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Mpl Ligand Increases P2Y1 Receptor Gene Expression in Megakaryocytes with No Concomitant Change in Platelet Response to ADP

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ABSTRACT

The $P2Y_1$ receptor is responsible for the initiation of platelet aggregation in response to ADP and plays a key role in thrombosis. Although this receptor is expressed early in the platelet lineage, the regulation of its expression during megakaryocyte differentiation is unknown. In the mouse megakaryocytic cell line Y10/L8057, we detected $P2Y_1$ mRNA of three sizes (2.5, 4.4, and 7.4 kb). These cells have previously been shown to respond to Mpl ligand, the pivotal regulator of megakaryocytopoiesis, by increasing their expression of differentiation markers. Mpl ligand enhanced levels of $P2Y_1$ mRNAs in Y10/L8057 cells and this effect was selective: the same cytokine did not increase levels of A2a adenosine receptor mRNA. Although Mpl ligand did not affect the short half-lives of the $P2Y_1$ mRNAs, it enhanced transcription of the *P2Y*$_1$ gene. It also increased cell size and the number of cell surface $P2Y_1$ receptors, but not $P2Y_1$ receptor density. Injection of Mpl ligand into mice upregulated $P2Y_1$ receptor mRNAs in megakaryocytes, as shown by in situ hybridization. However, platelets isolated from these mice did not exhibit a higher $P2Y_1$ receptor density or increased reactivity to ADP. This correlates with the finding that Mpl ligand increases GPIIb mRNA in megakaryocytes but not the density of the protein per platelet. Thus, the enhancement of $P2Y_1$ receptor expression induced by Mpl ligand in megakaryocytes may be an integral feature of their differentiation, whereas clinical use of this compound might not be associated with platelet hyper-reactivity to ADP.

ADP plays a key role in hemostasis by acting as an aggregating agent. In addition, through its secretion from platelet dense granules, it contributes to and reinforces the platelet aggregation induced by other agents such as collagen or thrombin (Mills, 1996; Gachet, 2001). Stimulation of platelets with ADP leads to an increase in intracellular calcium through mobilization of internal calcium stores, rapid calcium entry from the external medium and inhibition of adenylyl cyclase activity (Mills, 1996; Gachet, 2001). All these effects are caused by the interaction of ADP with specific P2 receptors that have been characterized over the past 3 years (Gachet, 2001). The metabotropic $P2Y_1$ receptor is expressed in a wide range of body tissues (Ralevic and Burnstock, 1998) and in blood platelets (Leon et al., 1997). This entity, initially described as a receptor for which ATP was an agonist (Schachter et al., 1996), was later demonstrated to be, in fact, an ADP receptor (Leon et al., 1997; Hechler et al., 1998c). Additional studies have demonstrated that the effect of ATP on the $P2Y_1$ receptor depends upon receptor density [i.e., at a low level of receptor abundance, ATP acts as an antagonist, whereas at higher receptor density, ATP could behave as a partial agonist (Palmer et al., 1998)]. Pharmacological studies, using selective $P2Y_1$ antagonists (Hechler et al., 1998b; Jin et al., 1998a), and investigations in $P2Y_1$ knock-out mice (Fabre et al., 1999; Leon et al., 1999) showed this receptor to be necessary for the initiation of platelet aggregation in response to ADP through the mobilization of internal calcium stores. Studies in $P2Y_1$ knock-out mice also pointed to its essential role in thrombotic states (Fabre et al., 1999; Leon et al., 1999). The $P2Y_1$ receptor is nevertheless insufficient to support full aggregation in response to ADP, and platelets also contain the newly identified $P2Y_{12}$ receptor, previously called P2Ycyc, $P2T_{AC}$, $P2Y_{AC}$ or $P2Y_{ADP}$ (Hollopeter et al., 2001), responsible for completion of the aggregation response

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ABBREVIATIONS: RT-PCR, reverse transcription-polymerase chain reaction; MGDF, megakaryocyte growth and differentiation factor; 2MeSADP, 2-methylthio-adenosine 5′-diphosphate; A2P5P, adenosine 2′-phosphate 5′-phosphate; PEG-rHuMGDF, pegylated recombinant human megakaryocyte growth and differentiation factor; PEG-rmMGDF, pegylated recombinant murine megakaryocyte growth and differentiation factor; SSC, standard saline citrate; bp, base pair(s); kb, kilobase pair(s); UTR, untranslated region; PE, phycoerythrin; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(Daniel et al., 1998; Hechler et al., 1998a). Finally, platelets contain a ligand-gated ion channel (MacKenzie et al., 1996; Vial et al., 1997; Scase et al., 1998; Sun et al., 1998; Takano et al., 1999), the $P2X_1$ receptor, which seems to play a discrete role in the aggregation induced by ADP (Takano et al., 1999). $P2Y_1$ mRNA has been characterized in several human leukemic cell lines with megakaryocytic features by reverse transcription-polymerase chain reaction (RT-PCR) methods (Leon et al., 1997; Jin et al., 1998b). In contrast, much less is known about the size of mouse $P2Y_1$ mRNA or its potential regulation by Mpl ligand.

Mpl ligand, also known as thrombopoietin or megakaryocyte growth and differentiation factor (MGDF), is the pivotal physiologic regulator of megakaryocytopoiesis and platelet production (Kaushansky, 1999). It stimulates both megakaryocyte progenitor cell proliferation and the maturation of megakaryocytes, a process involving the formation of platelet-specific granules and demarcation membranes, the expression of lineage-specific proteins, such as platelet factor 4, glycoprotein GPIIbIIIa, and the GPIb-IX-V complex and an increase in endomitosis (Kaushansky, 1999). Although Mpl ligand does not induce in vitro platelet aggregation, it enhances the effect of thrombin, collagen, or ADP on this process (Oda et al., 1996; Harker et al., 1996a; Oda et al., 1999). In other studies, platelets were derived from healthy human volunteers (Harker et al., 2000) or from nonhuman primates (Harker et al., 1996b) treated or nontreated with Mpl ligand. In each of these cases, the in vivo pretreatment with this cytokine did not affect platelet response to ADP in vitro. Because $P2Y_1$ is one of the key receptors of platelets, we wished to determine whether Mpl ligand could affect the expression of this receptor during megakaryocyte differentiation. In cell culture experiments, we used the mouse megakaryocytic cell line Y10/L8057, a subclone of the L8057 cell line (Ishida et al., 1993), which has been shown to respond to Mpl ligand through an increase in ploidy and specific megakaryocytic markers (Zhang et al., 1998; Thompson and Ravid, 1999). Subsequently, we examined the in vivo effects of Mpl ligand on levels of $P2Y_1$ receptor mRNA in spleen megakaryocytes and on platelet $P2Y_1$ receptor density and reactivity to ADP.

Levels of $P2Y_1$ receptor mRNA and protein in Y10/L8057 cells increased rapidly and significantly under Mpl ligand treatment and this effect was mediated by an increase in transcription of the *P2Y*$_1$ receptor gene. Moreover, the rise in $P2Y_1$ mRNA levels induced by Mpl ligand also appeared in vivo in spleen megakaryocytes, but without any repercussion on the density of the receptor on platelets or their responsiveness to ADP. This is in accordance with previous reports that Mpl ligand up-regulates GPIIbIIIa, GPIbIX, and GPV mRNA in megakaryocytes, but not the concentrations of these proteins in platelets (Harker et al., 1996b; O'Malley et al., 1996; Zauli et al., 1997; Thompson and Ravid, 1999). Hence, the Mpl ligand-induced increase in $P2Y_1$ receptor expression in megakaryocytes may be an integral feature of the megakaryocytic differentiation program. Because this receptor plays a key role in hemostasis and thrombosis (Gachet, 2001), the absence of enhanced platelet reactivity to ADP further suggests that clinical use of Mpl ligand to stimulate platelet production in patients with bone marrow failure might not be associated with adverse effects of platelet hyperreactivity.

Experimental Procedures

Materials. 2-Methylthio-adenosine 5′-diphosphate (2MeSADP) was from Sigma/RBI (Natick, MA) and human fibrinogen from Kabi (Stockholm, Sweden). ADP, adenosine 2′-phosphate 5′-phosphate (A2P5P), actinomycin D, and fatty-acid–free human serum albumin were from Sigma (St. Louis, MO). Pegylated recombinant human (or murine) MGDF [PEG-rHuMGDF (or PEG-rmMGDF)], recombinant polypeptides encompassing the amino-terminal sequence of human (or murine) Mpl ligand, were a generous gift of Amgen Inc. (Thousands Oaks, CA). Pegylation of MGDF increases its in vivo potency by roughly 10- to 20-fold, largely by delaying its clearance and thus prolonging plasma half-life (Harker et al., 1996b). AR-C69931 MX was kindly provided by AstraZeneca Charnwood (Loughborough, UK) and [^{33}P]2MeSADP (850 Ci/mmol) by PerkinElmer Life Sciences (Le Blanc Mesnil, France). Apyrase (adenosine 5′-triphosphate diphosphohydrolase, EC 3.6.1.5) was purified from potatoes as described previously (Hechler et al., 1998a). MRS2179 (N^6-methyl 2′-deoxy-adenosine 3′,5′-bisphosphate) was synthesized by P. Raboisson (Centre National de la Recherche Scientifique Unité Propre de Recherche 421, Faculté de Pharmacie, Illkirch, France).

Cell Culture. Y10/L8057 cells were maintained in Ham's F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (fetal calf serum, Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2 and the cells were subcultured every 3 days to a density of 3×10^5 cells/ml. In experiments using PEG-rHuMGDF, Y10 cells were cultured in Iscove's modification of Dulbecco's medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum.

Northern Blot Analyses. Y10/L8057 cells were harvested and total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instructions, electrophoresed on a 1.2% agarose-formaldehyde gel, and transferred onto a nylon membrane. The various probes were random labeled (NEBlot kit; New England Biolabs Inc., Beverly, MA) as specified in the figure legends. Hybridization was performed at 42°C for 20 h under agitation in 5× standard saline/phosphate/EDTA, 50% formamide, 5× Denhardt's solution containing 0.5% SDS, and 100 μg/ml heat-denatured salmon sperm DNA. The membrane was washed in 2× SSC, 0.1% SDS for 15 min at 42°C, in 1× SSC for 5 min at 60°C and in 0.1× SSC, 0.1% SDS for 5 min at 60°C, before exposure to an X-ray film for up to 72 h at −80°C.

$P2Y_1$ probes were as follows: (1) a DNA fragment (1293 bp) corresponding to the entire translated region of the mouse *P2Y*$_1$ gene was excised from the 12.6-kb mouse *P2Y*$_1$ gene fragment cloned in pBluescript KS$^+$ phagemid (kind gift of Dr Catherine Léon) (Léon et al., 1999) using the restriction endonucleases *Bss*HII and *Eco*RI; (2) an exon 2 fragment (670 bp) was prepared by digestion of the mouse *P2Y*$_1$ gene with *Nsi*I and *Xmn*I; (3) a 5′-UTR fragment (679 bp) was prepared by digestion of the mouse *P2Y*$_1$ gene with *Sac*II. The fragments were subsequently purified from a 1% agarose gel using a GeneClean kit (Bio 101, Vista, CA) and radiolabeled as described above. Rat A2a adenosine receptor cDNA was cloned by Matthew Jones in our laboratory by RT-PCR and its sequence was confirmed by DNA sequencing (GenBank accession number AF228684).

Nuclear Isolation and Run-On Assay. Nuclei were prepared from Y10/L8057 cells cultured in the absence or presence of 25 ng/ml PEG-rHuMGDF for 2 days. After isolation as described previously (Zhang et al., 1998), the nuclei were resuspended in 50 mM Tris, pH 8.3, containing 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol at a concentration of 1×10^7 nuclei/100 μl. In vitro transcription was initiated by adding 100 μl of the nuclear suspension to an equal volume of 2× reaction buffer [10 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.3 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 5 mM dithiothreitol, and 12.5 μl of (α-^{32}P)UTP (10 mCi/ml)] and was allowed to proceed for 30 min at 30°C. Products of the nuclear run-on reaction were extracted into 1 ml of TRIzol according to the manufacturer's instruc-

tions. The transcribed RNA was then resuspended in 20 mM HEPES, pH 7.5, containing 5 mM EDTA and denatured by addition of 200 mM NaOH for 15 min on ice. Denaturation was stopped by addition of 250 mM HEPES (free acid). RNA was precipitated with ethanol and 3 M potassium acetate, washed in 75% ethanol and finally resuspended in 500 μl of hybridization solution (50% formamide, 5× Denhardt's solution, 0.5% SDS, 5× standard saline/phosphate/EDTA, 200 μg/ml salmon sperm DNA). pUC-19 DNA and plasmids containing $P2Y_1$ or GAPDH cDNA, each at 10 μg, were applied to nitrocellulose with a slot-blot apparatus. The transcription reaction mixture (10^6 cpm/ml) was then added to the prehybridized membranes and hybridization was performed at 42°C for 24 h. After washing twice in 2× SSC, 0.1% SDS for 5 min at 42°C and once in 1× SSC, 0.1% SDS for 15 min at 60°C, the membranes were exposed to an X-ray film for up to 48 h at −80°C.

Binding of [^{33}P]2MeSADP to Y10/L8057 Cells and Washed Mouse Platelets. Y10/L8057 cells were washed twice by centrifugation (150 *g*, 20°C) in basal salt solution (25 mM HEPES, pH 7.3, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, and 0.1% fatty-acid–free human serum albumin) supplemented with 2 mM $CaCl_2$ and 0.2 U/ml apyrase (an ADP scavenger) and resuspended in basal salt solution containing calcium but no apyrase. At 20°C, a time course study of the incubation of [^{33}P]2MeSADP (0.6 nM, 10^6 d.p.m.) with Y10/L8057 cells showed that a steady state was achieved after 20 min and maintained for at least 30 min. In subsequent experiments, an incubation time of 30 min was employed for convenience. The temperature of 20°C was chosen to minimize the metabolism of nucleotides by ectoenzymes, a process that would be enhanced at 37°C. Because the radioactive phosphate of [^{33}P]2MeSADP is in the β position, possible degradation should not interfere with binding to the $P2Y_1$ receptor. Thus, the binding of 2MeSADP to nontreated or PEG-rHuMGDF-treated Y10/L8057 cells was evaluated by incubation of [^{33}P]2MeSADP (0.6 nM, 10^6 dpm) with Y10/L8057 cells (4×10^6/ml) for 30 min at 20°C in a final volume of 0.5 ml, in the presence or absence of 1 mM A2P5P, a selective $P2Y_1$ receptor antagonist (Boyer et al., 1996). Similarly, the binding of 2MeSADP to washed platelets from nontreated or PEG-rmMGDF (25 μg/kg) treated mice was determined (Hechler et al., 1998a) by incubating [^{33}P]2MeSADP (0.5 nM, 3×10^5 d.p.m.) with washed platelets in suspension (3×10^5 platelets/μl) for 5 min at 37°C in a final volume of 200 μl, in the presence or absence of 1 mM A2P5P.

Experiments were started by addition of Y10/L8057 cells or platelets to the reaction mixture and carried out in triplicate. The reaction was terminated by dilution in 4 ml of cold buffer and rapid filtration through Whatman GF/C glass fiber filters (Whatman, Clifton, NJ) under vacuum, after which the tubes and filters were rinsed 4 times with 2 ml of ice-cold buffer. Radioactivity bound to the cells on the filters was measured by scintillation counting (1409 Counter; PerkinElmer Wallac, Turku, Finland) and data were analyzed and plotted with the Ligand program (Rodbard et al., 1986). Nonspecific binding, measured in the presence of 1 μM unlabeled 2MeSADP, amounted to about 5% of the total binding. Saturation experiments were performed using a single concentration of [^{33}P]2MeSADP and increasing concentrations of unlabeled 2MeSADP.

Preparation of Y10/L8057 Cell Membranes and Binding Studies. Y10/L8057 cells were washed twice with PBS buffer and resuspended in buffer A (10 mM HEPES, 1 mM EDTA, 5 mM $MgCl_2$, 20 mM PMSF, pH 7.5) at a density of 10^7 cells/ml. After three freeze-thaw cycles, the cell suspension was homogenized with a Teflon pestle homogenizer on ice and centrifuged at 1,000*g* for 15 min at 4°C. The supernatant was then centrifuged at 45,000*g* for 15 min at 4°C; the resultant pellet was resuspended in buffer A, aliquoted, and stored at −80°C until use. Protein content was determined by the bicinchoninic acid method (Pierce, Rockford, IL), using bovine albumin as standard. Measurement of [^{33}P]2MeSADP binding to Y10/L8057 cell membranes (5 μg of protein/tube) was performed as described above for intact Y10/L8057 cells.

PEG-rmMGDF Injection and in Situ Hybridization Analyses of Spleen Tissues. PEG-rmMGDF diluted with 1% normal mouse serum in PBS was injected once into the lateral tail vein of 6- to 10-week-old FVB mice (50 μg/kg) as previously described (Zimmet et al., 1997). Mice injected with either PEG-rmMGDF or vehicle were sacrificed 3 days later and the spleens removed immediately and fixed in 4% paraformaldehyde overnight. Although megakaryocyte numbers and ploidy were both significantly increased, as reported elsewhere (Zimmet et al., 1997), the concentration of PEG-rmMGDF used in this protocol caused no significant change in platelet volume (Daw et al., 1998). The tissue samples were dehydrated in graded ethanol solutions, cleared with xylene, and embedded in paraffin. Sections (5 μm) were processed as described previously (Zimmet et al., 1997) before hybridization with the ^{35}S-labeled $P2Y_1$. Slides were stained with hematoxylin and eosin for analysis by bright field microscopy and radioactive signals were detected by autoradiography after 4 weeks' exposure.

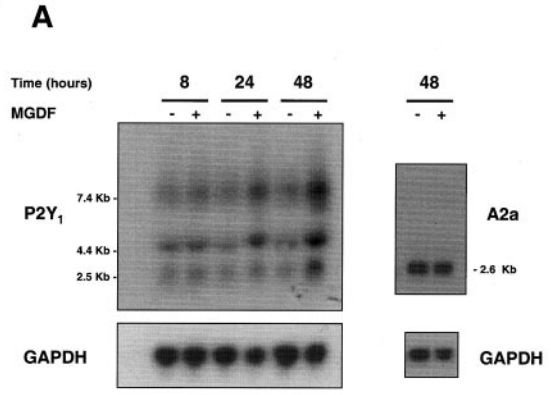
Preparation of Washed Mouse Platelets and Platelet Aggregation Studies. Six-week-old FVB mice were injected with PBS containing either 1% normal mouse serum or PEG-rmMGDF (25 μg/kg), a dose known to maximally increase platelet levels (Daw et al., 1998). Five days later, at the time of peak platelet production (Daw et al., 1998), blood was drawn from the abdominal aorta into acid-citrate-dextrose solution (1 volume of acid-citrate-dextrose solution/6 volumes of blood) and pooled (5 ml). Platelet suspensions, washed twice, were prepared as described previously (Hechler et al., 1998a) and the cell count was adjusted to $300 \times 10^3/\mu$l in the final suspension using an ACT Coulter Diff counter (Beckman-Coulter, Roissy, France). The final suspending medium was Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, and 5 mM HEPES, pH 7.3) containing 0.35% human serum albumin and 0.02 U/ml apyrase, a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. Platelets were stored at 37°C throughout experiments.

Aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, ON, Canada). A 450-μl aliquot of platelet suspension was stirred at 1100 rpm and activated by adding 50 μl of aggregating agent, after prior addition of fibrinogen (0.2 mg/ml) in the case of ADP.

Flow Cytometric Analysis of Lineage-Specific Platelet Surface Markers. Phycoerythrin (PE)-conjugated anti-mouse GPV (DOM2) monoclonal antibody, fluorescein isothiocyanate (FITC)-conjugated anti-GPIIb-IIIa (JON1) and FITC-conjugated anti-GPIbIX (POP1) (Bergmeier et al., 2000) monoclonal antibodies (mAbs) and PE- and FITC-conjugated irrelevant mAbs were kindly provided by Prof. Bernhard Nieswandt (Witten/Herdecke University, Wuppertal, Germany). Washed mouse platelets (1.5×10^6) in 15 μl of Tyrode's buffer containing 0.35% human serum albumin were incubated for 15 min with 0.5 μg of mAb. Samples were then diluted in 500 μl of the same buffer and analyzed on a FACSCalibur fluorescence cytometer (BD Biosciences, San Jose, CA). The fluorescence intensity of 10,000 cells was collected with a logarithmic gain and the binding of mAbs to mouse platelets was represented as the FL1 or FL2 fluorescence intensity relative to that of an irrelevant antibody.

Results

PEG-rHuMGDF Increases $P2Y_1$ Receptor mRNA levels in Y10/L8057 Cells. Northern blot analyses of total RNA from Y10/L8057 cells, using a radiolabeled probe covering the entire translated region of the mouse *P2Y1* gene, indicated the presence of three transcripts with sizes of 7.4, 4.4, and 2.5 kb (Fig. 1A, left). The 4.4-kb transcript seemed to be more abundant than the other two, which were of similar abundance. At 25 ng/ml, a concentration previously shown to



P2Y1 mRNAs	Fold increase by MGDF 8h	24h	48h
7.4 Kb	1.4 ± 0.1	2.5 ± 0.2	3.8 ± 0.2
4.4 Kb	1.4 ± 0.2	2.5 ± 0.2	3.4 ± 0.3
2.5 Kb	1.4 ± 0.1	1.9 ± 0.3	3.8 ± 0.3

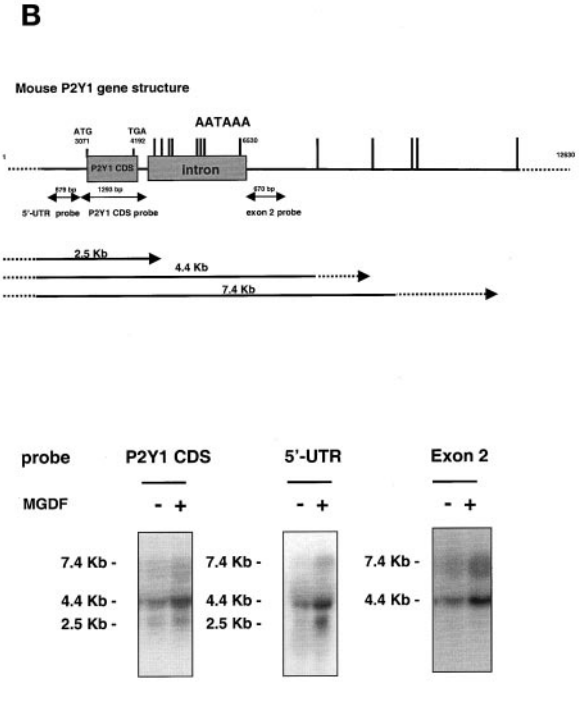


Fig. 1. PEG-rHuMGDF selectively enhances P2Y$_1$ receptor mRNA in Y10/L8057 cells. A, Y10/L8057 cells were cultured in the presence or absence of PEG-rHuMGDF (MGDF) (25 ng/ml) and harvested at the indicated times. Total RNA was extracted and analyzed on Northern blots using ^{32}P-labeled cDNA probes corresponding to the entire translated region of the mouse *P2Y$_1$* gene (left) or the A2a adenosine receptor gene (right). Equal loading of RNA (25 μg/lane) was checked with a cDNA probe encoding GAPDH. Levels of P2Y$_1$ mRNA and the fold increase induced by PEG-rHuMGDF were estimated with the electrophoresis documentation and analysis system. Results are the mean ± S.D. from three independent experiments and one representative experiment is shown. B, top, organization of the mouse *P2Y$_1$* receptor gene showing the location of the probes used for Northern blot analysis showed bellow. Bottom: Total RNA was extracted from Y10/L8057 cells cultured for 48 h in the presence or absence of PEG-rHuMGDF (25 ng/ml) and analyzed by Northern blotting. ^{32}P-labeled cDNA probes corresponded to the entire translated region (P2Y1 CDS probe), the 5′-untranslated region (5′-UTR probe), and the second exon (exon 2 probe) of the mouse *P2Y$_1$* gene.

maximally promote the differentiation of Y10/L8057 megakaryocytic cells (Zhang et al., 1998; Thompson and Ravid, 1999), PEG-rHuMGDF induced a gradual, time-dependent increase in these three bands (Fig. 1A, left). Levels of the three transcripts seemed to be significantly higher after 8 h stimulation, and we initiated our analyses at this time point because it corresponds to the time of detectable induction of megakaryocyte markers by PEG-rHuMGDF (Thompson and Ravid, 1999) (our equivalent data not shown). After 48-h treatment of Y10/L8057 cells with PEG-rHuMGDF, the three P2Y$_1$ mRNA bands increased about 3- to 4-fold compared with the corresponding bands in nonstimulated cells (see legend to Fig. 1 for the method of analysis), whereas prolonged incubation with PEG-rHuMGDF indicated that the rise in P2Y$_1$ mRNA was maximal at this time point (data not shown). Because A2a adenosine receptor mRNA, which is also expressed in Y10/L8057 cells, was not affected by PEG-rHuMGDF (Fig. 1A, right), its enhancement of P2Y$_1$ mRNA seemed to be selective.

To determine whether the three RNA transcripts detected by Northern blotting were related to hybridization of the P2Y$_1$ probe with homologous mRNA fragments, we checked whether probes corresponding to nontranslated regions of the P2Y$_1$ gene would hybridize with the same three transcripts. Thus, we constructed two radiolabeled probes corresponding to the 5′-untranslated region (5′-UTR probe) and to the second exon (exon 2 probe) of the *P2Y$_1$* gene (Fig. 1B top). The 5′-UTR probe hybridized with the three RNA transcripts detected using the probe covering the translated region of the gene (Fig. 1B, bottom). In contrast, the exon 2 probe hybridized with the 7.4- and 4.4-kb transcripts, but not with the 2.5-kb transcript. The results for the 5′-UTR probe confirm that all three transcripts are related to the same gene. Because the *P2Y$_1$* gene contains an intron and several polyadenylation sites downstream of the translation stop codon (Fig. 1B top) (Léon et al., 1999), these three P2Y$_1$ mRNA fragments could arise from alternative splicing of the intron and transcription arrest at various polyadenylation sites.

Stability of P2Y$_1$ Receptor mRNA in Y10/L8057 Cells. The increase in P2Y$_1$ receptor mRNA levels during megakaryocyte differentiation in the presence of PEG-rHuMGDF could reflect changes in mRNA stability caused by post-transcriptional modifications and/or changes in the gene transcription rate. Therefore, to better characterize the regulation of P2Y$_1$ mRNA levels by PEG-rHuMGDF, we assessed the stabilities of P2Y$_1$ mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in Y10/L8057 cells. Cells cultured for 48 h in the presence or absence of PEG-rHuMGDF (25 ng/ml) were incubated with the transcription inhibitor actinomycin D (5 μg/ml) for up to 10 h, before isolation of total RNA. Northern blot analyses indicated that levels of the three P2Y$_1$ mRNA transcripts decreased rapidly after addition of actinomycin D (Fig. 2A). Conversely, no decrease in GAPDH mRNA was observed during 10 h incubation with the transcription inhibitor (Fig. 2A). Thus P2Y$_1$ receptor mRNA levels were normalized at each time point to the corresponding GAPDH mRNA levels. The half-lives of the 7.4-, 4.4-, and 2.5-kb P2Y$_1$ mRNA transcripts calculated by this method were 1 h, 2.5 h, and 2 h, respectively, and were not modified by PEG-rHuMGDF (Fig. 2B).

PEG-rHuMGDF Induces de Novo Synthesis of $P2Y_1$ Receptor mRNA. To determine whether the increase in steady-state levels of $P2Y_1$ mRNA resulted from enhanced transcription of the $P2Y_1$ gene, de novo synthesis of $P2Y_1$ receptor mRNA was evaluated in nuclear run-on assays, using nuclei isolated from Y10/L8057 cells cultured for 48 h with or without PEG-rHuMGDF (25 ng/ml). Transcription of the *P2Y1* gene increased 2 fold in the presence of PEG-rHuMGDF (Fig. 3). Hence, transcription enhancement was totally responsible for the observed rise in $P2Y_1$ receptor mRNA levels during PEG-rHuMGDF treatment of Y10/L8057 megakaryocytic cells.

PEG-rHuMGDF Up-Regulates $P2Y_1$ Receptor Density on Y10/L8057 Cells. In the absence of any antibody recognizing the mouse $P2Y_1$ protein, its presence on the surface of Y10/L8057 cells and the effects of PEG-rHuMGDF on the surface density of the receptor were investigated with a radioligand binding assay. The radioligand was [^{33}P]2MeSADP, an ADP analog previously used to characterize ADP receptors on platelets (Hechler et al., 1998a). [^{33}P]2MeSADP binding sites corresponding specifically to the $P2Y_1$ receptor were quantified by measuring binding of the radioligand to Y10/L8057 cells treated or not with PEG-rHuMGDF in the presence or absence of the selective $P2Y_1$ antagonist A2P5P (1 mM) (Boyer et al., 1996). Nonlinear regression analysis of the data revealed 76,700 ± 7,000 [^{33}P]2MeSADP binding sites per cell, with an affinity of 73 ± 6 nM, on control cells (Fig. 4A). In the presence of A2P5P, the number of binding sites per cell decreased to 57,800 ± 5,400 and these residual sites displayed an affinity of 80 ± 19 nM. Hence, the "A2P5P-sensitive" sites, corresponding to binding of the radioligand to the $P2Y_1$ receptor, amounted to 18,900 sites per cell. In the presence of PEG-rHuMGDF (25 ng/ml), [^{33}P]2MeSADP binding sites increased to 94,100 ± 1,100 sites per cell with an affinity of 64 ± 5 nM. This additional binding corresponded to an increase in the density of $P2Y_1$ sites because it was displaced by 1 mM A2P5P, whereas the residual "A2P5P-insensitive" binding sites still amounted to 59,200 ± 13,600 sites per cell with an affinity of 81 ± 26 nM. Thus, the $P2Y_1$ binding sites on Y10/L8057 cells treated with PEG-rHuMGDF represented 34,900 sites per cell and PEG-rHuMGDF enhanced the $P2Y_1$ receptor density on these cells by a factor of 1.8.

The specific binding of [^{33}P]2MeSADP to Y10/L8057 cells was partially displaced by increasing concentrations of A2P5P and likewise by the selective and potent $P2Y_1$ antagonist MRS2179 (Nandanan et al., 2000) (data not shown), which reinforces the hypothesis that these [^{33}P]2MeSADP binding sites correspond to the $P2Y_1$ receptor. The residual "A2P5P-insensitive" sites were displaced equally by ATP or UTP, but not by AR-C69931 MX or 2 MeSAMP, two selective $P2Y_{12}$ receptor antagonists (Ingall et al., 1999; Jantzen et al., 1999; Hollopeter, 2001) (data not shown). Also, 2MeSADP was not able to induce an inhibition of forskolin-stimulated adenylyl cyclase activity in Y10/L8057 cells (our unpublished data).

Study of the binding of [^{33}P]2MeSADP to Y10/L8057 cell membranes indicated that the number of [^{33}P]2MeSADP binding sites sensitive to A2P5P, and thus corresponding to the $P2Y_1$ receptor (7.8 ± 0.5 pmol/mg protein), was not in-

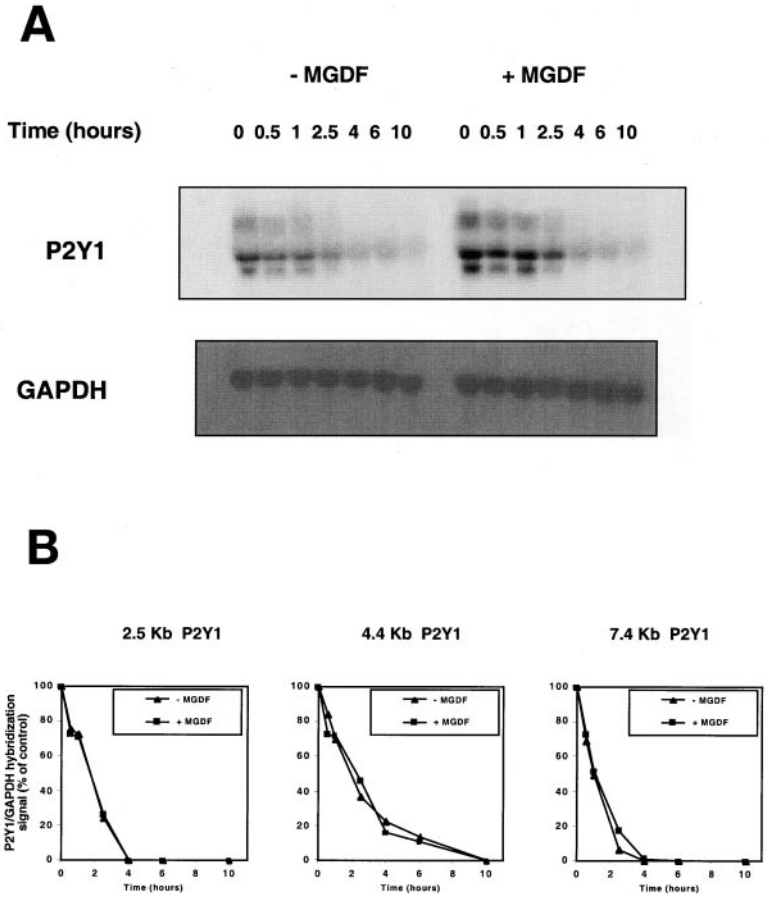


Fig. 2. PEG-rHuMGDF does not modify the half-lives of $P2Y_1$ mRNA transcripts. A, Y10/L8057 cells cultured for 48 h in the presence or absence of PEG-rHuMGDF (MGDF) (25 ng/ml) were treated with the transcription inhibitor actinomycin D (5 μg/ml) for up to 10 h. Total RNA was extracted and analyzed on Northern blots using cDNA probes encoding GAPDH or the entire translated region of the mouse *P2Y1* gene. B, hybridization bands in the Northern blots of A were quantified with the electrophoresis documentation and analysis system. At each time point, the $P2Y_1$ signals were normalized to the corresponding GAPDH signals and the normalized $P2Y_1$ receptor signals were then expressed as percentages relative to their values at time 0 (100%). One experiment is shown, representative of two experiments yielding identical results.

creased after PEG-rHuMGDF treatment (7.6 ± 1.2 pmol/mg protein) (Fig. 4B). This suggested that PEG-rHuMGDF-dependent increase in P2Y$_1$ receptor expression on megakaryocytes parallels the global increase in megakaryocyte size so that the density of P2Y$_1$ receptor per milligram of membrane proteins remains stable. Our previous studies indicated that the average size of Y10/L8057 cells treated with PEG-rHuMGDF is 2-fold greater than that of control cells, as assessed by forward-angle light scatter (Zimmet et al., 1997; Zhang et al., 1998).

In Situ Hybridization Analyses of Megakaryocytes from Mice Injected with PEG-rmMGDF. Our next step was to determine whether PEG-rmMGDF could similarly enhance *P2Y$_1$* gene expression in vivo in primary megakaryocytes. FVB mice were injected with PEG-rmMGDF (50 μg/kg) and levels of P2Y$_1$ receptor mRNA in spleen megakaryocytes were evaluated by an in situ hybridization technique (see *Experimental Procedures*). PEG-rmMGDF induces a significant increase in megakaryocyte ploidy and size under these conditions (data not shown; Zimmet et al., 1997). The in situ hybridization method allows one to compare relative levels of gene expression in individual primary megakaryocytes, which are easily recognized because of their size and morphology. P2Y$_1$ mRNAs were markedly enhanced in megakaryocytes from mice treated with PEG-rmMGDF, compared with control cells treated with vehicle (Fig. 5). Quantitative analyses of silver grains on sections showed averages of 190 ± 24 (n = 11) and 73 ± 12 (n = 11) grains per megakaryocyte in test and control animals, respectively ($p < 0.05$), which corresponds to a 2.6-fold induction of P2Y$_1$ receptor mRNA.

Studies of Platelets from Mice Treated with PEG-rmMGDF. Circulating platelet levels rose from 823 × 10^3 ± 125 × 10^3 (n = 17) to 1,600 × 10^3 ± 163 × 10^3 platelets/μl (n = 9) after administration of PEG-rmMGDF (25 μg/kg) to mice. Platelet P2Y$_1$ receptor densities were determined by measuring the binding of [^{33}P]2MeSADP to washed platelets from mice injected or not with PEG-rmMGDF (25 μg/kg), in the presence or absence of A2P5P (1 mM) (Hechler et al., 1998a). Saturation experiments revealed 1,075 ± 9 [^{33}P]2MeSADP binding sites per platelet, with an affinity of 11 ± 1 nM, on control platelets (Fig. 6A). In the presence of A2P5P, the number of binding sites per platelet decreased to 742 ± 23 and these residual sites displayed an affinity of 9 ± 1 nM. Hence, the P2Y$_1$ receptor density was calculated to be 333 receptors per platelet in control mice. Similarly, in mice receiving PEG-rmMGDF, [^{33}P]2MeSADP binding sites represented 975 ± 54 sites per platelet with an affinity of 9 ± 1 nM (Fig. 6A). Addition of A2P5P reduced [^{33}P]2MeSADP binding sites to 627 ± 72 sites per platelet with an affinity of 8 ± 1 nM (Fig. 6A), giving a calculated P2Y$_1$ receptor density of 348 receptors per platelet. Thus, injection of PEG-rmMGDF caused no significant change in platelet P2Y$_1$ receptor density in mice.

Interestingly, the affinity of the P2Y$_1$ receptor for 2Me-

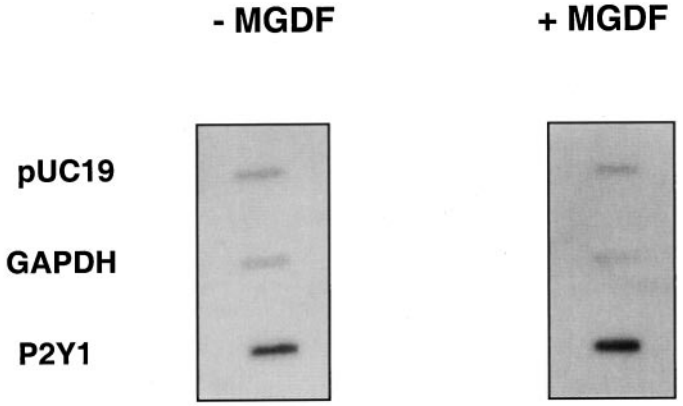


Fig. 3. PEG-rHuMGDF enhances transcription of the *P2Y$_1$* gene in Y10/L8057 cells. Nuclear run-on assays were performed on nuclear extracts from Y10/L8057 cells cultured for 48 h in the presence or absence of PEG-rHuMGDF (MGDF) (25 ng/ml), as described under *Experimental Procedures*. Radioactive newly synthesized mRNA was subsequently hybridized with a plasmid containing the entire translated region of the mouse *P2Y$_1$* gene, pUC-19, or cDNA for 18 S ribosomal RNA (10 μg each). Band intensities were quantified with the electrophoresis documentation and analysis system. Data are from one experiment representative of three performed and PEG-rHuMGDF increased the P2Y$_1$ signal 2-fold as assayed by this method.

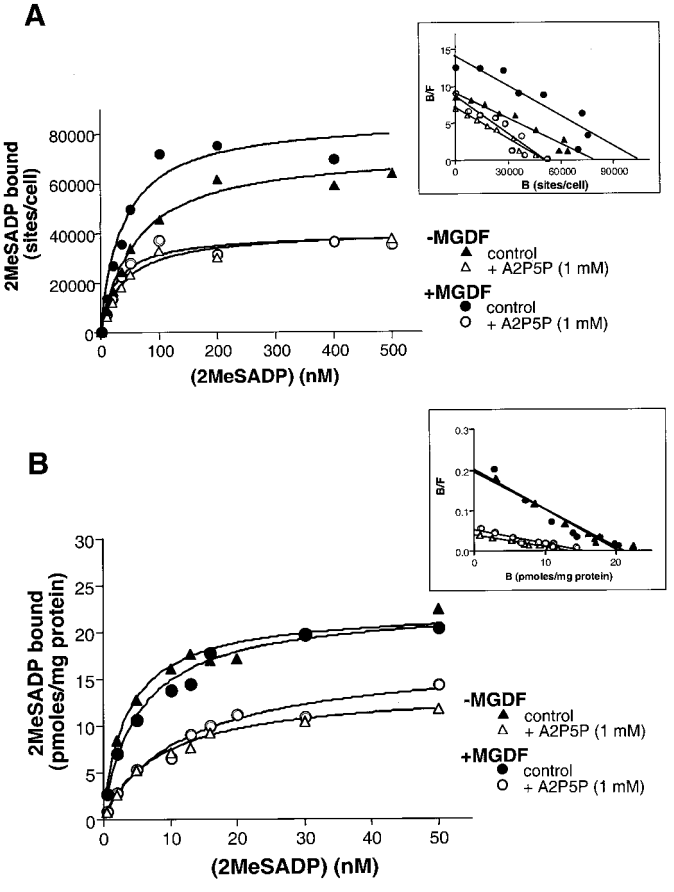


Fig. 4. PEG-rHuMGDF up-regulates P2Y$_1$ receptor density on Y10/L8057 cells but not in Y10/L8057 cell membranes. Y10/L8057 cells were cultured for 48 h in the presence or absence of PEG-rHuMGDF (MGDF) (25 ng/ml) and P2Y$_1$ receptor densities at the cell surface were evaluated in [^{33}P]2MeSADP binding studies, as described under *Experimental Procedures*. Saturation experiments in the presence or absence of A2P5P (1 mM), a selective P2Y$_1$ antagonist, were used to distinguish [^{33}P]2MeSADP binding sites corresponding specifically to the P2Y$_1$ receptor. A, incubation of Y10/L8057 cells with PEG-rHuMGDF increased their surface expression of the P2Y$_1$ receptor from 18,900 to 34,900 sites/cell. B, the number of P2Y$_1$ receptor binding sites remained similar in membranes of Y10/L8057 cells cultured in the absence (7.8 ± 0.5 pmol/mg protein) or presence of PEG-rHuMGDF (MGDF) (25 ng/ml) (7.6 ± 1.2 pmol/mg protein). Data were analyzed and plotted with the Ligand program (Rodbard et al., 1986) and the experiment shown is representative of three independent experiments performed in triplicate. Inset, Scatchard plot of the saturation experiments.

SADP was not the same in our cultured cell system (73 nM) as in mouse platelets (9 nM), probably because of differences inherent to the cell types. It is indeed possible that the ligand affinities of megakaryocytes differ from those of platelets through differences in the compositions of the coupling proteins. In addition, it cannot be excluded that the ectonucleotidase activity is higher on Y10/L8057 cells than on platelets. This could lead to more extensive degradation of 2MeSADP at the cell surface, resulting in a lower affinity of the $P2Y_1$ receptor for its ligand on Y10/L8057 cells. It must be emphasized, however, that the [^{33}P]2MeSADP binding sites on Y10/L8057 cells could be unambiguously assigned to the $P2Y_1$ receptor, because the radioligand was selectively displaced by the specific $P2Y_1$ antagonists A2P5P and MRS2179.

Aggregation studies using washed platelets from mice treated or not with PEG-rmMGDF (25 μg/kg) revealed no significant difference in the extent of aggregation in response to 5 μM ADP (Fig. 6B, top), whereas at a lower concentration of ADP (1 μM), the aggregation profile was the same in platelets from the two groups of animals (Fig. 6B, middle). To focus on the functional response specifically induced through activation of the $P2Y_1$ receptor by ADP, the $P2Y_{12}$ receptor was blocked with AR-C69931 MX (10 μM), a selective $P2Y_{12}$ antagonist (Ingall et al., 1999). The residual aggregation response induced by ADP (5 μM) was then identical between platelets from mice treated with PEG-rmMGDF and control cells (Fig. 6B, bottom).

Levels of expression of the platelet surface markers GPIbIIIa, GPIbIX, and GPV, as determined by flow cytometry, remained similar on platelets from mice injected or not with PEG-rmMGDF (25 μg/kg) (Fig. 7). This is an interesting observation because Mpl ligand has been reported to increase GPIIbIIIa mRNA and protein levels in megakaryocytes (Zauli et al., 1997; Thompson and Ravid, 1999) as it does $P2Y_1$ mRNA.

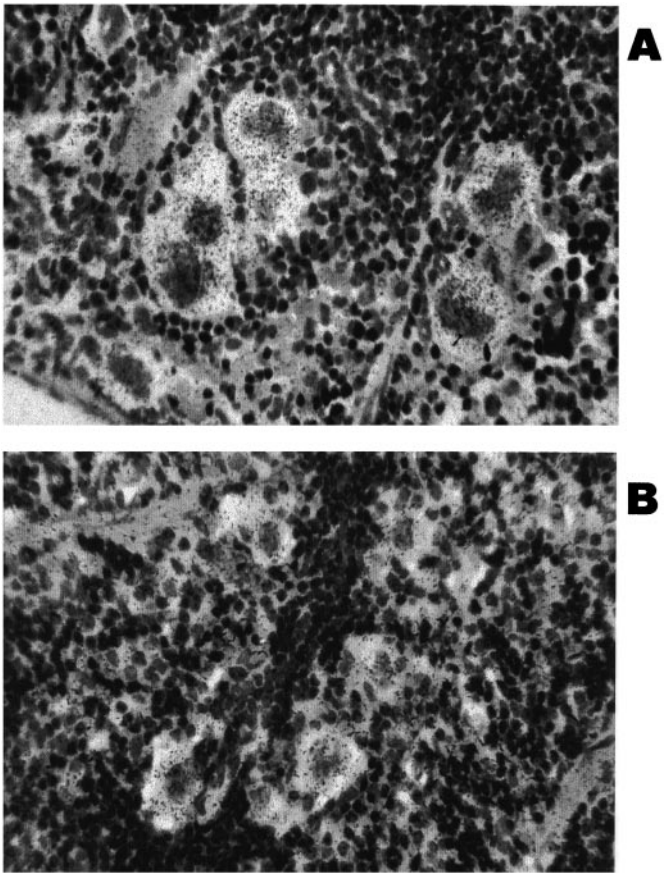


Fig. 5. PEG-rmMGDF up-regulates $P2Y_1$ mRNA in vivo in primary megakaryocytes of mice. Spleen sections from FVB mice injected with PEG-rmMGDF (50 μg/kg) or vehicle were hybridized with a ^{35}S-labeled antisense riboprobe for $P2Y_1$ and autoradiography was performed for 4 weeks. Control experiments using the sense riboprobe gave no signal (data not shown). Megakaryocytes were compared on sections from animals treated with PEG-rmMGDF (A) or vehicle (B). Calibration tools allowing the measurement of cell size (OpenLab Scientific Imaging software; Improvision, Lexington, MA) connected to the microscope (Olympus, Melville, NY) were employed in further analyses. However, this method of size analysis provides only a rough estimation, as the cells in these spleen sections are sectioned at different planes in the different samples. Slides were stained with hematoxylin and eosin before examination by bright field microscopy. Magnification, 300×.

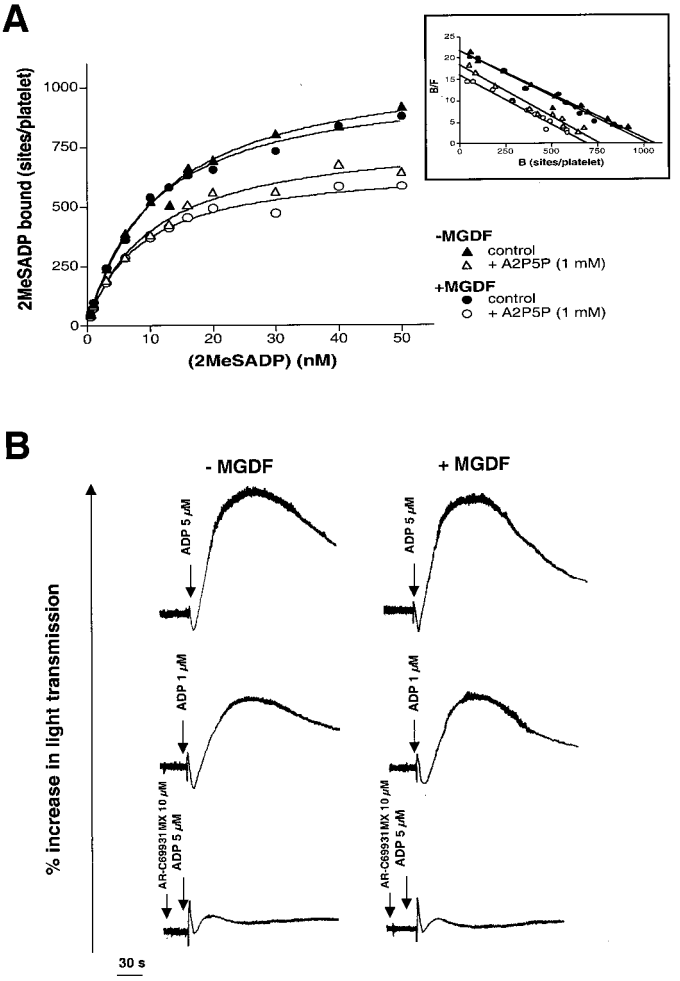


Fig. 6. PEG-rmMGDF does not alter platelet $P2Y_1$ receptor density or reactivity in mice. A, saturation binding of [^{33}P]2MeSADP to platelets from mice treated or not with PEG-rmMGDF (25 μg/kg), in the presence or absence of A2P5P (1 mM). Points are the means of triplicate determinations in a single experiment representative of two independent experiments. Inset, Scatchard plot. B, in vitro aggregation profiles of platelets from mice treated or not with PEG-rmMGDF (25 μg/kg). Aggregation in response to ADP (5 μM or 1 μM) was similar in control animals and mice receiving PEG-rmMGDF (top and middle). In the presence of the $P2Y_{12}$ antagonist AR-C69931 MX (10 μM), ADP (5 μM) induced identical aggregation of platelets from mice injected with PEG-rmMGDF and control cells (bottom).

Discussion

To determine whether Mpl ligand could affect expression of the $P2Y_1$ receptor during megakaryocytopoiesis, we performed studies in the mouse megakaryocytic cell line Y10/L8057. These cells can be generated in large quantities and have been shown to respond to a truncated form of Mpl ligand, PEG-rHuMGDF, through an increase in ploidy and enhanced expression of platelet specific proteins such as GPIIb (2- to 3-fold) and platelet factor 4 (6 fold) (Zhang et al., 1998; Thompson and Ravid, 1999), thus acquiring the characteristics of differentiated megakaryocytes. The in vivo effects of Mpl ligand on levels of $P2Y_1$ mRNA in primary megakaryocytes were also examined in mice.

In accordance with previous reports describing RT-PCR amplification of $P2Y_1$ cDNA from various human megakaryoblastic cell lines (Léon et al., 1997; Jin et al., 1998b; Kaushansky, 1999), $P2Y_1$ mRNA was found to be present in Y10/L8057 cells (Fig. 1). Because the phenotype of control untreated Y10/L8057 cells is megakaryoblastic, with a low percentage of acetylcholinesterase-positive cells and a ploidy distribution displaying a majority of 2N cells (Zhang et al., 1998), the $P2Y_1$ receptor seems to be expressed early in megakaryocyte maturation. Three different $P2Y_1$ mRNAs with sizes of 2.5 kb, 4.4 kb, and 7.4 kb were present in Y10/L8057 cells. The 4.4-kb $P2Y_1$ mRNA was the most abundant in resting cells, whereas the 7.4- and 2.2-kb $P2Y_1$ mRNAs were of similar abundance (Fig. 1). Northern blot analyses using probes corresponding to different regions of the mouse *P2Y₁* gene indicated that the different sizes could result from alternative splicing of the unique intron, together with use of various polyadenylation sites present in the gene. The production of $P2Y_1$ mRNA of three different sizes might play a role in modulating translation efficiency and therefore receptor expression levels. These three transcripts might also be involved in the tissue-specific distribution of the receptor. It should be pointed out, moreover, that the occurrence of three $P2Y_1$ transcripts is not a property of Y10/L8057 cells; three molecules were also detected by Northern blotting in mouse heart, brain, liver and kidney (C. Léon, personal communication).

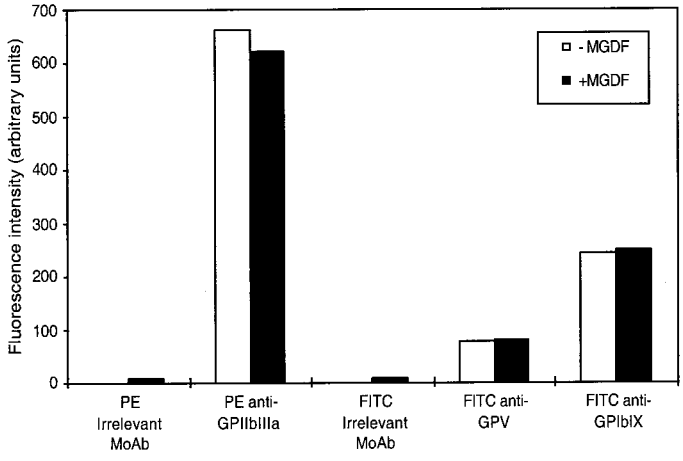


Fig. 7. Flow cytometric determination of lineage-specific markers on murine platelets. GPIIbIIIa, GPV, and GPIbIX expression on platelets from control mice (white bars) and mice injected with PEG-rmMGDF (25 μg/kg) (black bars) was detected with the JON1, DOM2, and POP1 mAbs, respectively. Flow cytometric data are expressed as the fluorescence intensity of the PE- (JON1) or FITC-conjugated mAb (DOM2 and POP1) and mean values were calculated from two separate experiments.

Although the half-lives of the three $P2Y_1$ mRNAs were not affected by PEG-rHuMGDF (Fig. 2), the very short lives of these transcripts (1 to 2.5 h) suggests that expression of the $P2Y_1$ receptor could be rapidly modulated by changes in gene transcription or mRNA stability. Moreover, because platelets are unable to transcribe new mRNA because they have no nuclei, young platelets may be able to synthesize $P2Y_1$ receptor protein from reminiscent megakaryocyte mRNA, whereas old platelets may not. Despite the current lack of experimental data concerning the stability of the platelet $P2Y_1$ protein, this means that the short half-life of $P2Y_1$ mRNA could be of functional importance in terms of platelet reactivity.

In the present study, we demonstrated that the increase in $P2Y_1$ mRNA levels in Y10/L8057 cells arises through a direct action of PEG-rHuMGDF on the rate of transcription of the *P2Y₁* gene (Fig. 3), which implies that the gene contains specific elements responsive to PEG-rHuMGDF. It was recently shown that several transcription factors including Sp1 (Zhang et al., 1998), PU.1/Spi-1 (Doubeikovski et al., 1997), and CREB (Zauli et al., 1997) are activated by Mpl ligand and are responsible for the cytokine-dependent transcriptional activation of genes, such as those of GPIIb (Doubeikovski et al., 1997; Zauli et al., 1997) and cyclin D3 (Zhang et al., 1998). It remains to be determined whether one or more of these transcription factors is responsible for the enhancement by PEG-rHuMGDF of transcription of the *P2Y₁* gene.

The rise in $P2Y_1$ receptor mRNA correlates with an increase in $P2Y_1$ protein levels at the surface of Y10/L8057 cells (Fig. 4A). This increase in $P2Y_1$ mRNA induced by PEG-rHuMGDF is not unique to a megakaryocytic cell line; it also occurred in the primary megakaryocytes of mice injected with the recombinant murine cytokine (Fig. 5). Although circulating platelet levels rose 2-fold in these mice, the platelet $P2Y_1$ receptor density (Fig. 6A) and aggregation profile in response to ADP (Fig. 6B) were not significantly modified by PEG-rmMGDF treatment. Thus, the enhanced $P2Y_1$ expression in megakaryocytes led to an increase in neither the density of the $P2Y_1$ receptor on platelets nor their reactivity to ADP. It is important to note that PEG-rHuMGDF did not increase the density of $P2Y_1$ receptors per milligram of protein of Y10/L8057 cell membranes. Thus, it seems that the rise in $P2Y_1$ receptor expression induced by Mpl ligand parallels the well documented global increase in megakaryocyte size and ploidy, the extensive development of demarcation membranes that precedes platelet production, and the rise in platelet levels (Kaushansky, 1999), so that the $P2Y_1$ receptor density on the resultant platelets remains stable. A similar phenomenon has been reported for GPIIb, which is enhanced in megakaryocytes under Mpl ligand treatment (Zauli et al., 1997), without any corresponding increase in levels of the protein in platelets (Harker et al., 1996b; O'Malley et al., 1996). Furthermore, expression of the platelet surface glycoproteins GPIIbIIIa, GPIbIX, and GPV remained comparable in the present work in mice treated with PEG-rmMGDF and control animals (Fig. 7).

Mpl ligand is currently under clinical investigation as a drug to stimulate platelet production in patients with bone marrow failure. Plasma levels of Mpl ligand are inversely related to the platelet count and it has also been clearly established in vitro that this cytokine potentiates platelet

aggregation in response to various agonists including ADP (Oda et al., 1996, 1999). If this were applicable in vivo, a cumulative effect would have been expected to create a risk of thrombotic complications in patients receiving Mpl ligand. However, a recent study reported no change in the ADP response of washed platelets isolated from healthy human volunteers treated with recombinant Mpl ligand (Harker et al., 2000), in agreement with an earlier finding in nonhuman primates (Harker et al., 1996b) and with our current findings in mice. Because the platelet $P2Y_1$ receptor plays a key role in hemostasis and thrombosis (Cattaneo and Gachet, 1999; Gachet, 2001), this apparent lack of increased platelet reactivity to ADP suggests that clinical use of Mpl ligand to promote platelet production might not in fact be associated with adverse effects of platelet hyper-reactivity.

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