Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells

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SUMMARY

In this study, we have characterized the early steps of hematopoiesis during embryonic stem cell differentiation. The immunophenotype of hematopoietic progenitor cells derived from murine embryonic stem cells was determined using a panel of monoclonal antibodies specific for hematopoietic differentiation antigens. Surprisingly, the CD41 antigen (cIIb, IIb integrin, platelet GPIIb), essentially considered to be restricted to megakaryocytes, was found on a large proportion of cells within embryoid bodies although very few megakaryocytes were detected. In clonogenic assays, more than 80% of all progenitors (megakaryocytic, granulo-macrophagic, erythroid and pluripotent) derived from embryoid bodies expressed the CD41 antigen. CD41 was the most reliable marker of early steps of hematopoiesis. However, CD41 remained a differentiation marker because some CD41- cells from embryoid bodies converted to CD41+ hematopoietic progenitors, whereas the inverse switch was not observed. Immunoprecipitation and western blot analysis confirmed that CD41 was present in cells from embryoid bodies associated with CD61 (β3 integrin, platelet GPIIIa) in a complex. Analysis of CD41 expression during ontogeny revealed that most yolk sac and aorta-gonad-mesonephros hematopoietic progenitor cells were also CD41+, whereas only a minority of bone marrow and fetal liver hematopoietic progenitors expressed this antigen. Differences in CD34 expression were also observed: hematopoietic progenitor cells from embryoid bodies, yolk sac and aorta-gonad-mesonephros displayed variable levels of CD34, whereas more than 90% of fetal liver and bone marrow progenitor cells were CD34+.

Thus, these results demonstrate that expression of CD41 is associated with early stages of hematopoiesis and is highly regulated during hematopoietic development. Further studies concerning the adhesive properties of hematopoietic cells are required to assess the biological significance of these developmental changes.

Key words: Embryonic stem cells, CD41, Hematopoiesis, Mouse

INTRODUCTION

During development, hematopoietic stem cells and progenitor cells reside at different sites. Primitive hematopoiesis starts in the yolk sac at 7.5 days post-coitus (dpc) in mice (Metcalfe and Moore, 1971), and generates primitive erythroid and macrophage progenitors (Palis et al., 1999). Intra-embryonic hematopoietic cell production begins in the para-aortic splanchnopleura (P-Sp) at 8.5 dpc (Godin et al., 1995). As development proceeds, the P-Sp further develops into the aorta-gonad-mesonephros (AGM), the region from which the first hematopoietic stem cells were isolated and characterized (Muller et al., 1994; Medvinsky and Dzierzak, 1996; Godin et al., 1999). Intra-embryonic stem cells migrate to the liver, and subsequently to the spleen and the bone marrow. Primitive hematopoiesis, which begins in the yolk sac, is thought to be transient and to differ from the definitive hematopoiesis that occurs in the AGM. Primitive hematopoiesis does not generate cells capable of reconstitution activity in the long term and the cells generated have a limited potential for differentiation. Long-term repopulating (LTR) activity begins at 10.5 dpc in the AGM region, 1 day earlier than in the yolk sac (Muller et al., 1994; Medvinsky and Dzierzak, 1996). This suggests that the cells endowed with LTR activity found in the 11.5 dpc yolk sac may originate from the AGM (Muller et al., 1994; Medvinsky and Dzierzak, 1996). However, these studies have been performed after establishment of circulation, so that the origin of yolk sac precursors cannot be definitely ascertained. More striking differences are observed in the maintenance and differentiation potentials of the yolk sac and intra-embryonic hemogenic sites, when they are separated before establishment of the circulation. Precursors derived from the yolk sac give rise only to erythro-myeloid progeny and lack LTR activity, whereas intra-embryonic precursors are capable of giving rise
to all hematopoietic lineages and display LTR activity (Cumano et al., 1996; Cumano et al., 2001). However, precursors present in the yolk sac at 8.5 dpc, before the development of blood vessels, that do not exhibit the features characteristic of hematopoietic stem cells during normal development may display such features if they are allowed to develop in an intra-embryonic environment (Matsuoka et al., 2001).

Antigens have been used as markers of specific subpopulations to determine more accurately the pattern of hematopoietic differentiation. Some studies have reported changes in antigen expression during development. For example, it has been shown that AA4.1 antigen expression on hematopoietic stem cells is associated with fetal liver hematopoiesis (Jordan et al., 1990; Trevisan and Iscove, 1995) and that the CD34 antigen is present on all fetal LTR cells, whereas it is detected on only a fraction of LTR cells in the adult (Sanchez et al., 1996; Ito et al., 2000; Tajima et al., 2001). This difference may be related to the stage of stem cell activation (Sato et al., 1999). There is also evidence that some lineage markers, such as Mac1, display different patterns of activation in the fetus and the adult, suggesting that expression of differentiation markers may change considerably during ontogenesis (Rebel et al., 1996). CD41 (αIIb chain, platelet GPIIb) is expressed on platelets and megakaryocytes. It is associated with CD61 (β3 integrin, platelet GPIIIa) in a complex to form the integrin GPIIb/IIIa (αIIbβ3), which plays a major role in platelet function, acting as a receptor for several adhesion molecules, including fibrinogen (Phillips et al., 1988). It is thought that CD41 is specific to the megakaryocytic/platelet lineage, but several reports have indicated that CD41 may be expressed on some hematopoietic progenitors in the embryo, fetus and adult of various species (Debili et al., 1992; Tronik-Le Roux et al., 1995; Murray et al., 1996; Tropel et al., 1997; Basch et al., 1999; Ody et al., 1999). Thus, this integrin may be a lineage-specific antigen that is also expressed during early stages of hematopoietic differentiation. In humans, CD41 is present on larger numbers of hematopoietic progenitor cells in cord blood than in adult hematopoietic tissues, suggesting a possible ontogenic regulation of CD41 expression (Debili et al., 2001).

The hematopoietic differentiation of murine embryonic stem cells (ES) is an interesting model for studying hematopoiesis at various stages of myeloid differentiation because progenitor cells and mature cells are easy to obtain in culture with this system. Moreover, the switch from primitive to definitive hematopoiesis occurs during culture (Keller et al., 1993), implying that hematopoiesis observed in ES cell cultures is similar to normal hematopoietic development.

In this study, we assessed the expression of various cell surface antigens, including CD41, on hematopoietic progenitor cells derived from ES cells during differentiation with the aim of characterizing in more details the mechanisms involved in their differentiation. We show that CD41 was the most reliable progenitor marker in ES cell differentiation and was also present on most hematopoietic progenitor cells in the AGM region and the yolk sac. By contrast, CD41 was found only on a small fraction of fetal liver and bone marrow progenitor cells. Our data provide evidence that CD41 is a differentiation marker that is regulated during development.

### MATERIALS AND METHODS

#### Cells

The SV129-derived D3 ES cell line was kindly provided by F. de Sauvage (Genentech, San Francisco, CA). Bone marrow cells were flushed from the femur and tibia of adult C57Bl/6 2-month old and SV129 mice. Yolk sac (9.5 dpc), 10.5 dpc AGM region and 13.5 dpc fetal liver were dissected from C57Bl/6 embryos.

#### Growth and differentiation of ES cells

ES cells were maintained in an undifferentiated state by culture on a monolayer of mitomycin-C-inactivated (17 μg/ml at 37°C for 2 hours) fibroblasts obtained from embryonic day 14 Swiss mice and cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with leukemia inhibitory factor (LIF) (1000 U/ml; ESGRO, AbCys, Paris, France), 15% fetal calf serum (FCS) (Invitrogen), 150 μM monothioglycerol (MTG, Sigma, Saint Quentin Fallavier, France). The culture medium was changed daily. Under these conditions, the ES cell line required passage every other day. ES cells were maintained at 37°C under air containing only 7% O2.

We used the method described by Wiles and Keller (Wiles and Keller, 1991) to obtain day 6 embryoid bodies (EB6), which were used to study hematopoietic differentiation from ES cells. Briefly, undifferentiated ES cells were treated with trypsin and cultured for 1 hour in DMEM without LIF in cell culture dishes to eliminate adherent embryonic fibroblasts. ES cells were then cultured in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) supplemented with 15% FCS, 450 μM MTG, 50 μg/ml ascorbic acid (Sigma) and 200 μg/ml transferrin (Sigma). Under these conditions, ES cells gave rise to EB.

All cultures were incubated at 37°C in air with a low concentration of O2 as described above.

#### Immunophenotyping

Fluorescein isothiocyanate (FITC)-conjugated rat monoclonal antibodies (MoAb) specific for the following murine antigens were used: CD34 (RAM34), Sca1 (E13-161.7), CD41 (MWR3230 clone), Gr1 (RB6-8C5), CD11b (or Mac-1, M1-70) and CD45R/B220 (RA3-6B2). R-phycocerythrin (R-PE)-conjugated anti-Thy1.2 (5-2.1), anti-Kit (3C1), anti-TER-119 (TER-119) and unconjugated anti-endothelial cell antibodies (MECA-32), anti-Fk-1 and anti-CD45 (30-F11.1), all purchased from Pharmingen (San Diego, CA). ER-MP12 (anti-CD31 antibody) was obtained from BMA (August, Switzerland) (Ling et al., 1997), AA4.1 and 4A5 were kindly provided by I. Lemieshka (Princeton, NJ) and S. Burstein (Oklahoma City, OK), respectively.

FITC-conjugated rat IgG2a, IgG2b and IgG1, R-PE-conjugated rat IgG2a and IgG2b and purified rat IgG2a and IgG2b (Pharmingen) were used as irrelevant control antibodies. The secondary reagent was a FITC-conjugated mouse anti-rat kappa light chain antibody (MARK 1; Immunotech, Lumigny, France).

EB6 were collected and mechanically dissociated by gentle pipetting. The resulting single-cell suspension was incubated with the conjugated MoAb for 30 minutes on ice. If unconjugated antibody was used, cells were washed and incubated with the secondary reagent for 30 minutes on ice. Finally, cells were washed and resuspended in phosphate buffered saline (PBS) containing 5% FCS and 7-aminoactinomycin D (7AAD; 1 μg/ml) (Sigma) to exclude dead cells. Flow cytometry analysis was carried out with a FACScan cytometer (Becton-Dickinson, San Jose, CA) to identify the molecules present at the cell surface.

#### Colony assays

Murine recombinant interleukin 1α (IL1α), and IL3 were purchased from AbCys; recombinant murine IL6 and Kit ligand (KL) were purchased from R and D Systems (Abingdon, UK); recombinant...
human granulocyte colony-stimulating factor (G-CSF) was obtained from Bellon Laboratory (Neuilly sur Seine, France). Recombinant human erythropoietin (EPO) was provided by Cilag (Levallois-Perret, France).

Cells were plated in 1% methylcellulose in IMDM supplemented with 10% FCS (Dominique Dutschke), 1% denatured serum albumin (Cohn fraction V) (BSA) (Sigma), 450 μM MTG, 10 μg/ml insulin, 5 μM EPO, 5 μM KL, 100 U/ml IL3, 10 ng/ml IL6, 10 ng/ml G-CSF and 1000 U/ml IL1β. Granulo-macrophagic (CUF-GM), erythroid (BFU-E), megakaryocytic (CFU-MK) and mixed (erythro-granulo-macrophagic or erythro-megakaryocytic; CFU-mix) colonies were counted with an inverted microscope seven days later.

**Purification of CD41+ cells**

EB6 were collected, rinsed and dissociated, as described for immunophenotyping. The resulting single-cell suspension was incubated with the unconjugated anti-CD41 MoAb for 30 minutes on ice. CD41+ cells were then isolated by the Miltenyi goat anti-rat immunomagnetic bead (Miltenyi Biotech, Paris, France) technique according to the manufacturer's protocol.

**Cell sorting**

EB6, 9.5 dpc yolk sac, 10.5 dpc AGM, 13.5 dpc fetal liver and bone marrow cells were sorted according to CD41 and CD34 expression. To prevent binding of platelets to hematopoietic cells, bone marrow and fetal liver cells were treated with neuraminidase at 0.2 U/ml (Sigma) for 1 hour at 37°C, washed and incubated with a mixture of FITC-conjugated anti-CD41 and biotinylated anti-CD34 MoAbs for 30 minutes at 4°C. Cells were washed and incubated with streptavidin-R-PE (DAKO, Trappes, France) for 30 minutes at 4°C. Cells were washed and incubated with FITC-conjugated anti-CD41 and biotinylated anti-CD34 MoAbs for 1 hour at 37°C, washed and incubated with a mixture of biotin-conjugated IgG1 and biotin-conjugated IgG2a (Pharmingen) were used as controls. Cells were sorted with a FACS Vantage cytometer (Becton-Dickinson) equipped with an argon laser (Coherent Radiation; Palo Alto, CA, USA) set to 485 nm, into four different populations: CD34+ CD41+, CD34+ CD41− and CD34− CD41−.

**Western blotting**

EB6 cells (9×10^6) were isolated by the immunomagnetic bead technique as described above. Unfractionated (7×10^6 cells), CD41+ (7×10^6 cells) and CD41− (2×10^6 cells) cell fractions were lysed at 4°C in a buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40 and 5% glycerol) to which protease inhibitors (CompleteTM Roche Diagnostics, Germany) were added. After one wash, cells were resuspended in culture medium supplemented with 5% FCS and 1 μg/ml 7AAD. FITC-conjugated IgG1 and biotin-conjugated IgG2a (Pharmingen) were used as controls. Cells were sorted with a FACS Vantage cytometer (Becton-Dickinson) equipped with an argon laser (Coherent Radiation; Palo Alto, CA, USA) set to 485 nm, into four different populations: CD34+CD41+, CD34−CD41+, CD34+CD41− and CD34−CD41−.

**Immunoprecipitation**

EB6, murine platelets, bone marrow cells from CD41 knockout mice and rabbit anti-human CD41 and CD61 polyclonal antibodies, which crossreact with the corresponding mouse proteins (obtained from Dr D. Pidard, Institut Pasteur, Paris, France) (Cramer et al., 1990), Loading proteins transfer were verified with a goat anti-mouse actin MoAb (AC-15, Sigma).

**RESULTS**

### Immunophenotypic analysis of EB6

Expression of hematopoietic antigen markers displayed on the surface of cells dissociated from EB6 was analyzed by FACS. Fig. 1 illustrates one typical experiment (n=3 to 15). The stem cell antigens Sca-1, Thy1.2 and AA4.1 (Jordan et al., 1990) were detected on a low percentage of cells (2%, 1% and 6%, respectively) (Table 1). By contrast, CD34 and Kit were expressed on a higher proportion of cells (16% and 33%, respectively). In addition, 36% of all cells were found to express CD31 and 52% Flk1. CD41, a marker usually considered to be specific of the megakaryocytic lineage, was expressed on a considerable proportion of cells (18% to 57%, respectively) (Table 1). The precipitate was washed in 20 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM EDTA and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% acrylamide gel, along molecular weight markers in reducing conditions. The proteins were then transferred onto nitrocellulose membrane (Amersham). The membrane was blocked with BSA, probed with rabbit anti-human CD41 and CD61 polyclonal antibodies and visualized by ECL (Amersham).

| Table 1. Immunophenotypic analysis of EB6 cells |
|-----------------|-----------------|-----------|-----------|
| **Monoclonal antibody** | **% of positive cells** | **MFI** | **Number of experiments** |
| Sca1 | 2±1 | 17±1 | 3 |
| Thy1.2 | 1±1 | 5±1 | 3 |
| AA4.1 | 6±1 | 23±3 | 4 |
| CD34 | 16±3 | 43±13 | 8 |
| c-Kit | 3±3 | 30±1 | 4 |
| CD31 | 4±1 | 17±3 | 7 |
| Flk-1 | 52±8 | 34±1 | 3 |
| CD41 | 52±11 | 5±24 | 15 |
| 4A5 | 8±1 | 23±7 | 5 |
| TER-119 | 22±5 | 24±5 | 3 |
| B220 | 3±3 | 17±10 | 4 |
| Gr-1 | 1±1 | 10±3 | 4 |
| Mac-1 | 4±2 | 10±5 | 4 |
| MECA | 3±1 | 16±1 | 3 |
| CD45 | 6±2 | 15±5 | 5 |

Immunophenotypic analysis was performed for EB6 cells. Single cell suspension of EB6 was incubated with conjugated or non conjugated rat monoclonal antibodies. Mean of percentage of cells expressing the corresponding antigen and mean of fluorescence intensity (MFI) of positive cells.
As we thought that it was likely that the CD41 antigen might also be expressed in early stages of hematopoiesis in EB6-derived cells, we immunomagnetically purified CD41+ cells and cultured them in methylcellulose to analyze their hematopoietic potential. In ten repeated experiments, in which the purity of the cell preparation was over 60%, we found that there was a mean of three times as many progenitors in the CD41+ cell population than in the unfractionated cell population. The total number of hematopoietic progenitor cells per 10^5 cells ranged from 140 to 2520 (mean=861) for the CD41+ cell population than in the unfractionated cell population. There was a mean of three times as many progenitors in the CD41+ cell fraction when compared with 23 to 407 (mean=161) for the CD41- cell fraction. This variability was in part related to the purity of the CD41+ cell population, which ranged from 60% to 98% (mean=81±10%) depending on the preparation. Almost all the clonogenic myeloid progenitors (95±3%) were found in the CD41+ cell fraction. However, the CD41- cell fraction was contaminated with CD41+ cells in all cases. Thus, in subsequent experiments, cells were sorted by flow cytometry, a more stringent method, to determine more accurately the hematopoietic potential of the CD41+ cell fraction.

To determine more precisely the hematopoietic potential of CD41+ cells, EB6 cells were co-labeled with the anti-CD41 and anti-CD34 MoAbs. This last marker was used because it

representative experiment are shown. At least three experiments were carried out for each marker; for CD41, 15 experiments were performed. Control immunoglobulins, broken lines; specific monoclonal antibodies, unbroken lines.
is considered as an important marker for hematopoietic progenitors and stem cells. The four cell subsets CD34+CD41+, CD34–CD41+, CD34+CD41– and CD34–CD41– accounted for a mean of 8%, 16%, 8% and 59% of the cells (Table 2) in four experiments (a typical experiment appears in Fig. 2). These subsets were sorted and cultured in methylcellulose. In six experiments, the cloning efficiency (the ratio of the number of colony-forming cells to the total number of cells plated) for the unfractionated cells and the subset of cells isolated on the basis of side and forward scatter, were not significantly different (0.3±0.2 and 0.4±0.3, respectively), demonstrating that cell sorting has no detrimental effect on hematopoietic progenitor activity. Cloning efficiency was high in the two cellular fractions expressing CD41 when compared with the CD41– populations, whether these cells express CD34 or not (Fig. 3). The two CD41+ cell fractions (CD34+CD41+ and CD34–CD41+) included 96% of all hematopoietic progenitors and gave rise to the various myeloid progenitors (CFU-Mix, CFU-GM, BFU-E and CFU-MK). No significant differences were found in the colony distribution between the two CD41+ populations (Fig. 4). As the CD34–CD41+ subset accounted for about 16% of all total cell population and the CD34+CD41+ cell subset for 8%, it contained a slightly larger number of hematopoietic progenitors (50±23% versus 46±10% of the progenitors contained in CD34–CD41+ subset) (Table 2). By contrast, the CD41– cell fractions were almost devoid of progenitors, irrespectively of the CD34 expression profile. The rare hematopoietic progenitor cells were present in the CD41– cell subsets essentially in the CD34–CD41– (9±14%) cell fraction, implying that the CD34+CD41– (1±2%) cell fraction may be enriched in endothelial cells (Table 2). We then investigated whether CD41 was also a marker of primitive erythropoiesis by counting primitive erythroid colonies at day 4 of culture. As shown in Fig. 5, progenitors that give rise to

<table>
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<th>Subset</th>
<th>EB6</th>
<th>Yolk sac</th>
<th>Fetal liver</th>
<th>Bone marrow</th>
<th>AGM</th>
</tr>
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<tbody>
<tr>
<td>CD34+CD41+</td>
<td>8</td>
<td>50±23</td>
<td>3</td>
<td>68±20</td>
<td>2</td>
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<tr>
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<td>CD34+CD41–</td>
<td>8</td>
<td>12</td>
<td>4</td>
<td>9±12</td>
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<tr>
<td>CD34–CD41–</td>
<td>59</td>
<td>9±14</td>
<td>88</td>
<td>5±7</td>
<td>81</td>
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Cells from EB6, yolk sac, AGM, fetal liver and bone marrow were sorted on the basis of CD34 and CD41 antigen expression. The mean of percentage CD34 and CD41 expression determined by flow cytometry in various CD34 and CD41 subsets (n=4), and the yield (calculated in Fig. 5) of hematopoietic progenitor in the various subsets defined as in Figs 2 and 8. The number of CD41+ hematopoietic progenitor cells increases by a factor of 10,000 from the yolk sac to the adult bone marrow.

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**Fig. 2.** Cell sorting of different CD34 and CD41 populations from EB6 cells. Double staining was performed with a FITC-conjugated anti-CD41 MoAb and a biotinylated anti-CD34 MoAb, using a streptavidin-R-PE as the secondary reagent. Four cell subsets (CD34+CD41+, CD34–CD41+, CD34+CD41– and CD34–CD41–) were sorted by flow cytometry in R1, R2, R3 and R4, respectively. The percentages are shown for one representative experiment (n=4).

**Fig. 3.** Hematopoietic colony formation in EB6 cells from the different CD34 and CD41 populations. Various EB6 cell fractions were sorted by flow cytometry according to CD34 and CD41 expression and cells were plated in methylcellulose for colony assays. Control 1 (C1) is the unfractionated cell population. Control 2 (C2) corresponds to EB6 cells sorted with the side scatter and forward scatter gating used for CD41 purification (n=6). Results are expressed as percentages (number of progenitors for 100 plated cells).
primitive erythropoiesis were found essentially in the CD41+ fractions, more specifically in the CD34–CD41+ cell fraction, this result demonstrates that CD41 expression is not restricted to definitive hematopoiesis.

In conclusion, this result demonstrates that CD41 is a reliable marker of hematopoietic progenitor cells during ES cell differentiation.

Characterization of the CD41 molecule produced by EB6 cells
To characterize the CD41 protein (platelet GPIIb) expressed by EB6 cells, we performed a western blot analysis with unfractionated EB6 cells and EB6-derived CD41+ and CD41− cells obtained after immunomagnetic bead purification. Mouse platelets were used as a control. The blots were probed with the same CD41 MoAb used for purification and cell sorting. CD41 from EB6 and murine platelets (Fig. 6A) was detected as a doublet at 125 kDa in reducing conditions. This doublet may result from detection of both the mature protein and the precursor (pro-GPIIb). These data were confirmed by experiments with a polyclonal antibody directed against human CD41 but crossreacting with the murine protein (data not shown).

In the CD41+ cell fraction obtained by separation on an affinity column, the 125 kDa band was much stronger than in unfractionated cells or the CD41− cell fraction which remained slightly contaminated with CD41+ cells because of an incomplete depletion by the magnetic method (Fig. 6A). Thus, our results demonstrate that CD41 (platelet GPIIb protein) is present in EB6 cells.

In platelets, CD41 is associated exclusively with the β3 integrin (CD61, GPIIIa). We investigated whether EB6 cells also contained CD61 by probing the blots with an anti-CD61 antibody. In platelets, CD61 had an apparent molecular weight of 90 kDa. A band with a similar molecular weight was detected with this antibody in the CD41+ cell fraction, which was much weaker in the CD41− cell fraction which remained slightly contaminated with CD41+ cells because of an incomplete depletion by the magnetic method (Fig. 6A). Thus, our results demonstrate that CD41 (platelet GPIIb protein) is present in EB6 cells.

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transcriptional activity (Tronik-Le Roux et al., 1995; Tropel et al., 1997). These data suggested possible ontogenic changes in CD41 expression and prompted us to evaluate its expression in hematopoietic tissues at various stages of development.

CD41+ cells from 9.5 dpc yolk sac, 10.5 dpc AGM, 13.5 dpc fetal liver and bone marrow were sorted and their hematopoietic potential was studied (Fig. 7A). Approximately 12% of all yolk sac cells expressed the CD41 antigen and 96±4% of the hematopoietic progenitor cells were contained in the CD41+ cell fraction (Fig. 7B). Progenitors that give rise to various myeloid colonies, including mixed colonies, were present in the CD41+ cell fraction (data not shown). By contrast, the rare progenitors present in the CD41− cell fraction gave rise predominantly to the granulo-macrophage lineage. Low CFU-S activity was also found in the 10.5 dpc yolk sac and was present in the CD41+ cell fraction (data not shown).

In the AGM, approximately 5% of cells expressed the CD41 antigen. As in the yolk sac, the majority (81±12%) of hematopoietic progenitor cells expressed this antigen.

Five experiments were performed on day 13.5 fetal liver, in which the percentage of CD41+ cells was lower than that in the yolk sac and the AGM (2% to 5%). The CD41+ cell fraction displayed greater enrichment in hematopoietic progenitor cells (sixfold) than did the CD41− cell population (Fig. 7B). As in the yolk sac and AGM, expression of CD41 was not associated to a lineage restriction and various myeloid progenitors were detected in the CD41+ cell fraction. However, owing to the rarity of CD41+ cells, most of the hematopoietic progenitor cells were CD41− (75±11%).

In the bone marrow, the percentage of CD41+ cells was similar to that observed in fetal liver and ranged from 1% to 4% in five experiments. The cloning efficiency was extremely variable (167 to 2050 per 10^5 cells) (mean=700) in the CD41+ cell fraction but was generally of the same order of magnitude as that in the CD41− cell fraction (213 to 264 per 10^5 cells) (mean=229) with the same types of progenitors present in both fractions. This demonstrates that the vast majority of hematopoietic progenitor cells (92±4%) were present in the CD41− cell fraction.

These results were obtained with C57Bl/6 mice. However, as ES cells were derived from Sv129 mice, we also studied bone marrow cells from Sv129 mice. Similar results were obtained confirming our findings (data not shown).

Because there is evidence that CD34 expression is also regulated during ontogeny (Ito et al., 2000), we subsequently investigated whether there were concomitant changes in the expression of CD41 and CD34. Cells from the yolk sac, AGM, fetal liver and bone marrow were sorted on the basis of CD34 and CD41 antigen expression, as previously carried out for EB6-derived cells (Fig. 8). In the yolk sac, a mean of 3% of...
cells were CD34+CD41+ and 2% were CD34-CD41+ (n=5). The majority of hematopoietic progenitor cells (68±20% of the total number of progenitors) was found in the CD34+CD41+ cell fraction, but a significant proportion (18±14%) was nonetheless found in the CD34+CD41- cell fraction. The various myeloid progenitors were present in either fraction with an almost identical distribution (Fig. 4). By contrast, 4% of cells were CD34+CD41- and the percentage of hematopoietic progenitor cells present in this subset was lower than in the two CD41+ cell fractions (9±12%). The CD34-CD41+ cell fraction accounted for 88% of cells and was almost devoid of progenitors (5±7%) (Table 2). As in EB6, the majority of hematopoietic progenitors giving rise to primitive erythropoiesis were contained in the CD34+CD41+ cell fraction (Fig. 5).

Far fewer cells were obtained from the AGM, and we were able to sort these cells only into three populations (CD41+, CD34+CD41+ and CD34-CD41-). The entire CD41+ cell population (irrespective of the CD34 expression) accounted for 2% of cells, 5% of the cells were CD34+CD41- and 90% of the cells were found in the CD34-CD41+ cell fraction (n=3).

Fig. 7. Cell sorting of CD41+ and CD41- cells from EB6, yolk sac, AGM, fetal liver and bone marrow cells. (A) Cells from yolk sac, AGM, fetal liver and bone marrow were sorted by flow cytometry according to CD41 antigen expression. The percentages of CD41+ and CD41- cells in one representative experiment are shown. The CD41+ and CD41- gates were slightly different in these various tissues. This is especially true for AGM, where a high autofluorescence is found in CD41+ cells. (B) Content in progenitor cells in the CD41+ and CD41- cell fractions. An immunomagnetic technique was used to separate EB6 into CD41+ and CD41- cell fractions. The results for EB6 cells are the mean of the ten experiments. 9.5 dpc yolk sac (YS) (n=3), 10.5 dpc AGM (n=3), 13.5 dpc fetal liver (FL) (n=5) and bone marrow (BM) (n=5) were purified by flow cytometry according to CD41 expression. The number of progenitors (yield) in each fraction (expressed as percentage) was calculated as the ratio between the numbers of progenitors contained in each cell fractions to the number of progenitors contained in the starting population.

The majority of hematopoietic progenitor cells (75±1%) was again found in the CD41+ cell fraction (Table 2).

Marked differences were observed in the fetal liver samples. Fetal liver CD34+ cell fractions accounted for 13% of the cells, 2% of which were CD41+. The CD34+CD41+ cell fraction, which accounted for 11% of cells, contained most of the hematopoietic progenitor cells (79±11%) (n=5), whereas the CD34-CD41+ cell fraction contained a lower but significant proportion of hematopoietic progenitor cells (12±9%) (Table 2).

The results obtained with bone marrow cells were similar to those obtained for the fetal liver. Most of the progenitors were present in the CD34+ cell subsets (92%) essentially in the CD34+CD41- (70±17%) fraction, which accounted for 2% of the cells. The CD34-CD41+ cell fraction representing 1% of the cells also contained a significant proportion of progenitors (22±16%). The CD34-CD41- cell fraction (2% of the cells) was almost devoid of progenitors (1±2%) (n=4). Whatever the ontogenetic stage, the various myeloid progenitors were detected in the CD41+ subsets (Fig. 4).

CD41+ and CD41- hematopoietic progenitor cells are not two different cell populations

Our results show that the pattern of CD41 expression on hematopoietic progenitor cells changes during ontogeny. This suggests that CD41 expression on hematopoietic progenitors is either a marker of some early emerging hematopoietic clones or a marker of differentiation regulated during ontogeny. In order to answer to this question, we investigated if CD41- cells could convert to CD41+ cells and vice versa.

We first studied changes in CD41 expression in culture. In two experiments, CD41+ cells from EB6 and yolk sac were isolated by flow cytometry. Cells were cultured in vitro in the presence of a combination of cytokines. After two days in culture, 95% of the cells had lost CD41 expression. Next, the CD41- cells from EB6 were sorted and cultured to investigate whether these cells could undergo conversion to CD41+. On day 2, about 45% of the cells expressed the CD41 antigen but this antigen disappeared during culturing with only 10% of cells positive on day 6. This result clearly indicates that CD41 is transiently expressed during hematopoietic differentiation.

To examine whether CD41 conversion could also occur in hematopoietic progenitors, the CD41+ and CD41- cell fractions...
from EB6 were sorted, cultured for 2 days and resorted into CD41+ and CD41− cell populations (n=3), and assayed for the presence of hematopoietic progenitors. The highest frequency of progenitors cells was reproducibly found in the cellular fraction expressing CD41 whether they originated from the CD41+ or CD41− cell populations. By contrast, the CD41− cell fractions isolated from EB6 or sorted from cultured CD41+ or CD41− cell populations showed low content in hematopoietic progenitor cells (Table 3). This result suggests that during EB6 differentiation, CD41+ hematopoietic progenitors cells arise from CD41− cells and that CD41 is a marker of differentiation. It remains to be determined if these CD41− cells, which give rise to CD41+ myeloid progenitors, are already committed to hematopoiesis or are pluripotent cells.

Table 3. CD41 conversion may occur in hematopoietic progenitors

<table>
<thead>
<tr>
<th>First sort</th>
<th>Number of progenitors/10^5 cells</th>
<th>Second sort after culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions</td>
<td>CD41+</td>
<td>CD41−</td>
</tr>
<tr>
<td>CD41+</td>
<td>2147±222</td>
<td>863±287</td>
</tr>
<tr>
<td>CD41−</td>
<td>20±20</td>
<td>6±6</td>
</tr>
</tbody>
</table>

The CD41+ and CD41− cell fractions from EB6 were sorted, cultured, for 2 days, and resorted into CD41+ and CD41− cells (n=3). These cell fractions were subsequently assayed for hematopoietic progenitors. CD41+ cells, which arise from the initial CD41+ and CD41− cell fractions, gave rise to hematopoietic progenitors.

**DISCUSSION**

The hematopoietic differentiation of ES cells in vitro is a powerful tool for studying the regulation of hematopoiesis and gene expression. This continuous-culture cell line has the potential to differentiate into multiple lineages, which is particularly interesting for studies of the effects of gene overexpression or gene knockout or knock-in, if homoygous ES cells are available.

We found that all hematopoietic progenitor cells derived from ES cells expressed CD41 on their surface, whereas other markers of hematopoietic progenitors, including CD34 are only present on a fraction of them. We also demonstrated by immunoprecipitation that CD41 (GPIIb) is associated with CD61 (β3 integrin, GPIIIa) in a complex (αIIbβ3 integrin, GPIIb/IIIa), as in platelets. CD41 was thought to be an integrin specific to the megakaryocyte/platelet lineage. However, there is increasing evidence that it is expressed on a fraction of hematopoietic progenitor cells, not only BFU-E and CFU-MK, but also CFU-GM and multipotent progenitor cells in bone marrow (Berridge et al., 1985; Tronik-Le Roux et al., 1995). In birds, CD41 is expressed on multipotent progenitor cells and on committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999). We recently detected CD41 on human NOD-SCID reconstituting cells and on all types of committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999). We recently detected CD41 on human NOD-SCID reconstituting cells and on all types of committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999). We recently detected CD41 on human NOD-SCID reconstituting cells and on all types of committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999). We recently detected CD41 on human NOD-SCID reconstituting cells and on all types of committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999). We recently detected CD41 on human NOD-SCID reconstituting cells and on all types of committed progenitors, including those of the T lineage, from the embryo to the adult (Ody et al., 1999).
progenitor cells of the yolk sac and AGM expressed CD41, whereas only a minority expressed CD41 in the fetal liver or bone marrow. Our data obtained with the AGM slightly differs from those of Ody et al., where only a fraction of the cells in the aortic clusters were found CD41 positive by immunostaining (Ody et al., 1999). This may be related to differences in species (chicken versus mouse) or in the methods (flow cytometry versus immunochemistry). At all stages of ontogeny, CD41 expression was not restricted to cells with the potential to differentiate into erythroid and megakaryocytic cells, as it was also detected on the majority of granulomonocytic progenitors. During ontogeny, we observed inverse patterns of expression for CD34 and CD41 on hematopoietic progenitors. Indeed, the percentages of the various CD34 and CD41 cell subsets were similar in yolk sac and bone marrow. However, hematopoietic progenitor cells were present in the two CD41+ cell populations (CD34+ or CD34−) in the yolk sac whereas they were present essentially in the CD34+CD41+ cell fraction in bone marrow. Thus, this result suggests that CD41 expression on hematopoietic progenitor cells is either directly regulated by ontology or that CD41 is a marker of differentiation expressed on a subset of hematopoietic progenitors that predominate in EB6, yolk sac and AGM.

There are two possible explanations for these results. The expression of CD41 on hematopoietic stem cells and progenitors may reflect the fact that several specific markers of hematopoiesis, such as myeloperoxidase and globin, have been detected in hematopoietic stem cells or progenitors (Hu et al., 1997). This may be the consequence of low levels of numerous hematopoietic transcription factors, leading to the priming of lineage-specific differentiation markers in stem cells (Hu et al., 1997). This activation of lineage-specific marker may be more potent in the fetus than in adult (Papayannopoulou et al., 2000). The differentiation program later becomes less flexible and genes of only one differentiation program can be fully activated. Alternatively, the presence of CD41 on early hematopoietic cells may be related to the biological function of this integrin. Indeed, CD41 is a very important adhesive receptor, which, unlike many other integrins, has several ligands, including fibrinogen, fibronectin, vitronectin and von Willebrand factor (Phillips et al., 1988). In hematopoiesis, it has been shown that the VLA-4 and VLA-5 integrins, which, in common with CD41, share fibronectin as a ligand, play a major role in the homing and mobilization of stem cells (Papayannopoulou and Nakamoto, 1993; Craddock et al., 1997). It is therefore possible that CD41 plays a similar role, especially in the embryo, replacing VLA-4 or VLA-5, or acting in combination with them. However, no defect in hematopoiesis has been described in individuals suffering from Glanzmann’s disease or in β3 integrin-deficient mice, although some homozygous embryos have died in utero (Hodivala-Dilke et al., 1999). These deaths were thought to result primarily from intrauterine bleeding in the pregnant β3 null female. In addition, hematological abnormalities have not been reported in CD41 knockout mice (Tronik-Le Roux et al., 2000). We also studied yolk sac hematopoiesis from a 9.5 dpc CD41 knockout embryo and found no quantitative or qualitative abnormality of hematopoietic progenitor cells. However, we cannot exclude the possibility that the absence of hematopoietic defects in knockout mice is related to redundant functions of integrins. In platelets, CD41 is normally nonfunctional and must be activated by an inside-out mechanism before it can bind fibrinogen. It has recently been demonstrated that NF-E2 is required for the agonist-induced activation of CD41 in megakaryocytes (Shiraga et al., 1999). NF-E2 is a heterodimer of a p45NF-E2 subunit, which is essentially present only in erythroid and megakaryocytic cells, and various ubiquitous subunits of the Maf family. Thus, the NF-E2 complex is essentially present in the erythroid and megakaryocytic cell lineages, suggesting that CD41 activation may occur only in these two cell lineages. However, there is also evidence for the existence of several P45NF-E2-related factors, such as NRF2 (Chan et al., 1996), and we cannot rule out the possibility that these factors may replace NF-E2 in hematopoietic progenitor cells and stem cells, in the activation of CD41.

In conclusion, this study shows that CD41 is a reliable marker of early steps of hematopoiesis during ES cell differentiation. It is also a marker of all the various progenitors present in the yolk sac and AGM. This result suggests that CD41 plays a role in differentiation or homing of murine hematopoietic cells during ontogeny.

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