

Megakaryocytopoiesis in the human fetus

J L ALLEN GRAEVE AND P A DE ALARCON

University of Iowa, Iowa City, USA

SUMMARY After immunohistochemical staining the size and maturational stage of fetal megakaryocytes from 20 human fetuses of 12 to 21 weeks' gestation were compared with those from normal adults. The mean diameter of fetal megakaryocytes was 14.0 microns when stained with antiglycoprotein IIb (Tab) and 15.2 microns when stained with antifactor VIII, which was significantly smaller than adult megakaryocytes, which were 18.4 microns when stained with Tab and 20.6 microns when stained with factor VIII. The proportion of immature (stage II) cells was higher—and the proportion of mature (stage IV) cells was lower—in the fetal tissue than in the adult.

The smaller size and shift to a less mature population indicated that there were differences at the non-mitotic phase of the development of megakaryocytes in the fetus. Such differences were probably associated with quantitative and qualitative platelet abnormalities in newborn infants. Understanding the physiology and regulation of megakaryocytopoiesis in fetuses and newborn infants will be invaluable in determining the pathophysiology of platelet dysfunction in the newborn.

Platelets in newborn infants are quantitatively and qualitatively different from those in older children and adults¹; The lowest normal platelet count may be as low as $100 \times 10^9/l$ in the newborn. Newborn infants are also vulnerable to thrombocytopenia, there being an incidence of 10 to 60% in ill newborn infants, and thrombocytopenic babies are at increased risk of intracranial haemorrhage and death.¹⁻³ Functional abnormalities in platelets from neonates include decreased adhesion to glass fibres, low platelet factor III activity, and hypoaggregability to adenosine diphosphate, epinephrine, thrombin, and collagen.^{1,4} There is also evidence of abnormal clot retraction and abnormal release of platelet factor IV in the newborn.^{5,6}

The metabolic capacity of the platelet—and perhaps its adaptability—is established in the precursor cell, the megakaryocyte,⁷ and it is likely that fetal megakaryocytes differ from adult megakaryocytes. Our hypothesis is that immature megakaryocytes produce platelets that differ in number and function from platelets produced by mature megakaryocytes. In this study the size and maturational stage distribution of fetal megakaryocytes were examined using an immunohistochemical staining procedure and then compared with normal adult marrow; there was morphological evidence of reduced megakaryocyte maturity in the fetus.

Material and methods

SPECIMENS

Human fetal tissue was obtained from 18 normal and two anencephalic aborted fetuses of 12 to 21 weeks' gestation after vacuum aspiration. Biopsy specimens of bone marrow were obtained from the femurs, and specimens from liver and spleen were collected when possible. Adult bone marrow trephine biopsy specimens were collected from the iliac crest of five normal volunteers after obtaining informed consent. All studies were approved by the Institutional Review Board.

Fixation and embedding were as described by Beckstead.⁸ Biopsy specimens were fixed in paraformaldehyde, serially dehydrated in acetone, then infiltrated and embedded in glycol methacrylate (JB4, Polysciences Inc). Sections were cut 3.0 microns thick with glass knives and mounted on glass slides precoated with 0.1% poly-L-lysine.

IMMUNOHISTOCHEMICAL STAIN

The staining procedure was modified from the procedure described by Beckstead *et al.*⁸ Biopsy sections mounted on glass slides were incubated in 0.25% trypsin, then in 3% normal sheep or goat serum using the same species as used for the secondary antibody. The sections were incubated in

primary antibody, mouse monoclonal antiplatelet glycoprotein IIb (Tab), or rabbit polyclonal anti-human factor VIII (Calbiochem-Behring Corporation) for 2 to 2½ hours at 37°C, and then in secondary antibody, biotinylated sheep antimouse IgG, or biotinylated goat antirabbit IgG (Organon Teknika-Cappel) for one hour at 37°C. Endogenous peroxidase was inhibited with 0.3% hydrogen peroxide in 30% methanol for three minutes, and then in avidin peroxidase conjugate diluted 1/80 at room temperature for one hour. The chromogenic substrate 3, 3-diaminobenzidine (DAB) 0.05% was added, and then a solution of 0.05% DAB, 0.1% hydrogen peroxide, imidazole, and sodium azide. Further colour development was achieved with 0.5% copper sulphate, and counterstained with haematoxylin before bluing in Scott's water.

Most sections of marrow and all specimens of liver and spleen were also stained with Wright-Giemsa stain or haematoxylin and eosin for comparison.

MEASUREMENT OF THE SIZE OF MEGAKARYOCYTES

Megakaryocytes were identified by specific brown DAB staining. The largest axis of every megakaryocyte was measured to the nearest micron with a calibrated eyepiece reticle at a magnification of 400. Megakaryocyte cytoplasmic fragments, and those without nuclei, were not measured.

ASSESSMENT OF THE MATURATIONAL STAGE OF MEGAKARYOCYTES

The approximate maturational stage of each megakaryocyte was assessed as stage I-IV, guided by criteria elaborated by Levine *et al.*⁹ Stage I cells were comparatively small with a high nuclear:cytoplasmic ratio and a single or bilobed nucleus. Stage II cells were larger with more complex nuclear lobulation than stage I cells; stage II cells also had a comparatively high nuclear:cytoplasmic ratio although it was less than in stage I cells. Stage III cells were larger with abundant cytoplasm and complex multilobulated nuclei with separated nuclear lobes. Stage IV cells were large with abundant cytoplasm and compact multilobulated nuclei.

STATISTICAL ANALYSIS

Megakaryocytes may be treated as spheres with minimum error, even when they look ellipsoidal.^{10 11} Harker has shown that the mean of the diameter measurements of biopsy sections gives a value that is 87% of the true diameter; that value increases to 93% of the true diameter if only cells containing nuclei are counted.¹¹ Measurements were therefore divided by 0.93 to represent the actual megakaryocyte diameter.

Because large cells are cut more times and

therefore represented in more sections than are smaller cells, the number of cells observed must be corrected for multiple counting. The data were grouped by size and pooled. The number (n) of cells at each size was divided by the correction factor (d/t+1), where d equals the corresponding diameter and t equals the section thickness (3 microns)^{10 11}; n corrected = n observed in section/(d/t+1).

The mean and standard deviation were calculated as for grouped data. Mean diameters were compared by the two sample *t* test for samples with equal variance or with unequal variance (equality of variances was assessed by the F test). Trends in size and stage distribution v fetal age were evaluated using regression analysis. Comparison of proportions of cells at various maturational stages were made with 2×2 contingency tables and χ^2 with Yates's correction.

Results

Twenty specimens of fetal marrow were stained with Tab antibody, and 15 with factor VIII antibody, both groups including two anencephalic fetuses, and spanning the gestational age group 12 to 21 weeks. Specimens of liver were obtained from 11 fetuses and of spleen from six fetuses. Five biopsy specimens of normal adult bone marrow were examined with Tab antibody and four with factor VIII antibody.

All megakaryocytes identified by immunostaining were counted and assessed. The median was 107 megakaryocytes/section, range 11–195. Femurs from the two youngest fetuses, of 12 and 13 weeks' gestation, had primarily loose mesenchymal tissue with immature cartilage and bone and only small sparse foci of haematopoiesis. In both fetal and adult specimens the distribution and size of the megakaryocytes varied through the specimen, with clusters of immature cells separated from clusters of mature cells. Almost all the megakaryocytes had diffuse cytoplasmic staining with increased intensity at the cytoplasmic membranes. Megakaryocytes in the adult specimens generally stained darker than fetal megakaryocytes. Although the staining procedure included a step to block endogenous peroxidase, slight non-specific staining in erythroid precursors occasionally occurred; the characteristic nuclear features of erythroid precursors and megakaryocytes were of use in differentiating between these small cells. Most osteoclasts also stained weakly with the immunohistochemical procedure, but the unique nuclear features, smooth outline, and proximity to bone identified them.

Megakaryocytes from the anencephalic fetuses

were of the same size and had the same stage distribution as those from normal fetuses.

SIZE

The mean (SD) diameter of fetal megakaryocytes identified with Tab antibody was 15.2 (1.4) microns, significantly smaller than the mean diameter of adult megakaryocytes, 20.6 (4.1) microns ($p=0.04$). The mean diameter of fetal megakaryocytes increased from 12.2 microns at stage I to 22.6 microns at stage IV. When each stage in the group stained with Tab was examined individually, at stages II, III, and IV, the fetal megakaryocytes were significantly smaller than adult megakaryocytes (table 1).

Similarly, the fetal megakaryocytes stained with factor VIII were smaller than the adult, mean diameters being 14.0 (1.2) and 18.4 (2.1) microns, respectively ($p<0.0001$). At all stages the fetal megakaryocytes were smaller than the adult megakaryocytes (table 1).

The size of the megakaryocytes did not vary significantly over the gestational age range of 12–21 weeks.

STAGE

In both the fetal and adult specimens the largest proportion of megakaryocytes was assessed as immature or stage I (table 2). The fetal specimens had a higher proportion of stage II immature megakaryocytes than the adult specimens, 28% and 22% ($p=0.004$) for those stained with Tab and 30%

and 21% ($p<0.001$) for those stained with factor VIII. There were significantly fewer stage IV mature cells in the fetal specimens than in the adult, 8% and 16% in the group stained with Tab and 9% and 19% in those stained with factor VIII ($p<0.001$). There was no significant association between gestational age and stage distribution for the gestational range examined.

Liver and spleen

Eleven liver biopsy specimens of 12–21 weeks' gestation and six biopsy specimens of spleen of 16–21 weeks' gestation showed nests of hematopoiesis with a decline in the hematopoietic population density with advancing gestation. Few megakaryocytes were present, their diameter and morphological appearances being similar to those in fetal marrow; few mature megakaryocytes were found. The immature megakaryocytes were most easily located by immunostaining and the mature megakaryocytes by Wright-Giemsa staining.

Discussion

This study shows that during the second trimester of gestation the fetal megakaryocytes are smaller than adult megakaryocytes. The mean adult megakaryocyte (SD) diameter in this study was similar to that found by Harker, being 20.8 (3.3) microns.¹¹ There is little information about megakaryocyte size in the fetus. Izumi *et al* examined the size of megakaryo-

Table 1 Mean (SD) diameter (microns) of megakaryocytes in biopsy specimens corrected for sampling method

	Stained with antiglycoprotein IIb			Stained with factor VIII		
	Fetal	Adult	p Value*	Fetal	Adult	p Value*
Stage I	12.2 (1.2)	15.2 (3.9)	0.2	11.0 (0.9)	12.4 (0.9)	0.01
Stage II	15.8 (1.1)	22.4 (2.4)	0.003	14.2 (1.0)	19.1 (2.9)	0.04
Stage III	20.7 (4.9)	29.2 (1.3)	<0.001	20.0 (1.8)	26.1 (3.0)	<0.001
Stage IV	22.6 (5.4)	29.3 (2.4)	<0.001	19.9 (5.9)	26.4 (1.5)	<0.001
Total	15.2 (1.4)	20.6 (4.1)	0.04	14.0 (1.2)	18.4 (2.1)	<0.001

*Two sample *t* test.

Table 2 Percentage distribution of megakaryocytes by age

	Stained with antiglycoprotein IIb			Stained with factor VIII		
	Fetal	Adult	p Value	Fetal	Adult	p Value
Stage I	50	49	0.7	48	46	0.5
Stage II	28	22	0.004	30	22	<0.001
Stage III	14	13	0.3	14	13	1.0
Stage IV	8	16	<0.001	8	19	<0.001
Total	100	100	<0.001*	100	100	<0.001*

* χ^2 test, 4×2 contingency table.

cytes over a range of ages and found that a 5 month old fetus had smaller cells than a 7 month old fetus; in turn, fetal megakaryocytes were smaller than infant, and infant smaller than child and adult megakaryocytes. The megakaryocytes reached adult size at about 1 year of age.¹²

The high proportion of stage I immature megakaryocytes was an unexpected finding with several possible explanations. Promegakaryoblasts are small and generally not morphologically distinguishable from lymphocytes or other blast cells, so specific staining techniques are needed to identify them. Previous reports may have used enrichment procedures which exclude the small, higher density cells, and diminish the immature population. In biopsy sections visual impression makes large, mature megakaryocytes seem predominant, and without correction for multiple counting the larger cells are disproportionately represented. With the DAB staining procedure used in this study endogenous peroxidase in erythroblasts may produce artifactual staining, which causes them to be counted as stage I megakaryocytes. Even if such an artifact exists, comparison of fetal with adult is still useful. When only stages II, III, and IV are evaluated there is a clear difference in maturational distribution between fetus and adult, with the balance being towards less mature forms in the fetus.

Stage distribution of megakaryocytes has not been widely reported. Rabellino *et al* studied marrow from human ribs and found the ratio of small cells to mature megakaryocytes was about 1:2; the small cells represented the early megakaryocytic differentiation compartment.¹³ Levine and Fedorko found that harvested guinea pig marrow cells in suspension contained an average of 25% immature, 50% intermediate, and 20% mature megakaryocytes; a concentrated population consisted of 21% immature, 62% intermediate, and 17% mature megakaryocytes.¹⁴

Evaluation of maturational stage in biopsy sections presented the following consideration: assessment of stage relies heavily on nuclear characteristics but only a random section of nucleus is represented on the slide, which is a potential source of error. The relative associations, however (stage I less mature than stage II, II less than III, and so on) would still be valid when applied to a large number of cells. For example, a small cell filled with a single lobed nucleus and a thin rim of cytoplasm is less mature than a cell with two nuclear lobes that only partially fill the cell.

Mature or advanced stage megakaryocytes are generally larger and have a higher DNA content, or ploidy. The quantity of DNA seems to be associated with the qualitative characteristics of platelets,

cytoplasmic volume, and quantitative platelet production.^{9 15-17}

The findings from this study indicate that fetal megakaryocytes are less mature than adult megakaryocytes. The morphological findings of smaller size and shift to a less mature population in the fetus are likely to be associated with quantitative and qualitative abnormalities in platelet of the newborn.

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Correspondence to Dr JL Allen Graeve, Pediatric Hematology/Oncology, University of Iowa, Iowa City, Iowa 52242, USA.

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