THE ACTIVITY OF α1,6-FUCOSYLTRANSFERASE DURING HUMAN MEGAKARYOCYTIC DIFFERENTIATION

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Abstract: α1,6-Fucosyltransferase (6FucT, E.C. 2.4.1.68) is one of the enzymes involved in the synthesis of N-linked glycans of the GpIIb/IIIa complex (CD41a) which is present on megakaryocytes (MKS) and platelets. In this study, we examined 6FucT activity in ex vivo cultures of immunoselected cord blood CD34⁺ cells grown in a medium promoting megakaryocytopoiesis. Our results show that the activity of 6FucT increased ahead of, and thereafter concomitantly with, cells expressing the CD41a antigen. When the CD41a⁺ subpopulation of cells was immunoselected (using anti-CD61 i.e. anti-GpIIIa antibodies), its 6FucT activity increased proportionally to the yield of CD61⁺ cells. Taking into account the heavy load of 6FucT in platelets and megakaryocytes, we regard this enzyme as a candidate for the earliest marker of MK-commitment in cultured hematopoietic stem cells. Such a marker should allow an earlier detection and earlier transplantation of patients’ own, ex vivo expanded, Mk progenitors.

Key Words: α1,6-fucosyltransferase, Glycoprotein IIb/IIIa, Megakaryocytes, CD34⁺ Cells

INTRODUCTION

Thrombocytopenia remains a significant clinical problem in cancer and hematological patients undergoing high-dose chemotherapy or radiotherapy [1]. Currently, allogenic platelet transfusion is a primary treatment for severe thrombocytopenia. The treatment is associated with a significant risk of
disorders, such as alloimmunization, transfusion reactions and transmission of blood-borne diseases [2]. Hence, there is an ongoing search for an alternative therapeutic strategy for thrombocytopenia. One of the latest approaches to the treatment of thrombocytopenia is the infusion of megakaryocyte (Mk) progenitors expanded in vitro from the patient’s own stem cells, thus eliminating all the aforementioned potential disorders [3]. Megakaryocytopoiesis is a complex multistep process involving cell division, endoreduplication and maturation, resulting in the release of platelets into the blood circulation. Therefore, any method for the expansion of Mk progenitors ex vivo requires the identification of their lineage-restricted proteins and glycoproteins. The earliest marker of megakaryocytopoiesis, appearing as early as at the CFU-Mk stage, is the GpIIb/IIIa (CD41a) complex [4]. In platelets, this complex functions as a receptor for fibrinogen, von Willebrand factor, fibronectin and vitronectin. Other lineage-restricted proteins, like Platelet Factor 4, β-tromboglobulin and the GpIb-V-IX complex, appear at later stages of Mk progenitor differentiation [5]. In this study, we tested 6FucT as a potential early marker of the Mk commitment of cells in culture to see if it would prove better than GpIIb/IIIa. 6FucT, an enzyme of glycoprotein synthesis, conveys fucose residues in an α1,6 linkage to the innermost N-acetylglucosamine residues of N-linked glycans. Glycans with this type of structure are present in both components of GpIIb/GpIIIa. The enzyme is heavily concentrated in the blood platelets; their 6FucT activity is over four times higher than that of much larger neutrophils [6]. In lymphocytes and erythrocytes, the enzyme is barely detectable. Megakaryocytes harvested from the bone marrow exhibit a very high 6FucT activity that is proportional to their platelet progenitor status (J. Koscielak, M. Ratajczak, unpublished). Platelets release 6FucT during blood coagulation [7], activation with agonists that make them change shape and aggregate [8], as well as after treatment with neutrophil proteases [9]. The biological significance of 6FucT secretion by platelets is unknown. Since 6FucT is a biosynthetic enzyme engaged in GpIIb/IIIa biosynthesis, it should appear in Mk-committed cells before GpIIb/IIIa is expressed. Thus, the determination of 6FucT activity in cultured cells should permit the earlier detection and transplantation of Mk progenitors. Unfortunately, the antibody against 6FucT is not yet available, and we had to rely on the enzymatic method for the determination of 6FucT activity.

MATERIALS AND METHODS

Isolation of CD34+ cells
CD34+ cells were isolated from cord blood nonadherent mononuclear cells via immunomagnetic bead selection using a Direct CD34 Progenitor Cell isolation Kit (Miltenyi Biotec GmbH) according to the manufacturer’s instructions. The purity of the enriched cell fraction was checked by flow cytometry after staining the cells with the anti-CD34-PE antibody.
Cell culture
The culture of CD34+ cells was performed according to the method described by Ratajczak et al. [10] with some modifications. The cells were cultured in IMDM medium at a concentration of 5x10^4/ml in 24-well plates. The culture medium was supplemented with 80 ng/ml thrombopoietin (TPO), 20 ng/ml IL1-β, 10 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml stem cell factor (SCF) and 40 ng/ml Flt-3 ligand (Flt-3). The cells were incubated for 14 days at 37°C in a fully humidified atmosphere containing 5% CO₂. After 3 days in culture, the cells were seeded in a fresh medium with all the cytokines except IL-3. It is well known that IL-3 diminishes the expression of GpIIb/IIIa on maturating Mks and at the same time maintains the proliferation of Mk progenitors, resulting in a higher-fold expansion of cell growth [11].

Phenotype analysis
The growth of megakaryocytic cells in liquid culture was monitored using a two-color flow cytometric technique (FACS). The cells were stained with different combinations of MoAbs FITC-anti-CD41a, PE-anti-CD42b, FITC-anti-CD61, PE-anti-CD14 and PE-anti-CD34. FITC-anti-IgG1, PE-anti-IgG1 and PE-anti-IgG2a were used as nonspecific binding controls. The cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

Immunoselection of CD61+ (GpIIIα+) cells
CD61+ cells were isolated from cultured cells by immunomagnetic bead selection using CD61 MicroBeads (Miltenyi Biotec GmbH) according to the manufacturer’s instructions. The purity of the enriched cell fraction was checked using flow cytometry after staining the cells with anti-CD61-PE antibody.

Determination of the activity of 6FucT
Cultured cells were harvested and solubilized. The determination of 6FucT activity in the cells was based on a count of the radioactivity of [14C]fucose transferred from GDP[14C]fucose to the acceptor, asialo-agalactotransferrin glycopeptide, which had been prepared by us from human apotransferrin [12].

Determination of the activity of β-4-galactosyltransferase 1 (GalT1, E.C. 2.4.1.38/90)
Cultured cells were harvested and solubilized. The activity of GalT1 was determined employing free N-acetylgalcosamine as substrate [13].

RESULTS
Cord blood CD34+ cells were purified via the immunomagnetic technique with a purity of 86.9% to 97.3% (mean 92.6% ±2.7%). The analysis of cells initiating the culture revealed that most of them were CD41a and CD42b negative (Fig. 1A). The culturing of cells in a medium supplemented with cytokines and
growth factors with thrombopoietic activity resulted in an increased expression of CD41a (Fig. 1B) and CD42b (α chain of GpIb protein) on the cell surface. At the end of the culture time the majority of cells were CD34 negative (Fig. 1C). The CD41a+ fraction represented from 49% to 63% of the expanded cells. Dot plot analysis indicated that on day 14, most of the CD41a+ cells were also CD42b positive.

Fig. 1. Flow cytometric analysis of in vitro expanded CD34+ cells in a medium promoting the expansion of Mk progenitors. (A) The expression of CD34 on expanded cells. (B) The expression of CD41a on expanded cells. The isotype control (___) is shown. (C) The expression of CD41a and CD42b on expanded cells. The numbers indicated in the top corners represent the % of cells in culture. Quadrants were set to exclude 99% of cells incubated with an irrelevant isotype-matched control antibody labeled with the corresponding fluorochrome. 10,000 events were acquired for each analysis. The results of one representative experiment of five are shown.
In order to avoid false positive results in the Mk cell phenotype analysis, all the steps were performed in the presence of 5mM EDTA for detaching platelets. The activity of 6FucT in the cultured cells was assessed on a daily basis. The activity of the enzyme in the cultured cells was found to increase along with the increased expansion of CD41α^+ cells (Fig. 2). The increase in 6FucT activity was highest between days 7 and 11 and preceded the increase in CD41α^+ cell percentage, which was most significant between days 11 and 13. The activity of GaT on day 0 and 14 was 119±12 fmol/h/10^3 cells and 261±9 fmol/h/10^3 cells respectively. These results show that during the 14 days of culture the activity of GaT increased 2.2 fold, while the activity of 6FucT increased 8 fold.

Fig. 2. The activity of 6 FucT in the cells correlated with the percentage of CD41α^+ cells during the in vitro culture of Mk progenitors. The data shown is the representative results of one of three experiments.

In order to demonstrate that the activity of 6FucT is specific for expanding megakaryocytes, the CD61^+ cells from the culture were immunoselected. The enriched, 90.3% of the cells in the CD61^+ fraction were CD61 positive (Fig. 3B), i.e. this fraction contained 1.9-fold more CD61^+ cells than the starting population of the cultured cells. Accordingly, the activity of 6FucT in the immunoselected fraction was 2.2-fold higher than in the total cultured cells. A monocyte marker (CD14) was used to check if dual-immunofluorescence could enhance the definition of Mk cells. It was shown that CD61^+ cells were CD14 negative (Fig. 3C). The May-Grünwald Giemsa staining of CD61^+ cells revealed the presence of large cells with multilobed nuclei characteristic for mature Mks (data not shown).
DISCUSSION

This is the first report showing the correlation of 6FucT activity with the expression of the platelet-specific antigen CD41a, during ex vivo Mks differentiation and maturation. The activity of 6FucT was lowest in the cells initiating the culture. Most of these cells were CD34+ and CD41a-CD42b-. The maximum activity of the enzyme was observed at the end of the culture when the majority of cells were CD34- and more than half of them were CD41a-CD42b-. Interestingly, the increase in 6FucT activity in Mks progenitors preceded that of CD41a expression, as predicted. Moreover, our results showed that the activity of 6FucT in a single, immunoselected CD41a- cell at the end of the culture was in quantitative terms comparable to that found in about 600 platelets (1.3 fmol/h/1000 platelets). Assuming that the cells analyzed are not fully mature, this result is in rough accord with the estimated number of 1000 to 3000 platelets that are produced on average from a single mature Mk [14]. It should be emphasized that the aforementioned 6FucT activity in 1000 platelets only applies to cells harvested from subjects with normal platelet counts. Under conditions of thrombocytopenia, the activity of 6FucT in circulating platelets is much increased [6]. We have also found that the increase in the activity of 6FucT in cultured cord blood CD34+ cells was almost 4-fold higher than that of the ubiquitous GaIT1, confirming the special status of 6FucT in megakaryocytopoiesis. This is so because, unlike 6FucT, GaIT1 is present in most cells of the body, and its product, the galactosylβ1,4N-acetylg glucosaminyl moiety, occurs in N-glycans, O-glycans and glycolipids.

In conclusion, the activity of 6FucT should be a useful marker of the early commitment of cultured hematopoietic stem cells into the megakaryocytic lineage. Our findings will allow the shortening of the expansion time and the
earlier transplantation of megakaryocyte progenitors to the patient, on the assumption that the transplanted cells would further mature in the recipient’s body.


REFERENCES


