

In Vitro Growth of Granulocytic Colonies From Circulating Cells in Human Cord Blood

By Søren Knudtzon

Human umbilical cord blood cells from 26 newborn infants and peripheral blood cells from 18 adults were cultured in vitro by using the agar-gel method of human hemopoietic cell culture. An increased concentration of colony-forming cells was seen in the cord blood cultures. Between 17 and 385 colonies, with a mean of 122, were formed in these cultures per 2×10^5 nucleated cells plated. The peripheral blood cell cultures from adults gave rise to 0–11 colonies, with a mean of 3, per 2×10^5 nucleated cells plated. The average number of cells per colony was 1000–1500 cells after 14 days of culture, predominantly granulocytic.

AN INCREASED CONCENTRATION of hemopoietic stem cells has been found in the blood of mouse embryos when compared with the concentration after birth.^{1,2} The method used for these experiments has been the spleen colony technique. A similar in vivo technique is not available for human studies, but a method for in vitro growth of human granulocytic progenitor cells has been described recently by Robinson and Pike.³ By this method granulocytic colonies are formed in an agar-medium layer when bone marrow cells are placed upon a feeder layer containing peripheral leukocytes.

The identity of the in vitro colony-forming cell is unknown, but similar studies in the murine system indicate that the in vitro colony-forming cell is a primitive member of the granulocytic cell line, a committed stem cell, more mature than the in vivo spleen colony-forming cell which is considered to be the multipotential stem cell.⁴ It is, however, still a matter of debate whether a difference exists between the in vitro colony-forming cell and the spleen colony-forming cells in the murine system.⁵

Evidence for in vitro colony-forming cells circulating in peripheral blood from normal adults has been reported, although in a small number compared with human bone marrow cultures.^{6,7}

In this study, the concentration of in vitro colony-forming cells in human umbilical cord blood was investigated and compared with the concentration in peripheral blood from normal human adults.

MATERIALS AND METHODS

Umbilical Cord Blood

Samples of umbilical cord blood were obtained from 26 newborn infants (16 males, 10 females), delivered at the obstetrical department YB, Rigshospitalet, Copenhagen. The deliveries were normal except in two cases where cesarian sections were done. Birth weights ranged between 1920

From the Department of Medicine, Finsen Institute, Copenhagen, Denmark.

Submitted April 10, 1973; revised August 27, 1973; accepted August 29, 1973.

Supported by grants from the Danish Hospital Foundation for Medical Research, The Danish Medical Research Council and the Hasselbalch Foundation.

Søren Knudtzon, M.D.: Research Associate, Department of Medicine, Finsen Institute, Copenhagen, Denmark.

© 1974 by Grune & Stratton, Inc.

and 4400 g, including seven infants with a birth weight of less than 2500 g. Two pairs of twins were included in the study. The cord was clamped after pulsation stopped in the umbilical vessels, and blood, usually 10-20 ml, was collected in 10-ml test tubes containing 100 U of heparin (LEO, Copenhagen). Samples of peripheral blood were collected in test tubes with heparin by venipuncture from 15 healthy adults and samples of maternal blood from three mothers were obtained within 24 hr after delivery. The cord blood and the peripheral blood from adults was allowed to stand for 2 hr at room temperature before removal of the leukocyte-rich supernatant. The number of nucleated cells was counted in a hemocytometer and a differential count made. The culture method is described in detail by Robinson and Pike.³ The medium used is a modified preparation of McCoy's 5A medium with 15% fetal calf serum. One milliliter of medium, 0.5% agar and 10^6 normal peripheral leukocytes, was placed into 35-mm Falcon plastic petri dishes as feeder layers. The blood cells from the umbilical cord or from adults were included in an upper layer containing a 1-ml mixture of 2×10^5 nucleated cells, 0.3% agar, and medium. The plates were then placed in a humid incubator at 37°C constantly flushed with 7.5% CO₂ in air.

The number of colonies was counted at day 14 using a binocular dissecting microscope ($\times 40$). Only colonies containing more than 50 cells were counted. The average number of cells in a colony was calculated by counting the number of cells in a hemocytometer after the removal of 50 colonies from the culture with a fine pipette and resuspension of the cells in 0.5 ml of saline.

For morphologic examination, colonies were removed from the upper layer with a pipette and the cells resuspended in ice-cold saline before spinning in a cytocentrifuge. The cell pellet was stained by May-Grünwald-Giemsa and for peroxidase.⁸

RESULTS

When blood cells from the umbilical cord were cultured, colonies began to appear at day 4-5. The number and size of the colonies gradually increased until day 14-17, the average number of cells per colony being at that time

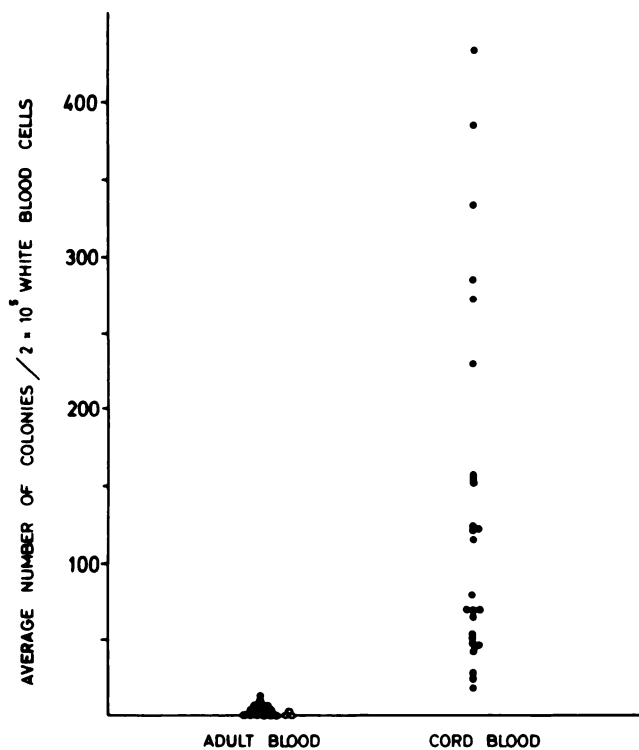


Fig. 1. Colony formation by cord blood cells and blood cells from adults including three mothers (open circles).

1000-1500. Thereafter the colonies began to degenerate and gradually disappeared after 4 wk of incubation. The colonies in a culture varied in size, but most of them were large and had a compact structure. The colonies formed when the peripheral blood from adults was cultured had the same size and structure.

The number of colonies formed from the cord blood and from peripheral blood of adults is shown in Fig. 1. When peripheral blood cells from adults were plated, 2×10^5 nucleated cells per plate, the number of colonies formed varied between 0 and 11, with a mean of 3. The number of colony-forming cells in the maternal blood was within this range. A considerably greater number of colonies was formed when cord blood cells were plated, the number varied between 17 and 385 with a mean of 122 per 2×10^5 nucleated cells plated. The concentration of nucleated red cells in cord blood varied between 0% and 26%. When corrected for this, 19-432 colonies were formed with a mean of 137 per 2×10^5 granulocytic and lymphocytic cells plated. The average number of colonies per ml of cord blood was 9200 (range: 1600-34,200). Adult blood contained an average number of 90 colonies/ml (range: 0-220). No correlation was found between birth weight and the number of colonies per milliliter.

In two experiments, half of the cells were washed three times in medium before plating. This did not change the colony number, thus excluding the possibility that admixture of cord plasma in the experiments might have any influence upon the number of colonies formed.

Morphologically, most of the cells in the colonies belonged to the granulocytic line, and, except for the macrophages, all cells were peroxidase-positive. At day 14 both kinds of cultures contained mostly neutrophilic myelocytes, metamyelocytes, and bands.

DISCUSSION

The finding reported here of an increased concentration of hemopoietic progenitor cells in human umbilical cord blood is well in accordance with the results from animal experiments. Peripheral blood in mice contains cells capable of reconstituting the hemopoietic tissue and thereby prolonging the survival after lethal irradiation.⁹ When peripheral blood cells from adult mice were used as donor cells, more than 10^7 cells were needed to restore the irradiated mouse, while 10^4 - 10^5 cells from the blood of fetal or neonatal mice were sufficient for the restoration.²

Evidence of circulating stem cells in man has been obtained through transfusion studies with peripheral leukocytes containing the Philadelphia chromosome. When these cells were given intravenously to patients with acute leukemia, evidence of temporary bone marrow grafts was obtained.¹⁰ When peripheral leukocytes from normal donors in a total dose of 10^{11} leukocytes were transfused into HL-A identical patients receiving chemotherapy, early marrow recovery was induced, suggesting that hemopoietic stem cells are circulating also in normal peripheral blood.¹¹

The origin and the function of the circulating stem cells during early embryogenesis in mice has been investigated by Moore and Metcalf.¹² Their work suggests that the yolk sac is the primary site of *in vivo* and *in vitro* colony-forming

cells and that these cells migrated through the blood and initiate hemopoiesis in the liver. The possible function of circulating in vitro colony-forming cells in human fetal blood is unknown. These cells might be of importance for the expansion of the hemopoietic tissue volume during the latter part of intrauterine life and after birth¹³ or they might merely represent an escape from the bone marrow into the circulation.

Cell separation studies in monkeys have indicated that the in vitro colony-forming cell possibly belongs to the group of cells named transitional lymphocytes.¹⁴ The same group of cells is considered to contain the multipotential stem cells.^{15,16} The concentration of transitional lymphocytes is known to be increased in human cord blood, and during midfetal life almost all the circulating lymphocytes are of the transitional type.¹⁷ This suggests that further studies with midfetal blood and the agar culture technique might be of value for the identification of the human colony-forming cell.

Whether the in vitro colony-forming cell in human bone marrow or in peripheral blood is the multipotential stem cell or a committed granulocytic precursor is yet to be decided, but the finding of an increased concentration of colony-forming cells in human cord blood comparable in number with human bone marrow cultures indicates that cord blood might be used as a source of hemopoietic stem cells for the restoration of bone marrow function in humans.

ACKNOWLEDGMENT

The author is indebted to Miss I. Klose for her excellent technical assistance and to Professor Mogens Osler and the personnel at the Department of Obstetrics YB, Rigshospitalet, Copenhagen, for supplying the cord blood samples.

REFERENCES

1. Barker JE: Embryonic mouse peripheral blood colony-forming units. *Nature (Lond)* 228:1305, 1970
2. Barnes DWH, Ford CE, Loutit JF: Haemopoietic stem-cells. *Lancet* 1:1395, 1964
3. Robinson WA, Pike BL: Colony growth of human bone marrow cells in vitro, in Stohlman F Jr (ed): *Hemopoietic Cellular Proliferation*. New York, Grune & Stratton, 1970, p 249
4. Bennett M, Cudkowicz G, Foster RS, Metcalf D: Hemopoietic progenitor cells of W anemic mice studied in vivo and in vitro. *J Cell Physiol* 71:211, 1968
5. Dicke KA, Platenburg MGC, Van Bekkum DW: Colony formation in agar: In vitro assay for haemopoietic stem cells. *Cell Tissue Kinet* 4:463, 1971
6. Chervenick PA, Boggs DR: In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. *Blood* 37:131, 1971
7. Kurnick JE, Robinson WA: Colony growth of human peripheral white blood cells in vitro. *Blood* 37:136, 1971
8. Sato A, Sekiya N: A simple method for differentiation of myeloid and lymphatic leukocytes in human blood. *Tohoku J Exp Med* 7:111, 1926
9. Goodman JW, Hodgson GS: Evidence for stem cells in the peripheral blood of mice. *Blood* 19:702, 1962
10. Levin RH, Whang J, Tjio JH, Carbone PP, Frei E, Freireich EJ: Persistent mitosis of transfused homologous leukocytes in children receiving antileukemic therapy. *Science* 142:1305, 1963
11. McCredie KB, Freireich EJ, Hersh EM, Curtis JE, Kaizer H: Early bone marrow recovery after chemotherapy following the transfusion of peripheral blood leukocytes in identical twins. *Proc Am Cancer Res* 11:54, 1970
12. Moore MAS, Metcalf D: Ontogeny of the haemopoietic system: Yolk sac origin of in vivo and in vitro colony forming cells in

the developing mouse embryo. *Br J Haematol* 18:279, 1970

13. Kalpaktsoglou PK, Emery JL: The effect of birth on the haemopoietic tissue of the human bone marrow. *Br J Haematol* 11:453, 1965

14. Moore MAS, Williams N, Metcalf D: Characterisation and purification of the in vitro CFC in monkey haemopoietic tissue, in Van Bekkum DW, Dicke KA (eds): *In Vitro Cul-*

ture of Hemopoietic Cells. Radiobiol Inst Rijswijk 1972, p 213

15. Murphy MJ, Bertles JF, Gordon AS: Identifying characteristics of the haemopoietic precursor cell. *J Cell Sci* 9:23, 1971

16. Yoffey JM: Experimental approaches to the stem cell problem in postnatal life. *Isr J Med Sci* 7:927, 1971

17. Winter GCB, Byles AB, Yoffey JM: Blood-lymphocytes in newborn and adult. *Lancet* 2:932, 1965