Effects of inhibitor of Src kinases, SU6656, on differentiation of megakaryocytic progenitors and activity of α1,6-fucosyltransferase

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Received: 18 March, 2008; revised: 06 September, 2008; accepted: 11 September, 2008
available on-line: 15 September, 2008

α1,6-Fucosyltransferase (FUT8) attaches fucose residues via an α1,6 linkage to the innermost N-acetylglucosamine residue of N-linked glycans. Glycans with this type of structure are present in GpIIb/GpIIa complex (CD41a) which is present on megakaryocytes (Mks) and platelets. CD41a is the earliest marker of megakaryocytopoiesis. The aim of this study was to analyse the morphology, phenotype, ploidy level and activity of FUT8 during induced differentiation/maturation of Mk progenitor cells in ex vivo culture. We used SU6656, a selective inhibitor of Src tyrosine kinases, as differentiation-inducing agent for Mks. The addition of SU6656 to the culture system of megakaryocytic progenitors from cord blood CD34+ cells and Meg-01 cell line induced their maturation towards later stages of Mk differentiation with increased activity of FUT8. We suggest FUT8 as a candidate for an early marker of differentiation and possibly of the ploidy level of Mks. We confirm a special status of FUT8 in megakaryocytopoiesis.

Keywords: CD34+ cells, megakaryocytes, Meg-01cell line, SU6656, FUT8

INTRODUCTION

Megakaryocytopoiesis is a complex multistep process involving stem cell commitment, mitotic amplification of committed progenitors, nuclear polyploidization, and cytoplasmic maturation leading to the production of platelets. Thrombopoietin (TPO or c-Mpl ligand) and a variety of pleiotropic hematopoietic growth factors synergistically promote the growth and maturation of megakaryocytes (Mks) (Guerriero et al., 1995). The ex vivo expansion of Mk progenitors may be the identification of their lineage-restricted proteins and glycoproteins. The earliest marker of megakaryocytopoiesis, appearing as early as the CFU-Mk stage, is the GpIIb/IIIa complex (CD41a) (Long, 1998). In platelets, this complex functions as a receptor for fibrinogen, von Willebrand factor, fibronectin and vimentin. The enzyme α1,6-fucosyltransferase (FUT8) is involved in GpIIb/ IIIa synthesis (Tsuji & Osawa, 1986). FUT8 catalyses the transfer of fucose residue from the donor substrate, guanosine 5’-diphosphate (GDP)-β-L-fucose, to GlcNAc of the core structure of asparagine-linked oligosaccharide via an α1,6-linkage. FUT8 is a typical type II membrane protein and a Golgi apparatus-resident glycosyltransferase. In human serum the activity of FUT8 is derived in about 95% from blood platelets (Koscielak et al., 1987b). Platelets release the enzyme during blood coagulation, activation with agonists that make them change shape and secrete components of vesicles (Antoniewicz et al., 1989). The serum activity of FUT8 is positively correlated with blood platelet concentration (Koscielak et al., 1987a). The question about the role of FUT8 in platelets remains open, but it is known that other glycosyltransferases have been found on the cell
surface. The best characterized sperm receptor for ZP3 (zona pellucida glycoprotein) is β1,4-galactosyltransferase (GalT), which functions in a lectin-like capacity (Nixon et al., 2001). α1,3-Fucosyltransferase (rFucT-IV) activity has been detected on the surface of mouse germ cells and rat Sertolli cells, and has been postulated to play a role in cell–cell interactions (Aucoin et al., 1998). FUT8 is localised in platelets not only in the membrane of the Golgi apparatus but also, probably in a soluble form, in α-granules. It was found that about 50% of the enzyme is contained in α-granules (Kaminska et al., 2001). Our earlier results let us form a hypothesis, that platelets with a high FUT8 activity are derived from high-ploidy megakaryocytes. Kościelak et al. (1995) suggested that platelet FUT8 may be a marker of the ploidy level of Mks since the enzyme activity is highly increased in platelets in thrombocytopenia. Mks expand not through mitosis but by endomitosis. It is highly likely, that under these conditions glycosyltransferases may accumulate in the cytoplasm. The highest FUT8 activity in thrombocytopenic platelets suggests that the biosynthesis of glycoproteins might be accelerated after each endomitotic division. Previously we reported that the activity of FUT8 could be a useful marker of the transition of cultured hematopoietic stem cells into the megakaryocytic lineage (Bany-Laszewicz et al., 2004). Taking into account the important role of FUT8 in megakaryocytogenesis, the aim of the study was to analyse the activity of FUT8 during differentiation and maturation of Mk progenitor cells induced by SU6656, the inhibitor of Src kinases. Src-family protein tyrosine kinases are proto-oncogenes that play key roles in cell morphology, motility, proliferation, and survival (Roskoski, 2004; Ingley, 2008). Six of the eight Src family kinases are present in primary Mks. Fyn and Lyn are expressed at the highest levels and these two kinases are both activated following thrombopoietin (TPO) stimulation of primary murine Mks. These data provide evidence for specificity of TPO signalling and suggest a probable role for Fyn and Lyn during megakaryocytogenesis (Lannutti et al., 2003). Src kinases inhibit differentiation of Mks (Lannutti & Drachman, 2004). The effect of SU6656 on TPO-induced growth and differentiation was reported by Lannutti et al. (2005). Remarkably, when SU6656 was added to a megakaryocytic cell line, UT-7/TPO, the cells ceased cell division but continued to accumulate DNA by endomitosis. During this interval, CD41 and CD61 expression on the cell surface increased. Similar effects on polyploidization and Mks differentiation were seen with expanded primary Mks, bone marrow from patients with myelodysplastic syndrome, and other cell lines, K562 and HEL, with Mk potential. The results suggest that SU6656 might be useful as a differentiation-inducing agent for Mks and is an important tool for understanding the molecular basis of Mk endomitosis (Lannutti et al., 2005).

In the present study we analysed the morphology, phenotype, ploidy, and activity of FUT8 during maturation of Mk progenitor cells induced by SU6656. Cord blood CD34+ cells cultured ex vivo in medium promoting megakaryocytogenesis, and megakaryocytic cell line Meg-01, were used in the experiments. These results have already been published in a preliminary form in conference proceedings (Kaminska et al., 2007).

**MATERIALS AND METHODS**

**Cells and cell culture.** Primary human CD34+ cells were isolated from umbilical cord blood non-adherent mononuclear cells via immunomagnetic bead selection using Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec GmbH) according to the manufacturer’s instructions. The CD34+ cells were cultured in serum-free StemSpan SFEM medium (StemCell Technologies Inc.) at a concentration of 5 × 10^5/mL in 24-well plates. The culture medium was supplemented with 50 ng/mL thrombopoietin (TPO; EuroClone), 10 ng/mL IL-3 (EuroClone), 10 ng/mL SCF (EuroClone). After 7 days in culture cytokines IL-3 and SCF were removed and the cells were suspended in StemSpan medium containing 30 ng/mL TPO and 0.1% dimethyl sulfoxide (DMSO), or 30 ng/mL TPO and 2.5 µM SU6656 (CalBiochem) in 0.1% DMSO.

The Meg-01 cell line (Ogura et al., 1985) derived from a Ph+ chronic myelogenous leukaemia (CML) patient in blast crisis was purchased from the American Type Culture Collection (ATCC). Meg-01 cells were cultured at 5 × 10^5/mL in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco) and antibiotic antimycotic solution (penicillin G, streptomycin sulphate, amphotericin B; Sigma).

**Determination of the activity of α1,6-fucosyltransferase (FUT8, EC 2.4.1.68).** Cultured cells were harvested and solubilized in buffer with 1% Triton CF54 (Sigma). The determination of FUT8 activity in the lysate was based on the radioactivity of ^14C]fucose transferred from GDP[^14C]fucose (Amersham) to the acceptor, asialo-agalactotransferase glycopeptide, which had been prepared by us from human apotransferrin (Sigma) (Kaminska et al., 1998).

**Determination of the activity of β1,4-galactosyltransferase (GalT).** Cultured cells were harvested and solubilized as above. The activity of 4GalT was determined employing free N-acetylglucosamine as substrate (Kaminska et al., 1999).

**Flow cytometric analysis. Phenotype analysis.** Cells in liquid culture were monitored using a
two-colour flow cytometric technique (FACS). The cells were stained with different combinations of moAbs PE-anti-CD34, FITC-anti-CD41a (Gpllb/IIIa), PE-anti-CD42b (α chain of GpIIb protein), PE-anti-CD184 (CXCR4, receptor for SDF1) (Becton Dickinson), FITC-anti-IgG1 and PE-anti-IgG1 (Becton Dickinson) were used as non-specific binding control. The cells were analysed on a Becton Dickinon FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

**Ploidy analysis.** The cell ploidy assessment by measurement of DNA content was performed after previous labelling of the cells with FITC-anti-CD41a antibodies and DAPI (Molecular Probes) using a Becton Dickinson flow cytometer (Vantage).

**RESULTS**

Human umbilical cord blood CD34+ cells were purified via the immunomagnetic method (mean purity 92.6%±2.7%) and expanded in StemSpan SFEM medium supplemented with cytokines promoting Mk development. The activity of enzymes, FUT8 and GalT, involved in the synthesis of GpIIb/IIIa complex (CD41a) in cultured CD34+ cells was determined. A very high correlation of the FUT8 activity (correlation coefficient = 0.78) and a weak correlation of GalT activity (correlation coefficient = 0.31) in Mks with the percentage of cells expressing the surface antigen CD41a during megakaryocytopoiesis was observed (Fig. 1). The selective inhibitor of Src family protein tyrosine kinase, SU6656, was used for investigation of the activity of FUT8 in megakaryocytopoiesis. After 7 days of CD34+ cells culture IL-3 and SCF were removed and only TPO together with SU6656 were added to the medium. SU6656 diminished the growth rate of cells in culture and simultaneously it induced Mks differentiation, as assessed by cell morphology and expression of specific differentiation markers. An increase of the cell size, multiplication of nuclei and higher number of granules in the cytoplasm, characteristic for mature Mks, were observed (Fig. 2A). Mk cells treated with SU6656 for 4 days had diameters of about 15 µm to 30 µm. The control cells had diameters of about 12 µm to 20 µm. After exposure of Mks to the inhibitor the percentage of cells expressing the surface antigens, CD41a, CD42b and CD184 increased. At the same time the percentage of CD34+ cells decreased (Fig. 2B). The activity of FUT8, an enzyme involved in CD41a synthesis, was enhanced in the Mk cells after exposure to SU6656 up to 411 ± 39 fmol/h per 10^5 cells on day 2 (control 299 ± 23 fmol/h per 10^5 cells) and up to 439 ± 34 fmol/h per 10^5 cells on day 4 (control 375 ± 31 fmol/h per 10^5 cells) (Fig. 3; P<0.05). A very high correlation of the FUT8 activity (correlation coefficient = 0.93) in Mks with the percentage of cells expressing the surface antigen CD41a during megakaryocytopoiesis was observed in cells cultured in the presence of SU6656. The inhibitor also induced polyploidization of Mk progenitors. After 4 days of exposure to the inhibitor we observed a higher percentage of CD41a+ cells with higher ploidy level: 8N (5.9-fold increase) and 16N (10-fold increase) (Fig. 2C). A substantial level of sub-G1 cells is visible in Fig. 2C-B. The high level of fragmented DNA indicates late phases of apoptosis in the cells cultured in the presence of SU6656.

The Meg-01 cells, megakaryoblasts in a relatively early stage, were analysed in culture after exposure to SU6656. The inhibitor diminished the growth rate of Meg-01 cells (Fig. 4) and simultaneously induced differentiation and maturation of megakaryocytic cells. Meg-01 cells grown in the presence of SU6656 showed changes of the morphology, phenotype, and ploidy (Fig. 5), and the activity of FUT8 (Fig. 6), when compared with cells grown without the inhibitor. The enlargement of the cells, multiplication of nuclei and higher number of granules in the cytoplasm, characteristics of mature Mks,
Figure 2. Analysis of morphology (A), surface phenotype (B) and ploidy level (C) of in vitro expanded CD34+ cells in presence of 2.5 µM SU6656 at day 4. Morphology of the expanded cells was analysed using inverted microscopy. Photographs show representative fields of preparations at x40 magnification. Expression of surface antigens: CD34 (a, b), CD41a (c, d), CD42b (e, f), and CD184 (g, h) was analysed by FACS. Ploidy of control Mk cells in the presence of 0.1% DMSO (a) and Mk cells treated with 2.5 µM SU6656 (b). Histograms of DNA content of cells double-labelled with FITC-anti-CD41a antibodies and with DAPI. Data show percentage ploidy distribution after gating CD41a+ cells. More details described under Materials and Methods. Representative experiments of three independent ones are presented.

Figure 3. Activity of FUT8 in primary Mk progenitor cells in presence of 2.5 µM SU6656. Cultured Mk cells were solubilized and activity of the enzyme was determined by isotopic method (as described under Materials and Methods). Data are means ±S.D. of four independent experiments. * P<0.05; P=0.00163 for results at 2nd day and P=0.03300 for results at 4th day of culture.
Effects of SU6656 on differentiation of megakaryocytic progenitors were observed after exposure to SU6656. The Meg-01 cells treated with the SU6656 for 2 and 4 days had a diameter of 12–35 µm and 12–46 µm, respectively. The control cells had diameters from 12 to 24 µm (Fig. 5A). The presence of SU6656 induced not only the higher percentage of CD41a⁺ cells (Fig. 5B), but simultaneously increased the activity of FUT8, involved in CD41a synthesis (Fig. 6). After 3, 4, and 6 days of exposure to the inhibitor the activity of FUT8 in Meg-01 cells, compared with the control (about 100 fmol/h per 10⁵ cells), increased 1.7-fold (to 170±21 fmol/h per 10⁵ cells), 2.4-fold (to 242±20 fmol/h per 10⁵ cells), and 3.3-fold (to 328±33 fmol/h per 10⁵ cells), respectively (Fig. 6). SU6656 also enhanced polyploidization of Meg-01 cells (Fig. 5C). After 4 days of exposure to the inhibitor we observed more CD41a⁺ cells with a higher ploidy level: 8N (1.7-fold increase), 16N (1.8-fold increase), and 32N (25-fold increase).

**DISCUSSION**

α-1,6-Fucosylation of N-glycan and FUT8 expression have important role in various biological events of physiological and pathological conditions. FUT8-deficient mice exhibit growth retardation, earlier postnatal death, and emphysema-like phenotype (Li et al., 2006). In a previous report we confirmed the special status of FUT8 during megakaryocytogenesis (Bany-Laszewicz et al., 2004). FUT8 is one
of the enzymes involved in the synthesis of the earliest marker of megakaryocytogenesis, the GpIIb/IIIa complex (CD41a). The activity of FUT8 in cultured cells increased with a simultaneously increased expression of the CD41a antigen. We postulated that the activity of FUT8 could be a useful marker of the early commitment of cultured hematopoietic stem cells to the megakaryocytic lineage. It was also found that the increase in the activity of FUT8 in cultured cord blood CD34⁺ cells was almost 4-fold higher, than that of the ubiquitous GalT (an enzyme also involved in GpIIb/IIIa synthesis), additionally confirming the special status of FUT8 in megakaryocytogenesis (Bany-Laszewicz et al., 2004). Taking into account the important role of FUT8 in megakaryocytogenesis the aim of the study was to analyse the activity of FUT8 during maturation of Mk progenitor cells induced by an Src kinases inhibitor, SU6656. The activity of FUT8 in cultured cord blood CD34⁺ cells was almost 4-fold higher than that of the ubiquitous GalT (an enzyme also involved in GpIIb/IIIa synthesis), additionally confirming the special status of FUT8 in megakaryocytogenesis (Bany-Laszewicz et al., 2004).

Cultured Meg-01 cells were solubilized and activity of the enzyme was determined by isotopic method as described under Materials and Methods. Data are means ±S.D. of three independent experiments.

Figure 6. Fold increase of FUT8 activity in Meg-01 cells in presence of 2.5 µM SU6656.

The human megakaryoblastic cell line Meg-01 may provide a useful model for the study of human megakaryocytogenesis. The activity of FUT8 also increased in Meg-01 cells after exposure to SU6656. The activity of FUT8 in Meg-01 is 4-fold higher than in the lymphoblastic cell line K562 (morphology — lymphoblast; CML Ph⁺; FUT8 = 25.7 ± 2.8 fmol/h per 10⁵ cells) or Jurkat cells (morphology — lymphoblast; lymphoma; FUT8 = 27.2 ± 3.0 fmol/h per 10⁵ cells), which confirms the special status of the enzyme in Meg-01 (not shown). Meg-01 cells are megakaryoblasts in a relatively early stage (Ogura et al., 1985). The
presence of SU6656 in culture medium clearly induced differentiation and maturation of Meg-01 cells. After exposure to the inhibitor we observed an arrest of proliferation (Fig. 4), enlargement of the cells, higher percentage of cells expressing the surface antigen CD41a (Fig. 5), and more CD41a+ cells with higher ploidy (8N, 16N and 32N) (Fig. 5C). The enhanced activity of FUT8 (3.3-fold at day 6) correlated with the differentiation of Meg-01 cells (Fig. 6). The anti-proliferative action of SU6656 on Meg-01 cells were very similar to those observed previously for STI-571 in Ph+ CML cells (Druker et al., 1996; Jakubowska et al., 2007) and with other Src kinases inhibitors in various cell lines with Mk potential. Similar effects of SU6656 on polyploidization and Mk differentiation were seen with cultured UT-7/TPO and the cell lines K562 and HEL with Mk potential (Lannutti et al., 2005). Treatment of the Ph+ CML cell lines, K-562 and Meg-01 with the pyrrolo-pyrimidine Src kinase inhibitors PP2 and A-419259 resulted in growth arrest and induction of apoptosis (Wilson et al., 2002). Using the inhibitors PP1 and PP2, it was shown that TPO-dependent proliferation of BaF3/MPL cells was enhanced (Lannutti & Drachman, 2004). SU6656 selectively inhibits Src family kinases, while the pyrrolo-pyrimidine Src kinases inhibitors, PP1 and PP2, are also potent inhibitors of the PDGF receptor (Blake et al., 2000). The inhibitors of Src kinases are a useful tool in the investigation of processes controlled by these enzymes, but also may play a therapeutic role. Disatinib is a novel, oral, multitargeted kinase inhibitor of Bcr-Abl and Src family kinases which induces complex hematologic and cytogenetic responses in patients with chronic myelogenous leukaemia (CML) in blast crisis (Cortes et al., 2007).

In our study we observed a positive correlation between the activity of FUT8 and the ploidy of Mks. Such results may confirm the hypothesis of Koscielak et al. (1995), presented in the Introduction, that FUT8 may be a marker of the ploidy level of Mks. The polyploidization is part of terminal Mk differentiation. Recently, Raslova et al. (2007) have demonstrated that Mk polyploidization leads to the coordinated expression of genes involved in the arrest of DNA replication and in platelet functions and production. Genes of the GpIIb/GpIIIa glycoprotein complex were up-regulated in polyploid Mks.

CONCLUSIONS

The addition of SU6656 to the culture system of megakaryocytic progenitors from cord blood CD34+ cells and megakaryocytic Meg-01 cell line induced their maturation towards later stages of Mk differentiation with increased activity of FUT8. The inhibitor diminished the growth rate of cells and induced changes in morphology, phenotype and ploidy level of Mk progenitor cells. We suggest FUT8 as a candidate for an early marker of differentiation and possibly of the ploidy level of Mks. In this report we confirm the special status of FUT8 in megakaryopoiesis.

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