Identification of the regulatory inputs that direct megakaryocytopoiesis and platelet production is essential for the development of novel therapeutic strategies for the treatment of thrombosis and related hematologic disorders. We have previously shown that primary human megakaryocytes express the N-methyl-D-aspartate acid (NMDA) receptor 1 (NR1) subunit of NMDA-type glutamate receptors, which appear to be pharmacologically similar to those identified at neuronal synapses, responsible for mediating excitatory neurotransmission in the central nervous system. However, the functional role of NMDA receptor signaling in megakaryocytopoiesis remains unclear. Here we provide evidence that demonstrates the fundamental importance of this signaling pathway during human megakaryocyte maturation in vitro. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of RNA extracted from CD34⁺-derived megakaryocytes identified expression of NR2A and NR2D receptor subunits in these cells, as well as the NMDA receptor accessory proteins, Yotiao and postsynaptic density protein 95 (PSD-95). In functional studies, addition of a selective NMDA receptor antagonist, MK-801 inhibited proplatelet formation, without affecting proliferation or apoptosis. Exposure of CD34⁺ cells to MK-801 cultured for 14 days in the presence of thrombopoietin induced a decrease in expression of the megakaryocyte cell surface markers CD61, CD41a, and CD42a compared with controls. At an ultrastructural level, MK-801-treated cells lacked α-granules, demarcated membranes, and multilobed nuclei, which were prominent in untreated mature megakaryocyte controls. Using immunohistochemistry on sections of whole tibiae from c-Mpl knockout mice we demonstrated that megakaryocytic NMDA receptor expression was maintained following c-Mpl ablation. These data support a fundamental role for glutamate signaling in megakaryocytopoiesis and platelet production, which is likely to be independent of thrombopoietin-mediated effects. (Blood. 2003;102:1254-1259)

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Introduction

Numerous cytokines including interleukin-1 (IL-1), IL-3, IL-6, IL-11, stem cell factor (SCF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) affect megakaryocyte development and platelet release from hematopoietic bone marrow stem cells. In particular, the c-Mpl ligand thrombopoietin (TPO) has now been identified as the principal regulator of megakaryocytopoiesis, by promoting megakaryocyte progenitor cell survival, proliferation, and terminal differentiation. Although c-Mpl-deficient (c-Mpl⁻/⁻) mice display a 90% reduction in the number of megakaryocytes, the megakaryocytes and platelets that remain are functionally normal, and c-Mpl⁻/⁻ mice do not exhibit severe bleeding abnormalities. In addition, a 5- to 7-day delay between TPO injection and peak circulating platelet number has been observed in vivo, suggesting that TPO is not required for the final stages of platelet release. Collectively these findings suggest that factors additional to TPO are also responsible for regulating megakaryocyte differentiation.

Recently we reported that the megakaryoblastic cell line MEG-01 expressed open channel–forming N-methyl-D-aspartate (NMDA)–type glutamate receptors. Further investigation indicated that phorbol myristate acetate (PMA)–induced differentiation of MEG-01 cells to a more megakaryocytic phenotype, which included increases in cell size, cell adhesion, and CD41a expression, was inhibited by the addition of the specific NMDA receptor antagonist MK-801. This was the first evidence of glutamate signaling in megakaryocytic cells and identified a potentially novel mechanism for regulation of megakaryocytopoiesis and platelet release.

Glutamate signaling was once thought to be restricted to the mammalian central nervous system (CNS), where it is involved in memory formation, learning, and synaptic development. The binding of glutamate to specific receptors on the postsynaptic membrane induces elevated intracellular calcium concentrations and activates diverse intracellular signal transduction cascades. The NMDA receptor belongs to a family of ionotropic glutamate receptors, which also include the α-aminoadroxy-5-methylisoxazole propionate (AMPA) and kainate receptors. In vivo, the NMDA receptor forms a functional channel as a heterotetramer or heteropentamer, composed of the essential NMDA receptor 1 (NR1) subunit and varying numbers of NR2 subunits (either A, B, C, or D). Recent evidence has accumulated to suggest that glutamate signaling, similar to that in the CNS, also exists in peripheral tissues such as bone, pancreas, and skin (for review see Skerry and Genever).

In this study, we determined the functional role of NMDA-type glutamate signaling during the differentiation of primary human megakaryocytes. Our findings suggest strongly that NMDA receptor–mediated signaling is centrally involved in the regulation of megakaryocytopoiesis, independent of TPO actions.

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Materials and methods

CD34+ stem cell separation and culture of megakaryocytes from umbilical cord blood

Megakaryocytes were generated from hematopoietic progenitor cells in umbilical cord blood obtained with informed consent. CD34+ cells were isolated using a magnetic immunoselection protocol (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in Iscoves modified Dulbecco medium (as supplied contains 500 μM L-glutamate) with 2 mM glutamine, 2 mg/mL sodium pyruvate, 1% minimum essential medium vitamin solution, 1% nonessential amino acids, 0.1 mM B-mercaptoethanol, and 2 mg/mL L-asparagine (Life Technologies, Paisley, United Kingdom). The medium was also supplemented with 10% cord blood plasma, 0.2% bovine serum albumin, and 25 ng/mL TPO (Calbiochem, Nottingham, United Kingdom). Cells were plated at 5 × 10^6 cells/mL in 24-well plates and replate at 1×10^7 cells/mL on human foreskin fibroblast feeder layers in culture. An additional 25 ng/mL TPO was added at this point and the cells were cultured for a further 7 days in culture. MK-801 (50 μM) was added to cultures on day 0 and day 7, 15 minutes prior to addition of TPO. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2/95% air.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted from CD34+-derived megakaryocytes after 14 days in culture, using TriZol reagent (Life Technologies). RNA from 12-week-old human fetal forebrain (Stratagene, Amsterdam, the Netherlands) was used as a positive control. cDNA was synthesized by first-strand cDNA synthesis (Life Technologies) and used for PCR with the following gene-specific primers: 5′-CCCCGCTGGTGTTCTCTC-3′ and 5′-AGTTCGGTTTGGATCTGGTCTCA-3′, amplifying a product of 457 bp base pair (bp) corresponding to positions 2875 to 3332 of human brain NMDAR2A; 5′-CTGCCGGACATCACCAACCAACAAAC-3′ and 5′-CATCAGCCGACCAACACCTTACC-3′, amplifying a product of 441 bp corresponding to positions 4283 to 4727 of human brain NMDAR2B; 5′-GAAGGCGATTTGGGAGGTGTA-3′ and 5′-CGTGTAGCTGGTCTG-3′, amplifying a product of 459 bp corresponding to positions 239 to 664 of human brain mammalian analog LIN-7; 5′-ACGGCGGGTTGAGCGACGGAGT-3′, amplifying a product of 474 bp corresponding to positions 1836 to 2310 of human brain NR2D, Y otiao, Chapsyn-110, and CASK was performed for 35 cycles of 94°C for 10 seconds, then at the relative annealing temperature for 10 seconds and 72°C for one minute. PCR of human NR2A, NR2C, PD-95, MALS, and SHANK was performed for 35 cycles of 94°C for 10 seconds and 72°C for 72 seconds and 72°C for 2 minutes.

Transmission electron microscopy

Day-14 megakaryocytes were washed twice in PBS before fixation in 4% paraformaldehyde for 5 minutes, washed 3 times in phosphate-buffered saline (PBS), and stained with hematoxylin (5 minutes) and 1% eosin (10 minutes). The preparations were then washed gently in tap water and mounted in glycerol/PBS. The cells were digitally quantified using Leica Quantimet image analysis system (Leica Q500win standard version 2.2, Leica Imaging Systems), and the mean of the area of individual cells in 6 random fields was determined. The mean number of cells in each field was 63.1 (range, 58–69 cells) for control and 63.8 (range, 56–71 cells) for MK-801–treated.

Immunohistochemistry

Tibiae from mice with a null mutation for the TPO receptor c-Mpl (c-Mpl<sup>−/−</sup>) and age- and sex-matched wild-type controls (wt) (samples were generously provided by Dr J. Tobias and Dr M. Perry, University of Bristol, United Kingdom) were dipped in 10% polyvinyl alcohol
(PVA; Sigma) and frozen in chilled isopentane (−70°C). The bones were mounted using 10% PVA on brass chucks and 7-µm sections were cut using a Bright cryostat (Bright Instruments, Huntingdon, United Kingdom). The sections were collected on polylysine slides (BDH) and stored at −35°C until examination. Sections were fixed in 4% paraformaldehyde for 5 minutes before the depletion of endogenous peroxide activity with 3% hydrogen peroxide (Sigma) for 30 minutes. Nonspecific avidin binding to endogenous biotin was prevented using the Vector Avidin/Biotin Blocking Kit (Vector Laboratories, Peterborough, United Kingdom) according to the manufacturer’s protocol. To minimize nonspecific staining due to endogenous mouse immunoglobulins, the Vector MOM Immunodetection Kit (Vector Laboratories) was used throughout antibody labeling according to the manufacturer’s instructions. Sections were incubated with anti-NR1 primary antibody (Pharmingen; 1 µg/mL; clone 54.1) for 30 minutes, followed by exposure to biotinylated anti–mouse IgG for 10 minutes before the application of Vectastain Elite ABC reagent (Vector Laboratories) for 5 minutes. The sections were counterstained with hematoxylin before mounting using 10% PVA on brass chucks and 70% ethanol before dehydration and xylene baths. Sections were collected on polysine slides (BDH) and stored at −70°C until use.

**Statistical analysis**

Where appropriate, results were analyzed using a one-way analysis of variance (ANOVA) or Student *t* test.

**Results**

mRNA expression of NMDA receptor subunits and associated proteins by primary human megakaryocytes

We have previously demonstrated that primary human megakaryocytes express the NR1 subunit of the NMDA-type glutamate receptor, however, the expression of related molecular components has not been determined in these cells. RT-PCR was used to identify mRNA expression of different NR2 subunits and associated protein expression by CD34+–derived megakaryocytes. Products corresponding in size to those in the positive human forebrain control were found for NR2A and NR2D subunits (Figure 1A), as well as the associated proteins Yotiao and PSD-95 (Figure 1B). In contrast mRNA expression of NR2B, NR2C, Chapsyn-110, SHANK, MALS, and CASK was either absent or expressed at very low levels.

Effects of NMDA receptor antagonism on megakaryocyte morphology and survival in vitro

To determine the effect of NMDA receptor inhibition on megakaryocyte number and morphology in vitro, a series of analytic experiments were performed blind, following predefined criteria. After 14 days in culture in the presence of the NMDA receptor antagonist MK-801, the percentage of cells forming proplatelet structures was reduced 9-fold compared with untreated controls (Figure 2A-B). Cell surface area was reduced by approximately 50% following MK-801 treatment, according to image analysis quantification of cytospin preparations (Figure 2C). However, megakaryocyte number was not significantly altered by treatment with MK-801 compared with untreated control cells (Figure 2D). To determine the effect of MK-801 on megakaryocyte survival, double staining for Annexin V and PI of day-14 cells was performed. There was no significant difference between MK-801–treated and control megakaryocytes in early apoptotic, late apoptotic, or necrotic cell populations (Figure 3A-B).

Effects of NMDA receptor antagonism on megakaryocyte differentiation in vitro

Expression of the differentiation-dependent megakaryocyte markers CD61, CD41a, and CD42a was determined by flow cytometry following exposure to MK-801. In the presence of TPO, MK-801 application reduced expression of CD61 by approximately 35%, CD41a by 28%, and CD42a by 43.5% compared with those cells treated with TPO alone (Figure 4). Ultrastructural characteristics of day-14 megakaryocytes were examined by transmission electron microscopy. Distinctive features of the mature megakaryocyte were present in the control cells. These included multilobed and indented nuclei, cytoplasmatic platelet α-granules, a dilated demarcation membrane system (DMS) toward the periphery of the cell, and clearly visible proplatelet structures (Figure 5A). However, MK-801–treated cells displayed a rounded, single-lobed nucleus, a complete lack of α-granules and DMS, an absence of proplatelet structures, and the presence of large open cytoplasmic cisternae (Figure 5B). These characteristics are not considered physiologic.

Megakaryocytic NMDA receptor expression in c-Mpl knock-out mice

NR1 expression by bone marrow megakaryocytes from c-Mpl knock-out mice (c-Mpl−/−) and age- and sex-matched controls (c-Mpl+/+) was determined by immunohistochemistry. Strong megakaryocytic expression of NR1 was detected in the wild-type
control bone marrow (Figure 6A), and although the number of megakaryocytes within the bone marrow compartment of c-Mpl⁻/⁻ mice was notably reduced, NR1 expression was maintained (Figure 6B). Staining was absent in antibody controls (Figure 6C).

**Discussion**

We have recently demonstrated that megakaryocytic cells express functional NMDA-type glutamate receptors and that antagonism of these receptors inhibits PMA-mediated differentiation of MEG-01 cells. Previous evidence demonstrated that NMDA receptors were also present on platelets. Franconi et al suggested that these receptors were not identical to those identified in the CNS. However NMDA and glutamate antagonized the aggregating activity of arachidonic acid (AA) and NMDA inhibited adenosine diphosphate (ADP) and platelet activating factor (PAF)-induced platelet aggregation. In addition NMDA increased intracellular-free calcium concentrations in platelets and completely inhibited synthesis of thromboxane B-2, an aggregation-promoting agent. Supersensitivity of platelet NMDA receptors has also been implicated in psychotic disorders, leading to the suggestion that platelets may be used as a peripheral marker of NMDA receptor dysfunction in these conditions.

We have now demonstrated that human primary megakaryocytes also express NR2 subunits and proteins associated with NMDA receptor-mediated signaling and that the NMDA receptor inhibition has profound functional effects on human megakaryocyte differentiation and proplatelet formation. We have confirmed expression of a range of NMDA receptor subunits and associated proteins in CD34⁻/⁻ derived primary human megakaryocytes, including NR2A, NR2D, Yotiao, and PSD-95, providing further insights into how these receptors might function on these cells. In the mammalian CNS, Yotiao binds protein kinase A II (PKAII) forming a protein complex with the NR1 subunit of the NMDA receptor and regulating intracellular signaling through cyclic adenosine monophosphate (cAMP)-dependent pathways. The PSD-95 family of proteins binds to postsynaptic NMDA receptors, causing receptor clustering to the plasma membrane and creating a scaffold for numerous downstream signaling cascades. However, mRNAs encoding other proteins associated with neuronal NMDA receptors, such as chapsyn-110, CASK, MALs, and SHANK, were not identified in primary megakaryocytes. Expression of only a subset of NMDA receptor subunits and associated proteins suggests that the molecular organization of megakaryocytic NMDA receptors is similar to but not identical to the neuronal...
receptor. This disparity in NMDA receptor composition may be due to differences in receptor function required for specialized cellular activities. For example, at the CNS synapse, the NMDA receptor plays a role in long-term potentiation (LTP) and long-term depression (LTD), by changes in receptor relocation and activation. This signaling mechanism is unlikely to be required in megakaryocyte differentiation. It is more probable that megakaryocytic NMDA receptor composition is aimed toward activation of currently unidentified downstream signaling events.

Blockade of NMDA receptors with the specific antagonist MK-801 significantly inhibited increases in progenitor cell size and expression of megakaryocyte-specific markers, and markedly reduced proplatelet formation in vitro. Interestingly, progenitor cell proliferation was not inhibited by MK-801, suggesting a role for glutamate signaling in megakaryocyte differentiation and platelet formation rather than stem cell proliferation and survival. This suggestion is supported by our evidence that MK-801 treatment prevented nuclear maturation, the development of demarcation membranes, and α-granule formation. Nuclear maturation has previously been demonstrated to be of great importance in normal megakaryocytopenesis as low ploidy cells (<8N) are unable to produce platelets (reviewed in Ravid et al25). It is possible, therefore, that NMDA receptor antagonism inhibits endomitosis in megakaryocytes; consequently the role of the NMDA receptor in megakaryocyte nuclear development would prove an extremely interesting topic for future investigation. It is unlikely that megakaryocytes with these morphologic characteristics would be able to form platelets, consistent with our findings that MK-801 treatment reduced proplatelet formation in vitro. It has recently been demonstrated that blocking the function of the tetraspanin CD9 results in strikingly similar ultrastructural characteristics as MK-801 treatment, specifically inducing disturbed DMS and the formation of large open cisternae. Clay et al suggested that this could be due to the involvement of CD9 in the membrane remodelling process during megakaryocytopenesis. These observations suggest that the relationship between CD9 function, NMDA receptor signaling, and membrane reorganization, warrant further investigation.

We have shown by immunohistochemistry on tibial sections of c-Mpl−/− and age/sex-matched controls, that NR1 expression by megakaryocytes is unaffected by c-Mpl ablation. It is therefore likely that NMDA receptor expression does not rely on TPO and c-Mpl receptor function, although this does not rule out the possibility of signaling cross-talk between the 2 systems to control megakaryocyte proliferation and differentiation. Recently, Levin et al demonstrated that circulating platelet number in c-Mpl−/− mice can be restored to normal by treatment with the myelosuppressive agent 5-fluorouracil, providing more evidence to support TPO-independent platelet production. It would be of interest to analyze NMDA receptor function and expression by megakaryocytes in disorders characterized by abnormal megakaryocytopenesis and platelet production, such as primary thrombocytopenia, myelofibrosis, and primary proliferative polycythemia, considering the profound effects inhibition of NMDA receptors had on megakaryocyte differentiation and proplatelet formation. It is possible that an aberration in this signaling mechanism may be implicated in the etiology of these disease states.

This work provides compelling evidence that NMDA receptor signaling is of crucial importance in human megakaryocytopenesis and platelet formation in vitro. Further studies will determine the functional importance of this newly identified signaling mechanism in vivo and how it interacts with other hematopoietic growth factors in the regulation of thrombocytopenesis.

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