appear to be activated. Finally, the lungs of $\beta6^{-/-}$ mice contain many more IL-4 secreting cells than do the lungs of $\beta6^{+/+}$ mice. Two recent studies in which airway hyperresponsiveness was transiently induced in ovalbumin-sensitized mice by inhalation of ovalbumin suggested that IL-4 played an important role in the induction of airway hyperresponsiveness. In one of these studies, IL-4 deficient mice were shown to be resistant to ovalbumin-induced airway hyperresponsiveness (Brusselle et al., 1995), and in the other study, ovalbumin-induced airway hyperresponsiveness was prevented by administration of an anti–IL-4 antibody (Corry et al., 1996). One feature that distinguishes the animals we describe from most people with asthma, and from ovalbumin-sensitized and challenged animals is the absence of eosinophils in the airways. Despite the absence of eosinophils, $\beta6^{-/-}$ mice demonstrated marked airway hyperresponsiveness, suggesting that eosinophils are not required for induction of this physiologic abnormality. These data are consistent with the recent report that treatment of ovalbumin-sensitized and challenged mice with anti–IL-5 antibody prevents airway eosinophilia but has no effect on the induction of airway hyperresponsiveness.

As discussed above, in the most widely studied experimental models of asthma, lymphocytes are recruited to the airways and activated in response to inhalation of specific allergens (Gavett et al., 1994). These models suffer from a high degree of variability and from the transient nature of the airway hyperresponsiveness induced. Moreover, many patients with asthma are not atopic, and most clinical exacerbations of asthma are provoked by nonimmunologic stimuli (Boushey et al., 1980). Many of these stimuli, including viral infection and inhalation of irritant gases, are

likely to directly affect the airway epithelium. Furthermore, one nearly universal feature of human asthma is an alteration in the amount and composition of extracellular matrix in the airway wall (Djukankovic et al., 1990; James et al., 1989; Laitinen and Laitinen, 1994). The results of the present study suggest that these changes in the matrix, detected by integrins, could modulate recruitment and activation of airway lymphocytes, and that genetic or environmental inactivation of the airway epithelial integrin, αvβ6, could induce or potentiate the airway lymphocytosis and airway hyperresponsiveness that characterize human asthma. Similarly, alterations in signals initiated through epithelial integrins could contribute to inflammatory diseases affecting other epithelial organs.

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