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Short Communication

Expression of αVβ3 integrin in the chick embryo aortic endothelium

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ABSTRACT The integrin chain αV, expressed in association with β3, by cells of the megakaryocytic/thrombocytic and endothelial lineages is thought to play an important role in angiogenesis. αVβ3 expression by endothelial cells is not constitutive but induced by various stimuli in avian and human models. Here the developmental pattern of αVβ3 expression was analysed in the chick embryo by immunocytochemistry, using a specific monoclonal antibody. On day 2 of development αVβ3 expression was restricted to rare cells in the blood stream, in the embryo proper and in the yolk sac blood islands. αVβ3 expression by endothelial cells became detectable on day 3 and was restricted to the dorsal aorta. Interestingly it was absent from the intra-aortic hemopoietic clusters (E3.5) which, as we have showed previously, express the αIIbβ3 integrin and display progenitor potentialities. However the endothelium underlying intra-embryonic hemopoietic clusters expressed this integrin. In contrast E6-7 para-aortic hemopoietic foci contained numerous αVβ3 positive cells. Both αVβ3 and αIIbβ3 were expressed in these latter hemopoietic sites, while αVβ3 was still selectively expressed by the aortic endothelium until E6. Thereafter, at E7 the pulmonary artery also expressed it. Since αIIbβ3 is expressed by avian and murine multilineage hemopoietic progenitors, we then studied the hemopoietic potentialities of αVβ3/αIIbβ3 double positive cells from embryonic bone marrow differentiating *in vitro* in erythro-myeloid conditions. Thrombocytic, erythroid and myeloid progenitor potentialities were found within the cell population expressing both β3 integrins.

KEY WORDS: *integrin, chick embryo, vitronectin receptor, aortic endothelium, hemopoiesis*

αV belongs to the large family of integrin proteins, which are heterodimers of α and β subunits that mediate cell adhesion between cells and extracellular matrix components. αV is known to bind several distinct β subunits such as β1, β3, β5, β6 to form receptors that can bind different ligands. The integrin αVβ3, also known as vitronectin receptor, is able to bind vitronectin, fibrinogen, thrombospondin, von Willebrand's factor and fibronectin. The β3 subfamily of integrins comprises αIIbβ3 and αVβ3 which are both expressed on the megakaryocytic/thrombocytic cells (Hynes, 1992). While αIIbβ3 is expressed only by hemopoietic cells (HC), αVβ3 is also expressed by endothelial cells (EC), osteoclasts and some metastatic melanomas. We analysed earlier the developmental expression pattern of αIIbβ3 in avian and murine embryos and showed that this integrin is present at sites of hemopoietic stem cell emergence. Moreover, we demonstrated, that the αIIbβ3 molecule is expressed by multilineage hemopoietic progenitor cells as well as by all cell types of the megakaryocytic/thrombocytic lineage (Ody *et al.,* 1999, Corbel and Salaün, 2002).

Although αVβ3 is expressed on activated EC, little is known about its expression by EC of small vessels compared with larger vessels and by venous EC compared with arterial EC. Such a differential phenotypic expression by arterial versus venous EC was recently demonstrated for two molecules, neuropilin-1 and ephrin-B2, whose genes are preferentially expressed in early embryonic arteries and not in veins (Herzog *et al.,* 2001; Moyon *et al.,* 2001; Wang *et al.,* 1998). Moreover, αVβ3 expression pattern during ontogeny was established neither in birds nor in other species. Drake *et al.* (1995) showed αVβ3 expression by the endothelium of the dorsal aorta of quail embryo. In their study, in which they used laser scanning confocal microscopy, whether αVβ3 was expressed in other vessels or by other cell types was not reported. Antibody blocking experiments in this study suggested that αVβ3 integrin plays a role in the development of the dorsal aorta (Drake *et al.,* 1995). αVβ3 induction of expression on blood vessels is correlated with angiogenesis induction, such as the chick chorioallantoic membrane upon stimulation by specific cytokines *in vitro*. In the later situation, the growth of new vessels was inhibited by mAb LM609 directed against αVβ3 while preexisting vessels were not perturbed (Brooks *et al.,* 1994a). αVβ3

Abbreviations used in this paper: HC, hemopoietic cell; EC, endothelial cell; En, n days of incubation.

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antagonists induced apoptosis of the proliferative angiogenic vascular cells and reduced the invasive behavior of tumors (Brooks *et al.,* 1994b). These data showed that this integrin is implicated in vascular development. However, in αV-null embryos i.e. in the absence of all five αV integrins vascular development proceeds mostly according to normal (Bader *et al.,* 1998).

In the present study, we used the anti-αVβ3 mAb, LM609, to determine the pattern of expression of this integrin in the developing embryo i.e. up to day 7 of incubation (E7). At the 22-somite stage, αVβ3 is only expressed by very few cells in the circulating blood (Fig. 1A). A few inner cells of yolk sac blood islands which contain primitive blood cells surrounded by a layer of EC expressed αVβ3. These cells are hemopoietic cells (HC), as shown at the 16- and 22-somite stages (Fig. 1B and Fig. 1C, respectively). At these early embryonic stages, EC expressed αVβ3 neither in the yolk sac nor in the embryo proper.

From E3, i.e., the 32-somite stage, while some αVβ3 positive cells in the blood stream could be attributed to the thrombocytic lineage, others were detected in a specific site, the endothelium of the dorsal aorta. As illustrated in Fig. 2 A,B, immunoreactivity was restricted to EC of the dorsal aorta while other vessels from the vascular tree, cardinal veins, endocardium, vitelline veins, primary vascular plexus of the yolk sac, and perineural vasculature were negative.

In the chick embryo, emerging hemopoietic stem cells can be detected at E3.5-4, in intra-aortic clusters and at E6-8 in para-aortic foci (Dieterlen-Lièvre and Martin, 1981). We demonstrated earlier that the aortic clusters and para-aortic foci contain some cells expressing integrin αIIbβ3, a molecule specific of the megakaryocytic/thrombocytic lineage (Ody *et al.,* 1999). We thus focused on these intra-embryonic sites and showed that clustered CD45 positive HC (Fig. 2C) were not stained for αVβ3 (Fig. 2B) although some of them were αIIbβ3 positive (data not shown, Ody *et al.,* 1999). Therefore, the two β3 integrins are differentially expressed at E3.5-4 intra-aortic sites of hemopoietic stem cell emergence, αVβ3 being a marker for EC while αIIbβ3 is a marker for hemopoietic progenitor cells.

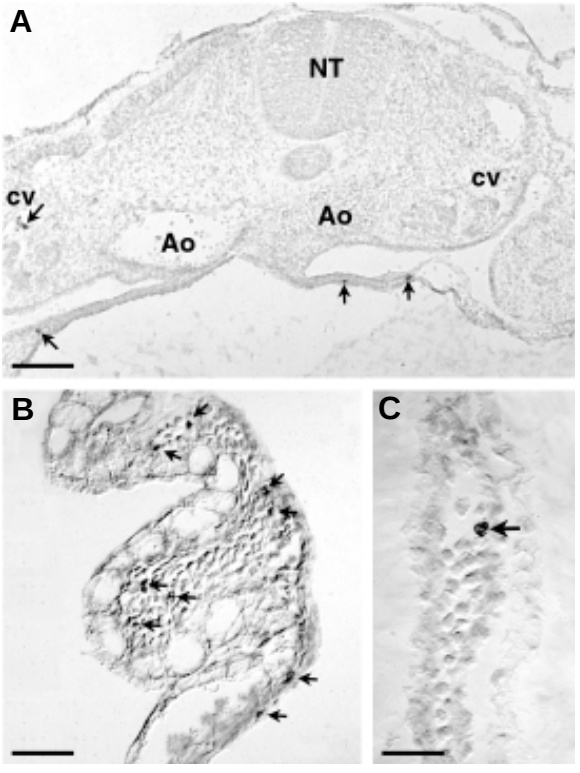

Fig. 1. αVβ3 **expression by HC, in the blood and yolk sac blood islands. (A)** *Transverse section of a 22-somite stage embryo immunostained with the anti–αVβ3 mAb LM609. Very few positive cells are present in the blood circulation as indicated by the arrows. No vessels are immunostained. Scale bar, 80 μm.* **(B,C)** *Yolk sac blood island at the 16- and 22-somite stages, respectively, contain αVβ3 positive HC (arrows). Scale bars, 160 and 320 μm respectively.*

Specific αVβ3 protein was still expressed by aortic EC at later stages studied (E6 and E7) as illustrated in Fig. 2D,F,H,I. At E6, anti-CD45 staining on consecutive sections showed that many circulating HC (Fig. 2 E,G) expressed αVβ3, for instance in the heart and inside the aorta (Fig. 2 D,F). Other cells lying free in the mesenchyme are also observed (Fig. 2E), which might correspond to the macrophages as described by Cuadros *et al.* (1992). Numerous HC expressing αVβ3 were also localized in the mesenchyme around the aorta. They belong to the para-aortic foci, constituted of HC of different stages from progenitors to differentiated cells (Dieterlen-Lièvre and Martin, 1981). E6 para-aortic foci contained αIIbβ3 positive cells (Ody *et al.,* 1999). Therefore, the two β3 integrins, αVβ3 and αIIbβ3, expressing HC were numerous at this location, while EC expressed αVβ3 only. Interestingly, one day later, at E7, the endothelium of the pulmonary arteries as well as the aorta expressed αVβ3 (Fig. 2 H,J).

We previously showed that multilineage hemopoietic progenitors, from both E3.5 intra-aortic clusters and bone marrow, express the αIIbβ3 integrin (Ody *et al.,* 1999). As E3.5 aortic HC do not expressed αVβ3 we performed double staining on E13 bone marrow cells with anti-αIIbβ3 (11C3) and anti-αVβ3 mAbs (Fig. 3A).

Semi-solid cultures of sorted embryonic bone marrow cells were performed under appropriate conditions to evaluate their hemopoietic progenitor potentialities. Colonies developed mainly from $\alpha V\beta3^{+}\alpha IIb\beta3^{+}$ cells. αIIbβ3 expression selected for myeloid and erythroid progenitors under both culture conditions (Fig. 3 B,C) since myeloid (M, M/G, G, Tb) and erythroid (Eb, Ec) colonies were recorded in both populations 1 and 3, $\alpha V\beta3^{+}\alpha IIb\beta3^{+}$ and $\alpha V\beta3^{-}\alpha IIb\beta3^{+}$ respectively. Colonies did not develop from $\alpha V\beta3^{+}\alpha IIb\beta3^{-}$ (population 2). Thus, αIIbβ3 positive myeloid and erythroid progenitors in the embryonic bone marrow co-express αVβ3.

The early development of EC, first step in the formation of the circulatory apparatus, has been studied in depth in the avian model. Herein we report that αVβ3 was not expressed by any of the earliest EC during the formation of the primitive vascular system. Expression by EC occurred at E3, but was restricted to the dorsal aorta. Specific location to this vessel persisted until E6 while at E7, the last time point of our investigation, an additional artery, the pulmonary artery also expressed it while the lack of expression by the other arteries and the veins is maintained. Therefore, αVβ3 cannot be used as a marker for the chick endothelial lineage. Unfortunately such a marker, which would labeled specifically the entire EC lineage in both embryos and adults, has not been yet identified for the chicken. In quail the QH1 marker is specific for both EC and HC lineages (Pardanaud *et al.,* 1989). The avian vascular endothelial growth factor receptor 2 (VEGFR2), is expressed by cells from the mesoderm of the early embryos at gastrulation stage and then by all EC only until E9 (Eichmann *et al.,* 1993, 1998; Wilting *et al.,* 1997). Endoglin, the transforming growth factor beta binding protein is expressed by

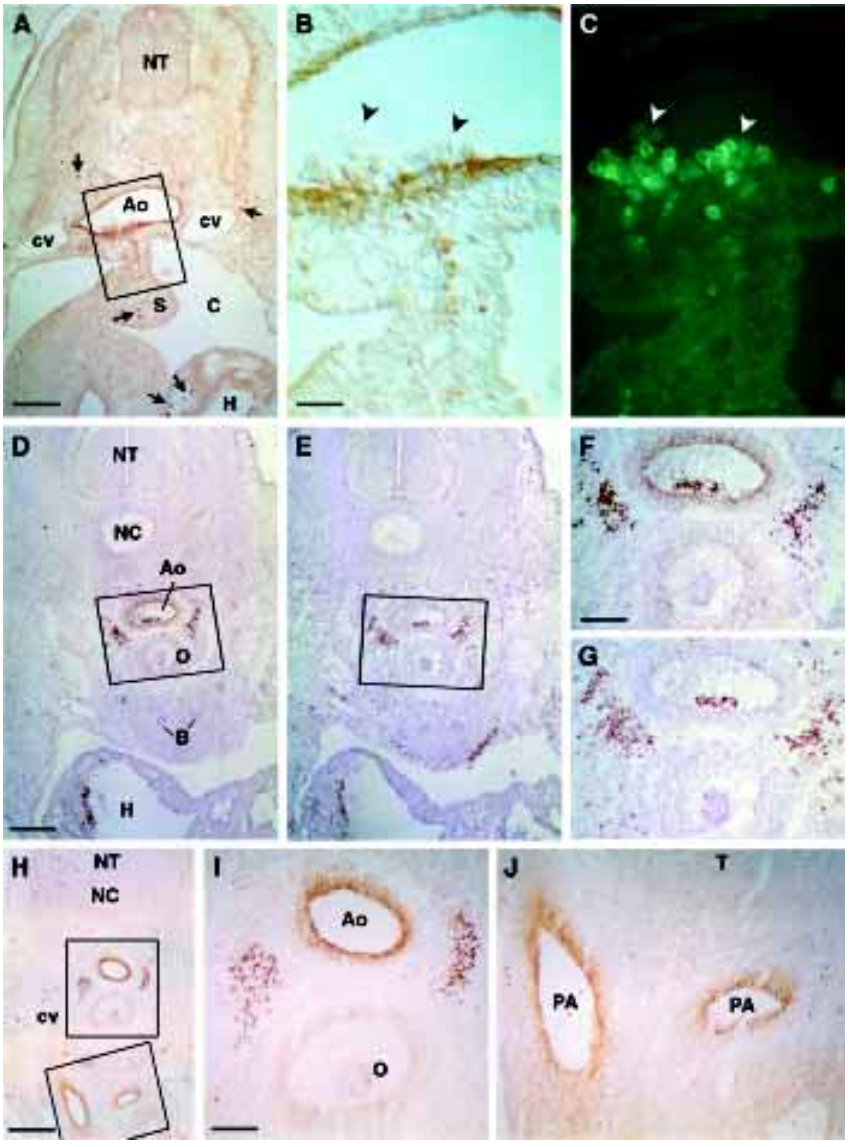

Fig. 2. αVβ3 expression by EC of the aorta and pulmonary arteries. (A) *Transverse section of an E3.5-4 embryo: immunoperoxidase staining with the anti–αVβ3 mAb. In the embryo proper, αVβ3 is strongly expressed by EC of the dorsal aorta and some HC in the blood stream (arrows). Scale bar, 72 μm.* **(B)** *Higher magnification of the field boxed in (A). The endothelium of the dorsal aorta is αVβ3 $^{+}$ while intra-aortic clusters (arrowheads) are αVβ3 $^{-}$.* **(C)** *The same section, double stained with anti-CD45 mAb and revealed by indirect immunofluorescence. The intra-aortic clusters contain CD45^{+} cells which do not express the αVβ3 integrin (arrowheads). Some CD45^{+} cells are present in the mesenchyme under the ventral part of αVβ3^{+} aortic endothelium. Scale bar, 144 μm. (D-G) Consecutive transverse sections of E6 embryo.* **(D,F)** *αVβ3 immunoperoxidase staining. Positive cells are scattered in blood vessels, more numerous in some, for instance in the heart and the lumen of the aorta. The only αVβ3 expressing EC are those from the dorsal aorta. Two para-aortic foci containing αVβ3 positive cells are shown in (F), a higher magnification from the field boxed in (D).* **(E,G)** *CD45 immunoperoxidase staining. Numerous isolated or aggregated CD45 positive cells are distributed throughout the embryo, in the blood vessels and the mesenchyme (para-aortic foci). The latter are illustrated in (G) a higher magnification from the field boxed in (E). (D,E) Scale bar, 32 μm; (F,G) Scale bar, 80 μm. (H-J) Transverse sections of an E7 embryo immunostained with the anti–αVβ3 mAb.* **(H)** *αVβ3 immunoperoxidase staining shows that dorsal aorta as well as pulmonary arterie EC are positive. Scale bar, 32 μm.* **(I)** *Higher magnification from the field boxed in (H) showing aortic EC expressing αVβ3 and also para-aortic area containing αVβ3 positive cells.* **(J)** *Higher magnification from the field boxed in (H) showing EC of pulmonary arteries expressing αVβ3. Scale bar, 74 μm. Abbreviations: Ao, aorta; B, Bronchi; C, Coelom; cv, cardinal vein; H, Heart; NC, notochord; NT, neural tube; O, Oesophagus; T, Trachea; S, Spleen.*

EC during early chick development (Raab *et al.,* 1999) but its embryonic pattern of expression is not known.

The early expression pattern of αVβ3 has not been studied in detail previously and our results are in agreement with those resulting of the genetic elimination of all αV integrins in mice. In such a case, most vascular development proceed normally (Bader *et al.,* 1998). During the course of this study, we have compared the distribution of the two β3 integrins, αVβ3 and αIIbβ3. The rare positive cells not integrated in the endothelium of the aorta, found during early ontogeny at E2-3, might be the first cells of the thrombocytic lineage. The earliest expression of αVβ3 by cells other than HC, in the embryo proper, was detected on day 3 of development and was specifically localized around the dorsal aorta. At the onset of intra-aortic cluster emergence (E3.5-4), HC did not express αVβ3 while the underlying endothelium did it. This pattern is different from that of VEGFR2 which disappears from this area (Jaffredo *et al.,* 1998).

We have shown that αIIbβ3 is expressed by intra-aortic hemopoietic clusters (Ody *et al.,* 1999 ; Corbel and Salaün, 2002). Therefore these two molecules, αIIbβ3 and αVβ3 are lineage specific i.e., hemopoietic and endothelial, respectively in this aortic area. Thereafter, when intraembryonic hemopoietic progenitors multiply and begin differentiate in the E6-8 para-aortic foci, αVβ3 is still expressed by the aortic EC. At E7, the pulmonary arteries expressed also αVβ3. Some HC express this integrin, these are circulating HC inside the aorta and HC within the foci. These αVβ3 positive HC appear as numerous as cells expressing αIIbβ3 (Ody *et al.,* 1999), which suggests that they belong to the thrombocytic lineage.

During avian ontogeny, hemopoietic progenitor cells are finally found in the embryonic bone marrow which remains the predominant hemopoietic site throughout life. Functional analysis showed that αVβ3 integrin expression by hemopoietic progenitors is acquired during late development, in E13 bone marrow. Altogether our data suggest that αVβ3 expressing hemopoietic progenitors present in the bone marrow could be more committed than progenitors from intra-aortic clusters which do not express αVβ3.

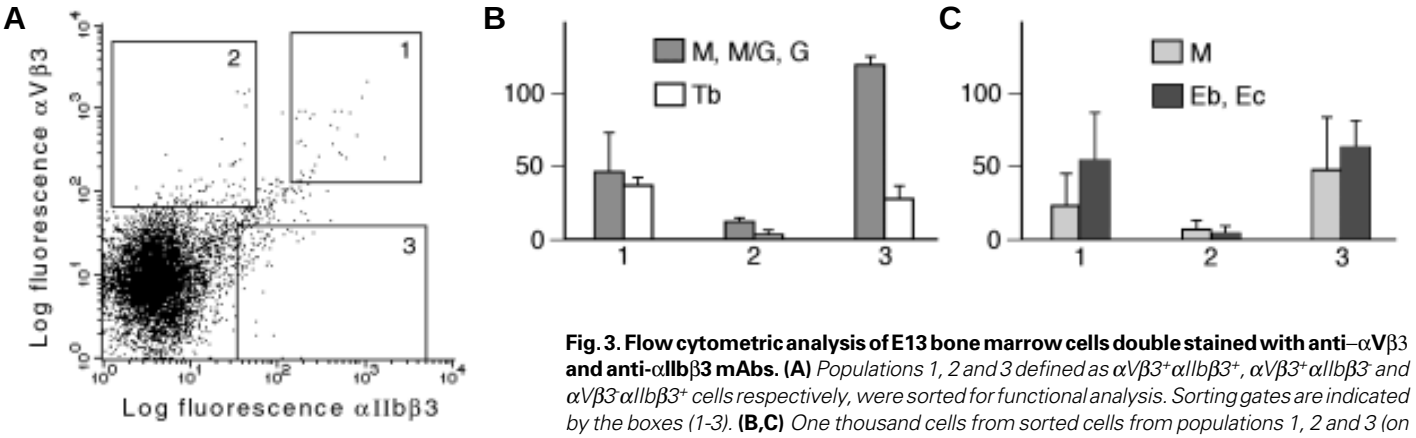

Fig. 3. Flow cytometric analysis of E13 bone marrow cells double stained with anti–αVβ3 and anti-αIIbβ3 mAbs. (A) *Populations 1, 2 and 3 defined as αVβ3⁺αIIbβ3⁺, αVβ3⁺αIIbβ3⁻ and αVβ3⁻αIIbβ3⁺ cells respectively, were sorted for functional analysis. Sorting gates are indicated by the boxes (1-3).* **(B,C)** *One thousand cells from sorted cells from populations 1, 2 and 3 (on abscissa) shown in (A) were cultured in myeloid (B) and erythroid (C) differentiation conditions in semisolid medium. Colonies were stained and scored: Macrophages (M), Macrophages/Granulocytes (M/G), Granulocytes (G), Thromboblasts (Tb), Erythroblasts (Eb) and Erythrocytes (Ec). The values on the ordinate represent the mean number of colonies developed in duplicate cultures and obtained in 3 sorting experiments ± SD.*

Experimental Procedures

E13 bone marrow cell suspensions, immunocytologic labeling, fluorescence-activated cell sorting analysis and sorting, as well as immunohistochemistry were performed as previously described (Ody *et al.,* 1999).

Different mAbs were used including: LM609, an anti-human αVβ3 (MAB 1976, Chemicon International, CA) which crossreacts with αVβ3 integrin of several species, including the chicken; 11C3, which detects the αIIbβ3 expressed by cells of the chicken thrombocytic lineage (Lacoste-Eleaume *et al.,* 1994) and multilineage hemopoietic progenitors (Ody *et al.,* 1999); LT40 which recognizes CD45, present on all leucocytes but not on erythrocytes and thrombocytes (Paramithiotis *et al.,* 1991).

For the double staining in cell suspensions, LM609 was detected with a goat anti-IgG1-PE, while 11C3 directly coupled to Alexa 588 was used. Alexa 588 coupled 11C3 was a kind gift from Dr. C. Ody (CMU, Geneva, Switzerland), who used the labelling kit from Molecular Probe, Eugene (Oregon, USA).

Hemopoietic progenitor cells were detected by their colony forming ability in semisolid cultures as previously described (Corbel *et al.,* 1992; ody *et al.,* 1999).

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