

Extensive Vasculogenesis, Angiogenesis, and Organogenesis Precede Lethality in Mice Lacking All α_v Integrins

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Summary

α_v integrins have been implicated in many developmental processes and are therapeutic targets for inhibition of angiogenesis and osteoporosis. Surprisingly, ablation of the gene for the α_v integrin subunit, eliminating all five α_v integrins, although causing lethality, allows considerable development and organogenesis including, most notably, extensive vasculogenesis and angiogenesis. Eighty percent of embryos die in mid-gestation, probably because of placental defects, but all embryos develop normally to E9.5, and 20% are born alive. These liveborn α_v -null mice consistently exhibit intracerebral and intestinal hemorrhages and cleft palates. These results necessitate reevaluation of the primacy of α_v integrins in many functions including vascular development, despite reports that blockade of these integrins with antibodies or peptides prevents angiogenesis.

Introduction

Integrins serve as major receptors for extracellular matrix (ECM)-mediated cell adhesion and migration, cytoskeletal organization, cell proliferation, survival, and differentiation (Hynes, 1992; Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Miyamoto, 1995). α_v integrins comprise a subset sharing a common α_v subunit combined with one of five β subunits (β_1 , β_3 , β_5 , β_6 , or β_8). All or most α_v integrins recognize the sequence RGD in a variety of ligands (vitronectin, fibronectin, osteopontin, bone sialoprotein, thrombospondin, fibrinogen, von Willebrand factor, tenascin, and agrin) (Felding-Habermann and Cheresch, 1993; Martin and Sanes, 1997) and, in the case of $\alpha_v\beta_8$, laminin and type IV collagen (Venstrom and Reichardt, 1995).

α_v integrins are widely expressed on multiple cell types, and during development, the different α_v -associated β subunits show distinct patterns of expression (Hirsch et al., 1994; Alfandari et al., 1995; Yamada et al., 1995). This, together with the wide range of potential ligands, differentially recognized by $\alpha_v\beta$ integrins, implies diverse functions for the different receptors and

their ligands. Significant expression of α_v integrins has been noted, in particular, in neural crest cells (Delannet et al., 1994), glial cells (Hirsch et al., 1994; Milner and French-Constant, 1994), muscle (Hirsch et al., 1994; McDonald et al., 1995; Martin and Sanes, 1997), osteoclasts (Väänänen and Horton, 1995), epithelia ($\alpha_v\beta_6$; Breuss et al., 1995; Huang et al., 1996), and blood vessels during development (Brooks et al., 1994a; Drake et al., 1995; Friedlander et al., 1995, 1996) or angiogenesis in response to tumors (Brooks et al., 1994a, 1994b, 1996, 1998; Varner et al., 1995).

Among the ligands for various $\alpha_v\beta$ integrins is fibronectin (FN), and results on mouse embryos lacking FN or FN receptor integrins suggest that α_v integrins might be important receptors for FN during early development. FN-null embryos fail to form notochord or somites (George et al., 1993; Georges-Labouesse et al., 1996), whereas embryos null for either (Yang et al., 1993, 1995) or both (Yang, B. B., and R. H., unpublished data) of two FN receptor integrin subunits, α_5 or α_4 , all form these mesodermal structures. This indicates the existence of one or more additional FN receptors functional during early organogenesis. Studies on embryonic cells lacking α_4 and/or α_5 show that FN receptor functions *in vitro* can be fulfilled by $\alpha_v\beta_1$ (Yang and Hynes, 1996). Other studies implicate $\alpha_v\beta_3$ in FN matrix assembly in the absence of β_1 integrins (Wennerberg et al., 1996). Therefore, α_v integrins could serve as FN receptors early in development, either by overlapping functionality or by compensation. To investigate that possibility we have ablated the α_v gene in mice.

A strain of mice deficient in α_v integrins also allows evaluation of the potential roles of α_v integrins in other biological processes. Of particular interest, both scientifically and clinically, are the potential roles of α_v integrins in implantation and placentation (Damsky et al., 1993, 1994; Cross et al., 1994), restenosis (Panda et al., 1997), bone remodelling, and angiogenesis. Particularly in the case of the last two, there is considerable interest in the possibility that blockade of α_v integrins could be useful clinically in treatments of osteoporosis (Engleman et al., 1997) or angiogenesis of tumors (Brooks et al., 1994b, 1995) or in the retina (Friedlander et al., 1996; Hammes et al., 1996). These initiatives employ antibodies, peptides, or peptidomimetics targeted against α_v integrins. It would be valuable to have independent genetic validation of the importance of α_v integrins in these processes.

For all these reasons, we have generated a strain of mice with a null mutation in the α_v integrin gene. As might have been expected, this mutation is lethal but, unexpectedly, a considerable degree of development proceeds almost normally in the absence of α_v integrins, some animals being born alive. Perhaps most notably, vasculogenesis and angiogenesis continue. Analyses of the development of α_v -null embryos provide significant new information bearing on the potential functions of α_v integrins.

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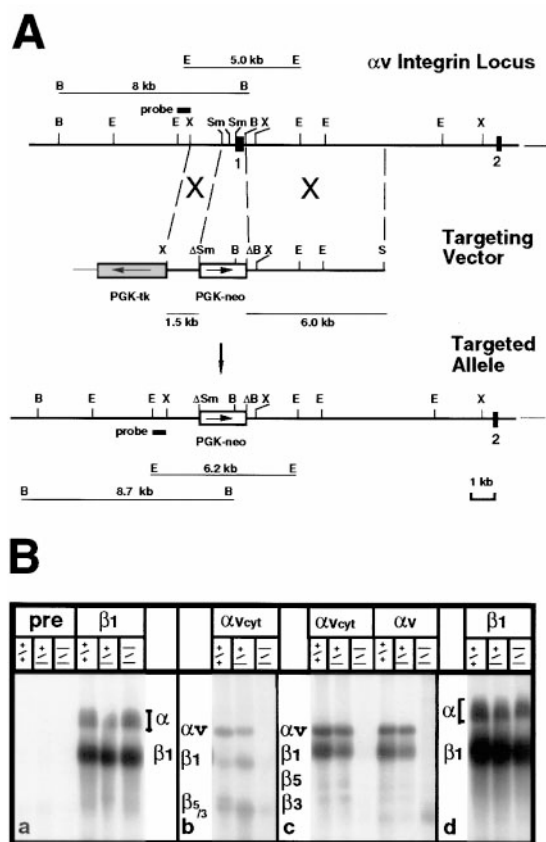


Figure 1. Disruption of the α_V Integrin Locus

(A) Strategy used to delete exon 1 and adjacent noncoding sequences. Solid rectangles represent exons 1 and 2. The PGK-neo^R-cassette and the PGK-tk-cassette are shown as open and stippled rectangles, respectively; arrows show gene orientations. Restriction fragments hybridizing to the 5' probe (heavy line) used for Southern blot analyses are indicated. B, BamHI; E, EcoRI; Sm, SmaI; S, Sall; X, XbaI.

(B) Expression of integrins on the surfaces of cells from embryos and newborns. Fibroblasts from E16 embryos (a and b) and dermal fibroblasts from newborns (c and d) were cell surface-labeled with ¹²⁵I. Cell lysates were immunoprecipitated with various integrin-specific antibodies as indicated at the top and analyzed by SDS-PAGE under nonreducing conditions. Genotypes of the cells are shown on the top of each lane. pre, rabbit preimmune serum; β_1 , β_1 integrin-specific rabbit serum; α_V cyt, α_V integrin-specific rabbit serum; α_V , α_V integrin-specific hamster monoclonal antibody. Migration positions of certain integrin subunits are indicated.

Results

Targeted Disruption of the α_V Integrin Gene

The α_V integrin gene was inactivated by homologous recombination deleting the first exon of the α_V integrin gene encoding 5' untranslated sequence, the signal peptide, and 30 amino acids of the mature protein, as well as 55 bp of the adjacent intron, and replacing these sequences by a neomycin-resistance (neo^R)-expression cassette (Figure 1A). Twelve independently targeted ES cell lines were obtained, injected into C57BL/6J blastocysts, and transferred to pseudopregnant females. Chimeric males from three lines gave germline transmission

of the mutated α_V integrin gene. Mice heterozygous for the mutation appeared normal and did not display any overt anatomical or behavioral abnormalities.

No mice homozygous for the mutation were detected among >300 weaned progeny from heterozygous intercrosses (Table 1). Analysis of newborn offspring detected eight homozygous mutant mice among 142 neonates (Table 1). However, these homozygotes showed intracranial and intestinal hemorrhage (see below; Figure 5H) and died perinatally. Therefore, the homozygous mutation is recessive lethal, and survival to the stage of perinatal lethality is only 20% of the expected Mendelian ratio.

Cells from Homozygous Mutant Embryos Do Not Express α_V Integrins

To verify the α_V integrin-null mutation, we cultured fibroblastic cells from E16 embryos and dermal fibroblasts from neonates and examined them for the presence of α_V integrins and other integrins by cell surface iodination and immunoprecipitation. While α_V and β_1 integrins (Figure 1B) and α_5 integrin (data not shown) were immunoprecipitated from both wild-type and heterozygous embryonic cells, no α_V integrin subunit was precipitated from homozygous mutant cells (Figure 1B, b and c). There was no indication that other integrins were upregulated in the absence of α_V integrins. These results show that the α_V integrin subunits are absent from the surfaces of cells derived from homozygous mutant embryos and that the mutation is a true α_V -null mutation. Consistent with this conclusion, α_V -null fibroblasts failed to adhere to vitronectin although adherence to FN was normal (data not shown). We also checked for α_V mRNA by RT-PCR using primers from regions of the gene 3' of the mutation and found no α_V mRNA (data not shown).

Mice Homozygous for the Mutant Allele Die in Two Waves: between 10 and 12 Days of Gestation and Perinatally

To determine when the rest of the homozygotes die, embryos were examined from E8.5 to birth (see Table 1). Between 8.5 and 10.5 days postcoitum, wild-type, heterozygous, and homozygous mutant embryos were represented in a normal Mendelian ratio of 1:2:1 (Table 1). α_V -null embryos at E8.5 and E9.5 were indistinguishable from wild-type and heterozygous littermates. Somite compaction in E8.5 α_V -null embryos appeared normal (Figures 2A and 2B). E9.5 α_V -null embryos formed brain vesicles, optic vesicles, branchial arches, otic pits, forelimb buds, a beating heart, and 20–25 somites (Figures 2C and 2D). Occasionally, E9.5 α_V -null embryos (~10% of the expected mutant embryos) were slightly smaller in size but did not exhibit morphological abnormalities compared with normal littermates. Therefore, development to day 9.5 of gestation is largely independent of α_V integrins.

By E10.5, defects began to appear in α_V -null embryos. Although they were still present in the expected proportions (Table 1) and all were alive as demonstrated by beating hearts, many appeared delayed in growth (smaller heads, underdeveloped branchial arches, and

Table 1. Genotypes of Progeny from Heterozygous Intercrosses

Stage	No. of Litters	αv Integrin Genotype (%)			-/- Viable ^a	% of Expected -/- Progeny
		+/+	+/-	-/-		
E8.5	8	16 (23)	36 (52)	17 (25)	17	98
E9.5	12	29 (27)	53 (49)	26 (24)	26	95
E10.5	11	25 (24)	55 (53)	24 (23)	24 ^b	90
E11.5	10	22 (25)	48 (55)	17 ^c (20)	4	17
E8.5-E11.5	41	92 (24)	192 (51)	84 (22)		
E12.5	12	20 (26)	46 (61)	10 ^c (13)	4	18
E13.5	10	20 (30)	42 (64)	4 (6)	2	10
E14.5	3	7 (37)	11 (58)	1 (5)	1	17
E15.5	4	8 (30)	15 (56)	1 (4)	1	13
E16.5	4	5 (23)	16 (73)	1 (4)	1	14
E17.5	8	14 (29)	32 (67)	2 (4)	2	13
E13.5-E17.5	29	54 (30)	116 (65)	9 (5)	7	12
P0-P1	24	41 (31)	83 (63)	8 (6)	8	19
P > 21	27	54 (32)	116 (68)	0 (0) ^d	0	0

E, embryonic day; P, postnatal day.

% of expected viable -/- calculated as % if sum of (+/+ + +/-) = 75%.

^a Heart is beating.

^b 5 embryos looked normal; 19 showed retardation and pericardial edema.

^c Many embryos deteriorating.

^d Total number of progeny now exceeds 300.

nasal processes, see Figures 2F and 2G). A significant number of the αv -null embryos also exhibited pericardial edema by E10.5 (Figure 2G). In contrast, a second, smaller class of αv -null embryos appeared normal at E10.5. By E11.5 this division into two classes was even

more evident. The minority class (II; ~20%) still appeared to be developing normally (Figures 6A and 6B) while the majority class (I; ~80%) were deteriorating (Figure 2H) or were already absorbed.

Therefore, homozygosity for the αv -null mutation leads

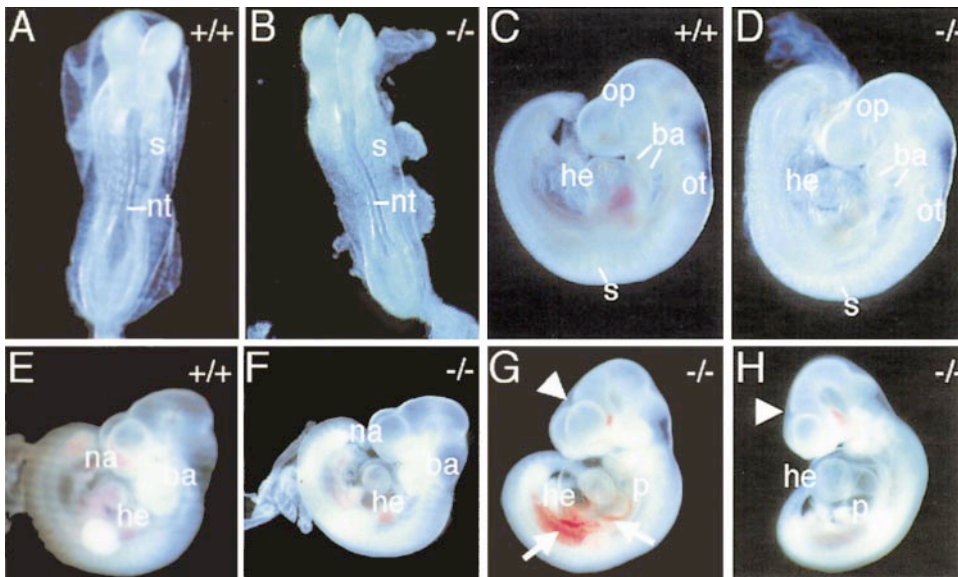


Figure 2. Wild-Type and αv -Null Embryos between E8.5 and E11 Showing the Early Defect Phenotype (Class I) of αv -Null Mice

(A and B) Dorsal views of E8.5 wild-type (A) and αv -null (B) embryos are similar; somites have formed and are compacted.

(C and D) Side views of E9.5 wild-type (C) and αv -null (D); still no significant difference between wild-type and mutant embryos.

(E and F) Side views of E10.5 wild-type (E) and αv -null (F); the mutant embryo is slightly retarded, the head is smaller, and the branchial arches and the nasal process are less developed; the heart appears slightly enlarged.

(G) Side view of an E10.5 αv -null embryo (class I). This more severely affected embryo shows significant pericardial edema and leaking of blood into the trunk region and pericardial sac (arrows).

(H) Side view of an E11 αv -null embryo (class I) shows a markedly enlarged heart, pericardial and systemic edema (arrowheads).

Abbreviations: ba, branchial arches; he, heart; na, nasal process; nt, neural tube; op, optic vesicle; ot, otic pit; p, pericardium; s, somites.

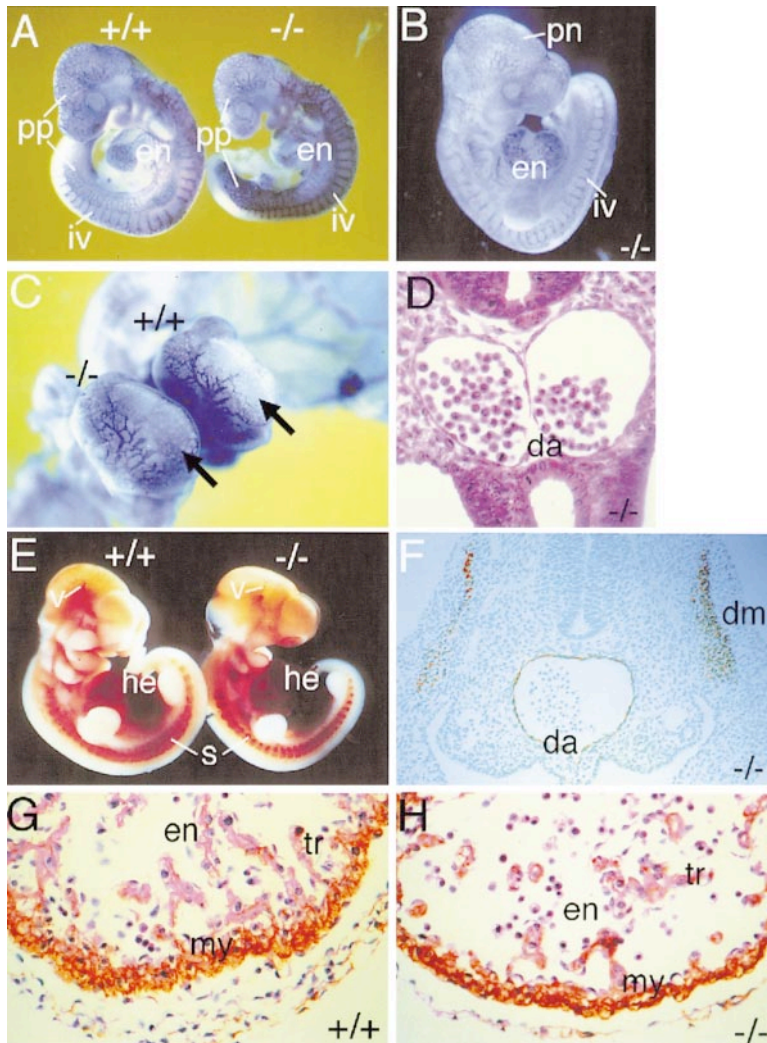


Figure 3. Whole-Mount Immunohistochemistry and Histology of E9.5 and E10.5 Embryos

Staining for PECAM-1 (A–C) visualized with alkaline phosphatase and for smooth muscle α -actin (SM α Ac). (E–H) visualized with HRP. (A) PECAM-1 staining of wild-type (left) and α v-null (right) E9.5 embryos. The vasculature is patterned correctly in α v-null embryos. (B) Enlargement of an α v-null E9.5 embryo stained for PECAM-1. Intersomitic vessels, endocardium, and perineural plexus are present and well developed.

(C) View of heads of wild-type (upper right) and α v-null (lower left) E10.5 embryos. Vessels of the perineural plexus (arrows) appear slightly distended in this class I α v-null embryo in comparison with wild-type. (D) Cross section of an E9.5 α v-null embryo showing the well developed paired dorsal aortae filled with blood cells.

(E) SM α Ac staining of wild-type (left) and α v-null (right) E10.5 embryos shows expression in the somites and the heart. Note staining of blood vessels in the head. (F) Cross section through an E10.5 α v-null embryo stained for SM α Ac. Note staining in the dermamyotome and around the dorsal aorta.

(G and H) Cross section through the heart of wild-type (G) and class I α v-null (H) E10.5 embryos stained for SM α Ac. In both embryos, staining can be found in the myocardium. Endocardium can be seen in both embryos. The myocardium of the α v-null heart appears thinner and less trabeculated.

Abbreviations: da, dorsal aorta; dm, dermamyotome; en, endocardium; he, heart; iv, intersomitic vessel; my, myocardium; pn, perineural plexus; pp, primary capillary plexus; s, somite; tr, trabeculae; v, blood vessel.

to two patterns of development. Class I α v-null embryos (~80%) die during an early crisis (E9.5–E11.5) characterized by pericardial edema and delayed growth and development. Class II α v-null embryos (~20%) survive this period of development without obvious developmental defects, survive the rest of gestation without further losses (Table 1), and give rise to the viable α v-null neonates. As mentioned, such pups are born with severe intracranial and intestinal hemorrhages and die at the day of birth. The intracerebral hemorrhage begins around E12.5 and becomes progressively worse during development (see below).

Embryonic Vascularization and Myogenic Development

Antibodies recognizing PECAM-1, a marker for endothelial cells, and smooth muscle α -actin (SM α Ac), an early marker for myogenic lineages, were used for whole-mount immunohistochemical staining of embryos at E9.5 and E10.5 (Figure 3). PECAM-1 staining revealed apparently normal vascular patterning in α v-nulls indistinguishable from that seen in control littermates (Figures 3A–3C). Cross sections of E9.5 α v-null (Figure 3D)

and E9.5 wild-type littermates (data not shown) demonstrate normally shaped paired dorsal aortae filled with red blood cells in the α v-null embryos. At E10.5 those class I α v-null embryos showing signs of pericardial edema also show a normal vascular pattern but generally exhibit a less complex primary perineural plexus. Those vessels appear somewhat distended (Figure 3C). In comparison, the wild-type plexus develops into a highly branched and intricate vascular network (Figure 3C) as is also the case in the class II α v-null embryos. Taken together, these data demonstrated clearly that the α v integrin gene is not required for the formation of the dorsal aortae and primary plexus (vasculogenesis) nor for capillary sprouting (angiogenesis) or remodelling of the capillary plexus into small and large vessels.

Whole-mount staining for SM α Ac of E10.5 embryos (Figure 3E) and cross sections (Figures 3F–3H) revealed that (1) somites in α v-null embryos can differentiate into the myogenic lineage (Figures 3E and 3F), (2) SM α Ac-expressing α v-null vascular smooth muscle cells (VSMC) surround the dorsal aorta (Figure 3F), and (3) in α v-null embryos, cardiac development initially is normal (data not shown) and SM α Ac-expressing cardiomyocytes are

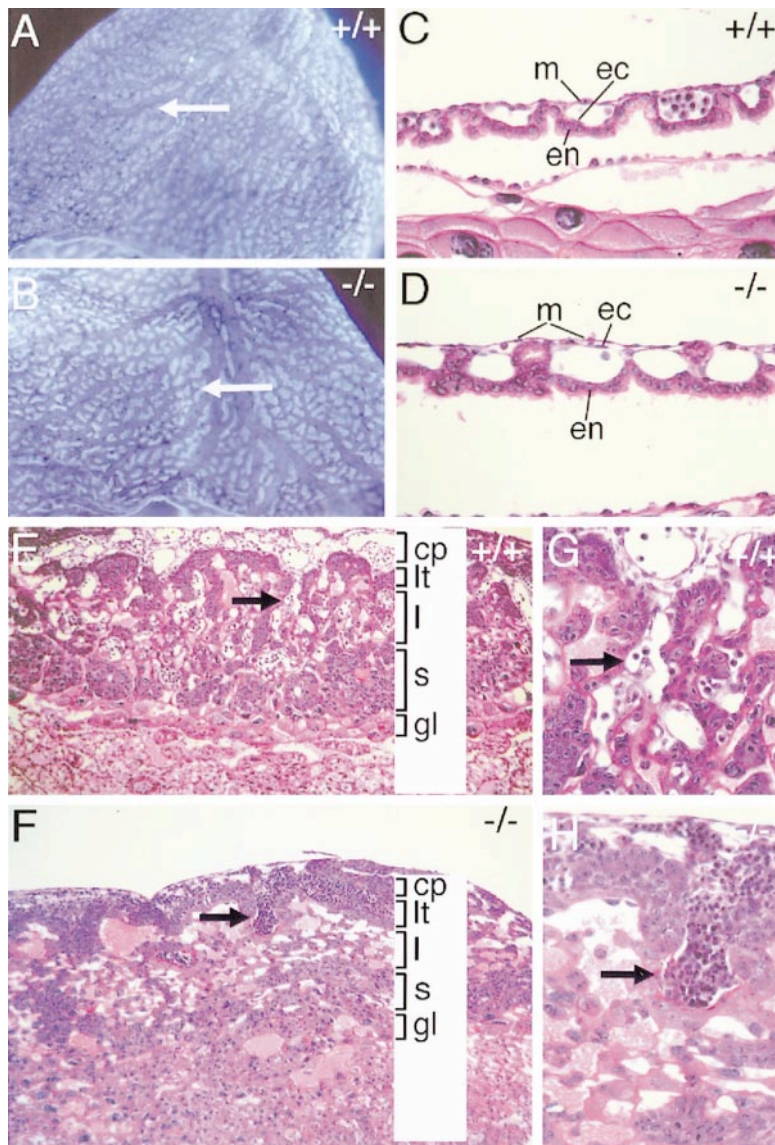


Figure 4. Yolk Sacs and Placentae of Wild-Type and αv -Null Littermate Embryos

(A and B) PECAM-1 staining of yolk sacs of E9.5 wild-type (A) and αv -null (B) shows elaborate vasculature with large and small vessels. Sprouting and branching vessels (arrows) are prominent in both wild type and mutant.

(C and D) Comparison of wild-type (C) and αv -null (D) yolk sacs; E10.5 embryos in utero. (D) Slightly distended vessels are occasionally observed in the αv -mutant yolk sac. Endothelial lining of blood vessels surrounded by mesodermal and endodermal constituents of the yolk sac are indicated.

Abbreviations in (A)–(D): ec, endothelial cell; en, endoderm; m, mesoderm.

(E–H) Transverse paraffin sections of placentae from E10.5 embryos; wild-type (E and G) and αv -null (F and H).

In αv -null placentae dilated placental sinuses filled with fetal blood cells (arrow) can be found in the chorionic plate and in the adjacent labyrinthine trophoblast layer (H) as compared with wild-type placental sinus (G). The labyrinthine trophoblast is more compact in mutant (F) than in wild-type placenta (E). The labyrinthine area appears reduced and fetal blood vessels intermingling deeply with maternal blood sinuses are sparse or absent in placentae of mutant embryos with early phenotype (F). The organization of the mutant giant cell layer and the spongiotrophoblast is not as distinct as in the wild-type placentae (E and F). Abbreviations: cp, chorionic plate; lt, labyrinthine trophoblast; l, labyrinthine layer; s, spongiotrophoblast layer; gl, giant layer.

present in the compact layer of the heart. However, at E10.5 the class I αv -null hearts became increasingly distended, the compact layer of the myocardium appeared thinner, and the myocardial trabeculae of the ventricles were less complex (Figure 3H). In contrast, the hearts of the class II αv -null embryos developed normally (see below).

Normal Yolk Sac Vasculature but Deficient Placentation

The vital connection between the developing heart and the yolk sac (the vitelline vessels) and the fusion of the allantois with the chorion were established in all αv -null embryos examined (data not shown). To assess the patterning and integrity of the extraembryonic vasculature and placentation of αv -null embryos, yolk sacs and placentae from wild-type and mutant embryos were analyzed. Whole-mount immunohistochemistry with anti-PECAM-1 antibodies of yolk sacs between E9.5 and

E13 (Figures 4A–4D and data not shown) showed an elaborate vasculature with larger and smaller vessels. Sprouting angiogenesis and branching vessels were prominent in both wild-type (Figure 4A) and αv -null E9.75 yolk sacs (Figure 4B). Histological analyses of E10.5 embryos and yolk sacs in utero (Figures 4C and 4D) demonstrated that in αv -null yolk sacs a complex vascular network formed and blood vessels of different diameters were present but occasionally appeared slightly distended compared with control littermates.

Placentae were also examined both after dissection and by histology in utero. Some αv -null placentae (~20%) appeared normal whereas the majority (~80%) showed some abnormalities. The concordance between the frequencies of abnormal placentae and class I αv -null embryos showing early lethality suggests that these two phenomena are linked. The most prominent differences between the defective αv -null and wild-type placentae were in the labyrinthine zone, which appeared

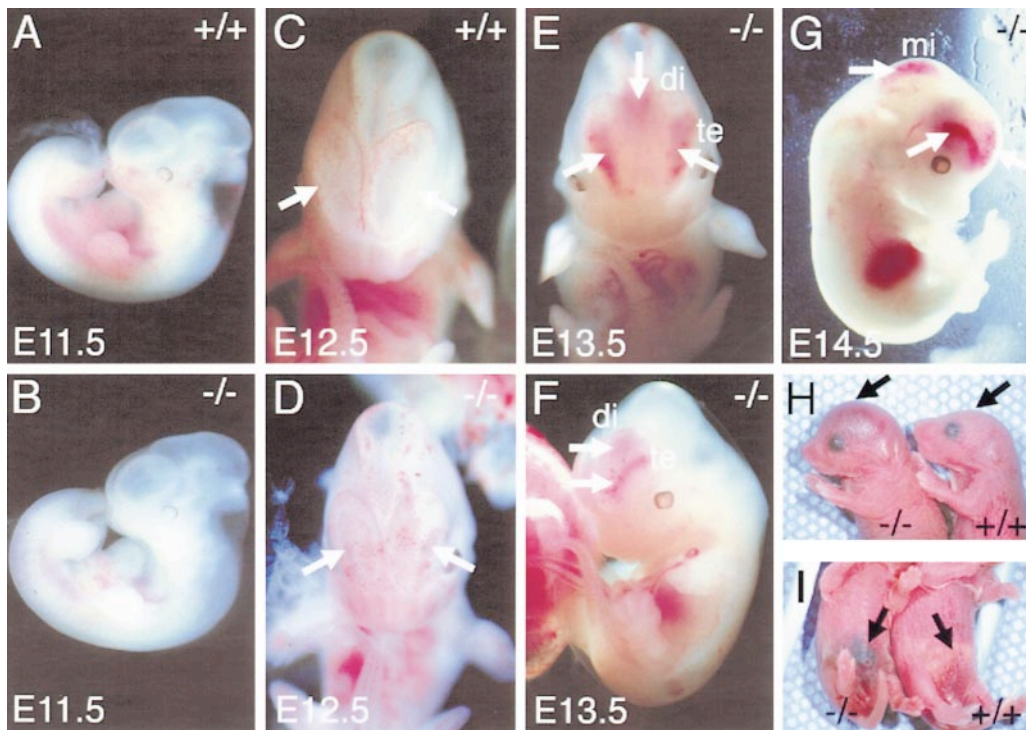


Figure 5. Wild-Type and αv -Null Embryos between E11.5 and E14.5 and Neonates Showing the Late Phenotype of αv -Null Class II Embryos
(A and B) Side views of E11.5 wild-type (A) and αv -null (B) show no significant differences.
(C and D) Frontal view of an E12.5 wild-type (C). Note the extracerebral vessels of the perineural mesenchyme. Frontal view of an E12.5 αv -null embryo (D). Hemorrhage is beginning in the ganglionic eminence of the forebrain. Arrows indicate comparable ventrolateral locations in the hemispheres, where hemorrhage occurs in αv -nulls, but not in wild-type animals.
(E and F) Frontal view of an E13.5 αv -null embryo (E) and side view of another αv -null E13.5 embryo (F). Intracerebral hemorrhage is more severe; blood can be found bilaterally in the forebrain, in the ganglionic eminence of the telencephalon, and in the diencephalon.
(G) Side view of an E14.5 αv -null embryo. Intracerebral hemorrhages progress (arrows), blood cells also accumulate in the cortex of both hemispheres and in the midbrain.
(H and I) Head (H) and trunk (I) regions of wild-type (right) and αv -null (left) neonate. Severe hemorrhage in head and intestine of the αv neonate are visible. Arrows indicate comparable locations.
 Abbreviations: di, diencephalon; mi, midbrain; te, telencephalon.

reduced. Fetal blood vessels intermingling deeply with maternal blood sinuses were sparse or absent in placenta of class I mutant embryos (Figures 4F and 4H) compared with wild-type placenta (Figures 4E and 4G). In addition, in αv -null placenta the allantoic and fetal placental blood sinuses were frequently dilated and typically filled with fetal red blood cells (Figure 4H). The mutant trophoblastic region was abnormally thick and compact. These abnormalities may impair the development of a functional placenta and lead to the early embryonic phenotype of αv -null embryos. In contrast, in the normally developing class II αv -null embryos the placenta appeared normal (data not shown).

Intracerebral Hemorrhage in αv -Null Embryos

The class II αv -null embryos, which did not show pericardial edema, appeared to develop largely normally until birth. As mentioned, these embryos develop prominent intracerebral hemorrhages. Whole-mount analyses between E11.5 and birth (Figure 5) illustrate these intracerebral hemorrhages. The most prominent late phenotype of the αv -null mutation was first visible at E12.5 bilaterally at a distinct region of the floor of the telencephalon,

the ganglionic eminence (Figure 5D). With increasing age (E13.5) the intracerebral bleeding became more severe, and hemorrhage could be seen also in the diencephalon (Figures 5E and 5F) and progressively in the cortex of the forebrain hemispheres and in the cortex of the midbrain (Figure 5G). At birth, αv -null heads appeared hydrocephalic (Figure 5H) due to the severe intracerebral hemorrhage and dilation of the lateral ventricles where blood accumulated (data not shown).

The intracerebral hemorrhage was evaluated by histology and immunohistochemistry (Figures 6 and 7 and data not shown). Transverse sections of E11.5 αv -null embryos compared to wild-type littermates showed no gross abnormalities (Figures 6A, 6B, 6E, and 6F) except slightly distended vessels in the forebrain, specifically in the subventricular zones of the ganglionic eminence (Figure 6F) and, less frequently, at E11.5 in the diencephalic subventricular zones of αv -null brains. Transverse (Figure 6) and coronal (Figure 7 and data not shown) sections of αv -null E12.5 embryos revealed the rapid progression of the distension of blood vessels in the forebrain (Figures 6D, 6H, and 6M) and striking bilateral cavitation in the subventricular zones of the ganglionic

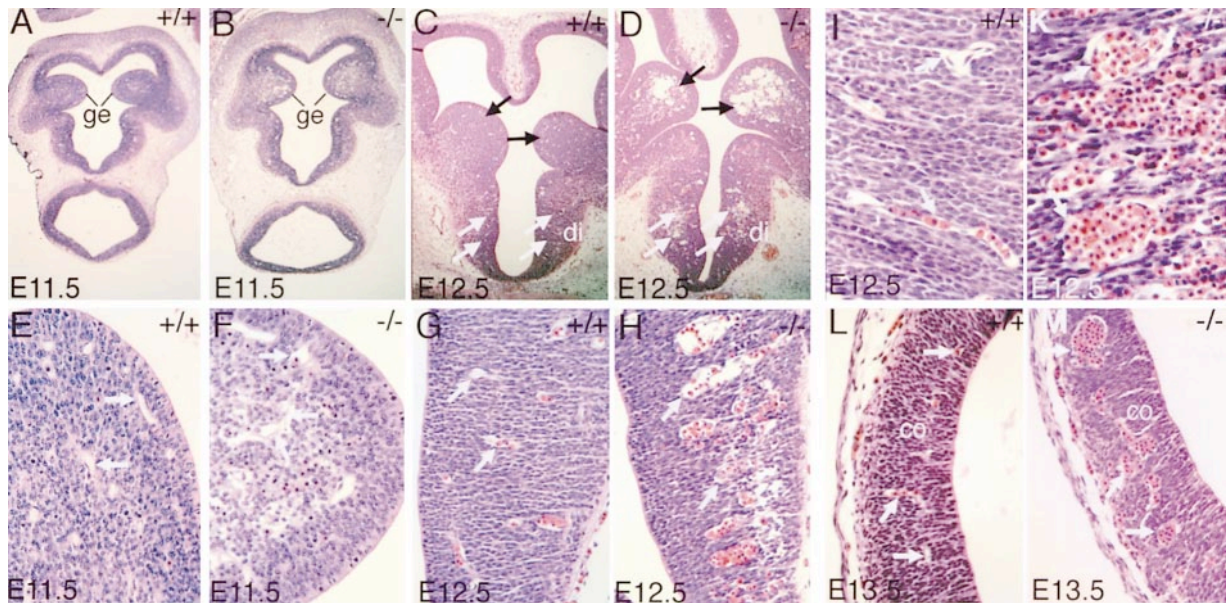


Figure 6. Histological Analysis of Wild-Type and αv -Null Brains at E11.5–E13.5

Transverse sections at the level of the interventricular foramen of Monroe. (A, B, E, and F) Transverse sections of E11.5 wild-type (A and E) and αv -null embryos (B and F). The ganglionic eminences of the telencephalon are prominent. (E and F) Enlargements of a region of the ganglionic eminence at E11.5 show the slightly distended vessels (arrows) in the mutant (F) as compared with wild-type (E). (C, D, G, H, I, and K) Transverse sections of E12.5 wild-type (C and G) and αv -null embryos (D and H); striking bilateral cavitation (arrows) in the subventricular zones of the mutant ganglionic eminence (D). In subventricular zones of the mutant diencephalon, strongly dilated blood vessels are also prominent bilaterally (D, H, and K). In (G and I) sections of a wild-type brain at comparable levels are shown for comparison. (I and K) Enlargement of wild-type (I) and mutant (K) blood vessels in the diencephalic subventricular zones. Note accumulation of nucleated red blood cells in highly distended vessels (arrows). (L and M) Transverse sections through wild-type (L) and αv -null (M) cortex of the hemispheres of E13.5 embryos. The vessels in the cortex of the αv -null embryo are markedly dilated and filled with nucleated red blood cells (M) in comparison with the wild-type cortex (L). Abbreviations: co, cortex; di, diencephalon; ge, ganglionic eminence.

eminence (Figure 6D). The αv -null blood vessels sprout deeply into the brain parenchyma but are distended in the subventricular region (Figure 6M). Notice the smaller diameter of wild-type blood vessels in a comparable region (Figure 6L). The distended blood vessels in the cortex of αv -null embryos are also highly packed with nucleated red blood cells (Figure 6K) compared to blood vessels in wild type (Figure 6I). Distended blood vessels subsequently rupture or leak, and blood cells exit into neuronal tissue (Figures 6D, 6H, 6K, and 6M and data not shown).

To analyze further the vascular development in the brains of αv -null and wild-type embryos (data not shown) serial coronal sections were stained for PECAM-1, FN, laminin-1, and entactin/nidogen (Figure 7). At E12.5 αv -null endothelial cells of perineural blood vessels sprout, penetrate the basement membrane of the neuroectoderm, and invade the brain parenchyma (Figure 7A). They form lumens and assemble various basement membrane proteins (Figures 7B–7D). Blood vessels branch in the subventricular zone of the forebrain most likely by sprouting angiogenesis (Figures 7E–7G). Distended vessels (Figure 7F) and vessels in regions where red blood cells have already leaked into the neuronal parenchyma also stained for laminin-1.

Therefore, endothelial cell proliferation and migration, tube formation, sprouting and branching, and basement membrane assembly all proceed in the absence of αv

integrins. The blood vessels formed are not entirely normal however; they are distended and subsequently herniate for unknown reasons (see Discussion).

Developmental Defects in Other Organs

Most other organ systems developed normally in the class II αv -null progeny. No overt abnormalities were observed in skin, skeletal muscle, heart, lung, cartilage, bone, kidney, pancreas, liver, and spleen of αv -null neonates. Organs were well vascularized, and no obvious hemorrhages were observed with the exception of the brain (see above) and the intestines of newborns and mild hemorrhage in the developing spinal cord (data not shown) and, less frequently, in the lamina propria of the urinary bladder (data not shown). Because of the defects seen in class I αv -null embryos, the hearts of the class II αv -nulls and control littermates at various stages were examined histologically (data not shown). At around E12, when the intracerebral hemorrhage is first visible, the αv -null hearts had a normal four-chambered structure with no indication of enlarged ventricles or other abnormalities.

Apart from the intracerebral hemorrhages described above and found in all class II αv -null embryos and neonates, malformation of the secondary palate and intestinal hemorrhage in newborns also occurred with 100% penetrance. Whole-mount analysis of the gastrointestinal tract of wild-type and αv -null pups showed no

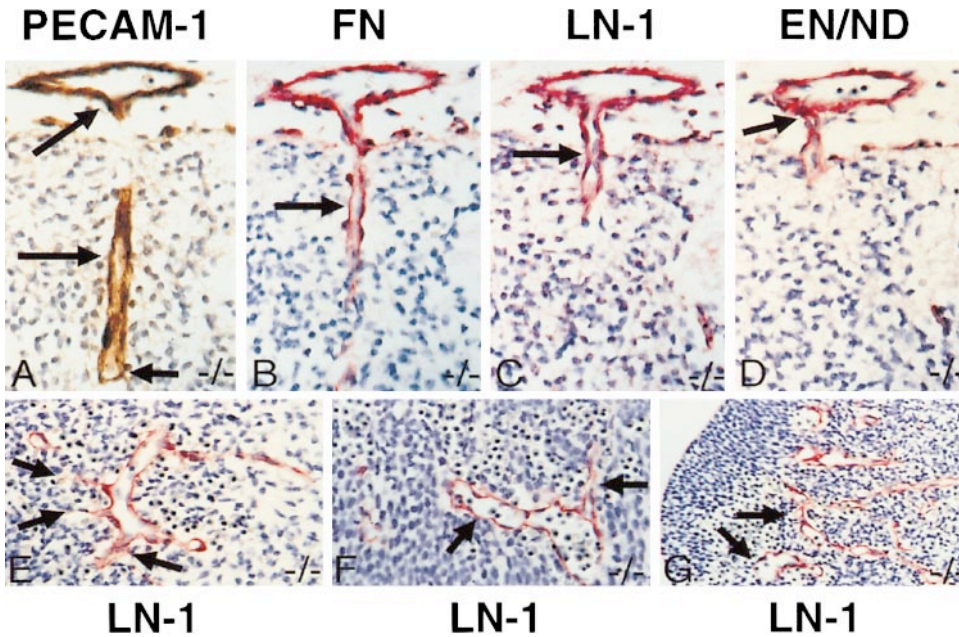


Figure 7. Immunohistochemical Analysis of the Brain Vasculature of αv -Null E12.5 Embryos

(A–D) Serial coronal brain sections at the level of the interventricular foramen stained for PECAM-1 (A) to visualize endothelial cells. Basement membrane proteins were stained with antibodies recognizing fibronectin (FN) laminin-1 (LN-1) or entactin/nidogen (EN/ND). (A–D) illustrates a mutant perineural blood vessel sprouting and penetrating (arrows) into the brain parenchyma. The vessel is surrounded by basement membrane proteins as in normal brains.

(E–G) Blood vessels in the ganglionic eminence show sprouting and branching (arrows). Areas where mild cavitation and hemorrhage occurred are shown.

obvious differences in size or length (data not shown), although the stomachs of αv -null neonates were not filled with milk as in control littermates. αv -null intestines had normal-looking intact mesenteric blood vessels. Thrombi were present in the proximal part of the duodenum but not in the stomach (data not shown). The original site of the bleeding is most likely of intestinal origin, although we could detect no overt signs of hemorrhage in the stomach or the lamina propria of the intestinal wall or in the intestinal villi.

A prominent anteroposterior cleft of the secondary

palate was observed in embryos and newborn αv -null mice (Figures 8B and 8D). Histological analysis of E14.5–E17 embryos and neonates revealed that the palatal shelves did elevate to a horizontal orientation. However, they were short stumps and did not meet in the midline. Ossification in the palatal shelf stumps of the αv -nulls was prominent and the nasal epithelium differentiated into pseudostratified ciliated columnar cells and the oral epithelia into stratified squamous cells, as in wild type.

Thus, most tissues and organs can develop normally in the absence of αv integrins. A few systems (e.g., brain

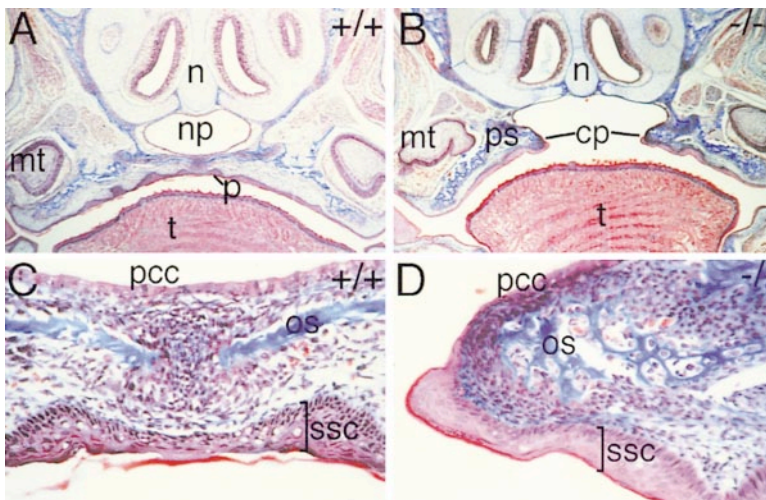


Figure 8. Histological Analysis of the Neonatal Palate

(A–D) Masson's trichrome-stained coronal sections at the level of the eyes from heads of wild-type (A and C) and αv -null neonates (B and D). In wild-type the lateral palatal processes have fused and form the hard and soft palates.

In αv -nulls elevated palatal shelves fail to fuse leaving an extensive cleft. The ossification in the palatal shelf stumps of the αv -nulls is prominent (B and D). Note differentiation of the nasal epithelium into pseudostratified ciliated columnar cells and the oral epithelium into stratified squamous cells in both wild-type and αv -null palate.

Abbreviations: cp, cleft palate; mt, upper molar tooth; n, nasal septum; np, nasal pharynx; os, ossification; p, palate; pcc, pseudostratified ciliated columnar cells; ps, palatal shelf stump; ssc, stratified squamous cells; t, tongue.

vasculature and palate) are dependent on α_v integrins for normal development, and others (e.g., placenta) show variable dependence. Most notably, the greater part of the vasculature develops normally in α_v -null embryos.

Discussion

The fact that 20% of α_v -null progeny survive until birth and that 100% develop normally until almost half way through gestation is a considerable surprise. Nearly all tissues and organs can develop normally or near normally in the complete absence of the entire family of α_v integrins, comprising five different adhesion receptors with a multiplicity of ECM ligands. It was already known that null mutations for most ECM ligands for α_v integrins are viable (Hynes, 1996; Hynes and Bader, 1997) and that mutations in several of the individual β subunits, β_3 (Hodivala-Dilke et al., 1997), β_5 (Huang and Sheppard, personal communication), and β_6 (Huang et al., 1996), are also viable. These results could be rationalized since each of the ECM ligands is known to have multiple integrin receptors, and the ligand specificities of different α_v integrins overlap extensively. However, elimination of all five α_v integrins, which have been implicated in a wide variety of developmental processes (see Introduction), was expected to yield early embryonic lethality. In actuality, most of these processes can clearly proceed in the absence of all α_v integrins, although clear defects do occur in placental development, development of the intracerebral vasculature and formation of the palate. Although the α_v -null mutation is recessive lethal, the extensive development occurring in the absence of α_v integrins necessitates considerable reevaluation of the importance of α_v integrins in numerous developmental events.

Implantation and Placentation

The normal Mendelian ratio and normal development of α_v -null embryos up to early postimplantation stages (E7.5–E9.5) clearly shows that zygotic expression of α_v integrins is not necessary for implantation. α_v integrins are expressed by mouse blastocysts (Sutherland et al., 1993) and can mediate their adhesion *in vitro*, and $\alpha_v\beta_3$ is expressed in a cycle-regulated fashion in human uterus, being expressed only at the time of optimum uterine receptivity (see references in Cross et al., 1994). Our data clearly do not argue against (or for) a role for α_v expression, either in the uterus or from maternal mRNA in the embryo, but they do rule out an essential role for zygotic α_v in implantation. Embryonic α_v integrins are also nonessential for subsequent development of the placenta since 20% of α_v -null embryos develop to term with overtly normal and functional placentae. However, 80% of the α_v -null embryos do show defects in the development of the placenta with poor development of the labyrinthine layer and poor interdigitation of fetal and maternal vessels. A similar percentage of the α_v -null embryos dies between E10 and E12, the stage at which placental function becomes necessary for further development. It seems likely that these observations are linked and that the defects in placental development cause the embryonic lethality.

Several α_v integrins are expressed on trophoblast-derived cells during placentation (Fisher et al., 1989; Damsky et al., 1992, 1993, 1994). Our data suggest that they may play an important, although nonessential, role in placentation. The placental defect could arise from defective invasion by the trophoblastic cells and/or from defective development of the fetal blood vessels (see later). The defects we observe are reminiscent of the human disorder preeclampsia, responsible for failure of 7%–19% of first pregnancies and also characterized by an abnormally shallow placenta and defects in development of the labyrinthine layer of the placenta. Damsky and colleagues have reported altered expression of adhesion molecules in preeclampsia (Zhou et al., 1997a, 1997b). It will be of some interest to analyze further the involvement of α_v integrins in placentation in both mouse and human (Damsky et al., 1993; Cross et al., 1994).

The reasons for the incomplete penetrance of the placental defects are unclear. Most of the mice analyzed are on a mixed genetic background (129Sv/C57BL6), raising the possibility of modifier genes. However, preliminary data with a pure 129 strain also show both phenotypes (classes I and II) in similar proportions as reported here (Table 1), suggesting that the variability in the placental defect is not caused by strain-specific modifiers.

Organogenesis

All α_v -null embryos develop normally up to E9.5; they form a notochord, somites, and heart. Thus, they are clearly much less defective than FN-null embryos (George et al., 1993) or even than α_5 -integrin-null embryos (Yang et al., 1993; Goh et al., 1997), which show few somites and a defective notochord. Therefore, α_v integrins are not essential FN receptors for early development and morphogenesis. This does not mean that they cannot function as FN receptors, either overlapping in function with $\alpha_5\beta_1$ in normal embryos or compensating for the absence of $\alpha_5\beta_1$ in α_5 -null embryos. α_v integrins can serve as FN receptors *in vitro* in α_5 -null cells (Yang and Hynes, 1996) or β_1 -null cells (Wennerberg et al., 1996). It remains to be tested whether α_5/α_v doubly deficient embryos show enhanced defects akin to those of FN-null embryos and that analysis is underway (Yang, B. B., and R. H., unpublished data).

Notwithstanding their involvement (or not) with FN, it is clear that α_v integrins are not required for somite formation or compaction, nor for the subsequent development of the skeletal myogenic lineage. Muscle-specific actin is detected in the dermamyotome and skeletal muscles develop normally as evidenced by gross anatomy and histology and by movements of neonatal α_v -null pups. Smooth muscle cells also develop associated with blood vessels and in organs such as the intestinal tract and cardiac development appears normal in the class II α_v -null embryos. The myocardium of the class I α_v -null embryos undergoing crisis is thinned, but we believe that to be a secondary consequence of the placental defects discussed above.

Thus, our data do not support an essential role for α_v integrins in any of the muscle lineages. That is not to

say that αv integrins may not play some more subtle role in the development, function, or maintenance of muscle cells. Similarly, to the extent analyzed, namely histology of developing embryos, cartilage, and bone develop normally in αv -null embryos. However, there is considerable bone remodelling postnatally, and our data do not address the possible role of αv integrins, particularly $\alpha v\beta 3$, in osteoclast function (see Introduction).

One structure in which we did detect consistent defects was the secondary palate. This defect was observable as early as E14.5. The palate forms from the maxillary processes, which comprise both craniopharyngeal ectoderm and cranial neural crest cells (Ferguson, 1988). The failure of palate closure seen in the αv -null pups could arise from defects in either of these cell populations and could occur in any of several aspects of palate development. Perhaps the most interesting possibility is that there is a deficit in neural crest-derived mesenchymal cells, since neural crest cells are known to express αv integrins, which play a role in their adhesion and migration *in vitro* (Delannet et al., 1994). This and other aspects of neural crest development in the αv -null embryos will need further investigation.

Overall, it is notable that development of most organs and tissues proceeds apparently normally in the absence of αv integrins. If this were to be because of compensation by other adhesive systems for the loss of all five αv integrins, it would require a remarkable degree of compensation in many different cell types. We cannot rule out overlapping functions of, or compensation by, other adhesion receptors in any given situation in these embryos. However, there is no evidence for upregulation of other integrins in embryonic or neonatal fibroblasts.

Vascular Development

Arguably the most surprising and interesting observation is that most vascular development proceeds normally in the absence of all five αv integrins. In particular, $\alpha v\beta 3$ and $\alpha v\beta 5$ have been implicated in vascular development both by their expression at appropriate times and places (see references in Introduction) and by experiments blocking vasculogenesis and angiogenesis in embryos (Drake et al., 1995), neovascularization in the retina (Friedlander et al., 1996; Hammes et al., 1996), and angiogenesis stimulated by angiogenic growth factors or tumors (Brooks et al., 1994a, 1994b, 1996, 1998). Those data have suggested that these αv integrins play crucial roles in vascular development, both normal and pathological, and have stimulated a drive to develop antagonists of αv integrins as therapeutic agents.

In marked contrast with those data, our results suggest that most vascular development (although not all, see below) proceeds normally in the complete absence of αv integrins. This includes both vasculogenesis to form major vessels such as the dorsal aorta and the primary vascular plexuses of the yolk sac and around the brain. αv -null embryos show normal patterns of initial vascular plexus formation, angiogenesis, sprouting, and remodelling to generate vessels of different sizes. In most cases, the pattern of the vasculature is indistinguishable from that of wild-type embryos, although

class I αv -null embryos do exhibit some dilation of the vessels at the time of crisis (E10–E11). This is probably a secondary consequence of other defects, since class II αv -null embryos do not show these abnormalities.

Vascular beds in virtually all organs develop normally in αv -null embryos. The major location in which αv integrins appear to be essential for normal vascular development is the CNS (brain and spinal cord). Here the vessels must arise by angiogenesis, sprouting from the vessels surrounding the neural tube, since there are no angioblasts within the neural parenchyma (Breier and Risau, 1996; Risau, 1997). However, sprouting, invasion, and branching of vessels all occur in the αv -null embryos. The vessels do become dilated and eventually herniate leading to leakage of blood into the neural parenchyma. However, many endothelial functions necessary for formation of a vascular system within the CNS clearly do occur. These must include endothelial cell proliferation and migration and formation and branching of tubes. We do not observe an increase in apoptosis of endothelial cells, although that could have been missed if the cells slough off into the circulation. These results are all unexpected in light of earlier data using blocking agents targeted against αv integrins. The dilation and eventual leakage of the intracerebral vasculature might suggest that intercellular adhesions, the basement membrane, or other supporting structures may be defective in the absence of αv integrins. However, by immunohistochemistry, the basement membrane of αv -null brain vessels contains normal components arranged in a normal fashion around the vessels, and the analyses to date do not suggest a basement membrane defect. Clearly it is possible that other basement membrane components may be missing or misarranged, and it will be necessary to examine the organization of both the ECM and the endothelial layer (e.g., cell–cell junctions) at the ultrastructural level.

Another possibility is that accessory cells normally associated with the brain vasculature (e.g., pericytes or glial cells) fail to associate properly in the absence of αv integrins. In some respects, the dilation of brain capillaries in αv -null embryos resembles that seen in PDGF-B-null embryos (Lindahl et al., 1997). In the latter case, the defects arise from failure of migration of pericytes, which depend on PDGF signaling by the endothelial cells. However, the defects we observe occur earlier than those seen in the PDGF-B-null embryos, suggesting they are not due solely to failure of pericyte immigration and interaction. Another cell type that interacts closely with brain capillaries is the astrocyte (Marin-Padilla, 1985), and there might be defects in establishment of perivascular glial processes.

Suffice it to say, the development of the αv -null brain vasculature proceeds much more normally than would have been predicted for an angiogenic process on the basis of prior publications. Further work will be necessary to elucidate precisely what goes awry, but it is apparently not the basic endothelial cell biological steps of angiogenesis in which αv integrins had been previously implicated.

This discrepancy raises several questions. Could different angiogenic processes exhibit differential dependencies on integrins? The antibody and peptide blocking

data have largely been obtained for postnatal angiogenic responses to growth factors or tumors (Brooks et al., 1994a, 1994b, 1996, 1986; Friedlander et al., 1995) or during retinal neovascularization (Friedlander et al., 1996; Hammes et al., 1996). Tumor angiogenesis could be different from developmental angiogenesis, with the former being dependent on α_v integrins and the latter not. Even during development, yolk sac vasculogenesis is clearly α_v -independent but is equally clearly dependent on $\alpha_5\beta_1$ integrin (Yang et al., 1993) and FN (George et al., 1993; 1997). Earlier results on avian embryos have suggested that α_v integrins play important roles in development of the dorsal aorta (Drake et al., 1995) whereas they are clearly not essential in mice. Here, there is a clear conflict in the implications of the different results.

How can one explain such discrepancies between antibody or peptide blocking experiments and genetic elimination? One possibility is that the genetic experiments underestimate the contribution of α_v integrins as a consequence of overlapping functions of, or compensation by, other adhesion receptors. Although we have no evidence for this (e.g., no upregulation of other integrins in fibroblasts), we cannot rule it out. Equally likely is the possibility that the blocking experiments are overestimating the contributions of α_v integrins. This could arise for in vitro experiments if the in vitro systems are biased toward dependence on α_v integrins but could also arise, either in vitro or in vivo, because of transdominant inhibition of other integrins by interference with one set of integrins (see, Diaz-Gonzalez et al., 1996). Parallel blocking experiments have implicated $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins in angiogenesis (Senger et al., 1997), so clearly α_v integrins are not the only integrins involved in angiogenesis, even based on the interpretation of blocking data. At this point, it is not possible to draw definitive conclusions as to the relative importance of different integrins in different angiogenic processes. However, the data presented here clearly raise questions as to the primacy of α_v integrins.

Recent research on the control of vasculogenesis and angiogenesis has implicated VEGF and angiopoietins and their receptors (Hanahan, 1997) as key regulators of the various steps. Other growth factor/receptor pairs have also been implicated (e.g., Beck and D'Amore, 1997; Yancopoulos et al., 1998). Integrins and their ligands are likely downstream effectors regulated by these diverse signaling molecules. The data presented here and reviewed above suggest the involvement of several integrins, perhaps differing among different vascular developmental processes, and it will be of considerable interest to unravel the regulatory controls connecting these various players in the complex processes necessary for forming a well-ordered vascular network (Risau and Flamme, 1995; Risau, 1997).

Experimental Procedures

Generation of α_v Integrin-Null Mice

A ~ 700 bp EcoRI/BglI DNA fragment of human α_v integrin cDNA (Suzuki et al., 1987) was used to screen a genomic library derived from a mouse D3/129 ES cell line (S. Tonegawa, MIT) to isolate α_v integrin genomic clones. To construct the targeting vector (Figure 1A), a 1.5 kb XbaI/SmaI fragment located 5' of the initiation codon was subcloned into pBluescript II (SK; Stratagene, La Jolla, CA) and

then fused to a PGK-tk-cassette; the 3' arm consisting of a 6 kb BamHI/SalI DNA fragment was subcloned into pBluescript II, and a PGK-neo^R-cassette was ligated to the 3' arm. These two constructs were then combined to give the final targeting construct (Figure 1A).

Electroporation, selection, and blastocyst injection of D3/129 ES cells were performed essentially as described (George et al., 1993). For Southern blot analyses a ~ 500 bp EcoRI/XbaI DNA fragment located 5' of the left arm of the targeting construct was used (Figure 1A). Among 480 G418-resistant ES cell clones examined, 12 were found to have undergone homologous recombination. Chimeric progeny were identified by coat color, and chimeric males were bred to C57BL/6J and 129/Sv females. Transmission of the targeted α_v integrin locus was confirmed by Southern blotting.

Antibodies

Rabbit polyclonal antisera against various integrin subunits: α_5 and β_1 (160, 363; Yang and Hynes, 1996), α_v (AB1930; Chemicon International, Inc., Temecula, CA), β_3 (8275, a gift of Dr. Mark Ginsberg). Biotinylated hamster monoclonal antibodies 8B3 and 82D recognized α_v and β_3 integrin subunits (Gerber et al., 1996). Rabbit antisera against rat FN (61.1; Yang and Hynes, 1996), mouse collagen IV (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA), mouse laminin-1 (L-9393; Sigma, St. Louis, MO), entactin/nidogen (a gift from A. Chung, University of Pittsburgh, PA), rat anti-mouse PECAM-1/CD 31 antibody MEC13.3 (Pharmingen, San Diego, CA), and mouse anti-human smooth muscle α -actin (SM α Ac) antibody directly conjugated with horseradish peroxidase (HRP; clone 1A4; DAKO, Carpinteria, CA).

Iodination and Immunoprecipitation of Integrins

Fibroblasts were surface-labeled with 0.5 mCi/10 cm plate of Na(¹²⁵I) (NEN, Boston, MA) using lactoperoxidase and washed and lysed with 1 ml cold lysis buffer (200 mM octyl- β -D-glucopyranoside, 50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2 mM PMSF, 0.02 mg/ml aprotinin, and 0.0125 mg/ml leupeptin). Lysates were preabsorbed with 100 μ l of protein A-Sepharose beads (Pharmacia LKB, Piscataway, NJ) for 1 hr. For immunoprecipitations with biotinylated hamster antibodies, lysates were preabsorbed with 25% volume Ultralink-immobilized streptavidin beads (Pierce, Rockford, IL). The beads were removed by centrifugation, the resulting supernatants were used for immunoprecipitation, and immunoprecipitates were analyzed by nonreducing SDS-PAGE on 6% or 7.5% gels with 3% stacking gel (Marcantonio and Hynes, 1988).

Whole Embryo Immunohistochemistry

Staged embryos were dissected in PBS; amnion and a small piece of each embryo were used for genotyping by PCR. Yolk sacs and embryos were fixed overnight in Dent's fixative (80% methanol: 20% DMSO). Samples were treated with 6% H₂O₂ in methanol for 1 hr at room temperature to quench endogenous peroxidases. Samples were rehydrated to PBS with 0.1% Tween-20, incubated in antibody buffer (10% goat serum, 5% BSA in PBS) 2 \times 1 hr, and exposed to primary antibody overnight at 4°C. After 5–7 hr wash in 0.1% Tween-20 in Tris-buffered saline, samples were incubated with secondary antibodies overnight at 4°C.

For endothelial staining, PECAM-1-specific rat monoclonal antibody MEC 13.3 and alkaline phosphatase (AP)-conjugated goat anti rat IgG (H + L) (Pierce) and the NBT/BCIP (Boehringer Mannheim, Indianapolis, IN) detection system were used. SM α Ac was detected with the monoclonal anti-human SM α Ac antibody and DAB as chromogenic substrate. Stained embryos and yolk sacs were fixed with 0.1% glutaraldehyde/4% paraformaldehyde in PBS, examined, and photographed. Further histological analyses were as described below.

PCR Analyses of Genotypes

Yolk sac DNA was used for PCR analyses with primers specific for the wild-type and targeted alleles. PCR conditions were as described by the manufacturer of AmpliTaq Polymerase (Perkin-Elmer Cetus, Norwalk, CT) except that the PCR reactions contained 5% DMSO and 1 mM MgCl₂.

Reaction conditions were: 1 min at 95°C, 60°C for 2 min, and 72°C for 3 min for 35 cycles.

Primer set A: mouse αv integrin: forward 5'-GAC GCC TTC AAC CTG GAC-3'; reverse 5'-CTG GAT GCT GAG TGT CAG GT-3'; neoR gene: forward 5'-AGG ACA TAG CGT TGG CTA CC-3'. The amplified PCR products were analyzed on 2% agarose gels to separate the amplified wild-type (291 bp) and targeted allele (890 bp) fragments.

Embryonic tissue scraped from several paraffin sections was pooled in 20 μ l dd H₂O, incubated with 0.5 mg/ml Proteinase K (55°C for 20 min, 95°C for 10 min), and used for two sequential PCR reactions with different primer sets (A and B). PCR reactions with primer set A were performed for 20 cycles, and 1/10 of the product served as template for primer set B. (PCR conditions and analysis as above).

Primer set B: mouse αv integrin: forward 5'-GAA AGT CCC GCC GAG TAT G-3'; reverse 5'-TTC ATC CAT GCC AGA CCT G-3'; neoR gene: forward 5'-ATA TTG CTG AAG AGC TCG GCG-3'. Wild-type (230 bp) and targeted allele (860 bp) fragments.

Culture of Embryonic and Neonatal Dermal Fibroblasts

To culture embryonic fibroblasts, heads and inner organs of E16 embryos were removed and the remainder washed in PBS, dissociated in ES cell medium without LIF (MEF-medium), and plated in a 10 cm plate precoated with 0.1% gelatin. After 4 days, cells were subcultured to obtain a more homogeneous fibroblastic cell culture.

Dermal fibroblasts were prepared from neonatal mice. Skins were removed from the torso and limbs and minced in HEPES-buffered (20 mM) Minimum Essential Medium plus 0.25% collagenase (Collaborative Research) for 30 min at 37°C, followed by an equal volume of 0.25% trypsin (GIBCO-BRL) and further incubation for 15 min at 37°C. Tissue chunks were dissociated and plated on gelatin-coated plates in MEF-medium. After 3 days, cultures were trypsinized and passed through a sterile 100 μ m nylon mesh (Falcon, Becton Dickinson, Mountain View, CA). Dermal fibroblasts were grown further as described above.

Morphological and Histological Analysis

Whole-mount embryos were examined and photographed on a dissecting microscope. For histology, embryos were fixed in Carnoy's solution. Paraffin sections (6–8 μ m) were either stained with Harris hematoxylin and/or eosin, methyl green, or Masson's trichrome or used for immunohistochemistry.

Immunohistochemistry

Sections were dewaxed by standard techniques. To improve staining, dewaxed sections were treated with 0.1% trypsin (GIBCO-BRL) in PBS for 10–30 min at 37°C. After a 15 min wash with PBS, nonspecific binding sites were blocked with antibody buffer (10% heat-inactivated FN-depleted goat serum, 0.05% Tween-20 in PBS) for 1 hr at room temperature or overnight at 4°C. Staining procedures and chromogenic reactions were carried out according to the protocols of the Vectastain ABC Kit protocol (Vector Laboratories) or the Renaissance TSA-System for Immunohistochemistry-Kit (NEN Life Science Products, Boston, MA).

Primary antibodies were applied for 1 hr at room temperature. Secondary antibodies conjugated with either AP or HRP were applied for 1 hr at room temperature.

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