# Thrombopoietin in Patients With Congenital Thrombocytopenia and Absent Radii: Elevated Serum Leveis, Normal Receptor Expression, But Defective Reactivity to Thrombopoietin

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The pathophysiology of thrombocytopenia in the syndrome of thrombocytopenia with absent radil (TAR) is not yet un derstood. We examined thrombopoietin (TPO) serum leveis and the in vitro reactivity of platelets to TPO in five patients affected with TAR syndrome. We found elevated TPO serum leveis in all patients tested, excluding a TPO production de fect as cause for thrombocytopenia in TAR syndrome. In addition, we found similar expression of the TPO receptor c-MpI on the surface of platelets from TAR patients (5 of 5) and a similar molecular weight of the receptor as compared with healthy controls (4 of 4). Platelet response to adenosine diphosphate or thrombin receptor agonist peptide SFLLRN

(TRAP) was normal in TAR patients. However, in contrast to results with healthy controls we could show absence öf in vitro reactivity of platelets from TAR patients to recombi nant TPO as measured by testing TPO synergism to adenine diphosphate and TRAP in platelet activation. TPO induced tyrosine phosphorylation of platelet proteins was com pletely absent (3 of 4) or markedly decreased (1 of 4). Our results indicate that defective megakaryocytopoiesisl thrombocytopoiesis in TAR syndrome is not caused by a defect in TPO production but a lack of response to TPO in the signal transduction pathway of c-Mpl.



THROMBOCYTOPENIA WITH absent radii (TAR) is a rare congenital defect wit« hypomegakaryocytic

thrombocytopenia and bilateral radial aplasia. Although first described in 1929,1 it was defined as a syndrome by Hall et al in l969.² TAR seems to be inherited in an autosomal recessive manner.3 The pathogenesis of TAR is poorly un derstood. Bone marrow aspirates from TAR patients show either decreased or absent megakaryocytopoiesis, indicating that the thrombocytopenia is caused by a defective platelet production.

There are inconsistent reports about "thrombopoietin like" activity and megakaryocyte colony-stimulating activ ity (Mega-CSA) in the serum of TAR patients.4~7

Uhe presence of colony forming units for megakaryocytes (CFU-Mega) in the bone marrow of TAR patients is controversial, too. Whereas some investigators did not find any growth of megakaryocytic colonies from the bone marrow of TAR patients,4,6 de Alarcon et a17 showed normal CFU Mega counts with an abnormal colony morphology. Cells in these colonies were sma1ler, and the number of cells per colony was much higher than in normal CFU-Mega-derived colonies.

Platelet function seems to be normal in TAR patients,3 but thei-e are also some reports about abnormal platelet mor phology and functioh, 8,9

In 1994 several groups purified and cloned a new hemato poietic factor: thrombopoietin (TPO).10-14 Since then, this

cytokine has been ~hown to be the major reuulator of mega karyocytopoiesis and thrombocytopoiesis. Investigations of the in vitro and in vivo effects of TPO showed that it acts as a Mega-CSF as weil as a megakaryocyte maturation factor and pösitive regulator of platelet produetion in vivo.15 The receptor for TPO, the product of the proto-oncogene c-mpl, 1' 6s expressed in the megakaryocytic lineage from progenitor cells to platelets.17 c-mpl, a cellular homologue of the v-mpl oncogene,' 8was found to be a member of the cytokine receptor superfamily 16,19 that share common signal transduc tion pathways.20 Binding of the ligand leads to tyrosine phosphorylation of some cellular proteins in cell lines and platelets.21~23

Our aim was to elucidate a possible relationship between the TAR-related thrombocytopenia and the TPO-c-Mpl sys tem. Here we present the first report about TPO serum levels in TAR patients and about the in vitro reactivity of platelets from TAR patients to recombinant human (rh) TPO. Our results indicate that defective megakaryocytopoiesislthrom bocytopoiesis in TAR syndrome is

not caused by a defect in TPO production but by a lack of response to TPO in the signal transduction of c-Mpl.

## MATERIALS AND METHODS

Patients. We examined samples from five unrelated chudren with TAR syndrome. The patients' characteristics are described in detail in Table 1. lt is important to note that four of the patients required platelet transfusions.

Patient 1 was a 3-year-old girl with bilateral absent radii and hypoplastic, curved ulna and with platelet counts usually about 50,000 /µL. She needed three platelet transfusions during the first year of life. Aside from this, a clinically irrelevant ventride septum defect (VSD) was stated.

Patient 2 was a 3-year-old gin with i~Iateral absent radii and short ulna with platelet counts usually approximately 20,000 /µL. During infections she required platelet transfusions because of bleedings from nose and rnouth.

Patient 3 was a 6-week-old boy at first examination. He showed bilateral absent radii, a short ulna right, an ulna aplasia leif, and a bilateral hip Üranslocation. Because of a severe thrombocytopenia he needed platelet transfusions twice a month for the first 3 months of life. Platelet counts were between 25,000 at the time of the first examination and 72,000 at the age of 10 months.

Patient 4 was a 6-month-old baby gin. Bilateral absent radii and

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**Table 1. TAR Patients**

| Patient No. | Sex | Absent Radii | Requirement of Platelet Transfusions | Age | WBC ($\mu L^{-1}$) | Hgb (g/dL) | Plt ($\mu L^{-1}$) | Tests Performed* |
|---|---|---|---|---|---|---|---|---|
| 1 | F | + | + | 2 yr 6 mo | 8,100 | 11.7 | 57,000 | 1, 2, 3, 4, 5 |
| | | | | 3 yr 7 mo | 6,700 | 12.1 | 87,000 | 3, 6 |
| 2 | F | + | + | 1 yr 11 mo | 15,000 | 8.6 | 28,000 | 1, 3, 4, 5 |
| | | | | 2 yr 9 mo | ND | ND | 31,000 | 1, 2, 3, 4, 6 |
| 3 | M | + | + | 6 wk | 10,800 | 8.0 | 25,000 | 1, 5 |
| | | | | 10 mo | 11,900 | 13.6 | 72,000 | 1, 2, 3, 4, 6 |
| 4 | F | + | – | 6 d | ND | ND | ND | 7 |
| | | | | 6 mo | 27,000 | 8.7 | 10,000 | 1, 2, 3, 5 |
| 5† | F | + | + | 6 yr 11 mo | 12,000 | 10.7 | 12,000 | 1, 2, 6 |
| | | | | 7 yr 8 mo | 8,400 | 8.4 | 12,000 | 1, 2, 3, 4, 5 |

Abbreviations: WBC, white blood count; Hgb, Hemoglobin; Plt, platelets; ND, not determined.

* Code for the different examinations: 1, TPO serum level; 2, IL-11, IL-6, LIF level; 3, c-Mpl (flow cytometry); 4, c-Mpl (Western-blotting); 5, TPO reactivity (flow cytometry); 6, TPO reactivity (Tyrosine phosphorylation); 7, CFU-mega.

† This patient has been described in an earlier abstract.[6]

short ulnae, as weil as a severe thrombocytopenia. lead to the diagno- sis of TAR. Megakaryoeytes were completely absent in a bone mar row sample from the first week of life. This sample was used for the clonogenic assay presented here.

Patient 5, a 7-year old girl, has been described in an earlier publi- cation by us.6 She showed absent radii and short u1nae~ thumb hypo- plasia and shoulder-girdle involvement. in addition, a VSD was stated. In contrast with other reports about TAR-syndrome. bleeding problems continued up to now. For instance, in the last vear she had problems with petechiae and bleeding w ith platelet counts between 10,000 and 2O,OOO /µL.

Matenais. rhTPO was provided by Dr A. Shimosaka Kinn Brewery (Tokyo, Japan). rh granulocyte colony-stimulating factor (G-CSF) and rh stern cell factor (SCF) were provided by Amgen (Thousand Oaks, CA). rh granulocyte-macrophage colony-stimuiat ing tactor (GM-CSF) and rh interleukin-3 (IL-3) were a gift frorn Behringwerke (Marburg, Germany), rh erythmpoietin (EPO) was obtained from Boehringer Mannheim (Mannheim Germanv). Aden osine diphosphate (ADP), prostaglandin El (PGEI), acetyl salicvlic acid, apyrase (type VIII), and bovine serum albumine (BSA) were purchased from Sigma (Deisenhofen, Germany). The thrombin re ceptor agonist

peptide (serine-phenylaiani ne-leucine-ieucine-arginine-asparagine, TRAP) was purchased from Bachem (Heidelberg Gerrnany). Monodonal anti bodies against CD62 and CD4 1, as weil as the IgG isotype control were purchased ftom Immunotech (Ham burg, Germany), the monodonal antibody against human c-Vlpl was obtained from Genzyme (Rüsselsheim Germany), the horse-radish peroxidase (HRP)-conjugated recombinant antiphosphotyrosine anti body RC2O was purchased from Transduction Laboratories (Lexing ton, KY). The reagents for the enhanced chemiluminescence (ECL) and the 3H-thymidine were obtained from Amersham (Braun schweig, Germany). Ccli culture media and fetal calf serum were purchased from Life Technologies (Eggenstein, Germany).

TPO serum leveis. TPO serum leveis were measured in a bioas say using the murine IL-3-dependent 32D (elone 23) cells24 trans fected with the human c-mpl.' 2 Serum samples were preabsorbed with the parental 32D (done 23) cells to remove compiement activ ity. For the assay, 32D/MpI ceils were washed free of IL-3 and plated (3,000 ceils, 100 µL total volume per weil) in flat-bottomed 96-weil piates (Nunc, Wiesbaden, Germany:) in RPMI 1640 medium suppiemented with 5% heat-inactivated fetal caif serum in 100 µL serial dilutions of test sera in assay medium. After 72 hours, the microtiter cultures were pulsed with 3H-thymidine (0.5 ,iCi/weil, specific activity 5 Ci/mmol) for another 4 hours. The radioactive uptake was determined in a liquid scintillation counter. Serial dilu tions of rhTPO in preabsorbed human serum were used as standards

the concentrations of the samples were calcuiated from the standard curve by probit analysis. The sensitivity of the assay was 100 pg/ mL.

Senirn leve/s of JL-6, IL-Il, aiid leitkeinia inhibitorvfactoi (LIF). Serum levels of IL-6 and IL- 11 were measured in commerciaily available enzyme-linked immunosorbent assay (ELISA) systems (Quantikine. R&D systems, Abingdon. UK). Detection iimits were 8 pg/mL for IL 6 and 30 pg/mL for IL 11 Serum leveis ot LIF were measured in a sandwich ELISA.²5 Briefly, a monodonal antibody (MoAb) against LIF served tor capturin a polvclonal rabbit antise rum was used tor detection. The detection limit of this assay was 30 pg/mL.

Assay for CFUs. Bone marrow mononudear edis (BM-MNCs, 105) obtained by densitv gradient centrifugation with Ficoii were cultured in dup1icates in 1 mL aliquots in a semi solid medium containing 0 7% methyl cellulose (vlethocel A4C WAK Chemie Bad Homburo Germanv), 30% human tresh trozen plasma (Blood Bank Medica1 Schoo1 Hannover Germany), 0.5 x 10-9) mol/L 2 mercaptoethanol in Iscove' s modified Dulbecco' s medium in 35- mm culture dishes. Hematopoietic growth factors were added as specified in the Results section. The cultures were incubated at 370C in an atmosphere of 5% C02 and 100% humidity for 14 days. After this time, colonies were analyzed and counted in an inversion micro scope. Megakaryocytic colonies were picked, spinned on sudes, and May-GrünwaldlGiemsa stained for verifying the megakaryocytic morphology of the cells.

Costiinukition of' J(1teiets with TPO. Blood of heaithy volun teers or TAR patients was obtained from an antecubital vein through a 19-gauge needle with oniy a light toumiquet into a piastic syringe containing trisodium citrate (10 mmol/L final concentration). Stimu lation experiments were

started within 15 minutes after blood collec tion. Experiments were usually performed with whole blood, and in some cases (platelet counts < 50,000/µL) we used platelet-rich plasma (PRP). For preparing PRP, whoie blood was centrifuged at 200g for 20 minutes and the supematant PRP was removed. All incubations were done at 370C. Five to 10 µL aliquots of blood or PRP containing2 X 105 to 2 x 106 piatelets were added to polysty rene tubes containing 60 µL of phosphate-buffered sahne (PBS; 130 mmol/L NaCI, 10 mmol/L sodium phosphate, pH 7.5) with various concentrations of rhTPO (20 nglmL, unless indicated otherwise). After a preineubation of 5 minutes, the platelet activators ADP (final concentration 50 µmol/L) or TRAP26 (final concentration 5 µmol/ L) were added. The stimulation was stopped by addition of 1 rnL of a solution of 1 % formaldehyde in PBS. Unstimulated platelets serving as a negative control were fixed immediately after blood collection or preparation of PRP, respectively. The samples were



stored for 30 minutes on ice before they were stained for flow cytometric analysis.

Flow cytomnetry. Fluorescei n isothiocyanate (FITC)-conj ugated MoAb anti P-selectin (CD62; clone CLB-Thromb/6) as anactiva tion-dependent antibody and phycoerythrine (PE)-conjugated anti gplIbIIIIa (CD4 1; clone P2) as a pan-platelet marker were used for determination of platelet activation. FITC- and PE-labeled isotype control antibodies (Immunotech, Hamburg, Germany) were used as control. Fixed platelets were pelleted (5 minutes, 2,00g), washed two times in FACS-buffer (PBS supplemented with 0.1 % BSA and 0,1 % sodium azide and then resuspended in 40 µL of a diluted human Ig solution (10 mg/mL GammaGard; Baxter. Un terschleißheim, Germany) for blocking the Fc-receptors. After a short preincubation 10 µL of each antibody solution was added followed by a 20-minute incubation on ice. To determine the c-Mpl expression on patients' platelets we used an anti-c-Mpl MoAb (vi 1.17 Genzyme): Piatelets were fixed and preincubated with human Ig as described before. Cells were incubated with the M 1 antibody (10 µL in the Ig-solution) or a nonspecific isotype control antibody for 30 minutes on ice. Cells were washed in FACS-buffer and then incubated with FITC-conjugated rabbit antimouse Ig (Dako, Glostrup, Denmark) according to manufacturer' sinstructions.

After staining, platelets were washed two times in FACS-buffer and analyzed on a FACScan flow cytometer (Becton-Dickinson). FITC ~uorescence was detected with a 530/30 nm and PE fluores cence was detected with a 585/42 nm band pass filter. Light scatter and fluorescence data were obtained with gain settings in the ioga rithmic mode. Platelets were distinguished from other cells, debris, and machine noise" on the basis of their scatter profile. In some cases a fluorescence threshold was set to analyze oniy those blood cells tliat had bound the PE-conjugated anti-CD4 1 antibody.

Platelet preparation for gel electrophoresis and ilnmliloprecipitation. PRP from whole blood was prepared as described above. PRP was incubated with acetyl salicylic acid (2 mmol/L) for 30

minutes at room temperature. Then PGE 1 (1 μmol/L) was added from a 1- mmol/L stock solution in absolute ethanol. A soft pellet was obtained by centrifugation of the PRP at 800g for 10 minutes. The peilet was resuspended in a modified HEPES-Tyrode buffer as recently described21 also containing apyrase (2 U/mL) to avoid stimulation of platelets by ADP and washed only once to minimize additional physical stress. The platelets were resuspended in the same buffer, which was recalcified containing 1 mmoi/L CaCi2 at 370C.

Gel eiectrophoresis and Western blotting. The stimulation of platelets was terminated by adding an equal volume of 2x-concen- trated sample buffer (10% giycerol, 1 % sodium dodecyi sulfate [SDS], 5% 2-mercapto-ethanol, 50 mmol/L Tris-HC1 pH 6.8, 10 mmolIL EGTA and 1 mmol/L sodium orthovanadate and 0.002% bromophenol blue). SDS-gel electrophoresis of platelet proteins was run on a 7.5% polyacrylamide gel after boiling the samples for 5 minutes.

The proteins were transferred onto a nitrocelluiose membrane by semidry Western blotting with a buffer containing 50 mmol/L Tris, 40 mniol/L glycine, 0.04% SDS, and 17.5% methanol for 1.5 hours at room temperature.

For blocking unoccupied binding sites the membrane was incu bated in PBS-T (0.1% Tween 20 in PBS) with 1% BSA (blocking buffer). For the detection of phosphotyrosine or c-Mpl the membrane was incubated in blocking buffer with MoAb against c-Mpl (done M 1,1:1,000) or with HRP-conjugated recombinant MoAb against phosphotyrosine (RC2O, 1:2 ,500) overnight at 40C with gentle motion.

In the case of c-Mpl the MoAb was removed and the membrane was washed four times with PBS-T and incubated with HRP-conju gated second antibody (dii uted 1:1,000 in PBS-T) for 1 hour at room temperature with gentle motion.

After washing the blots in PBS-T four times the antibody reactions were detected by chemiluminescence with the ECL reagents (Amer sham) according to the manufacturer' s instructions.

hnmunoprecipitation. The platelet stimulation was terminated by adding an equal amount of lysis buffer [15 mmol/L HEPES, 150 mmolIL NaC1, 1 mmol/L phenylmethylsulfonyl fluoride (PVISF), 10 mmol/L EGTA, 1 mmol/L sodium orthovanadate 0.8 mg/mL leupeptin, 2% (vol/vol) Triton X-100, pH 7.4). The samples were incubate d for 20 to 30 minutes on ice and atterwards the debns was pelleted at l0,OOOg for 20 minutes at 40C. The supernatant was removed and precieared by adding 50 μL of 50% slurry of protein A-agarose-beads (Upstate Biotechnology, New York. NY) for 1 liour in an orbital shaker at 40C. The beads were centrifuged at 10,000g for 10 minutes, and the supematant was transferred into a new tube. The MoAb against human c-Mpl (Genzyme) was added and incu bated for 4 hours or overnight in an orbital shaker at 40C. Then the complex was captured adding protein A-agarose-beads (50 μL of 50% slurry) for another 2 to 4 hours.

The immune complexes were washed three times with ice-cooled immunoprecipitation-buffer (50 mmol/L Tris-CI: pH 7.4;1% Noni det P-40; 150 mmol/L NaCT; 1 mmol/L EGTA; 1 nimoliL PMSF:

1 µg/mL of the protease inhibitors aprotinin. leupeptin, and pep statin; 1 mmol/L sodium vanadate; and 1 mmol/L sodium fluoride). resuspended in an equal volume of 2X-sample buffer, and detected by Western biotting after SDS-PAGE.

## RESULTS

Senim leveis of TPO, IL-6, IL-I1, and LIF. In healthy control persons TPO serum leveis normally were not detect able in the bioassay (detection Jimit: 100 pg/mL). In contrast, TPO serum levels were above the detection limit in all serum samples from TAR patients with the exception of the first serum sample of patient 2 (Table 2).

Leveis of other cytokines known to influence thrombocy topoiesis were evaluated in the sera of the TAR patients. Serum levels of IL-6 and LIF were undetectable in all cases (detection limits 8 respectively 30 pg /mL). In contrast IL 1 1 serum levels were elevated above 300 pglmL in three out of five TAR patients (Table 2).

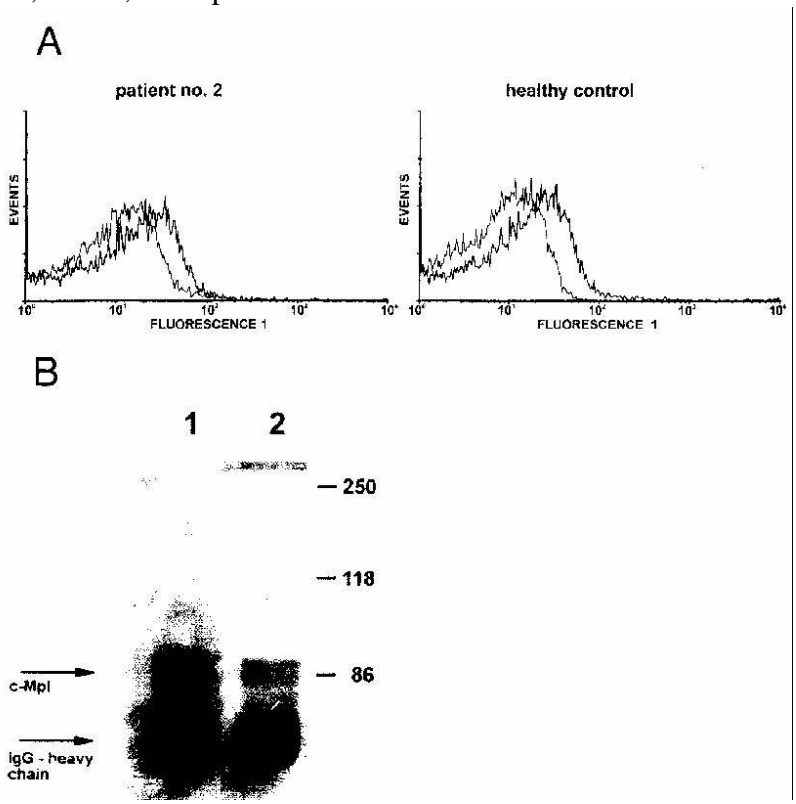
CFU-mega assay. Colony forming assays were per formed with bone marrow cells of one patient only (no. 4). We had no consent of the other patients' pæents for bone marrow evaluation and there was no clinical need for bone marrow evaluation. In the patient tested, no megakaryocytic colonies were observed after stimulation of BM-MNC with rhTPO (10 ng/mL; Table 3). For comparison, in heaithy controls tlie median number of megakaryocytic colonies was 11 (n = 12). In contrast, the numbers of CFU-GM and BFU E colonies grown from the TAR patients' bone marrow were elevated as compared with healthy controls (Table 3;).

Expression of c-Mpl on piatelets. Expression of the TPO receptor c-Mpl on platelets was determined by flow cytome try in all patients and by Western blotting in four out of five TAR patients (patients no. 1, 2, 3, and 5) (Fig 1). Because the receptor is presumably expressed on all cells of the mega karyocytic lineage, we used platelets as an example for c Mpl expression in TAR patients. We could show a specific binding of a MoAb against the c-Mpl on platelets of all patients tested. Fluorescence intensity after staining with the anti-c-Mpl in platelets from the TAR patients was in the range of normal controls (Fig lA). The molecular weight of

The TAR syndrome is a rare eongenital disorder eharaeter ized by the absenee of radb on both arms and severe throm boeytopenia during the first years of life. Reeent studies showed that the

thromboeytopenia seen in TAR patients is eaused by a defeet in the megakaryocytopoiesis/thromboey topoiesis.4~7 Different possible meehanisms for this bone marrow failure ean he postulated: (1) absenee of humoral or eellular stimulators of megakaryoeytopoiesis, (2) absenee of megakaryoeytie progenitor eells, (3) eellular defeets in megakaryoeytie preeursors (eg, reeeptor defeets), or (4) pres

Fig 1. Expressicn of c-MpI on piatelets from TAR patients. IAI c-MpI expression on the surface of plateiets es measured by flow cytometry see Materials and Methodsl. Grav. isotype Gontrol; black. anti c-Mpi. IB Detection of c-Mpl in pletelet lysates after immunoprecipitetion and Western blot analysis with anti-c.Mpl entibody Ml lchemo luminescence detection, See Meterjeis and Meth odsl. Lene 1, heelthv donor; lene 2, TAR patient na. 1.




enee of humoral or edlular inhibitors of megakaryoeyto poiesis.

There are eontroversial reports eonceming Mega-CSA or "TPO-like" aetivities in the sera of TAR patients. Our group6 and others4 found high Mega-CSA in the sera of TAR patients. However, de Alareon et a17 reported Mega-CSA and "TPO-like" activities in the serum of one TAR patient. whieh were within the range of normal and below the high levels seen in amegakaryoeytie subjects. Miehaleviez et a15 even found an inhibitory effeet of plasma of one TAR patient on the growth of multipotent and megakaryoeytie progeni tors. After the diseovery of TPO and its reeeptor e-Mpl we were able to measure the aetivity of this major humoral regulator of megakaryoeytopoiesis and thrombocytopoiesis in the sera of TAR patients. We found elevated levels of bioaetive TPO in sera from all TAR patients tested, exelud ing a TPO produetion defeet as the eause of thrombocyto penia in TAR syndrome. lt is a eommon phenomenon that

lineage speeifle hematopoietic growth factors are inversely regulated with the number of

eorresponding mature eells in peripheral blood. This has been shown for G-CSF,28 EPO.29,30. and TPO31,32 and was found to be the ease in a number of eongenital eytopenias as the severe eongenital neutropenia. also.33 The first determination of TPO serum levels of patient 2 yielded no elevated level of this eytoldne. We have no explanation of that diserepancy. However, the first determi nation has been made with a relatively old serum sample and we eannot exelude a loss of activity during inappropriate storage. On the other hand, variations of TPO levels in TAR syndrome might be the reason for differenees in former re ports.

The presence of eolony forming units megakaryoeyte (CFU-Mega) in the bone marrow of TAR patients is another pomt of eontroversy in the literature. Whereas most groups, like us, did not find any growth of megakat'yocytic eolonies from TAR patients.4' 6 el Alareon et a17 showed normal CFU

**Table 2. Levels of TPO, IL-6, IL-11, and LIF in Sera of TAR Patients**

| Patient No. | Age | Platelet Count | TPO (pg/mL) | IL-6 (pg/mL) | IL-11 (pg/mL) | LIF (pg/mL) |
|---|---|---|---|---|---|---|
| 1 | 2 yr 6 mo | 57,000 | >1,000 | <8 | <30 | <30 |
| 2 | 1 yr 11 mo | 28,000 | <100 | nd | nd | nd |
| | 2 yr 9 mo | 31,000 | >1,000 | <8 | 380 | <30 |
| 3 | 6 wk | 25,000 | >1,000 | nd | nd | nd |
| | 10 mo | 72,000 | 500-1,000 | <8 | 490 | <30 |
| 4 | 6 mo | 10,000 | 500-1,000 | <8 | <300* | <30 |
| 5 | 6 yr 11 mo | 12,000 | 500-1,000 | <8 | 660 | <30 |
| | 7 yr 8 mo | 34,000 | 100-500 | <8 | 330 | <30 |
| Normal range | | | <100 (n = 36) | <8† | <30† | <30 (n = 50) |

Abbreviation: nd, not determined.
* Because of the little volume of the serum sample of patient 4 we had to dilute the serum for IL-11 determination.
† Information provided by the manufacturer of the ELISA system.

the c-Mpl on the platelets from the TAR patients was found tu be 86 kD as determined bv SDS-PAGE and was not different from that of healthy controls (Fig 1B).

Costiruulotjou of p/atelets with TPO. TPO svnereizes with ADP or thrombin receptor agonists to activate platelets as measured by increased expression of P-seleetin (CD62P(.2 We used this fact for in vitro testing of TPO reaetivitv of platelets from TAR patients. (CD62P).²7 served as an aetivation-dependent marker. Using this method, platelets from normal individuals showed a synergism of rhTPO (5 - 10 ng/mL) with the platelet aetivators ADP and TRAP. AI though there was a great variety in the reacüvity of platelets tu the activators ADP and TRAP, platelets from all TAR patienis showed reactivity in the range of normal controls (Fig 2 and Table 4). However, we eould not deteet the ex peeted synergism of these platelet aetivators with rhTPO up to enneentrations of 1 µg/mL in these patients. The results of one representative expetiment are shuwn in Fig 2. To quantitate the synergistic effeet of TPO on platelet activation we ealeulated the ratio between the amount of CD62P-posi tive eells afier TPO costimulation (with ADP or TRAP) and the amount of CD62P-positive eells after control stimulation without TPO (Table 4). TPO preineubation (20 ng/mL) lead tu a l.6-fold (~0.3) enhaneement of ADP stimulation (50 µmol/L) or 1 4-fold (~0.3) enhaneement of TRAP stimula tion (5 µmol/L) in platelets of normal individuals (n = 6). In enutrast, TPO stimulation indiees in TAR patients were

1.0 0.1 for ADP and 1.0 0.2 for TRAP. respeetively

(Table 4).

Table 3. CFU Assay (Day 14)

| | Patient No. 4 | | | Normal Values* Median (range) | | |
|---|---|---|---|---|---|---|
| | CFU-GM | BFU-E | CFU-Mega | CFU-GM | BFU-E | CFU-Mega |
| Control | 0 | 0 | 0 | 0 (0-3) | 0 (0-2) | 0 (0) |
| TPO† | 2 | 0 | 0 | 0 (0-1) | 0 (0-3) | 11 (4-40) |
| G-CSF,† GM-CSF,† IL-3,† SCF,† EPO† | 428 | 507 | nd‡ | 125 (68-240) | 180 (91-431) | nd‡ |

Colony numbers are based upon $10^5$ BM-MNC. Data represent the mean of duplicates.
* Obtained in our laboratory from 12 healthy individuals, using the same experimental conditions.
† 10 ng/mL each, except for EPO (1 U/mL).
‡ Counting Mega-colonies was not possible in dishes with "maximum stimulation" because of the high numbers of colonies.

na/ transducrior of c-Mpl after TPO binding. Becemse platelets express e-Mpl they are used as a mode for investigating signal transduetion pathways of TPO. c-Mpl is a member of the cytokine reeeptor superfamily, whieh leads tu tyrosine phosphorylation of eellular proteins.

We could show that stimulation of platelets with hieb enneentrations of rhTPO for 5 minutes indueed phosphoryla tion of several proteins, espeeially at 86 kD and higher (Fig 3). Stimulation with only 50 ng/mL rhTPO lead tu phosphor vlation of a 1 l0- protein (data not shown). In Contrast, platelets from three nut of four TAR patients (no. 1,2. and 5) ineubated wuh rhTPO enneentrations up tu 12 µg/mL showed no difference in tyrosine phosphorylation as eom pared with unstimulated eells (Fig 3), one other patient (no. 3) showed a weak phosphorylation of the 1 l0-kD protein oniv after stimulation with rhTPO enneentrations hieher than 5 µg/mL.

DISCUSSION

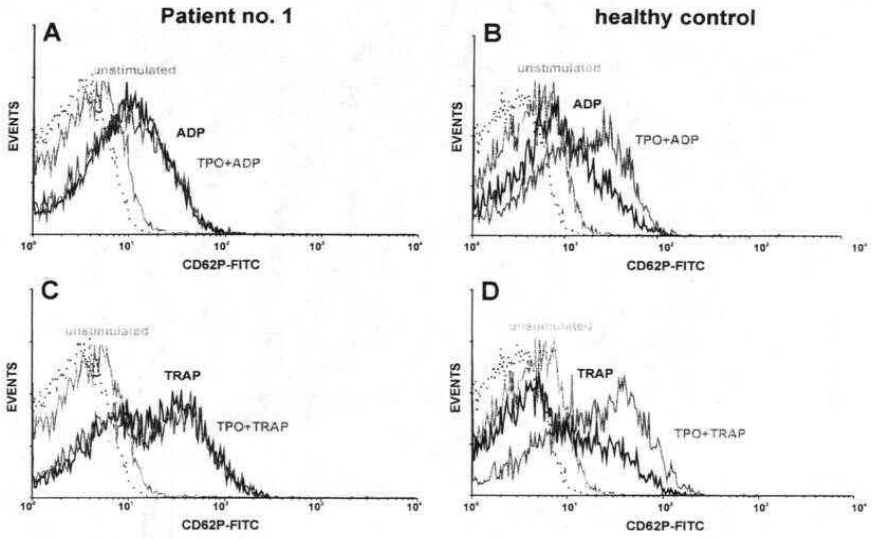



Fig 2. Costimulation of platelets with rhTPO: TAR patient versus healthy control. Platelets of TAR patient no. 1 (A and C) and of a healthy control (B and D) were stimulated with either ADP (50 µmol/L; A and B) or TRAP (5 µmol/l; C and D) with or without preincubation with rhTPO (20 ng/mL). Flow cytometric analysis of platelet activation was performed using a MoAb against CD62P. CD62P expression of unstimulated platelets as well as the isotype control (dotted line) are shown in each histogram.

Mega counts with an abnormal colony morphology. In these colonies cells were smaller and the number of eetls per colony was mueh higher than in normal CFU-Mega derived colonies. In addition to the patient we tested earlier,6 we were ahle to test the presence of CFU-Mega in the bone marrow From orily one TAR patient, a newborn baby-gin whose bone marrow was aspirated for diagnostic purposes.

We could not detect any megakaryocyte colony growth after stimulation with TPO, indicating that megakaryoeyte devel opment is markedly irnpaired. Ilowever, more patients liave tu be tested to malte a final statement conceming megakaryo eytic colony growth in vitro. In contrast, the numbers of nonmegakaryocytic colonies (CFU-GM and BFU-E) stimulated by other growth factors (G-CSF GM-CSF IL-3 SCF

**Table 4. TPO Synergism in Platelet Activation**

| Patient No. | 1 ADP | 2 TPO + ADP | 3 TPO + ADP / ADP | 4 TRAP | 5 TPO + TRAP | 6 TPO + TRAP / TRAP |
|---|---|---|---|---|---|---|
| 1 | 26.8 | 23.3 | 0.87 | 48.6 | 45.4 | 0.93 |
| 2 | 43.9 | 45.3 | 1.03 | 68.5 | 65.6 | 0.96 |
| 3 | 24.9 | 24.0 | 0.96 | 23.9 | 19.9 | 0.83 |
| 4 | 9.8 | 10.9 | 1.11 | 6.8 | 8.6 | 1.26 |
| 5 | 27.3 | 28.7 | 1.05 | 29.6 | 31.4 | 1.06 |
| TAR (mean ± SD; n = 5) | 27 ± 12 | 26 ± 12 | 1.00 ± 0.09 | 36 ± 24 | 34 ± 22 | 1.01 ± 0.16 |
| Healthy control (mean ± SD; n = 10) | 25 ± 11 | 38 ± 10 | 1.61 ± 0.28 | 39 ± 25 | 50 ± 28 | 1.43 ± 0.33 |

Platelets of TAR patients and of 10 healthy controls were stimulated with either ADP (50 μmol/L, columns 1-3) or TRAP (5 μmol/L, columns 4-6) with (columns 2 and 5) or without (columns 1 and 4) preincubation with rhTPO (20 ng/mL) and analyzed for CD62P expression by flow cytometry. The percentage of CD62P-positive cells after stimulation with or without rhTPO preincubation, as well as the ratio between these data (columns 3 and 6), are shown. Values are the percentage of $CD62P^+$ cells in all columns except 3 and 6.

Abbreviation: SD, standard deviation.

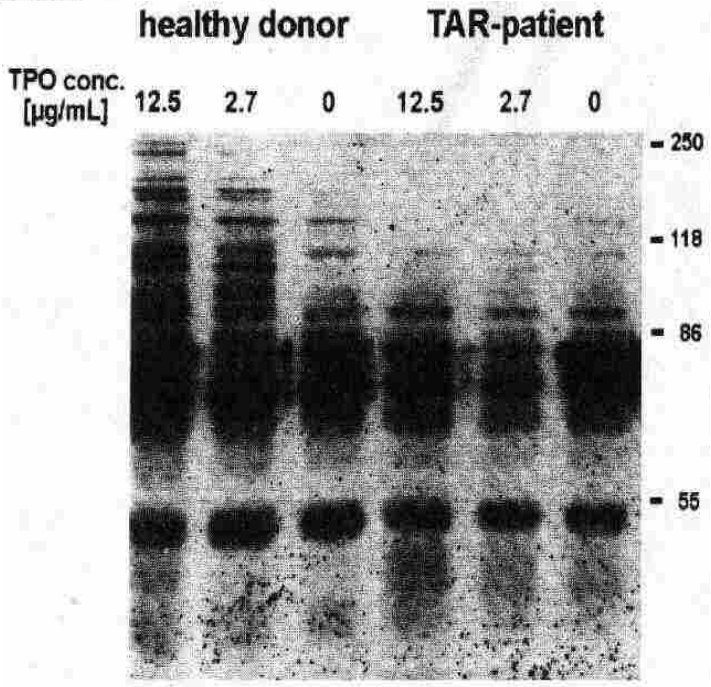



**Fig 3. Induction of tyrosine phosphorylation of platelet proteins after stimulation with rhTPO. Platelets (patient no. 5) were preincubated with different concentrations of rhTPO before lysis and Western-blot analysis with HRP-conjugated antiphosphotyrosine antibody (chemoluminescence detection) (see Materials and Methods)**

and EPO) were increased. This is in aeeordance with reports of episodes of leukocytosis and leukemoid reaetions with white cell counts greater than 35,000/μL and a left shift of myeloid eells in infants with TAR syndrome.2~3 These reie tions eould be caused by elevated leveis of other hematopoi efle growth faetors. In fact, we found elevated levels of IL 11 in at least three out of five patients affected with the TAR syndrome. IL- 11 is a cytokine that is reported tu promote the growth of myeloid and erythroid precursors. 34 However, elevation of this cytokine also could have occurred indepen dent of thrombocytopenia seen in these patients.

Platelet funetions were reported tu be normal in patients with TAR syndrome,3 although few patients had ahnormal platelet aggregation and storage pool defects. 8,9 Beeause of the high blood

volume required in testing platelet aggrega tion horn thrombocytopenic patients. we only measured the expression of the activation-dependent antigen CD62P on platelets after stimulation with ADP or TRAP. Under these experimental conditions. we found normal platelet activation in all TAR patients tested.

We examined the in vitro reactivity of platelets tu TPO. Although TPO by itself does not cause an inerease in a- granule seeretion as assayed by CD62P expression. it upregulates the response tu ADP or thrombin receptor ago nists.27~35~36 However, TPO by itseif causes signifleant tyro sine phosphorylations in a number of different proteins in platelets.21~35~37 In contrast, platelets from TAR patients showed no or very little reaetivity tu TPO. We found no synergism of TPO tu the platelet aetivators ADP and TRAP. Furthermore there was no (3 of 4) or very wealt (1 of 4) additional tyrosine phosphorylation of platelet proteins after stimulation with TPO. From this defeetive reactivity of plate lets in response tu TPO we assume a defeet of all cells of the megakaryocytopoiesis ineluding megakatyocyte progenitor cells. This defeetive response might be the cause ofthrombo

cytopenia in the TAR syndrome. Signal transduction path ways downstream of c-Mpl should be essentially similar in platelets and their progenitors in the bone marrow. Regardless of whether the signal transduction in platelets is a simple remnant horn the megakaryocytes or whether there is a phys iological role of TPO in platelet activation. Miyakawa et a121'37 showed phosphorylation of the signal transduction molecules Jak2, Shc, Stat3. and Stat5 in platelets in response to TPO. These data correspond tu those obtained with cell (ines normally expressing or engineered tu express c Mpl.22.23 38. We could show that c-Mpl is expressed on the surface of platelets horn TAR patients in similar amounts as in healthy controls. In addition the molecular weight of the receptor corresponds tu that of normal donors. However. puint muta tions in c-Mpl of TAR patients cannot be excluded as a cause for the defeet in signal transduction. This seenarirt resembles very much the situation in congenital neutropenia patients in which we could show elevated G-CSF serum levels and normal G-CSF receptor expression but defeetive response tu G-CSF in vitro and in vivo.39 Nevertheless, cnn genital neutropenia patients responded weIl tu pharmacologi cal dosages of G-CSF with an inerease of neutrophils associ ated with a reduetion of bacterial infeetions. suggesting that treatment of TAR patients with rhTPO might help tu inerease platelet numbers and improve elinical problems associated with thrombocytopenia.

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