Animal Model

The α_2 Integrin Subunit-Deficient Mouse

A Multifaceted Phenotype Including Defects of Branching Morphogenesis and Hemostasis

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The $\alpha_2\beta_1$ integrin is a collagen/laminin receptor expressed on platelets, endothelial cells, fibroblasts, and epithelial cells. To define the role of the $\alpha_2\beta_1$ integrin *in vivo*, we created a genetically engineered mouse in which expression of the $\alpha_2\beta_1$ integrin was completely eliminated. Mice deficient in the $\alpha_2\beta_1$ integrin are viable, fertile, and develop normally with no excess lethality of homozygotes. Both $\alpha_2\beta_1$ -integrin protein and α_2 mRNA were undetectable in the α_2 -null mice. Gross and histological evaluation of the heart, lungs, kidneys, gastrointestinal tract, pancreas, skin, and reproductive tracts revealed no abnormalities. However, quantitative analysis of mammary gland branching morphogenesis demonstrated that branching complexity is markedly diminished in the α_2 -deficient animals. Studies in the α_2 -deficient animals do not support the proposed roles for the $\alpha_2\beta_1$ integrin on fibroblasts and keratinocytes in wound healing. When compared to platelets from wild-type littermates, platelets from α_2 -null mice failed to adhere to type I collagen under either static or shear-stress conditions. Although platelets from α_2 -deficient animals aggregated in response to collagen, they did so with prolonged lag time and lessened intensity. The $\alpha_2\beta_1$ integrin-null mouse thus exhibits diverse, sometimes subtle, phenotypes consistent with the widespread pattern of $\alpha_2\beta_1$ integrin expression. (*Am J Pathol* 2002, 161:337–344)

The $\alpha_2\beta_1$ integrin functions as a collagen receptor on platelets and fibroblasts and as both a collagen and laminin receptor on endothelial cells and epithelial cell

types.^{1–3} The $\alpha_2\beta_1$ integrin has been implicated in a variety of complex biological processes. An abundant literature supports an important role for the $\alpha_2\beta_1$ integrin in platelet function and hemostasis.⁴ Extensive *in vitro* studies of renal, pulmonary, gastrointestinal, and breast epithelia suggested that signals downstream of $\alpha_2\beta_1$ -integrin ligation contribute to branching morphogenesis.^{5–8} Experiments using explant cultures and three-dimensional collagen gels have implicated the $\alpha_2\beta_1$ integrin in keratinocyte migration and fibroblast contraction during wound healing.^{9–11}

To define better the role of the $\alpha_2\beta_1$ integrin *in vivo*, we created a genetically engineered mouse in which expression of the $\alpha_2\beta_1$ integrin was eliminated. We now show that mice deficient in the $\alpha_2\beta_1$ integrin are viable and fertile. However, $\alpha_2\beta_1$ -integrin-deficient mice show defective hemostasis and abnormalities of branching morphogenesis, but apparently normal healing of skin wounds.

Materials and Methods

The Targeting Construct

Screening of a P1 129/Svj DNA genomic library (Genomic Systems, St. Louis, MO) by polymerase chain reaction (PCR) using primers directed against bp –15 to +6 and 165 to 186 of exon 1 of the murine α_2 -subunit cDNA sequence¹² yielded a 100-kb P1 clone. The targeting vector [*loxP neo/TK* vector (a gift from Dr. Robert Schreiber, Washington University, St. Louis, MO)] was prepared from an 8.0-kb *EcoRI* restriction enzyme fragment from the P1 genomic clone containing exon 1 (Figure 1A).

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Figure 1. Targeting of the α_2 integrin gene. **A:** The targeting construct was prepared from an 8.0-kb *EcoRI* genomic clone containing exon 1 (black box). The targeting vector contained a 2.0-kb *Bam*HI fragment that included 5' UTR of the α_2 integrin gene upstream of the 5' *loxP* (triangle) site. A 600-bp *Bam*HI fragment containing exon 1 and flanking sequence was placed between the two downstream *loxP* sites. A 2.4-kb *Aval/EcoRI* fragment containing intronic sequence was placed between the 3' *loxP* site and the PGK-TK cassette. The fragment used for Southern blot hybridization (probe a), primers (1 and 2) for PCR genotyping, restriction enzyme sites, the 5' UTR, and intron 1 are indicated. After homologous recombination, transient transfection with a plasmid encoding Cre-recombinase resulted in removal of both the *neo*-cassette and exon 1. A single *loxP* site remained. Restriction enzyme sites: B, *Bam*HI; A, *Aval*; R, *EcoRI*; X, *Xho*. **B:** Southern blot analysis of wild-type, heterozygous, and α_2 -deficient animals. Tail DNA from mice obtained from the heterozygous intercrosses was digested with *EcoRI* and *XhoI*, electrophoresed, and hybridized with probe a. The wild-type allele generates a 6.5-kb fragment and the targeted allele generates a 4.5-kb fragment. **C:** PCR genotyping of tail DNA. The genotype was confirmed by PCR analysis of tail DNA using primer 1, directed against the 5' UTR of the α_2 integrin gene and primer 2 against intron 1. The wild-type allele generated an 870-bp product and the mutant allele generated a 320-bp product.

A 2.0-kb *Bam*HI fragment consisting of 5' untranslated region of the α_2 integrin gene was inserted upstream of the 5' *loxP* site. The contiguous 600-bp *Bam*HI fragment containing exon 1 and flanking sequence was placed between the two downstream *loxP* sites. A 2.4-kb *Aval/EcoRI* fragment containing intronic sequence was placed between the 3' *loxP* site and the PGK-TK cassette, as shown in Figure 1A.

Generation of α_2 -Deficient Mice

R1 embryonic stem cells derived from a 129/Sv X 129/Sv-CP F1 3.5-day blastocysts from the Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada, were electroporated with 25 μ g of linearized targeting construct and selected in both G418 (26 μ mol/L) and gancyclovir (0.2 μ mol/L). Genomic DNA from resistant clones was digested with *XhoI*, and analyzed by Southern blot hybridization with the 1.7-kb *Bam*HI/*XhoI* genomic fragment (probe a in Figure 1A). In a second step embryonic stem cell clones that had undergone homologous recombination were transfected with 25 μ g of Cre-recombinase-expressing plasmid and selected for G418. Clones in which both the *neo*-cassette and exon 1 were deleted were identified by Southern blot analysis and PCR and injected into C57BL/6 blastocysts. PCR was performed using oligonucleotides directed against the 5' UTR [primer 1 (5'-GCGTTGGGGGGTGGAGGGAAGT-GCC-3')] and intron 1 [primer 2 (5'-CTGGCTCCACGAA-GCCTGG)]. Male chimeric mice were bred to C57BL/6 females to obtain heterozygous α_2 -deficient mice. Heterozygous mice were intercrossed to obtain wild-type, heterozygous, and α_2 -deficient animals.

Morphological and Histological Analyses

Mice at ages 9 to 11 weeks were euthanized by CO₂ asphyxiation. Tissues were fixed in 4% formaldehyde and paraffin-embedded. Sections (4- μ m) were stained with Harris' hematoxylin and eosin.

Whole mounts of the mammary gland were prepared from the no. 4 inguinal gland by spreading the fatpad onto a precleaned glass slide, air-drying for 10 minutes, and fixing in acetic acid:ethanol (1:3) for 1 hour at room temperature. The tissue was dehydrated in 70% ethanol and ductal structures were stained with carmine solution (0.2% carmine and 0.5% aluminum potassium sulfate). The gland was dehydrated through graded ethanol, defatted in acetone, clarified in toluene, and mounted.

Reverse Transcriptase (RT)-PCR Analysis

Total RNA from whole mammary glands was isolated by the guanidine isothiocyanate extraction technique. First-strand cDNA synthesis of total RNA was performed using an oligonucleotide primer complementary to bp 3141 to 3161 of the murine α_2 cDNA. RT-PCR was performed with the same PCR primer and one complimentary to bp 2320 to 2341 using standard PCR protocol.

Immunoblot Analysis

Purified platelet protein preparations or mammary glands were lysed in sodium dodecyl sulfate sample buffer [10% (v/v) glycerol/62.5 mmol/L Tris (pH 6.8)/2% sodium dodecyl sulfate/2% 2-mercaptoethanol], subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and electroblotted onto Immobilon P transfer membrane (Millipore, Bedford, MA). Immunoblots were incubated

with the appropriate dilution of each primary antibody and secondary horseradish peroxidase-conjugated donkey/anti-rabbit IgG antibody (Amersham, Arlington Heights, IL). The enhanced chemiluminescence system (Amersham) was used for visualization. The polyclonal antiserum against a portion of the carboxy terminus of the murine α_2 integrin has been previously described.¹² The goat polyclonal antiserum against actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Platelet Preparation, Adhesion, and Aggregation Assays

Murine platelets were purified from citrated whole blood and resuspended in Ca^{2+} -free solution to a final concentration of 1×10^8 platelets per ml, as previously described.¹³ When fixation was required, an equal volume of 2% paraformaldehyde in platelet buffer was added to the platelet suspensions, and the mixture was incubated at 37°C for 1 hour. Platelets were quantitated on a Hemavet 850 (CDC Technologies, Inc., Oxford, CT) and showed <0.1% contamination with leukocytes or erythrocytes.

Static adhesion assays were performed as previously described.¹⁴ Platelets purified from wild-type, heterozygous, and α_2 -integrin-deficient animals were prepared as described above and aliquotted into flat-bottom cells of a 96-well plate coated with either type I collagen or bovine serum albumin. The platelets were allowed to adhere for 1 hour at 37°C. Nonadherent platelets were removed by washing and the remaining attached platelets quantitated, as described.¹⁴

Platelet adhesion to substrates prepared with 100 $\mu\text{g/ml}$ of equine tendon collagen (Helena Laboratories, Beaumont, TX) was assessed in a parallel-plate flow chamber apparatus as previously described.¹⁵ Platelets ($1 \times 10^8/\text{ml}$) were perfused through the chamber at a shear rate of 150 seconds⁻¹ for 5 minutes. The surface area covered by adherent platelets at the end of each experiment was determined (Image Pro Plus software) and expressed as a percentage of wild-type platelet adhesion. All experiments were performed in duplicate on different days.

Platelet aggregation was performed in siliconized glass cuvettes at 37°C with constant stirring (1200 rpm) using a dual-channel aggregometer (Payton Scientific, Buffalo, NY) using washed murine platelets ($2 \times 10^8/\text{ml}$). Aggregation was initiated by either the addition of 2.5 or 5 $\mu\text{g/ml}$ of soluble equine tendon collagen (Helena Laboratories) or 0.5 U/ml of murine thrombin (Sigma Immunochemicals, St. Louis, MO).

Flow Cytometry

Suspensions of paraformaldehyde-fixed platelets were incubated with the appropriate monoclonal antibody, washed with phosphate-buffered saline containing 1% bovine serum albumin and 5 mmol/L ethylenediaminetetraacetic acid, and subsequently incubated with fluorescein isothiocyanate-labeled secondary antibody (Zymed

Laboratories, Inc., South San Francisco, CA) for 30 minutes at room temperature. The platelets were again washed and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The following previously described antibodies to murine antigens were obtained from BD PharMingen (San Diego, CA): HM α_2 (anti-CD49b, hamster IgG), MEC 13.3 (anti-CD31, rat IgG2a), and isotype-matched control (rat anti-IgG2a). NAD-1 (anti-CD41/61, mouse anti-IgG) and anti-glycoprotein VI (GPVI) were generated to murine GPIIb/IIIa complex and murine GPVI, respectively, using standard hybridoma techniques.¹⁶

Cutaneous Wounds

α_2 -deficient and wild-type mice were anesthetized with ketamine, dorsal hair was removed, and a 100-mm² full-thickness wound was created by excising the skin. The wounds were photographed on day 0 and on every third day until they were completely healed. The size of the wound was digitally quantitated using Scion Image software.

Results

Targeted Disruption of the α_2 Integrin Gene in Embryonic Stem Cells

To generate α_2 -integrin-null animals, we used a Cre-loxP targeting approach. The targeting vector was designed to flank exon 1 of the α_2 -integrin gene by two loxP sites and to place the PGK-neomycin resistance gene cassette upstream of exon 1 and the PGK-thymidine kinase gene downstream in the first intron. Embryonic stem cell clones with a targeted allele were transiently transfected with a Cre-recombinase encoding plasmid. Clones with deletion of both exon 1 and the neo-cassette were injected into host 129/Svj blastocysts. Chimeric animals with germline transmission of the targeted allele were obtained. Heterozygous mice were intercrossed to obtain mice homozygous for the α_2 mutation. Genotypic analysis confirmed that mice lacking exon 1 of the α_2 -integrin gene were obtained (Figure 1, B and C).

α_2 -Deficient Mice Are Viable and Fertile

Mice deficient in the $\alpha_2\beta_1$ integrin are viable and fertile and develop normally. Matings of heterozygous progeny yielded 22% wild-type, 58% heterozygous, and 21% homozygous mutant animals, consistent with typical Mendelian inheritance with no excess lethality of homozygotes. α_2 -null females produced viable litters of the expected sizes and nursed their young.

To verify that α_2 -integrin gene disruption generated a null allele and that the α_2 -null mice were deficient in expression of the $\alpha_2\beta_1$ integrin, platelets purified from the blood of wild-type, heterozygous, and homozygous mutant animals were evaluated by flow cytometric analysis using a fluorescein isothiocyanate-conjugated anti-murine α_2 (CD49b) integrin subunit antibody. As shown in

Figure 2. Expression of the α_2 integrin subunit protein and mRNA. **A:** Flow cytometric analysis to detect extracellular expression of the $\alpha_2\beta_1$ integrin, CD31, and CD41/61 was performed on purified platelets from wild-type, heterozygous, and α_2 -deficient mice using fluorescein isothiocyanate-conjugated monoclonal anti-CD49b, CD31, or CD41/61 antibodies or isotype control. **B:** Immunoblot analysis of purified protein lysates from platelets or mammary glands derived from wild-type, heterozygous, and α_2 -deficient animals was performed with polyclonal rabbit antiserum directed against the carboxy-terminal 20 amino acids of the murine α_2 integrin cytoplasmic domain or against actin. **C:** RT-PCR was performed on total RNA isolated from the mammary gland of wild-type, heterozygous, and α_2 -deficient mice. mRNA from wild-type and heterozygous animals generated an 840-bp product.

Figure 2A, the $\alpha_2\beta_1$ integrin was highly expressed by platelets from wild-type animals, as expected. In contrast, there was no detectable expression of the $\alpha_2\beta_1$ integrin on the platelets of α_2 -deficient animals. Intermediate levels of the $\alpha_2\beta_1$ integrin were detected on the surface of platelets from heterozygous littermates. Expression of CD31 and CD41/61 ($\alpha_{IIb}\beta_3$, GPIIb/IIIa, the most abundant integrin on platelets) (Figure 2A) and GPVI on α_2 -deficient platelets was comparable to that on the platelets of wild-type and heterozygous littermates.

Western blot analysis of platelet lysate was also performed with a polyclonal rabbit antiserum directed against the carboxy-terminal 20 amino acids of the murine α_2 -integrin cytoplasmic domain. Neither full-length nor truncated α_2 -integrin subunit protein was detected from platelets of α_2 -null animals (Figure 2B). The α_2 -integrin subunit was detected at intermediate level from platelets of heterozygous littermates and at high level from the wild-type littermates.

Western blot analysis and RT-PCR of protein lysates and mRNA of virgin female mammary glands established

that the α_2 integrin subunit was also effectively deleted from epithelial tissues. As shown in Figure 2, B and C, the wild-type mammary gland expressed the α_2 -integrin subunit protein and mRNA, as expected from previous studies.¹⁷ In contrast, the α_2 -integrin subunit was not detected in protein lysates (Figure 2B) or total RNA (Figure 2C) from the α_2 -null mammary gland.

α_2 Integrin Subunit-Deficient Mice Develop Normally

Analysis of *in vitro* models of pulmonary, mammary, gastrointestinal, and renal development suggested a role for the $\alpha_2\beta_1$ integrin in epithelial differentiation and branching morphogenesis.^{5-8,18} However, α_2 -deficient mice appear grossly normal, are fertile, and are able to nurse their young. Gross and histological evaluation of a number of organs including the heart, lung, gastrointestinal tract, liver, kidney, pancreas, and reproductive tract failed to detect differences between the wild-type, heterozygous, and α_2 -deficient animals. Although the organization of the lungs, gastrointestinal tract, kidney, and mammary gland was entirely normal by histological sections in the α_2 -null animals (Figure 3A, top), whole-mount preparations of virgin female mammary gland revealed marked differences in branching complexity. As shown in Figure 3A, middle and bottom panels, the branching was much more extensive throughout the gland as well as at the terminal ducts in the wild-type mammary gland than in the α_2 -null gland. The number of branch points per terminal duct was 4 ± 1.7 in the wild-type gland versus 1.6 ± 0.2 ($P = 0.0008$) in the α_2 -null mammary gland, as shown in Figure 3B. These findings support a role for the $\alpha_2\beta_1$ integrin in branching morphogenesis *in vivo*.

Effects of α_2 Integrin Deficiency on Platelet Function

Many studies have suggested that the $\alpha_2\beta_1$ integrin is a critical mediator of platelet adhesion to collagen within the vessel wall after vascular injury. Tail vein bleeding times in wild-type, heterozygous, and α_2 -deficient mice were similar (data not shown). Platelets purified from wild-type animals adhered under static conditions to collagen substrates in a Mg^{2+} -dependent manner, as expected for $\alpha_2\beta_1$ integrin-mediated adhesion (Figure 4A). In contrast, platelets purified from α_2 -deficient mice failed to adhere to type I collagen. Platelets from heterozygous littermates exhibited intermediate adhesion. Platelets from all animals adhered to fibronectin in a comparable manner (data not shown), but failed to adhere to bovine serum albumin.

As shown in Figure 4, B and C, purified platelets from wild-type mice also effectively adhered to type I collagen at shear rates of 150 seconds⁻¹, a flow rate in which exogenous Von Willebrand factor is not required. In contrast, platelets from α_2 -deficient mice failed to adhere under shear stress. Platelets from heterozygous mice again adhered to an intermediate extent, indicative of a

Figure 3. Branching morphogenesis of the mammary gland. **A:** Histological sections of mammary glands of wild-type (+/+) and α_2 -deficient (-/-) animals 8 to 12 weeks of age were evaluated (**top**). Whole-mount preparations of 8- to 10-week-old virgin wild-type (+/+) and α_2 -deficient (-/-) mammary glands were stained with carmine solution. Terminal ducts were photographed at $\times 100$ (**middle**) and $\times 1000$ (**bottom**). **B:** The number of branch points per terminal duct was quantitated morphologically. The mean and SEM are shown.

gene dosage effect and suggesting that there are few, if any, spare $\alpha_2\beta_1$ collagen receptors. As also shown in Figure 4B, platelets from both wild-type and heterozygous mice formed small platelet thrombi on the collagen substrates under conditions of flow.

Platelets from wild-type and heterozygous animals comparably aggregated in response to low concentrations of collagen (2.5 and 5 $\mu\text{g/ml}$) (Figure 4D). Although platelets from α_2 -null animals aggregated in response to type I collagen, they did so with a significantly prolonged lag phase and a decreased rate of aggregation.

$\alpha_2\beta_1$ Integrin and Wound Healing

The $\alpha_2\beta_1$ integrin is highly expressed by the basal cell layer of the skin and moderately expressed by dermal fibroblasts. *In vitro* and explant studies of matrix contraction and wound healing suggested that the $\alpha_2\beta_1$ integrin may serve a role in the normal organization of the dermis and an obligatory role in wound healing.^{9,10} Histological evaluation of full-thickness skin biopsies of wild-type and α_2 -null animals identified no detectable differences (Figure 5A). In both cases the epidermis was composed of layers of stratified squamous epithelium attached to the basal cell layer. The dermis in the α_2 -null and wild-type animals contained comparable numbers of fibroblasts and well-organized collagen bundles.

To determine whether the $\alpha_2\beta_1$ integrin was required for wound healing, the ability to close a 100-mm² full-thickness wound was evaluated. Compared to wild-type animals, α_2 -deficient mice demonstrated no defect in wound repair and all animals displayed a similar temporal pattern of wound healing (Figure 5B). In fact, wound healing on day 12 was slightly enhanced in the α_2 -null animals compared to wild-type littermates. The morphology of the wounds failed to demonstrate any difference in keratinocyte migration or angiogenesis into the wound site (data not shown).

Discussion

The expression of the $\alpha_2\beta_1$ integrin by diverse cell types raised many questions regarding the role of the $\alpha_2\beta_1$ integrin *in vivo*. Indeed, the $\alpha_2\beta_1$ -integrin-null mouse exhibits a multifaceted phenotype. In some cases, such as platelets and mammary epithelial cells, the null phenotype was consistent with predictions based on the expression pattern of the integrin and consistent with the *in vitro* data. In other instances, the *in vivo* findings suggest alternative mechanisms for biological processes such as wound healing, and reject the proposed obligatory role for the $\alpha_2\beta_1$ integrin.

Although the $\alpha_2\beta_1$ integrin is expressed at high level in the placenta and in many other tissues during embryogenesis,¹² its expression is not required for normal murine development. Deficiency of the $\alpha_2\beta_1$ integrin had no effect on offspring viability or fertility of the α_2 -deficient animals.

The $\alpha_2\beta_1$ integrin is highly expressed at sites of terminal duct branching in the developing mammary gland and lung.^{12,17} The significant decrement in branching complexity observed in the mammary glands of $\alpha_2\beta_1$ integrin-deficient animals supports an important role for the integrin in branching morphogenesis *in vivo*.⁵⁻⁸ The results of the *in vivo* studies, although striking, were less dramatic than the complete absence of branching morphogenesis observed in the absence or inhibition of the $\alpha_2\beta_1$ integrin in earlier *in vitro* studies in three-dimensional collagen gels. The *in vitro* models were designed to study a single integrin interaction with a single matrix molecule. Mammary epithelial cells express a wide variety of integrin and nonintegrin adhesion receptors that interact with

Figure 5. $\alpha_2\beta_1$ integrin and wound healing. **A:** Photomicrographs of full-thickness skin biopsies from wild-type or α_2 -null mice revealed no differences in skin development. The dermis of both animals contained comparable numbers of fibroblasts and organized collagen bundles. **B:** The size of a 100-mm full-thickness wound was digitally quantitated from day 0 to day 18, when all wounds were healed. Both wild-type and α_2 -null animals exhibit similar rates of wound healing.

multiple components of the extracellular matrix *in vivo* and may mitigate the consequences of $\alpha_2\beta_1$ integrin deficiency.

The mechanism by which branching is impaired in the α_2 -deficient mouse is not yet understood. One possibility is defective proliferation of $\alpha_2\beta_1$ -integrin-deficient mammary epithelial cells. Recent studies have established that the integrin is an important determinant of growth factor-dependent cell cycle progression of mammary epithelial cells.^{18,19} Whether the differences seen at weeks 9 to 11 are because of defective branching per se, or rather to a delay in development, is uncertain. Future studies will evaluate the mammary gland during all stages of maturation including puberty, gestation, and involution. However, the α_2 -deficient mammary gland is capable of lactation and the α_2 -deficient animals can adequately nurse their young despite the deficit in branching complexity. Litters of α_2 -deficient females do

Figure 4. Platelet adhesion and aggregation. **A:** Platelets purified from wild-type (+/+), heterozygous (+/-), and α_2 -deficient (-/-) animals were assayed for adhesion to type I collagen. The assays were performed for 1 hour in phosphate-buffered saline with 2 mmol/L of Mg^{2+} or ethylenediaminetetraacetic acid. **B:** Platelet adhesion to type I collagen at shear rates of 150 seconds⁻¹ was assayed in a parallel-plate flow chamber apparatus. Micrographs of the collagen substrate and the deposition of platelets from wild-type (+/+), heterozygous (+/-), and α_2 -deficient (-/-) mice are depicted. **C:** The surface area covered by adherent wild-type, heterozygous, or α_2 -deficient platelets was quantitated and expressed as a percentage of wild-type platelet adhesion. **D:** Platelet aggregation of heterozygous (+/-) or α_2 -deficient (-/-) platelets was initiated by either the addition of 2.5 or 5 μ g/ml of soluble equine tendon collagen or 0.5 U/ml of thrombin.

not show a growth defect. Our earlier studies established a strong correlation between diminished $\alpha_2\beta_1$ integrin expression and a less differentiated phenotype in breast cancer.^{20,21} The $\alpha_2\beta_1$ -integrin-deficient mouse should afford a system to further assess the relationship between $\alpha_2\beta_1$ integrin expression and the development and phenotype of breast carcinomas.

The lack of obvious abnormalities in other organs such as the lung, gastrointestinal tract, and kidney may be because of the subtlety of lesions in these organs not detected by standard histopathological evaluation. In the mammary gland, the differences were most obvious in whole-mount preparations, but not readily apparent in cross sections of the mammary gland. More detailed examinations of other organs are required to exclude this possibility.

In vitro studies of human skin have suggested that the $\alpha_2\beta_1$ integrin is required for wound healing.⁹⁻¹⁰ Inhibitory antibodies against the $\alpha_2\beta_1$ integrin prevent keratinocyte migration on type I collagen and across wounded dermis and block fibroblast contraction of three-dimensional collagen gels.⁹⁻¹⁰ However, deficiency of the $\alpha_2\beta_1$ integrin failed to alter wound healing *in vivo* and had no effect on the migration of keratinocytes over exposed dermis (data not shown) suggesting that $\alpha_2\beta_1$ integrin does not play an obligatory role in wound healing. There are at least two possible explanations for the discrepancy. One arises from the use of two different organisms that may not be mechanistically equivalent as model systems. Second, on binding the integrin, inhibitory antibodies may stimulate signals downstream of the integrin that are responsible for the observed phenotype. Thus, antibodies could produce effects that differ from those because of lack of integrin expression.

The profound deficit in platelet adhesion to collagen observed with platelets from α_2 integrin subunit-deficient animals is in accord with earlier studies addressing the role of the $\alpha_2\beta_1$ integrin as a collagen receptor on platelets by less direct approaches.⁴ The lack of $\alpha_2\beta_1$ integrin expression did not effect the expression of the other major platelet integrin, $\alpha_{IIb}\beta_3$ (CD41/61). The magnitude of the deficit under both static conditions and conditions of flow clearly indicates that the $\alpha_2\beta_1$ integrin is the dominant adhesive receptor for collagen on platelets.

Other putative receptors are unable to support significant adhesion in the absence of the $\alpha_2\beta_1$ integrin not only under conditions of flow, but also under the less rigorous static conditions. The dramatic reduction in adhesion of heterozygous platelets under both static and flow conditions is consistent with a gene dosage effect and suggests that the level of $\alpha_2\beta_1$ integrin expression profoundly influences the adhesive functions of platelets on collagenous substrates. This provides an animal model to evaluate the molecular mechanisms by which allelic differences in the α_2 integrin gene and different levels of $\alpha_2\beta_1$ integrin expression are associated with increased risk of myocardial infarction, diabetic retinopathy, and stroke.²²⁻²⁵ The adhesive decrement in heterozygotes suggests that the receptor is not present in significant excess.

The results of the platelet studies are primarily consistent with the two-step, two-site model of collagen-induced platelet activation.²⁶ In this model, adhesion is

mediated by the $\alpha_2\beta_1$ integrin, whereas collagen-induced activation and aggregation is mediated by a second, low-affinity signal-transducing co-receptor. Recent studies suggest that GPVI serves the role of the signal-transducing co-receptor. Under the extremely low shear conditions of the aggregation assay, as opposed to the more stringent conditions of the adhesion assays, our data suggest that GPVI is sufficient to support activation and aggregation although at a somewhat reduced level. Collagen is only one of several physiologically relevant activators of platelets. The presence of other potent activators, such as thrombin, at the sites of vascular injury *in vivo*, likely accounts for the relatively normal tail vein bleeding times observed and the absence of an overt bleeding diathesis.

Some comparison with the recently published study of collagen-induced platelet aggregation and adhesion in platelets deficient in the β_1 integrin subunit is warranted.²⁷ The findings regarding collagen-induced platelet aggregation and adhesion to substrates of monomeric collagen are primarily in accord. The studies of adhesion to fibrillar substrates described by Nieswandt and colleagues²⁷ were likely confounded by the highly thrombogenic nature of fibrillar collagen substrates and the relatively intact aggregation response to collagen exhibited by platelets deficient in the $\alpha_2\beta_1$ integrin. It should also be noted that differences between $\alpha_2\beta_1$ -deficient and control platelets were minimized by reconstituting washed platelets in media containing 1 mmol/L of Ca^{++} thereby suppressing $\alpha_2\beta_1$ integrin functions. The model presented above, but not that of Nieswandt and colleagues,²⁷ is also consistent with the findings of studies with human platelets deficient in GPVI. GPVI-deficient platelets exhibit markedly impaired collagen-induced aggregation and activation, little or no defect in adhesion per se, and a modest decrement in total platelet deposition (adhesion plus aggregation) on collagen substrates measured under conditions of flow that was attributable to the loss of platelet-platelet interactions.^{28,29}

In addition to profoundly deficient adhesion to collagen, the platelets deficient in the $\alpha_2\beta_1$ integrin of a patient described by Nieuwenhuis and colleagues^{30,31} also exhibited markedly impaired (essentially undetectable) collagen-induced platelet aggregation. This finding is at variance with both our study of the α_2 -integrin subunit null mouse and the study of Nieswandt and colleagues²⁷ of the β_1 -integrin subunit-deficient mouse. It seems likely that the platelets of the patient described by Nieuwenhuis and colleagues^{30,31} exhibit an additional defect resulting in the absence of collagen-induced platelet aggregation. Alternately, the relative contributions of the $\alpha_2\beta_1$ integrin, glycoprotein VI, and perhaps other receptors and signaling pathways differ in human and murine platelets.

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